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Identification of Single *Meloidogyne* Juveniles by Polymerase Chain Reaction Amplification of Mitochondrial DNA[1]

T. S. Harris, L. J. Sandall, and T. O. Powers[2]

Abstract: Polymerase chain reaction (PCR) was used to amplify a specific 1.8-kb sequence of mitochondrial DNA from single juveniles and eggs from 17 populations of *Meloidogyne incognita, M. hapla, M. javanica,* and *M. arenaria.* Approximately 2 μg amplified product were produced per reaction. Restriction digestion of the amplified product with *Hin*fI permitted discrimination of clonal lineages of the four species. *Meloidogyne javanica,* however, could not be separated from *M. hapla* by the enzymes used in these experiments. Various amplification conditions and nematode lysis procedures were examined in order to optimize the speed and quality of identifications.

Key words: diagnostics, *Meloidogyne* spp., mitochondrial DNA, polymerase chain reaction (PCR), root-knot nematode.

The pre-adult stages of plant-parasitic nematodes have been difficult to identify because of their small size and lack of distinguishing morphological features. Identification of these stages is important since they include the infective and overwintering stages of many nematode species. Whereas some biochemical methods have been successful in identifying the enlarged female stages (4), they lack the sensitivity to detect the relatively smaller vermiform and egg stages. Our objective was to adapt the highly sensitive technique of polymerase chain reaction (PCR) for species identification of single *Meloidogyne* eggs and individual second-stage juveniles.

The polymerase chain reaction is an in vitro method for the primer-directed enzymatic amplification of DNA (3,10,12). The process is essentially similar to in vivo DNA replication. The standard reaction consists of repetitions of three steps. First, the double-stranded DNA is denatured by incubation at a high temperature. Then extension primers, a set of short, specific oligonucleotide sequences, are annealed to the single-stranded DNA template and subsequently extended by DNA polymerase. After every amplification cycle the number of copies of a specific DNA sequence are doubled. This amplified product can then serve as the template for the next amplification cycle. Theoretically, a single template molecule present in an overwhelming abundance of nontarget sequences may be amplified, with over a millionfold amplification reported in some cases (3). Although relatively new, PCR has been quickly applied to forensic science (6), genetics (8,18,19), and evolutionary biology (7).

In this study we have used PCR to amplify a specific mitochondrial DNA (mtDNA) sequence from four *Meloidogyne* species. Evaluation of the amplified product indicates that restriction site polymorphism within the mtDNA sequence can be used to identify clonal lineages of *Meloidogyne.*

Materials and Methods

MtDNA isolation and cloning and DNA hybridization: MtDNA was isolated as reported previously (13). Purified mtDNA from *Meloidogyne incognita* race 2 (NCSU #E1135) was digested according to manufacturer's recommendations (Bethesda Research Laboratories) with *Hin*dIII, and the resulting mitochondrial fragments were cloned into the plasmid vector pBR322 (9). The cloned *Hin*dIII fragments were tested as hybridization probes for mtDNA extracted from 20 different *Meloidogyne* iso-

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lates in order to detect interspecific restriction site polymorphism. MtDNA from these isolates was digested with *Hin*fI and *Eco*RI, the digest was electrophoretically fractionated on 0.7, 1.0, or 1.2% agarose gels (0.09 M Tris, 0.09 M borate, 2.0 mM EDTA buffer, pH 8.0; 3.5 hours at 100 V), and the DNA fragments were transferred to nitrocellulose filters (21). Hybridization probes were nick-translated (16) with [α-^{32}P]dATP and hybridization reactions were conducted overnight at 60 C in 4 × SSC (9) followed by four 45-minute washes in 2 × SSC at 60 C. A 1.8-kb *Hin*dIII fragment that displayed restriction site polymorphism among nematode isolates was subcloned into the cloning vector pUC18. Clones with the fragment oriented in opposite directions were selected to obtain sequence information from both ends of the fragment.

Oligonucleotide primers and sequencing: Approximately 100 nucleotides from the *Hin*dIII restriction sites delimiting the 1.8-kb fragment were sequenced by the dideoxynucleotide method (20) on purified double-stranded plasmid DNA (11). Sequencing and autoradiography were performed with 6% denaturing gels by standard techniques (1,2). The extreme A+T richness of the mitochondrial fragment (83% adenines and thymines for sequenced regions) necessitated the selection of primer sites that maximized the number of guanines and cytosines in order to reduce the possibility of nonspecific binding or self-annealing of the primers (17,22). Two 20-bp sequences were selected for PCR amplification primers: 5′-TAAATCAATCT GTTAGTGAA-3′, and 5′-ATAAACCAG TATTTCAAACT-3′. Primers were synthesized and purified by the University of Florida DNA Synthesis Facility.

Enzymatic amplification of mtDNA: For initial evaluation of PCR amplification conditions, each primer at a final concentration of 1.0 μM and the reagents (10 × reaction buffer, 200 μM of each dNTP, and 2.5 units of *Taq* DNA polymerase) of the GeneAmp DNA Amplification Kit (Perkin Elmer Cetus) were added to 1 ng of purified *M. incognita* mtDNA. Amplification products were detected by fluorescence on ethidium bromide-stained agarose gels. Optimum amplification conditions were determined by systematically varying one parameter while holding all other factors constant. One nanogram of purified *M. incognita* mtDNA was the template. The parameters evaluated were denaturation temperature, annealing temperature, primer extension temperature, the sequential increase of extension times, and number of amplification cycles. A range of temperatures was tested for each of the steps of the PCR cycle. Five denaturation temperatures, decreasing from 94 C by 5-C increments, were evaluated by observing the intensity of stained amplified product on agarose gels. Seven annealing temperatures, increasing from 37 C by 4–5-C increments, were evaluated. Extension temperatures ranging from 68 to 84 C were examined at 4-C increments. The sequential lengthening of extension time (the autoextension function) was also evaluated at 5-second increments. PCR cycle numbers of 25–40 were evaluated at 5-cycle increments.

Amplification from single juveniles and eggs: A single, freshly hatched *M. incognita* juvenile was placed in a 15-μl drop of distilled water on a coverslip. The nematode was manually disrupted with a micropipet tip, and the suspension was transferred to a cold 0.5-ml microfuge tube. Addition of oligonucleotide primers and PCR reagents brought the final reaction volume to 100 μl.

Different nematode lysis procedures were examined for amplification experiments using single juveniles or eggs. These conditions included disrupting the nematode with a micropipet tip on a coverslip, boiling it for 10 minutes in sterile double distilled H_2O, incubating the nematode in 0.6 mg/ml Proteinase K in double distilled H_2O for 10 minutes at 37 C prior to boiling, and 1–3 freezing and thawing cycles prior to boiling. Lysis, in each case, was followed immediately by addition of primers and amplification reagents.

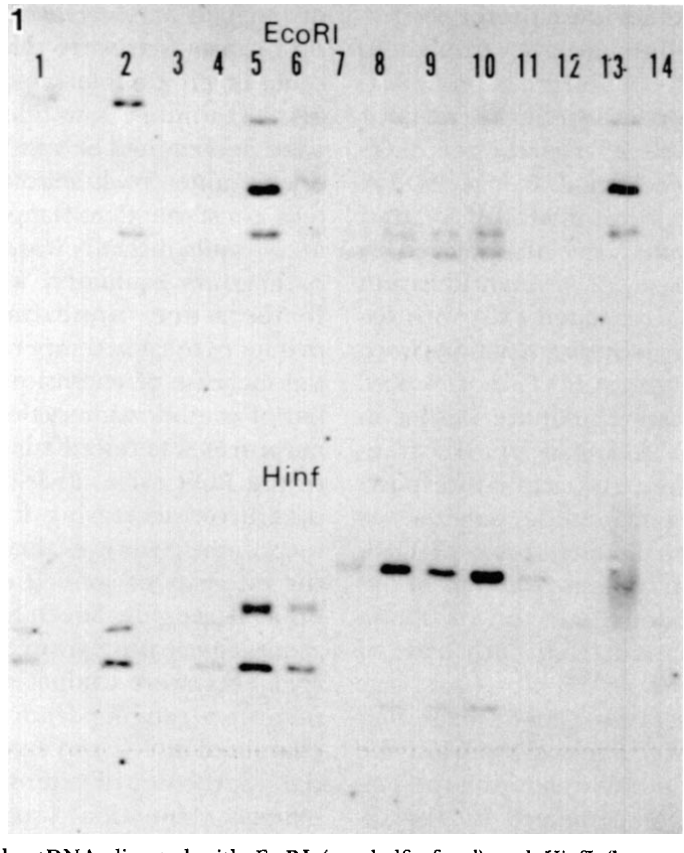


FIG. 1. Purified mtDNA digested with *Eco*RI (top half of gel) and *Hin*fI (bottom half). MtDNA was hybridized with the cloned 1.8-kb *Hin*dIII fragment from *M. incognita*. *Meloidogyne* isolates are *M. arenaria* (lanes 1–6), *M. javanica* (lanes 7–11), and *M. incognita* (lanes 12–14).

Nematode isolates: All nematodes were maintained on greenhouse-grown tomato (*Lycopersicon esculentum* L. cv. Rutgers). Each of these isolates has been characterized wholly or in part by host range (5), perineal patterns (5), or esterase phenotype (4), and each is consistent with the specific designation. Original isolations are presented in Table 1.

RESULTS

In hybridization patterns (Fig. 1) of 14 mtDNA isolates digested with *Eco*RI and *Hin*fI and hybridized with the cloned 1.8-kb *Hin*dIII fragment, mtDNA isolated from five *M. javanica* populations (lanes 7–11) displays hybridization patterns distinct from those of *M. incognita* (lanes 12–14) and *M. arenaria* (lanes 1–6). Both enzymes appear to recognize restriction sites and produce DNA fragments that, when hybridized with the *Hin*dIII fragment, identify subsets of *Meloidogyne* mitochondrial genomes.

PCR amplification: Successful amplification of a 1.75-kb fragment was demonstrated by restriction site comparisons with the original 1.8-kb cloned fragment. The slight size difference resulted from the selection of primer binding sites approximately 25 bp internal to the flanking *Hin*dIII sites. In subsequent experiments to determine the optimum conditions for amplification, denaturation at 94 C produced the greatest quantity of amplified DNA. The optimum annealing and extension temperatures were 50 C and 68 C, respectively. No apparent increase in product yield was achieved by lengthening extension times by more than 5 seconds per cycle. Similar high levels of DNA amplification resulted from 35 and 40 PCR cycles. Subsequent experiments

with single nematodes were therefore performed under the following conditions: denaturation at 94 C for 1 minute, annealing at 50 C for 2 minutes; extension at 68 C for 3 minutes, auto-extension for 5 seconds, and PCR for 35 cycles.

To shorten the total amplification time, annealing and extension times were decreased by 1 minute each and auto-extension was eliminated. A 5-minute incubation at 72 C to allow completion of partially extended amplification products followed the 35th cycle. There was no obvious reduction in the amount of amplified product. With the parameters established in the optimization experiments, the DNA from a single juvenile or egg was amplified. In an ethidium bromide-stained agarose gel (Fig. 2) with 10% of the undigested amplified product applied directly to the gel following amplification, a single 1.75-kb fragment is evident. DNA concentration of the total amplified product from a single juvenile was estimated by spectrophotometry to be approximately 2 μg. Lanes E, J, and M are amplified products from single eggs, juveniles, and an adult male, respectively. In numerous amplification experiments, assayable quantities of amplified product were consistently obtained from both single eggs and juveniles. The mtDNA from juveniles, however, routinely produced more amplified product than that from eggs.

Other lysis techniques were less reliable. The mtDNA from less than 50% of the juveniles was amplified when they were lysed by boiling for 10 minutes. A 10-minute incubation in Proteinase K (0.6 mg/ml) did not improve lysis. A single freezing and thawing cycle resulted in erratic lysis. Consistent DNA amplification was obtained only when we verified lysis by direct observation of disrupted nematodes.

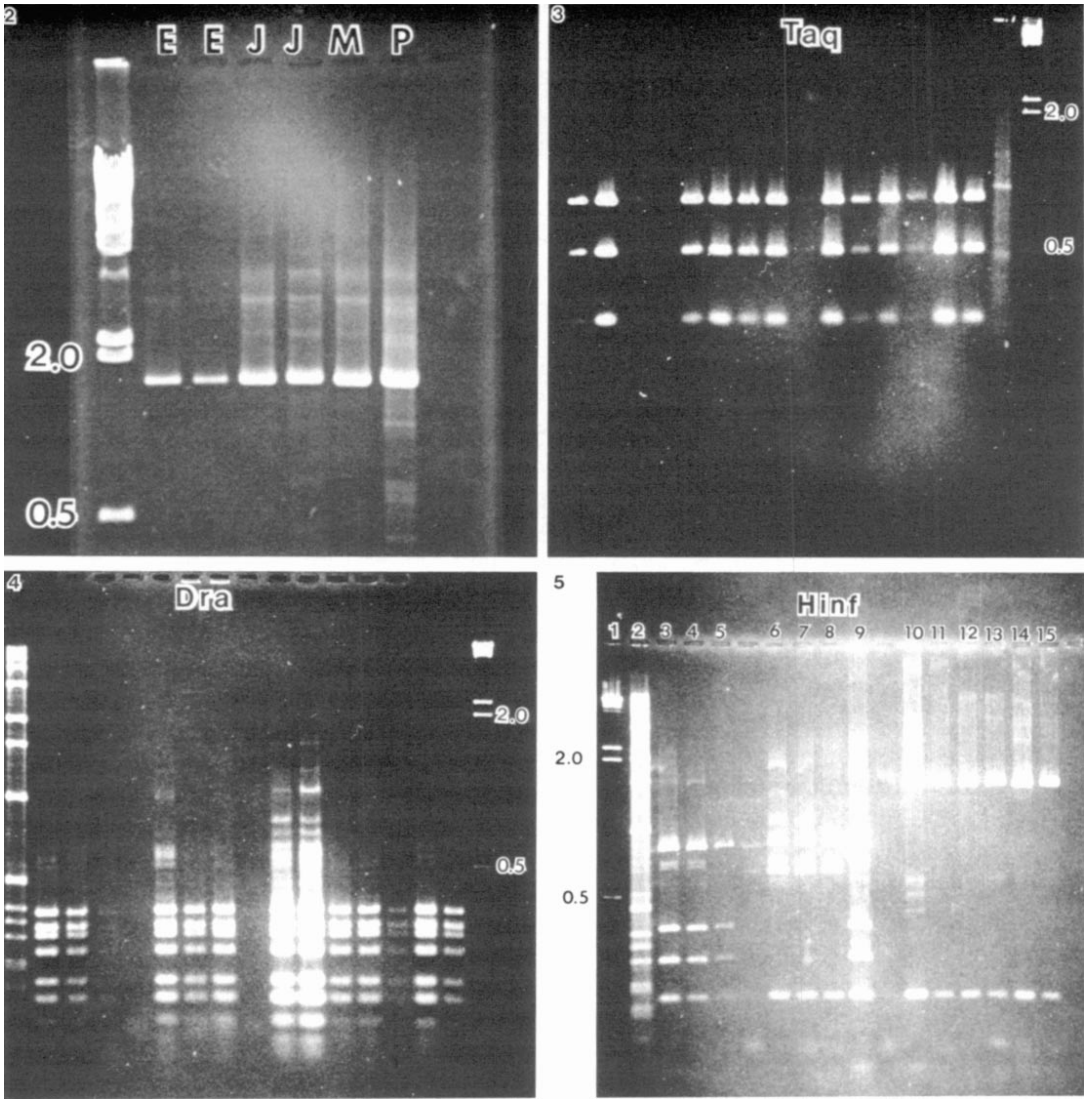
Amplification of mtDNA from single juveniles was attempted with 20 populations of *Meloidogyne*, representing the four major species (Table 1), and one population of *Heterodera glycines*. The 1.75-kb *Hin*dIII fragment was amplified from juveniles in 17 of the *Meloidogyne* populations. The quantity of amplified product did not vary among species although intraspecific sample-to-sample variation occurred. Amplification was unsuccessful with four populations—*M. arenaria* 576, 351, and *M. hapla* (E-20) from Wisconsin and *H. glycines*—despite repeated attempts with less stringent annealing conditions.

Diagnostic potential of the amplified product: In restriction digestions of the amplified products from 13 populations, 10% of the amplified product was digested with a restriction enzyme, fractionated on a 1.5% agarose gel, and stained with ethidium bromide. Digestion with *Taq*I (Fig. 3) and *Dra*I (Fig. 4) produced 3 and 7 fragments, respectively, but no polymorphism was evident among samples. *Hin*fI digestion (Fig. 5), however, produced restriction fragment polymorphism displaying a species-specific pattern among the 13 populations. *Meloidogyne incognita* (lanes 3–5, 9) was characterized by four DNA fragments 900, 400, 290, and 175 bp in size. *Meloidogyne arenaria* (lanes 6–8) lacked the restriction site between the 290-bp and 400-bp fragment, creating a new 700-bp fragment. *Meloidogyne javanica* and *M. hapla* (lanes 10–15) could not be distinguished from each other by use of this enzyme, but they were separated from the other two species by the absence of the restriction site dividing the 700-bp and 900-bp fragment of *M. arenaria*, thereby creating a new 1,600-bp fragment.

Discussion

Polymerase chain reaction is a powerful new technique that promises to revolutionize molecular diagnostics. The experiments described in this study demonstrate how nematode identification may be conducted on a single egg or juvenile in less than 8 hours. The steps involved—nematode lysis, amplification, and PCR product evaluation—are technically simple and require few experimental manipulations.

DNA hybridization experiments were conducted to select a region of the mitochondrial genome for PCR amplification. Ideally, such a region should contain di-



FIGS. 2–5. Amplification products from mtDNA. 2) The 1.75-kb amplification products from two single *Meloidogyne incognita* eggs (lanes E), two single second-stage juveniles (*M. incognita* and *M. javanica,* lanes J), and one single *M. incognita* male (lane M). Lane P is amplified product from approximately 1.0 ng of the cloned 1.8-kb fragment. 3–5) Amplified products from mtDNA from single *Meloidogyne* juveniles digested with the restriction enzymes *Taq*I (Fig. 3), *Dra*I (Fig. 4), and *Hin*fI (Fig. 5). In *Taq*I and *Dra*I digestions all amplification products from mtDNA from single juveniles produced the same number of fragments in every *Meloidogyne* isolate. Fragments greater than 400 bp in *Dra*I digestions are caused by incomplete digestion of the amplified product. Figure 5 *Meloidogyne* isolates are *M. incognita* NCSU #E1135, NCSU #108, race 4, and Riverside in lanes 3–5 and 9, respectively; *M. arenaria* Arizona, NCSU #436, and Nevada in lanes 6–8; *M. hapla* Kincaid and Tennessee in lanes 10 and 11; *M. javanica* Riverside, NCSU E#781, NCSU #610, and NCSU #525 in lanes 12–15. Incomplete digestions are evident in lanes 3, 4, 6, 7, 9, and 10. In all cases 10% of the amplified product was digested. Lanes 1 and 2 are size markers.

agnostic restriction site polymorphisms and be small enough (0.5–3.0 kb) to be readily amplified by PCR. A 1.80-kb *Hin*dIII fragment, the smallest *Hin*dIII fragment generated when purified mtDNA from *Meloidogyne incognita* is digested by the enzyme, was selected on the basis of these criteria. *Hin*fI restriction site polymorphism in this fragment appears to be concordant with traditional species designations. However, we were unable to amplify this region from three *Meloidogyne* populations and an iso-

TABLE 1. Origins of *Meloidogyne* isolates used in PCR amplification.

Species	Origin
M. incognita	California (NCSU E1135)
M. incognita	Alabama (NCSU #108)
M. incognita	(NCSU) (Race 4)
M. incognita	Riverside, California
M. arenaria	Arizona
M. arenaria	California (NCSU #576)
M. arenaria	Florida (NCSU #351)
M. arenaria	Georgia (NCSU #436)
M. arenaria	Georgia (NCSU E1084)
M. arenaria	Nevada
M. arenaria	South Dakota (Nursery)
M. javanica	Riverside, California
M. javanica	Arizona (NCSU E781)
M. javanica	North Carolina (NCSU #610)
M. javanica	(NCSU)
M. javanica	Texas (NCSU #525)
M. hapla	Tennessee
M. hapla	Wisconsin (Kincaid)
M. hapla	Wisconsin (Leach)
M. hapla	Wisconsin (E-20)

late of *Heterodera glycines*. This failure may be due to a loss of a primer annealing site in these genomes. The location of the 1.8-kb fragment is adjacent to an A+T-rich region of the mitochondrial genome, which is the site of frequent size polymorphism in *Meloidogyne* (14, Powers, unpubl.). By comparing nucleotide sequences of the primer region from *M. arenaria*, we should be able to select a suitable primer site for the amplification of these genomes.

Another explanation for lack of amplification could be extreme nucleotide sequence divergence in the region of the primer binding sites of these populations. Mitochondrial nucleotide sequence divergence between *H. glycines* and *H. schachtii*, two sibling species, has been estimated to be approximately 15% (15). It is likely that sequence divergence between *Meloidogyne* and *Heterodera* greatly exceeds this value. For DNA amplification over a broader taxonomic range, it may be necessary to construct primers for a more conserved region of the genome.

The potential applications of PCR in nematology are numerous. Regulatory nematologists could quickly determine species status of vermiform nematodes from soil and plant samples and avoid lengthy quarantine periods. Ecologists could examine nematode population dynamics in fields with a mixture of congeneric species. Evolutionary biologists and geneticists could study their gene of interest without having to culture large quantities of nematodes or construct genomic libraries. Furthermore, comparative studies can be conducted on small quantities of fixed material from exotic nematodes that would be difficult to transport because of regulatory concerns.

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