sequences. We hypothesized that the variable length of rDNA IGS sequences could be used to separate species of root-knot nematodes. Our objective was to design primers that would amplify the IGS regions of *M. hapla* and *M. chitwoodi,* and the newly described species, *M. fallax,* and identify these species using the variable length of the amplified IGS fragment.

Materials and methods

Nematodes

The populations of *M. chitwoodi, M. hapla,* and *M. fallax* used in this study are listed in Table 1. Genomic DNA was purified from nematode cultures as previously described (Vrain *et al.,* 1992; Zijlstra *et al.,* 1995).

Table 1. *Populations of* Meloidogyne chitwoodi, M. hapla, *and* M. fallax *used in the study.*

Species	Code	Origin	Reference
M. hapla	Ham	Queensland, Australia	Zijlstra *et al.,* 1995
M. hapla	He	Drouwenerveen, NL	"
M. hapla	Hi	Smilde, NL	"
M. hapla	Hbq	Hungary	"
M. chitwoodi	Cbf (ORMC8)	Oregon, USA	Mojtahedi *et al.,* 1988
M. chitwoodi	Cbh (CAMC2)	California, USA	Mojtahedi & Santo, 1994
M. chitwoodi	Cp	Sterksel, NL	Zijlstra *et al.,* 1995
M. fallax	Cae	Roggel, NL	"
M. fallax	Cc	Baexem, NL	"
M. fallax	Cg	Vredepeel, NL	"

Polymerase chain reaction

Amplifications of DNA targets greater than 5 kb were performed using 2 μl eLONGase Enzyme Mix (GibcoBRL, Burlington, ON, Canada) in 30 μl PCR reactions containing 60 mM Tris-SO_4 (pH 9.1), 15 mM $(NH_4)_2SO_4$, 1.6 mM $MgSO_4$, 200 μM dNTPs, 500 nM each primer, and 100 ng genomic DNA. Cycling parameters consisted of 95 °C denaturing for 15 s, 50 °C annealing for 30 s, and 72 °C extension for 8 min. DNA targets of less than 3 kb were amplified using 1 unit of Taq DNA polymerase (Appligene-Oncor, France) in 30 μl PCR reactions containing 10 mM TrisHCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X100, 0.2 mg/ml BSA, 65 μM dNTPs, 500 nM each primer, and 10 ng genomic DNA. Cycling parameters consisted of 95 °C denaturing for 30 s, 46 °C annealing for 45 s (3 cycles), 52 °C annealing for 45 s, (37 cycles), and 72 °C extension for 2 min. All PCR reactions were performed in a robocycler gradient 96 (Stratagene, La Jolla, CA, USA).

Oligonucleotide synthesis

Primer sequences previously employed for amplification of the rDNA ITS region (Vrain *et al.,* 1992) were used as a basis for designing a new primer set. The inverted primers G : 5'-TCCCTTAGTAACGGCGAGTG-3', and B : 5'-TGTACAAAGGGCAGGGACG-3' (Fig. 1) were capable of amplifying a portion of the rDNA cistron (an approximately 5.8 kb DNA fragment) that included the IGS. Additional primers to the 3' end of the 28 S gene (283-N : 5'-TTCGAGTAAGCGCGGGTAAACG-3'), and to the 5' end of the 18 S gene (185-0 : 5'-CAGTTCAGGCAGGATCAAC-3'; 185-1 : 5'-GTGAACACCACTCTCATC-3') were used to amplify the IGS region alone (Fig. 1). All oligonucleotide primers were synthesized by phosphoramidite chemistry using the Oligo1000M (Beckman).

Cloning

Amplified DNA from *M. chitwoodi* was separated from the genomic DNA template and primers by electrophoresis using 1 % (w/v) low-melting point agarose

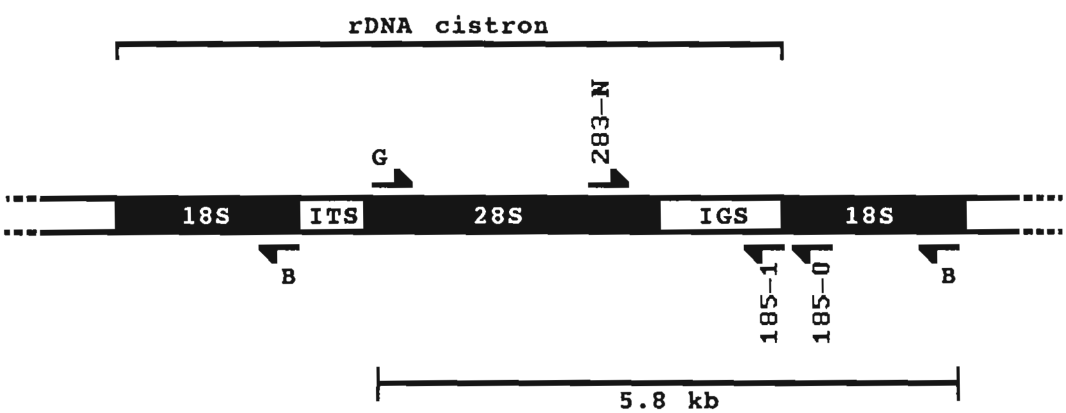

Fig. 1. *Structural map of the rDNA cistron of* Meloidogyne chitwoodi. *The hybridization positions of oligonucleotide primers are shown on the map, and the orientation of each primer for amplifying the IGS via PCR is indicated by an arrow.*

(BRL) gels containing 0.5 ug/ml ethidium bromide electrophoresed in Tris-acetate EDTA buffer at 80 V for 1 h. DNA bands were excised from gels with a sterile scalpel and purified from the agarose using a freeze-thaw procedure (Qian & Wilkinson, 1993). The purified fragments were cloned into the *SmaI* site of pSK$^-$ (Stratagene, La Jolla, CA, USA) by blunt-end cloning using T4 DNA ligase (GibcoBRL, Brulington, ON, Canada) according to manufacturer's directions. Ligation products were transformed into $CaCl_2$ competent *Escherichia coli* by a brief heat shock at 42 °C, and cells selected for both resistance to ampicillin and inability to utilize the chromogenic substrate X-gal (*lacZ*$^-$) (Miller, 1972). Alkaline lysis was used to purify recombinant plasmids from single colonies grown overnight in Terrific Broth (Sambrook *et al.*, 1982).

DNA SEQUENCING

Cloned restriction fragments were further purified for sequencing using Qiaquick spin columns (Qiagen Inc., Chatsworth, CA, USA), eluted in 10 mM Tris-HCl, pH 8.0, and stored frozen until further use. Sequencing was accomplished using the PRISM^{TM} DyeTerminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Mississauga, ON, Canada) and analyzed by ABI310 capillary electrophoresis system according to the manufacturer's directions. The sequencing data obtained for the 28S, IGS, and 18S regions was compared with other nematode sequences in the Genbank database (http://www.ncbi.nlm.nih.gov) to find homologous regions so that additional primers could be designed to more efficiently amplify the IGS region.

Results

Reorientation and modification of the ITS primers allowed successful PCR amplification of a 5.8 kb region of the rDNA cistron of *M. chitwoodi* when a proofreading *Taq* polymerase was used. Restriction mapping of the cloned fragments obtained from amplifications of both *M. chitwoodi* race 2 and *M. chitwoodi* race 3 allowed further subcloning and DNA sequencing of fragments contained within the 5.8 kb amplicon. Comparisons of nucleotide sequences with other nematodes or related rDNA sequences present within the Genbank database revealed regions to which primers might be designed for amplification of the IGS region from a variety of nematode species.

Upon testing of the newly designed primers 283-N and 185-0 (Fig. 1), successful PCR amplification of an approximately 2.6 kb fragment from all three described *M. chitwoodi* races was achieved (Fig. 2). This amplification utilized a more common form of thermostable *Taq* polymerase, and reaction times were reduced by nearly 75 %. Additionally, amplified product yields were substantially increased, allowing product from amplification of single juveniles to be visualized by ethidium bromide staining of reactions separated by gel electrophoresis. The rDNA IGS region from several *Meloidogyne* species was amplified and compared using the 283-N/185-0 primer combination. Separation of the amplified fragments obtained from *M. chitwoodi, M. fallax,* and *M. hapla* demonstrated size differences in the amplified product (Fig. 2). The products from *M. chitwoodi* and *M. fallax* differ by approximately 100 bp (2.6 *vs* 2.7 kb), while the corresponding band from *M. hapla* is much smaller (600 bp). These amplified fragment length polymorphisms were reproducibly obtained from three or more populations of each species tested.

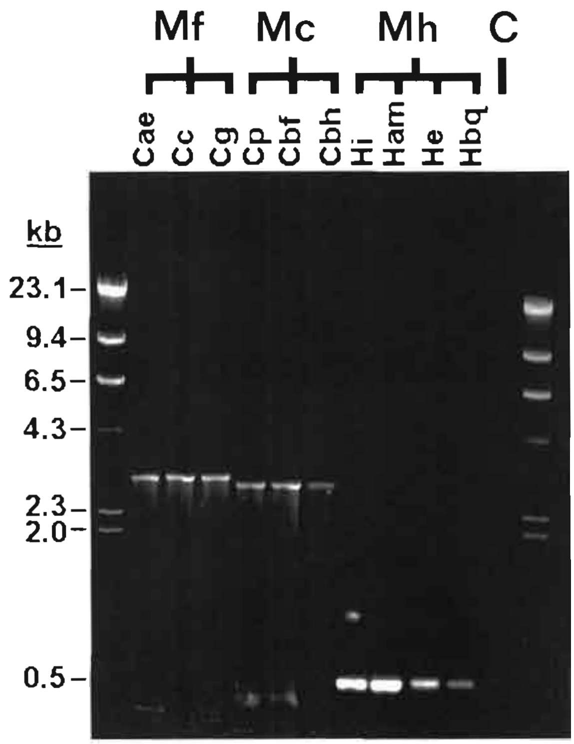

Fig. 2. *Length polymorphisms within the IGS region. PCR products obtained by amplification of 8 ng template DNA using primers 283-N and 185-0 as described in materials and methods were separated by electrophoresis through 0.7 % agarose gels (70 V, 2 h). Fragment sizes of products from* Meloidogyne fallax *(Mf),* M. chitwoodi *(Mc), and* M. hapla *(Mh) were calculated using the lambda-HindIII digested size markers in the two outside lanes (size, in kilobases, is shown at left). The control reaction (no template) is also shown (C).*

To confirm that each fragment corresponded to the correct amplicon, two further tests were performed as follows. A fifth primer (185-1) was synthesized, hybridizing approximately 200 bp upstream of the 18S 5′ terminus (Fig. 1), to amplify the IGS region when employed in combination with the 283-N primer. For each species, a band approximately 200 bp smaller than the original 283-N/185-0 product was obtained (Fig. 3). This result was expected if the amplified regions were

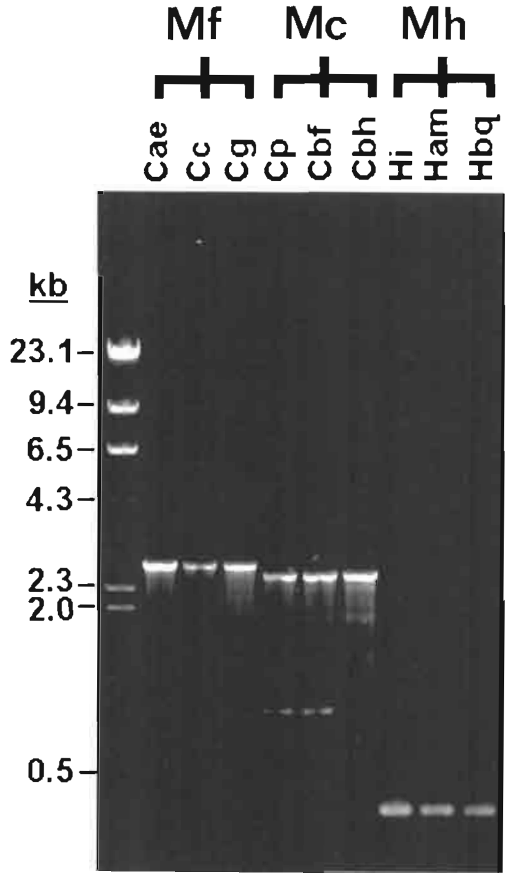

Fig. 3. Length polymorphisms within the IGS region. PCR products obtained by amplification of 8 ng template DNA using primers 283-N and 185-1 as described in materials and methods were separated by electrophoresis through 0.7 % agarose gels (70 V, 2 h). Fragment sizes of products from Meloidogyne fallax *(Mf),* M. chitwoodi *(Mc), and* M. hapla *(Mh) were calculated using the lambda-HindIII digested size marker (size, in kilobases, is shown at left).*

indeed the IGS for which the primers were designed. In addition, we also sequenced the terminal regions of each amplified fragment. The DNA sequences revealed that each band was indeed the product of amplification using both primers contained in the PCR reaction, and that the amplified region exhibited some homology to rDNA sequences of nematodes or other organisms contained within the Genbank database.

Discussion

The study of RFLPs in nematode taxonomy routinely involves combining PCR amplification of the rDNA ITS with subsequent restriction enzyme digestion. Our procedure advances upon this technique in three main aspects : *i)* amplified fragment length polymorphisms can allow single step discrimination of species identity; *ii)* processing time is substantially reduced since restriction enzyme incubation periods are avoided, and only one gel need be run; and *iii)* the procedure is less costly since no restriction enzymes are employed, and lower agarose concentrations may be used for electrophoretic detection.

Using the single step protocol, we confirmed the recent reclassification of Baexem-type isolates as a new species (*M. fallax*) distinct from *M. chitwoodi* (Zijlstra *et al.,* 1995). Hence, the sensitivity of molecular methods can identify genetic distinctions even though physiological or host range differences are not yet described. Previously, the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* were directly distinguished by PCR amplification of the spliced leader RNA and 5S rRNA spacer region (Stratford & Shields, 1994). Amplified fragment length polymorphisms were also observed within mitochondrial DNA of several *Meloidogyne* species, but did not distinguish *M. chitwoodi* from *M. hapla* (Powers & Harris, 1993). We now routinely use the size differences in the rDNA IGS as a diagnostic tool to differentiate these two species. Sequencing of the rDNA IGS is currently underway in order to produce species-specific probes (Petersen *et al.,* 1995) for detection of quarantined nematodes on imported agricultural commodities.

The presence of a 5S gene within the rDNA IGS may contribute to size differences between species (Blok *et al.,* 1997); however, the small size of the *M. hapla* IGS amplification product suggests a large deletion. Genetic changes in the rDNA IGS are not uncommon; they are readily accomodated by the host since this DNA region is not transcribed (Gerbi, 1985). Indeed, IGS deletions have already been found in *M. arenaria* (Vahidi & Honda, 1991). The presence of such a deletion, found in *M. hapla* isolates from two different continents, is in agreement with morphometric and phylogenetic classification of *M. hapla* as evolving separately from *M. chitwoodi* and *M. fallax* (Golden *et al.,* 1980; Zijlstra *et al.,* 1995). Nevertheless, our results do not negate the possibility of full length cistrons present within *M. hapla,* since variations within the tandemly repeated rDNA copies can exist within individuals (Williams *et al.,* 1985; Gerbi, 1985; Vahidi & Honda, 1991), and the kinetics of the PCR reaction can favor smaller amplicons, resulting in their dominating the final reaction products. Analysis of additional populations and *Meloidogyne* species is necessary to determine whether IGS size polymorphisms are truly species-specific.

The inverse of primers B and G (Fig. 1) that have been used in several published studies of nematode ITS variability were originally designed from homologous sequences between several nematodes and other organisms (Vrain *et al.,* 1992). We have not been able to amplify the IGS region of nematode species from several genera (*Xiphinema, Globodera, Heterodera, Steinernema,* and *Pratylenchus;* data not shown) with primers

283-N and 185-0. These primers were designed from sequences at the 3′ end of the 28S gene and the 5′ end of the 18S gene of *M. chitwoodi*, regions lacking the high sequence conservation observed at the other end of each gene. Therefore, we are presently acquiring the nucleotide sequences, at the 3′ end of the 28S and the 5′ end of the 18S genes of the genera of interest, to design common primers that can amplify the IGS of all species to be compared.

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