DIFFERENTIATION OF *MELOIDOGYNE FLORIDENSIS* FROM *M. ARENARIA* USING HIGH-FIDELITY PCR AMPLIFIED MITOCHONDRIAL AT-RICH SEQUENCES

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ABSTRACT

Jeyaprakash, A., M. S. Tigano, J. Brito, R. M. D. G. Carneiro and D. W. Dickson. 2006. Differentiation of *Meloidogyne floridensis* from *M. arenaria* using high-fidelity PCR amplified mitochondrial AT-rich sequences. Nematropica 36:1-12.

Standard polymerase chain reaction (PCR) and high-fidelity PCR procedures were compared for amplifying DNA sequences from five root-knot nematode species. High-fidelity PCR was found to be at least four orders of magnitude more efficient than standard PCR. High-fidelity PCR was used to amplify mitochondrial DNA sequences located between the cytochome oxidase subunit II (*COII*) and 16S rRNA genes from the following root-knot nematode species; *Meloidogyne mayaguensis* (0.7 kb), *M. floridensis* (1.1 kb), *M. arenaria* (1.1 kb), *M. incognita* (1.5 kb), and *M. javanica* (1.6 kb). This region includes the non-coding "control" region, which is also referred to as AT-rich region and tRNA-His gene. The high-fidelity PCR products were cloned and sequenced. The *COII* 3' partial, tRNA-His, and 16S rRNA 5' partial sequences were found to display few nucleotide differences, but the AT-rich region displayed extensive nucleotide and length variations in all five species; *M. mayaguensis* (167 bp), *M. arenaria* (573 bp), *M. floridensis* (603 bp), *M. incognita* (963 bp), and *M. javanica* (1110 bp). A stem and loop structure, which has been characterized from insects and other higher organisms, was detected in a conserved block of AT-rich sequences from *M. floridensis*, *M. arenaria*, *M. incognita*, and *M. javanica*. Similar stem and loop structures were also detected from the AT-rich sequences previously reported from *Caenorhabditis elegans* and *Ascaris suum* and compared to the *M. floridensis* stem and loop structure. Even though the high-fidelity PCR products amplified from *M. floridensis* and *M. arenaria* were about the same size (1.1 kb), their AT-rich sequences were found to contain nucleotide differences at several locations. Hence, digesting the 1.1 kb high-fidelity PCR products from these species with the restriction enzyme *Ssp* I produced two distinct banding patterns that distinguished *M. floridensis* from *M. arenaria*.

Key words: high-fidelity PCR, *Meloidogyne*, mitochondrial AT-rich region, molecular diagnosis, stem and loop structure.

RESUMEN

Jeyaprakash, A., M. S. Tigano, J. Brito, R. M. D. G. Carneiro and D. W. Dickson. 2006. Diferenciación de *Meloidogyne floridensis* y *M. arenaria* utilizando secuencias mitocondriales ricas en AT amplificadas por PCR de alta fidelidad. Nematropica 36:1-12.

Se comparó el procedimiento tradicional de reacción en cadena de la polimerasa (PCR) con PCR de alta fidelidad para amplificar secuencias de ADN de cinco especies de nematodo del nudo radical. Se encontró que la PCR de alta fidelidad es por lo menos cuatro órdenes de magnitud más eficiente que la PCR tradicional. Se utilizó PCR de alta fidelidad para amplificar secuencias de ADN localizadas entre la subunidad II de la citocromo oxidasa (*COII*) y los genes 16S rRNA de las siguientes especies de nematodo del nudo radical: *Meloidogyne mayaguensis* (0.7 kb), *M. floridensis* (1.1 kb), *M. arenaria* (1.1 kb), *M. incognita* (1.5 kb) y *M. javanica* (1.6 kb). Esta región incluye la región de "control" no codificada, también conocida como región rica en AT y gen tRNA-His. Se clonaron y secuenciaron

los productos de la PCR de alta fidelidad. Se encontró que las secuencias parciales de *COII* 3', tRNA-His y 16S rRNA 5' poseían pocas diferencias en nucleótidos, pero que la región rica en AT poseía gran variación en nucleótidos y en longitud en las cinco especies: *M. mayaguensis* (167 bp), *M. arenaria* (573 bp), *M. floridensis* (603 bp), *M. incognita* (963 bp) y *M. javanica* (1110 bp). Se detectó una estructura de tallo y bucle, caracterizada previamente en insectos y otros organismos, en un bloque conservado de sequencias ricas en AT de *M. floridensis*, *M. arenaria*, *M. incognita* y *M. javanica*. Se compararon estructuras similares de tallo y bucle en secuencias ricas en AT detectadas en *Caenorhabditis elegans* y *Ascaris suum* con la estructura encontrada en *M. floridensis*. Aunque los productos amplificados por PCR de alta fidelidad de *M. floridensis* y *M. arenaria* fueron del mismo tamaño (1.1 kb), se encontraron diferencias en nucleótidos en varias posiciones de la región rica en AT. La digestión con la ezima de restricción *Ssp* I de los productos de 1.1 kb obtenidos por PCR de alta fidelidad produce patrones de bandas que permiten distinguir a *M. floridensis* de *M. arenaria*.

Palabras clave: diagnóstico molecular, estructura de tallo y bucle, *Meloidogyne*, PCR de alta fidelidad, región mitocondrial rica en AT.

INTRODUCTION

The root-knot nematode, *Meloidogyne floridensis*, was recently described from an isolate originally collected from peach roots (*Prunus persica*) in Gainesville, Florida (Handoo *et al.*, 2004). This nematode was initially characterized as *M. incognita* race 3 (Sherman and Lyrene, 1983), and later recognized as a different species based on preliminary characterization studies (Nyczepir *et al.*, 1998).

Currently, *M. floridensis* is identified using morphological and morphometric characters. This can be tedious, time consuming and requires considerable technical skill. Additionally, *M. floridensis* is similar to *M. incognita*, *M. christiei*, *M. graminicola*, and *M. hispanica* (Handoo *et al.*, 2004), which makes identification more difficult. Isozyme studies showed that the esterase phenotype of *M. floridensis* is species-specific (Handoo *et al.*, 2004), however this analysis can only be performed using females. Analyses of the ribosomal RNA genes intergenic spacer sequences displayed divergences <1.1% between *M. floridensis*, *M. arenaria*, *M. incognita*, and *M. javanica* (Handoo *et al.*, 2004). This is considered low and insufficient for easily distinguishing *M. floridensis* from other root-knot nematodes.

However, amplifying mitochondrial DNA sequences from the cytochrome oxidase subunit II (*COII*) to the 16S rRNA genes that includes the variable AT-rich non-coding "control" region, which is also referred to as AT-rich region, and the tRNA-His gene by standard polymerase chain reaction (PCR) and digestion of the amplified product with restriction endonuclease *Hin*f I or *Dra* I has allowed discrimination of five major *Meloidogyne* species, namely *M. incognita*, *M. javanica*, *M. arenaria*, *M. hapla*, and *M. chitwoodi*, due to distinct restriction fragment length polymorphism (RFLP) patterns (Powers and Harris, 1993; Orui, 1998). Recently, sequences of this region were used to confirm the identification of *M. mayaguensis* populations (Blok *et al.*, 2002; Brito *et al.*, 2004).

Correct identification of *M. floridensis* is of particular concern because of its virulence on the root-knot nematode resistant rootstock in peach cv Nemared and Okinawa. These cultivars have resistance to *M. incognita* and *M. javanica* (Sharpe *et al.*, 1969). Identification of *M. floridensis* can be critical for crop management, plant breeding, and nematode regulatory programs.

Hence, a molecular detection method would serve to screen *M. floridensis* specimens collected from field and nursery plants.

The objective of this study is to analyze the mitochondrial AT-rich region from *M. floridensis*, *M. arenaria*, *M. mayaguensis*, *M. incognita*, and *M. javanica*, and to design a molecular method for distinguishing *M. floridensis* from these root-knot nematode species.

MATERIALS AND METHODS

Nematode Species

Four separate isolates of *M. floridensis* were collected from peach (Alachua County), tomato (Indian River and Collier Counties) and cucumber (Hendry County) in Florida, USA. Six separate isolates of *M. arenaria* were collected including one from *Impatiens* sp. (Osceola County, Florida, USA), three from banana (Guiana, Guadalupe and Martinica, Brazil), and one each from pumpkin and soybean (Brasil, Brazil). One isolate each of *M. mayaguensis* from guava (Petrolina, Brazil), *M. incognita* from coffee (Londrina, Brazil), and *M. javanica* from soybean (Londrina, PR, Brazil) were collected. None of these isolates were reared on tomato and the collected specimens were used directly for further analysis. Identification of taxa was made using morphological and morphometric characters of all stages, and isozyme electrophoresis of young egg-laying females (Carneiro *et al.*, 1998, 2000, 2004).

DNA Extraction

Genomic DNA was extracted by grinding 40 females with their egg masses together in 300 µl PUREGENE cell lysis solution provided by the manufacturer (Gentra Systems, Minneapolis, MN), digesting with 1 mg Proteinase K (Roche Applied Science, Indianapolis, IN) for 16 h at 37°C, and then precipitating the proteins using 100 µl of PUREGENE protein precipitation solution for 10 min over ice. After centrifugation at 14,000 rpm for 5 min, the supernatant was removed and genomic DNA precipitated by centrifugation at 14,000 rpm for 20 min using 300 µl isopropanol, washed with 70% ethanol, and resuspended in 50 µl sterile water.

Similarly, genomic DNA was extracted from individual egg masses using the same PUREGENE procedure and resuspended in 10 µl sterile water. Genomic DNA was extracted from specimens collected from Brazil using 300 µl of egg masses following a method described by Randig *et al.* (2002), and resuspended in 25 µl sterile water. DNA extracted in Brazil was transported in a cooled package to Florida, USA. Finally, all DNA preparations were stored at -20°C.

Primers

A forward primer (C2F3, 5'-GGTCAATGTTCAGAAATTTGTGG-3') and a reverse primer (1108, 5'-TACCTTTGACCAATCACGCT-3'), designed by Powers and Harris (1993) to amplify mitochondrial DNA sequences, were synthesized at GenoMechanix LLC (Gainesville, FL). Additionally, a forward primer (gfp-F, 5'-GTGTGGAATTGTGAGCGGATAAC-3') and a reverse primer (gfp-R, 5'-GAGCTGCATGTGTCAGAGGTTTT-3') were designed to amplify a 980 bp DNA segment containing the green fluorescent protein (GFP) sequences carried by plasmid pGFP DNA (3.3 kb) (Clonetech, Inc., Palo Alto, CA).

Standard PCR

Standard PCR was performed in a 25 µl volume containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM dATP, dGTP, dTTP, dCTP, 200 pM primers, 1 µl of DNA preparation, and 0.8 unit *Taq* DNA

polymerase (Roche Applied Science, Indianapolis, IN) (Saiki, 1989). Standard PCR was performed for 35 cycles with each cycle consisting of initial denaturation at 94°C for 30 s, annealing for 30 s (temperature varied depending on the primers, 48°C for mitochondrial primers or 63°C for GFP primers), and extension at 72°C (time varied depending on the gene sequences amplified, 1 min for GFP or 2 min for mitochondrial DNA sequences).

High-fidelity PCR

High-fidelity PCR (also known as Long PCR) was performed in a 50 µl volume containing 50 mM Tris (pH 9.2), 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 µM dNTPs, 400 pM primers, 1 µl of DNA preparation, 0.2 unit of *Tgo* DNA polymerase and 5 units of *Taq* DNA polymerase (Roche Applied Science, Indianapolis, IN) (Barnes, 1994). Amplification was carried out using a linked profile consisting of three different cycles (i) one cycle of initial denaturation at 94°C for 2 min, (ii) 10 cycles of denaturation at 94°C for 10 s, annealing temperature varied depending on the primers (see above) for 30 s, and extension at 68°C for 1 min or 2 min, and (iii) 25 cycles of denaturation at 94°C for 10 s, annealing temperature varied depending on the primers (see above) for 30 s, and extension at 68°C for 1 min or 2 min plus 20 s was added for every consecutive cycle. The extension time varied depending on the primers (see above).

Recombinant DNA Techniques

The high-fidelity PCR products were cloned into plasmid pCR2.1-TOPO using the reagents provided by the manufacturer (Invitrogen, Carlsbad, CA). DNA sequencing was performed at the University of Florida Interdisciplinary Core Facility for Biotechnology Research using a Perkin-Elmer Applied Biosystem ABI Prism Automated DNA Sequencer (The Perkin-Elmer Corporation, Norwalk, CT). To differentiate between specimens of *M. floridensis* and *M. arenaria*, amplified high-fidelity PCR products from both species were purified using a QIAquick PCR purification column (QIAGEN, Valentia, CA), and then restriction digested with *Ssp* I (Roche Applied Science, Indianapolis, IN). Digestion was carried out using a mixture of 10 µl high-fidelity PCR products, 1 unit of the restriction enzyme and 1 µl of the 10× recommended buffer for 4 hours at 37°C. Electrophoresis was performed using 2% TBE-agarose gel.

Sequence Analyses

DNA sequences were aligned using CLUSTAL W and manually adjusted in MacClade 4.02 (Sinauer Associates, Sunderland, MA). Sequence divergences were calculated using PAUP 4.0b10. Mfold was used to analyze the mitochondrial AT-rich region sequences for detecting a stem and loop (or hairpin) structure using default parameters (Zuker, 2003). Mfold is available on the World Wide Web (http://www.bioinfo.rpi.edu/applications/mfold).

RESULTS AND DISCUSSION

Sensitivity of Standard and High-fidelity PCR Procedures

Initially, the standard PCR procedure was tried for amplifying *Meloidogyne* mitochondrial DNA sequences using the primers C2F3 and 1108 designed from the highly conserved *COII* and 16S rRNA sequences, respectively (Powers and Harris, 1993). However, the amplification failed consistently, possibly because *Taq* DNA polymerase makes errors during amplification and lacks 3' to 5' exonuclease activity to remove such errors (Barnes, 1994).

Hence, a high-fidelity PCR procedure developed by Barnes (1994), which possesses the required 3' to 5' exonuclease activity, was tried for amplifying sequences from root-knot nematodes.

In order to compare directly the efficiency of standard and high-fidelity PCR procedures, a sensitivity analysis was performed by mixing 10 ng of *M. arenaria* PUREGENE genomic DNA with a known amount of serially diluted plasmid pGFP DNA (ranging from 10 ng to 1 fg), and then amplifying a 980 bp region containing the green fluorescent protein sequences carried by the plasmid using the primers gfp-F and gfp-R. Standard PCR amplified only from the four highest plasmid DNA dilutions tested (ranging from 10 ng to 10 pg) and the amplified 980 bp DNA band was found to be faint (Fig. 1A). In contrast, high-fidelity PCR amplified the 980 bp DNA band successfully from all plasmid DNA dilutions tested (ranging from 10 ng to 1 fg) and the band produced was bright (Fig. 1B). High-fidelity PCR appears to be at least 4 orders of magnitude more efficient than standard PCR, which is significant and could improve amplification dramatically.

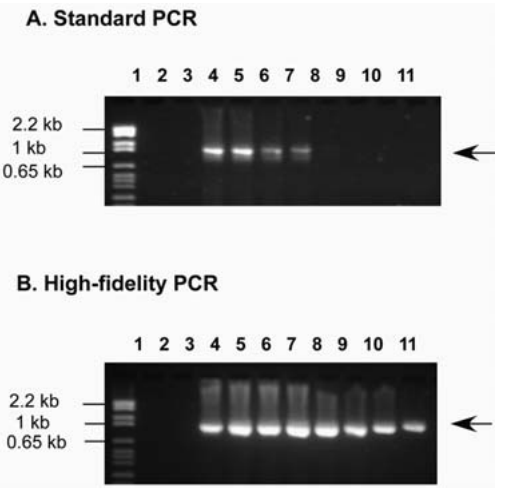

Fig. 1. Sensitivity analysis of standard and high-fidelity PCR procedures performed by adding a serially diluted plasmid pGFP DNA to *M. arenaria* genomic DNA and then amplifying a 980 bp segment from the plasmid using GFP primers. (A) Standard PCR and (B) High-fidelity PCR: lane (1) DNA marker VI (Roche Applied Science, Indianapolis, IN); lane (2) no DNA-control; lane (3) 10 ng *M. arenaria* DNA-control; lanes (4) to (11) 10 ng *M. arenaria* DNA + 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg pGFP DNA, respectively. Arrow indicates the amplified DNA band.

High-fidelity PCR Amplifies Mitochondrial AT-Rich Sequences

Initially, one isolate was selected from each of five different root-knot nematode species; *M. mayaguensis* from guava (Petrolina, PE, Brazil), *M. arenaria* from *Impatiens* sp., (Osceola County), *M. floridensis* from tomato (Indian River County, Florida, USA), *M. incognita* from coffee (Londrina, PR, Brazil), and *M. javanica* from soybean (Londrina, PR, Brazil). Genomic DNA was extracted from pooled specimens of these isolates and amplified using the mitochondrial primers C2F3 and 1108 and high-fidelity PCR procedure. A single amplicon was obtained from each species screened: 0.7 kb from *M. mayaguensis*, 1.1 kb from *M. arenaria* and *M. floridensis*, 1.5 kb from *M. incognita*, and 1.6 kb from *M. javanica* (Fig. 2). The size of DNA band obtained from *M. mayaguensis* (0.7 kb) agrees with the size reported previously from this nematode (Blok *et al.*, 2002; Brito *et al.*, 2004).

High-fidelity PCR utilizes both *Taq* and a proof-reading DNA polymerase (*Pfu*, *Pwo* or *Tgo*), which exhibits 3' to 5' exonuclease activity and removes the errors generated by *Taq* during amplification (Barnes, 1994). Previously, this procedure has been shown to work very well for amplification of mitochondrial DNA sequences from mites, which are also small in size (<0.5 mm) and known to yield trace amounts of genomic DNA (<10 ng per

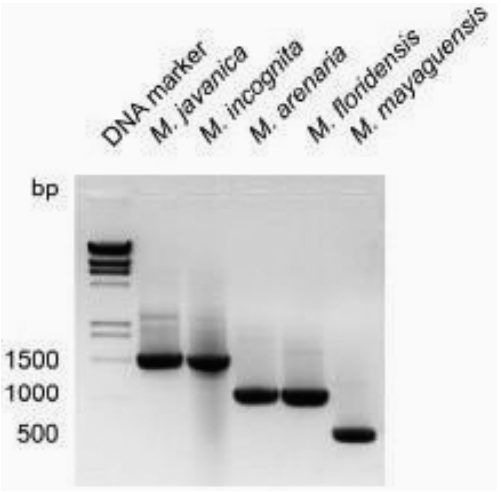

Fig. 2. Mitochondrial DNA amplified from *Meloidogyne* spp. The DNA marker used was Lambda DNA/*Hin*d III Plus Marker (Continental Lab Products, San Diego, CA).

specimen) (Jeyaprakash and Hoy, 2002). Even trace amounts of microbial DNA (<1 pg) present mixed in with insect genomic DNA was amplified by high-fidelity PCR (Jeyaprakash and Hoy, 2000). Hence, this procedure is ideal for amplifying DNA sequences from nematodes.

Sequence Analyses

The high-fidelity PCR products obtained were cloned and at least two or more independent clones were completely sequenced from each species. The sequences obtained varied in length as follows: 705 bp from *M. mayaguensis* (GenBank accession no. AY635613), 1,112 bp from *M. arenaria* (AY635610), 1,141 bp from *M. floridensis* (DQ228697), 1,502 bp from *M. incognita* (AY635611), and 1,640 bp from *M. javanica* (AY635612). *Meloidogyne incognita* had been previously reported as having populations producing products of two sizes, ca. 1,500 and 1,700 bp (Blok *et al.*, 2002; Powers and Harris, 1993), but only a 1,502 bp product was cloned from the *M. incognita* isolate that we analyzed. Similarly, *M. javanica* was reported to produce a product of 1,700 bp (Blok *et al.*, 2002; Powers and Harris, 1993), but a smaller product of 1,640 bp was cloned from the *M. javanica* isolate we analyzed.

Sequences obtained from *M. mayaguensis*, *M. floridensis*, *M. arenaria*, *M. incognita*, and *M. javanica* (AY635610 to AY635613 and DQ228687) were aligned using CLUSTAL W and a total of 1,644 aligned characters were obtained (data not shown).

The partial *COII* (102 characters) and 16S rRNA sequences (387 characters), and the complete tRNA-His sequences (53 characters) were found to be highly conserved in *M. arenaria*, *M. floridensis*, *M. incognita*, and *M. javanica*, with sequence divergences ranging from 0% to 2% (Table 1). However, *M. mayaguensis* displayed high sequence divergences ranging from 7.8% to 13.2%, when compared to the other four species.

In contrast, AT-rich region sequences, located between the *COII* and tRNA-His genes, show greater length variations amongst all root-knot isolates screened: *M. mayaguensis* (167 bp), *M. arenaria* (573 bp), *M. floridensis* (603 bp), *M. incognita* (963 bp), and *M. javanica* (1,100 bp). Previously, it has been shown that the AT-rich region was completely absent from this locus in *M. hapla* isolates (Hugall *et al.*, 1997). Since the *M. mayaguensis* AT-rich region was short in length (167 bp), it is possible that this region could exist in a different location of the mitochondrial genome in both *M. mayaguensis* and *M. hapla*. Currently, no *Meloidogyne* mitochondrial genome has been completely sequenced and it is not possible to predict the location of AT-rich region for *M. mayaguensis* and *M. hapla* at the present time. Hence, *M. mayaguensis* AT-rich sequences obtained were excluded from further analyses.

Table 1. Pair-wise sequence divergences between *Meloidogyne javanica* (Mj), *M. incognita* (Mi), *M. floridensis* (Mf), *M. arenaria* (Ma), and *M. mayaguensis* (Mm) for the 3' portion of *COII,* AT-rich region, tRNA-His and 5' portion of 16S rRNA sequences of mitochondrial DNA obtained using PAUP 4.0b10 with the criteria set for Distance and Uncorrected "P" parameters. Distance measured used a scale of 0 to 1.

Mitochondrial DNA	Species	Mj	Mi	Mf	Ma	Mm
COII 3' partial	Mj	—				
	Mi	0.010	—			
	Mf	0.020	0.010	—		
	Ma	0.000	0.010	0.020	—	
	Mm	0.088	0.078	0.088	0.088	—
AT-rich region	Mj	—				
	Mi	0.015	—			
	Mf	0.008	0.015	—		
	Ma	0.007	0.012	0.007	—	
	Mm	0.158	0.142	0.158	0.143	—
tRNA-His	Mj	—				
	Mi	0.000	—			
	Mf	0.000	0.000	—		
	Ma	0.000	0.000	0.000	—	
	Mm	0.132	0.132	0.132	0.132	—
16S rRNA 5' partial	Mj	—				
	Mi	0.003	—			
	Mf	0.003	0.000	—		
	Ma	0.000	0.003	0.003	—	
	Mm	0.120	0.123	0.123	0.120	—
All	Mj	—				
	Mi	0.011	—			
	Mf	0.007	0.008	—		
	Ma	0.003	0.008	0.006	—	
	Mm	0.125	0.121	0.120	0.118	—

The A and T content of AT-rich region was high, as expected, in all four remaining root-knot nematode species; *M. javanica* (82.4%), *M. incognita* (82.4%), *M. floridensis* (85.2%), and *M. arenaria* (85.9%).

Previously, the AT-rich region sequences from the fruitfly, *Drosophila yakuba* had been shown to contain a characteristic stem and loop structure (Clary and Wolstenholme, 1985). Similar stem and loop structures also were reported from AT-rich region sequences of several other insects (Zhang *et al.*, 1995), and D-loop region sequences of mammals including mouse and human (Clayton, 1982; Saccone *et al.*, 1987). Mitochondrial DNA replication was

shown to initiate within or close to this stem and loop structure (Zannis-Hadjopoulos *et al.*, 1988). In order to detect this stem and loop structure, *M. floridensis*, *M. arenaria*, *M. incognita*, and *M. javanica* AT-rich region sequences were aligned using CLUSTAL W and a total of 1,102 aligned characters were obtained (Fig. 3). Four blocks of sequences: block A (47 characters containing 44 conserved nucleotides), B (118 characters containing 117 conserved nucleotides), C (108 characters containing 104 conserved nucleotides), and D (149 characters containing 144 conserved nucleotides), were found to be highly conserved between these root-knot nematode species in the 5' segment of these sequences (Fig. 3). All four blocks of conserved sequences from all these root-knot nematode species were individually subjected to analysis using Mfold. This program employs the Zuker-Stiegler algorithm, which calculates energy for the growing fold and finally, predicts a stem and loop structure requiring the lowest energy (Zuker, 2003). The block C sequences from these *Meloidogyne* species were found to contain such a stem and loop structure. The loop has a run of nine Ts in all four species including *M. floridensis* (Figs. 3 and 4), but differences were detected in the three nucleotide positions of the stem sequences immediately following the loop sequences in all *Meloidogyne* species analyzed (Fig. 3). The reason for this enhanced mutation rate in the stem sequences is unknown.

The mitochondrial AT-rich sequences have been previously reported from two other nematodes; *Caenorhabditis elegans* (GenBank accession no. X54252) and *Ascaris suum* (X54253). The *C. elegans* AT-rich region (467 bp) was located between the tRNA-Ala and tRNA-Pro genes, whereas the *A. suum* AT-rich region (887 bp) was located between the tRNA-Ser and tRNA-Asn genes (Okimoto *et al.*, 1992). A CLUSTAL W alignment was made using the *C. elegans*, *A. suum* and *M. floridensis* AT-rich sequences (data not shown here). Mfold was used to analyze a 148 bp segment of *C. elegans* and a 133 bp segment of *A. suum* AT-rich sequences that aligned with the conserved 108 bp segment of *M. floridensis* block C sequences. A stem and loop structure was detected by Mfold from both these species in this region (Fig. 4). Sequences of *C. elegans* and *A. suum* AT-rich sequences outside this region were also analyzed using Mfold, but no clear stem and loop structure could be detected. Despite the fact that these three nematode species are widely divergent, the stem and loop structure is conserved and located in the same general segment of the AT-rich sequences. The loop sequences were found to be short in both *C. elegans* and *A. suum*: two Ts and one A detected in the *C. elegans* loop, whereas three Ts and one G detected in the *A. suum* loop (Fig. 4). The stem sequences were found to contain mismatches, but more were detected in *M. floridensis* than in *C. elegans* and *A. suum* (Fig. 4). The reason for this increased mutation rate in *Meloidogyne* spp. is unknown. Further, the reason for numerous nucleotide differences detected outside this important stem and loop structure in *Meloidogyne* spp., which has resulted in extensive length variations, is also unknown.

High-fidelity PCR-RFLP Analysis

Given that the mitochondrial AT-rich region displays numerous nucleotide differences in all *Meloidogyne* species investigated, it is possible to exploit this information and design a molecular method to differentiate the high-fidelity PCR products of *M. floridensis* and *M. arenaria* (1,141 and 1,112 bp in length, respectively). The restriction map generated using these two sequences for the enzyme *Ssp* I revealed different sized DNA

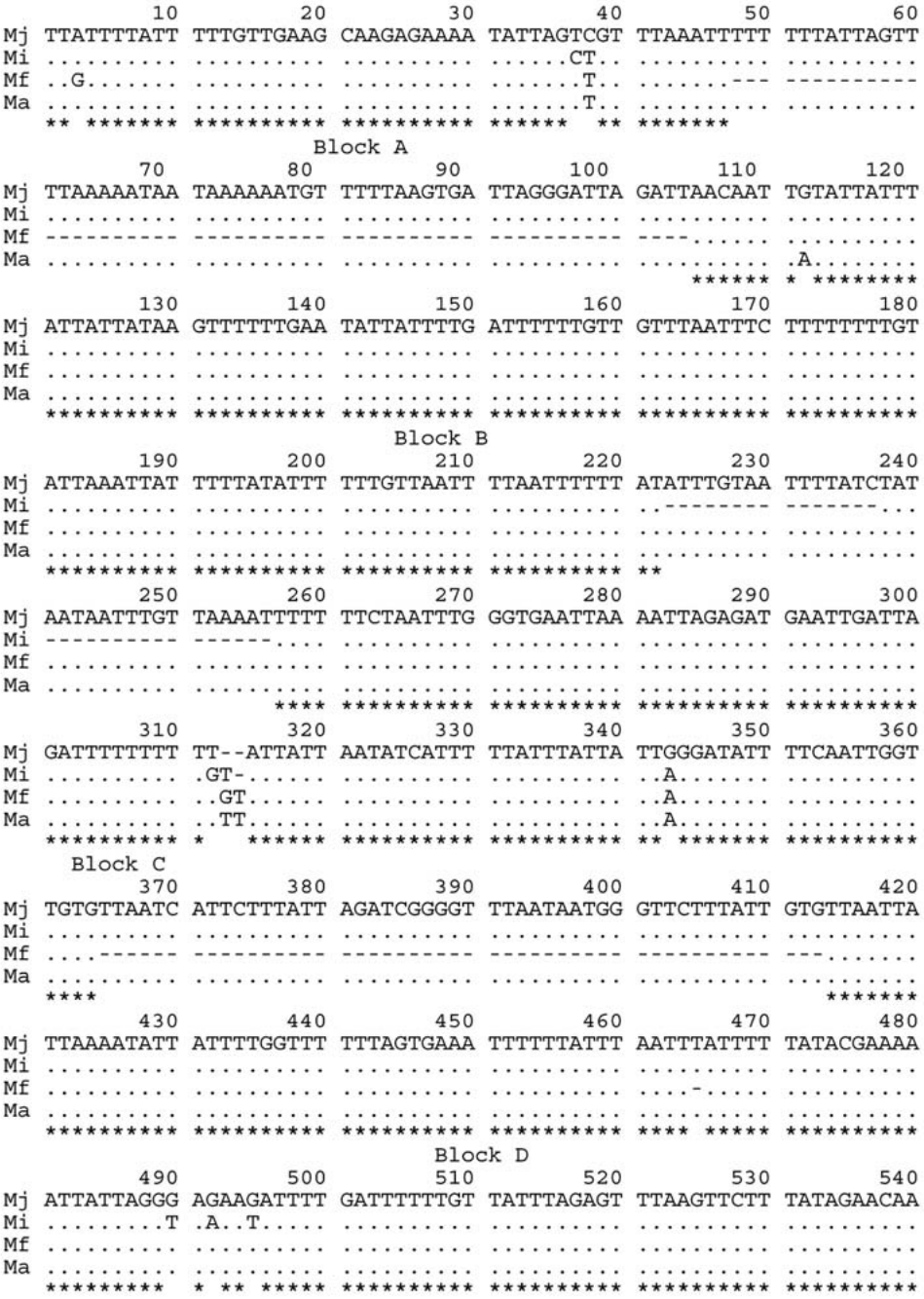

Fig. 3. Mitochondrial DNA AT-rich region sequences from four species of *Meloidogyne* were aligned using CLUSTAL W. A dot denotes a matching nucleotide and a hyphen denotes a deletion. Numbers above each line of sequence represent nucleotide position number. Conserved sequences between the species are underscored by an asterisk and they occur in four different segments shown here as block A, B, C, and D.

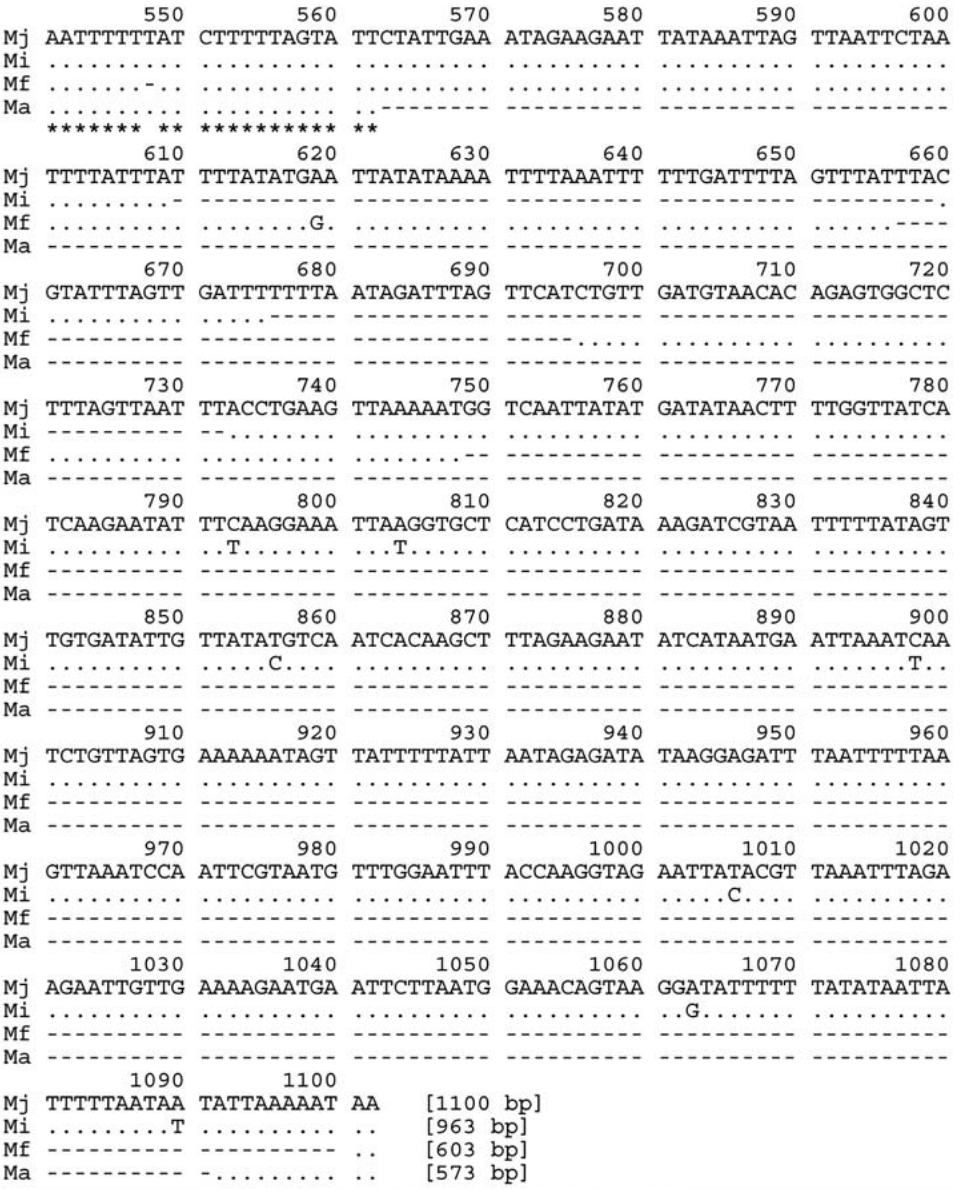

Fig. 3. (Continued) Mitochondrial DNA AT-rich region sequences from four species of *Meloidogyne* were aligned using CLUSTAL W. A dot denotes a matching nucleotide and a hyphen denotes a deletion. Numbers above each line of sequence represent nucleotide position number. Conserved sequences between the species are underscored by an asterisk and they occur in four different segments shown here as block A, B, C, and D.

fragments: 53, 97, 131, 211, 237 and 412 bp DNA fragments for *M. floridensis*, and 97, 110, 131, 212, 276, and 286 bp DNA fragments for *M. arenaria*. DNA preparations from pooled specimens of four separate isolates of *M. floridensis* and six separate isolates

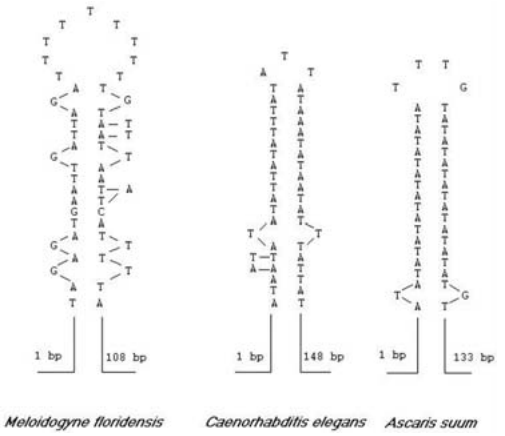

Fig. 4. Stem and loop structure generated from three species of nematodes using their AT-rich region sequences in Mfold are shown.

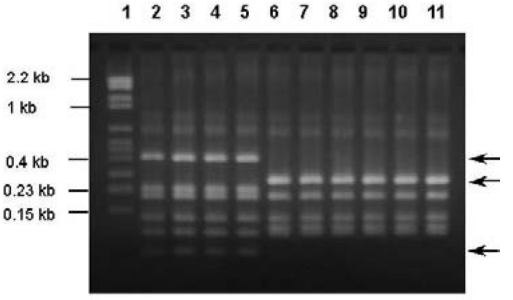

Fig. 5. Amplified products from mitochondrial DNA digested with the restriction enzyme *Ssp* I and separated on a 2% TBE-agarose gel: lane (1) DNA Molecular Weight Marker VI (Roche Applied Science, Indianapolis, IN); lanes (2) to (5) *M. floridensis* collected from tomato (Collier County), peach (Alachua County), tomato (Indian River County) and cucumber (Hendry County); lane (6) *M. arenaria* collected from *Impatiens* sp., (Osceola County, Florida, USA); lanes (7) to (11) *M. arenaria* collected from pumpkin, soybean (Brasil, Brazil) and banana (Guiana, Guadalupe and Martinica, Brazil). Arrows indicate the species-specific diagnostic DNA band.

of *M. arenaria* were amplified using high-fidelity PCR and an expected 1.1 kb DNA band was obtained from each isolate. The fragments predicted by the sequences were confirmed by digesting the high-fidelity PCR products from all these isolates with *Ssp* I (Fig. 5). *Meloidogyne floridensis* isolates can be distinguished clearly due to the band produced by 412 bp DNA fragment. Similarly, *M. arenaria* isolates can be distinguished clearly due to the doublet band produced by 276 and 286 bp DNA fragments. The unique 53 bp DNA band in *M. floridensis* was too faint to be used for diagnosis.

Finally, in order to evaluate the efficiency of this high-fidelity PCR procedure, DNA preparations from individual egg masses of *M. floridensis* and *M. arenaria* were amplified and an expected 1.1 kb DNA band was successfully obtained from each isolate screened (data not shown here), indicating that this procedure is efficient and could work even when trace amounts of genomic DNA (<10 ng) is available.

Hence, it is now possible to distinguish *M. floridensis* from *M. arenaria*, *M. mayaguensis*, *M. incognita*, and *M. javanica* by the size of the high-fidelity PCR products and the RFLP banding patterns generated using *Ssp* I. Finally, high-fidelity PCR is recommended for amplifying sequences from nematodes instead of standard PCR.

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