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Comparison of Sequences from the Ribosomal DNA Intergenic Region of *Meloidogyne mayaguensis* and Other Major Tropical Root-knot Nematodes

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Abstract: The unusual arrangement of the 5S ribosomal gene within the intergenic sequence (IGS) of the ribosomal cistron, previously reported for *Meloidogyne arenaria,* was also found in the ribosomal DNA of two other economically important species of tropical root-knot nematodes, *M. incognita* and *M. javanica.* This arrangement also was found in *M. hapla,* which is important in temperate regions, and *M. mayaguensis,* a virulent species of concern in West Africa. Amplification of the region between the 5S and 18S genes by PCR yielded products of three different sizes such that *M. mayaguensis* could be readily differentiated from the other species in this study. This product can be amplified from single juveniles, females, or egg masses. The sequences obtained in this region for one line of each of *M. incognita, M. arenaria,* and *M. javanica* were very similar, reflecting the close relationships of these lineages. The *M. mayaguensis* sequence for this region had a number of small deletions and insertions of various sizes, including possible sequence duplications.

Key words: Meloidogyne arenaria, Meloidogyne incognita, Meloidogyne javanica, Meloidogyne mayaguensis, PCR, rDNA, root-knot nematode.

Different regions of the ribosomal cistron have been used for phylogenetic studies of relationships in the plant, animal, and bacterial kingdoms. Several such studies of plant-parasitic nematodes have been based on the internal transcribed spacer (ITS) region in the ribosomal repeats. Differences in the ribosomal cistron have been used to separate species and races of *Aphelenchoides* (Ibrahim et al., 1994) *Ditylenchus* spp. (Ibrahim et al, 1994; Wendt et al., 1993) and various *Heterodera* and *Globodera* spp. (Ferris et al., 1993, 1994, 1995). Considerable intraspecies variation also has been observed in the *Xiphinema americanum* group by examining restriction fragment length polymorphism (RFLP) in the polymerase chain reaction (PCR) products from the rDNA (Vrain et al., 1992; Vrain, 1993). In contrast, Xue et al. (1993) reported that no inter- or intraspecies variation in the ITS1 and ITS2 regions was observed among *Meloidogyne incognita* (Kofoid and White) Chitwood, *M. arenaria* (Neal) Chitwood, *M. javanica* (Treub) Chitwood, and *M. hapla* Chitwood, following digestion with restriction enzymes or when sequences adjacent to the 28S gene were compared. However, Zijlstra et al. (1995) have subsequently distinguished isolates of *M. hapla* and *M. chitwoodi* from each other and from *M. incognita* and *M. javanica* using other restriction enzymes.

The intergenic spacer (IGS) region of the ribosomal repeats has been reported to be more variable than the ITS region, with little conservation between distantly related species (Gerbi, 1985). Vahidi et al. (1988, 1991) reported sequences homologous to 5S RNA in the IGS region of *M. arenaria,* an arrangement that had not previously been reported in other higher eukaryotes, though prokaryotes and certain "lower eukaryotes" (protozoa and fungi) have been found to have both the 5S and larger ribosomal genes within a single rDNA repeat. This unusual occurrence of the 5S gene sequence within the IGS region raised questions about the origin of this arrangement of ribosomal genes and the evolutionary relationship of *M. arenaria* to other nematodes. The IGS region in *M. arenaria* includes 129 bp repeats (Vahidi and Honda, 1991) that vary in number. Interpretation of variability of these repeat units is further complicated by the polyploid nature of the *Meloidogyne* genome and

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the complexity involved in examining the structure of tandemly repeated genes, possibly distributed over several chromosomes. In this study reported, the IGS regions between the 18S and 5S genes of the most economically important tropical root-knot nematodes (*M. incognita, M. javanica,* and *M. arenaria*) were compared with several populations of *M. mayaguensis* Rammah and Hirschmann, 1988, and one of *M. hapla* by means of PCR and sequence analysis.

Materials and Methods

Nematode populations: Each of the 27 lines used in this study were established from single egg masses (Fargette et al., 1996) and have been maintained for several years on susceptible tomato plants (cv. Moneymaker) in glasshouse cultures. Juveniles were collected as described by Blok et al. (1996). The lines used in this study are shown in Table 1 along with their countries of origin.

Table 1. Single egg mass lines of *Meloidogyne* spp., their codes, and country of origin.

Species	Line Number	Country
M. javanica	22	Burkina Faso
	23	Burkina Faso
	24	Spain
	25	Portugal
M. arenaria	10	Ivory Coast
	26	Portugal
	28	French West Indies
	29	French West Indies
	31	French West Indies
	32	French West Indies
	34	French West Indies
M. incognita	9	Ivory Coast
	11	USA (Race 3)
	12	USA (Race 4)
	15	Thailand (Race 1)
	16	Senegal
	17	Burkina Faso
	18	Chad
	19	French West Indies
	20b	Guyana
	27	USA
M. mayaguensis	3	Ivory Coast
	5	Ivory Coast
	7	Ivory Coast
	13	Puerto Rico
	30	Burkina Faso
M. hapla	33	The Netherlands

Four of these lines were used in the sequence analysis of *M. incognita* (line 19), *M. arenaria* (line 26), *M. javanica* (line 23), and *M. mayaguensis* (line 13).

DNA extraction: For population studies, DNA was extracted from 5-µl packed-cell volume of juveniles in a 1-ml glass homogenizer (Burkard Scientific, Ltd.) using the method of Pastrik et al. (1995) but with the addition of phenol:chloroform and chloroform extraction steps following the isopropanol precipitation stage. Suspensions of DNA (2 ng/µl) were stored at 4 °C and the concentrated suspensions at −20 °C. DNA was quantified by fluorometry. Single juveniles, egg masses, or females were placed in a 1-ml glass homogenizer in 10 µl H_2O and ground for 5 min. The extract was removed into a 0.5-ml microcentrifuge tube, the homogenizer washed with 5 µl H_2O, and the wash added to the extract.

PCR reactions: Primers 5S (5′-TTAACTTGCCAGATCGGACG-3′) and 18S (5′-TCTAATGAGCCGTACGC-3′) were designed to amplify the intergenic region between the 18S and 5S genes (Fig. 1) using sequence information from Vahidi et al. (1988). Amplification reactions using nematode DNA extracted from juveniles included 10 ng of DNA in 50 µl of 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl; 200 µM each of dATP, dCTP, dGTP, and dTTP; and 1 unit of Taq polymerase (Boehringer). Amplifications with 45 cycles of 94 °C for 30 sec, 53 °C for 30 sec, and 72 °C for 90 sec were done with a Perkin-Elmer 480 thermal cycler. The products from these PCR reactions were separated by electrophoresis in TRIS-Borate-EDTA (TBE) buffered 1% agarose gels (Sambrook et al., 1989) and the products visualized with UV illumination after ethidium bromide binding to the DNA fragments.

Cloning of PCR products: PCR products were purified from a piece of agarose excised from a 1% TBE buffered gel using the Wizard PCR Prep kit (Promega) and cloned into the vector pGEM-T (Promega). Two clones from each species line (*M. incognita* line 19 [in19a and b], *M. javanica* line 23

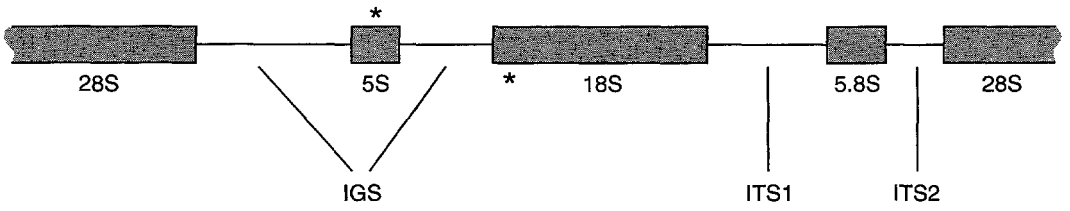

FIG. 1. Diagram of the ribosomal cistron as observed in *M. arenaria*. "*" indicates the position of the PCR primers used to amplify the intervening IGS between the 18S and 5S genes.

[ja23a and b], *M. arenaria* line 26 [ar26a and b], and *M. mayaguensis* line 13 [ma13a and b]) were sequenced.

DNA purification for sequencing: Double-stranded DNA was prepared with the Wizard Midipreps DNA Purification System (Promega).

Sequencing: Double-stranded DNA was sequenced by cycle sequencing (Applied Biosystems) both strands. Sequence was determined from all clones using M13 forward (5′-GTTTTCCCAGTCACGAC-3′) and M13 reverse (5′-AACAGCTATGACCATG-3′) primers. Primers melrib1 (5′-GCGGGAATGGTTTAAAGG-3′) and melrib2 (5′-AATTACCGAGACTGCTGG-3′) were used for internal sequencing of clones in 19a; in 19b, ja23a, ja23b, ar26a, and ar26b, primers marib1 (5′-GCGGAATGGTTTAAGGC-3′) and marib2 (5′-GCGGGTAATTAAGCTGTC-3′) were used for internal sequencing of ma13a and ma13b. Sequence information was assembled using the Staden (1982) program package, and further analysis done with the STADEN, UWGCG, and FASTA (Devereux et al., 1984) packages available through Daresbury Laboratory, Warrington, England WA4 4AD.

RESULTS

Three size classes of PCR product were produced from the IGS region between the 18S and 5S genes, as shown in Fig. 2. Amplification of products from the five species indicated that the 5S gene occurs in the IGS region of the ribosomal cistron of all these species, and this was confirmed by sequencing PCR products from each of these species. The products from the five lines of *M. mayaguensis* were larger, and those from the one *M. hapla* line were smaller, than those produced from the lines of the other three species (*M. incognita, M. javanica,* and *M. arenaria*). When PCR was done with disrupted single juveniles, single females, or single egg masses, products of identical sizes were produced (results not shown).

The sequences of the PCR products produced from one line each of *M. incognita, M. javanica, M. arenaria,* and *M. mayaguensis* are shown in Figure 3. The 715 bp of sequences determined for *M. incognita, M. javanica,* and *M. arenaria* were similar and unlike that of *M. mayaguensis,* which differs from them at 116 bases, with five deletions (three of single and two of two bases) and seven insertions (three of single nucleotide and one each of 5, 19, and 41 nucleotides). The two clones from *M. mayaguensis* were identical as were the two from *M. incognita.* The two *M. arenaria* clones differed from each other at base 180 (A/C) and a single nucleotide deletion at position 552 (G/–). The *M. javanica* clones also differed from each other at bp 156 (C/A), 444 (C/T), 605 (T/A), 706 (G/A), and by deletion of two nucleotides at bp 470–471 (TA/––). The two *M. incognita* sequences differed from the *M. javanica* and *M. arenaria* sequences by a deletion at base 115 and an A at base 116.

Small stretches of repeated sequences were found in the insertions in the *M. mayaguensis* sequence and in the sequence common to the other species, i.e., TTTTTTGAAGAAT (212–224 of *M. mayaguensis* and 270–282 of the other three species), TAAGCT (297–302 of *M. mayaguensis* sequence and from 261–266 in the other three sequences); within the largest insertion of the *M. mayaguensis* sequence, AGCTTTATT occurs twice (340–348 and 366–374).

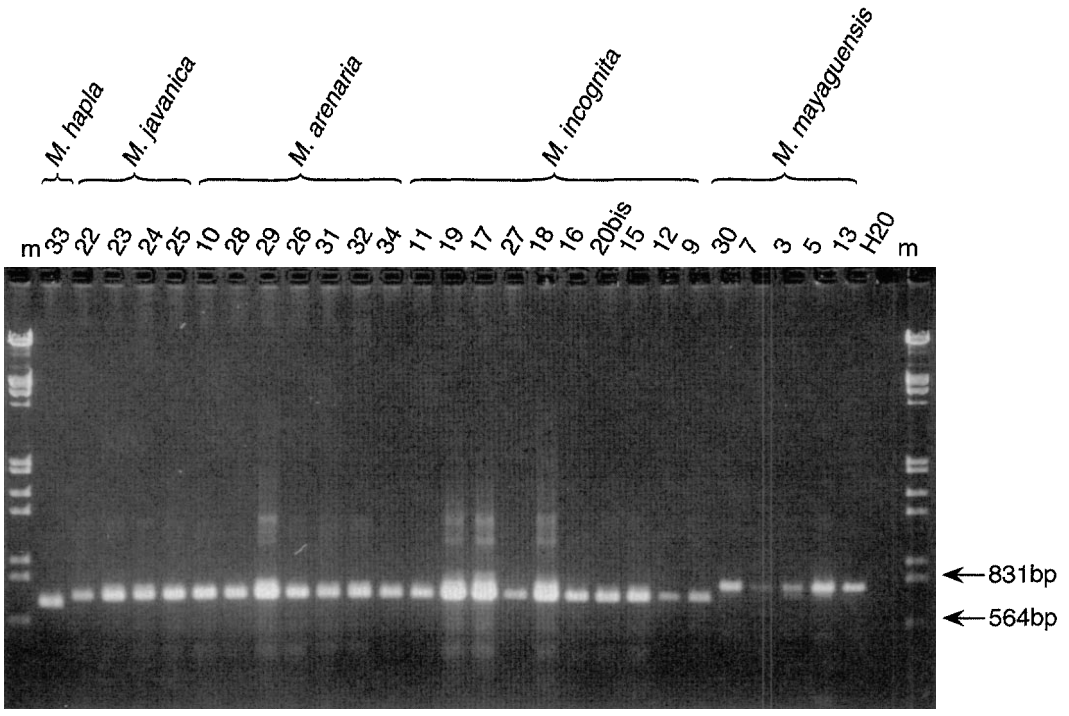

FIG. 2. PCR products using primers specific for the 18S and 5S genes that amplify the intervening IGS region. The marker (m) used was lambda DNA HindIII/EcoRI.

DISCUSSION

No obvious size polymorphisms were evident in the PCR products produced from the group of five *M. mayaguensis* lines or within the second, distinct-size group comprising *M. arenaria, M. javanica,* and *M. incognita* lines. The three size groups of products were readily differentiated on the 1% agarose gels; hence, this PCR approach provides a means of easily differentiating *M. mayaguensis* from the other three species of tropical root-knot nematode, as single juveniles, single females, or individual egg masses successfully generated PCR products. This approach could be used where the amount of material is limited and a quick differentiation of *M. mayaguensis* from the other tropical root-knot nematodes species is required. The PCR product produced from the *M. hapla* line was smaller than that produced from any of the other lines in this study, but because only one isolate of *M. hapla* was used, it is not possible to conclude whether this is typical of this species. Results of Zijlstra et al. (1995) have shown that within the ITS regions of *M. hapla* there are differences in the restriction patterns between individuals, indicating intraspecies variability. This intraspecific variation has not been observed with the three species of tropical root-knot nematodes, *M. incognita, M. javanica,* and *M. arenaria* (Xue et al., 1993; Zijlstra et al., 1995).

A PCR-based detection method is one of the preferred techniques for species identification. When the insert of ma13a was used as a probe in a dot blot of nematode DNA, detection sensitivity was insufficient to clearly distinguish *M. mayaguensis* from the other species despite the sequence differences between species in this region (Blok, unpublished results). Whether better sensitivity could be achieved with a short probe based on the unique insert region of the *M. mayaguensis* sequence was not explored as the PCR approach was simple to use and effective in distinguishing *M. mayaguensis* from the other species used in this study. The amount of nematode DNA used in the dot

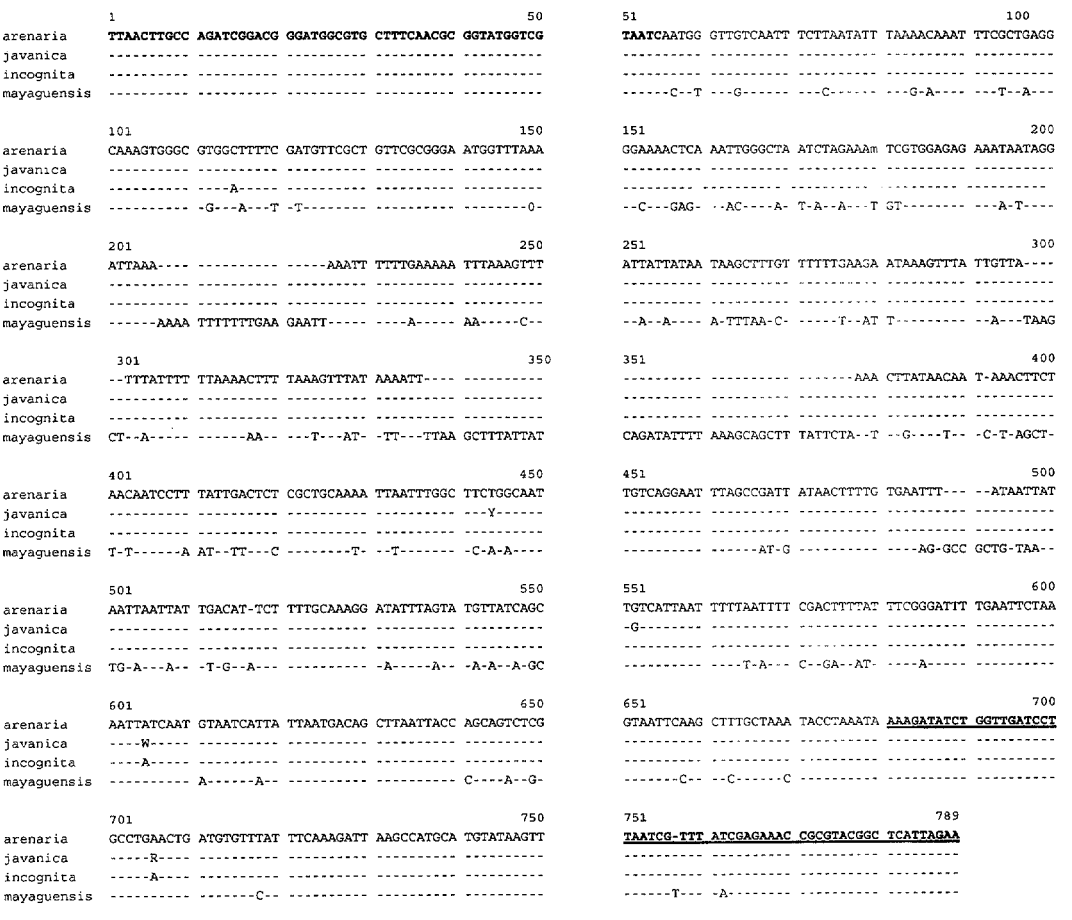

FIG. 3. Alignment of sequence obtained from the PCR products produced with the 18S and 5S primers from one line of each of *M. mayaguensis, M. arenaria, M. javanica,* and *M. incognita.* The symbol "-" indicates identity for sequence listed below *M. arenaria,* except for deletions in *M. mayaguensis* at positions 187, 462, 463, 572, 592, and 593. All deletions between *M. incognita, M. arenaria,* and *M. javanica* are indicated in the text. Sequence from the 5S gene is highlighted in bold, and that from the 18S sequence is in bold and underlined.

blot procedure (25 ng) was greater than that used for PCR (10 ng), and PCR can be performed with single individuals. Use of probes to detect lower quantities of nematode DNA has been reported by Chacón et al. (1993). They used a short probe to a highly repeated sequence that enabled them to detect 2 ng of *M. incognita* DNA and distinguish *M. incognita* from several other *Meloidogyne* spp. The ribosomal primers described in this study were routinely more reliable in producing an amplification product than primers that amplify a region of the mitochondrial genome (Powers and Harris, 1993) when using single juveniles, females, or egg masses (Blok, unpublished results).

The presence of the 5S gene in the IGS region of *M. arenaria* was reported (Vahidi and Honda, 1991; Vahidi et al., 1991), and this study has shown that this arrangement is common to the three tropical root-knot species as well as *M. mayaguensis* and *M. hapla.* To determine whether this arrangement of ribosomal genes is characteristic of all *Meloidogyne* spp. and other related species requires further study. Drouin and Desa (1995) examined various reports of the 5S rRNA being linked to tandemly repeated multigene families and suggested that this has occurred on a number of occasions during evolution through fortuitous recombination events. An examination of the occurrence of the 5S gene in the IGS region in the phylum Nematoda would be of interest for

understanding the molecular basis for evolutionary relationships.

Within the rDNA repeats from a wide variety of organisms, the IGS region is the least conserved. However, in this study little sequence variation was found in the region between the 18S and 5S genes for the group comprising *M. incognita, M. arenaria,* or *M. javanica,* despite the populations originating from widely dispersed geographic regions, including the West Indies, Portugal, and Burkino Faso, respectively. This raises questions as to the relationships of these lineages, their dispersal, and when they differentiated.

Assessing the importance of small differences in sequence between clones of a PCR product is difficult. Differences in sequence between clones from a single line either could reflect genuine sequence variants between repeats or could be artifacts arising through the amplification, cloning, and sequencing procedure. To establish whether variability between repeats truly occurs in a parthenogenetically reproducing line would require a large number of clones from a particular line to be sequenced. Given the low variation in sequence between clones from some lines, the small number of differences in sequence between species, and that sequence was only obtained from one line of each of species, ranking the relative similarities of the species with confidence is not possible, nor is distinguishing the species from each other based on the sequence from the IGS region. Regions of the mtDNA have proved more useful for species identification and examination of intraspecific relationships of tropical *Meloidogyne* (Harris et al., 1990; Hugall et al., 1994; Powers and Sandall, 1988), as have RFLP (Curran et al., 1986; Fargette et al., 1996; Gárate et al., 1991; Xue et al., 1992), RAPD (Blok et al., 1996; Cenis, 1993; Guirao et al., 1995), and other studies using similar techniques (Baum et al., 1994) that survey the nuclear genome.

Populations of *M. mayaguensis* have been identified in West Africa that are able to overcome resistance in cultivars as diverse as tomato cv. Rossol, sweet potato cv. CDH, and soybean cv. Forrest (Fargette and Braaksma, 1990; Prot, 1984). Consequently, *M. mayaguensis* poses a serious agricultural threat, and monitoring its spread and developing effective quarantine procedures have been suggested. For this, reliable differentiation of *M. mayaguensis* from the other tropical RKN will be necessary. PCR of the IGS region offers a simple method for identification of this species.

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