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Variation in ribosomal genes in *Meloidogyne arenaria*

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Summary – *Meloidogyne* spp. are serious pests of crops worldwide. Several laboratories have used rDNA to investigate their phylogenetic relationships and to develop diagnostic tests for these species, yet these genes are known to be unusually variable in *M. arenaria*. We have analyzed three rDNA clones from *M. arenaria*, two of which contain large deletions. Based on these and other available *M. arenaria* rDNA sequences, the sizes of the *M. arenaria* 18S and 28S rRNAs are estimated at 1788 and 3645 bases, respectively. Seven rearrangements were characterized. All affected the ITS or IGS regions, which are frequently used for phylogenetic studies and diagnosis. Two different sequences were obtained for the intergenic spacer region (IGS), both of which were shown by PCR amplification to be present in individual nematodes. © Orstom/Elsevier, Paris.

Résumé – ***Variabilité dans les gènes ribosomiques chez* Meloidogyne arenaria** – Les espèces du genre *Meloidogyne* sont de véritables fléaux pour les cultures du monde entier. Certains laboratoires de recherche ont utilisé des ADNr afin d'étudier les relations phylogéniques et développer des tests de diagnostic pour ces espèces, bien que ces gènes soient très variables spécialement chez *M. arenaria*. Nous avons analysé trois clones d'ADNr de *M. arenaria*, deux d'entre eux contenant de larges délétions. D'après nos résultats et les séquences d'ADNr de *M. arenaria* disponibles, les tailles des ARNr 18S et 28S de *M. arenaria* ont été estimées respectivement à 1788 et 3645 bases. Sept réarrangements ont été caractérisés. Tous affectent les régions ITS et IGS qui sont fréquement utilisées pour des études phylogéniques et pour des diagnostics. Deux séquences différentes ont été obtenues pour la région IGS ; toutes deux ont pu être amplifiées par PCR chez chaque individu testé. © Orstom/Elsevier, Paris

Keywords: *Meloidogyne arenaria*, ribosomal DNA, root-knot nematode.

The major root-knot nematode (RKN) species, *Meloidogyne incognita, M. javanica, M. arenaria,* and *M. hapla*, are geographically widespread and capable of infecting and damaging a wide range of plant hosts, making them economically important agricultural pests (Eisenback & Triantaphyllou, 1991). They are facultative or obligate parthenogens (Eisenback & Triantaphyllou, 1991), so a single individual can establish a population, a useful attribute in species that exploit disturbed ecosystems (agroecosystems, for example). Like many parthenogens, the major RKN species are usually polyploid (Triantaphyllou, 1985) and are conceivably of hybrid origin (Hyman & Powers, 1991).

In recent years, a number of laboratories have undertaken molecular studies of RKN ribosomal genes, both to elucidate their phylogenetic relationships and to develop simple methods for their identification (Xue *et al.*, 1993; Zijlstra *et al.*, 1995, 1997; Petersen & Vrain, 1996; Al-Banna *et al.*, 1997; Blok *et al.*, 1997; Petersen *et al.*, 1997; Zijlstra, 1997). These genes have been a popular target, because highly conserved sequences are interspersed with less conserved regions, enabling phylogenetic studies at various taxonomic levels and across a range of time scales (Hillis & Dixon, 1991). This interspersed pattern of conserved and less conserved sequences allows the design of primers that can be used to amplify homologous segments from rather distantly related organisms, and also obtain sequences that have a reasonable likelihood of varying even among more closely-related species. In addition, because the ribosomal genes are present in the genome in multiple copies that tend to evolve in concert (Dover, 1982), relatively little material is needed to analyze them (Hillis & Dixon, 1991).

Because of the interest in using ribosomal genes for inferring phylogenetic relationships and identifying species of RKN, it is a matter of some concern that our laboratory and others have found these genes to be variable both between (Carpenter *et al.*, 1993) and within (Hiatt *et al.*, 1995) populations of *M.arenaria*, with Vahidi *et al.* (1991) even reporting heterogeneities among adjacent (but apparently non-functional) rDNA repeats. They suggested that some of the observed size polymorphism could be due to variation in the number of copies of a 129 bp subrepeated element in the intergenic spacer (IGS), but they also found that some of the differences involved rRNA coding sequence, specifically the 3' end of the 28S rRNA sequence (Vahidi *et al.*, 1988, 1991). *M. arenaria* is the most variable morphologically, cytologically, and biochemically of the major RKN species (Esbenshade & Triantaphyllou, 1985); however,

variation in ribosomal DNA has also been reported in *M. incognita* and *M. javanica* (Georgi *et al.*, 1986) and *M. hapla* (Zijlstra *et al.*, 1995). In order to investigate further the nature of variability in the ribosomal DNA in *M. arenaria*, we undertook the sequence analysis of rearranged portions of two clones containing ribosomal RNA genes from this species.

Materials and methods

Strains and sequence analysis

Escherichia coli strain LE392 was used to propagate phage clones. Phage DNA was purified by standard methods (Sambrook *et al.*, 1989), using equilibrium centrifugation in CsCl. For sequencing, DNA fragments were subcloned into pUC119 (Viera & Messing, 1987) or pBluescriptSK(-) (Stratagene) plasmids and propagated in *E. coli* strain DH5α. Plasmid DNA was prepared using Qiagen-tip 20 columns or an alkaline lysis/PEG method recommended by ABI, and was sequenced using ABI's Taq DyeDeoxy terminator cycle sequencing kit and an ABI 373A DNA Sequencer. Nucleotide sequences were obtained from both DNA strands. In order to sequence the larger fragments, oligonucleotide primers were synthesized, preferentially utilizing sequences that were conserved, *i.e.*, identical or nearly identical to the corresponding sequence from *Caenorhabditis elegans*. Oligonucleotides were synthesized using phosphoramidite chemistry and a Milligen Cyclone Plus DNA Synthesizer. Following cleavage/deprotection with ammonium hydroxide and lyophilization, synthetic oligonucleotides were resuspended in water or TE (10 mM Tris, 1 mM Sodium EDTA, pH 8) and desalted using G25 Sephadex spun columns. For the convenience of others who may have a need for ribosomal gene primers, the sequences of primers used in this study are listed in Table 1, noting which are conserved between *M. arenaria* and *C. elegans*. Sequence compilation and comparisons were performed using IntelliGenetics programs GEL and FASTDB, respectively.

Plasmid pE1.6A is a pUC8 recombinant plasmid containing a 1.6 kb *M. arenaria* ribosomal DNA fragment identified as detecting restriction fragment length differences between (Carpenter *et al.*, 1992) and within (Hiatt *et al.*, 1995) *M. arenaria* race 2 populations. As described by Vahidi and Honda (1991), the IGS in this species is interrupted by 5S rRNA coding sequence. Our clone contains the 3' end of the 28S rRNA coding sequence, IGS I (including several copies of the 129-bp subrepeated element), 5S rRNA coding sequence, and part of IGS II. The 28S fragment and a copy of the 129-bp subrepeat were subcloned and designated BA#1 and BA#2, respectively, in accordance with published guidelines for naming recombinant molecules containing nematode DNA (Bird & Riddle, 1994).

Genomic library construction and screening

RKN genomic DNA was prepared as described (Hiatt *et al.*, 1995). DNA from *M. arenaria* race 2, Govan population was partially digested with Sau3A I, phosphatased, and ligated into the BamH I sites in lambda EMBL3. The ligated DNA was packaged using Stratagene Gold, and approximately 20 000 primary recombinants were obtained. After library amplification, approximately 20 000 plaques were plated, and duplicate lifts prepared using Hybond N+ (Amersham), following the manufacturer's recommended procedure. Plasmid inserts were amplified from BA#1 and BA#2 using PCR and were labeled by random priming (Feinberg & Vogelstein, 1983) using 15 μCi α-^{32}PdCTP per reaction. Hybridizations were conducted at 65 °C overnight in 6× SSPE (1.08 M NaCl, 0.06 M sodium phosphate, and 6 mM EDTA pH 7,7), 10× Denhardt's, 1 % SDS, and 20 μg/ml boiled salmon sperm DNA (20). Filters were rinsed four times in 2× SSPE or 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate) at 65 °C for 20 min each and one rinse in 0.3× SSPE or 0.3× SSC at 65 °C for 20 min (Hiatt *et al.*, 1995). Plaques were purified that hybridized with BA#1 and BA#2, or with BA#2 but not BA#1.

PCR amplification from single *M. arenaria* females

Individual *M. arenaria* females were dissected from infested tomato roots and frozen at -80 °C in 0.5 ml microfuge tubes. The nematodes were lysed and subjected to PCR as described by Williams *et al.* (1992) for *C. elegans*, except that no gelatin was added either at the lysis step or for the subsequent PCR. Primers F1 and R6 (Table 1) were used at 25 pmol per 25 μl reaction to amplify part of the IGS II from individual worms and from approx. 0.7 ng of cloned BA#3 and BA#4 DNA; a reaction with no added template was included as a control. Thirty cycles of amplification (95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s), followed by a final extension at 72 °C for 7 min, were performed in a Perkin-Elmer DNA thermal cycler 480. PCR reactions were extracted with phenol and chloroform and ethanol precipitated prior to restriction digestion and agarose gel electrophoresis.

Results

Isolation and sequence analysis of rDNA clones

Of approximately 20 000 plaques plated from a *M. arenaria* genomic library, over 400 were found to hybridize with BA#2 (a 129-bp repeat from the IGS region). Of these, all but six also hybridized with

Table 1. *Primers used in sequence and PCR analysis of* Meloidogyne arenaria *rDNA, listed in order of their appearance in the gene, beginning with IGS I.*

Name	Sequence	Orientation *	
R5	TCCAGGCTAAGTTGACT	forward	
5SINV-2	GTTGCTTAACTTGCCAG	forward	conserved**
F2	GACCATACCGCGT	reverse	
R6	ACGCGGTATGGTCGTAATC	forward	
F1	GCAGCGAGAGTCAATAA	reverse	
18S#4	CCGCGAACGGCTCATTA	forward	
18S1906-22	GATACCGCCCTAGTTCT	forward	conserved
18S2090-74	GAGTCAAATTAAGCCGC	reverse	conserved
18S2190-2206	GTGGAGTGATTTGTCTG	forward	conserved
18S2650-66	GTCGTAACAAGGTAGCT	forward	conserved
5.8SF	CTACCCTTATCGGTGGA	forward	
28S#3	TAGAGTCGGGTTGTTTG	forward	
28S#6	CAACTTTCCTTCACAGT	reverse	
28S4355-71	CCGACCCGTCTTGAAAC	forward	conserved
28S4371-55	GTTTCAAGACGGGTCGG	reverse	conserved
28S4584-600	GAAGCCAGAGGAAACTC	forward	conserved
28S4694-78	GGAACCAGCTACTAGAT	reverse	conserved
28S4870-86	TTGGTAAGCAGAACTGG	forward	conserved
28S5244-63	GGAGAAGGGTTCCACGTGAA	forward	conserved
28S5266-50	CTGTTCACGTGGAACCC	reverse	conserved
28S#5	TATTAACCCACTTCCCT	reverse	
28S5637	AGGTCTCCAAGGTGAAC	forward	conserved
28S5721R	CTTTTCCCGAAGTTACG	reverse	conserved
28S6151	CGAAACCACAGCCAAGG	forward	conserved
28S6181R	TGCCAAGCCCGTTCCCT	reverse	conserved
F5	GGATTCTGACTTAGAGGCG	reverse	

* With respect to the 18S, 5.8S and 28S rRNA sequence

** Sequence is identical to corresponding sequence in *Caenorhabditis elegans*; with the exception of primer 5SINV-2, numbering of "conserved" primers refers to the *C. elegans* sequence, GenBank Accession No. X03680.

BA#1 (a fragment from the 3' end of the *M. arenaria* 28S coding sequence); two of the six (BA#3, Fig. 1C and BA#4, Fig. 1B) were purified to homogeneity. A third clone (BA#5, Fig. 1A) that hybridized with both probes was isolated. BA#5 contained an insert of approximately 12 kb comprising one rDNA unit and part of a second, with no obvious deletions. BA#4 contained an insert of approximately 13.5 kb comprising two rDNA units and a part of a third (all three missing approximately 2.6 kb from the 3' end of the 28S coding sequence). BA#3 contained an insert of approximately 14.2 kb comprising six partial rDNA units, with a variety of deletions, including a deletion of approximately 2.0 kb from the 3' end of both copies of the 28S coding sequence. Although every part of the 18S sequence appears to be represented in BA#3, the clone contains no single intact copy of the sequence. By piecing together sequences obtained from these three clones and pE1.6A (Hiatt, 1991), plus GenBank accession number X57223 (Vahidi & Honda, 1991), preliminary estimates were obtained for the length of the *M. arenaria* 18S rRNA (1788 bases) and 28S rRNA (3645 bases).

In both deleted clones (BA#3 and BA#4), the truncated end of the 28S sequences is followed within a few bases by 129-bp subrepeat sequence (Fig. 2, *cuDf2, cuDf1*). The 129-bp subrepeat sequence differs by one base between BA#3 and BA#4: at residue 23 (counting from a Sau3A I site that cuts the 129-bp repeat into unit lengths) the former has a G whereas the latter has an A. This difference was found consistently in all copies of the subrepeat we sequenced. While both ends of the break differ between the two clones, within a clone all copies of the 28S gene share

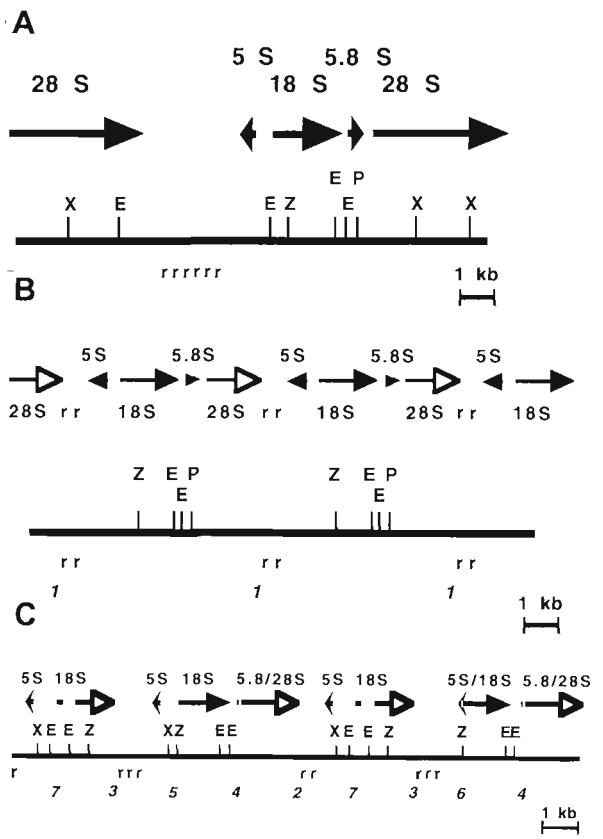

Fig. 1. Maps of rDNA clones from Meloidogyne arenaria. *A: BA#5; B: BA#4; C: BA#3. Numbers in italics (1, 2, 3, 4, 5, 6, 7) refer to deletions* cuDf1, cuDf2, cuDf3, cuDf4, cuDf5, cuDf6 *and* cuDf7, *respectively (see also Fig. 2). Abbreviations: E:* EcoR I*; P:* Pst I*; X:* Xba I*; Z:* Xho I*; rr: subrepeat(s).* EcoR I *fragments of BA#5; Hind III fragments of BA#4; and Hind III fragments;* EcoR I *fragments; and* Xba I *fragments of BA#3 were subcloned and sequenced in whole or in part.*

the same rearrangement. In BA#4, the bases between the truncated 28S sequence and regular subrepeat sequence are a duplication, in the same orientation, of nearby subrepeat sequence (underlined in Fig. 2, *cuDf1*). The origin of the bases in BA#3 (*cuDf2*) between the end of recognizable 28S sequence and 129-bp subrepeat sequence is less obvious. We have sequenced a variant copy of the subrepeat (not shown) from another region of BA#3 (downstream of *cuDf3,* Fig. 1C) that also contains the sequence TCCCCCATTAAA, apparently due to the loss of 51 bp between the run of C's and the first A. Assuming that this variant subrepeat flanks *cuDf2*, this still leaves a number of bases to be accounted for between the 28S and subrepeat sequences (either four [CACG] or nine [CAGTTCACG], depending on whether CAGTT is considered to derive from 28S sequence). FASTDB found no perfect match with the nine base fragment in either strand in the available ribosomal sequences, however, the sequence matched the complement of the adjacent truncated 28S sequence at eight of nine positions (Fig. 2). This source for the inserted sequence is preferred purely on the basis of propinquity over two other, more distant sites that also match eight out of nine bases, both in the complementary strand: one is near the 5' end of 18S sequence, the other near the 3' end of 28S sequence.

One additional rearrangement, present twice in BA#3, involved 129-bp subrepeat sequence (Fig. 2, *cuDf3*). In this rearrangement, the 5' end of the break lies in 18S sequence, the duplicated intervening bases come from nearby subrepeat sequence but in the opposite orientation, and, strikingly, the 3' end of the break is at the same position in the 129-bp subrepeat as in the 28S deletion in BA#4.

None of the remaining rearrangements in BA#3 directly involves 129-bp subrepeat sequence. One (Fig. 2, *cuDf4*), present twice in the clone, deleted most of the 5.8S sequence (including a unique Pst I site), all of the ITS II, and 331 bp from the 5' end of the 28S coding sequence, juxtaposing sequences that are base-paired in stem 8 of the large ribosomal subunit (Ellis *et al.*, 1986).

Another rearrangement (Fig. 2, *cuDf5*), present in one rDNA unit in BA#3, deleted over 600 bp of IGS II and 18S coding sequence, breaking at points in the IGS II and 18S sequences where the two share a 5 bp stretch of sequence. An additional rearrangement (Fig. 2, *cuDf6*), also present once, deleted all but the 3' end of the 5S sequence and about 90 bp more from the 5' end of 18S coding sequence than the rearrangement just mentioned.

The final rearrangement (Fig. 2, *cuDf7*), present in two non-adjacent positions in the cloned DNA (Fig. 1C), is roughly the inverse of the two preceding rearrangements. It contains a splice between 18S coding sequence and IGS II, producing an rDNA unit that is less than 0.6 kb. To determine whether this rearrangement was present in the DNA from which the library was constructed, or was a cloning artifact, PCR using primers F1 and 18S#4 (see Table 1) was performed on 54 ng of genomic *M. arenaria* DNA extracted from a population of worms, on sixteen individual *M. arenaria* females, and on the BA#3 clone itself. These two primers should be separated by several kb in functional rDNA units, but were predicted to amplify a 204 bp fragment from BA#3. A fragment of the expected size was amplified from BA#3 and from bulk genomic DNA, but not from the individual females, which did, however, show some individual differences in the sizes of minor amplified

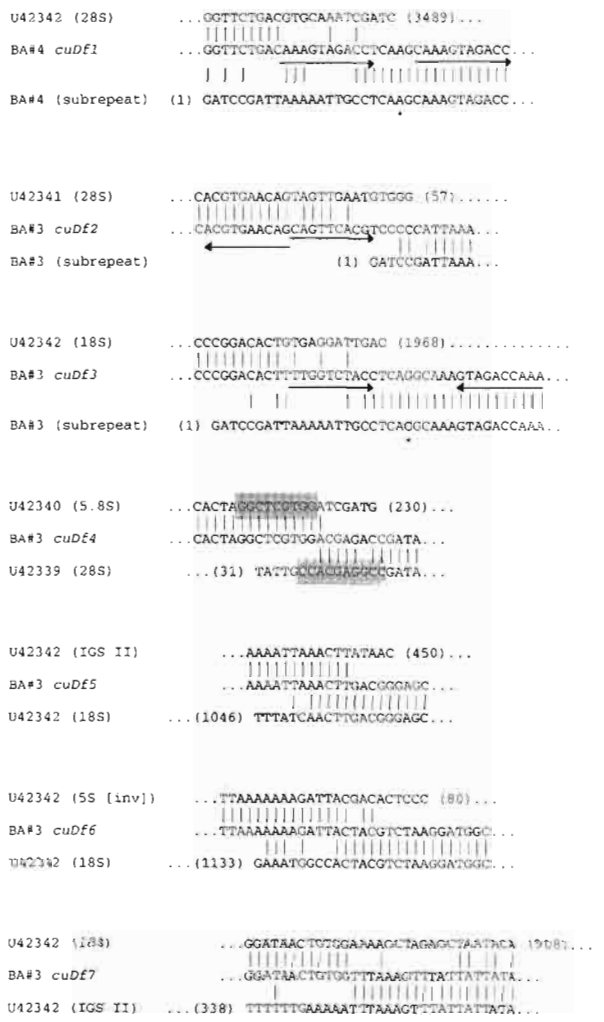

Fig. 2. *Sequence comparisons for rearranged regions of clones BA#3 and BA#4. See Fig. 1 for locations (deletions* cuDf1, cuDf2, cuDf3, cuDf4, cuDf5, cuDf6 *and* cuDf7 *correspond to italicized numbers [1, 2, 3, 4, 5, 6, 7], respectively, in Fig. 1). The middle sequence in each group of three is the rearranged sequence, the upper sequence is non-rearranged upstream sequence, and the lower sequence is non-rearranged downstream sequence. Non-rearranged sequences derive from the same clone as the rearrangements wherever possible, and from other clones when necessary. The source for each sequence fragment (Accession Number or clone designation) is provided to the left of the sequence, followed by a short descriptor (28S,* e.g.*) or the official designation for the rearrangement. A number in parenthesis following a sequence is the number of the last base shown; a number in parenthesis preceding a sequence is the number of the first base shown in that sequence. Vertical lines mark identities between sequences. Arrowed lines mark insertion sequences and the location and orientation of nearby matching sequences. Asterisks in* cuDf1 *and* cuDf3 *mark a single base difference between subrepeat sequences from BA#3* (cuP2[1]) *and BA#4* (cuP2[2]). *Shaded bases in 5.8S and 28S sequences are paired in the large ribosomal subunit to form stem 8; the base in the 28S sequence that differs between BA#3 and BA#4 is looped out of this structure (Ellis* et al., *1986).*

fragments between approx. 1.2 and 2.6 kb. Minor fragments in this size range were also amplified from genomic DNA if the extension step of the cycle was increased to 3 min (results not shown).

Single female PCR

In addition to the rearrangements, there are notable differences in sequence between BA#3 and BA#4. In order to determine whether these variants were present in the same animal or represented forms present in different clonal lines, we used primers F1 and R6 (Table 1) to amplify part of the IGS II, from the 5' end of the 5S sequence to the far side of a Hind III site present in BA#3 (*cuP1[1]*) but (due to a single base pair change) not in BA#4 (*cuP1[2]*, Fig. 3). This pair of primers amplified a larger product from BA#4 than from BA#3 (404 bp in BA#4 *vs* 323 bp in BA#3). Hind III digestion cuts the BA#3 amplification product in two (207 and 116 bp); sequence similarity between BA#3 and BA#4 is concentrated in the 207 bp fragment. All amplifications from individual nematodes contained both the 323 bp and 404 bp products, both of which hybridized with a probe consisting of labeled amplification product from BA#4 and exhibited the predicted pattern on digestion with Hind III (Fig. 4). The presence of amplification products larger than 404 bp suggests the existence of additional variant forms.

Discussion

Ribosomal DNA clones BA#3 and BA#4 were selected for analysis because they hybridized with a 129 bp repeat from the IGS but not with a fragment from the 3' end of the 28S coding sequence. On one level, these clones cannot be viewed as representative of rDNA in *M. arenaria*, as they were selected because they were rearranged; however, Southern hybridization shows that this species displays an unusual degree of variability in its ribosomal DNA, even within clonal lines (Hiatt *et al.*, 1995). Furthermore, Vahidi *et al.* (1988) have shown that a major variant form of the rDNA in *M. arenaria* contains a deletion in the 3' end of the 28S coding sequence. Thus, the mere presence of rearrangements does not make these clones atypical of rDNA in this species; and the screening method used in the present work was an efficient means of selecting altered copies of the ribosomal genes. Nonetheless, even where there is no obvious defect, the sequences of these clones (including BA#5) may not exactly match the sequence of functional ribosomal genes.

Two very different sequences were obtained for the IGS region, and PCR amplification of this region provided evidence that both forms are present in individual *M. arenaria* females. One of the sequences (as cloned in BA#3) is identical to that obtained by Blok

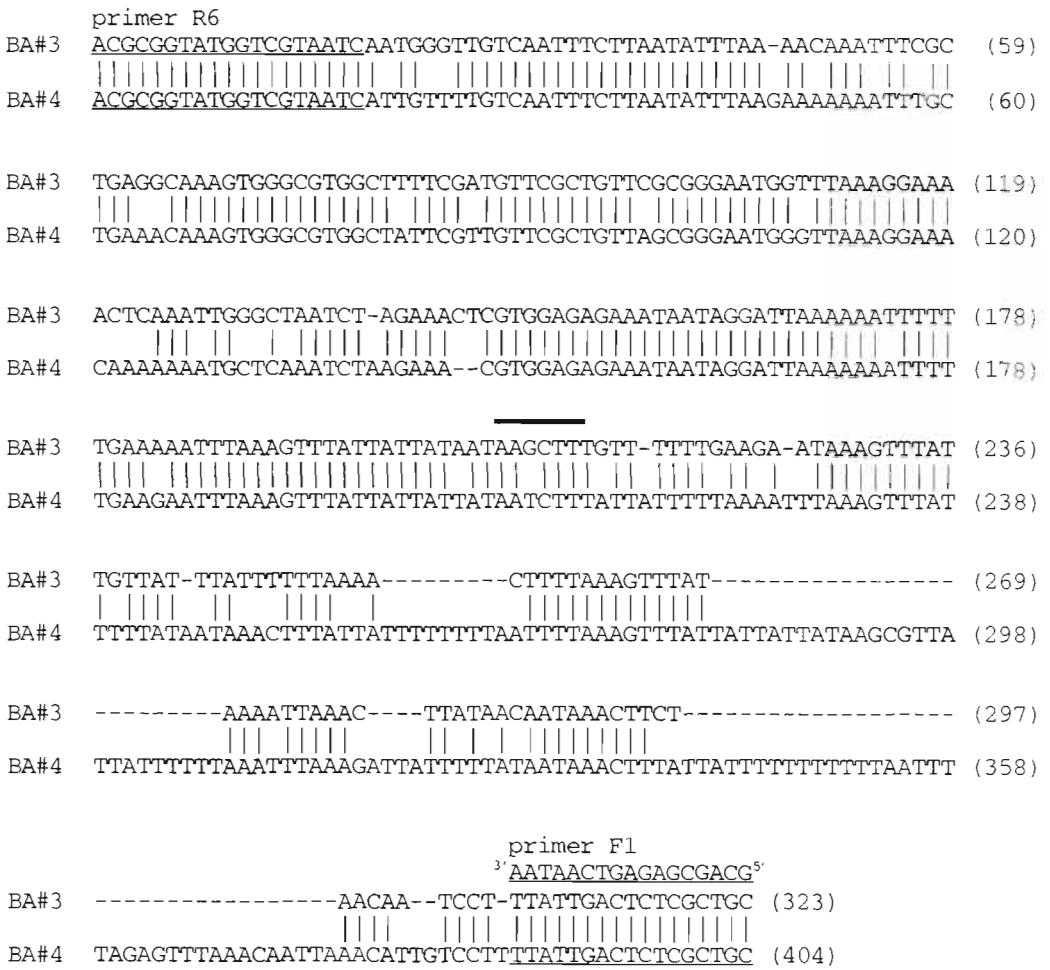

Fig. 3. *Alignment of partial IGS II sequences from BA#3 and BA#4, extending from the 5' end of the 5S sequence approximately two-thirds of the distance to the start of the 18S sequence (amplified by primers R6 and F1, underlined). The heavy overscore marks a* Hind III *site present in the former* (cuP1[1]) *but not the latter* (cuP1[2]). *Vertical lines mark identities between sequences; gaps in the alignment are indicated by dashes.*

et al. (1997) from *M. arenaria* and *M. javanica*, and differs at only one position from the sequence they obtained from *M. incognita*. In short, there is far greater sequence divergence between two forms of the IGS in *M. arenaria* than there is among sequences corresponding to one of the forms from three different species. One explanation for this observation is that *M. arenaria* arose by interspecific hybridization, and one (at least) of the parental species is shared with *M. javanica* and *M. incognita*. Descendants of the other parental species have yet to be identified, as the *M. arenaria* sequence cloned in BA#4 is a poor match with sequence from *M. mayaguensis* (Blok *et al.*, 1997). All of the clones examined had one or the other of the two forms; the two forms were never found together on the same clone. Although these clones represent a very small sample of the total rDNA, this result is consistent with the two forms being present in separate clusters in the genome, as one would expect if *M. arenaria* arose by interspecific hybridization. If so, we would expect the two forms to reside on different chromosomes. In *Ascaris lumbricoides*, however, although there are two rDNA forms in separate clusters, the two clusters appear to be on the same chromosome (Back *et al.*, 1984; Müller *et al.*, 1992). The situation in *M. arenaria* might be resolved using pulsed field gel electrophoresis (PFGE) or *in situ* hybridization.

The impact of variant rDNA units on the use of rDNA in PCR-based diagnostics depends on the rela-

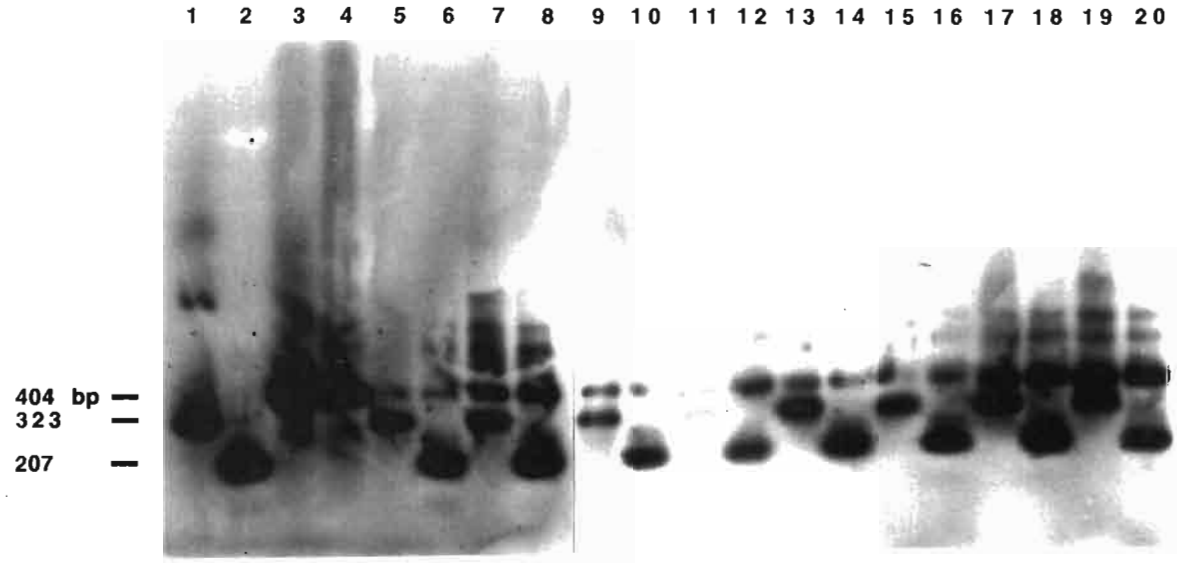

Fig. 4. *Southern blot of fragments amplified from single* Meloidogyne arenaria *females using primers F1 and R6, probed with the fragment amplified from BA#4 using the same primers. Odd lanes: undigested products; Even lanes: products digested with* Hind III. *The smaller fragment released by* Hind III *digestion of the 323 bp product is not visualized with this probe. Lanes 1 and 2: amplified from BA#3; lanes 3 and 4: amplified from BA#4; lanes 5 through 20: amplified from eight individual* M. arenaria *females.*

tive abundance of these variants in the genome and on whether the primer binding sites have been preserved. Rare variants will tend not to be seen because they will be eclipsed by the major amplification products or will be dismissed as PCR artifacts. The existence of variants missing primer binding sites will be completely overlooked, because they obviously will not amplify (though the normal copies present in the sample will result in an amplified product of the expected size). All of the sequence variants characterized here affected the ITS (*cuDf4*, Figs 1, 2) or IGS regions (*cuDf1*, *cuDf2*, *cuDf3*, *cuDf5*, *cuDf6*, *cuDf7* [Figs 1, 2], and the two sequence forms *cuP1[1]* – represented by clones BA#3, BA#5, and the sequence published by Blok *et al.* [1997] – and *cuP1[2]* – represented by clone BA#4 [Fig. 3]). It hardly needs mentioning that these regions are frequently used for phylogenetic studies and diagnosis. We do not know how common the ITS II deletion *cuDf4* is, however, it lacks the binding sites for the primers used by Vrain *et al.* (1992) and Ferris *et al.* (1993). Although Vahidi *et al.* (1988) found that units truncated in the 3' end of the 28S coding sequence represented a significant proportion of the total rDNA, our library screening results indicate that this class of variant is distinctly in the minority. Likewise, the failure to detect an amplification product of the size expected for *cuDf7* in sixteen individual *M. arenaria* females suggests that this variant is also not common. In any case, both *cuDf1* and *cuDf2* lack the binding site for the 28S primer 283-N used by Petersen and Vrain (1996). Likewise the absence of this primer sequence would preclude amplification of variants *cuDf3*, *cuDf5*, *cuDf6*, and *cuDf7*; additionally, *cuDf5* and *cuDf6* lack the binding site for the 18S primer 185-0 (Petersen & Vrain, 1996).

The remaining sequence variant, however, is easily detectable and amplifiable by primers flanking the IGS region. The presence of two forms of the IGS in a single RKN species (indeed, in a single individual of that species), plus the appearance of one of the forms in several different species, complicates the use of the IGS in species identification, to say the least. Thus it is premature to utilize this region for diagnostic purposes, although it has intriguing possibilities for phylogenetic analysis, potentially enabling the identification of species that are related to the progenitors of the polyploid (hybrid) RKN species. It should also be noted that the clone we used to obtain "normal" *M. arenaria* rDNA sequence, BA#5, appears to have an unusually large IGS (Fig 1).

There are several differences between our *M. arenaria* sequences and the sequences of primers others have used for amplifying RKN rDNA genes. The 18S (reverse) primer described by Blok *et al.* (1997) differs at two positions from our sequences, which were identical in this region for both BA#3 and BA#4 (5'-tCtaatgagccgtAcgc-3' *vs* 5'-tGtaatgagccgtTcgc-3').

Whilst the sequence of primer 185-0 (Petersen & Vrain,1996) is identical to our *M. arenaria* sequence, there are three mismatches between primer 283-N and our BA#5 sequence, clustered at the 5' end of the primer (5'-TtCgaGtaagcgcgggtaaacg-3' *vs* 5'-CtTgaTtaagcgcgggtaaacg-3'). Sequences corresponding to primers 185-1 (Petersen & Vrain,1996) and 1839 (Petersen *et al.*, 1997) were not found in the corresponding regions of our *M. arenaria* sequences. Likewise the 28S (reverse) primer of Ferris *et al.* (1993) differs at two positions from our BA#5 sequence (5'-tcctccgctAaAtgatatg-3' *vs* 5'-tcctccgctTaCtgatatg-3'); oddly, Zijlstra (1997) cites Ferris *et al.* (1993) without comment for the 28S primer sequence, while showing 28S sequence identical to ours. The 28S (reverse) primer of Vrain *et al.* (1992) differs from our sequence at three positions (5'-TttcactcgccgttactAAgg-3' *vs* 5'-GttcactcgccgttactGGgg-3'). Evidently, the minor differences are not fatal, and perhaps primers 185-1 and 1839 are conserved only in the northern RKN species.

In many organisms, size variation in rDNA units is due to variation in copy number of subrepeated elements. Vahidi *et al.* (1988) showed that this is the case in *M. arenaria*, though they also discovered the 3' end deletion alluded to previously. They suggested that the nearby subrepeated elements (in this species a 129-bp repeat) might also be involved in this rearrangement. The subrepeated elements are thought to act as enhancers of transcription. In *Saccharomyces cerevisiae*, the enhancer and initiator regions of the ribosomal genes were shown to stimulate recombination (Voelkel-Meiman *et al.*, 1987), apparently by increasing transcription by RNA Polymerase I, and not by their direct participation in rearrangements. It should be noted, however, that the enhancer in *S. cerevisiae* does not consist of obvious subrepeated elements (Jemtland *et al.*, 1986). Vahidi *et al.* (1988) did not report the sequence of the lesion in their 3' deleted clone, so we do not know whether theirs, like the ones presented here, resulted in the juxtaposition of the subrepeated elements with 28S coding sequence.

The seven rearrangements described here fall into three categories that differ so markedly in their attributes as to suggest strongly that they arose through different mechanisms of recombination or repair. One category, represented by three rearrangements, involved subrepeated elements and contained short insertions at the sites of the rearrangements; the inserted sequences appear to have derived from nearby sequences. This sort of variation is more complicated than simple expansion or contraction due to alteration in subrepeat copy number, but the subrepeat does seem to be important. A second category, represented by a single rearrangement, involved sequences that form a base-paired stem in the ribosome (Gerbi, 1985; Ellis *et al.*, 1986). The Polymerase Chain Reaction could be used to search for additional independent rearrangements of this type, and the results of such a search might suggest whether this rearrangement represents a class in which stem-loop structures are mechanistically involved, or whether the presence of such a structure in this rearrangement is purely coincidental. At least three of the four rearrangements in these first two categories were homogenized among adjacent gene copies and might be older, evolutionarily speaking, than rearrangements that are not homogenized, if only because the process of homogenization takes time. Finally, there were three rearrangements with no obvious features in common: there were no substantial sequence similarities at the breakpoints, nor did they form base-paired structures in mature rRNA. Perhaps these rearrangements represent crude repairs to breaks that occurred in already non-functional gene copies. Given that meiotic recombination frequencies typically are substantially higher than mitotic recombination frequencies (*cf.* Keil & Roeder, 1984, in yeast), it is conceivable that the homogenized rearrangements predate the adoption of a mitotic mode of reproduction by *M. arenaria*, while the non-homogenized rearrangements arose afterwards. This possibility is also suggested by an analysis by Piotte *et al.* (1994) of satellite sequences from a meiotic population of *M. hapla* and a population of *M. incognita*, which, like *M. arenaria*, is mitotically parthenogenetic (Castagnone-Sereno *et al.*, 1993). The overall percentages of divergence from the consensus sequences were similar in the two species, but the proportion of variants at a given position showing the same nucleotide substitution was an order of magnitude higher in *M. hapla* than in *M. incognita* (Piotte *et al.*, 1994). The authors attributed this to a higher activity of an unspecified homogenizing process in the meiotic species (Piotte *et al.*, 1994).

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