A New AluI Satellite DNA in the Root-Knot Nematode Meloidogyne fallax: Relationships with Satellites from the Sympatic Species M. hapla and M. chitwoodi

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A highly abundant satellite DNA comprising 20% of the Meloidogyne fallax (Nematoda, Tylenchida) genome was cloned and sequenced. The satellite monomer is 173 bp long and has a high A+T content of 72.3%, with frequent runs of A's and T's. The sequence variability of the monomers is 2.7%, mainly due to random distribution of single-point mutations. A search for evidence of internal repeated subunits in the monomer sequence revealed a 6-bp motif (AAATTTT) for which five degenerated repeats, differing by just a single base pair, could be identified. Pairwise comparison of the M. fallax satellite with those from the sympatic species Meloidogyne chitwoodi and Meloidogyne hapla revealed a high sequence similarity (68.39%) with one satellite DNA subfamily in M. chitwoodi, which indicated an unexpected close relationship between them. Given the high copy number and the extreme sequence homogeneity among monomeric units, it may be assumed that the satellite DNA of M. fallax could have evolved through some recent and extensive amplification burst in the nematode genome. In this case, its relatively short life would not yet have allowed the accumulation of random mutations in independent amplified repeats. Considering the morphological resemblance between the two species and their ability to produce interspecific fertile hybrids under controlled conditions, these results indicate that M. fallax may share a common ancestor with M. chitwoodi, from which it could have diverged recently. All these data suggest that M. fallax could be the result of a recent speciation process and show that Meloidogyne satellite DNAs may be of interest to resolve phylogenetic relationships among closely related species from this genus.

Introduction

Repetitive sequences are ubiquitous genomic components of all eukaryotic species. Among them, tandemly arranged DNA sequences known as satellite DNA are usually concentrated in substantial blocks at the centromeric and telomeric regions of chromosomes and form the principal component of constitutive heterochromatin. Because of their high rate of evolutionary change, they often display great variability in reiteration frequency, sequence, monomer length, and chromosomal distribution, even in closely related species (Miklos 1985). Nevertheless, satellite DNAs are apparently not as variable in array size within populations as other classes of tandemly repeated noncoding sequences, such as micro- or minisatellites (Wevrick and Willard 1989), even though substantial differences in array size are often observed between isolated populations and closely related species (Bachmann and Sperlich 1993). In a number of cases, satellite DNA sequences have been described to be species-specific and cannot be detected even in sibling species. However, in some occurrences, conserved satellite DNA families have been found either in a group of closely related species (Cremisi et al. 1988), or even spread among evolutionarily distant groups (Abad et al. 1992). Thus, this class of tandem repetitive elements is potentially of interest for the study of the phylogenetic relationships among related organisms.

Root-knot nematodes, Meloidogyne species, are plant obligate parasites able to reproduce on both monocotyledons and dicotyledons and on both herbaceous and woody plants, but the host ranges vary largely between the nematode species. There are over 60 species identified, but only four of them—M. arenaria, M. incognita, M. javanica, and M. hapla—are of worldwide major economic importance. Some other species, like M. chitwoodi, are also of known economic importance but have a more restricted distribution. Recently, a deviating group of populations of M. chitwoodi was distinguished mainly on the basis of host and morphological differences (van Meggelen et al. 1994) and further characterized as a new species, M. fallax (Karssen 1996). To date, populations of M. fallax are found in the southeastern part of the Netherlands near the borders of Germany and Belgium, and their distribution pattern seems to coincide largely with that of M. chitwoodi (Karssen 1996). Moreover, M. hapla is very often found to be associated with both species under temperate climates. Beyond their geographic association, these three species resemble each other from a morphological point of view and share a number of host plants, including potato. In order to get additional detailed information on the evolutionary relationships between M. fallax and the sympatic species M. hapla and M. chitwoodi, a fast-evolving marker was needed. As there are several examples showing that satellite DNA may be suitable for this purpose (Bachmann et al. 1993), the presence of such sequences in the genome of M. fallax was to be investigated.

In this paper, we present a novel repetitive sequence detected in the genome of M. fallax, analyzed with respect to structure, abundance, and organization. The characterized satellite DNA has been compared with known satellites from the sympatic species M. hapla and M. chitwoodi. High sequence similarities have been detected among the satellite from M. fallax and one satellite subfamily from M. chitwoodi, which
raises the possibility that *M. fallax* may share a common ancestor with *M. chitwoodi*, from which it could have diverged recently.

**Materials and Methods**

**Nematodes**

The *M. fallax* isolate (originating from the Netherlands) was maintained on tomatoes grown at 20°C in a greenhouse. It was specifically identified based on morphology as well as esterase and malate dehydrogenase phenotypes from single females.

**Purification of Genomic DNA**

Eggs were collected from infested roots and placed on a 10-µm pore sieve at 20°C to allow them to hatch for 3 weeks. *Meloidogyne fallax* second-stage juveniles were collected, concentrated by centrifugation at 2,000 × g for 2 min in a 30% sucrose solution, washed in distilled water, and pelleted in a microcentrifuge. The pellet was transferred to a mortar, frozen by liquid nitrogen, and ground. From the resulting fine powder, total DNA was extracted as previously described (Zijlstra et al. 1995).

**DNA Analysis**

Standard procedures were used for restriction endonuclease digestion, electrophoresis, transfer to nitrocellulose or nylon membranes, radioactive labeling, and hybridization (Sambrook, Fritsch, and Maniatis 1989). Hybridizations were performed overnight at 65°C. Conditions for washing consisted of 65°C in a 1 × SSC, 0.1% SDS final solution.

**Cloning of Satellite DNA**

Genomic DNA of *M. fallax* was digested with a set of restriction endonucleases, separated on a 1% agarose gel, and stained with ethidium bromide. To ensure complete digestion, incubations were performed for 4 h at 37°C with 10 U of enzyme/µg of DNA. Putative satellite DNA restriction fragments were recovered from the gel according to Dretzen et al. (1981), ligated into the plasmid vector pUC19, and used to transform competent *Escherichia coli* DH1 cells according to standard procedures (Sambrook, Fritsch, and Maniatis 1989). The transformants were selected on ampicillin (100 µg/ml) agar plates containing X-gal (80 µg/ml) and IPTG (120 µg/ml) and screened by colony hybridization (Grunstein and Hogness 1975) using the DNA fragments isolated from the gel as probes.

**Sequence Analysis and Comparison**

The nucleotide sequences of inserts from recombinant clones selected at random and corresponding to putative monomers were determined by the dideoxy chain termination method (Sanger, Nicklen, and Coulson 1977) using double-stranded plasmid as template and a Sequenase kit (U.S. Biochemical Corp.). Multiple-sequence alignments and tree construction were performed using CLUSTAL W (Thompson, Higgins, and Gibson 1994), and the significance of the results was assessed by bootstrap analysis (Felsenstein 1985). DNA sequence data were compared with the EMBL and GenBank databases by using the NCBI BLAST server (Altschul et al. 1990).

**Results**

**Detection and Cloning of a Satellite DNA Family in *Meloidogyne fallax***

Digestion of *M. fallax* genomic DNA and subsequent electrophoresis revealed the presence of a strong band of approximately 170 bp in *Alu*I digests (not shown). The *Alu*I band was purified from the gel, 32P-labeled, and used as a probe in Southern blot analysis. The autoradiograph revealed the presence of prominent 170-bp repeats in the *Alu*I digest accompanied by faint bands up to the pentamer size (not shown). Presumably, these arise from occasional loss of recognition sites due to random nucleotide mutations within the monomer sequences. To further examine the arrangement of these sequences, DNA fragments produced after time course digestion of *M. fallax* DNA with *Alu*I were hybridized with the probe described above. The autoradiography showed a typical ladder pattern (fig. 1), characteristic for digestion of repeated sequences arranged in tandem arrays. The 170-bp *Alu*I fragments isolated from the gel were subcloned into pUC19 vector. Ten positive clones, named pMIFd, were selected at random for further analysis. Under our experimental conditions, it was not possible to detect any other satellite DNA family in the genome of *M. fallax*. The method we used to identify the *Alu*I repetitive sequences tends to indicate that it is the only one present with such a high copy number, assuming that the range of restriction endonucleases tested was large enough.

**Quantitation and Copy Number in the Genome**

The relative abundance of the *Alu*I repetitive sequence was assessed from dot blot experiments. Increasing amounts of a cloned monomer (pMIFd1.9) and *M. fallax* genomic DNA were blotted onto nitrocellulose membrane and hybridized with the DNA insert released from the same clone (fig. 2). PUC19 was used as a background control, and *Caenorhabditis elegans*, *Drosophila melanogaster*, and calf thymus DNAs were used as negative controls (data not shown). The satellite DNA family appears to make up to 20.0% of the nematode genomic DNA as calculated from values obtained by scintillation measurements. Assuming that the genome size of *Meloidogyne* is about 51 Mb (Pablo and Triantaphyllou 1989), and based on an average monomer size of 173 bp, this fraction corresponds to approximately 57,800 copies per haploid genome.

**Sequence Organization and Variability of Monomeric Units**

The nucleotide sequence from each of 10 cloned monomers was determined in order to elucidate the internal organization of the repeats (fig. 3). The lengths of these sequences were found to be quite homogeneous, ranging from 172 to 177 bp. An unambiguous consensus sequence of 173 bp was derived from the complete data set and is listed at the top of figure 3. It was deposited
in the EMBL Nucleotide Sequence Database under the accession number AJ223457. Its A+T content is high (72.3%), with frequent stretches composed exclusively of A's and/or T's.

Analysis of primary structure among the 10 monomers in relation to the consensus sequence revealed a high homogeneity of this reiterated DNA family, with an overall sequence variability of only 2.7%. Compared with the consensus sequence, a total of 31 variable positions were observed (17.9%). Single-point substitutions contributed to more than half of all mutations (53%). In general, the observed changes were randomly spread over the whole sequences, although nucleotide substitutions appeared to be shared between two or more monomers in six positions (positions 59, 60, 98, 100, 163, 166; see fig. 3). Identical mutations shared among monomers can usually be explained as the result of partial gene conversion in the repetitive family in contrast to independent mutational events (Drouin and Dover 1990). The remaining mutations consisted of single-nucleotide deletions and of single- and double-nucleotide insertions (fig. 3).

A search for evidence of internal repeated subunits in the pMiFd consensus sequence did not reveal any motif of particular interest. However, a more careful analysis allowed the detection of a short 6-bp motif (AAATTT, in positions 52–57) for which five degenerated repeats, differing by just a single base pair, could be identified (positions 8–13, 21–26, 77–82, 96–101, and 111–116; see fig. 3).

A search in the EMBL and GenBank nucleic acid databases revealed no significant similarity with any recorded sequence, thus suggesting that the pMiFd family
represents a novel satellite DNA family in root-knot nematodes.

Sequence Comparisons

In the closely related root-knot nematode species *M. hapla* and *M. chitwoodi*, satellite DNAs have previously been characterized (Piotte et al. 1994; Castagnone-Sereno et al. 1998). Their most important features, together with those from *M. fallax*, are summarized in table 1. Unlike *M. chitwoodi*, in the genome of which six satellite DNA subfamilies have been identified, *M. hapla* and *M. fallax* each have a single sequence of monomeric units.

Pairwise comparisons between monomer consensus sequences of satellite DNA from the three *Meloidogyne* species (including consensus for each of the six subfamilies of *M. chitwoodi*) revealed sequence similarities ranging from 51.11% to 77.33% (table 2). Interspecies similarity was rather low between *M. hapla* and the two other species: 55.84% with *M. fallax*, and from 51.58% to 54.77% (average 52.98%) with *M. chitwoodi*. The same level of similarity was observed between *M. fallax* and *M. chitwoodi* (from 51.11% to 57.21%, with an average of 53.87%), except for the comparison with the consensus sequence from subfamily 2a. Surprisingly, the two sequences shared 68.39% similarity, suggesting a close relationship between the satellite DNA from *M. fallax* and satellite DNA subfamily 2a of *M. chitwoodi* (table 2). The pairwise genetic distances between the monomer consensus sequences of satellite DNA from the three *Meloidogyne* species were calculated according to Kimura’s (1980) two-parameter method, and the resulting data were used according to the method of Saitou and Nei (1987) to build an unrooted neighbor-joining tree (fig. 4). The consensus sequences appeared to be clustered in two main groups: one of them consisted of the four *M. chitwoodi* consensus sequences from subfamily 1 (pMcCo1a, 1b, 1c, and 1d), and the other one brought together the consensus sequences from *M. hapla* (pMhM), *M. fallax* (pMiF), and *M. chitwoodi* subfam-

Table 1
Summary of the Characteristics of Satellite DNAs from Three Different *Meloidogyne* Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Satellite Code</th>
<th>Abundance (%)</th>
<th>Copies/ Haploid Genome</th>
<th>Monomer Length (bp)</th>
<th>A+T Content (%)</th>
<th>Average Variability (%)</th>
<th>Variable Positions (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. hapla</em> .....</td>
<td>pMhM</td>
<td>5.0</td>
<td>15,000</td>
<td>169</td>
<td>68.0</td>
<td>3.0</td>
<td>15.9</td>
<td>Piotte et al. (1994)</td>
</tr>
<tr>
<td><em>M. chitwoodi</em></td>
<td>pMcCo</td>
<td>3.5</td>
<td>11,400</td>
<td>146</td>
<td>75.3</td>
<td>2.2</td>
<td>8.2</td>
<td>Castagnone-Sereno et al. (1998)</td>
</tr>
<tr>
<td>Subfamily 1a</td>
<td>146</td>
<td>75.3</td>
<td>2.2</td>
<td>8.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Subfamily 1b</td>
<td>143</td>
<td>68.5</td>
<td>0.7</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Subfamily 1c</td>
<td>169</td>
<td>66.9</td>
<td>3.2</td>
<td>9.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subfamily 1d</td>
<td>147</td>
<td>69.4</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Subfamily 2a</td>
<td>153</td>
<td>69.3</td>
<td>0.9</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
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<td>Subfamily 2b</td>
<td>180</td>
<td>78.9</td>
<td>5.4</td>
<td>12.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. fallax</em> .....</td>
<td>pMiF</td>
<td>20.0</td>
<td>57,800</td>
<td>173</td>
<td>72.3</td>
<td>2.7</td>
<td>17.9</td>
<td>This study</td>
</tr>
</tbody>
</table>
ily 2 (pMcCo2a and pMcCo2b). Moreover, as could be expected from the sequence comparison data, sequences pMIFd and pMcCo2a were closely grouped together, and this clustering occurred with no exception in all 1,000 repeats of the bootstrap procedure (fig. 4).

Discussion

The satellite DNA isolated from the genome of *M. fallax* shows structural characteristics common to satellite DNAs from other *Meloidogyne* species. Overall, it has a high A+T content, with frequent stretches of A's and/or T's. In the closely related species *M. hapla* and *M. chitwoodi*, A+T-rich satellite sequences have also been detected (Piotte et al. 1994; Castagnone-Sereno et al. 1998). In fact, such a feature seems to be quite usual for parasitic nematodes, since it has been reported for both plant-parasitic (Táres et al. 1993) and animal-parasitic species (Callaghan and Beth 1994). In a number of cases, A+T enrichment during satellite DNA evolution has been suggested to be the result of C-to-T and G-to-A transitions being more frequent than the reverse transitions (Ugarkovic, Plohl, and Gamulin 1989; Rojas-Rousse, Bigot, and Periquet 1993). From this point of view, parasitic nematodes in general, and root-knot nematodes in particular, should be considered as ancient organisms. Based on isoenzymatic data, it has been suggested that the divergence between *M. hapla* and *M. chitwoodi* might have occurred approximately 35 MYA, although this value may be an overestimate of actual evolutionary time (Esbenshade and Triantaphyllou 1987). More recently, a relatively high mtDNA diversity for *M. hapla* compared with other *Meloidogyne* species has been reported, which is in favor of the argument that *M. hapla* is old (Hugall, Stanton, and Moritz 1997). However, no definitive datation is currently available. Moreover, although the functional significance of satellite DNA remains unclear, the common high A+T composition of these sequences in nematodes also suggests that such a feature may be selectively constrained in this group.

A second remarkable trait of the pMIFd family is the very high degree of sequence similarity displayed among monomeric units. The same characteristic has also been observed for *M. hapla*, for which satellite DNA monomers showed only 3% average divergence from their consensus sequence (Piotte et al. 1994). Such an unusual sequence homogeneity could be achieved as a consequence of some highly effective homogenization mechanism, as has been proposed for a number of homogenous satellite DNA families recently isolated from the insects *Eyprepocnemis plorans* (Lopez-Leon et al. 1995) and *Nicrophorus* spp. (King and Cummings 1997). Among the genetic mechanisms that can affect interrepeat variability, unequal exchange (Krüger and Vogel 1975; Smith 1976) is thought to be actively involved in the low level of interrepeat variability, even though it is considered a relatively rare event (Charlesworth, Sniegowski, and Stephan 1994). Indeed, simulation experiments confirmed that unequal crossing over is a dominant long-range ordering force which keeps arrays homogeneous even in regions of very low recombination rates, such as at satellite DNA loci (Stephan and Cho 1994). However, an alternative view to this hypothesis is that the pMIFd repeats belong to a family that may have recently appeared in the genome of *M. fallax*. Considering its high copy number (about 20% of the nematode genome) compared with the pMcCo family found in *M. chitwoodi* (about 3.5% of the nematode genome), this satellite DNA could have arisen through some recent and extensive amplification burst. In this case, the relatively short life of the satellite DNA has not yet allowed the accumulation of mutations in the amplified repeats.

Another characteristic which is not restricted to *M. fallax* satellite DNA is the occurrence of short AAATTT subrepeats (one perfect and five 1-bp degenerated motifs) present in the monomer consensus sequence. The same AAATTT motif has also been detected in *M. chitwoodi* satellite DNA in both direct and inverted orientations, up to nine times in the consensus sequence, depending on the satellite subfamily considered (Castagnone-Sereno et al. 1998). Although no clear arrangement of these subrepeats can be found, and even though most of them appeared (slightly) degenerated in *M. fallax*, the fact that they are shared by both species and repeated within each consensus sequence also suggests

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>pMcCo 1a</th>
<th>pMcCo 1b</th>
<th>pMcCo 1c</th>
<th>pMcCo 1d</th>
<th>pMcCo 2a</th>
<th>pMcCo 2b</th>
<th>pMIFd</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. hapla</em></td>
<td>52.54</td>
<td>51.96</td>
<td>51.58</td>
<td>54.19</td>
<td>52.81</td>
<td>54.77</td>
<td>55.84</td>
</tr>
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<td>pMhM</td>
<td>65.90</td>
<td>68.39</td>
<td>63.89</td>
<td>51.88</td>
<td>54.26</td>
<td>52.20</td>
<td>57.21</td>
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<tr>
<td><em>M. chitwoodi</em></td>
<td>77.33</td>
<td>63.43</td>
<td>67.31</td>
<td>55.0</td>
<td>58.92</td>
<td>54.40</td>
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<td>pMcCo 1a</td>
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<td>pMcCo 1b</td>
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<td><em>M. fallax</em></td>
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<tr>
<td>pMIFd</td>
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</table>
that the evolution of the two satellites may have occurred through successive amplification events from a common ancestral sequence.

Surprisingly, the dendrogram derived from the sequence comparison of satellite DNAs from *M. fallax* and the sympatric species *M. hapla* and *M. chitwoodi* data did not isolate each species in a separate cluster, but clearly distinguished between sequences from *M. hapla*, *M. fallax*, and *M. chitwoodi* subfamily 2 on one hand, and sequences from *M. chitwoodi* subfamily 1 on the other. Obviously, this clustering is not in agreement with the genetic relationships currently assumed for these

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**Fig. 4.—** Unrooted neighbor-joining dendrogram of relationships between the nucleotide consensus sequences of satellite DNAs from three *Meloidogyne* species. Scale bar represents a genetic distance \( D \) of 0.043 as the frequency of nucleotide substitutions in a pairwise comparison of two sequences according to Kimura’s (1980) two-parameter method.
species, i.e., the specific status of *M. chitwoodi* and the early divergence of *M. chitwoodi* and *M. hapla* (see above). At first sight, such a discrepancy between the evolution of satellite DNA and genetic or cytogenetic characters of the species tends to imply that this kind of sequence is not suitable for phylogenetic reconstruction. From this point of view, this result does not seem to be consistent with the conclusions of a number of studies, e.g., a recent comparative analysis of satellite DNAs from several species of the genus *Tribolium*, in which phylogenetic relationships deduced from satellite sequences agreed with those based on karyological, chemotaxonomic, and hybridization data (Ugarkovic, Podnar, and Plohl 1996).

However, interesting features can be deduced from the phylogeny of these sequences. First, based on this result, it could be assumed that the differentiation between satellite subfamilies 1 and 2 in *M. chitwoodi* is possibly an ancient event relative to the divergence between *M. chitwoodi* and the other two species. This is consistent with the hypothesis that *M. chitwoodi* should hold a position close to the ancestral forms of root-knot nematodes, in relation with its cytogenetic characteristics (Triantaphyllou 1985) and the strongly structured sequence variation detected in its *Alu* satellite DNA family (Castagnone-Sereno et al. 1998). Second, the analysis also revealed an unexpected close relationship between the satellite DNA from *M. fallax* and satellite DNA subfamily 2a of *M. chitwoodi*. Recently, it has been proposed that the strong structuration of *M. chitwoodi* satellite DNA could be the result of the independent evolution of an ancestral *Alu* sequence in isolated populations and subsequent hybridization events between them, thus leading to the current occurrence of different (and more or less related) subfamilies within the genome of a single *M. chitwoodi* population (Castagnone-Sereno et al. 1998). Given the high sequence similarity between these two sequences (68.39%) and the significant support of their relatedness (clustering of the consensus sequences pMiFd and pMcCo 2a is present in 100% of the 1,000 bootstrap replicates), it is reasonably possible that *M. fallax* may share a common ancestor with (at least one population of) *M. chitwoodi*, from which it would have diverged recently.

Several arguments favor of this hypothesis. First, *M. chitwoodi* and *M. fallax* highly resemble each other from a morphological point of view. Besides, *M. fallax* had originally been (mis)identified as a new race of *M. chitwoodi* for this reason (van Miggelen et al. 1994) and was recognized as a new species only very recently (Karssen 1996). Moreover, the two species also appear to be rather similar, compared with other root-knot nematode species, on the basis of the molecular analyses performed so far. For example, differences in ITS or IGS ribosomal DNA sequences from *M. chitwoodi* and *M. fallax* are quite scarce, although both species are easily separated from *M. hapla* (Zijlstra et al. 1995). But the strongest evidence supporting the phylogenetic relationships between *M. chitwoodi* and *M. fallax* was reported very recently as the result of hybridization experiments (van der Beek and Karssen 1997). Successful interspecific hybridization was obtained, and the resulting hybrids produced egg masses. In eggs, cell division was observed, but most of the eggs were without clear differentiation and consequently were sterile. Hatched F2 juveniles were few in number, were not viable, and showed morphological distortions. However, although a stable progeny could not be observed under these experimental conditions, the fact that some hybridization events occurred between *M. chitwoodi* and *M. fallax* clearly indicates that the two species share cytogenetic characteristics. Moreover, although the extreme sequence homogeneity among pMiFd repeats could be partly achieved as a consequence of some unequal exchange mechanism, it may also suggest that this repetitive DNA family evolved quite recently through some amplification events, thus preventing the accumulation of random mutations in independent monomeric units. Together, all these data are congruent with the hypothesis that *M. fallax* could be the result of a recent speciation process and show that *Meloidogyne* satellite sequences may be able to reflect some evolutionary trends within this genus.

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