Evolution of the AT-Rich Mitochondrial DNA of the Root Knot Nematode, *Meloidogyne hapla*

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Mitochondrial DNA of the root knot nematode *Meloidogyne hapla* was investigated for intraspecific diversity and divergence from other parthenogenetic root knot nematodes. A 1,900-bp fragment containing COII, tRNAts, 16S rRNA, ND3 and Cyt b genes has been cloned and sequenced from one individual and an 1,188-bp region within this region was sequenced from four other Australian isolates. *M. hapla* mtDNA is more than 80% AT-rich, like other *Meloidogyne* spp. Nucleotide diversity within *M. hapla* is some 10-fold higher than across three other parthenogenetic species of root-knot nematode (*M. arenaria*, *M. javanica*, and *M. incognita*), implying an earlier origin for *M. hapla*. Nucleotide divergence between *M. hapla* and its congener *M. javanica* is as great as that between *Ascaris suum* and *Caenorhabditis elegans*, members of different nematode subclasses, while amino acid sequence divergence between *Meloidogyne* is more than twice as great. This is interpreted as an AT-bias-induced acceleration of the amino acid substitution rate, over and above saturation of nucleotide divergence in the strongly AT-biased DNA, on three lines of evidence: (1) in conserved blocks in 16S rDNA congeneric *Meloidogyne* have no more differences than between *A. suum* and *C. elegans*; (2) the *Meloidogyne* lineage has more amino acid changes relative to the *Ascaris/Caenorhabditis* lineage with respect to four of five outgroups, the exceptional outgroup being the only species (*Apis*) as AT-rich as *Meloidogyne*; and (3) between the two *Meloidogyne* there are more first and second but fewer third codon position changes than between the other nematode species. *M. hapla* is also found to contain a 102-bp tandem repeat of at least 40 copies; a size, arrangement, and position the same as in *M. javanica*, but sequence comparisons did not demonstrate homology between the two repeats.

Introduction

Mitochondrial DNA has been characterized for only a handful of nematode genera, and those that have been examined reveal consistently high AT content and substantial divergences, even between congeners (Thomas and Wilson 1991; Okimoto et al. 1992; Powers, Harris, and Hyman 1993). Very high AT content, also seen in some insects (e.g., honeybees *Apis mellifera*; Crozier, Crozier, and MacKinlay 1989), can introduce strong bias in nucleotide and amino acid substitution processes that complicate analyses of variation and phylogeny among species (Thomas and Wilson 1991; Jermiin and Crozier 1994b; Lockhart et al. 1994). In particular, amino acid composition seems to be correlated to AT content and may be influenced by it (Jukes and Bushan 1986; Thomas and Wilson 1991). This potentially affects rates of amino acid substitution because of reduced redundancy and greater dependence of synonymous sites on transitions among predominant codons (Jukes and Bushan 1986; Jermiin and Crozier 1994a). The asymmetry of substitution processes associated with strong base content bias can also lead to low saturation ceilings, especially for transitions, a situation that can only be aggravated by the reduced redundancy (e.g., Holmqvist 1983; DeSalle et al. 1987; Zhu et al. 1994). Martin (1995) has suggested that AT bias may also be positively correlated with the rate of oxidative DNA damage and hence mutation rate, further compromising phylogenetic inference.

Nonetheless, patterns of mtDNA variation have been informative at lower taxonomic levels of nematodes, revealing, for example, low diversity within *Caenorhabditis elegans* and large divergences among species of *Caenorhabditis* (Thomas and Wilson 1991). In comparison, different parthenogenetic species of the root-knot nematode *Meloidogyne* were found to have very low levels of sequence divergence (Powers and Sandall 1988; Hugall et al. 1994) with the exception of one species, *M. hapla*. Mitochondrial DNA from the latter did not cross hybridize with that from the other species (Powers et al. 1986) and showed divergence in RFLP pattern so extensive that sequence divergence could not be estimated reliably (Powers, Sandall, and Powers 1986; Hugall et al. 1994). Conflicting reports of low mtDNA divergence between *M. hapla* and the other species (Powers and Sandall 1988; Harris, Sandall, and Powers 1990) apparently result from contamination of laboratory stocks (Peloquin et al. 1993).

The present study assesses mtDNA sequence variation within *M. hapla* and between *M. hapla* and other parthenogenetic *Meloidogyne* for several different sections of the mtDNA; 16S rRNA, protein-coding genes (ND3 and sections of COII and Cytochrome b) and a 102-bp noncoding repeat. AT content and inferred nucleotide and amino acid divergence between *Meloidogyne* species is compared to that between two other nematodes from different subclasses, *Ascaris suum* and *Caenorhabditis elegans*.

Materials and Methods

Samples

From our collection of 110 Australian field isolates, five were identified as *M. hapla* on the basis of diag-

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nostic esterase and malate dehydrogenase phenotypes (Esbenshade and Triantaphyllou 1990). Four of the five isolates of *M. hapla* examined here were described previously (Hugall et al. 1994); the fifth, isolate 150, was characterized subsequently by the same methods.

Mitochondrial DNA was prepared either by isopycnic CsCl ultracentrifugation from egg masses, or as chelax extracts of individual females teased from host roots and macerated in 15 μl of 1% triton X-100 (see Hugall et al. 1994).

Hybridization and Cloning of mtDNA Fragments

Mitochondrial DNA restriction fragments containing sequences homologous to those characterized previously in other species were cloned from *M. hapla*. Purified mtDNA from *M. hapla* (isolate 113) was digested with a series of restriction endonucleases and probed separately with PCR fragments containing *M. javanica* 16s rRNA and ND3/Cyt b genes amplified using primers described by Stanton, Hugall, and Moritz (1996). This identified a 1.8-kb *Bcl I* fragment containing homologous sequences, which was then cloned into pBluescript II KS+ vector (Stratagene) and sequenced. An additional *EcoRI* library of mtDNA from *M. hapla* isolate 113 was made to clone copies of a 102-bp tandem repeat that contains an *EcoRI* site (see Hugall et al. 1994).

PCR Amplification and Sequencing

Mitochondrial DNA sequences were amplified from purified mtDNA and from chelax (Bio-Rad) extracts in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.01% Tween 20, 0.01% NP40 with 0.2 μM of each primer and 1.5 mM MgCl₂, using *Taq* DNA polymerase I and an annealing temperature of 50°C. The primers used are described by Stanton, Hugall, and Moritz (1996). D. Wolstenholme (personal communication) provided *M. javanica* sequence for primer design. Both PCR products and plasmid DNA of clones were sequenced by cycle sequencing using primers end labeled with [*γ-33P*]ATP.

Analyses of 102-bp *EcoRI* Repeats

The number of copies of the *EcoRI* repeat was estimated using a two-dimensional scintillation counter that directly determines cpm from the dried gel, averaging triplicate lanes of [*32P*]-end-labeled *EcoRI* digests. Appropriate sections were chosen to determine background radiation and cpm in single-copy bands. The tandem arrangement of the *EcoRI* repeats was demonstrated through time course partial digests of isolate 113 mtDNA with 0.5 units of *EcoRI* at 5°C.

Statistical Analysis of DNA Sequence

Nucleotide and protein sequences were aligned with the assistance of CLUSTAL V (Higgins, Bleasby, and Fuchs 1992) using the alignment of *A. suum* and *C. elegans* as given by Okimoto et al. (1991). Sequence statistics were determined with the MEGA (Kumar, Tamura, and Nei 1993) and PHYLIP (Felsenstein 1991) computer packages. Nucleotide diversities were estimated by REAP (McElroy et al. 1992). Most parsimonious distribution of changes among *M. hapla* mtDNA isolates was determined with the aid of MacClade (Madison and Maddison 1992). Gene designations and boundaries concord with those of other authors (Okimoto et al. 1991; Wolstenholme 1992 and personal communication; Powers, Harris, and Hyman 1993). Protein-coding sequences were translated using the *Drosophila* mtDNA genetic code as used by Okimoto et al. (1991) and Powers, Harris, and Hyman (1993). Nucleotide and protein sequences of the outgroups are from the GenBank database. Neighbor-joining trees are from a distance matrix of amino acid changes and were generated using MEGA. Deviations in rates of amino acid sequence change between the *Meloidogyne* spp. and the *Ascaris/Caenorhabditis* lineage relative to a series of outgroups were evaluated using the chi-square test of Tajima (1993, eq. 4). Sequences used in this study are deposited in the GSDB database; accession numbers: *M. javanica* L76261, *M. hapla*, I.76262.

Results

Alignment and Characteristics of Sequences

The sequence from the 1.9-kb clone of *M. hapla* DNA was aligned to corresponding sequences obtained by PCR from two isolates (60 and 78 in Hugall et al. 1994) of *M. javanica* and homologous regions of previously published sequences of *A. suum* and *C. elegans* (Okimoto et al. 1992). This alignment (fig. 1) and the corresponding translations (fig. 2) demonstrated that the clone contained ≈356 bp of the 3' end of COII; the entire tRNAHis, 16s rRNA, and ND3 genes; and ≈375 bp of the 5' end of the Cyt b gene.

As inferred from variation in length of PCR fragments (Powers, Harris, and Hyman 1993), the mtDNA of *M. hapla* lacks a ≈340-bp open reading frame and adjacent ≈680-bp noncoding region found in *M. javanica* (Okimoto et al. 1991; Wolstenholme 1992 and personal communication). Otherwise the gene arrangement is the same in the two species of *Meloidogyne*. Some gene boundaries, however, are difficult to determine precisely; for example, the boundaries between the 16s rRNA, ND3, and Cyt b genes. The *Meloidogyne* ND3 genes appear to be 8–9 amino acids shorter at the amino terminus than those from the other species of nematode, whereas the COII gene in *M. javanica* appears to be eight amino acids longer. To achieve similar 5' lengths for Cyt b requires overlapping ND3. In view of the uncertainty about the location of the start codon, analyses do not include the first 24 codons of Cyt b. The true length of the transcribed 16s rRNA gene in *Meloidogyne* is not known, but the maximum length, determined by approximate boundaries with the adjacent genes, appears to be ≈820 bp in *M. hapla* and a similar size in *M. javanica*. Powers, Harris, and Hyman (1993) estimated a similar length for *M. incognita*. This is substantially shorter than the corresponding genes in *A. suum* and *C. elegans*, previously considered to be the smallest metazoan 16s rRNA genes (Okimoto, MacFarlane, and Wolstenholme 1994). The alignments of 16s rRNA sequences between *Meloidogyne* spp. and be-
between A. suum and C. elegans are straightforward, whereas between the two pairs of nematode species has numerous uncertainties of alignment. The alignment shown maximizes matches in the universal conserved blocks (underlined in fig. 1) previously identified for A. suum and C. elegans by Okimoto, MacFarlane, and Wolstenholme (1994).

Both M. hapla and M. javanica have extremely AT-rich sequences (83.4% and 84.7% overall, respectively), even higher than the bias over the same sequences in A. suum (72.8% AT) and C. elegans (76.5% AT). The bias toward AT varies among genes, being strongest for ND3 and the 16S rRNA gene and weakest for the 3’ segment of COII (table 1). Within the protein-coding sequences, AT content at sites with synonymous differences (81%) is slightly less than for sites with nonsynonymous differences (85%). Comparison of base contents over alignable regions from a wider range of metazoans (table 2) reveals that both species of Meloidogyne, like the other nematodes, have a very low proportion of C on the sense strand. This contrasts with usual paucity of G evident in both AT-rich and unbiased genomes and may signal transcription of the alternative H strand (cf. Tanaka and Ozawa 1994; Jerinmi, Graur, and Crozier 1995).

mtDNA Diversity Within M. hapla Relative to Other Meloidogyne

An 1,188-bp region covering the complete 16S rRNA and ND3 genes was amplified and sequenced for
Fig. 2.—Predicted amino acid sequence alignments of the three coding genes shown in figure 2. Alignments are the same as in Powers, Harris, and Hyman (1993) and Okimoto et al. (1992). The bottom line shows C. elegans with respect to A. suum. Analyses in table 1 use sequences 1–117 for COI, 101–113 for ND3, and 23–114 for Cyt b.
four other isolates of *M. hapla* (114, 48, and 102 from Hugall et al. 1994, and 150). This revealed substantial polymorphism including base substitutions at 26 sites toward transitions (17 vs. 11 transversions) and three of indels at another three sites. There was a weak bias extensive relative to that observed for the same region in other species. Similarly, estimated nucleotide diversity within *M. hapla* varied from 0.2% to 2.0%, compared to a maximum value of 0.34% across the other species. Thus, the total number of nucleotide changes between the two groups (12 to 17 of 33) is threefold less than for the *M. hapla* repeats.

### Table 1

<table>
<thead>
<tr>
<th>Nematode Mitochondrial Gene Alignment Analyses</th>
<th>Differences</th>
<th>%AT</th>
<th>% Divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st/2nd/3rd</td>
<td>Nucleotide</td>
<td>Amino Acid</td>
</tr>
<tr>
<td><strong>Table 1</strong></td>
<td></td>
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<tr>
<td><strong>Gene Comparison</strong></td>
<td><strong>BP/AA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COII (3' end)</td>
<td>351/117</td>
<td></td>
<td></td>
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<tr>
<td><em>M. hapla</em> to <em>M. javana</em></td>
<td>24/17/45</td>
<td>86</td>
<td>39 (29/10)</td>
</tr>
<tr>
<td><em>C. elegans</em> to <em>A. suum</em></td>
<td>14/7/52</td>
<td>73</td>
<td>12 (9/3)</td>
</tr>
<tr>
<td>ND3 (complete)</td>
<td>306/102</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. hapla</em> to <em>M. javana</em></td>
<td>30/11/29</td>
<td>70</td>
<td>38 (23/15)</td>
</tr>
<tr>
<td><em>C. elegans</em> to <em>A. suum</em></td>
<td>15/7/52</td>
<td>74</td>
<td>18 (12/6)</td>
</tr>
<tr>
<td>Cytochrome b (5' end)</td>
<td>276/92</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. hapla</em> to <em>M. javana</em></td>
<td>16/11/27</td>
<td>54</td>
<td>25 (18/7)</td>
</tr>
<tr>
<td><em>C. elegans</em> to <em>A. suum</em></td>
<td>12/6/51</td>
<td>69</td>
<td>16 (11/5)</td>
</tr>
<tr>
<td>16s rRNA (complete)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. hapla</em> to <em>M. javana</em></td>
<td>822/882</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. elegans</em> to <em>A. suum</em></td>
<td>960/953</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Coding sequence analyses are based on positions in figure 1 of 3-535 for COII, 1456-1760 for ND3, and 1809-2054 for Cyt b.*

**Table 2**

| Base Content (%) of the Combined Sections of COII, ND3, and Cytochrome b Genes |
|---------------------------------|-----------------|---------------|
|                                  | A               | T             | C             | G             | AT            | 1st | 2nd | 3rd |
| **M. hapla**                    | 31.2            | 50.6          | 5.5           | 12.7          | 81.8          | 73.4 | 78.6 | 90.0 |
| **M. javanaica**                | 30.1            | 52.6          | 5.0           | 12.3          | 82.7          | 75.9 | 78.2 | 90.8 |
| **A. suum**                     | 19.5            | 50.3          | 7.1           | 23.1          | 69.8          | 64.3 | 68.4 | 74.2 |
| **C. elegans**                  | 30.5            | 44.0          | 7.9           | 17.5          | 75.4          | 67    | 68   | 86.0 |
| **A. mellifera**                | 36.9            | 42.0          | 12.2          | 8.8           | 78.9          | 71.4 | 69.9 | 96.6 |
| **S. purpureatus**              | 26.5            | 30.4          | 25.0          | 18.1          | 56.9          | 51.2 | 62   | 56.8 |
| **L. terrestris**               | 28.4            | 31.6          | 21.8          | 18.2          | 60.0          | 49.6 | 62.4 | 68.6 |
| **A. gambiae**                  | 31.9            | 39.8          | 13.9          | 14.4          | 71.7          | 59    | 65   | 90.2 |
| **G. gallus**                   | 25.8            | 23.9          | 35.4          | 15.0          | 49.7          | 46.3 | 57.5 | 45.8 |

*%AT content of the 1st, 2nd, and 3rd codon positions separately.*
last can be assumed to be saturated, implying saturation of the differences within pairs also. As expected for comparisons of divergent AT-rich regions, the base substitutions between the *Meloidogyne* spp. in protein-coding genes are dominated by transversions (TV) (142 vs. 68 transitions [Ts], TV/Ts ratio 2.09), whereas the other, less AT-rich nematodes have similar numbers of transversions and transitions (108 vs. 107, TV/Ts ratio 1.01).

The patterns of amino acid substitution between the nematode genomes are of particular interest. A high proportion of base substitutions occurs in first (33%) and second (18%) codon positions (table 1), and there is a correspondingly large ratio of substitutions per non-synonymous site ($K_A$) to substitutions per synonymous site ($K_S$) ($K_A/K_S = 0.54$ vs. $K_A/K_S = 0.15$ between *A. suum* and *C. elegans* and similar values for the comparisons among nematode CO11 genes in Thomas and Wilson [1991]). Particularly notable is that the number of amino acid differences between the *Meloidogyne* spp. (102 over 311 codons) is more than twice that observed between *A. suum* and *C. elegans* (46), in contrast to the similar levels of nucleotide divergence between the two pairs. The ratio of conserved to nonconserved amino acid changes is similar across the different genes (ratio varies from 2–3) and between the two pairs of species.

Each gene can be expected to be under different patterns of amino acid substitution constraint; nevertheless, analyzing each gene separately, the higher the AT content, the greater the $K_A/K_S$ ratio and the higher the ratios of transversions to transitions and of two-fold to four-fold redundant codons (correlation coefficient > 0.9). To investigate this correlation of AT bias to substitution rate all three genes are pooled in subsequent analyses, although we recognize that there are slight differences in the extent of AT content among genes (table 1).

Relative Rates of Amino Acid Substitution

Given the disparity between amounts of nucleotide and amino acid differences between the congeneric *Meloidogyne* versus those between the other pair of nematodes, we tested for accelerated rates of amino acid substitution in the former lineage using a variety of outgroups with varying AT content (table 2). This was based on an unambiguous alignment over 264 codons from COII, ND3, and Cyt b (not shown but available on request); some sections of ND3 were difficult to align across this broader group and were therefore excluded.

Two examples of neighbor-joining trees of amino acid differences, using either *Lumbricus terrestris* (%AT = 60.0) or *Apis mellifera* (%AT = 78.9) as an outgroup, are shown in figure 4. Chi square tests of branch length differences (using eq. 4 in Tajima 1993) for all four combinations of nematode sequences against five outgroups are presented in table 3. These comparisons suggest a significant ($P < 0.05$) acceleration of amino acid substitution in the *Meloidogyne* lineage relative to the other nematodes for comparisons with four of the five outgroups (sea urchin, *S. purpuratus*; worm, *L. terrestris*; mosquito, *A. gambiae*; and chicken, *G. gallus*). In contrast, no acceleration in *Meloidogyne* is evident.
when using the honeybee, *Apis mellifera*, as an outgroup. The reason for this difference is apparent from figure 4; use of the similarly AT-rich *Apis mellifera* as an outgroup shifts the root of the tree toward the *Meloidogyne* species and thereby equalizes overall branch lengths. The extent of the estimated difference in rates of amino acid substitution, indexed by the $\chi^2$ values in table 3, is influenced by the AT bias of the outgroup; the greater the difference in %AT between the outgroup, the greater the apparent disparity in rates.

**Discussion**

Implications for Evolution of *Meloidogyne hapla*

Two observations from our study of mtDNA variation are relevant to understanding the evolutionary history of parthenogenetic *M. hapla*. The first is the extreme divergence of mtDNA sequences from *M. hapla* relative to homologous sequences from the other species. The second is that *M. hapla* was found to have high intraspecific diversity relative to the other four parthenogenetic species of *Meloidogyne* combined.

*M. hapla* is generally supposed to represent a separate origin of parthenogenesis in *Meloidogyne* relative to other lineages because of the presence of both facultative meiotic and obligate mitotic parthenogenesis in the species (Triantaphyllou 1985) and divergent profiles for both allozymes (Esbenshade and Triantaphyllou 1987) and nuclear genes (Castagnone-Sereno et al. 1993; Castagnone-Sereno, Vanlerberghe-Masutti, and Leroy 1994). This view is supported by the divergence of mtDNA from *M. hapla* versus the other parthenogenetic species, reflecting separate origins from evolutionarily distant sexual females. Based on observed genetic distance for allozymes, Esbenshade and Triantaphyllou (1987) suggested that the ancestors of *M. hapla* separated from those of other parthenogenetic *Meloidogyne* at least 43 MYA. The similarity of levels of mtDNA sequence divergence between *M. hapla* and other parthenogenetic *Meloidogyne* and that between *C. elegans* and *A. suum*, whose separation is tentatively dated at 80 Myr (Okimoto et al. 1992), suggest that the lineages leading to the two groups of parthenogenetic *Meloidogyne* might be far older than previously thought. Two conflicting caveats need to be added: (1) AT bias may point to much higher mutation rate in *Meloidogyne* and so suggest a younger age (see Martin 1995) and (2) AT bias may mask the true divergence and so suggest an older age. These matters are addressed further in the final section.

The high mtDNA diversity of *M. hapla* relative to the other four parthenogenetic species combined could reflect multiple origins of parthenogenesis from a variable stock of sexual females (e.g., Moritz and Heideman 1991). Studies of nuclear genes (repetitive DNA analysis of Castagnone-Sereno et al. 1993) and gene products (isozyme studies of Esbenshade and Triantaphyllou 1987) have revealed much lower diversity in *M. hapla* than in the other parthenogenetic species. The combination of relatively high mtDNA diversity and low nuclear gene diversity tends to favor the argument that *M. hapla* is old. However, surveys of mtDNA and nuclear gene diversity in the facultative meiotic forms from which the parthenogens are supposed to have arisen (Triantaphyllou 1985) are needed to resolve this question.

**Effect of AT Bias on Nucleotide and Amino Acid Sequence Divergence**

Relative to differences between *A. suum* and *C. elegans*, the two species of *Meloidogyne* show a similar level of overall nucleotide sequence divergence, but a two-fold increase in inferred amino acid sequence difference. This disparity could be due to one or more of three causes: (1) underestimation of actual nucleotide divergence between *Meloidogyne* because of earlier sat-

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**Table 3**

$\chi^2$ Values of Tests for Heterogeneity of Rates of Amino Acid Substitution

<table>
<thead>
<tr>
<th>OUTGROUP</th>
<th>%AT DIFFERENCE $^a$</th>
<th>C. elegans</th>
<th>A. suum</th>
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<tbody>
<tr>
<td>Tests to <em>M. hapla</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. mellifera</em> . . . .</td>
<td>1.3</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td><em>A. gambiae</em> . . . .</td>
<td>9.5</td>
<td>5.8*</td>
<td>4.4*</td>
</tr>
<tr>
<td><em>L. terrestris</em> . . . .</td>
<td>20.4</td>
<td>9.0**</td>
<td>6.5**</td>
</tr>
<tr>
<td><em>S. purpuratus</em> . . . .</td>
<td>74.3</td>
<td>9.0**</td>
<td>6.7**</td>
</tr>
<tr>
<td><em>G. gallus</em> . . . .</td>
<td>30.9</td>
<td>15.0**</td>
<td>12.0**</td>
</tr>
</tbody>
</table>

| Tests to *M. javanica* |                      |            |         |
| *A. mellifera* . . . . | 2.5 | 0.3 | 0.1 |
| *A. gambiae* . . . . | 10.7 | 7.41** | 5.7* |
| *L. terrestris* . . . . | 21.6 | 6.3* | 4.2* |
| *S. purpuratus* . . . . | 25.5 | 10.37** | 7.68** |
| *G. gallus* . . . . | 32.1 | 6.81** | 5.12* |

$^a$ Difference in AT content between the *Meloidogyne* taxon and the outgroup, from table 2.

$^b$ Based on equation 4 of Tijima (1993). One-way tests of significance only.

* Significant at $P = 0.05$.

** Significant at $P = 0.01$. 

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**Fig. 4.—**Neighbor-joining tree of amino acid differences for nematodes with outgroups (A) *L. terrestris* and (B) *A. mellifera*. Numbers on branches are the estimated numbers of amino acid differences (changes). This is based on an alignment (not shown) using 264 codons with all gaps excluded.
uration of base substitution in their more AT-rich sequences (due to either earlier divergence or higher mutation rate), (2) accelerated rates of amino acid substitution in the more AT-rich sequences of *Meloidogyne*, or (3) positive selection for amino acid substitutions in *Meloidogyne*. The third hypothesis is difficult to test with the available data, but does not seem consistent with the consistency of patterns across the three protein-coding genes examined.

Under hypothesis 1, the true value of nucleotide sequence difference between the two species of *Meloidogyne* is actually greater than that between *A. suum* and *C. elegans*, but the difference is not apparent because of greater saturation effects associated with strong AT bias. Supporting this interpretation, the proportional sequence difference between the *Meloidogyne* species is greater than that for the other pair of nematodes in the gene with the lowest A1 content (COII, table 1). Differences in four-fold sites in conserved amino acids should give an estimate of divergence less biased by amino acid constraints and mutation bias, and differences in these sites appear to have reached saturation levels among all comparisons.

Two lines of evidence against greater saturation between *Meloidogyne* spp. than between *A. suum* and *C. elegans* are: (1) Of the variable codons, *Meloidogyne* spp. have a lower proportion of codons with multiple differences (49/99) than do *A. suum* and *C. elegans* (32/44). (2) In the conserved blocks of 16S rRNA gene (fig. 1), the within-pairs differences (22–23) are a third of those between the two groups (62–66). Altogether these observations suggest that sequence divergence between *Meloidogyne* spp. may not be sufficiently in excess of that between *A. suum* and *C. elegans* to explain all of the observed differences in K_s/K_s.

The second hypothesis suggests that amino acid substitution is accelerated by the high AT content of the *Meloidogyne* lineage, as postulated for rapid evolution in the similarly AT-rich honeybee mtDNA (Crozier, Crozier, and MacKinlay 1989, Jermiin and Crozier 1994a). AT content correlates with amino acid content; for example, shifting away from Gly, Ala, Arg, and Pro toward Phe, Leu, Tyr, Met, Ile, Asn, and Lys. This represents a shift from four-fold to two-fold degenerate codons (Clary and Wolstenholme 1985, Jukes and Wilson 1966) and so a greater dependency on transitions for synonymous mutations.

These effects are evident in the comparisons here, with *Meloidogyne* mtDNA having more two-fold (242 vs. 213) and fewer four-fold (59 vs. 96) synonymous sites than the other species, and also a higher equilibrium transition-to-transition ratio (3.6/2.7, see Holmquist 1983). Thus, randomly located mutations, especially transversions, are more likely to be nonsynonymous in *Meloidogyne* and while there are similar numbers of third-position transversions (68 vs. 74), twice as many of these (49 vs. 26) are nonsynonymous in *Meloidogyne*.

However, the loss of degeneracy in third positions cannot alone explain directly all the difference in K_s/K_s. While *Meloidogyne* spp. have three times as many codons with only a third position nonsynonymous difference as between *A. suum* and *C. elegans* (15 vs. 6), they also have eight times as many codons with only a first-position nonsynonymous difference (24 vs. 3), half of these being Met→Leu and Val→Ile changes.

Another line of evidence supporting the second hypothesis comes from the relative rate tests, which demonstrated accelerated amino acid substitution using all outgroups except for the extremely AT-rich *Apis mellifera*. The discrepancy appears to reflect AT-driven convergence in amino acid sequences of *Meloidogyne* and *Apis*. This would effectively draw the root node toward *Meloidogyne* and away from *A. suum* and *C. elegans*. This sequence convergence from composition convergence is slight (data not shown), with coincidental matches becoming a false phylogenetic signal only at high proportions of differences, and so would be most apparent among distant comparisons.

The above effect of AT bias on amino acid sequences was not apparent in the more extensive study by Jermiin and Crozier (1994a). They found a nonsignificant correlation between AT content and amino acid sequence divergence among insect orders for the COII data of Liu and Beckenbach (1992). Of the 114 Cyt b sequences analyzed by Jermiin and Crozier (1994a) (range of 49%–81% AT), the values seen in *Meloidogyne* and *Apis mellifera* (and *Romanormermis culicivorax*, see Powers, Harris, and Hyman 1993; Hyman and Azevedo 1996) are the highest, whereas the AT contents for *C. elegans*, *A. suum*, and most of the insects used by Jermiin and Crozier (1994a) are just above the middle of the range. What limit there is to the AT richness of coding sequences and whether the taxa studied here are near that limit remain to be seen.

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**LITERATURE CITED**


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