Journal of Nematology 34(4):319–327. 2002.
© The Society of Nematologists 2002.

Phylogenetic Analyses of *Meloidogyne* Small Subunit rDNA

Irma Tandingan De Ley,[1] Paul De Ley,[1] Andy Vierstraete,[2] Gerrit Karssen,[3] Maurice Moens,[4] and Jacques Vanfleteren[2]

Abstract: Phylogenies were inferred from nearly complete small subunit (SSU) 18S rDNA sequences of 12 species of *Meloidogyne* and 4 outgroup taxa (*Globodera pallida, Nacobbus abberans, Subanguina radicicola,* and *Zygotylenchus guevarai*). Alignments were generated manually from a secondary structure model, and computationally using ClustalX and Treealign. Trees were constructed using distance, parsimony, and likelihood algorithms in PAUP* 4.0b4a. Obtained tree topologies were stable across algorithms and alignments, supporting 3 clades: clade I = [*M. incognita* (*M. javanica, M. arenaria*)]; clade II = *M. duytsi* and *M. maritima* in an unresolved trichotomy with (*M. hapla, M. microtyla*); and clade III = (*M. exigua* (*M. graminicola, M. chitwoodi*)). Monophyly of [(clade I, clade II) clade III] was given maximal bootstrap support (mbs). *M. artiellia* was always a sister taxon to this joint clade, while *M. ichinohei* was consistently placed with mbs as a basal taxon within the genus. Affinities with the outgroup taxa remain unclear, although *G. pallida* and *S. radicicola* were never placed as closest relatives of *Meloidogyne.* Our results show that SSU sequence data are useful in addressing deeper phylogeny within *Meloidogyne,* and that both *M. ichinohei* and *M. artiellia* are credible outgroups for phylogenetic analysis of speciations among the major species.

Key words: 18S, *Globodera, Meloidogyne* spp., *Nacobbus,* nematode, phylogeny, rDNA, root-knot nematode, SSU, *Subanguina, Zygotylenchus.*

Root-knot nematodes are worldwide in distribution, and morphologically and genetically diverse, and four polyphagous species (*M. arenaria, M. hapla, M. incognita,* and *M. javanica*) among more than 80 nominal species on record (Eisenback, 1997; Karssen and van Hoenselaar, 1998) are considered of major economic importance. With the exception of the meiotic parthenogen *M. hapla* race A, these four species are mitotic (=ameiotic) parthenogens, although all exhibit diversity of ploidy within species (Triantaphyllou, 1966). Other species like *M. chitwoodi* and *M. fallax,* which are important on fewer agricultural crops, are meiotic parthenogens. Strictly amphimictic species that either have limited host range (*M. carolinensis;* Eisenback, 1982) or show certain host preferences (*M. microtyla*; Townshend et al., 1984) were previously hypothesized to be ancestral to the parthenogenetic forms (Triantaphyllou, 1985). Molecular methods now allow the investigation of evolutionary relationships on the basis of characters that are independent from host range or reproductive behavior. In this paper, we present the first rRNA-based phylogenetic analysis of a reasonably diverse set of root-knot nematodes, including not only the four species of greatest economic importance but also eight species with less extensive or no recorded impact on agriculture.

Significance of rRNA in phylogenetic inference: As with other eukaryotes, the nematode rDNA cistron typically consists of several hundred tandemly repeated copies of the transcribed units (small subunit or SSU or 18S; large subunit or LSU or 28S; 5.8S; internal and external transcribed spacers) and an external nontranscribed or intergenic spacer (Hillis and Dixon, 1991). The highly conserved gene sequences (SSU and LSU) and highly variable spacer sequences (NTS and ITS) are considered most useful for phylogenetic inference between very distant species for the former, and very closely related species for the latter (Page and Holmes, 1998). Extent of sequence divergence is used as a parameter to estimate relatedness of taxa (Files and Hirsh, 1981). SSU sequence information has been used to estimate the phylogenetic history of phylum Nematoda (Blaxter et al., 1998) and infer affinities between genera within Tylenchida (Szalanski et al., 1997).

Phylogenetic relationships in Meloidogyne: The evolutionary relationships of *Meloidogyne* have been inferred from several types of data. Triantaphyllou (1966, 1985) inferred the phylogeny of root-knot nematodes from cytogenetic data and outlined the evolutionary pathway from amphimixis to mitotic parthenogenesis. It was hypothesized that in the process various levels of ploidy are established, with mitotic parthenogenesis arising directly from meiotic parthenogenesis following the suppression of the meiotic process during oocyte maturation.

Meloidogyne proteins and isozymes have also been characterized and phenotypes have been used in species diagnostics as well as for phylogenetic inference. One of the earliest studies involving the use of protein analysis showed that *M. arenaria* and *M. javanica* are more closely related to each other than to *M. incognita,* and that *M. hapla* is more distantly related to *M. arenaria, M. javanica,* and *M. incognita* (Dalmasso and Bergé, 1978; Dickson et al., 1971). As inferred from 27 enzymes, *M. hapla, M. microtyla, M. chitwoodi, M. naasi, M. graminis,* and *M. graminicola* are assigned to a monophyletic clade, while the mitotic species *M. incognita,*

Received for publication 13 February 2002.
[1] Department of Nematology, University of California, Riverside, CA 92507.
[2] Department of Biology, University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium.
[3] Plant Protection Service, Geertjesweg 15, 6706 EA Wageningen, Netherlands.
[4] Agricultural Research Center, Burg. Van Gansberghelaan 96, B-9820, Merelbeke, Belgium.
The authors thank J. G. Baldwin, E. C. Bernard, W. Bert, J. Bridge, R. Hussey, A. McGuidwin, and J.-C. Prot for providing nematodes. We are also grateful to J. G. Baldwin, P. Roberts, and the anonymous reviewers for constructive comments.
E-mail: *itdeley@citrus.ucr.edu*
This paper was edited by B. C. Hyman.

M. javanica, M. microcephala, and *M. arenaria* belong to another (Esbenshade and Triantaphyllou, 1987).

On the basis of percentage nucleotide base substitution estimates obtained from DNA hybridization studies of total genomic DNA using Eco RI (Xue et al., 1992), and of *Bam*HI -digested DNA fragments of total genomic DNA (Castagnone-Sereno et al., 1993), *M. arenaria* and *M. javanica* are more closely related to each other than they are to *M. incognita*. Together, the clustering of these three species agreed with relationships inferred from DNA amplification fingerprinting (DAF) involving 400 DNA fragments (Baum et al., 1994) and from 400 proteins and 192 amplified fragments of total genomic DNA (Van der Beek et al., 1998). With both types of data, *M. hapla* race A and race B together belong to one clade, while the 3 species *M. incognita, M. javanica,* and *M. arenaria* form another, and the Gramineae-specialized species *M. naasi, M. chitwoodi,* and *M. fallax* are distant from the rest, with high similarity between *M. chitwoodi* and *M. fallax*. All but the mitochondrial DNA data (Hyman and Powers, 1991; Powers and Sandall, 1988) were congruent with respect to the inferred relationships between the three major ameiotic species.

More recently, De Ley et al. (1999) and Hugall et al. (1999) reported that Internal Transcribed Spacer (ITS) sequences were polymorphic within individual nematodes of several *Meloidogyne* species. Comparison of ITS phylogenies with mitochondrial markers led Hugall et al. (1999) to suggest that these polymorphisms may have resulted from at least two hybridization events. These difficulties with ITS phylogeny of *Meloidogyne* are compounded by the absence of outgroup sequences that are easy to align, and because of extensive divergence between the ITS sequences of *Meloidogyne* species compared to those of other Tylenchida (unpubl. data). To assess ancient molecular relationships within *Meloidogyne,* and to discover better clues to their relationships with other Tylenchida, the present work investigated a more slowly evolving ribosomal locus, the SSU rRNA gene (De Ley et al., 2001) of an ecologically wider and more representative range of root-knot nematodes.

Materials and Methods

Many of the amphimictic species studied by Triantaphyllou (1966, 1985) are no longer available in culture or from original sampling localities. Therefore, to maximize diversity in our dataset, we have included several recently described species from various natural habitats, selected on the basis of their current availability and favoring morphological disparity. Fourteen SSU rDNA gene sequences were determined by direct DNA sequencing in the senior author's laboratory, while four (*Globodera pallida, Subanguina radicicola, M. arenaria,* and *M. incognita*) were downloaded from GenBank. We studied 14 isolates representing 13 species of *Meloidogyne* (Table 1) with considerable diversity in reproductive strategy, natural host preferences, and geographical locations. Where needed, identity was verified based on perineal patterns and morphometrics of adult females, morphometrics of adult males and second-stage juveniles (J2), and isozyme electrophoresis of young egg-laying females (Esbenshade and Triantaphyllou, 1985; Karssen et al., 1995; data not included).

DNA extraction and amplification protocols: For each isolate, 10 J2 from one hatched egg mass, or a single male or female, were cut in 20 µl worm lysis buffer (50 mM KCL, 10 mM Tris-Cl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, and 0.45% Tween 20) on a sterile glass slide. Each lysate was transferred to a microcentrifuge tube and digested with 60 µg/ml proteinase K before freezing at −80 °C for at least 10 minutes. Incubation was then performed at 65 °C for 1 hour, followed by proteinase K inactivation at 95 °C for 10 minutes. Centrifugation was done for 3 minutes at 13,000 rpm.

Genomic DNA (5 µl) was used directly as template for double-stranded PCR in 50 µl reaction volume containing 5 µl of 10X reaction buffer, 2.5 mM of $MgCl_2$, dNTP-mix at 0.2 mM each, 1 µM each of the primers G18S4 and 18P (Table 2), and 0.02 U/µl of Taq polymerase (Goldstar, Eurogentec, Belgium). PCR mixtures were covered with 10 µl of mineral oil and DNA was amplified for 40 cycles, each involving denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 2 minutes, followed by a 5-minute polymerization at 72 °C. Amplified PCR product was electrophoretically fractionated in 40 ml 1X TAE buffer in 1% agarose gel (Ultrapure Gibco BRL Life Technologies, UK) and visualized by staining with 0.003% ethidium bromide (0.02 µg/ml). A 1 Kbp DNA ladder (BRL Life Technologies, UK) was used as size markers. Gels were viewed on a UV transilluminator.

DNA sequencing: PCR products (3 µl) were purified with 1.2 µl each of exonuclease I (10 U/µl) and shrimp alkaline phosphatase (2 U/µl) by incubating the mixture for 15 minutes at 37 °C, followed by enzyme inactivation for 15 minutes at 80 °C. In addition to the amplification primers, eight sequencing primers (Table 2) were used to ensure proper overlapping of fragment products from both strands to obtain sequences of the entire SSU region. Sequencing reactions were performed using Ampli-Taq FS DyeDeoxy Terminator Cycle Sequencing kits (PE Applied Biosystems, UK) following the manufacturer's protocol, and implementing 40 cycles, each consisting of denaturation at 96 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 60 °C for 4 minutes, prior to fractionation using an ABI Prism ™ 377 automated sequencer (Perkin-Elmer/Cetus, Foster City, CA). Sequences obtained in our laboratory included the whole length of 5′ to 3′ of SSU; however, GenBank-downloaded sequences were shorter, and so sequences in the final dataset were

TABLE 1. *Meloidogyne* isolates/species, host plants and sources, and outgroup nematode taxa.

Species/Isolates	Plant host/cultivar	Origin	Source of material
M. arenaria 2	*Lycopersicon esculentum* Mill. cv Moneymaker [originally from *Glycine max* (L) Merr.]	Georgia, USA	R. Hussey
M. artiellia	*Brassica oleracea* L.	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. chitwoodi (Lok A)	*Solanum tuberosum* (Bintje)	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. duytsi	*Elymus farctus* (Viv.) Meldris	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. exigua	*Coffea arabica* L.	(no information)	J. Bridge IIP, St. Albans, UK
M. graminicola (WB)	*Oryza sativa* L.	Batangas, Philippines	J.C. Prot IRRI
M. hapla A (16–18)	*Lycopersicon esculentum* Mill. cv Moneymaker	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. hapla B (C4900)	*L. esculentum* Mill. cv Moneymaker	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. ichinohei	*Iris laevigata*	Japan	G. Karssen Plt. Prot. Service, Netherlands
M. incognita	*L. esculentum* Mill. cv Moneymaker [originally from *G. max* (L.) Merr.]	Georgia, USA	R. Hussey
M. javanica	*L. esculentum* Mill. cv Moneymaker	China	G. Karssen Plt. Prot. Service, Netherlands
M. maritima	*Ammophila arenaria* (L.) Link	The Netherlands	G. Karssen Plt. Prot. Service, Netherlands
M. microtyla	(no information)	USA	A. MacGuidwin Madison, Wisconsin, USA
M. trifoliophila	*Trifolium repens* L.	USA	E.C. Bernard University of Tennessee, USA
Globodera pallida	—	—	GenBank accession #AF036592
Nacobbus abberans	*L. esculentum* Mill. cv Moneymaker	Argentina	J.G. Baldwin University of California, USA
Subanguina radicicola	—	—	GenBank accession #AF202164
Zygotylenchus guevarai	*Pistacia vera* L.	Iran	W. Bert RUG, Belgium

reduced by 40 bp downstream of 5′ G18S4 and 105 bp upstream of 3′ 18p primer. These sequences (accession #s AF442189-AF442200, AF535867, AF535868) and the secondary structure alignment (NCBI REF 1107784) are available in GenBank.

Sequence alignments: Boundaries of the SSU gene were determined by comparison with sequences (*Caenorhabditis elegans* and *M. arenaria*) previously published and deposited in GenBank. Identical sequences were obtained for *M. hapla* A and *M. hapla* B, while *M. trifoliophila* and *M. graminicola* differed by just a single base. To maximize computational speed, only one sequence of each pair was included in subsequent analyses. Four outgroup taxa (*S. radicicola, G. pallida, Z. guevarai,* and *N. aberrans*) were selected on the basis of their possible close relationship to *Meloidogyne* spp. as inferred from SSU ribosomal sequences (Blaxter et al., 1998; De Ley and Vanfleteren, unpubl. data). All four sequences were aligned with the 12 retained *Meloidogyne* sequences. A secondary structure alignment was constructed using Dedicated Comparative Sequence Editor (DCSE v. 3.4) (De Rijk and De Wachter, 1993), based on the unweighted SSU rRNA model of Van de Peer et al. (1998) derived from base-pairings detected as compensating mutations. Stems and loops were given equal weights in the secondary structure alignment because it

TABLE 2. List of amplification and sequencing primers used in this study.

Primer	Sequence	Location based on *Meloidogyne arenaria* U42342 and *Caenorhabditis elegans* (in parentheses)
G18S4	5′ GCT TGT CTC AAA GAT TAA GCC 3′	777–790 (Forward, 37–50)
9FX	5′ AAG TCT GGT GCC AGC AGC CGC 3′	1289–1309 (531–550)
9R	3′ GTC GGC GCC ATT AAG GTC GA 5′	1303–1322 (545–564)
26R	3′ GCT TTC GTA AAC GGA AGA ATG 5′	1669–1689 (902–922)
4F	5′ TCA AGG ACG AAA TC AGA GG 3′	1700–1719 (933–952)
4R	3′ GRT CGA MGG CGA TTA GAT AC 5′	1719–1738 (952–971)
2FX	5′ GGA AGG GCA CCA CCA GGA GTG G 3′	1882–1903 (1118–1139)
23F	5′ ATT CCG ATA ACG AGC GAGA 3′	2046–2064 (1272–1290)
13R	3′ ATT GTC CAG ACA CTA CGG G 5′	2154–2172 (1379–1397)
18P	3′ CAC TTG GAC GYC KWC CTA GT 5′	2505–2524 (Reverse, 1732–1751)

has been demonstrated that differential phylogenetic weighting in terms of stems and loops is unsatisfactory (Hickson et al., 1996). Secondary structures thus inferred for SSU and LSU rDNA were recently confirmed by experimental X-ray diffraction results (Wuyts et al., 2001).

Two computer alignment programs were used to generate data matrices. CLUSTALX v. 1.64 (Jeanmougin et al., 1998) was used to generate a total of 22 automated alignments of the primary structure. The first dataset had combinations of gap opening penalties (GOP) 5, 15, and 30 and gap extension penalties (GEP) 3, 6.66, and 10 and with smaller values of GOP and GEP (1, 1). Another set of GOP and GEP combinations similar to those of Morrison and Ellis (1997) were used to generate data matrices: values were increased logarithmically by choosing GOPs of 4, 8, and 16 and GEPs 1, 2, 4, and 8. In a study of SSU rDNA data of Apicomplexa, these GOPs produced trees that were most similar to that of the secondary structure alignment, while GEP values below 0.1 had little effect for all GOPs tested (Morrison and Ellis, 1997). Limited manual editing for all alignments was done with GeneDoc (Nicholas and Nicholas, 1997; Nicholas et al., 1997). Sequence formats were converted using Forcon 1.0 (Raes and Van de Peer, 1999).

The second alignment program used was TREEALIGN (Hein, 1989), a multiple-sequence alignment program that simultaneously builds trees and aligns the DNA sequences using a combination of distance matrix and approximate parsimony methods. Alignments and tree construction are results of modifying gap penalty function (gk = a + k*b) variables, where k = length of indel, and *a* and *b* are GOP and GEP, respectively. Default values used and gap weight combinations (default: 8 + 3k) were the same as those used in CLUSTALX alignments (except when GOP/GEP combination did not meet the program's requirement).

Phylogenetic analyses: We tested the model of DNA substitution that best fits the secondary structure-aligned dataset by computing and comparing log likelihoods using Model Test (Posada and Crandall, 1988). GTR + I + Γ was the best fit, where GTR is general-time reversible model, I represents the proportion of invariable sites, and Γ is the gamma distribution shape parameter. A two-step procedure was then performed on all datasets to reconstruct phylogeny based on the above evolutionary model.

Molecular phylogenetic relationships were reconstructed using distance-based (neighbor joining, NJ; Saitou and Nei, 1987), and discrete character-based (maximum parsimony and maximum likelihood, respectively MP and ML) algorithms implemented in the test versions of PAUP* m4.0b10 (Swofford, 2000). During the analyses involving alignment from the secondary structure, phylogeny trees were rooted alternately with the four outgroup taxa to compare tree topologies. Subsequent analyses with computer-generated alignments were carried out with *S. radicicola* as the root. Gaps were always treated as missing data.

To discover the most parsimonious trees, MP was performed with a heuristic search (3,000 replicates of random addition sequences, tree-bisection-reconnection branch swapping algorithm, and steepest descent not in effect). Starting trees were obtained by stepwise addition. To search for the best likelihood and distance trees and to establish realistic assumptions of evolution within the framework of GTR (Waddell and Steel, 1997; Yang et al., 1994), NJ calculations and ML heuristic searches were performed as follows.

Approximation/Estimation step. NJ distance calculations were subjected to 3,000 bootstrap replications to estimate overall support for monophyly. Multiple substitutions for NJ were corrected using the Log Determinant distance measure (Lockhart et al., 1994). MP heuristic analysis was performed twice: (i) with 3,000 bootstrap replicates using the fast but less accurate default settings, and (ii) without bootstrapping to allow more accurate but computationally more demanding settings (i.e., 100 replicates of random branch addition). Finally, a non-bootstrap heuristic ML search was also conducted with 100 replicates of random branch addition (PAUP*4.0b4a does not allow ML bootstrapping with more than 12 taxa, as this exceeds current computational capabilities). All resulting MP, NJ, and ML trees were used to estimate the substitution rate (six substitution types), relative base frequencies, gamma distribution shape parameter, and proportion of invariable sites. The trees were then compared statistically using the Kishino and Hasegawa (1989) pairwise tests with

ML and MP optimality criteria, and Templeton test with MP optimality criterion.

Optimization step. During a second step, the best trees obtained were then used as starting trees to optimize the NJ and ML settings, by assigning the previously obtained parameter estimates as set values for the second round of ML heuristic searches and NJ tree construction (both with 3,000 bootstrap replicates). Finally, optimized NJ and ML trees were compared with all previously generated trees using the same pairwise tests as before.

The following measures of homoplasy were computed: Consistency index (CI) (Kluge and Farris, 1969), Retention index (RI) (Farris, 1989), re-scaled CI (Farris, 1989), and the skewness test statistic ($g1$) (Hillis and Huelsenbeck, 1992; Sokal and Rohlf, 1981). The latter was determined by generating 10^6 random trees and measuring the skewness of the resulting tree-length distribution as an estimator of phylogenetic signal. Obtained $g1$ values were compared with critical values listed by Hillis and Huelsenbeck (1992).

Data matrices were further analyzed with Quartet puzzling maximum likelihood (Puzzle 4.0.2) (Strimmer and von Haeseler, 1996). This method infers phylogeny by dividing the set of sequences into quartet, finding the optimal tree for each quartet, and combining most of the four-taxa maximum likelihood trees to construct an overall approximation of the best maximum likelihood tree. These analyses involved a total of 1,000 puzzling steps and exact parameter estimates using the HKY (Hasegawa et al., 1985) model of substitution and gamma distributed rate of heterogeneity.

Phylogenetic trees were examined with TREEVIEW 1.6 (Page, 1996) and converted into graphic files for Adobe Illustrator 9 (Adobe Systems Inc., San Jose, CA).

Results

Sequence analyses: The nearly complete SSU sequences obtained from the 16 included taxa consisted of 1,577 (*M. incognita* and *M. microtyla*) to 1,597 (*M. artiellia*) bp. The CLUSTALX alignments contained 1,613-1,710 positions, of which 325-382 (19–24%) were variable and of which 159-227 (9–14%) were parsimony-informative. The secondary structure alignment comprised 1,625 positions, of which 365 (22%) were variable with 218 (13%) informative characters. This alignment resulted in six positions more than the CLUSTALX default alignment (with GOP = 15 and GEP = 6.66), having an equal number of informative characters, and one less variable position. Eighteen nucleotide ambiguities were found in *M. javanica,* three in *M. arenaria,* two in *M. hapla,* and one in *Nacobbus aberrans.* Sequence divergence within the ingroup ranged from complete identity between *M. hapla* A and *M. hapla* B to 10% between *M. ichinohei* and the species *M. maritima, M. arenaria,* and *M. exigua. Meloidogyne trifoliophila* and *M. graminicola* differed by only one substitution. A + T content ranged from 49% in *M. graminicola* to 53% in *G. pallida.* All performed X^2 tests confirmed a high level of homogeneity ($P > 0.999$) in base frequencies across taxa (data not included). This indicates that the sequences satisfied the basic assumptions of base composition equilibrium for phylogenetic models of inference.

*Secondary structure alignment and PAUP*4.0b4a:* Tree topologies within *Meloidogyne* were fairly consistent irrespective of algorithm and outgroup taxon, with a single best tree each produced by MP and ML during the parameter estimation step. In all these analyses, MP pairwise tests (Kishino and Hasegawa, 1989) scored MP and ML trees as equally parsimonious while the NJ tree was not significantly worse. On the other hand, ML pairwise tests (Kishino and Hasegawa, 1989) supported NJ as the most likely tree. The same best trees were obtained during the optimization step.

Topology within the ingroup was fairly consistent in all best trees that were generated, and is represented by the parsimony tree in Figure 1. *Meloidogyne javanica* and *M. arenaria* are sister taxa with 89–92% bootstrap support (bs) in all the NJ and MP analyses, both belonging to a clade that had *M. incognita* as a basal taxon. This group, henceforth referred to as clade I, had 100% bootstrap support and was a sister clade to a less resolved group (clade II) that included one poorly supported clade comprised of *M. duytsi* and *M. maritima*; and a strongly supported (91–98% bs) pair of sister

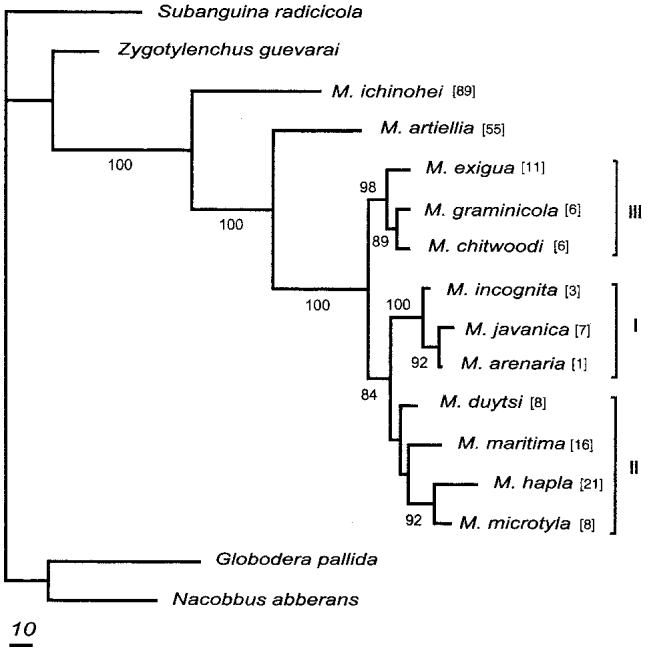

Fig. 1. Maximum parsimony tree based on the secondary structure alignment. This tree also represents the congruent topologies obtained from different CLUSTALX alignments combined with neighbor joining, maximum parsimony, and maximum likelihood analyses. *Subanguina* was designated as outgroup; numbers next to branches are bootstrap values obtained with MP of the secondary structure alignment; numbers in brackets are autapomorphies.

taxa, *M. microtyla* and *M. hapla*. NJ trees showed a very weak support (56–57% bs) for *M. duytsi* and *M. maritima* as sister taxa, placing the two in a trichotomy with clades I and II. Although MP and ML trees consistently placed *M. duytsi* as basal taxon to the other three species (*M. maritima; M. microtyla* and *M. hapla*), this topology had less than 50% bs and was interpreted as an unresolved polytomy with clades I and II. However, monophyly of clade I and this less-resolved group has moderate-to-strong support (71–84% bs) and was placed as sister to highly supported clade III (98–100% bs), which included *M. graminicola* and *M. chitwoodi* as sister taxa (89–96% bs) with *M. exigua* as basal taxon. *Meloidogyne artiellia* was consistently placed with maximal support (100% bs) as sister taxon to this joint clade, while *M. ichinohei* was consistently placed as basal taxon within the genus, also with maximal support.

Among the outgroups, *Z. guevarai* was never placed in polyphyly with *Meloidogyne*. Variations in tree topology were mainly attributed to the changes in placement of outgroup taxa and had no effect on the basic tree topology within *Meloidogyne*.

*Clustal alignments and PAUP*4.0b4a:* MP and ML each produced a single best tree with the computer-aligned datasets. Two MP trees were generated as the GOP-GEPs increased from 30-3 to 30-10. MP pairwise tests (Kishino and Hasegawa, 1989) supported MP trees, while ML pairwise tests (Kishino and Hasegawa, 1989) supported ML trees. As GOP-GEPs were increased to 30-6.66 and 30-10, ML trees were found equally parsimonious with best MP trees, and both ML and MP were equally likely. Similarly, all the remaining computer alignments produced one best tree each for MP and ML analyses. As can be expected, ML trees were best supported by ML pairwise tests, while MP trees were supported by MP pairwise comparisons in most of the analyses involving computer-aligned sequences.

A congruent tree topology was observed between trees obtained from the computer-generated alignments and those of the secondary structure alignment as shown in Figure 1.

Quartet puzzling maximum likelihood: All 21 ML trees generated by Puzzle were congruent with the basic tree topologies derived with PAUP*4.0b4a from secondary structure alignment and the CLUSTALX alignments (Fig. 2). All three individual clades, as well as the monophyly of ((I, II) III), were strongly supported. In addition, within clade II (*M. duytsi, M. maritima*) received variable degrees (68–80 %) of bootstrap support as sister taxon to (*M. hapla, M. microtyla*).

Treealign: Sixteen trees generated from 16 gap penalty combinations had mutually inconsistent tree topologies in terms of the placement of *M. duytsi* and *M. maritima* and the assignment of basal species within clades I and II (Fig. 3). Basal taxa within clade I and clade II were, respectively, *M. arenaria* (vs. *M. incognita*) and *M. chitwoodi* (vs. *M. exigua*). The poorly resolved

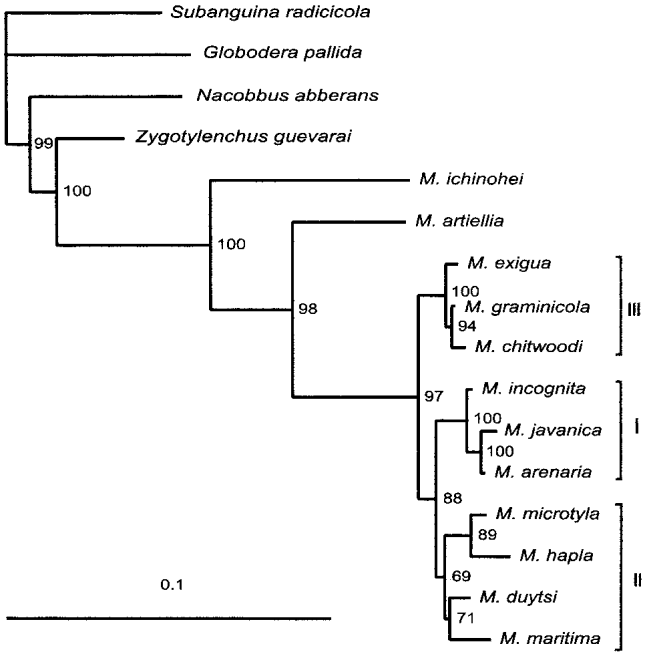

FIG. 2. Quartet (Maximum Likelihood) Puzzle tree based on CLUSTALX alignment (default values GOP = 5, GEP = 6.66). This tree also represents the congruent topologies obtained from the secondary structure and other CLUSTALX alignments. *Subanguina* was designated as outgroup; numbers are bootstrap values.

species *M. duytsi* and *M. maritima* were placed in clades I and II, and as basal taxa within the joint clades I, II, and III. Two penalty combinations (gk = 5 + k*3 and gk = 4 + k*4) resulted in tree topologies similar to the MP and ML trees obtained from the secondary structure and CLUSTALX alignments. The tree obtained from default values (gk= 8 + k*3) varied only slightly from the previous ones in that *M. maritima* (vs. *M. duytsi*)

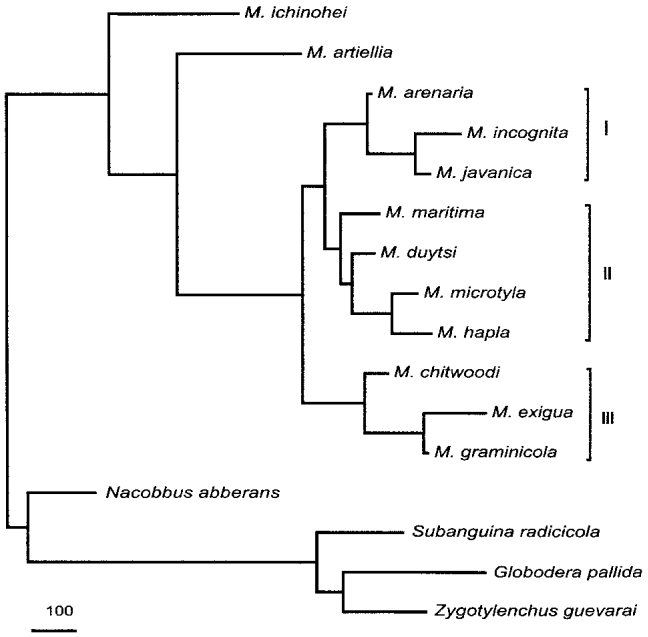

FIG. 3. Phylogeny based on default values of Treealign, 8 + 3k. Rooting was calculated by Treealign.

diverged earlier within clade II. Both *M. ichinohei* and *M. artiellia* were placed in the same positions relative to the joint clades I, II, and III.

Discussion

Our results suggest that SSU rDNA sequence data perform well in resolving deeper relationships within *Meloidogyne*. Tree topology with PAUP*4.0b4a was fairly consistent across different combinations of alignments and tree-construction algorithms, regardless of the designated outgroup taxon. Also, there is an unequivocally high phylogenetic signal in our dataset as revealed by the skewness of tree length distribution, with g_1 values ranging from −0.972 to −1.099. (Hillis and Huelsenbeck, 1992; −0.12 [P = 0.05] to −0.15 [P = 0.01] for 15 taxa, 500 characters). As such, the SSU rDNA gene is useful in providing information about the evolutionary history of *Meloidogyne*. In addition, ambiguities representing possible sequence polymorphism occur in 1% or less of the nucleotides of each sequence.

Neither *Globodera pallida* nor *Subanguina radicicola* were resolved as a sister taxon to *Meloidogyne* in any of our analyses. Although the number of included outgroup taxa was limited, the observed absence of a sister taxon relationship between *G. pallida* and the *Meloidogyne* clade strengthens the case against inclusion of root-knot nematodes in family Heteroderidae (Baldwin, 1992; Geraert, 1997; Wouts and Sher, 1971). This is also in agreement with previous SSU phylogenies (Szalanski et al., 1997; De Ley and Vanfleteren, unpubl. data). *Zygotylenchus guevarai* and *N. aberrans* are both representatives of the family Pratylenchidae, and their placement in the trees generated in the current study is compatible with the suggestion of a closer relationship between family Pratylenchidae and subfamily Meloidogyninae (Geraert, 1997).

Three clades are particularly strongly supported: clade I includes the mitotic parthenogens (*M. incognita, M. arenaria,* and *M. javanica*); clade II has obligatory amphimictic (*M. microtyla*), meiotic and mitotic parthenogens (respectively, *M. hapla* A and B), as well as two species of unknown reproductive strategy (*M. duytsi* and *M. maritima*); and clade III comprises three meiotic parthenogens (*M. exigua, M. graminicola,* and *M. chitwoodi*).

Our analysis placed the ameiotic species on a separate clade that is distantly related to either the obligate amphimictic or meiotic species. This agrees with previous phylogenies of Castagnone-Sereno et al. (1993), Van der Beek et al. (1998), and, aside from a few exceptions, with Esbenshade and Triantaphyllou (1987). Our present analyses robustly support separate branching of meiotic parthenogens in clade III from the obligate amphimictic *M. microtyla,* along with *M. hapla* A (and B), *M. duytsi,* and *M. maritima*. Previous isozyme studies by Esbenshade and Triantaphyllou (1987) placed *M. chitwoodi* and Gramineae-specialized species (*M. graminicola, M. graminis,* and *M. naasi*) in the same clade with *M. microtyla* and *M. hapla* (Fig. 2). Similarly, relationships between the three major species are in agreement with phylogenies estimated from cytogenetic, isoenzyme, or other molecular data. Only mitochondrial DNA data (Powers and Sandall, 1988) placed *M. arenaria* in apposition to sister taxa *M. incognita* and *M. javanica*. Hyman and Powers (1991) subsequently revised some of the identifications in the previous study but retained *M. arenaria* as the more divergent species, and hypothesized that it could actually represent a hybrid lineage. Discordance between the mitochondrial results and those obtained with other data may be due to some or all of the methodological differences in types of characters (e.g., fragment lengths vs. chromosome numbers or nucleotides), sources of data (e.g., rapidly evolving mitochondrial loci vs. a highly conserved ribosomal locus), numbers of included taxa, and tree construction methods (clustering algorithms in UPGMA and NJ vs. optimality criterion in MP, ML or minimum evolution). If hybridization did indeed occur in *Meloidogyne,* then this would be another probable cause of disagreement between mitochondrial and ribosomal trees (Hugall et al., 1999).

Although Puzzle gave moderate support for sister taxon status to the sympatric species *M. duytsi* and *M. maritima* (Karssen et al., 1998), most analyses placed both species in unresolved trichotomy with the sister species *M. hapla* and *M. microtyla*. The inclusion of more species and more loci in these analyses may help resolve relationships in this group.

Previous analyses using ITS sequences did not resolve the position of either *M. ichinohei* or *M. artiellia* as the basal taxon (De Ley et al., 1999). Our attempt in the present study to address deep-level phylogeny within *Meloidogyne* suggested that *M. ichinohei* diverged earlier than the rest of the species included herein. Comparisons based on spermatheca morphology in Tylenchida (Bert et al., 2002) also indicated *M. ichinohei* to be highly distinct from the other species within the genus in terms of the number and arrangement of cells. Interestingly, *M. ichinohei* also failed to infect or establish in plant hosts other than *Iris laevigata* (Karssen, unpubl. data).

Overall, the evolution of reproductive modes as hypothesized by Triantaphyllou is still largely compatible with the tree, but if his assumption of amphimixis as a pleisiomorphic character is correct, then the position of *M. microtyla* as sister taxon to *M. hapla* implies that at least one homoplastic change must have occurred. Therefore, a detailed character mapping analysis must await data on reproductive mode of the other species (*M. ichinohei, M. artiellia, M. duytsi,* and *M. maritima*).

Our study constitutes the first report of phylogenetic inference within the genus *Meloidogyne* using the SSU rDNA locus. Based on our findings, this gene is diver-

gent within the genus and has a high signal-to-noise ratio for phylogenetic reconstruction of deeper relationships. This is not to say that we are fully convinced of the reliability of our molecular phylogenies; the possible occurrence of multiple hybridization events in the evolution of root-knot nematodes (Hugall et al., 1999) limits the confidence that should be placed in any analysis based on a single nuclear locus. Nevertheless, potential nucleotide polymorphisms in our SSU rDNA sequences are rare to absent, and we can cautiously conclude from the robustness of our results that SSU rDNA data do not present the obvious analytical problems encountered with the *Meloidogyne* ITS region. Among the taxa included here, *M. ichinohei* and *M. artiellia* are placed consistently as species with earlier divergence from all others, suggesting that they are appropriate choices for rooting trees in analyses of more rapidly evolving loci. Also, a number of species of lesser economic significance are sufficiently divergent to contemplate diagnostic applications based on SSU rDNA sequence data.

Literature Cited

Baldwin, J. G. 1992. Evolution of the cyst and noncyst-forming Heteroderinae. Annual Review of Phytopathology 30:271–290.

Baum, T. J., P. M. Gresshoff, S. A. Lewis, and R. A. Dean. 1994. Characterization and phylogenetic analysis of four root-knot nematode species using DNA amplification fingerprinting and automated polyacrylamide gel electrophoresis. Molecular Plant-Microbe Interactions, 7:39–47.

Bert, W., G. Karssen, R. Van Driessche, and E. Geraert. 2002. The cellular structure of the female reproductive system within the Heteroderinae and Meloidogyninae (Nematoda). Nematology, in press.

Blaxter, M. L., P. De Ley, J. R. Garey, L. X. Liu, P. Scheldeman, A. Vierstraete, J. R. Vanfleteren, L. Y. Mackey, M. Dorris, L. M. Frisse, J. T. Vida, and W. K. Thomas. 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392:71–75.

Castagnone-Sereno, P., C. Piotte, J. Uijthof, P. Abad, E. Wajnberg, F. Vanlerberghe-Masutti, M. Bongiovanni, and A. Dalmasso. 1993. Phylogenetic relationships between amphimictic and parthenogenetic nematodes of the genus *Meloidogyne* as inferred from repetitive DNA analysis. Heredity 70:195–204.

Dalmasso, A., and J. G. Bergé. 1978. Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp.: Application to the taxonomy of *Meloidogyne*. Journal of Nematology 10:323–332.

De Ley, I. T., G. Karssen, P. De Ley, A. Vierstraete, L. Waeyenberge, M. Moens, and J. Vanfleteren. 1999. Phylogenetic analyses of internal transcribed spacer region sequences within *Meloidogyne*. Journal of Nematology 31:530–531 (Abstr.).

De Ley, I. T., P. De Ley, A. Vierstraete, G. Karssen, M. Moens, and J. Vanfleteren. 2001. Phylogenetic analyses of *Meloidogyne* SSU rDNA. Phytopathology 91 (Supplement): S133 (Abstr.).

De Rijk, P., and R. De Wachter. 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. Computer Applications in the Biosciences 9:735–740.

Dickson, D. W., D. Huisingh, and J. N. Sasser. 1971. Dehydrogenases, acid and alkaline phosphatases, and esterases for chemotaxonomy of selected *Meloidogyne, Ditylenchus, Heterodera,* and *Aphelenchus* spp. Journal of Nematology 3:1–16.

Eisenback, J. D. 1982. Description of the blueberry root-knot nematode, *Meloidogyne carolinensis* n. sp. Journal of Nematology 14:303–317.

Eisenback, J. D. 1997. Root-knot nematode taxonomic database. Wallingford, UK: CAB International.

Esbenshade, P. R., and A. C. Triantaphyllou. 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species. Journal of Nematology 17:6–20.

Esbenshade, P. R., and A. C. Triantaphyllou. 1987. Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). Journal of Nematology 19:8–18.

Farris, J. S. 1989. The retention index and the rescaled consistency index. Cladistics 5:417–419.

Files, J. G., and D. Hirsh. 1981. Ribosomal DNA of *Caenorhabditis elegans*. Journal of Molecular Biology 149:223.

Geraert, E. 1997. Comparison of head patterns in the Tylenchoidea (Nematoda). Nematologica 43:283–294.

Hasegawa, M., H. Kishino, and T-A. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160–174.

Hein, J. 1989. A new method that simultaneously aligns and reconstructs ancestral sequences for any number of homologous sequences, when phylogeny is given. Molecular Biology and Evolution 6:649–668.

Hickson, R. E., C. Simon, A. Cooper, G. S. Spicer, J. Sullivan, and D. Penny. 1996. Conserved sequence motifs, alignment, and secondary structure for the third domain of animal 12S rRNA. Molecular Biology and Evolution 13:150–169.

Hillis, D. M., and M. T. Dixon. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quarterly Review of Biology 66:411–453.

Hillis, D. M., and J. P. Huelsenbeck. 1992. Signal, noise, and reliability in molecular phylogenetic analysis. Journal of Heredity 83: 189–195.

Hugall, A., J. Stanton, and C. Moritz. 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. Molecular Biology and Evolution 16:157–164.

Hyman, B. C., and T. O. Powers. 1991. Integration of molecular data with systematics of plant parasitic nematodes. Annual Review of Phytopathology 29:89–107.

Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. 1998. Multiple sequence alignment with ClustalX. Trends in Biochemical Science 23:403–405.

Karssen, G., A. van Aelst, and W. H. van der Putten. 1998. *Meloidogyne duytsi* n. sp. (Nematoda: Heteroderidae), a root-knot nematode from Dutch coastal foredunes. Fundamental and Applied Nematology 21:299–306.

Karssen, G., and T. van Hoenselaar. 1998. Revision of the genus *Meloidogyne* Göldi, 1892 (Nematoda: Heteroderidae) in Europe. Nematologica 44:713–788.

Karssen, G., T van Hoenselaar, B. Verkerk, and R. Janssen. 1995. Species identification of cyst and root-knot nematodes from potato by electrophoresis of individual females. Electrophoresis 16:105–109.

Kishino, H., and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. Journal of Molecular Evolution 29:170–179.

Kluge, A. G., and J. S. Farris. 1969. Quantitative phylogenetics and the evolution of anurans. Systematic Zoology 18:1–32.

Lockhart, P. J., M. A. Steel, M. D. Hendy, and D. Penny. 1994. Recovering evolutionary trees under a more realistic model of sequence evolution. Molecular Biology and Evolution 11:605–612.

Morrison, D. A., and J. T. Ellis. 1997. Effects of nucleotide sequence alignment on phylogeny estimation: A case study of 18S rDNAs of Apicomplexa. Molecular Biology and Evolution 14:428–441.

Nicholas, K. B., and H. B. Nicholas, Jr. 1997. GeneDoc v. 2.6.000. Multiple Sequence Alignment Editor and Shading Utility.

Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield, II. 1997. GeneDoc: Analysis and Visualization of Genetic Variation. EMBNET.NEWS 4:14

Page, R. D. M. 1996. TREEVIEW: An application to view phylogenetic trees on personal computers. CABIOS 12:357–358.

Page, R. D. M., and E. C. Holmes. 1998. Molecular evolution: A phylogenetic approach. Oxford, UK: Blackwell Science Ltd.

Posada, D., and K. A. Crandall. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817–818.

Powers, T. O., and L. J. Sandall. 1988. Estimation of genetic divergence in *Meloidogyne* mitochondrial DNA. Journal of Nematology 20: 505–511.

Raes, J., and Y. Van De Peer. 1999. Forcon: A software tool for the conversion of sequence alignments. EMBnet. News 6:1; http://vulcan.rug.ac.be/~jerae/Forcon/index.html.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry, 2nd ed. San Francisco: W. H. Freeman.

Strimmer, K., and A. von Haeseler. 1996. Quartet puzzling: A quartet maximum-likelihood method for reconstructing tree topologies. Molecular Biology and Evolution 13:964–969.

Swofford, D. L. 2000. "PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods)", Sunderland, MA: Sinauer Associates, Inc.

Szalanski, A. L., B. J. Adams, and T. O. Powers. 1997. Molecular systematics of Tylenchida using 18S ribosomal DNA. Journal of Nematology 29:607–608 (Abstr.).

Townshend, J. L., J. W. Potter, and T. R. Davidson. 1984. Some monocotyledonous and dicotyledonous hosts of *Meloidogyne microtyla.* Plant Disease 68:7–10.

Triantaphyllou, A. C. 1966. Polyploidy and reproductive patterns in the root-knot nematode *Meloidogyne hapla.* Journal of Morphology 118:403–414.

Triantaphyllou, A. C. 1985. Cytogenetics, cytotaxonomy, and phylogeny of root-knot nematodes. Pp. 113–126 *in,* J. N. Sasser and C. C. Carter, eds. An advanced treatise on *Meloidogyne,* vol. I. Biology and control. Raleigh, NC: North Carolina State University Graphics.

Van De Peer, Y., E. Robbrecht, S. de Hoog, A. Caers, P. De Rijk, and R. De Wachter. 1998. Database on the structure of small subunit ribosomal RNA. Nucleic Acid Research 271:179–183.

Van der Beek, J. G., R. Folkertsma, C. Zijlstra, P. H. G. Van Koert, L. Poleij, and J. Bakker. 1998. Genetic variation among parthenogenetic *Meloidogyne* species revealed by AFLPs and 2D-protein electrophoresis contrasted to morphology. Fundamental and Applied Nematology 21:401–411.

Waddell, P. J., and M. A. Steel. 1997. General time-reversible distances with unequal rates across sites: Mixing Γ and inverse Gaussian distributions with invariant sites. Molecular Phylogenetics and Evolution 8:398–414.

Wouts, W. M., and S. A. Sher. 1971. The genera of the subfamily Heteroderinae (Nematoda: Tylenchoidea) with a description of two new genera. Journal of Nematology 3:129–144.

Wuyts, J., Y. Van de Peer, and R. De Wachter. 2001. Distribution of substitution rates and location of insertion sites in the tertiary structure of ribosomal RNA. Nucleic Acids Research 29:2017–5028.

Xue, B., D. Baillie, K. Beckenbach, and J. Webster. 1992. DNA hybridization probes for studying the affinities of three *Meloidogyne* populations. Fundamental and Applied Nematology 15:35–41.

Yang, Z., N. Goldman, and A. Friday. 1994. Comparison of models for nucleotide substitution used in maximum-likelihood phylogenetic estimation. Molecular Biology and Evolution 11:316–324.