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Phylogeny of Criconematina Siddiqi, 1980 (Nematoda: Tylenchida) based on morphology and D2-D3 expansion segments of the 28S-rRNA gene sequences with application of a secondary structure model

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Summary – The suborder Criconematina is a large group of ecto- and endoparasitic nematodes, including several species of major agricultural importance. The D2-D3 expansion segments of the 28S nuclear ribosomal RNA gene were amplified and sequenced from 23 nominal and six unidentified species from the genera *Mesocriconema*, *Criconemoides*, *Ogma*, *Criconema*, *Xenocriconemella*, *Hemicriconemoides*, *Hemicycliophora*, *Paratylenchus*, *Tylenchulus*, *Trophonema* and *Sphaeronema*, together with outgroup taxa from Tylenchidae (*Aglenchus*) and Atylenchidae (*Eutylenchus*). A sequence alignment optimised using the secondary structure model was analysed using maximum parsimony, maximum likelihood and Bayesian inference approaches under two models. All analyses yielded a similar topology with differences primarily in the position of poorly supported clades. Although some molecular trees differ from the previous morphologically based hypotheses of criconematid phylogeny, maximum likelihood tests did not yield statistically significant differences between some of the tested classical morphological and molecular topologies. DNA data support monophyly for the genera *Mesocriconema*, *Hemicriconemoides* and *Criconema* and reject the hypothesis of a single origin of criconematids with a cuticular sheath or 'double cuticle'. Application of the complex model of rRNA evolution, considering paired nucleotides for the stem and unpaired nucleotides for the loop region, resulted in a majority rule consensus Bayesian tree with unresolved relationships between main clades. This lack of resolution is expected by the low number of independently evolving nucleotides. Sequence divergence in this DNA segment between populations of *Mesocriconema xenoplax*, *M. sphaerocephalum* and *Hemicriconemoides cocophillus* suggest the presence of several sibling species under these taxa names.

Keywords – Bayesian analysis, Criconematidae, evolution, LSU, molecular, rRNA, secondary structure, systematics.

The suborder Criconematina Siddiqi, 1980 includes more than 750 species of ecto- and semi-endo plant-parasitic soil-inhabiting nematodes. Several species, including the citrus nematode *Tylenchulus semipenetrans* and the ring nematode *Mesocriconema xenoplax* are considered to be of major agricultural importance for many countries.

The taxonomy of criconematids has been intensively revised since 1960 (Mehta & Raski, 1971; Khan *et al.*, 1975; Andrássy, 1979; Ebsary, 1981). Unfortunately, the group was revised independently and more or less simultaneously by several nematologists; the confusing result including conflicting definitions of genera as well as contradictions in proposed species synonyms. Recently,

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Opinion 2046 of the International Commission of Zoological Nomenclature (ICZN, 2003) was published in response to Case 3185 (Loof *et al*., 2001). This adjudication, by establishing the type of *Criconema* Hofmänner & Menzel, 1914 as *Eubostrichus guernei* Certes, 1889, allowed *Criconemoides* Taylor, 1936 to be re-established as a valid genus with *Criconema morgense* Hofmänner *in* Hofmänner & Menzel, 1914 as type. Previously this species had been used both by Micoletzky (1925) as the type of *Criconema* and subsequently by Taylor (1936) as the type of *Criconemoides*, the latter, sharing the same type as the senior genus, therefore becoming a junior synonym. As combinations to *Criconemella* De Grisse & Loof, 1965 and then to *Mesocriconema* Andrássy, 1968 were based on the fact that *Criconemoides* had not been validly established, Opinion 2046 also has implications for the validity of *Mesocriconema*.

In the present study we have essentially followed the view of Siddiqi (see Siddiqi, 2000, pp. 556-559) and maintain both *Criconemoides* (species with a closed vulva and submedian pseudolips – which appear as lobes – not separate) and *Mesocriconema* (open vulva and true submedian lobes present and separate) as valid genera. We have not, however, followed Siddiqi's recognition of *Macroposthonia* de Man, 1880 as a valid genus, accepting instead the current concensus that *Macroposthonia* is a *genus dubium* and therefore recognising *Mesocriconema*, the next available name, in its stead. We propose herein to further test the monophyly of *Mesocriconema* and other genera, a test that ultimately contributes to a new basis for future taxonomic revision whilst fully addressing nomenclatural issues.

Due to morphological peculiarities, including structure of the pharyngeal basal bulb, males being degenerate with respect to the feeding system, and the putative absence of phasmids, the criconematids have been considered as a separate lineage within the order Tylenchida. There is a general assumption of their monophyly, although a clear set of unique morphological synapomorphies has not been articulated and assumptions about their origin and relationship to other tylenchids remain controversial.

Based on the pharyngeal structure, Siddiqi (1980) stated that Criconematina might have originated from an ancestor with a broad corpus and strong stylet, as is found in infective females of some species in the suborder Hexatylina. Maggenti *et al*. (1987) suggested that the origin of criconematids was likely to be from "Tylenchidae-like" nematodes and assumed that they diverged at a very early stage of evolution of Tylenchida. The distinction of criconematids from other Tylenchida is reflected in the classification of Maggenti *et al.* (1987) that places them with the rank of superfamily Criconematoidea Taylor, 1936 (compared only to Tylenchoidea Örley, 1880 within Tylenchida). In Siddiqi's (2000) classification they are placed with the rank of suborder Criconematina beside four other suborders within Tylenchida. Despite disagreement of these authors with respect to ranking and including diverse numbers and placement of families and subfamilies, in both systems two lineages of criconematids are identified based on morphological and biological features, *viz*, Tylenchulidae and Criconematidae (Maggenti *et al*., 1987) or Tylenchuloidea and Criconematoidea with Hemicycliophoroidea (Siddiqi, 2000).

With respect to cladistic analysis of Criconematina one attempt has been made with a few morphological characters by Siddiqi (2000). DNA sequence data have never been explored to reconstruct the phylogeny of this group. The eukaryotic large subunit (LSU) of nuclear ribosomal RNA (rRNA), containing highly variable regions termed as divergent domains (D) or expansion segments, has specific advantages for phylogenetic studies. The D2 and D3 expansion segments are the longest expansion fragments in the LSU. They are easily amplified and often used for phylogenetic studies of different groups. Among nematodes this region has been used to infer phylogenetic relationships among species of the genera *Pratylenchus* (Al-Banna *et al.*, 1997; Duncan *et al*., 1999), *Acrobeloides* (De Ley *et al*., 1999), *Steinernema* (Stock *et al*., 2001), *Longidorus* (Rubtsova *et al*., 2001), *Meloidogyne* (Castillo *et al*., 2003) and between different nematode orders (Litvaitis *et al*., 2000).

The primary goal of the present study was to analyse phylogenetic relationships of the main groups or lineages presently recognised in Criconematina based on morphological characters and the partial LSU rRNA sequences. Our analysis includes 36 populations of 22 nominal and six unidentified species and two outgroup taxa from the superfamily Tylenchoidea. We tested the monophyly of taxa using the maximum likelihood test. Because different methods available for building a phylogenetic tree can affect the outcome of the analysis, we analysed our data using maximum parsimony, maximum likelihood and Bayesian inference. The resulting tree topologies were analysed including measurements of support for specific clades.

Determining homology between nucleotides in rRNA is a significant issue for any phylogenetic study. Because rRNA function is mainly determined by its structure,

which is more highly conserved among taxa than is its nucleotide composition, the best way for maximising nucleotide identity in alignment is to use the secondary structure model for this procedure. In our study we reconstruct the secondary structures of the LSU for all studied species and apply structural information to improve the alignment of sequences. We present the information content of D2 and D3 segments and the contribution of the loop or stem regions in terms of phylogenetic information for the suborder Criconematina. In this study, we also test the efficacy of the Bayesian approach using a complex model of RNA evolution based on the secondary structure model.

Materials and methods

Nematode populations, DNA extraction, PCR and sequencing

Original DNA sequence data were collected from 38 nematode samples (Table 1). Nematodes were identified by their morphology and morphometrics. One species from the family Tylenchidae (*Aglenchus agricola*) and one species from the Atylenchidae (*Eutylenchus excretorius*), were selected, based on our unpublished data, as outgroups.

Several nematode specimens of each sample were transferred to an Eppendorf tube containing 16 μl ddH_2O, 2 μl 10X PCR buffer and 2 μl proteinase K (600 μg/ml) (Promega, Benelux, The Netherlands) and crushed for 2 min with a Vibro Mixer microhomogeniser (Zürich, Switzerland). The tubes were incubated at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR, cloning and automated sequencing are described by Tanha Maafi *et al*. (2003). The forward D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCTACTA-3′) primers were used for amplification and sequencing of the fragment of the 28S rRNA gene. The sequences reported here have been deposited in the GenBank database under the accession numbers given in Table 1.

Secondary structure prediction and alignment

The secondary structure model of the D2 and D3 expansion segments of rRNA was constructed for each sequence. It was predicted for each helix using the energy minimisation approach as applied in the Mfold software program Version 3 (http://www.bioinfo.rpi.edu/~zukerm/). The entire structural model for each sequence was manually reconstructed from optimal and suboptimal models for helices using comparative sequence analysis (Cannone *et al*., 2002). The secondary structure models were visualised using the RnaViz (De Rijk & De Wachter, 1997) and drawn using Adobe Illustrator®. Thirty six sequences of ingroups and two outgroup taxa were aligned using ClustalX 1.64 with default parameters for gap opening and gap extension. The final alignment was manually created using GenDoc 2.5 software and knowledge of the structural model of these gene fragments. The multiple alignment is available from the first author.

Phylogenetic analysis of molecular data

Three types of phylogenetic analyses were applied to analyse the alignment: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). MP and ML analyses were performed with PAUP* 4b4a (Swofford, 2003). We conducted equally weighted MP analyses. The gaps were coded as missing data and molecular characters were assessed as independent and unordered. Heuristic search settings were 60 replicates of random taxa, addition, tree bisection-reconnection, branch swapping, multiple tree retained and without steepest descent. The g1 statistics were computed to estimate the amount of phylogenetic signal available for parsimony analysis by generation of 10 000 random trees in PAUP. Partition homogeneity tests comparing morphological and molecular datasets were performed with 100 replicates with ten random additions. For ML, all necessary parameters were estimated from the data using ModelTest based on Akaike Information Criterion (Posada & Crandall, 1998). Robustness of the clades was assessed by the bootstrap analysis yielding bootstrap percentage (BS) for each node estimated from 1000 and 100 replicates for MP and ML analyses, respectively.

A Bayesian inference analysis (BI) of the data set was conducted using MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). We applied BI under different models. For the first analysis a general-time-reversible (GTR) model of nucleotide substitution was used with a proportion of invariable site (I) and a gamma distribution (G) of among-site rate heterogeneity with six rate categories estimated as the best-fit model by ModelTest to the present data set. The second analysis was conducted under the complex model: the doublet model with 16 states of nucleotide doublets (Huelsenbeck & Ronquist, 2001) for the stem region and the standard model of DNA substitution with four nucleotide states for loops and bulges and a gamma distribu-

Table 1. *List of nematode species and populations used in the study.*

Genera and species	Locality	Host	GenBank number	Collector and Identifier
***Mesocriconema* Andrássy, 1965**				
M. sphaerocephalum (Taylor, 1936) Loof & De Grisse, 1989 'haplotype A'	Maracay, Venezuela	*Saccharum* hybrid	AY780950	R. Crozzoli
M. sphaerocephalum 'haplotype B'	Serranova, Italy	Unknown	AY780951	N. Vovlas
M. ornatum (Raski, 1958) Loof & De Grisse, 1989	Maracay, Venezuela	*Paspalum* sp.	AY780968	R. Crozzoli
M. xenoplax (Raski, 1952) Loof & De Grisse, 1989	Fresno, California, USA	*Salix* sp.	AY780963	S.A. Subbotin
M. xenoplax	Münster, Germany	Unknown	AY780961	D. Sturhan
M. xenoplax	Italy	*Vitis vinifera*	AY780966	N. Vovlas
M. xenoplax	Trentino, Italy	Unknown	AY780965	N. Vovlas
M. xenoplax	Valenzano, Italy	*V. vinifera*	AY780964	N. Vovlas
M. xenoplax	Vite-Stefano-Di Monte, Italy	Unknown	AY780962	N. Vovlas
M. solivagum (Andrássy, 1962) Loof & De Grisse, 1989	Moscow, Russia	Unknown	AY780969	S.A. Subbotin and D. Sturhan
Mesocriconema sp.	Bari, Italy	*Prunus* sp.	AY780967	N. Vovlas
***Ogma* Southern, 1914**				
O. civellae (Steiner, 1914) Raski & Luc, 1987	Bajo Seco, Venezuela	*Paspalum fasciatum*	AY780955	R. Crozzoli
***Criconema* Hofmänner & Menzel, 1914**				
C. mutabile (Taylor, 1936) Raski & Luc, 1985	Bajo Seco, Venezuela	weeds	AY780954	R. Crozzoli
Criconema sp. 1.	Bajo Seco, Venezuela	weeds	AY780953	R. Crozzoli
Criconema sp. 2.	Laghi di Monticchio, Italy	Unknown	AY780952	N. Vovlas
***Hemicycliophora* de Man, 1921**				
H. poranga Monteiro & Lordello, 1978	Bajo Seco, Venezuela	weeds	AY780975	R. Crozzoli
H. chilensis Brzeski, 1974	Cuyagua, Venezuela	*Theobroma cacao*	AY780977	R. Crozzoli
H. typica de Man, 1921	Brake, Germany	unknown	AY780973	D. Sturhan
H. thienemanni (Schneider, 1925) Loos, 1948	Trentino, Italy	unknown	AY780976	N. Vovlas
Hemicycliophora sp.	Terovo, Epirus, Greece	*Urtica dioica*	AY780974	N. Vovlas
***Hemicriconemoides* Chitwood & Birchfield, 1957**				
H. strictathecatus Esser, 1960	Maracay, Venezuela	*Musa* sp.	AY780956	R. Crozzoli
H. strictathecatus	Maracay, Venezuela	*Mangifera indica*	AY780958	R. Crozzoli
H. strictathecatus	Cuyagua, Venezuela	*T. cacao*	AY780957	R. Crozzoli
H. cocophillus (Loos, 1949) Chitwood & Birchfield, 1957) 'haplotype A'	Caracas, Venezuela	*Paspalum* sp.	AY780949	R. Crozzoli
H. cocophillus 'haplotype B'	Cuyagua, Venezuela	weeds	AY780947	R. Crozzoli
H. alexis Vovlas, 1980	Epirus, Greece	Unknown	AY780959	N. Vovlas
H. ortonwilliamsi Ye & Siddiqi, 194	M. di Savoia, Italy	Unknown	AY780948	N. Vovlas
***Xenocriconemella* De Grisse & Loof, 1965**				
X. macrodora (Taylor, 1936) De Grisse & Loof, 1965	Italy	Unknown	AY780960	N. Vovlas
***Criconemoides* Taylor, 1936**				
C. informis (Micoletzky, 1922) Taylor, 1936	Epirus, Greece	Unknown	AY780970	N. Vovlas
***Trophonema* Raski, 1957**				
T. arenarium (Raski, 1956) Siddiqi, 1999	Serranova, Italy	Unknown	AY780971	N. Vovlas
***Tylenchulus* Cobb, 1913**				
T. semipenetrans Cobb, 1913	Egypt	*Citrus* sp.	AY780972	V. Chizhov
***Sphaeronema* Raski & Sher, 1952**				
S. alni Turkina & Chizhov, 1986	Münster, Germany	*Alnus glutinosa*	AY780978	D. Sturhan
***Paratylenchus* Micoletzky, 1922**				
P. bukowinensis Micoletzky, 1922	Monopoli, Italy	Unknown	AY780943	N. Vovlas

Table 1. *(Continued).*

Genera and species	Locality	Host	GenBank number	Collector and Identifier
P. nanus Cobb, 1923	Niebüll, Germany	Unknown	AY780946	D. Sturhan
Paratylenchus sp. 1.	Bari, Italy	*Laurus nobilis*	AY780944	N. Vovlas
Paratylenchus sp. 2.	Fresno, CA, USA	*Salix* sp.	AY780945	S.A. Subbotin
Outgroup taxa				
Eutylenchus excretorius Ebsary & Eveleigh, 1981	Brake, Germany	Unknown	AY780979	D. Sturhan
Aglenchus agricola (de Man, 1884) Meyl, 1961	Merelbeke, Belgium	Unknown	AY780980	S.A. Subbotin and D. Sturhan

tion (G) of among-site rate heterogeneity with six rate categories. All Bayesian analyses were initiated with random starting trees and were run with four chains for 1.0×10^6 generations. Sampling the Markov chains was made at intervals of 100 generations. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades.

Phylogenetic analysis of morphological and biological dataset

Nineteen morphological and two biological characters were scored and used in phylogenetic investigations. The presence or absence of phasmids in the genera was scored according to recent data by Sturhan and Geraert (2005). Seven characters were scored as variable. The matrix was analysed for the 11 ingroup and two outgroup genera (Tables 2, 3) by using MP analysis as implemented in PAUP* 4b4a. Recognising the need for future more detailed character analysis, tentatively, and for this preliminary analysis, all characters were considered unordered and equally weighted. The robustness of the clades was estimated for 1000 bootstrap replicates and calculation of Bremen indices.

Results

Sequence analysis and secondary structure of the D2 and D3 expansion fragment of the LSU

The DNA fragment including D2 and D3 used for analysis ranged in length from 502 bp (*Trophonema arenarium*) to 554 bp (*Sphaeronema alni*). ClustalX generated an alignment of 573 positions. Manual correction and improvement of alignment according to secondary structures resulted in alignment with 632 positions. Percentage sequence divergences ranged from 0 to 31.3% among ingroup species and to 39.7% among all species. Interspecific variation was detected for *Mesocriconema xenoplax* (D2 segment: 0-3.1%; 0-12 substitutions; D3 segment: 0-1.8%; 0-3 substitutions), *Hemicriconemoides cocophillus* (6.2%, 23; 1.8%, 3, respectively) and *M. sphaerocephalum* (4.9%, 19; 3.6%, 6, respectively). The number of transitions and transversions were plotted against uncorrected percentage sequence divergence (p-distance) and no saturation was found (data unpubl.).

The sequences and predicted secondary structures of D2 and D3 expansion fragments of the LSU rRNA of *M. xenoplax* are shown in Figure 1. A largely similar folding pattern was observed for all studied species; homology includes folding of the D2 domain into several helices C1-C1/e4 and the D3 includes folds D2-D6 as named by Wuyts *et al.* (2001). More than 75% of nucleotides were paired and helices contained 85% parsimony informative characters. The base frequencies were uniform across taxa (as measured by a chi-square test of base homogeneity across taxa; chi-square = 67.60, $P = 0.99$). Nucleotides containing C and G were predominant (57.5%). It has been observed that nucleotide composition differs between stems and unpaired regions including bulges and terminal loops. The percentage of G and C was higher in stems than in loops and bulges (27% G, 37% C *vs* 18% G, 21% C). Most base pairings involved C-G combinations with relatively fewer G-U and A-U pairs. The Bayesian analysis provides marginal posterior mean probability for Watson-Crick doublets for the stem region, which are C:G = 0.231; G:C = 0.197; A:U = 0.104; U:A = 0.100, and for G:U = 0.091 and U:G = 0.122.

Table 2. *Morphological and biological character states for the Criconematina and outgroup species.*

Character number	Character trait	Character state
1	Shape of mature female	Four states: 0 – vermiform; 1 – spindle or sausage shaped; 2 – elongate-saccate; 3 – subspherical
2	Isthmus	Two states: 0 – slender isthmus not amalgamated with basal bulb; 1 – broad isthmus amalgamated with basal bulb
3	Cuticle of females and juveniles	Two states: 0 – thin; 1 – thick
4	Retrorse annules of cuticle of female and/or juveniles	Two states: 0 – absent; 1 – present
5	Cuticle outgrowths in females or/and juveniles	Two states: 0 – absent; 1 – present
6	Body sheath in females	Two states: 0 – absent; 1 – present
7	Body sheath in juveniles	Two states: 0 – absent; 1 – present
8	Stylet length	Two arbitrary chosen states: 0 – short (<18 μm); 1 – long ($\geqslant 18$ μm)
9	Shape of stylet base	Two states: 0 – anchor shaped; 1 – spheroid shaped
10	Length of stylet conus	Two states: 0 – equal or shorter than shaft; 1 – much longer than shaft
11	Position of excretory pore	Two states: 0 – $<68\%$ of body length; 1 – $\geqslant 68\%$ of body length
12	Structure of vulva	Two states: 0 – open; 1 – closed
13	Phasmids	Two states: 0 – present; 1 – absent
14	Structure of bursa	Two states: 0 – present; 1 – indistinct or absent
15	Length of spicules	Two arbitrary chosen states: 0 – short (<25 μm); 1 – long ($\geqslant 25$ μm)
16	Stylet and pharynx in males	Two states: 0 – developed; 1 – degenerated
17	Deirids	Two states: 0 – absent; 1 – present
18	Shape of postcorpus	Two states: 0 – postcorpus not amalgamated with precorpus; 1 – postcorpus amalgamated with precorpus
19	Tail shape of female	Four states: 0 – filiform; 1 – elongate-conoid; 2 – cylindroid, conically pointed to rounded; 3 – absent
20	Type of parasitism	Two states: 0 – migratory ectoparasites; 1 – sedentary semi-endoparasites
21	Type of feeding cell	Three states: 0 – one cell; 1 – modified nurse cells, 2 – syncytium

Table 3. *Matrix of morphological and biological characters.*

Genus	Character																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Mesocriconema	1	1	1	1	0	0	0	1	0	1	0	0	1	0	1	1	0	1	2	0	0
Criconemoides	1	1	1	1	0	0	0	1	0	1	0	1	1	0	1	1	0	1	2	0	0
Xenocriconemella	1	1	1	1	0	0	0	1	0	1	0	1	1	0	0	1	0	1	2	0	0
Criconema	1	1	1	0/1	0/1	0	0	1	0	1	0	1	1	0	1	1	0	1	2	0	0
Ogma	1	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	1	2	0	0
Hemicriconemoides	1	1	1	1	0	1	0	1	0/1	1	0	0/1	1	0	1	1	0	1	2	0	0
Hemicycliophora	0	1	1	0	0	1	1	1	1	1	0	0	1	0	1	1	0	1	0/1/2	0	0
Paratylenchus	0/1	1	0	0	0	0	0	0/1	0/1	1	0	0	1	1	0	1	1	1	2	0	0
Trophonema	2	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	1	1	1	2
Tylenchulus	2	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	1	1	2	1	1
Sphaeronema	3	0	1	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	3	1	2
Aglenchus	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
Eutylenchus	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	0

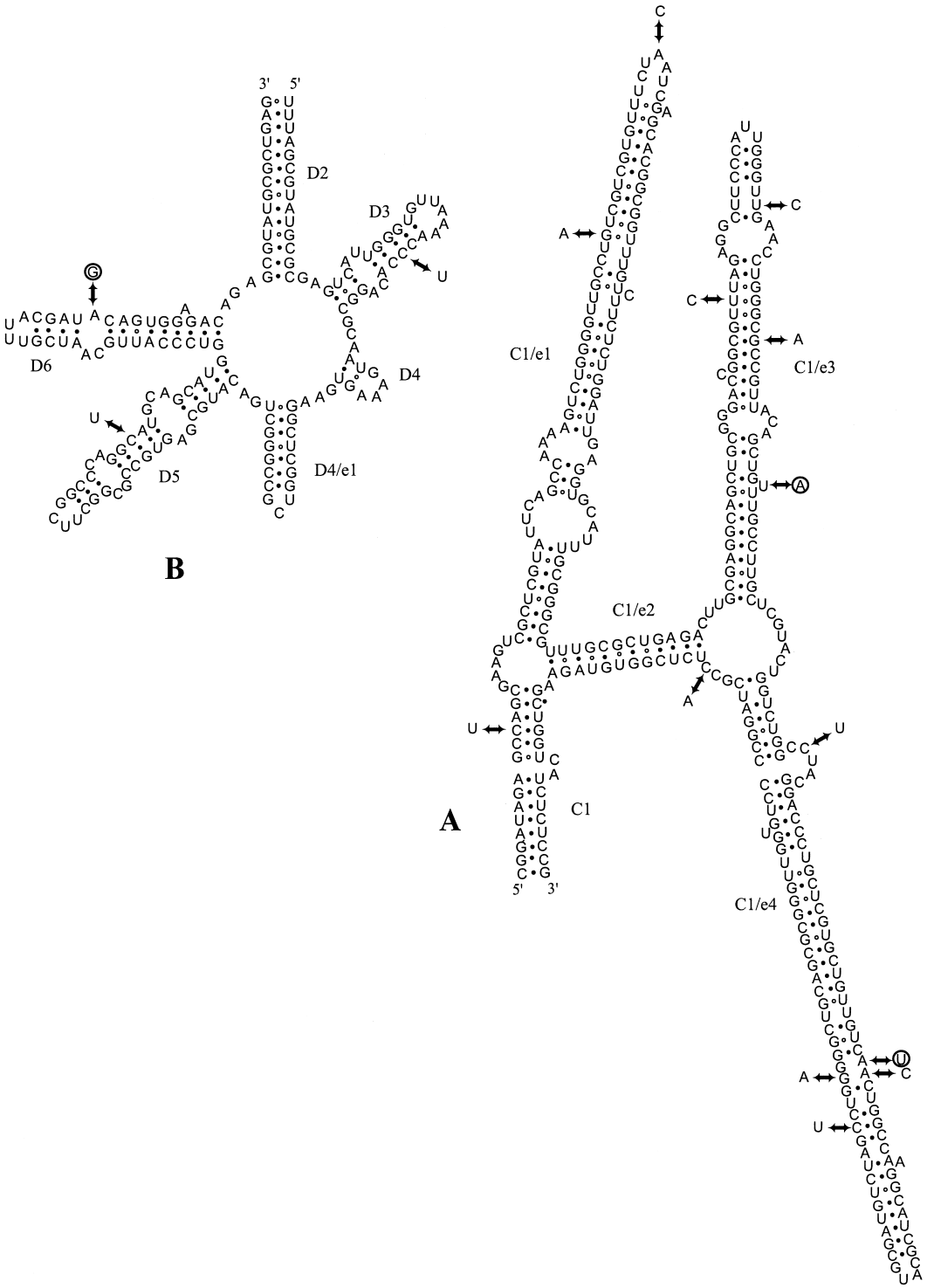


Fig. 1. *Putative secondary structure of D2 (A) and D3 (B) expansion fragments of the LSU rRNA for* Mesocriconema xenoplax. *Arrows indicate substitution changes for sequences from different populations. Nucleotides in circles vary among European populations.*

Sequences of *M. xenoplax* vary among European populations in three nucleotides; all of these are in unpaired position or situated at the edge of bulges, whereas between European and American population six of 13 mutations occur in paired regions (Fig. 1). Compensatory and semi-conservative changes are detected in helices of the D2 extension segment for *Hemicycliophora* and *Mesocriconema* species (Fig. 2). Length changes in C1/e1 and C1/e4 helices for these nematode groups are due to insertion/deletion of C-G or cover different positions. Some indels in the criconematid sequences occur in unpaired nucleotides include in loops and bulges, which reflect differences in length in primary sequences (Fig. 2). The largest deletion was observed in helices C1/e1 of *Trophonema*, and this helix is marked as most variable among criconematids (Fig. 2).

Phylogenetic relationships within the Criconematina

Analysis of the morphological and biological data matrix

Parsimony analysis of the 21 morphological and biological characters produced 24 shortest trees with 32 steps (Fig. 3). The sedentary nematodes (*Trophonema*, *Sphaeronema*, *Tylenchulus*) comprise a well-supported (BS = 94%) basal lineage. *Paratylenchus* is supported as a sister taxon to the Hemicycliophoroidea + Criconematoidea clade but with low BS (66%). *Paratylenchus* forms a cluster with other Tylenchoidea in trees one step longer than the MP tree. The superfamily Criconematoidea constitutes a monophyletic assemblage with BS = 68% and Bremen index = 2. Consistency index suggests that presence of the body wall sheath, cuticle, bursa, or deirids, as well as spicule and stylet length, are homoplastic (CI = 0.50-0.75), whereas all other characters had CI = 1.00.

Maximum parsimony analysis of molecular data

Analysis of the D2-D3 alignment produced 72 equally parsimonious trees with a length of 1647 distributed in one tree island. The skewness of a sample of 10 000 random trees indicated significant phylogenetic signals in the dataset: g1 = −0.559. *Sphaeronema alni* occupies the most basal position within the Criconematina with moderate BS support (Fig. 4). The strict consensus tree provides some insight into relationships between extant families and genera. In the MP tree the genera *Paratylenchus* and *Hemicycliophora* are monophyletic, whereas *Mesocriconema* and *Criconema* appear to be paraphyletic. MP analysis of the D3 fragment only produced 2203 equally MP trees with a length of 299 from thirteen tree islands and a skewness value of g1 = −0.489616. The D3 trees provide less phylogenetic resolution among genera and species than for those from the D2-D3 data set (Fig. 5). The BS support for monophyly of the genera *Paratylenchus* and *Hemicycliophora* (see above) decreased from 100 to 87 and from 94 to 67, respectively. Searching the constrained MP D2-D3 tree topology under D3 data set resulted trees in 11 steps longer than maximum parsimony trees.

Combined molecular and morphological data set

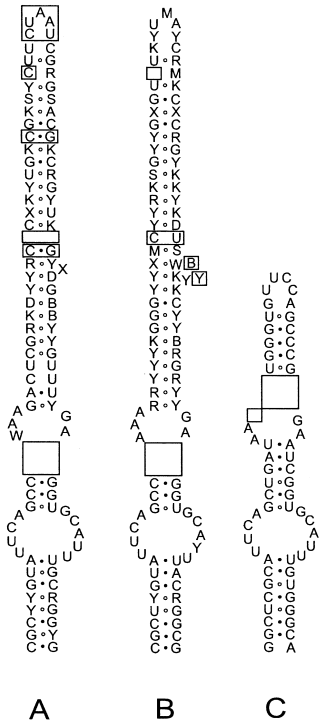
Results of the partition homogeneity test support the combinability of the morphological character set with the molecular sequence data ($P = 0.830$). The combined morphological and LSU gene dataset produced 54 most parsimonious trees with a length of 1687. The topology resembles that obtained from the MP analysis of molecular data (Fig. 4), but with higher resolution among genera of Tylenchuloidea and slightly higher bootstrap support for monophyly of *Hemicycliophora* (98 *vs* 94) and *Hemicriconemoides* (60 *vs* 44).

Maximum likelihood analysis

ModelTest estimated the GTR + G + I model as the best fit to present the molecular dataset. ML analyses using this model generated two trees with the best likelihood score of −7446.20, one of them being shown in Figure 6. The Sphaeronematidae occupy the most basal family, followed by *Tylenchulus*, *Paratylenchus* and *Trophonema* with very low BS support. BS support for monophyly of *Paratylenchus* and *Hemicycliophora* is 87% and 76%, respectively. The genus *Ogma* is placed as a sister to *Criconema* (BS = 88%). The ML tree provides little information about family relationships between Hemicycliophoridae and Criconematidae or between the genera of Criconematidae.

Bayesian analysis

The majority-rule consensus tree of the BI (standard model) analysis (Fig. 7A) also shows *Sphaeronema* as occupying the most basal position (as sister to the remainder of the criconematid clade), followed by *Tylenchulus. Hemicycliophora* forms a highly supported clade with *Mesocriconema* and *Criconemoides* (PP = 98%). Relationships between *Hemicriconemoides*, *Criconema* and *Xenocriconemella* clades are unresolved. The 50% majority consensus BI tree under the complex model was much less resolved for relationships between families and genera (Fig. 7B). The clade Hemicycliophoridae and *Mesocriconema* with *Criconemoides* is weakly supported (PP = 63%).



Test of tree topology and alternative hypotheses

Tree comparison using the Shimodaira-Hasegawa test did not find any significant differences among trees generated from ML, MP, or both BI analyses for D2 and D3 alignment and the combined data set (Table 4). Constraining *Mesocriconema* and *Criconema* to be monophyletic results in trees not significantly different from the ML tree. Validity of a single origin of a cuticular sheath in criconematids is rejected.

Discussion

PHYLOGENY BASED ON MORPHOLOGY

MP analysis of morphological and biological characters treated as unordered and equally weighted generated a topology different from that proposed by Siddiqi (2000), especially with respect to the position for *Paratylenchus*, which did not form a clade with other Tylenchuloidea. Relationships of *Paratylenchus* with Criconematidae statistically were not well-supported. Within Criconematina and outgroups, morphological characters have not yet been fully investigated from the perspective of accurate coding,

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H.typica              [CGCTTGTA]TTCA[GCC---]AAA[GACTCGAGGCTGC-CTGTTGTCGTGT-TT]TTAAT[CGAGCACGGCGGCTT-GTC--TGCTTCGTTTC]GA[---GGT]GCATT[TGCGGGCG]
Hemicycliophora sp.   [CGCTCGTA]TTCA[GCC---]AAA[GACTCGGGGCCGC-CTGCTGTCGTGT-TT]CTAAT[CGAGCACGGCGGTTG-GTG--GGCTCTGTTTC]GA[---GGT]GCATT[TGCGGGCG]
H. poranga            [CGCTCGTA]TTCA[GCC---]AAA[GACTCGGTGTCRC-CGGCTGTCGGGT-TT]CTAAT[CGAGCACGGCAGTTG-GTT--GGCGCTGTTTC]GA[---GGT]GCATT[TGCGGGCG]
H. thienemanni        [CGCCCGTA]TTCA[GCC---]AAA[GACTCGGGTCTGA-CAGCTGTCGTGT-TT]CTAAT[CGAGCACGGCAGTTG-CCA--AGGCCTGTTTC]GA[---GGT]GCATT[TGCGGGTG]
H. chilensis          [CGCTTGTA]TTCA[GCC---]TAA[GACTCGGGACTG--CCTCTGG-GGCCCTT]-----[CGGGGAC-TCAGTTG--TG--TGTCTCGTTTT]GA[---GGT]GCATT[TGCAGGCG]
M. xenoplax           [CGCTCGTA]TTCA[GCC---]AAA[AGTCTGGGGTTGC-CTGTCGTCGTGT-TT]CTAAT[CGAGCACGGCGGTTT-GTC--TTCTCTGGATT]GA[---GGT]GCATT[TGCGGGCG]
Mesocriconema sp.     [CGCTTGTA]TTCA[GCC---]AAA[GACCCGGGGCTAC-CTGTCGTCGTGT-TT]CTAAT[CGAGCACGGCGGCTT-GTCTCTGCTCCGGGCC]GA[---GGT]GCATT[TGCAGGCG]
M. solivagum          [CGCTCGTA]TTCA[GCC---]AAA[AGCCTTGGGTCCC-CCGTGGTTGCGT-TT]CTAAT[CGAGCGCGGCTGCGG-GATC-GACTCGAGGCC]GA[---GGT]GCACT[TGCGGGCG]
M. ornatum            [CGCTCGTA]TTCA[GCC---]AAA[GGCCCGGGGCTCA-TCGTCGTCGTGT-TT]CTAAT[CGAGCACGGCGGCGA-CTC--TGCTCTGGGTT]GA[---GGT]GCATT[TGCGGGCG]
M. sphaerocephalum "A" [CGCTCGTA]TTCA[GCC---]AAA[GGCTCGGGGCTTCCCTGGCGTTGAGT-TG]CTAAC[CACTCTCAGCGTTGGTGTGTTGTCCTTGGGTT]GA[---GGT]GCATT[TACGGGCG]
M. sphaerocephalum "B" [CGCTCGTA]TTCA[GCC---]AAA[GGCTCGGGGCTTCCCTGGCGCTGGGT-TG]TTAAC[CACTCCCAGTGTTGGTGTGTTGTCCTCGGGTT]GA[---GGT]GCATT[TACGGGCG]
T. arenarium          [GGCTCGCA]TTCA[GCTGAT]AA-[---TGGGTGTT----------CCAGCCCG---]GA[ATCGGT]GCATT[TGTGGGCA]
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D

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H.typica               CGCTTGTATTCAGCCAAAGACTCGAGGCTGCCTGTT-GTCGTGTTTTTAATCGAGCACGGCGGCTTG--TCTG-CTTCGTTTCGAGGTGCATTTGCGGGCG
Hemicycliophora sp.    CGCTCGTATTCAGCCAAAGACTCGGGGCCGCCTGCT-GTCGTGTTTCTAATCGAGCACGGCGGTTGG--TGGG-CTCTGTTTCGAGGTGCATTTGCGGGCG
H. poranga             CGCTCGTATTCAGCCAAAGACTCGGTGTCRCCGGCT-GTCGGGTTTCTAATCGAGCACGGCAGTTGG--TTGG-CGCTGTTTCGAGGTGCATTTGCGGGCG
H. thienemanni         CGCCCGTATTCAGCCAAAGACTCGGGTCTGACAGCT-GTCGTGTTTCTAATCGAGCACGGCAGTTGC--CAAG-GCCTGTTTCGAGGTGCATTTGCGGGTG
H. chilensis           CGCTTGTATTCAGCCTAAGACTCGGGACTGCCT-CT-GGGGCCCTT-----CGGGGACT-CAGTTG---TGTG-TCTCGTTTTGAGGTGCATTTGCAGGCG
M. xenoplax            CGCTCGTATTCAGCCAAAAGTCTGGGGTTGCCTGTC-GTCGTGTTTCTAATCGAGCACGGCGGTTTG--TCTT-CTCTGGATTGAGGTGCATTTGCGGGCG
Mesocriconema sp.      CGCTTGTATTCAGCCAAAGACCCGGGGCTACCTGTC-GTCGTGTTTCTAATCGAGCACGGCGGCTTGTCTCTG-CTCCGGGCCGAGGTGCATTTGCAGGCG
M. solivagum           CGCTCGTATTCAGCCAAAAGCCTTGGGTCCCCCGTG-GTTGCGTTTCTAATCGAGCGCGGCTGCGGGA-TCGA-CTCGAGGCCGAGGTGCACTTGCGGGCG
M. ornatum             CGCTCGTATTCAGCCAAAGGCCCGGGGCTCATCGTC-GTCGTGTTTCTAATCGAGCACGGCGGC--GACTCTG-CTCTGGGTTGAGGTGCATTTGCGGGCG
M. sphaerocephalum "A" CGCTCGTATTCAGCCAAAGGCTCGGGGCTTCCCTGGCGTTGAGTTGCTAACCACTCTCAGCGTTGGTGTGTTGTCCTTGGGTTGAGGTGCATTTACGGGCG
M. sphaerocephalum "B" CGCTCGTATTCAGCCAAAGGCTCGGGGCTTCCCTGGCGCTGGGTTGTTAACCACTCCCAGTGTTGGTGTGTTGTCCTCGGGTTGAGGTGCATTTACGGGCG
T. arenarium           GGCTCGCATTCAGCTGA---TAATGGGT----------GTTCCAG--------CCCGGAATCG-------------------GTGCATTTGTGGGCA
```

E

Fig. 2. *Putative consensus structure for C1/e1 helices of the D2 segment for species of* Hemicycliophora *(A),* Mesocriconema *(B) and* Trophonema arenarium *(C). Nucleotides in which substitutions have occurred are indicated in blocks as are insertions/deletions. Two alignments of this helix are presented: one manually constructed based on the secondary structure model (D), in which square brackets indicate positions included in helices; and one generated by Clustal using default options (E).*

Table 4. *Results of Shimodaira-Hasegawa (SH) tests of tree topologies and alternative hypotheses of the phylogeny of the Criconematina.*

Topology evaluated	Tree numbers	Best log likelihood*	Δ lnL	*P**
ML tree	2	−7446.20518	best	–
MP trees (D2 and D3 expansion fragments)	72	−7454.17954	7.97436	0.978
MP trees (combined data set)	54	−7454.17889	7.97371	0.978
BI trees (GTR model)	9900	−7447.59391	1.38872	1.000
BI trees (complex model)	9900	−7448.56944	2.36426	0.999
Monophyly of *Mesocriconema*	40	−7456.99512	10.78994	0.900
Monophyly of *Hemicriconemoides*	66	−7454.17854	7.97336	0.978
Monophyly of *Criconema*	45	−7467.76344	21.55825	0.542
Validity of Tylenchulidae *sensu* Raski *et al.* (1987) or Tylenchuloidea *sensu* Siddiqi (2000) (*Paratylenchus* + *Tylenchulus* + *Trophonema* + *Sphaeronema*)	100	−7484.47116	38.26598	0.124
Validity of Tylenchulinae *sensu* Raski & Luc (1987) (*Tylenchulus* + *Trophonema* + *Sphaeronema*)	24	−7460.50384	14.29865	0.828
Validity of Criconematoidea *sensu* Siddiqi (2002) or Criconematinae *sensu* Raski & Luc (1987)	24	−7453.66934	17.46416	0.726
Validity of Macroposthoniinae *sensu* Siddiqi (2000) (*Mesocriconema* + *Criconemoides* + *Xenocriconemella*)	42	−7489.94297	43.73778	0.083
Monophyly of Criconematina having a cuticular sheath or double cuticle (*Hemicycliophora* + *Hemicriconemoides*)	6	−7508.57321	62.36803	0.020*
Monophyly of Criconematina inducing syncytium (*Trophonema* + *Sphaeronema*)	71	−7454.17854	7.97438	0.978

* Tree significantly worse that the best tree at $P < 0.05$.

delineating the full range of character states, character homologies, and character independence. Nevertheless, we included in this analysis widely recognised characters, as a starting point to better recognise, for example, questions of convergence that can be further tested by additional comparative morphological research.

Phylogenetic information content of sequences of D2 and D3 fragments of the LSU

The D3 expansion segment alone and in combination with the D2 segment has been recently used to study phylogenetic relationships of a wide range of nematode genera and species (Al-Banna *et al.*, 1997; De Ley *et al.*, 1999; Rubtsova *et al.*, 2001; Stock *et al.*, 2001; Castillo *et al.*, 2003). In many cases the D3 segment allows differentiation of species, although very often it does not contain sufficient information to produce reliable phylogenies at different levels. Our study of strict consensus tree obtained from the D3 dataset does not resolve relationships between most lineages. The combination of D3 with D2 segment, which is 2.5 times longer, provided 3.7 times the number of phylogenetically informative characters, and thus improved tree resolution. However, none of the phylogenetic analyses of the D2-D3 dataset allowed resolution of relationships between main lineages. This finding is consistent with knowledge that expansion segments are a useful source of phylogenetic information for relatively recent evolutionary events and specifically for resolving relationships between closely related species. Combining sequences of D2 and D3 segments with other fragments of LSU may increase the number of informative characters and likely provide greater resolution and support for relationships at higher taxonomic levels.

Structural aspects of rRNA offer some additional opportunities for phylogenetic understanding. Specifically, unequal rates of substitution changes between loops and bulges and conserved regions such as stems, if not considered, could undermine phylogenetic resolution. Our analysis reveals that phylogenetic signals are distributed almost equally in both paired and unpaired fragments. In preliminary analyses, exclusion of unpaired nucleotides from the analysis did not result in more highly supported trees or in significantly different topologies.

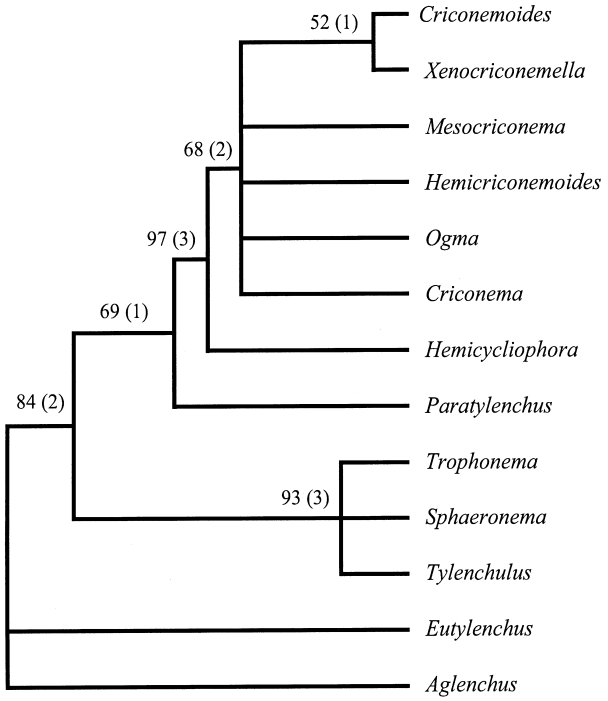

Fig. 3. *The strict consensus of 24 equally parsimonious trees of the Criconematina resulting from the morphological and biological matrix (tree length = 32, CI = 0.8125; HI = 0.1875; RI = 0.8776; RC = 0.7130; number of parsimony informative characters = 18). Bootstrap values greater than 50% are given on appropriate clades, Bremen index is given in brackets.*

THE USEFULNESS OF THE SECONDARY STRUCTURE MODEL FOR ALIGNMENT AND PHYLOGENETIC ANALYSIS

The secondary structure model for the taxa used in this study and the D2-D3 fragments of large subunit rRNA is a good fit for the model proposed by Wuyts *et al.* (2001) for eukaryotic organisms and later reconstructed for the parasitic nematode *Labiostrongylus bipapillosus* by Chilton *et al.* (2003). Regardless of nucleotide variability, the structural model for all studied taxa had the same helix numbers and similar conserved features, indicating a strong evolutionary functional constraint in the structural pattern of this part of rRNA molecule.

Aligning sequences represents a major challenge in phylogenetic studies using molecular data where differences in lengths of the sequences are compared. Several studies demonstrate that different sequence alignments produce different tree topologies (Morrison & Ellis, 1997; Subbotin *et al.*, 2001). We advocate using alignment based on secondary structure with optimised positional homology. However, construction of an alignment based on the structural model is complex and time consuming. After careful consideration of secondary structure model we introduced 59 gaps in a generated automatic alignment to find the best nucleotide homology. In some places this estimation remains somewhat arbitrary because there were slight shifts of loops and bulges in some sequence regions and there were also uncertainness with respect to the helix boundary.

Knowledge of secondary structure is also critical for applying a model of evolution to RNA molecules. Most substitution models treat substitution changes along nucleotide sequences as independent (Smith *et al.*, 2003). It has been shown that the mode of evolution within the helical region of the LSU is *via* compensatory mutations. Thus, the evolution of bases in structurally related positions is highly dependent. This process was also clearly observed in our study during analysis of sequence groups during early phases of evolutionary divergence. Where phylogenetic studies treat the substitution changes as independent, phylogenetic signals and confidence levels of support of clades on a tree may be significantly overestimated. Models of evolutionary dependence between sites have been described and recently developed and these provide the rationale to consider the basic unit of evolution as pairs of, rather than single, sites (Schöniger & von Haeseler, 1994; Savill *et al.*, 2001; Jow *et al.*, 2002; Smith *et al.*, 2003). As we expected, the phylogenetic BI tree obtained using a complex model that considers paired nucleotides in stems and unpaired in loops showed reduced statistical support compared to other analyses. This loss of resolution is expected by the consideration of a reduced number of independently evolving characters. Thus, the knowledge of the secondary structure allowed us to apply the more complex model and, as consequence, to generate a more realistic picture of the relationships of criconematids as inferred from the D2-D3 dataset.

TAXONOMIC IMPLICATIONS

We provide the first information on intra and interspecific sequence variations in D2 and D3 segments of the LSU gene among criconematids. Although we recognise that the delimitation of species on the basis rDNA is controversial because of potential problems associated with incomplete lineage sorting and pseudogene amplification, the present data give some indication on possible presence of cryptic species within this nematode group. It is noteworthy that substantial sequence divergence exceeding the range of intraspecific variation in these segments was ob-

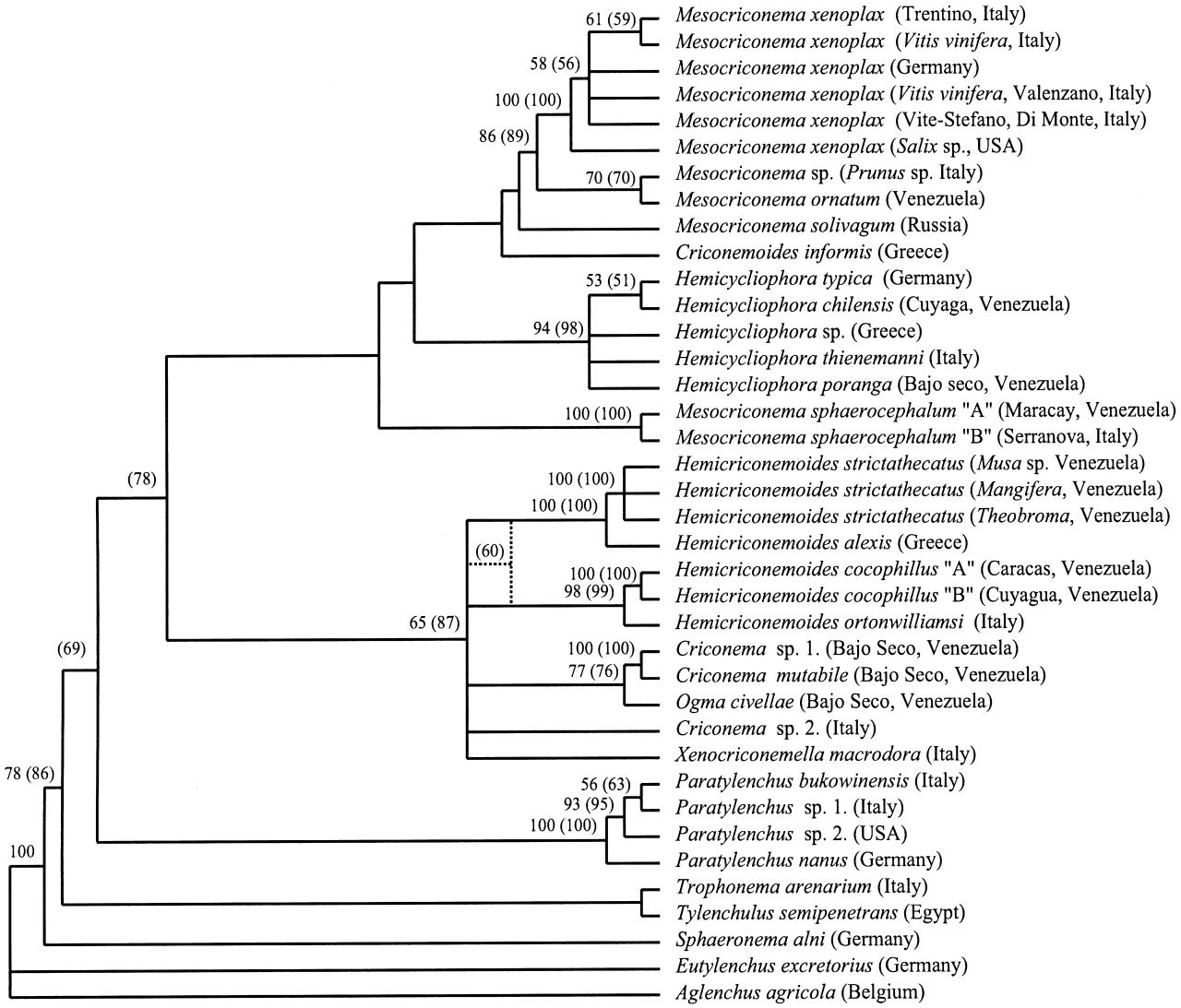

Fig. 4. *Strict consensus of 72 equally parsimonious trees resulting from the analysis of the D2-D3 sequence alignment of the Criconematina and two outgroup taxa (tree length = 1647; CI = 0.4563; HI = 0.5537; RI = 0.6104; RC = 0.2724; number of parsimony informative characters = 301). Bootstrap values (in brackets for combined data) greater than 50% are given on appropriate clades. Branches for MP tree obtained from the combined data set are indicated by dashed lines.*

served between two populations identified as *M. sphaerocephalum* but originating from different continents. *Mesocriconema sphaerocephalum* was first described from the island of Trinidad in what was then the British West Indies. Presently, this species is reported from North and South America, Europe, tropical Africa and Asia. After analysing intraspecific morphological and morphometric variation, De Grisse and Loof (1970) found that populations of this species from the temperate zones have a slightly longer stylet than those from tropical countries. Our preliminary morphometric analysis of representative populations from the geographic regions used in the present study separates these into two distinct groups. Thus, combined morphological and molecular data appear to indicate that *M. sphaerocephalum*, as presently defined, consists of two sibling species. Further analyses of these populations are required to confirm this hypothesis.

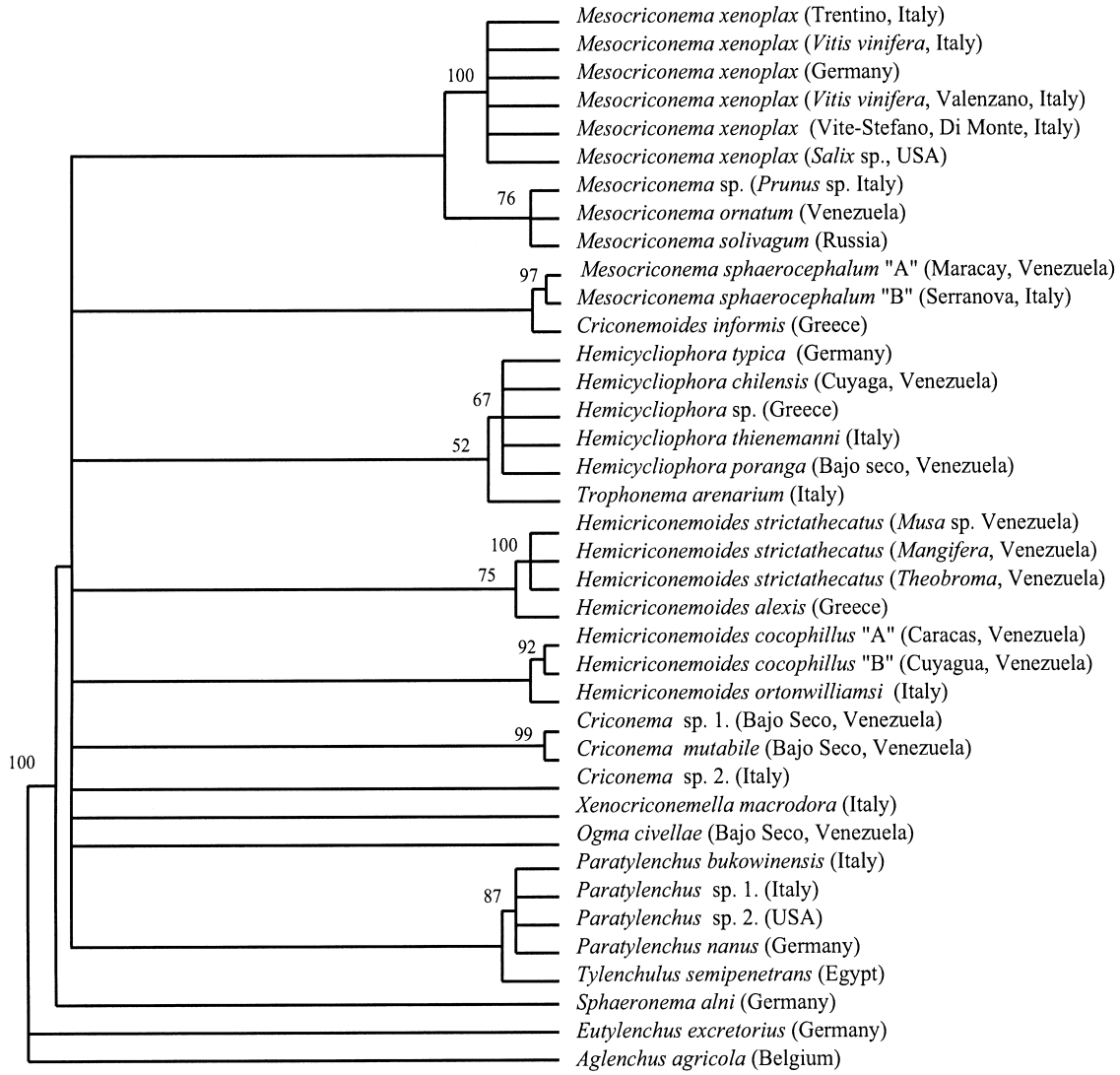

Fig. 5. *Strict consensus of 2203 equally parsimonious trees resulting from analysis of the D3 sequence alignment of the Criconematina and two outgroup taxa (tree length = 299; CI = 0.4783 HI = 0.5217; RI = 0.6774; RC = 0.3241; number of parsimony informative characters = 64). Bootstrap values more than 50% are given on appropriate clades.*

Our study also reveals sequence differences between one American and several European *M. xenoplax* populations. Although after analysis of morphological variation of *M. xenoplax* populations De Grisse and Loof (1970) concluded that there was no correlation with geographical distribution, more detailed morphological analysis is required to clarify the taxonomic status of the studied populations.

Sequence variation in the D2-D3 segments also occurs among populations of *H. cocophillus*. This species is distributed in subtropical and tropical regions (Sri Lanka, India, Indonesia, Philippines, Egypt, Iraq, Venezuela, *etc*.). The morphometrics of the two populations included in the present studies agree with the original description (see Crozzoli & Lamberti, 2003), although these populations show considerable variation in body length and tail shape, thereby suggesting the need for more careful morphological analysis of the species.

Paraphyly of *Mesocriconema* and *Criconema* is suggested by some phylogenetic trees, but ML tests did not

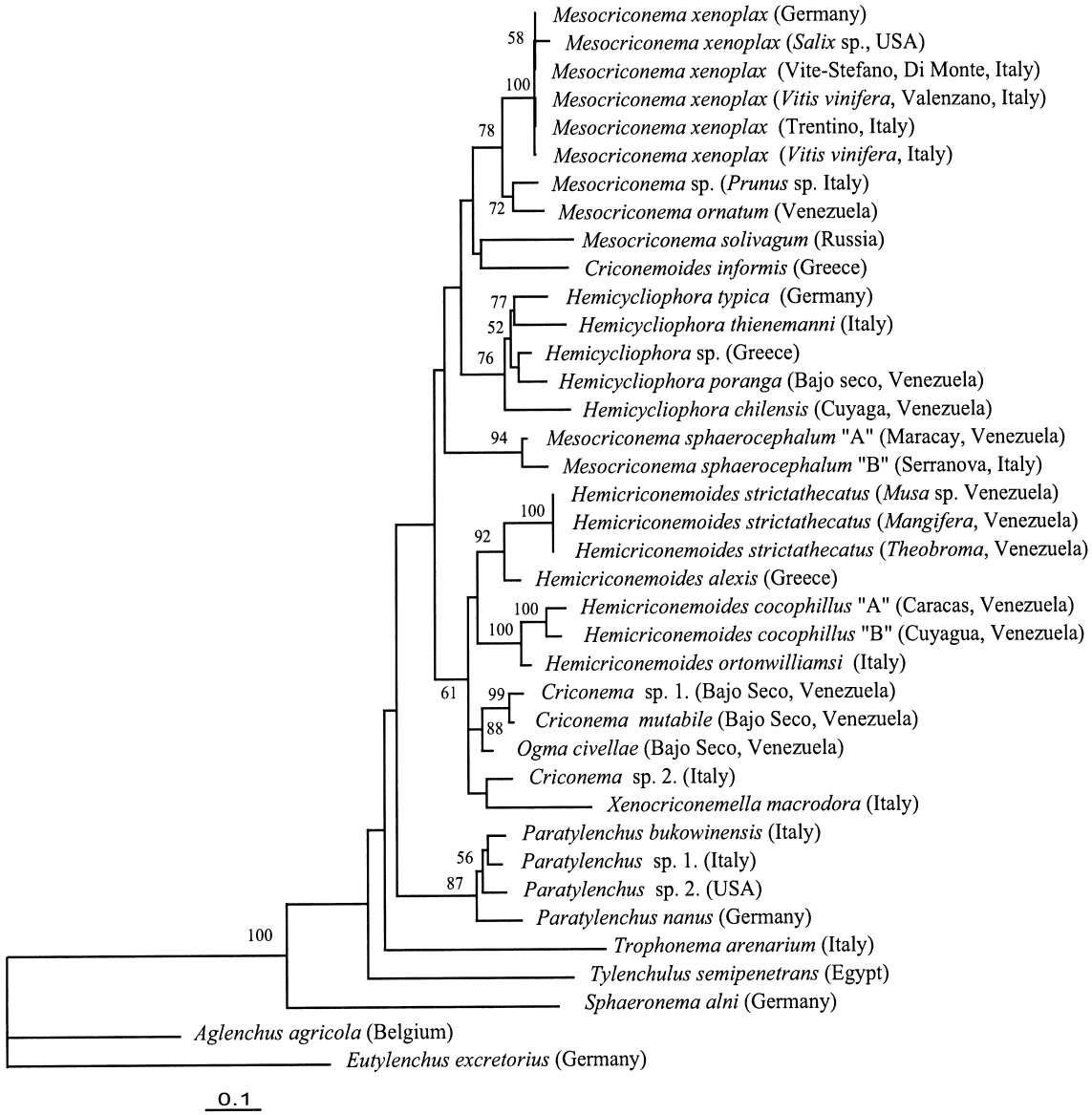

Fig. 6. *One of two maximum likelihood tree resulting from the analysis of the D2-D3 sequence alignment of the Criconematina and two outgroup taxa under GTR + I + G model (lnL = −7446.20518; freqA = 0.1646; freqC = 0.2386; freqG = 0.3368; freqT = 0.2600; R(a) = 0.6244; R(b) = 2.1470; R(c) = 1.0702; R(d) = 0.4315; R(e) = 4.2002; R(f) = 1.0000; Pinvar = 0.1557; Shape = 0.7123). Bootstrap values exceeding 50% are given on appropriate clades.*

reject constrained monophyly of these genera. One possible explanation is unequal evolutionary rates between lineages. Another possibility is that *Mesocriconema* and/or *Criconema* are indeed paraphyletic and comprised of species sharing similar characters but different histories. Based on morphology, Loof and De Grisse (1974) noted that *Mesocriconema* could be resolved into several subgroups and Siddiqi (2000) distinguished several subgenera within *Criconema*. These issues may be resolved by including additional species of these genera in new phylogenetic analyses.

The taxonomic status of *Trophonema arenarium* has long been debated. Raski (1957) erected the genus *Trophonema*, to which *Sphaeronema arenarium* Raski, 1956

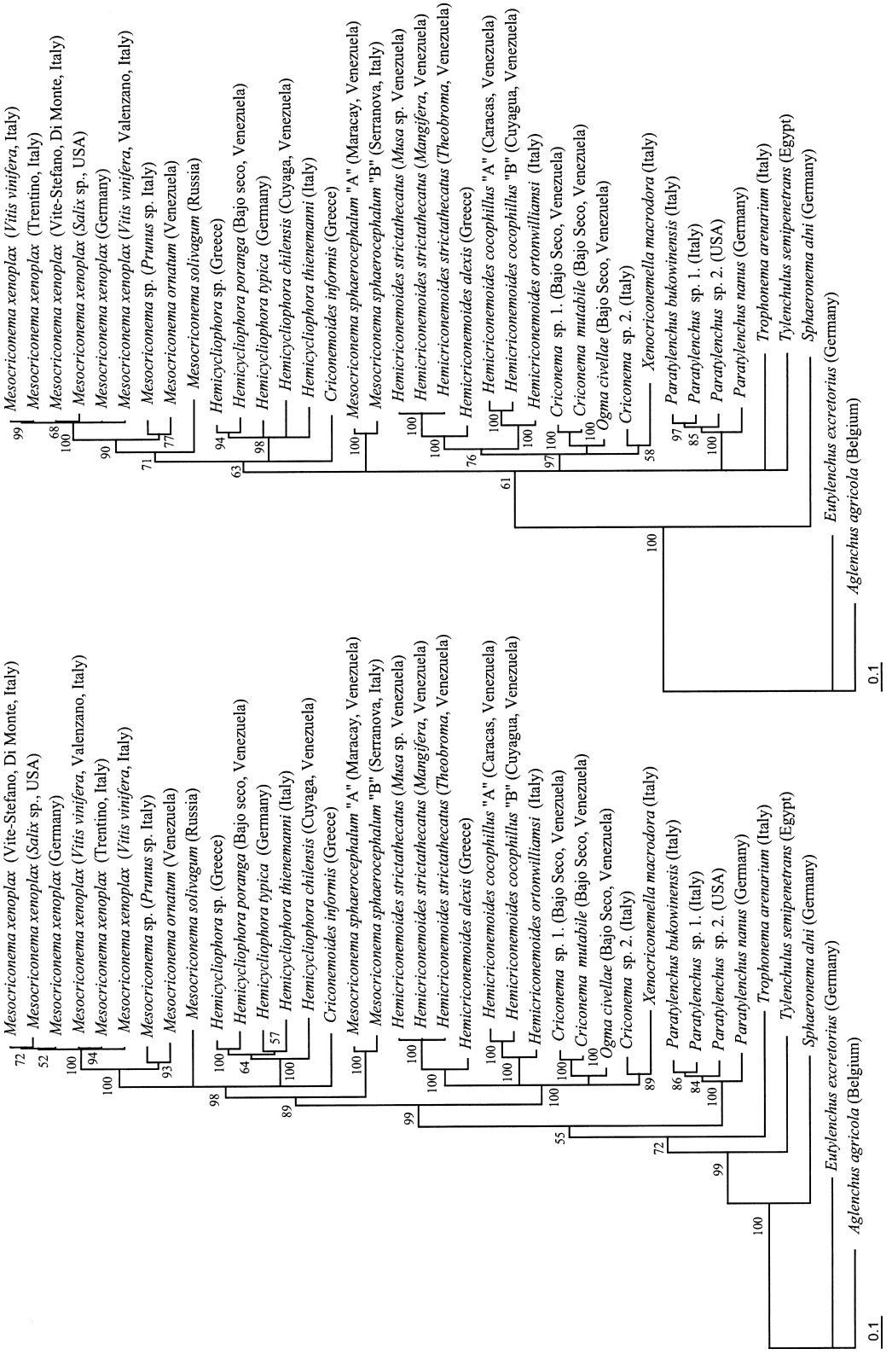

Fig. 7. *The 50% majority rule consensus trees from Bayesian analysis generated with (A) the GTR + I + G model and (B) the complex model: 4 × 4 model for loops and 16 doublets model for stems of the secondary structure.*

was transferred. *Trophonema* was considered a genus of the subfamily Sphaeronematinae and distinguished from *Trophotylenchulus* by the anterior position of the excretory pore. Siddiqi (1999) considered the position of the excretory pore to be variable and therefore synonymised *Trophonema* with *Trophotylenchulus*. Earlier, Geraert (1966) synonymised *Trophonema* with *Paratylenchus* because of similar body shape of both genera. In the present study, phylogenetic analyses either failed to resolve relationships of *Trophonema* with other genera, or placed *Trophonema* as the next lineage after *Tylenchulus*.

The presence of a body sheath as a second cuticle for adults and juveniles is the most important feature of nematodes from the family Hemicycliophoridae, a feature that distinguishes them from all other tylenchids. Adults of the genus *Hemicriconemoides* also have a double cuticle and this genus is proposed as being intermediate between *Criconemoides* and *Hemicycliophora.* Brzeski (1974) assigned, for the first time, the genus *Hemicriconemoides* to the subfamily Hemicycliophorinae. Raski and Luc (1987) remarked that a cuticular sheath and weak differentiation of the anterior annuli were reminiscent of *Hemicycliophora* and for this reasons some species of *Hemicriconemoides* have been transferred to *Hemicycliophora.* They also noted, however, that many more features link *Hemicriconemoides* to genera classified under the Criconematinae. Our molecular analyses strongly suggested that *Hemicriconemoides* and *Hemicycliophora* are not closely related taxa.

The monophyly of Tylenchuloidea (with Paratylenchidae, Tylenchulidae and Sphaeronematidae) was not supported in any molecular trees, although the ML test could not reject validity of this superfamily. All analyses of molecular data place *Sphaeronema* at a basal position with varying levels of statistical support relative to all other ingroup species. Positions of the other genera of Tylenchuloidea were less clearly defined on most trees, but where it was resolved, the genus *Tylenchulus* was the second basal lineage. Thus, if further phylogenetic analyses including those based on a longer LSU fragment or combination of several genes clearly do support monophyly of Tylenchuloidea, the classification proposed by Geraert (1966) distinguishing five lineages (Criconematidae, Hemicycliophoridae, Paratylenchidae, Sphaeronematidae and Tylenchulidae) should be seriously considered.

Phylogeny of Criconematina is essential to developing a consistent classification as well as ultimately to understand genes and processes involved in plant parasitism. The present study, while advancing understanding of criconematid phylogeny, points to unresolved questions and the need for expanded studies. Greater phylogenetic resolution will require representatives of additional genera and species, additional genes and increasingly precise DNA models. Whereas the present study provides important congruence tests with morphology suggesting convergence, for example, of the 'double cuticle', additional detailed comparative morphological and developmental studies are needed to recognise those characters most informative for recovering phylogenetic history and to better understand complex issues of morphological homology, character-state coding and character-independence.

Acknowledgements

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References

AL-BANNA, L., WILLIAMSON, V. & GARDNER, S.L. (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetics and Evolution* 7, 94-102.

ANDRÁSSY, I. (1979). Revision of the subfamily Criconematinae Taylor, 1936 (Nematoda). *Opuscula Zoologica, Budapestinensis* 16, 11-57.

BRZESKI, M.W. (1974). Taxonomy of Hemicycliophorinae (Nematoda, Tylenchida). *Zeszyty Problemowe Postępów Nauk Rolniczych* 154, 237-330.

CANNONE, J.J., SUBRAMANIAN, S., SCHNARE, M.N., COLLETT, J.R., D'SOUZA, L.M., DU, Y., FENG, B., LIN, N., MADABUSI, L.V., MULLER, K.M., PANDE, N., SHANG, Z., YU, N. & GUTELL, R.R. (2002). The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* 32, 1-32.

CASTILLO, P., VOVLAS, N., SUBBOTIN, S. & TROCCOLI, A. (2003). A new root-knot nematode: *Meloidogyne baetica* n. sp. (Nematoda: Heteroderidae) parasitizing wild olive in Southern Spain. *Phytopathology* 93, 1093-1102.

CHILTON, N.B., HUBY-CHILTON, F. & GASSER, R.B. (2003). First complete large subunit ribosomal RNA sequence and secondary structure for a parasitic nematode: phylogenetic and diagnostic implications. *Molecular and Cellular Probes* 17, 33-39.

CROZZOLI, R. & LAMBERTI, F. (2003). Species of *Criconemoides* Taylor, 1936, *Discocriconemella* De Grisse & Loof, 1965 and *Hemicriconemoides* Chitwood & Birchfield, 1957 occurring in Venezuela, with description of *Criconemoides*

tiaraensis sp. n. (Nematoda: Criconematidae). *Russian Journal of Nematology* 11, 67-79.

DE GRISSE, A. & LOOF, P.A.A. (1970). Intraspecific variation in some Criconematidae (Nematoda). *Mededeelingen Faculteit Landbouwetenschappen Rijksuniversiteit Gent* 35, 41-63.

DE LEY, P., FELIX, M.-A., FRISSE, L.M., NADLER, S.A., STERNBERG, P.W. & THOMAS, W.K. (1999). Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). *Nematology* 2, 591-612.

DE RIJK, P. & DE WACHTER, R. (1997). RnaViz, a program for the visualization of RNA secondary structure. *Nucleic Acids Research* 25, 4679-4684.

DUNCAN, L.W., INSERRA, R.N., THOMAS, W.K., DUNN, D., MUSTIKA, I., FRISSE, L.M., MENDERS, M.I., MORRIS, K. & KAPLAN, D.T. (1999). Molecular and morphological analysis of isolates of *Pratylenchus coffeae* and closely related species. *Nematropica* 29, 61-80.

EBSARY, B.A. (1981). Generic revision of Criconematidae (Nematoda): *Crossonema* and related genera with a proposal for *Neocrossonema* n. gen. *Canadian Journal of Zoology* 59, 103-114.

GERAERT, E. (1966). The systematic position of the families Tylenchidae and Criconematidae. *Nematologica* 12, 362-368.

HUELSENBECK, J.P. & RONQUIST, F. (2001). MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754-755.

ICZN (2003). Opinion 2046 (Case 3185). *Criconema* Hofmänner & Menzel, 1914 (Nematoda): *Eubostrichus guernei* Certes, 1899 designated as the type species. *Bulletin of Zoological Nomenclature* 60, Part 3.

JOW, H., HUDELOT, C., RATTRAY, M. & HIGGS, P.G. (2002). Bayesian phylogenetics using an RNA substitution model applied to early mammalian evolution. *Molecular Biology and Evolution* 19, 1591-1601.

KHAN, E., CHAWLA, M.L. & SAHA, M. (1975). Criconematoidea (Nematoda: Tylenchida) from India, with descriptions of nine new species, two new genera and a family. *Indian Journal of Nematology* 5, 70-100.

LITVAITIS, M.K., BATES, J.W., HOPE, W.D. & MOENS, T. (2000). Inferring a classification of the Adenophorea (Nematoda) from nucleotide sequences of the D3 expansion segment (26/28S rDNA). *Canadian Journal of Zoology* 78, 911-922.

LOOF, P.A.A. & DE GRISSE, A. (1974). Interrelationships of the genera of Criconematidae (Nematoda: Tylenchida). *Mededeelingen Faculteit Landbouwetenschappen Rijksuniversiteit Gent* 38 (1973), 1303-1328.

LOOF, P.A.A., ANDRÁSSY, I., LUC, M., RASKI, D.J., SIDDIQI, M.R. & WOUTS, W.M. (2001). *Criconema* Hofmänner & Menzel, 1914 (Nematoda): proposed designation of *Eubostrichus guernei* Certes, 1899 as the type species. *Bulletin of Zoological Nomenclature* 58, 179-181.

MAGGENTI, A.R., LUC, M., RASKI, D.J., FORTUNER, R. & GERAERT, E. (1987). A reappraisal of Tylenchina (Nemata). 2. Classification of the suborder Tylenchina (Nemata: Diplogasteria). *Revue de Nématologie* 10, 135-142.

MEHTA, U.K. & RASKI, D.J. (1971). Revision of the genus *Criconema* Hofmänner and Menzel, 1914 and other related genera (Criconematidae: Nematoda). *Indian Journal of Nematology* 1, 145-198.

MICOLETZKY, H. (1925). Die freilebenden Süsswasser- und Moornematoden Dänemarks nebst Anhang über Amöbosporidien und andere Parasiten bei freilebenden Nematoden. *Kongelige Danske Videnskabernes Selskabs Skrifter, 10th series* 8, 57-310.

MORRISON, D. & ELLIS, J. (1997). Effect of nucleotide sequence alignment on phylogenetic estimation: A case study of 18S rDNAs of Apicomplexa. *Molecular Biology and Evolution* 14, 428-441.

POSADA, D. & CRANDALL, K.A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 917-818.

RASKI, D.J. (1957). *Trophotylenchus* and *Trophonema*, two new genera of Tylenchulidae n. fam. (Nematoda). *Nematologica* 2, 85-90.

RASKI, D.J. & LUC, M. (1987). A reappraisal of Tylenchina (Nemata). 10. The superfamily Criconematoidea Taylor, 1936. *Revue de Nématologie* 10, 409-444.

RUBTSOVA, T.V., SUBBOTIN, S.A., BROWN, D.J.F. & MOENS, M. (2001). Description of *Longidorus sturhani* sp. n. (Nematoda: Longidoridae) and molecular characterisation of several longidorid species from Western Europe. *Russian Journal of Nematology* 9, 127-136.

SAVILL, N.J., HOYLE, D.C. & HIGGS, P.G. (2001). RNA sequence evolution with secondary structure constraints: comparison of substitution rate models using maximum-likelihood methods. *Genetics* 157, 399-411.

SCHÖNIGER, M. & VON HAESELER, A. (1994). A stochastic model and the evolution of autocorrelated DNA sequences. *Molecular Phylogenetics and Evolution* 3, 240-247.

SIDDIQI, M.R. (1980). Taxonomy of the plant nematode superfamily Hemicycliophoroidea, with a proposal for Criconematina, new suborder. *Revue de Nématologie* 3, 179-199.

SIDDIQI, M.R. (1999). *Trophotylenchulus colbrani* sp. n. and *T. cunctus* sp. n. (Criconematina: Tylenchulidae) from Mbalmayo forest in Cameroon. *International Journal of Nematology* 8, 179-184.

SIDDIQI, M.R. (2000). *Tylenchida parasites of plants and insects*. Wallingford, UK, CABI Publishing, 833 pp.

SMITH, A.D., LUI, T.W. & TILLER, E.R.M. (2003). Empirical model for substitution in ribosomal RNA. *Molecular Biology and Evolution* 21, 419-427.

STOCK, P., CAMPBELL, J.F. & NADLER, S.A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology* 87, 877-889.

STURHAN, D. & GERAERT, E. (2005). Phasmids in Tylenchulidae (Tylenchida: Criconematoidea). *Nematology* 7, 249-252.

SUBBOTIN, S.A., VIERSTRAETE, A., DE LEY, P., ROWE, J., WAEYENBERGE, L., MOENS, M. & VANFLETEREN, J.R. (2001). Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of ribosomal DNA. *Molecular Phylogenetics and Evolution* 21, 1-16.

SWOFFORD, D.L. (2003). *PAUP*: Phylogenetic analysis using parsimony (* and other methods)*, *version 4.0b 10*. Sunderland, Mass., USA, Sinauer Associates.

TANHA MAAFI, Z., SUBBOTIN, S.A. & MOENS, M. (2003). Molecular identification of cyst-forming nematodes (Heteroderidae) from Iran and a phylogeny based on the ITS sequences of rDNA. *Nematology* 5, 99-111.

TAYLOR, A.L. (1936). The genera and species of the Criconematinae, a sub-family of the Anguillulinidae (Nematoda). *Transactions of the American Microscopical Society* 55, 391-421.

WUYTS, J., VAN DE PEER, Y. & DE WACHTER, R. (2001). Distribution of substitution rates and location of insertion sites in the tertiary structure of ribosomal RNA. *Nucleic Acids Research* 29, 5017-5028.