

Phylogenetic analysis of Tylenchida Thorne, 1949 as inferred from D2 and D3 expansion fragments of the 28S rRNA gene sequences

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Summary – The evolutionary relationships of 82 species of tylenchid and aphelenchid nematodes were evaluated by use of sequence data of the D2 and D3 expansion fragments of the 28S ribosomal RNA genes. Nine automatic and one culled sequence alignments were analysed using maximum parsimony and Bayesian inference approaches. The molecular data sets showed that the order Tylenchida comprises lineages that largely correspond to two suborders, Hoplolaimina and Criconematina, and other taxonomic divisions as proposed by Siddiqi (2000). Several significant results also derived from our study include: *i*) the basal position of groups that include entomoparasitic nematodes within tylenchid trees; *ii*) paraphyly of the superfamily Dolichodoroidea *sensu* Siddiqi (2000); *iii*) evidence for a *Pratylenchus*, *Hirschmanniella* and *Meloidogyne* clade; and *iv*) lack of support for widely held traditional placement of *Radopholus* within Pratylenchidae and placement of this genus within Hoplolaimidae or Heteroderidae. Congruence and incongruence of molecular phylogeny and traditional classifications and morphological-based hypotheses of phylogeny of tylenchids are discussed.

Keywords – cyst-forming nematodes, evolution, LSU rRNA, phylogeny, *Radopholus*, root-knot nematodes.

The order Tylenchida Thorne, 1949 includes the largest and most economically important group of plant-parasitic nematodes. As plant parasites they have diverged to exploit all plant parts including foliage, flowers and seeds, but mostly they attack roots. The order also includes mycophagous species, and a large group parasitises the haemocoel of insects and mites. Several classifications and phylogenies have been proposed for tylenchids (Chitwood & Chitwood, 1950; Maggenti, 1971, 1981; Andrásy, 1976; Skarbilovich, 1978, 1980; Siddiqi, 1980, 1986, 2000; Ryss & Krall, 1981; Luc *et al.*, 1987; Maggenti *et al.*, 1987; Chizhov & Berezina, 1988; Chizhov & Kruchina, 1988, 1989, 1992; Ryss, 1993; Chizhov, 2004). At present, two prominent classifications of Tylenchida differ by subdivision into suborders. That is, Maggenti *et al.* (1987) recognised four suborders, the Tylenchina, Aphelenchina, Sphaerulariina and Hexatyliina; whereas Siddiqi (1980, 1986, 2000) recognised four sub-

orders, the Tylenchina, Hoplolaimina, Criconematina and Hexatyliina but also regarded the Aphelenchida as a distinct order.

The present classifications reflect controversy regarding the origin of Tylenchida and estimation of different evolutionary trends in this group (Baldwin *et al.*, 2004a). Filipjev (1934) was the first to propose that tylenchids originated from a rhabditid-diplogastrid ancestry. Paramonov (1962, 1970) pioneered more detailed work on phylogeny of Tylenchida, suggesting that through a thorough study of ecological-morphological characters and ontogeny of contemporary nematodes it is possible to deduce the phylogenetic relationships of various groups. Paramonov (1970) proposed, on the basis of evolutionary trends, that Tylenchida evolved from fungus-feeding ancestors and suggested a hypothetical ancestor close to the modern *Psilenchus*. This point of view was later shared by Chizhov and Berezina (1988) and Ryss

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(1993). Another concept of tylenchid evolution was developed by Siddiqi (1980, 1986, 2000), who employed clado-evolutionary methods to understand the mutual relationships of the Tylenchida groups. He believed that tylenchids originated from a “Cephalobida-Oxyurida complex”, and considered *Hexatyclus* as being closest to the hypothetical ancestor of the Tylenchida. He also presented a cladogram suggesting relationships between tylenchid suborders. Although Chizhov and Kruchina (1988) and Ryss (1993) generally accepted Siddiqi’s classification, they differed in their proposal of phylogenetic relationships between the main tylenchid groups. More recently, classical information and molecular data have been used as a basis for a classification. Although resolution for tylenchids is limited, this approach nevertheless provides testable hypotheses of monophyly in tentatively proposing an infraorder, Tylenchomorpha, with the superfamilies Aphelenchoidea, Criconematoidea, Sphaerularioidea, Tylenchoidea and Myenchoidea (De Ley & Blaxter, 2002).

Phylogenetic relationships among the main tylenchid groups based on molecular or morphological data sets have not been precisely studied using rigorous cladistics or other approaches. A phylogenetic reconstruction of Tylenchida based on a partly sequenced fragment of the SSU gene for more than 35 species was recently presented in the review by Baldwin *et al.* (2004a). Although in this study Tylenchida rRNA phylogeny was not fully resolved, several trends were distinct, including convergent evolution of sedentary endoparasitism and feeding cells in cyst and root-knot nematodes. Skantar and Carta (2004) analysed phylogenetic relationships within five tylenchid genera based on nucleotide sequences of the Hsp90 gene. Scholl and Bird (2005) employed an EST mining strategy to recover robust phylogeny using 47 orthologous genes although only three genera of sedentary tylenchid nematodes were included in this study. Thus, the general picture of phylogenetic relationships within tylenchids has not been addressed. As noted by Siddiqi (2000) the reconstruction of phylogeny, although extremely difficult for Tylenchida, is essential for creating a stable, hierarchical, system of classification. It is also essential to select meaningful tylenchid model representatives for recognising and ultimately manipulating the diverse pathways for plant pathogenesis (Baldwin *et al.*, 2004a).

In this paper, for purposes of clarity and consistency, we use terminology consistent with taxonomic categories of Tylenchida as proposed by Siddiqi (2000), including Aphelenchida as a separate order. We present a phylogenetic analysis of the D2-D3 expansion region of the LSU

gene sequences of 77 species of Tylenchida as well as five species of Aphelenchida Siddiqi, 1980, chosen here, among several possibilities, as the most plausible outgroup taxon. There is general agreement that Tylenchida is monophyletic and, whereas there is some disagreement of rank and discussion of polyphyly (Baldwin *et al.*, 2004), typically Aphelenchida is regarded as a sister taxon to Tylenchida. More recent primarily molecular-based literature treats Tylenchida and Aphelenchida as rooted within the Cephalobioidea and/or the Panagrolaimoidea (De Ley & Blaxter, 2002; Nadler *et al.*, 2006). Thus, for this study, selecting the most informative and closest representatives of the outgroup of Tylenchida requires difficult choices depending on classical, morphologically-based, perspectives, while also considering weak or lacking resolution of such deeper levels by published molecular phylogenies. For the present study we consider representative species of Aphelenchida as the best possible choice of outgroup while nevertheless recognising this as a testable hypothesis in conjunction with further resolution of the tylenchid tree and its deeper phylogenetic context.

The species included in this study were selected to represent the breadth of known taxonomic and morphological diversity of the Tylenchida. Our analysis is based on maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). The main goals of this study are: *i*) to estimate the phylogenetic relationships within Tylenchida at the levels of suborders, superfamilies, and families; and *ii*) to test congruence of extant morphologically and biologically-based tylenchid classifications with phylogenies inferred from molecular data.

Materials and methods

TAXON SAMPLING

In this study we obtained original sequences from 49 species of tylenchids and from three species of aphelelenchids (Table 1). All nematodes were identified by their morphological characters. Several sequences from our previous publications (Castillo *et al.*, 2003; Zheng *et al.*, 2003; Subbotin *et al.*, 2005) and from other authors (Duncan *et al.*, 1999; Inserra *et al.*, 2001), as well as sequences from GenBank were included in our analyses. New sequences reported here have been deposited in GenBank under the accession numbers given in Table 1.

Table 1. List of species from the Orders Tylenchida and Aphelenchida used in the study.

Classification: genera and species*	Country	GenBank accession	Reference or Collector and/or identifier
Order Tylenchida Thorne, 1949			
Suborder Tylenchina Chitwood in Chitwood & Chitwood, 1950			
Superfamily Tylenchoidea Örley, 1880			
Family Tylenchidae Örley, 1880			
Subfamily Tylenchinae Örley, 1880			
<i>Aglenchus agricola</i> (de Man, 1884) Meyl, 1961	Belgium	AY780980	Subbotin <i>et al.</i> (2005)
<i>Coslenchus costatus</i> (de Man, 1921) Siddiqi, 1978	Germany	DQ328719	This study, D. Sturhan
Subfamily Boleodorinae Khan, 1964			
<i>Basiria gracilis</i> (Thorne, 1949) Siddiqi, 1963	USA	DQ328717	This study, J. Baldwin
<i>Boleodorus</i> sp.	Germany	DQ328718	This study, D. Sturhan
Family Atylenchidae Skarbilovich, 1959			
Subfamily Eutylenchinae Siddiqi, 1986			
<i>Eutylenchus excretorius</i> Ebsary & Eveleigh, 1981	Germany	AY780979	Subbotin <i>et al.</i> (2005)
Superfamily Anguinoidea Nicoll, 1935			
Family Anguinidae Nicoll, 1935			
Subfamily Anguininae Nicoll, 1935**			
<i>Anguina tritici</i> (Steinbuch, 1799) Filipjev, 1936	Unknown	DQ328723	This study, M. Moens
<i>Subanguina radicolica</i> (Greeff, 1872) Paramonov, 1967	Belgium	DQ328721	This study, S.A. Subbotin
<i>Subanguina chilensis</i> Vovlas, Troccoli & Moreno, 2000	Chile	DQ328724	This study, N. Vovlas
<i>Mesoanguina millefolii</i> (Löw, 1874) Chizhov & Subbotin, 1985	Russia	DQ328722	This study, V.N. Chizhov
<i>Heteroanguina graminophila</i> (Goodey, 1933) Chizhov, 1980	Russia	DQ328720	This study, V.N. Chizhov
Subfamily Ditylenchinae Golden, 1971**			
<i>Ditylenchus destructor</i> Thorne, 1945	Russia	DQ328727	This study, V.N. Chizhov
Suborder Hoplolaimina Chizhov & Berezina, 1988			
Superfamily Hoplolaimoidea Filipjev, 1934			
Family Hoplolaimidae Filipjev, 1934			
Subfamily Rotylenchoidinae Whitehead, 1958			
<i>Helicotylenchus multicinctus</i> (Cobb, 1893) Golden, 1956	Sudan	DQ328745	Subbotin <i>et al.</i> (unpubl.)
<i>Helicotylenchus pseudorobustus</i> (Steiner, 1914) Golden, 1956	Germany	DQ328751	Subbotin <i>et al.</i> (unpubl.)
<i>Helicotylenchus vulgaris</i> Yuen, 1964	Italy	DQ328759	Subbotin <i>et al.</i> (unpubl.)
<i>Rotylenchus uniformis</i> (Thorne, 1949) Loof & Oostenbrink, 1958	Belgium	DQ328738	Subbotin <i>et al.</i> (unpubl.)
<i>Rotylenchus goodeyi</i> Loof & Oostenbrink, 1958	Spain	DQ328756	Subbotin <i>et al.</i> (unpubl.)
Subfamily Hoplolaiminae Filipjev, 1934			
<i>Scutellonema brachyurus</i> (Steiner, 1938) Andrassy, 1958	Italy	DQ328753	Subbotin <i>et al.</i> (unpubl.)
<i>Hoplolaimus seinhorsti</i> Luc, 1958	Israel	DQ328752	Subbotin <i>et al.</i> (unpubl.)
<i>Peltamigratus perscitus</i> Doucet, 1980	Spain	DQ328744	Subbotin <i>et al.</i> (unpubl.)
Family Rotylenchulidae Husain & Khan, 1967			
<i>Rotylenchulus reniformis</i> Linford & Oliveira, 1940	Brazil	DQ328713	This study, M. Moens
<i>Rotylenchulus macrodoratus</i> Dasgupta, Raski & Sher, 1968	Italy	DQ328711	This study, N. Vovlas
Family Pratylenchidae Thorne 1949			
Subfamily Pratylenchinae Thorne, 1949			
<i>Pratylenchus coffeae</i> (Zimmermann, 1898) Filipjev & Schuurmans Stekhoven, 1941	Martinique	AF170430	Duncan <i>et al.</i> (1999)

Table 1. (Continued).

Classification: genera and species*	Country	GenBank accession	Reference or Collector and/or identifier
<i>Pratylenchus dunensis</i> de la Pena, Moens, Van Aelst & Karssen, 2006	The Netherlands	AJ890461	de la Pena <i>et al.</i> (2006)
<i>Pratylenchus jaehni</i> Inserra, Duncan, Troccoli, Dunn, Maria dos Santos, Kaplan & Vovlas, 2001	Brazil	AF170427	Inserra <i>et al.</i> (2001)
Subfamily Hirschmanniellinae Fotedar & Handoo, 1978			
<i>Hirschmanniella</i> sp.	Viet Nam	DQ328686	This study, C.N. Nguyen
Subfamily Radopholinae Allen & Sher, 1967			
<i>Radopholus</i> sp.	Viet Nam	DQ328712	This study, C.N. Nguyen
Family Meloidogynidae Skarbilovich, 1959			
<i>Meloidogyne incognita</i> (Kofoid & White, 1919) Chitwood, 1949	USA	AF435794	De Ley <i>et al.</i> (2005)
<i>Meloidogyne exigua</i> Goeldi, 1892	UK	AF435804	Tenente <i>et al.</i> (2004)
<i>Meloidogyne chitwoodi</i> Golden, O'Bannon, Santo & Finley, 1980	The Netherlands	AF435802	Tenente <i>et al.</i> (2004)
<i>Meloidogyne hapla</i> Chitwood, 1949	Moldova	DQ328685	This study, V.N. Chizhov
<i>Meloidogyne baetica</i> Castillo, Vovlas, Subbotin & Troccoli, 2003	Spain	AY150367	Castillo <i>et al.</i> (2004)
Family Heteroderidae Filipjev & Schuurmans Stekhoven, 1941			
Subfamily Heteroderinae Filipjev & Schuurmans Stekhoven, 1941			
<i>Heterodera litoralis</i> Wouts & Sturhan, 1996	New Zealand	DQ328690	This study, W. Wouts
<i>Heterodera sorghi</i> Jain, Sethi, Swarup & Srivastava, 1982	India	DQ328689	This study, J. Rowe
<i>Heterodera cynodontis</i> Shahina & Maqbool, 1989	Pakistan	DQ328698	This study, F. Shahina
<i>Heterodera oryzicola</i> Rao & Jayaprakash, 1978	India	DQ328693	This study, J. Rowe
<i>Heterodera salixophila</i> Kirjanova, 1969	Belgium	DQ328689	This study, S.A. Subbotin
<i>Heterodera auclandica</i> Wouts & Sturhan, 1995	New Zealand	DQ328688	This study, W. Wouts
<i>Heterodera latipons</i> Franklin, 1969	Syria	DQ328687	This study, U. Scholz
<i>Heterodera glycines</i> Ichinohe, 1952	USA	DQ328691	This study, R. Robbins
<i>Heterodera cajani</i> Koshy, 1967	India	DQ328692	This study, J. Rowe
<i>Heterodera goettingiana</i> Liebscher, 1892	Germany	DQ328697	This study, J. Rowe
<i>Heterodera urticae</i> Cooper, 1955	Belgium	DQ328696	This study, S.A. Subbotin
<i>Heterodera zea</i> Koshy, Swarup & Sethi, 1971	USA	DQ328695	This study, Z. Handoo
Subfamily Punctoderinae Krall & Krall, 1978			
<i>Cactodera cacti</i> (Filipjev & Schuurmans Stekhoven, 1941) Krall & Krall, 1978	Germany	DQ328702	This study, D. Sturhan
<i>Punctodera punctata</i> (Thorne, 1928) Mulvey & Stone, 1976	Belgium	DQ328699	This study, S.A. Subbotin
<i>Globodera millefolii</i> (Kirjanova & Krall, 1965) Behrens, 1975	Estonia	DQ328700	This study, E. Krall
Unidentified species of cyst-forming nematode ***	New Zealand	DQ328701	This study, D. Sturhan
Subfamily Meloidoderinae Golden, 1971			
<i>Meloidodera alni</i> Turkin & Chizhov, 1986	Belgium	DQ328706	This study, S.A. Subbotin
Subfamily Ataloderinae Wouts, 1973			
<i>Cryphodera brinkmani</i> Karssen & van Aelst, 1999	Japan	DQ328705	This study, G. Karssen
<i>Rhizonema sequoiae</i> Cid del Prado Vera, Lownsbery & Maggenti, 1983	USA	DQ328703	This study, I. Cid del Prado Vera
<i>Atalodera crassicrustata</i> (Bernard, 1981) Moreira de Souza & Huang, 1994	Russia	DQ328704	This study, A. Eroshenko

Table 1. (Continued).

Classification: genera and species*	Country	GenBank accession	Reference or Collector and/or identifier
Superfamily Dolichodoroidea Chitwood in Chitwood & Chitwood, 1950			
Family Belonolaimidae Whitehead, 1960			
<i>Belonolaimus longicaudatus</i> Rau, 1958	USA	DQ328710	This study, O. Becker
Family Telotylenchidae Siddiqi, 1960			
Subfamily Telotylenchinae Siddiqi, 1960			
<i>Tylenchorhynchus dubius</i> (Bütschli, 1873) Filipjev, 1936	Russia	DQ328707	This study, D. Sturhan
<i>Trophurus sculptus</i> Loof, 1956	Germany	DQ328709	This study, D. Sturhan
Subfamily Macrotriphurinae Fotedar & Handoo, 1978			
<i>Macrotriphurus arbusticola</i> Loof, 1958	Germany	DQ328708	This study, D. Sturhan
Subfamily Merliniinae Siddiqi, 1971			
<i>Nagelus leptus</i> (Allen, 1955) Siddiqi, 1979	Russia	DQ328715	This study, D. Sturhan
<i>Amplimerlinius icarus</i> (Wallace & Greet, 1964) Siddiqi, 1976	Belgium	DQ328714	This study, D. Sturhan
Family Psilenchidae (Paramonov, 1967) Khan, 1969			
<i>Psilenchus</i> sp.	USA	DQ328716	This study, J. Baldwin
Suborder Criconematina Siddiqi, 1980			
Superfamily Criconematoidea Taylor, 1936			
Family Criconematidae Taylor, 1936			
Subfamily Criconematinae Taylor, 1936			
<i>Criconema mutabile</i> (Taylor, 1936) Raski & Luc, 1985	Venezuela	AY780954	Subbotin <i>et al.</i> (2005)
<i>Ogma civellae</i> (Steiner, 1914) Raski & Luc, 1987	Venezuela	AY780955	Subbotin <i>et al.</i> (2005)
Subfamily Macroposthoniinae Skarbilovich, 1959			
<i>Mesocriconema xenoplax</i> (Raski, 1952) Loof & De Grisse, 1989	Germany	AY780961	Subbotin <i>et al.</i> (2005)
<i>Mesocriconema sphaerocephalum</i> (Taylor, 1936) Loof & De Grisse, 1989	Venezuela	AY780968	Subbotin <i>et al.</i> (2005)
<i>Criconemoides informis</i> (Micoletzky, 1922) Taylor, 1936	Greece	AY780970	Subbotin <i>et al.</i> (2005)
<i>Xenocriconemella macrodora</i> (Taylor, 1936) De Grisse & Loof, 1965	Italy	AY780960	Subbotin <i>et al.</i> (2005)
Superfamily Hemicycliophoroidea Skarbilovich, 1959			
Family Hemicycliophoridae Skarbilovich, 1959			
<i>Hemicycliophora typica</i> de Man, 1921	Germany	AY780973	Subbotin <i>et al.</i> (2005)
Superfamily Tylenchuloidea Skarbilovich, 1947			
Family Tylenchulidae Skarbilovich, 1947			
<i>Tylenchulus semipenetrans</i> Cobb, 1913	Egypt	AY780972	Subbotin <i>et al.</i> (2005)
<i>Trophonema arenarium</i> (Raski, 1956) Siddiqi, 1999	Italy	AY780971	Subbotin <i>et al.</i> (2005)
Family Sphaeronematidae Raski & Sher, 1952			
<i>Sphaeronema alni</i> Turkina & Chizhov, 1986	Germany	AY780978	Subbotin <i>et al.</i> (2005)
Family Paratylenchidae Thorne, 1949			
<i>Paratylenchus bukowinensis</i> Micoletzky, 1922	Italy	AY780943	Subbotin <i>et al.</i> (2005)
Suborder Hexatyliina Siddiqi, 1980			
Superfamily Sphaerularioidea Lubbock, 1861			
Family Sphaerulariidae Lubbock, 1861			
<i>Sphaerularia bombi</i> Dufour, 1837	Russia	DQ328726	This study, V.N. Chizhov
Family Allantonematidae Pereira, 1931			
Subfamily Allantonematinae Pereira, 1931			
<i>Bradynema rigidum</i> (von Siebold, 1836) zur Strassen, 1892	Russia	DQ328730	This study, V.N. Chizhov
<i>Howardula phyllotretae</i> Oldham, 1933	Russia	DQ328728	This study, V.N. Chizhov

Table 1. (Continued).

Classification: genera and species*	Country	GenBank accession	Reference or Collector and/or identifier
Subfamily Contortylenchinae Rühm, 1956 <i>Contortylenchus</i> sp.	Russia	DQ328731	This study, V.N. Chizhov
Superfamily Iotonchioidea Goodey, 1953			
Family Iotonchiidae Goodey, 1953			
Subfamily Iotonchiinae Goodey, 1953 <i>Skarbilovinema lyoni</i> Chizhov & Zakharenkova, 1991	Russia	DQ328733	This study, V.N. Chizhov
Family Parasytylenchidae Siddiqi, 1986			
Subfamily Heterotylenchinae Siddiqi, 1986 <i>Wachekitylenchus bovi</i> (Wachek, 1955) Slobodyanyuk, 1986	Russia	DQ328732	This study, V.N. Chizhov
Subfamily Parasytylenchinae Siddiqi, 1986 <i>Parasytylenchus</i> sp.	Russia	DQ328729	This study, V.N. Chizhov
Unidentified entomoparasitic tylenchid nematode****	Russia	DQ328725	This study, V.N. Chizhov
Order Aphelenchida Siddiqi, 1980 (outgroups)			
Family Aphelenchoididae Skarbilovich, 1947			
<i>Aphelenchoides besseyi</i> Christie, 1942	Russia	DQ328684	This study, V.N. Chizhov
<i>Aphelenchoides fragariae</i> (Ritzema Bos, 1890) Christie, 1932	USA	DQ328683	This study, J. Baldwin
<i>Aphelenchoides</i> sp.	Unknown	DQ328682	This study, J. Baldwin
Family Parasitaphelenchidae Rühm, 1956			
<i>Bursaphelenchus xylophilus</i> (Steiner & Buhrer, 1934) Nickle, 1970	China	DQ356002	Zheng <i>et al.</i> (2003)
<i>Bursaphelenchus mucronatus</i> Mamiya & Enda, 1979	China	DQ356001	Zheng <i>et al.</i> (2003)

* For purposes of clarity and consistency, taxonomic categories referred to in this work are those of Siddiqi (2000) unless otherwise indicated.

** Division of the family Anguinidae into subfamilies according to Chizhov and Kruchina (1992).

*** We anticipate that this cyst-forming nematode, previously identified as *Dolichodera* sp. (Subbotin *et al.*, 2002), will be described as the type of a new genus.

**** Only juveniles were recovered from beetles.

DNA EXTRACTION, PCR AND SEQUENCING

To obtain DNA, several nematode specimens of each sample were transferred to an Eppendorf tube containing 16 μ l ddH₂O, 2 μ l 10X PCR buffer and 2 μ l Proteinase K (600 μ g/ml) (Promega Benelux, Leiden, The Netherlands) and crushed with an ultrasonic homogeniser. 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 as described by Tanha Maafi *et al.* (2003). The forward D2A (5'-ACAAGTACCGTGAGG-GAAAGTTG-3') and reverse D3B (5'-TCGGAAGGAA-CCAGCTACTA-3') primers were used for amplification and sequencing of the fragment of the 28S rRNA gene.

SEQUENCE ALIGNMENTS AND PHYLOGENETIC ANALYSIS

Nucleotide insertions or deletions are commonly found in the ribosomal RNA gene and its spacer regions, and these may create a problem for construction of an optimal alignment. Several approaches have been proposed and tested using nematode sequence data to minimise the influence of possible incorrect alignment procedures on phylogenetic inference (Adams *et al.*, 1998; Subbotin *et al.*, 2001; Spiridonov *et al.*, 2004). To approach this problem in our study, we generated nine alignments using ClustalX with gap open penalty parameters of 10, 15 (default), and 20 and gap length penalty parameters of 3, 6.66 (default), and 10 as well as culled alignment (Table 2). A culled alignment was created using GenDoc 2.5.0 after manually removing the

Table 2. Alignment parameters and tree statistics for maximum parsimonious trees generated from D2-D3 sequences of species from the orders Tylenchida and Aphelenchida.

No	Gap	Gap	Aligned	Informative	Constant	Tree	Tree	CI	HI	RI	RC	g1
computer	open	length	length	characters	characters	length	number	(w/o uninf)	(w/o uninf)			
alignment	penalty	penalty										
	Culled		629	415	167	4917	63	0.2077	0.7923	0.5555	0.1202	-0.308
1	10	3	936	514	340	5979	94	0.2138	0.7862	0.5504	0.1248	-0.379
2	10	6.66	898	515	295	6113	1	0.2248	0.7752	0.5444	0.1224	-0.369
3	10	10	814	525	226	6196	6	0.2235	0.7865	0.5463	0.1121	-0.372
4	15	3	910	506	301	6213	12	0.2220	0.7780	0.5487	0.1218	-0.374
5	15	6.66	897	509	293	6258	45	0.2044	0.7956	0.5464	0.1193	-0.372
6	15	10	807	512	226	6299	41	0.2183	0.7817	0.5494	0.1199	-0.366
7	20	3	896	503	291	6322	24	0.2178	0.7822	0.5486	0.1195	-0.377
8	20	6.66	897	508	295	6371	6	0.2152	0.7848	0.5472	0.1178	-0.370
9	20	10	798	517	212	6401	8	0.2176	0.7824	0.5477	0.1192	-0.395

Table 3. Base composition and length of D2-D3 expansion fragment for tylenchids and outgroup taxa.

Taxa	D2 and D3 expansion fragments				
	A%	C%	G%	T%	Length
Hoplolaimina	17.2 (14.5-25.6)	23.5 (16.3-28.0)	34.1 (23.4-37.2)	25.0 (21.5-27.6)	553 (533-561)
Criconematina	16.7 (15.5-19.1)	25.3 (23.7-26.5)	33.5 (31.3-36.9)	24.4 (22.9-26.7)	537 (501-548)
Tylenchidae	18.0 (15.1-21.1)	23.2 (20.9-26.4)	33.2 (30.4-36.2)	25.5 (22.2-27.7)	546 (545-549)
Anguinidae (without <i>D. destructor</i>)	21.3 (21.0-22.8)	20.9 (19.3-22.1)	31.0 (30.5-31.5)	26.7 (25.6-27.6)	555 (553-557)
Allantonematidae	18.1 (15.5-20.7)	21.4 (19.0-22.5)	32.6 (29.8-36.1)	27.8 (25.7-30.2)	582 (573-593)
Iotonchioidea (<i>Wachekitylenchus</i> + <i>Skarbilovinema</i>)	30.1, 33.7	13.0, 13.2	19.4, 18.1	37.2, 34.9	749, 712
Tylenchida	18.2 (14.5-33.7)	22.9 (13.0-28.0)	32.9 (18.1-36.9)	25.8 (19.2-37.2)	557 (501-749)
Aphelenchida (outgroup)	16.9 (14.5-20.6)	21.7(18.8-22.2)	33.9 (31.2-35.9)	27.3 (25.0-29.3)	551 (534-557)

most ambiguous positions from an automatic alignment generated with default parameters. All these alignments were compared by use of MP and BI approaches. We are interested in the branching orders of clades and their statistical support within each analysis (Table 3). For testing congruence of new molecular phylogenies with extant tylenchid morphological classifications and phylogenies we took two alignments including the culled alignment and automatic alignment generated with default options. In order to reduce computing time for the ML testing procedures, we used these alignments with half of the species (41 species) selected to broadly represent the main clades.

Alignments with the full set of taxa were analysed using MP and BI approaches. MP was performed with PAUP* 4b4a (Swofford, 2003). For MP the gaps were coded as missing data and molecular characters were assessed as independent, unordered, and equally weighted. Heuristic

search settings were ten replicates of random taxa, addition, tree bisection-reconnection, branch swapping, multiple tree retained and without steepest descent. Robustness of the clades was assessed by bootstrap analysis yielding bootstrap percentage (BS) for each node estimated from 1000 replicates. 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. A Bayesian interference analysis (BI) of the data set was conducted using MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). We applied a general-time-reversible (GTR) model of nucleotide substitution 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. Bayesian analyses were initiated with random starting trees and were run with four chains for 1.0×10^6 generations. Markov chains were sampled 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.

ML analysis of the culled alignment and automatic alignment (default options) with reduced numbers of species was performed using PAUP*. All necessary parameters for alignments were estimated from the data using ModelTest based on the Akaike Information Criterion (Posada & Crandall, 1998). Alternative topology was tested by the ML method of Shimodaira-Hasegawa (SH) and Kishino-Hasegawa (KH) as implemented in PAUP* with RELL bootstrap for 1000 replicates.

Results

SEQUENCE ANALYSIS

The entire fragment of the 28S rRNA gene used in the present analysis varied in tylenchids from 577 bp to 854 bp. For the D2-D3 fragments the longest length of 749 bp was revealed for *Wachekitylenchus boviensis* and the shortest (501 bp) was in *Trophonema arenarium*. A broad range of G + C content was observed among studied tylenchids and base frequencies differed significantly across taxa. Maximal content of T + A was found in two species from the superfamily Iotonchioidea (Table 3). Chi-square test of base frequency stationary indicated significant variation among tylenchid taxa in base composition ($\chi^2 = 877.70$, d.f. = 255, $P < 0.0001$). Because the substantial nucleotide compositional bias could potentially affect the phylogenetic results, we analysed the D2-D3 data set for automatic alignment 5 and the culled alignment with LogDet method as implemented in PAUP* to assess the effect of compositional bias on the resulting phylogeny. The topology of the neighbour-joining tree resulting from the LogDet analysis was largely in agreement with those of Bayesian analyses (unpubl.), and we concluded that this base bias did not significantly affect our phylogenetic results.

PHYLOGENETIC ANALYSES

Culled alignment

A culled alignment was manually created from automatic alignment 5 after removing 268 nucleotide positions. Phylogenetic signal in the data set was significant

($P < 0.01$) based on the value of the $g1$ statistic (-0.308) (Table 2). The BI majority consensus tree indicated the division of Tylenchida into seven main clades (Fig. 1). Clade I (PP = 99%) included two representatives of the superfamily Iotonchioidea and Clade II (PP = 99%) comprised three species of the family Allantonematidae and *Parasitylenchus* sp. These clades occupied a basal position within tylenchids. Clade III (PP = 99%) consisted of most species from the family Anguinidae (excluding *Ditylenchus destructor*) and an unidentified nematode associated with an insect. Relationships of *Eutylenchus excretorius* or *D. destructor* with other Anguinidae were not resolved. Representatives of the families Tylenchidae (Clade V) and Psilenchidae with subfamily Merliniinae (Clade IV) form a highly supported clade (PP = 96%). Clade VI (PP = 99%) includes all species of the suborder Criconematina with early divergence separating *Sphaeronema alni* from others. Clade VI (PP = 96%) consists of all Hoplolaimina, except Psilenchidae and subfamily Merliniinae. Monophylies of Heteroderidae, Hoplolaiminae, Pratylenchinae, Meloidogynidae were strongly suggested (PP > 90%). Relationships within species of the Rotylenchoidea remain unresolved. *Radopholus* sp. was clustered with *Meloidodera* and *Cryphodera* (PP = 84%). *Meloidogyne*, *Pratylenchus* and *Hirschmanniella* grouped in a highly supported clade (PP = 99%). Their sister relationship with Telotylenchinae + Macrotriphuriinae were strongly evident (PP = 97%). The grouping of Clades VI and VII was robustly supported (PP = 96%). MP analysis resulted in 63 maximum parsimonious trees (unpubl.), with high support for Clades I (BS = 100%), IV (BS = 92%), VI (BS = 99%) and moderate support for Clade VII (BS = 75%). Relationships between the main clades in the MP tree were not well resolved compared with those observed in the BI tree.

Automatic alignments

The $g1$ statistic indicated significant phylogenetic signal in all alignments (Table 2). The general trend to form the seven main clades within tylenchids was observed across phylogenetic analyses of the nine automatic alignments, although with different levels of statistical support (Fig. 2). Posterior probabilities and bootstrap values for BI and MP trees, respectively, for selected clades obtained after analyses of nine automatic alignments are given in Table 4. The phylogenetic pattern depended on alignment parameters and applied phylogenetic methods are summarised below. In BI trees constructed from alignments generated with the lowest gap open and gap length penalty parameters, Allantonemati-

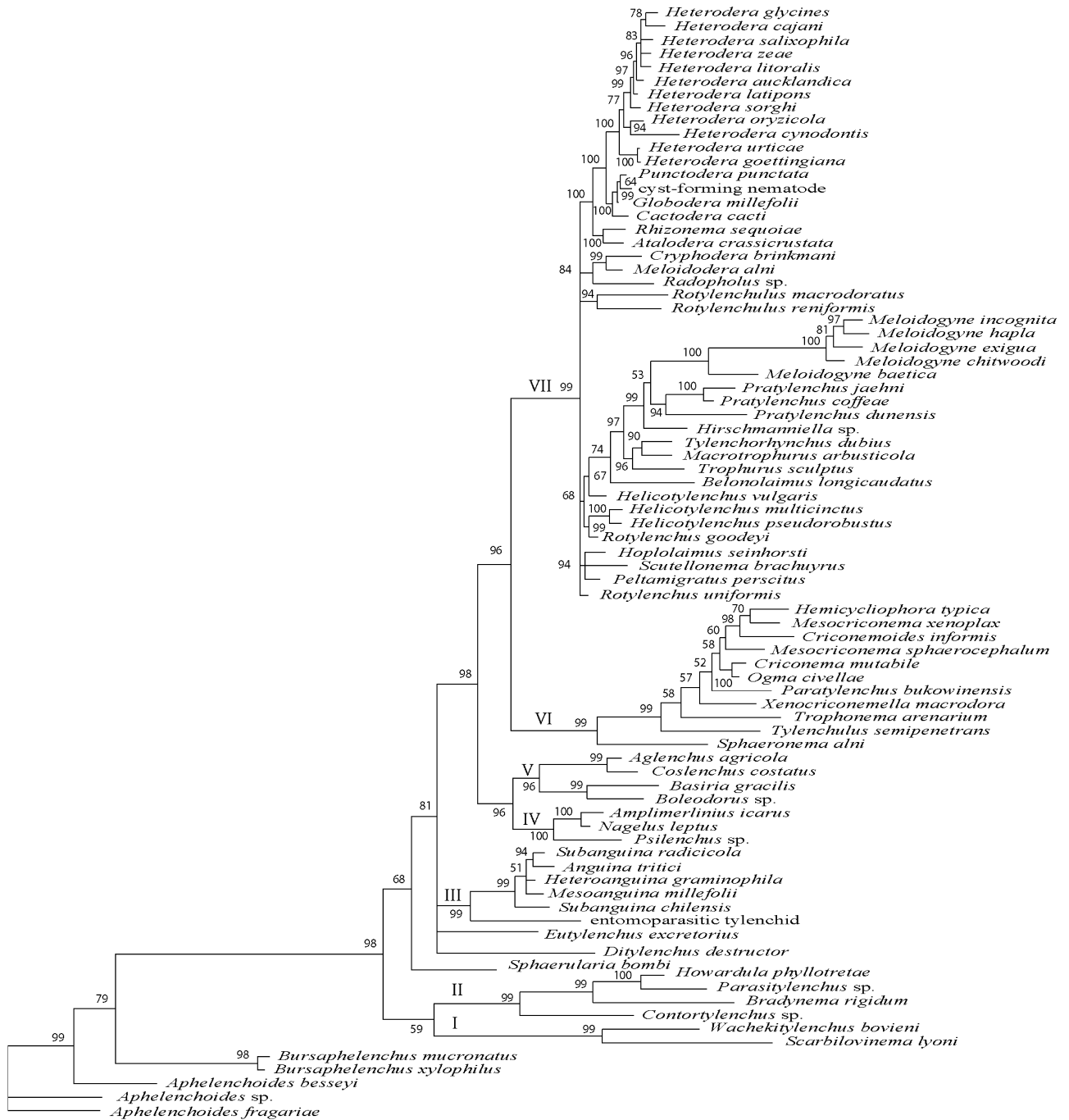


Fig. 1. The 50% majority rule consensus trees from Bayesian analysis generated with the GTR + I + G model from culled alignment of 82 sequences of the D2-D3 expansion region of the 28S-rRNA gene from nematodes of the Tylenchida and Aphelenchida. Posterior probability is given as a percentage for each appropriate clade.

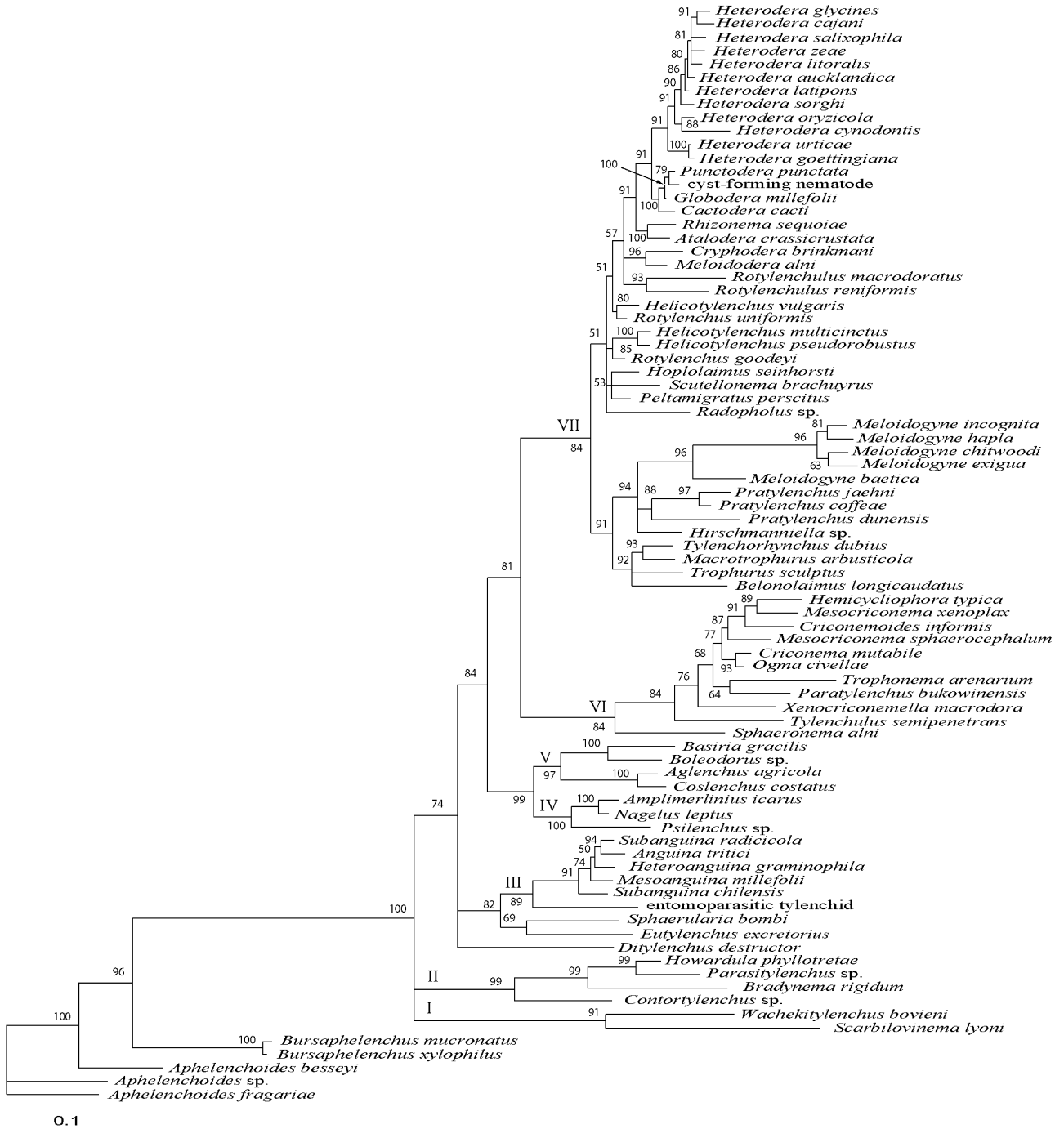


Fig. 2. The 50% majority rule consensus trees from Bayesian analysis generated with the GTR + I + G model from automatic ClustalX alignment (with default options) of 82 sequences of the D2-D3 expansion region of the 28S-rRNA gene from nematodes of the Tylenchida and Aphelenchida. Posterior probability is given as a percentage for each appropriate clade.

Table 4. Occurrence of selected clades in the topologies resulted from maximum parsimony and Bayesian inference analyses of the tylenchid D2-D3 expansion segments of rRNA gene sequences.

Clade	Culled		1		2		3		4		5		6		7		8		9		
	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	MP	BI	
Clade I (Iotonchiidae + Heterotylenchinae)	100	99	100	84	100	98	100	100	100	86	100	91	100	99	100	98	100	98	100	100	97
Clade II (Allantonematidae with <i>Parasitylenchus</i> sp.)	65	99	61	77	-	-	85	95	76	56	67	99	86	95	82	85	69	64	70	96	
Allantonematidae + Iotonchiodea	-	59	54	82	74	96	-	-	-	81	-	-	-	-	-	-	-	-	-	-	-
Clade III (Anguininae* + unidentified entomoparasitic tylenchid)	72	99	72	65	79	98	74	98	63	94	70	89	66	85	55	89	51	84	62	61	
Anguininae* + unidentified entomoparasitic tylenchid + <i>Eutylenchus excretorius</i> + <i>Sphaerularia bombi</i>	-	-	63	99	-	-	66	-	91	97	-	82	92	92	70	97	-	92	65	-	
<i>Eutylenchus excretorius</i> + <i>Sphaerularia bombi</i>	-	-	-	92	-	-	-	53	-	88	-	69	-	98	-	97	-	98	-	78	
Anguininae*	100	99	100	99	100	99	100	99	100	98	100	91	100	96	100	97	100	99	100	73	
Clade IV (Merlininae + Psilenchidae)	92	100	91	98	78	99	67	100	78	99	89	100	90	99	94	99	87	98	87	98	
Clade V (Tylenchidae)	53	96	-	92	58	99	65	99	63	98	-	97	-	98	-	83	-	50	-	52	
Merlininae + Psilenchidae + Tylenchidae	-	96	-	89	-	-	-	99	62	98	59	99	-	92	-	-	-	-	-	-	
Tylenchinae (<i>Aglenchus</i> + <i>Costlenchus</i>)	100	99	100	100	100	100	100	99	100	99	100	100	100	99	100	99	100	98	100	100	
Boleodorinae (<i>Basiria</i> + <i>Boleodorus</i>)	69	99	82	100	71	100	87	99	78	99	78	100	91	99	68	100	86	98	88	100	
Clade VI (Criconematina)	99	99	100	99	99	93	98	99	99	98	99	84	100	99	98	93	97	99	98	72	
Basal position of <i>Sphaeronema alni</i> within Criconematina	91	99	84	99	92	93	87	99	88	98	79	84	90	99	82	90	79	99	85	97	
Clade VII (Hoplolaimoidea + Belonolaimidae + Telotylenchinae + Macrotylphurinae)	75	99	64	82	90	92	77	99	60	85	82	84	76	98	88	93	87	98	94	73	
Meloitogynidae + Hirschmanniellinae + Pratylenchinae + Belonolaimidae + Telotylenchinae + Macrotylphurinae	-	74	-	82	58	97	-	88	-	94	-	91	-	97	-	-	-	59	-	-	
Cyst nematodes (Heteroderinae + Punctoderinae)	68	100	88	97	90	95	91	99	93	85	85	91	86	98	78	97	87	98	89	73	
Heteroderinae (<i>Heterodera</i>)	59	100	87	97	84	95	86	99	87	85	81	91	81	98	80	97	85	98	90	73	
Punctoderinae (<i>Punctodera</i> + <i>Globodera</i> + <i>Cactodera</i> + unidentified cyst-forming nematode)	81	100	84	99	80	99	81	100	78	99	74	100	76	100	86	100	76	100	80	99	
Heteroderinae + Punctoderinae + Aialoderinae	50	100	76	97	68	95	66	99	70	85	60	91	63	98	-	97	64	98	65	73	
<i>Cryphodera</i> + <i>Meloitodera</i>	74	99	90	100	81	100	78	100	85	100	85	96	85	100	79	97	81	100	82	100	
<i>Rhizonema</i> + <i>Atalodera</i>	78	100	90	100	88	100	85	100	79	99	78	100	76	100	75	100	79	100	78	99	
Hoplolaiminae (<i>Scutellonema</i> + <i>Hoplolaimus</i> + <i>Peltamigratus</i>)	-	94	-	-	-	-	-	-	-	-	-	53	-	-	-	61	-	-	-	-	
Hoplolaiminae + <i>Radopholus</i>	-	-	-	65	52	64	-	85	-	83	-	-	-	63	-	-	-	60	-	62	
Heteroderidae + Hoplolaimidae + Rotylenchulidae + <i>Radopholus</i>	-	-	69	76	52	91	59	88	51	73	-	51	-	74	-	88	66	97	67	73	
<i>Meloitogyne</i>	67	100	61	81	85	94	66	96	100	67	77	96	71	99	78	99	81	99	83	75	
<i>Meloitogyne</i> + <i>Pratylenchus</i> + <i>Hirschmanniella</i>	-	99	57	81	86	95	76	88	-	96	67	94	65	99	66	97	75	87	78	74	
Belonolaimidae + Telotylenchinae + Macrotylphurinae	-	-	68	97	65	90	57	99	63	97	65	92	62	97	-	50	-	-	-	-	
<i>Tylenchorhynchus</i> + <i>Macrotylphurus</i>	84	90	85	88	77	92	78	98	84	97	87	93	83	95	87	98	91	96	90	98	
Clade VI + Clade VII	-	96	-	80	-	95	-	88	-	81	-	95	-	95	-	92	-	94	-	60	

*Anguininae as interpreted herein includes *Subanguina*, *Anguina*, *Mesoanguina* and *Heteroanguina*.

- no clade or BS or PP < 50%.

dae and Iotonchioidea form a distinct group with moderate or high PP; in other BI trees they were not sister taxa and Iotonchioidea (without *Parasitylenchus* sp.) was placed in a basal position within tylenchids. Clade III including the plant-parasitic Anguinidae and an unidentified entomoparasitic tylenchid, as well as *Eutylenchus excretorius* and *Sphaerularia bombi*, appeared in MP and BI trees respectively from three and four alignments. A sister relationship of Anguininae and the unidentified entomoparasitic nematode was evident from all analyses but with differing levels of support. Clade IV (Merliniinae, Psilenchidae and Tylenchidae) was moderately to highly supported (PP = 89-99%) in BI trees from five automatic alignments and weakly supported in MP trees from two alignments. In other trees these interrelationships were not observed. A sister relationship between Tylenchinae and Boleodorinae (Clade V) was supported in BI trees across all alignments, whereas it was found only in the MP trees from three alignments with low bootstrap support. Sister relationship between Merliniinae and Psilenchidae (Clade IV) were highly supported among most trees. Clade VI, including all representatives of the suborder Criconematina, was moderately to highly supported by MP and BI analyses. The basal position of *Sphaeronema alni* to all other Criconematina was evident in all trees. Clade VII, including nematodes from the suborder Hoplolaimina (excluding Psilenchidae and Merliniinae), was weakly, moderately or highly supported, depending on alignment parameters and tree-building methods (Table 4). Molecular data suggested monophyly for the subfamilies Heteroderinae and Punctoderinae *sensu* Wouts (1985), and monophyly for *Heterodera* as well as for *Meloidogyne*. Sister relationship between the genera *Cryphodera* and *Meloidodera*, and between *Rhizonema* and *Atalodera* were strongly suggested (Table 4). In most trees *Meloidogyne*, *Pratylenchus* and *Hirschmanniella* grouped together, however, interrelationships between these genera were not well resolved in most data sets. *Radopholus* sp. typically clustered with Hoplolaimidae or Heteroderidae, although in many cases its relationship with other genera was left uncertain. MP analyses gave lower support and did not allow resolution of relationships among the main tylenchid groups.

TESTING OF ALTERNATIVE HYPOTHESES

In this study we used the constrain option in PAUP to generate several trees which support some traditional views on tylenchid relationships from culled and automatic alignments. These trees were then tested. ML

tests for both datasets strongly rejected a number of traditional hypotheses: *i*) monophyly of the superfamily Dolichodoroidea *sensu* Siddiqi (2000); *ii*) placement of *Radopholus* within the family Pratylenchidae; *iii*) the sister relationship between cyst-forming (Heteroderidae) and root-knot nematodes (Meloidogynidae); and *iv*) *Eutylenchus* as a sister taxon of *Macrotriphurus* (Table 5). Furthermore, ML tests of trees from the culled alignment rejected the hypothesis of a sister relationship of *Psilenchus* and *Basiria*.

Results of the KH test for the other four hypotheses chosen *a priori* were also significant, whereas the SH test did not reject them (Table 5). The KH test rejected: *v*) the placement of *Eutylenchus* in the family Tylenchidae; *vi*) the sister relationship of *Psilenchus* and *Basiria*; *vii*) *D. destructor* as a sister taxon to the Anguininae; and *viii*) the basal position of *Psilenchus* within tylenchids. A tree constraining the monophyly of Hexatylinea *sensu* Siddiqi (2000) cannot be rejected by ML testing using either the culled or automatic alignment (Table 5).

Discussion

PHYLOGENETIC CONTENT OF D2-D3 DATA SET

Phylogenetic inference can be confounded by various evolutionary factors, including heterogeneity of nucleotide composition among taxa. The data obtained in this study revealed significant variation in base composition among taxa. When assessing the effect of compositional bias on the resulting phylogeny using the LogDet model for culled and automatic alignment we found that these trees were largely congruent with that of BI analyses. Absence of a compositional bias effect on our phylogenetic results also can be supported by the rather high variation of observed nucleotide composition within clades. As a recent simulation study showed, the base composition bias needed to mislead phylogenetic methods in simulated data sets is far higher than that normally found in nature (Conant & Lewis, 2001) and higher than in our data.

The accuracy of phylogenies deduced from molecular data depends critically on the accuracy of sequence alignment and the process of identifying areas of ambiguous alignment in order to include or exclude them is somewhat subjective. The approach to reduce mistakes in this step is to apply secondary structure rRNA information for alignment contractions. Recently we demonstrated the influence of alignment reconstruction based on

Table 5. Results of Shimodaira-Hasegawa (SH) and Kishino-Hasegawa (KH) tests of tree topologies and alternative hypotheses of the phylogeny of the Tylenchida.

Topology evaluated	Culled alignment					Automatic alignment N 5 (default options)				
	Tree numbers	Best log likelihood	Δ lnL	KH-test <i>P</i>	SH-test <i>P</i>	Tree numbers	Best log likelihood	Δ lnL	KH-test <i>P</i>	SH-test <i>P</i>
ML tree	1	13098.853	best	–	–	1	16155.808	best	–	–
MP tree	2	13122.881	24.028	0.073	0.412	2	16179.382	23.574	0.095	0.393
Monophyly of Hexatyulina <i>sensu</i> Siddiqi (2000)	2	13122.739	23.886	0.074	0.419	5	16183.235	27.427	0.077	0.289
Clade with all entomoparasitic nematodes	2	13123.963	25.110	0.073	0.382	2	16193.020	37.212	0.017*	0.123
Monophyly of Dolichodoroidea <i>sensu</i> Siddiqi (2000)	1	13193.440	94.587	0.000*	0.000*	1	16240.667	84.859	0.000*	0.004*
Clade <i>Eutylenchus</i> + Tylenchidae <i>sensu</i> Siddiqi (2000)	10	13133.841	34.988	0.028*	0.188	1	16211.372	55.564	0.003*	0.034*
Clade <i>Eutylenchus</i> + <i>Macrotriphurus</i> (Tylozorinae) <i>sensu</i> Maggenti <i>et al.</i> (1988)	3	13189.770	90.917	0.000*	0.001*	1	16253.832	98.023	0.000*	0.000*
Clade <i>Psilenchus</i> and <i>Basiria</i> (Boleodorinae) <i>sensu</i> Maggenti <i>et al.</i> (1988)	3	13154.765	55.912	0.000*	0.023*	2	16198.844	43.036	0.005*	0.077
<i>Psilenchus</i> at the basal position in Tylenchida	3	13148.423	49.570	0.001*	0.052	1	16192.467	36.659	0.010*	0.152
Clade <i>Ditylenchus destructor</i> + Anguinidae	2	13130.754	31.902	0.027*	0.198	2	16188.039	32.231	0.036*	0.211
Clade <i>Radopholus</i> sp. + all other Pratylenchidae	3	13173.096	74.243	0.003*	0.007*	1	16252.810	97.002	0.000*	0.000*
Clade Heteroderidae + Meloidogynidae <i>sensu</i> Maggenti <i>et al.</i> (1988)	1	13156.836	57.982	0.002*	0.026*	2	16216.692	60.884	0.001*	0.013*

* Hypothesis is rejected at level $P < 0.05$.

secondary structure of D2-D3 expansion fragments applied to an inferred phylogeny of Criconematina (Subbotin *et al.*, 2005). The primary disadvantage of this approach is that it is time consuming and does not guarantee unambiguous alignments for highly divergent sequences as are also found in our present data set. To overcome this problem we applied the strategy of analysing a series of automatic alignments generated under different gap length and gap open penalties. Although the position of certain clades was dependent on alignment parameters, several trends in taxonomic grouping were evident from all data sets as well as from the results of different tree-building methods.

Our results of MP analyses were generally equivalent to those of the Bayesian analyses. Some simulation studies suggest that Bayesian analyses may sometimes accord unduly high support values to questionable or incorrect

branches. On the other hand, BI may often provide more accurate estimates of phylogeny than parsimony because it incorporates explicit models of DNA sequence evolution and may be less sensitive to long-branch attraction (Alfaro *et al.*, 2003).

PHYLOGENY OF TYLENCHIDA

In many aspects, the phylogenetic pictures of tylenchid relationships that we obtained from analyses of partly sequencing the LSU are consistent with morphological evidence. Our results in MP and BI analyses show that the order Tylenchida comprises seven main lineages that largely correspond to taxonomic divisions proposed by Siddiqi (2000). These include the suborder Hoplolaimina (Clade VII) and suborder Criconematina (Clade VI), as well as the family Iotonchiidae with *Wachekitylenchus* (Clade I) and the family Allantonematidae with *Parasity-*

lenchus sp. (Clade II), the family Tylenchidae (Clade V), the family Psilenchidae with representatives of the subfamily Merliniinae (Clade IV), and the family Anguinidae (in our case excluding *Ditylenchus destructor* and with an unidentified entomoparasitic tylenchid nematode) (Clade III).

Pro-tylenchids

Two main hypotheses concerning the ancestral form of tylenchids have been proposed and discussed. The hypothesis that modern psilenchids most closely resemble the tylenchid ancestor was proposed by Paramonov (1967, 1970) and then later developed by Chizhov and Berezina (1988), Chizhov and Kruchina (1988) and Ryss (1993). Chizhov and Berezina (1988) studied the female genital system of Tylenchida and proposed *Psilenchus* as the most primitive tylenchid form, with ancestral characters including a didelphic genital system. Luc *et al.* (1987) also suggested that *Psilenchus* appears closest in form to the ancestors of the Tylenchina. Siddiqi (2000) believed that *Psilenchus*-like forms may be considered as ancestors of Hoplolaimina but not for other suborders. This point of view is congruent with our results showing that molecular data strongly reject a basal position of *Psilenchus* within tylenchids. According to Siddiqi's (1980, 2000) hypothesis, *Hexatylys* may be closest to the hypothetical ancestor of the Tylenchida. Although in the present study we did not analyse representatives of the genus *Hexatylys*, a basal position in our trees of other entomoparasitic nematodes may be significant relative to Siddiqi's hypothesis.

Hexatylina

Hexatylina are highly adaptive with respect to morphology and in having complicated generation life cycles. Siddiqi (2000) distinguished two superfamilies of Hexatylina: Sphaerularioidea and Iotonchioidea. Several findings add support to distinguish not two, but three, main evolutionary lineages within Hexatylina. Chizhov (2004) suggested three superfamilies: Sphaerularioidea, Allantonematoidea and Iotonchioidea, which are generally congruent with our molecular trees. Unfortunately, relationships between the three main tylenchid lineages of entomoparasitic nematodes still remain unclear based on the D2-D3 dataset, and even the hypothesis of a single origin of entomoparasitism among tylenchids cannot be rejected by ML tests. Additional taxa as well as other

gene fragments should be included in subsequent analyses to create a more reliable phylogeny for entomoparasitic tylenchids as well as to test their origin. As Siddiqi (2000) remarked, there is the possibility that parasitism of the insect haemocoel originated independently, and that Sphaerulariina, Allantonematina and Heterotylenchina may prove to be valid clades and justifiably considered as suborders.

Anguinoidea

Siddiqi (1986) classified Anguinoidea within Hexatylina but subsequently placed this superfamily separate from Tylenchoidea in a new infraorder, the Anguinata (Siddiqi, 2000). The basis for distinction of Anguinoidea from Tylenchoidea was considered to include a direct life cycle lacking an insect phase, and the presence of postdeirids (= 'prophasms' of Siddiqi, 2000). In the tylenchid cladogram (Siddiqi, 2000) Anguinata is suggested to share several synapomorphies with Hexatylina. Maggenti *et al.* (1987), Chizhov and Kruchina (1988) and Ryss (1993) concur that there is a close relationship of entomopathogenic hexatylin and Anguinoidea. In several of our BI trees Anguinoidea clustered in a basal position with Hexatylina, and the possibility of a sister relationship between the two groups cannot be ruled out by our data set.

Phylogenetic relationships among genera and species of gall-forming nematodes of the subfamily Anguininae were recently analysed using the ITS-rRNA gene sequences (Powers, 2001; Subbotin *et al.*, 2004). In this study, among other anguinids, we included *Subanguina chilensis*, which is unique among Anguininae because it induces galls on leaves of a deciduous tree, *Nothofagus obliqua*. Relationships within Anguininae are not well resolved by either the D2-D3 data or a previous ITS tree (Subbotin *et al.*, 2004), nevertheless, *S. chilensis* occurs within anguinids at the basal position in most trees. Surprisingly, in our data *D. destructor* does not appear as a sister taxon to Anguininae, although this placement is statistically conclusive based on the KH test, the SH test does not preclude this relationship. It has been shown that the SH test is more appropriate than the KH test for comparing multiple trees (Goldman *et al.*, 2000).

Criconematina

The MP and BI trees from the present analysis are fully congruent with our MP and BI tree constructed under a general-time-reversible (GTR) model of nucleotide substitution from an extended dataset for Criconematina (Subbotin *et al.*, 2005). It was shown in that study that the monophyly of Tylenchuloidea (with classical systems including Paratylenchidae, Tylenchulidae and Sphaeronematidae) was not supported in any molecular trees, although the ML test still could not reject validity of this superfamily. All analyses placed *Sphaeronema* at a basal position with varying levels of statistical support relative to all other in-group 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 after *Sphaeronema*. Application of different models, including a doublet model with 16 states, did not allow resolution of relationships within Criconematoidea. Subbotin *et al.* (2005) concluded that, if further phylogenetic analyses including those based on a longer LSU fragment or on a combination of several genes clearly support monophyly of Tylenchuloidea, the classification proposed by Geraert (1966) distinguishing five lineages (Criconematidae, Hemicycliophoridae, Paratylenchidae, Sphaeronematidae and Tylenchulidae) should be seriously considered.

Hoplolaimina

Nematodes of the suborder Hoplolaimina (Clade VII), composed of the superfamilies Dolichodoroidea and Hoplolaimoidea, include plant-parasitic nematodes that are often considered to have the most sophisticated host-parasite relationships and the greatest economic significance to agriculture. The Hoplolaimina clade is moderately or highly supported across most analyses, but not in its traditional composition. Of considerable significance is support for the paraphyly of the superfamily Dolichodoroidea by the exclusion of representatives of Merliniinae and Psilenchidae, which are instead resolved within Clade IV.

Merliniinae and Psilenchidae

In the present study, *Amplimerlinius* and *Nagelus* (Merliniinae) are sister taxa forming a clade with *Psilenchus* (Psilenchidae), and these three (all didelphic) often occur in some trees within a clade in a deeper position (*Basiria*,

Boleodorus, *Aglenchus*, *Coslenchus* – all monodelphic). This is contrary to traditional classifications. Within Tylenchoidea Maggenti *et al.* (1987) recognised the family Belonolaimidae to include the subfamilies Telotylenchinae and Belonolaiminae. This classification did not recognise the subfamily Merliniinae *sensu* Siddiqi (1971). Although Siddiqi (2000) included Merliniinae in the family Telotylenchidae (Dolichodoroidea) he noted that the accessory genital structures suggested that Merliniinae are distinctive among Telotylenchinae. Moreover, Ryss (1993) considered this taxon to be of family rank, *i.e.*, Merliniidae Siddiqi, 1971. While the placement of Merliniinae representatives outside Hoplolaimina is strongly supported by this D2-D3 data set, as well as by SSU sequence data (Subbotin *et al.*, unpubl.), the monophyly of this diverse group must be tested with additional representatives to justify adjustments in classification to more specifically reflect phylogeny.

Maggenti *et al.* (1987) considered *Psilenchus* to be within Boleodorinae (also including *Boleodorus* and *Basiria*) of the Tylenchidae/Tylenchoidea, but Siddiqi (1986, 2000) and Ryss (1993) recognised a separate family, Psilenchidae, within Dolichodoroidea. Psilenchidae has members that resemble Tylenchidae, including a weak stylet and elongated tail, but differ by other characters noted below. In the present study a sister relationship between Psilenchidae and Merliniinae is supported across most trees, challenging a clear understanding of morphological evolution that would further reflect this relationship. Relative to sister taxa, *Amplimerlinius*, *Nagelus* and *Psilenchus* are distinctive by didelphy, but they are united with many Tylenchoidea by, for example, the presence of deirids and distinct phasmids on the tail. Notably Ryss (1993), in an effort to understand deep level tylenchid phylogeny, defined a morphological lateral complex (amphid, phasmid, deirid, lateral field and head sensory organs) and suggested that there are only a few genera which, by his definition, have a complete set of these structures. *Psilenchus*, *Atetylenchus* and *Antarctenchus* in Psilenchidae, and genera such as *Merlinius*, *Amplimerlinius*, *Geocenamus*, *Hexadorus* (subsequently synonymised with *Geocenamus*), *Nagelus* and *Scutylenchus* were included with *Pratylenchoides* in Merliniidae by Ryss (1993). While the morphological approaches by Ryss clearly have merit, a more robust phylogenetic framework is needed to address the difficulties of recognising convergence and of establishing character polarity to infer synapomorphies.

Macrotrophurus

The present study supports the placement of *Macrotrophurus arbusticola*, consistent with Siddiqi (1986), within a clade shared by other Dolichodoridae also including *Trophurus*, *Tylenchorhynchus* and *Belonolaimus*. More recently, Siddiqi (2000) placed *Macrotrophurus* in a subfamily separate from that of *Trophurus* but nevertheless within Dolichodoridae. This is in contrast to Luc *et al.* (1987) where *Macrotrophurus* was ‘tentatively’ placed within the Tylenchidae (Geraert & Raski, 1987). Morphological characters that may influence testing the uncertain position of *Macrotrophurus* might include the relatively posterior and elongate amphids openings relative to the more anterior small rounded amphid openings of most taxa of Belonolaimidae *sensu* Luc *et al.* (1987).

Radopholus

A significant result of our study is the indication for placement of *Radopholus* within Hoplolaimidae or Heteroderidae, and lack of support for its widely held traditional placement in Pratylenchidae (Siddiqi, 1986, 2000; Maggenti *et al.*, 1987; Chizhov & Kruchina, 1992). Luc (1987) noted that Pratylenchidae is an euryomorphic family, because its genera differ from each other by a rather large number of characters, and that *Radopholus* is set apart from other genera in Pratylenchidae by its strong secondary sexual dimorphism and the distinctive lip pattern as understood by comparative SEM. Clustering *Radopholus* with Hoplolaimidae and Heteroderidae was evident from our unpublished analyses of the 18S gene sequences for tylenchids (Subbotin *et al.*, unpubl.). The relationships of this genus with hoplolaimids and heteroderids should be further studied.

Heteroderidae and Meloidogynidae

The D2-D3 data give additional insight into the long-debated origin of Heteroderidae (including cyst-forming) and *Meloidogyne* (root-knot nematodes) that is pertinent to ongoing model systems for understanding pathways for pathogenesis (Baldwin *et al.*, 2004a). Our molecular data adds strong support to the argument that these groups do not have a unique common ancestor and we argue that this should be reflected in a revised classification.

Although from 1909 to 1949 (Chitwood, 1949) root-knot and cyst nematodes were typically included in a sin-

gle genus, subsequent information increasingly suggests that putative similarities are the result of convergence rather than common ancestry (Baldwin, 1992). Divergence of root-knot and cystoid nematodes was elegantly articulated by Wouts (1973) who proposed placing them in separate families, the Meloidogynidae and Heteroderidae, yet by maintaining these within the same superfamily, the resulting classifications nevertheless seemed to reflect common ancestry as indeed did the system of Luc *et al.* (1988) that reduced the ranks to Meloidogyninae and Heteroderinae within Heteroderidae. Others who, by accepting the two families with the lack of an exclusive higher rank, better reflected the independent evolution of these diverse groups (Siddiqi 1986, 2000; Chizhov & Kruchina, 1992).

The morphological similarity of *Rotylenchulus* (Hoplolaimidae) with respect to Heteroderinae has long been noted (Wouts, 1973, 1985) and particularly so since there appears to be a morphocline in female shape within Heteroderinae that basally includes taxa that are relatively elongate and sometimes even kidney-shaped (*e.g.*, *Verutus*, see Figure 3 in Baldwin *et al.*, 1989; Baldwin, 1992). Morphological congruence with the present molecular findings might suggest that the morphocline extends beyond classical heteroderids to *Rotylenchulus*. This can be more clearly tested and the relationship of heteroderids to hoplolaimids more fully resolved when additional heteroderids including *Verutus* are available to be included in an expanded molecular phylogenetic analysis.

Whereas common ancestry between Hoplolaimidae and Heteroderidae has long been proposed, consideration in combination with parphyly of Heteroderidae with the Meloidogynidae raises the question of what then is the sister taxon to root-knot nematodes. In the present study, support for a relationship of *Meloidogyne* with Pratylenchidae (*Pratylenchus* and *Hirschmanniella*) was observed across most trees. This result is congruent with a hypothesis suggested by Ryss (1988), that among Hoplolaimoidea, pratylenchids are most closely related to *Meloidogyne* by details of the lip region and pharyngeal structure. Ryss believed these morphological similarities to be indicative of common ancestry between Meloidogynidae and Pratylenchidae. We have noted, however, that even with the limited sampling of the present study, the classical interpretation of Pratylenchidae is not supported as monophyletic, including the position of *Radopholus* outside this clade.

While previously considered to be intermediate between *Heterodera* and *Meloidogyne* (Chitwood *et al.*,

1956), *Meloidodera* has since been recognised to be within Heteroderidae. In the present study, *Meloidodera* forms a clade with *Cryphodera* and these occupy a basal position within Heteroderidae. Among Heteroderidae Wouts (1985) distinguished six subfamilies: Verutinae; Meloidoderinae; Cryphoderinae; Heteroderinae; Ataloderinae; and Punctoderinae. Baldwin and Schouest (1990), maintaining the lower rank of Heteroderinae, distinguished the tribes Verutini, Meloidoderini, Cryphoderini, Sarisoderini, Ataloderini and Heteroderini, but Baldwin (1992) later proposed classifying the family using only the tribes Heteroderini, Ataloderini and Sarisoderini. Siddiqi (2000), on the other hand, recognised within Heteroderidae the three subfamilies Heteroderinae, Ataloderinae and Meloidoderinae. Our molecular data strongly suggest monophyly of cyst-forming nematodes and also two distinct groups justifying Heteroderinae and Punctoderinae as also supported by the ITS-rRNA phylogeny (Subbotin *et al.*, 2001). Interestingly, in our molecular trees an unidentified cyst-forming nematode species, parasitising the rush *Eleocharis gracilis* at a brackish lagoon in the South Island of New Zealand and morphologically similar to *Dolichodera*, has some support as a sister of *Punctodera*. We anticipate that this nematode will be described as the type of a new genus.

In all our analyses the non-cyst-forming Heteroderidae (*Atalodera* and *Rhizonema*) are resolved as a sister group to the cyst-forming nematodes. This relationship is in part congruent with a morphology-based phylogeny proposed by Baldwin and Schouest (1990) describing a clade that reflected monophyly of an Ataloderini-Heteroderini tribe. Previously, *Rhizonema* was synonymised to *Thecavermiculatus* by Siddiqi (1986), but reinstated by Luc *et al.* (1988) and Siddiqi (2000) on the grounds that *Rhizonema* is distinctive by the presence of a vulval cone, D-layer of the body wall cuticle, absence of male tail and the ability to induce a single, uninucleate, giant feeding cell. Two other heteroderid genera, *Meloidodera* and *Cryphodera*, are shown in the present study as sister taxa, although the validity of the subfamily Cryphoderinae, as questioned by Krall and Krall (1978), requires further testing including analysis of sequences of from additional non-cyst-forming heteroderids.

Tylenchoidea

As defined by Siddiqi (2000) Tylenchoidea exhibits a broad range of biological and morphological characters including monodelphy and presence of postdeirids. In this

study, although we included only a few representatives of Tylenchoidea, their clustering based on molecular data is, with the exception of *Eutylenchus*, congruent with morphological groupings by Siddiqi (2000). The placement of *Eutylenchus* out of Tylenchoidea is notable considering that the genus, together with *Atylenchus* (Atylenchidae), is distinctive within Tylenchida by the presence of four cephalic setae (Siddiqi, 2000). The phylogenetic position of Atylenchidae requires further resolution through the study of additional genes and taxa.

MORPHOLOGICAL CONSIDERATIONS

Molecular phylogenies provide an independent character set against which to test morphologically-based phylogenies and this test of congruence between diverse character sets is particularly significant in unravelling the complex phylogenetic patterns of Tylenchida. Although to a large degree morphologically-based phylogenies, including recognition of at least four major subgroups of the order (Siddiqi, 1980, 1986, 2000), are supported by molecular-based schemes, it is not surprising to discover classical morphological characters that might have mislead some aspects in traditional classifications. Such errors in interpreting phylogeny may be due in part to misinterpretations of characters (for morphology, often due to the limits of light microscope resolution) and character homologies, defining taxa on the basis of shared plesiomorphies, lack of recognition of wide-ranging convergence, and encountering the morphologies decoupled from apparent evolutionary roots by rapid rates of evolution and extinctions. In face of these difficulties, molecular phylogenies can provide a preliminary framework to discover morphological polarity (*i.e.*, the direction of character evolution) and the tests of congruence can distinguish those morphological character sets that directly track evolution, *versus* those that converge and reverse (Baldwin *et al.*, 2004a). In the present study, examples of convergence which were previously reported but herein underscored, include: independent origin of sedentary plant parasitism occurring at least twice; inclusion of heteroderids and *Meloidogyne* within Hoplolaimina; and possibly several times within Criconematina. What has been misleading is, at least in the case of heteroderids and *Meloidogyne*, that there are large sets of shared characters such as swollen females, vermiform males lacking caudal alae and multinucleate host syncytia. Whereas such large sets also are interpreted to support shared evolution on the basis of congruence, Wouts and Sher (1971)

and Baldwin (1992) argue that these sets of characters are linked. Additional examples of convergence might include the low anteriorly flattened head region and the tendency toward monodelphy, which together with other characters have been used to define Pratylenchidae. However, there is increasing evidence from the present study and from assessment of additional taxa and genes, that this family may be paraphyletic, for example, with respect to the position of *Radopholus* outside this group (Baldwin *et al.*, 2004b; Subbotin *et al.*, unpubl.). A particular difficulty occurs in interpreting morphological relationships among Tylenchida which share characters that are arguably plesiomorphic (deirids, didelphy, pharyngeal basal bulb), and while the temptation might be to group these taxa on the basis of these similarities, there may in fact be members that are basal to diverse clades. In this regard, *Amplimerlinius* and *Nagelus* superficially appear to be typical stunt nematodes, similar, for example, to *Tylenchorhynchus* and other genera in Dolichodoroidae/Dolichodoridae *sensu* Siddiqi (2000). Molecular resolution of *Amplimerlinius* and *Nagelus* as a sister taxon of *Psilenchus*, Tylenchoidea/respectively Tylenchida and Psilenchidae *sensu* Siddiqi, (2000), results in a group that retains the plesiomorphic didelphy but that shares a clade with monodelphic *Basiria*, *Boleodorus*, *Aglenchus* and *Coslenchus*. *Amplimerlinius*, *Nagelus* and *Psilenchus* share with these Tylenchidae the presence of a deirid and their removal from Dolichodoridae may result in a revised clade (*Tylenchorhynchus*, *Macrotrophurus*, *Trophurus*, *Belonolaimus*) united (and ultimately including perhaps all of Clade VI and VII) by the apparent absence of a deirid. Characters that appear to be highly mosaic (convergent) in defining these clades may include tail length and shape, some aspects of lip patterns as viewed with SEM, as well as, to some degree, position and shape of amphid openings. The Tylenchoidea have been defined in part by the presence of postdeirids (= prophasms *sensu* Siddiqi) vs typical phasms in Dolichodoroidae, but placement of Merliniinae (with typical phasms) within Tylenchoidea suggests the need for careful consideration of the polarity and potential convergence of these structures. Molecular phylogenies of Tylenchidae point to intriguing questions of morphological evolution and challenge us to employ emerging new tools in a comparative framework, in order to unravel these complex patterns in support of a refined and improved classification.

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