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Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda: Rhabditida)

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ABSTRACT

Tylenchina are a morphologically and functionally diverse group of nematode species that range from free-living bacteriovores, over transitory grazing root-hair feeders to highly specialized plant-parasites with complex host associations. We performed phylogenetic analyses of small subunit rDNA sequences from 97 species including an analysis that account for the RNA secondary structure in the models of evolution. The present study confirms the sister relationship of the bacteriovore Cephalobidae with the predominantly plant-parasitic Tylenchomorpha. All analyses appoint the fungal-feeding Aphelenchidae and Aphelenchoididae as being polyphyletic but the morphology based hypothesis of their monophyly could not be significantly rejected. Within the Tylenchomorpha, the families that exclusively parasitize higher plants are joined in a single clade. However, only the monophyletic position of the (super)families Hoplolaimidae and Criconematoidea were supported; Anguinidae, Tylenchidae, Belonolaimidae and Pratylenchidae appeared to be paraphyletic or polyphyletic. Parsimony and likelihood ancestral state reconstruction revealed that burrowing endoparasitism and sedentary endoparasitism each evolved, respectively, at least six and at least three times independently, mostly from migratory ectoparasitic ancestors. Only root-knot nematodes have evolved from burrowing endoparasitic nematodes. Traditional classifications are partially misled by this convergent evolution of feeding type and associated morphology. Contrastingly, mapping attributes of the gonoduct cellular architecture, including newly obtained data of 18 species belonging to the Aphelenchoidea, Criconematoidea, Anguinidae and Panagrolaimidae, revealed a broad congruence of the gonoduct characters and the molecular phylogenetic hypothesis. Yet, the presence of an offset spermatheca and proliferation of uterus cells has evolved multiple times, the latter associated with derived endoparasitic feeding specialization and resulting reproduction mode. Ancestral state reconstruction further revealed that the gonoduct of the morphologically and ecologically dissimilar tylenchid and cephalobid nematodes evolved from a common ancestor.

1. Introduction

Nematodes of the suborder Tylenchina *sensu* De Ley and Blaxter (2002) include an ecologically and morphologically diverse array of species that range from soil dwelling bacteriovores to highly specialised plant-parasites. De Ley and Blaxter (2002) recognised four infraorders in Tylenchina: Panagrolaimorpha, Cephalobomorpha, Drilonematomorpha and Tylenchomorpha. Panagrolaimorpha include insect pathogens (Steinernematidae), amphibian–reptilian parasites (Rhabdiasidae), parasites of vertebrates (Strongyloididae) and highly opportunistic bacteriovores notable to survive in extreme environments, such as vinegar (Panagrolaimidae). Cephalobomorpha are bacteriovores, not closely associated with animal hosts or arthropod vectors, and include some of the most widespread opportunists, but also numerous specialists of sandy soils and extreme temperatures. In desert and mountain soils, Cephalobomorpha may constitute more than half of the total nematode density (De Ley, 1992). Drilonematomorpha are relatively rare and are mainly parasites of earthworms. The Tylenchomorpha, the most intensively investigated infraorder within the Tylenchina, comprises the largest and economically most important group of plant-parasitic nematodes. They have exploited all plant organs including flowers and seeds, but they attack mostly roots. The evolution of plant-parasitic Tylenchomorpha is of particular interest because associations range from transitory grazing by root-hair feeders to the highly complex host-pathogen interactions of gall-inducing nematodes and their hosts. Non-plant-parasitic Tylenchomorpha feed on fungi, algae, lichens, mosses, insects, mites, leeches or frogs (Siddiqi, 2000). However, the evolution of this diversity of complex feeding traits is not yet fully understood (Subbotin et al., 2006).

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Despite their remarkably diverse ecological traits, Tylenchina exhibit relatively simple morphologies and superficial similarities may be the result of convergent evolution. Hence, the limited number of characters and especially the lack of objective criteria for assessing homology of these characters has hampered the reconstruction of their phylogeny. Furthermore, the ontogeny of nematodes is poorly understood and there is a lack of an informative fossil record (e.g. Poinar, 2003). Such difficulties have led to the erection of multiple, at least partially conflicting classifications. Traditional long-standing classifications have unified (e.g. Andrássy, 1976) the parasitic strongyloids and steinernematids together with the bacteriovore cephalobs and panagrolaims within the predominantly bacteriovorous order Rhabditida, while the mainly plant-parasitic aphelenchs and tylenchs were considered as a separate order Tylenchida. However, major parasitic groups were downgraded by De Ley and Blaxter (2002) in their hierarchical position to a level which conforms better to their phylogenetic history than to their previously anthropocentrically determined position. In this new classification the morphologically dissimilar panagrolaims, steinernematids, strongyloids, tylenchs and cephalobs were included in a single suborder Tylenchina because of moderate support of their common origin in small subunit (SSU) rDNA inferred phylogenies. This support is affirmed in recent phylogenetic analyses (Holterman et al., 2006; Meldal et al., 2007), although, a phylogeny based on large subunit (LSU) rDNA analysis rejects inclusion of *Steinernema* in Tylenchina (Nadler et al., 2006a).

Holterman et al. (2006), Meldal et al. (2007) produced phylum-wide SSU rDNA phylogenies and their identification of the major clades is based on a wider sampling compared to the first molecular phylogenetic framework of the phylum Nematoda (Blaxter et al., 1998). Within Tylenchina, LSU rDNA sequences have been used to infer relationships among Cephalobomorpha and Panagrolaimorpha (Nadler et al., 2006a), and Subbotin et al. (2006) inferred the relationships within the Tylenchomorpha (however without the Aphelenchoidea) based on D2–D3 expansion region of LSU rDNA. A multiple gene approach derived from an EST mining strategy has identified the relationships between the plant-parasitic genera *Meloidogyne*, *Heterodera* and *Globodera* (Scholl and Bird, 2005). Other phylogenies within Tylenchina, mainly based on nrDNA sequences or the internal transcribed spacers, are restricted to individual (super)families or genera. Recent studies include analyses of Heteroderidae (Subbotin et al., 2001), Anguinidae (Subbotin et al., 2004), Criconematoidea (Subbotin et al., 2005), *Meloidogyne* (De Ley et al., 2002; Tenente et al., 2004; Tigano et al., 2005), *Strongyloides* (Dorris et al., 2002), *Steinernema* (Nadler et al., 2006b) and Hoplolaimidae (Subbotin et al., 2007). In the majority of these rDNA based phylogenies, alignment of the sequences is guided by considering the RNA secondary structure. But, except for the individual (super)families Criconematoidea and Hoplolaimidae (Subbotin et al., 2005, 2007), accounting for this secondary structure in the models of evolution used in phylogeny reconstruction is largely ignored. However, because the functionality of RNA molecules lies in their secondary structure, which is mediated by base pairing between sometimes distant regions of the RNA molecule, there is a selective pressure for maintenance of the rRNA secondary structure. Substitutions affecting stem nucleotides have a different probability of fixation as compared to a nucleotide in a loop. The pairing between the stem nucleotides has thus important consequences for their evolution which differs from that of unpaired loop sequences. Considering the widely accepted view that employing more realistic models of sequence evolution should lead to more accurate phylogenies, the differences in evolution between stem and loop regions of rDNA should ideally be accounted for. Several studies have indeed shown the superiority of considering base pair correlation in RNA stems over methods assuming independent evolution of nucleotides (e.g. Savill et al., 2001; Murray et al., 2005; Telford et al., 2005), although other studies have only shown minor effects of different evolutionary models in phylogenetic reconstruction (Russo et al., 1996; Leliaert et al., 2007).

Nematodes are very attractive in evolutionary biology given the species richness of the phylum and the easiness with which several of these species can be cultured under laboratory conditions (Sommer, 2005). The reproductive system is herein of crucial importance for the study of fundamental problems in cell biology and developmental biology (Hubbard and Greenstein, 2000). Hence, the somatic gonad or gonoduct is intensively studied in the model organism *Caenorhabditis elegans* (Kimble and Hirsh, 1979; Hubbard and Greenstein, 2000) and the satellite model organism *Pristionchus pacificus* (Rudel et al., 2005). However, multiple species comparisons of detailed gonoduct characteristics are limited to vulval development analysis (e.g. Felix et al., 2000). Thus, data on the reproductive structure that cover an adequate part of the Tylenchina are largely limited to fragmentised light-microscopic data. The reproductive system is a complex organ composed of both somatic and germ line tissues and exhibits a variation in form (Chitwood and Chitwood, 1950), and its cellular architecture appears to be stable for a certain species (Geraert, 1983). Whereas in rhabditid nematodes the gonoduct structure varies significantly across genera (Geraert et al., 1980), the oviduct and uterus structure in Tylenchomorpha is more conserved. The number and spatial arrangement of spermatheca cells have been regarded as valuable characters to differentiate genera or species in the latter group (Geraert, 1983; Bert et al., 2002, 2003, 2006). Typically the mainly plant-parasitic Tylenchomorpha have a mono- or didelphic reproductive system with an oviduct comprising two rows of four cells, a distinct spermatheca comprising 10–20 cells, and a uterus composed of mostly regular rows of cells. The bacteriovorous Cephalobomorpha, which are considered to have a sister group relation with the Tylenchomorpha (Holterman et al., 2006; Meldal et al., 2007), appeared to have a Tylenchomorpha-like gonoduct structure (Bert et al., 2007) albeit their remarkable difference in general morphology. However, although the nematode reproductive system includes characters that are taxonomically useful or are possibly phylogenetically informative, evolutionary patterns explaining this variability are unknown. Intriguingly, the morphology of the gonoduct is likely to have a different selective pressure compared to the traditionally sampled nematode morphological characters; these are mostly feeding related and seem to show an apparent ubiquity of homoplasy in their morphology. The current study based on a large dataset will thus provide insights in the evolution of internal, reproduction related, morphological structures in Tylenchina that combine extreme ecological diversity with morphological flexibility.

Our main goals include (1) to infer the relationships within Tylenchina based on SSU rDNA sequences of 97 species, of which 17 were newly generated, (2) to trace the evolution of feeding types to obtain a minimal ecological framework of this extremely diverse array of species, (3) to describe the cellular structure of 18 additional nematodes belonging to the Panagrolaimidae, Aphelenchoidea, Criconematoidea, Anguinidae in order to complete the knowledge of the female reproductive system within the Tylenchina, and (4) to map the gonoduct characters on the phylogenetic tree to analyse the evolution of reproduction related structures.

2. Materials and methods

2.1. Taxon sampling

Sample information is listed in Table 1. We sampled 93 nematode species that are bacteriovores, entomopathogens, fungiphagous or plant-parasites within the Tylenchina, with main focus

Table 1
List of species, accession number, origin, summary of morphological data and (literature) source

Species and classification (Species associated with new accession number are in bold)	GenBank Accession no.	Origin/culture new material (either to obtain SSU sequence, morphological data or bot)	Cellular architecture[a]							Source morphological observations/**new** obtained morphological data
			Oviduct			Spermatheca		Uterus		
			1	2	3	4	5	6	7	
Suborder Rhabditina, Infraorder Rhabditomorpha, Family Rhabditidae										
Rhabditis blumi	**U13935**		2	N (ovi. + sp.: 2 × 12–15 cells)				N[b]	N	Geraert et al. (1980),
Caenorhabditis elegans	**X03680**		2	2	4	18–22	no	No	50	White (1988), Bert et al. (2007)
Infraorder Diplogasteromorpha, Family Diplogasteridae										
Pristionchus lheritieri	**AF036643**		2	2	5	4–5	No	No	12	Geraert et al. (1980), see also Rudel
Tylopharynx foetidus	**EU306343**	Pigeon aviary, Belgium (P. De Ley)	2	2	3	N	No	No	16	et al. (2005), Geraert et al. (1980)
Suborder Tylenchina, Infraorder Panagrolaimomorpha, Family Steinernematidae										
Steinernema carpocapsae	**AF036604**		2	Variable	18–25	N	No	No	>200	Zograf et al. (2008)
Family Strongyloididae										
Strongyloides ratti	**AF036605**		2	2	6	14–16	No	3	16	Triantaphyllou and Moncol (1977) & new
Strongyloides stercoralis	**AF279916**		2	N	N	N	N	N	N	(incomplete)[c]
Family Panagrolaimidae										
Baujardia mirabilis	**AF547385**		1	N	N	N	N	N	N	
Panagrellus redivivus	**AF036599**		1	N	5	30–50	No	4[d]	80–100	Geraert et al. (1980) **new**, see also
Panagrobelus stammeri	**AF202153**	PDL24 (P. De Ley)	1	2	2	10–12 offset	Yes	2d	N	Stock et al. (2002)
Panagrolaimus rigidus	**DQ285636**	AF40 (CGC)	1	2	11–13	N	No	No	60	**New**
Plectonchus hunti	**AF202154**		1	2	2	12	Yes	2[d]	N	Stock et al. (2002)
Turbatrix aceti	**AF202165**		1	2	3–4	16–21	No	4[d]	N	Geraert et al. (1980)
Infraorder Cephalobomorpha Family Cephalobidae										
Acrobeloides bodenheimeri	**AF202159**		1	2	4	12–14	Yes	2	N	Bert et al. (2007)
Acrobeloides maximus	**EU306344**	DF5048 (P. De Ley)	1	2	4	12–14	Yes	2	±35	Bert et al. (2007)
Cephalobus cubaensis	**AF202161**		1	2	4	12	Yes	3	N	Bert et al. (2007)
Zeldia punctata	**U61760**		1	2	4	10	Yes	4	±30	Bert et al. (2007)
Infraorder Tylenchomorpha, Superfamily Aphelenchoidea, Family Aphelenchoidea										
Aphelenchoides bicaudatus	**AY284643**		1	Possibly not homologous structures, coded as different						Geraert (1973)
Aphelenchoides blastophtorus	**AY284644**		1							Geraert (1973)
Aphelenchoides fragariae	**AJ966475**		1							**New**
Laimaphelenchus penardi	**EU306346**	Bark *Salix* sp. Belgium (A. Ryss)	1							**New**
Family Aphelenchidae										
Aphelenchus sp.	**AY284641**		1	Possibly not homologous structures, coded as different						**New** & Geraert (1973)
Aphelenchus avenae	**EU306347**	Maize field, Belgium	1							
Paraphelenchus sp.	**AY284642**		1							Geraert (1973)
Superfamily Sphaerularioidea, Family Neotylenchidae										
***Deladenus* sp.**	**EU306345**	P. De Ley	1	2	7	±30	No	4	N	Geraert (1976) & **new**[e]
Family Anguinidae										
Anguina tritici	**AY593913**		1	N	N	N	No	N	N	
Ditylenchus acris	**AY593914**		1	N	N	N	No	N	N	
Ditylenchus angustus	**AJ966483**	Fungal culture, UK	1	2	5	16	No	4	16	**New** (incomplete)
Ditylenchus destructor	**AY593912**	Pea field, Belgium	1	2	5	16	No	4	16	**New**
Pseudhalenchus minutus	**AY284638**		1	N	N	N	No	N	N	
Superfamily Tylenchoidea, Family Tylenchidae										
Boleodorus thylactus	**AY993976**		1	2	5	16	Yes	4	N	Bert et al. (2006)
B. thylactus	**AY593915**		1	2	5	16	Yes	4	N	Bert et al. (2006)
Cephalenchus hexalineatus	**AY284594**		1	2	5–7	12	No	4	28–32	Bert et al. (2006)

Coslenchus costatus	**AY284581**		1	2	4	10–12	No	4	20	Bert et al. (2006)
Coslenchus franklinae	**AY284583**		1	N	N	N	No	N	N	
Filenchus filiformis	**AY284592**		1	2	3–4	14–16	Yes	4	N	Bert et al. (2006)
Filenchus thornei	**AY284591**		1	2	3	14–16	Yes	4	16	Bert et al. (2006)
Neopsilenchus magnidens	**AY284585**		1	2	5	16	Yes	4	N	Bert et al. (2006)
Tylenchus arcuatus	**EU306348**	Moss, Belgium	1	2	4	12–14	Yes	4	16	Bert et al. (2006)
T. arcuatus	**EU306349**	Lawn, Belgium	1	2	4	12–14	Yes	4	16	Bert et al. (2006)
Tylenchus davainei	**AY284588**		1	2	3–4	12–14	Yes	4	16	Bert et al. (2006)
Tylenchus sp.	**AY284589**		1	2	N	12–16	N	4	N	Bert et al. (2006)
Family Tylenchidae (see Maggenti et al., 1987) or BeloNolaimidae (see Siddiqi, 2000)										
Psilenchus sp.	**AY284593**		2	2	5	16	No	3–4	N	Geraert (1981) & Bert et al. (2006)
Macrotrophurus arbusticola	**AY284595**		2	N	N	N	No	N	N	
Family Belonolaimidae										
Geocenamus brevidens	**AY284597**		2	2	4	12	No	3	12–15	Bert et al. (2006)
G. quadrifer	**AY993977**		2	2	4	12	Yes	3	9–21	Bert et al. (2006)
Nagelus obscurus	**EU306350**	*Phragmites australis*, Belgium	2	2	4	12–14	Yes	3	12	Bert et al. (2006)
Amplimerlinius icarus	**EU306351**	Lawn, Belgium	2	2	4	12–14	No	3	N	Bert et al. (2006)
Tylenchorhynchus maximus	**AY993979**		2	2	4	8–10	No	3	21–27	Bert et al. (2006)
T. dubius	**EU306352**	Maize field, Belgium	2	2	4	12–14	No	3	12–15	Bert et al. (2006)
Family Pratylenchidae										
Hirschmanniella loofi	**EU306353**	*Phragmites australis, Belgium*	2	2	4	10–12	No	3	21–27	Bert et al. (2003)
Hirschmanniella sp.	**AY284614**		2	N	N	N	No	N	N	
Pratylenchoides magnicauda	**AF202157**		2	2	4	12	No	3	18	Bert et al. (2003)
P. ritteri	**AJ966497**		2	2	4	12	Yes	3	18	Bert et al. (2003)
Nacobbus aberrans	**AF442190**		1	2	4	±14	No	No	>200 cells	Bert et al. (2003)
Pratylenchus sp. 1	**AY279545**		1	N	N	N	N	N	N	
Pratylenchus sp. 2	**AY286310**		1	N	N	N	N	N	N	
Pratylenchus sp. 3	**AY279544**		1	N	N	N	N	N	N	
P. crenatus	**AY284610**		1	2	4	12	No	3	12	Bert et al. (2003)
P. goodeyi	**AJ966498**		1	N	N	N	No	N	N	
P. penetrans	**AY286308**		1	2	4	12	No	3	12	Bert et al. (2003)
P. scribneri	**AY286309**		1	2	4	12	No	3	12	Bert et al. (2003)
P. vulnus	**AY286312**		1	2	4	12	No	3	12	Bert et al. (2003)
Radopholus similis	**AJ966502**		2	2	4	12	No	3	12	Bert et al. (2003)
Zygotylenchus guevarai	**AF442189**		2	2	4	12	No	3	12	Bert et al. (2003)
Family Hoplolaimidae										
Afenestrata koreana	**EU306357**	Bamboo, USA (R. Inserra)	2	2	4	20–26	No	3	>200	Bert et al. (2002)
Globodera pallida	**AF036592**		2	2	4	12	No	3[d]	>200	Bert et al. (2002)
G. rostochiensis	**AY593880**		2	2	4	14	No	3[d]	>200	Bert et al. (2002)
Helicotylenchus dihystera	**AJ966486**		2	2	4	12	Yes	3	12	Bert et al. (2006)
H. pseudorobustus	**AY284606**		2	2	4	12	Yes	3	12	Bert et al. (2006)
H. varicaudatus	**EU306354**	Riverbank, Belgium	2	2	4	12	Yes	3	12	Bert et al. (2006)
H. vulgaris	**AY284607**		2	N	N	N	Yes	N	N	
Heterodera schachtii	**EU306355**	Culture PSB, UGent, Belgium	2	2	4	12	No	3	12	Bert et al. (2002)
Rotylenchulus reniformis	**EU306342**	Culture PPS, The Netherlands (G. Karssen)	2	2	4	N	No	N	N	Bert et al. (2006)
Rotylenchus uniformis	**EU306356**	*Ligustrum* hedge, Belgium	2	2	4	12	No	3	12–15	Bert et al. (2006)
Scutellonema bradys	**AJ966504**		2	2	4	12–14	No	3	N	Bert et al. (2006)
Family Meloidogynidae										
Meloidogyne arenaria	**U42342**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. artiellia	**AF442192**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. chitwoodi	**AF442195**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. exigua	**AF442200**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. fallax	**AY593895**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. graminicola	**AF442196**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. hapla	**AF442194**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. ichinohei	**AF442191**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. incognita	**U81578**		2	2	4	16–18	No	3	>200	Bert et al. (2002)

(*continued on next page*)

Table 1 (*continued*)

Species and classification (Species associated with new accession number are in bold)	GenBank Accession no.	Origin/culture new material (either to obtain SSU sequence, morphological data or bot)	Cellular architecture[a]							Source morphological observations/**new** obtained morphological data
			Oviduct			Spermatheca		Uterus		
			1	2	3	4	5	6	7	
M. javanica	**AF442193**		2	2	4	16–18	No	3	>200	Bert et al. (2002)
M. microtyla	**AF442198**		2	2	4	18–26	No	3	>200	Bert et al. (2002)
Meloidogyne sp. 1	In process		2	2	4	16–18	No	3	>200	Bert et al. (2002)
Meloidogyne sp. 2	In process		2	N	N	N	N	N	N	
Superfamily Criconematoidea, Family Criconematidae										
Criconema sp.	**AJ966480**		1	2	4	10–15	No	No	N	**New**
Hemicriconemoides pseudobrachyurus	**AY284622**		1	N	N	N	No	N	N	
Mesocriconema xenoplax	**AY284625**		1	2	4	12–14	No	No		**New**
Family Hemicycliophoridae										
Hemicycliophora conida	**AJ966471**		1	2	4	10–12	No	No	16–18	**New**
H. thienemanni	**EU306341**	PPS, The Netherlands (G. Karssen)	1	2	4	10–12	Yes	No	16–18	**New**
Family Tylenchulidae										
Tylenchulus semipenetrans	**AJ966511**		1	N	N	N	No	No	N	**New** (incomplete)
Paratylenchus microdorus	**AY284632**	Lawn, Belgium	1	2	4	12–14	No	No	N	**New**
P. straeleni	**AY284630**		1	N	N	N	No	N	N	

[a] 1: number of gonoduct branches (mono- *vs.* didelphic); 2: number of oviduct cell rows;3: number of cells per oviduct cell row; 4: number of spermatheca cells; 5: spermatheca being offset *vs.* not offset; 6: number of uterus cell rows, "No" if not arranged in rows; 7: number of cells per uterus cell row.

[b] N: morphological observations absent.

[c] Morphological data are deduced from *in toto* observations of *S. ratti* combined with literature data of extruded gonads of *S. ransomi* (free-living generation).

[d] Somewhat irregular arrangement of cell rows.

[e] Data based on *D. siricidicola* (see Geraert, 1976) and incomplete own data on *Deladenus* sp.; all mycetophagous generations.

on Tylenchomorpha. Four representatives of the Rhabditina (Rhabditomorpha and Diplogasteromorpha) were selected as outgroup taxa based on existing hypotheses of their affinities with the Tylenchina (Blaxter et al., 1998; Holterman et al., 2006; Meldal et al., 2007). Seventeen nearly complete SSU rDNA sequences are novel; the remaining sequences were obtained from GenBank, mainly from Holterman et al. (2006), Meldal et al. (2007). Feeding type attributes were based on literature data (mainly Baldwin et al., 2004a); gonoduct attributes and their states were based on previous research and by analysing 18 additional species obtained from soil samples or cultures (see Table 1).

2.2. Molecular analysis

2.2.1. DNA amplification and sequencing

The extraction and selection of nematodes from soil samples was as described in Meldal et al. (2007). Nematode individuals were transferred into 25 ml worm lysis buffer (50 mM KCl; 10 mM Tris, pH 8.3; 2.5 mM MgCl2; 0.45% NP 40 (Tergitol Sigma); 0.45% Tween 20; 60 μg/ml proteinase K), cut into pieces and transferred into a 0.5 ml tube. The tubes were incubated at −80 °C (10 min), 65 °C (1 h) and 95 °C (10 min) consecutively. After centrifugation (1 min; 16,000*g*), 5 μl of the DNA suspension was added to the PCR mixture (*Taq DNA Polymerase*, Qiagen, Germany) with the G18S4 (5′-GCT TGT CTC AAA GAT TAA GCC-3′) and 18P (5′-TGA TCC WMC RGC AGG TTC AC-3′) primers. The PCR conditions were 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C for 40 cycles.

Sequencing was performed using an Applied Biosystems ABI 3130XL Genetic Analyser (Foster City, California, USA). Excess primer and dNTP were removed with ExoSAP-IT® (USB Corporation; Cleveland, Ohio, USA) for 15 min at 37 °C, followed by 15 min at 80 °C to inactivate the enzymes. This material was then used for cycle sequencing without any further purification, using the ABI Prism BigDye V 3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). The sequencing conditions were 30 s at 96 °C, 15 s at 50 °C and 1 min at 60 °C for 27 cycles. Primers used for sequencing are: G18S4, 18P, 2FX (5′-GGA AGG GCA CCA CCA GGA GTG G-3′), 23R (5′-TCT CGC TCG TTA TCG GAA T-3′), 13R (5′-GGG CAT CAC AGA CCT GTT A-3′), 23F (5′-ATT CCG ATA ACG AGC GAG A-3′), 9FX (5′-AAG TCT GGT GCC AGC AGC CGC-3′), 9R (5′-AGC TGG AAT TAC CGC GGC TG-3′), 26R (5′-CAT TCT TGG CAA ATG CTT TCG-3′) and 22R (5′-GCC TGC TGC CTT CCT TGG A-3′). Cycle sequence products were precipitated by adding 25 μl of 95% ethanol and 1 μl of 3 M sodium acetate, pH 4.6 to each cycle sequencing reaction (10 μl). The samples were placed at room temperature for 15 min and centrifuged at 14,000 rpm for 15 min. The pellet was additionally washed with 125 μl of 70% ethanol and dried in a Speedvac concentrator, redissolved in formamide and run on 50 cm capillaries with POP7 polymer. Sequences were edited and assembled with Seqman 7.0 (DNASTAR Lasergene; Madison, WI, USA).

2.2.2. Sequence alignment and phylogenetic analyses

The SSU rDNA sequences were aligned on the basis of their rRNA secondary structure information with DCSE v2.6 (De Rijk and De Wachter, 1993). The rationale for using secondary structure models for aligning rDNA sequences is based on the fact that the conservation of secondary structures exceeds that of nucleotides (Kjer, 1995). The SSU rDNA sequences of several representatives of Tylenchina and Rhabditina incorporated in the European Ribosomal RNA Database (http://www.psb.ugent.be/rRNA/) was used as an initial model for building the SSU alignment. The alignment of the variable helices 6, 8, 10, E10_1, 11, E23_1, E23_4, E23_7, E23_14, 29, 43, 44, 45, 46 and 49 of the SSU rRNA [see Van de Peer et al. (1999) and Appendix 1 (Supplementary data) for RNA secondary structure nomenclature] was refined and aided by folding

the sequences of representative samples using the Mfold software (http://www.bioinfo.rpi.edu/) (Zuker, 2003). Foldings were conducted at 20 °C using a search within 5% of thermodynamic suboptimality. The different optimal and suboptimal secondary structures were screened for common motifs. The alignment including the secondary structure annotation and phylogenetic trees can be obtained from WB on request and are available at TreeBASE (http://www.treebase.org, study number: SN3718).

The detection of saturated regions in the alignment was guided by screening the alignment for regions with elevated substitution rates. Site-specific substitution rates were calculated with HyPhy (Pond et al., 2005). The identified variable regions were then tested for substitutional saturation by analysing the measure of skewness (Hillis and Huelsenbeck, 1992) and the I_{ss} statistic (Xia and Xie, 2001) (see Supplementary data, Appendix 1).

Bayesian phylogenetic inference (BI) was performed with MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Two approaches of model selection were implemented. Firstly, a general time-reversible model with rate variation across sites and a proportion of invariable sites (GTR + I + G), as estimated by PAUP/MrModeltest 1.0b (Nylander, 2004), was selected for the entire alignment (from hereon referred to as BI-unpartitioned analysis). Secondly, the datasets was partitioned into stem and loop regions. We used the Xstem software (Telford et al., 2005) to extract the RNA secondary structure information from DCSE to a nexus format readable in MrBayes. Different substitution models were then selected for the two partitions. For the loop regions a GTR + I + G (a 4-state, single-nucleotide substitution model) was selected by PAUP/MrModelTest, while for the paired stem regions, the doublet model (a 16-state RNA stem substitution model, originally formulated by Schöniger and von Haeseler, 1994) was selected as recommended by Telford et al. (2005). A 16-state RNA substitution models consider pairs of nucleotides (16 possible pairs that can be formed with 4 bases) as their elementary states rather than single sites as in 4-state DNA substitution models. This analysis will be further referred to as BI-partitioned analysis. Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach with sampling according to the Metropolis-Hastings algorithm. For all analyses, two independent, simultaneous analyses were run for 3 million generations, each starting from different random trees and sampled every 1000th generation. Each analysis used four chains, one cold and three incrementally heated. Summary statistics and trees were generated using the last 1 million generations, well beyond the point of convergence between the two runs as confirmed by the average standard deviations of split frequencies between the two analyses which approached zero.

Maximum parsimony (MP), maximum likelihood (ML) and LogDet-transformed distance analyses were performed using PAUP* 4.0b10 (Swofford, 2002). Distance analyses using log determinant distances (LogDet; Lockhart et al., 1994) were especially used to cope with possible effects of compositional heterogeneity across taxa (CG%: 37–53%). ML analyses consisted of a heuristic search with 3000 random sequence addition replicates and Tree Bisection Reconnection (TBR) with the option Multrees. The optimal model of nucleotide substitution for ML was determined in Modeltest 3.6 according to the Akaike Information Criterion (Posada and Crandall, 1998): a GTR + I + G model (base frequencies and substitution rates see Table 2; gamma distribution shape parameter (G) = 0.71; proportion of invariable sites (I) = 0.25). MP and LogDet-transformed distance analyses were performed under the same heuristic search settings as ML, with a proportion of invariant sites that was set to 0.25 for the distance analysis. Robustness of the inferred MP, ML and distance trees were tested using nonparametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates for MP and LogDet criteria and 250 pseudoreplicates for ML.

The potential effect of long branch attraction, with special reference to the relative positions of the Aphelenchidae and Aphelenchoididae clades, on the tree topology was assessed by rerunning the analyses by selectively excluding divergent taxa: (1) only including the ingroup sequences, i.e. excluding *Rhabditis*, *Caenorhabditis*, *Diplogaster* and *Tylopharynx* (2) only including the Tylenchomorpha and Cephalobidae, and (3) excluding all taxa with long branches from the analyses. The likelihood of alternative topologies was tested against the optimal ML topology using Shimodaira–Hasegawa (SH) tests as implemented in PAUP using RELL optimization and 1000 bootstrap replicates (Shimodaira and Hasegawa, 1999).

2.3. Morphological analysis

Extraction and examination of the female reproductive system was based on the method of Geraert (1973). Four to ten live females of every studied population were individually transferred to a drop of tap water on a glass slide. Bisecting at the vulva region with a small scalpel induced expulsion of gut and reproductive tissue. The preparations were covered with a coverslip, sealed with nail polish, and observed directly with an Olympus BX 51 DIC microscope (Olympus optical, Tokyo, Japan). This procedure was repeated until at least 20 preparations could be observed for each population. Six features describing the cellular architecture of the female gonoduct were used: (1) number of gonoduct branches (mono- *vs.* didelphic); (2) number of oviduct cell rows; (3) number of cells per oviduct cell row; (4) number of spermatheca cells; (5) spermatheca being offset *vs.* not offset; (6) number of uterus cell rows; (7) number of cells per uterus cell row. Illustrations were prepared using a camera lucida; the drawings were prepared using Illustrator CS software (Adobe Systems, Mountain View,

Table 2
Specifications of the SSU rDNA alignment, summary of model parameters obtained, and estimated likelihoods/score/steps of the BI, ML, MP and distance analyses

Alignment	1925 characters, 97 taxa
Analyzed (saturated excluded)	1925/1715[a]
Parsimony informative sites	993/835[a]
Empirical base frequencies[b]	A = 0.25, C = 0.20, G = 0.25, T = 0.30
Substitution rates[b]	A–C = 1.37, A–G = 2.88, A–T = 1.58, C–G = 0.92, C–T = 4.27, G–T = 1.00
Probabilities for base pairs in loop regions[c]	CG/GC = 0 .24/0.20, AU/UA = 0.21/0.21, GU/UG = 0.04/0.04
BI (GTR + I + G) $-\ln L$	34505.6/26617.8[a]
BI (GTR + I + G model & doublet model) $-\ln L$	32148.8/24260.0[a]
ML (GTR + I + G)[b] $-\ln L$	34362.1/2648.6[a]
MP steps	7074/5157[a]
Distance score	3.83520/3.12678[a]

[a] Saturated sites included/excluded.
[b] Estimated by the Akai information criterion (AIC) implemented in ModelTest (Posada and Crandall, 1998).
[c] Posterior mean probabilities for base pairs in the RNA loop regions, as determined by summarizing the parameters from the MCMC in the Bayesian analysis.

California). The morphology was also recorded as video clips that mimic multifocal observation through a light microscope following the Video Capture and Editing procedures developed by De Ley and Bert (2002). The resulting virtual specimens are available on the web at: http://www.nematology.ugent.be/VCE.htm.

2.3.1. Character evolution

Feeding types and gonoduct character states was traced along the Bayesian phylogeny (Figs. 1 and 2) using parsimony and maximum likelihood reconstructions (Cunningham et al., 1998) implemented in Mesquite v1.11 (Maddison and Maddison, 2006). The character states at the internal nodes were also reconstructed using ML as the optimality criterion (Pagel, 1999) with the 'reconstruct ancestral states' module implemented in Mesquite. This likelihood reconstruction finds, for each node, the state assignment that maximises the probability of arriving at the observed states at the terminal taxa under an explicit model of character evolution. ML methods of ancestral state reconstruction are valuable because they use branch length information and quantify uncertainty in character state evolution (Pagel, 1999). The model used (Mk1) is a one-parameter Markov k-state model (Lewis, 2001). The likelihood values are expressed as proportional likelihood (pLh). An ancestral state at a given node was considered significant (appointed with $*$) and preferred over the other if its likelihood value was higher by at least two log units than the likelihood value of the other character state (likelihood decision threshold values [T] set to two by default in Mesquite). Because this ancestral states module in a likelihood framework does not allow multiple states, the coding used for parsimony was replaced by "unknown" for the very few characters with a multiple state.

3. Results

3.1. Sequence information and saturation

Specifications of the SSU rDNA dataset, and BI, ML, distance and MP scores are given in Table 2. The predicted consensus secondary structure of the SSU rDNA of the Tylenchina sequences, mapped on the sequence of the tylenchid nematode *Meloidogyne arenaria* is shown in supplemented material (Appendix 1). This secondary structure model was used for guiding the sequence alignment, and for partitioning the dataset into stems and loops. Saturation analysis revealed five highly variable regions, corresponding to the E23_1 and E23_7 helices and the terminal loops of helices 29, 43 and 49 (sites indicated in red in the RNA secondary structure model, Supplementary data, Appendix 1). Although the measure of skewness indicated that the complete alignment was significantly more structured than random data, calculation of the I_{ss} statistic clearly indicated that these variable regions were highly saturated and thus unsuitable for phylogenetic analyses (Supplementary data, Appendix 1). These saturated regions were therefore excluded from further analyses, although inclusion of these regions proved to have only minor effects on tree topology and resolution (Supplementary material, Appendix 1, Table S3).

3.2. Molecular phylogenetic analyses

The different phylogenetic analyses (BI-partitioned see Fig. 1; BI-unpartitioned, ML, MP, LogDet-distance, see Appendix 2 in Supplementary data) did not reveal essential conflicts in the resulting tree topologies, except for the position of certain individual taxa (*Steinernema carpocapsae*, *Cephalenchus hexalineatus* and *Psilenchus* sp.) and the position of the Aphelenchidae in respect to Cephalobidae (for a summary of conflicts see Table 3). However, compared to the BI-partitioned analysis, the number of resolved nodes (Table 3; Supplementary material, Appendix 1, Table S3) was slightly lower in the BI-unpartitioned and considerably lower in the ML, MP and LogDet distance results. Further enumeration of results, character analysis and discussion are mainly based on the tree resulting from the BI-partitioned analysis (Fig. 1).

The phylogenetic analysis recovered a tree with significantly different branch lengths among certain lineages. Especially *Strongyloides*, the sampled Panagrolaimidae and Aphelenchoididae had particularly long branches.

Based on our sampled taxa, the ingroup Tylenchina was strictly monophyletic with the entomopathogenic species *Steinernema carpocapsae* being sister to the remaining Tylenchina. Early divergences within the Tylenchina were not completely resolved, especially with respect to the relationship between the Panagrolaimidae, Aphelenchoidea and the remaining of the ingroup clade. Yet, the monophylies of Aphelenchoididae and Panagrolaimidae were maximally supported (PP = 100). The primarily bacteriovore Cephalobomorpha were consistently sister to the subdominant plant-parasitic Tylenchomorpha without Aphelenchoididae (PP = 100) (this well supported clade is here further appointed as the Cephalobomorpha–Tylenchomorpha clade). Aphelenchid nematodes (Aphelenchoidea: Aphelenchoididae + Aphelenchidae) appeared thus to be polyphyletic. Ingroup analysis, including only Cephalobomorpha and Tylenchomorpha, and excluding all long branches from the analyses did not alter the polyphyletic nature of the Aphelenchidae and Aphelenchoididae (results not shown). However, Shimodaira–Hasegawa tests did not reject ($p = 0.38$) the morphologically based hypothesis that Aphelenchoidea are monophyletic.

Tylenchid nematodes (=nematodes with tylenchid stylet; = Tylenchida *sensu* Siddiqi, 2000; =Tylenchomorpha without Aphelenchoidea) were found to be strictly monophyletic. The relationship between clades that include Tylenchidae, Anguinidae and *Deladenus* (tylenchs with ancestral morphological characters) were weakly supported. The primarily fungal-feeding Anguinidae and the entomopathogenic *Deladenus* formed a maximally supported clade that had a weakly supported sister relationship (PP = 64) with the Boleodorinae clade (Tylenchidae; mainly root-hair, epidermal cell or lower plant feeders). The remaining Tylenchidae formed a maximally supported clade, however without *Psilenchus* and *Cephalenchus*. The plant-parasites of higher plants (Belonolaimidae, Pratylenchidae, Hoplolaimidae and Meloidogynidae) plus *Psilenchus* sp. (Tylenchidae or Belonolaimidae) and *Cephalenchus hexalineatus* (Tylenchidae) formed a well supported clade (PP = 97). However, certain basal divergences within this "higher parasites clade" were weakly supported; *Psilenchus*, the Belonolaimidae (part 1) plus the genus *Pratylenchoides*, *Cephalenchus hexalineatus* and the monophyletic Criconematoidea had an uncertain position in respect to each other. Both the clade containing the ectoparasitic genera *Geocenamus*, *Nagelus*, *Amplimerlinius* (Belonolaimidae, part 1) plus the burrowing endoparasitic *Pratylenchoides* and the clade containing Criconematoidea were maximally supported.

The families Hoplolaimididae, Pratylenchidae (except *Pratylenchoides*) and Meloidogynidae, which comprises the economically most important plant-parasites, plus the genera *Tylenchorhynchus* and *Macrotrophurus* formed a relatively well supported clade (PP = 94). The burrowing endoparasitic species *Radopholus similis*, a notorious pest in banana and citrus, grouped with the Hoplolaimidae (ectoparasites and cyst-forming endoparasitic nematodes) (PP = 100). *Meloidogyne* (root-knot nematodes), *Pratylenchus*, *Zygotylenchus* and *Hirschmanniella* (burrowing endoparasites) were grouped in a highly supported clade (PP = 99). Their sister relation-

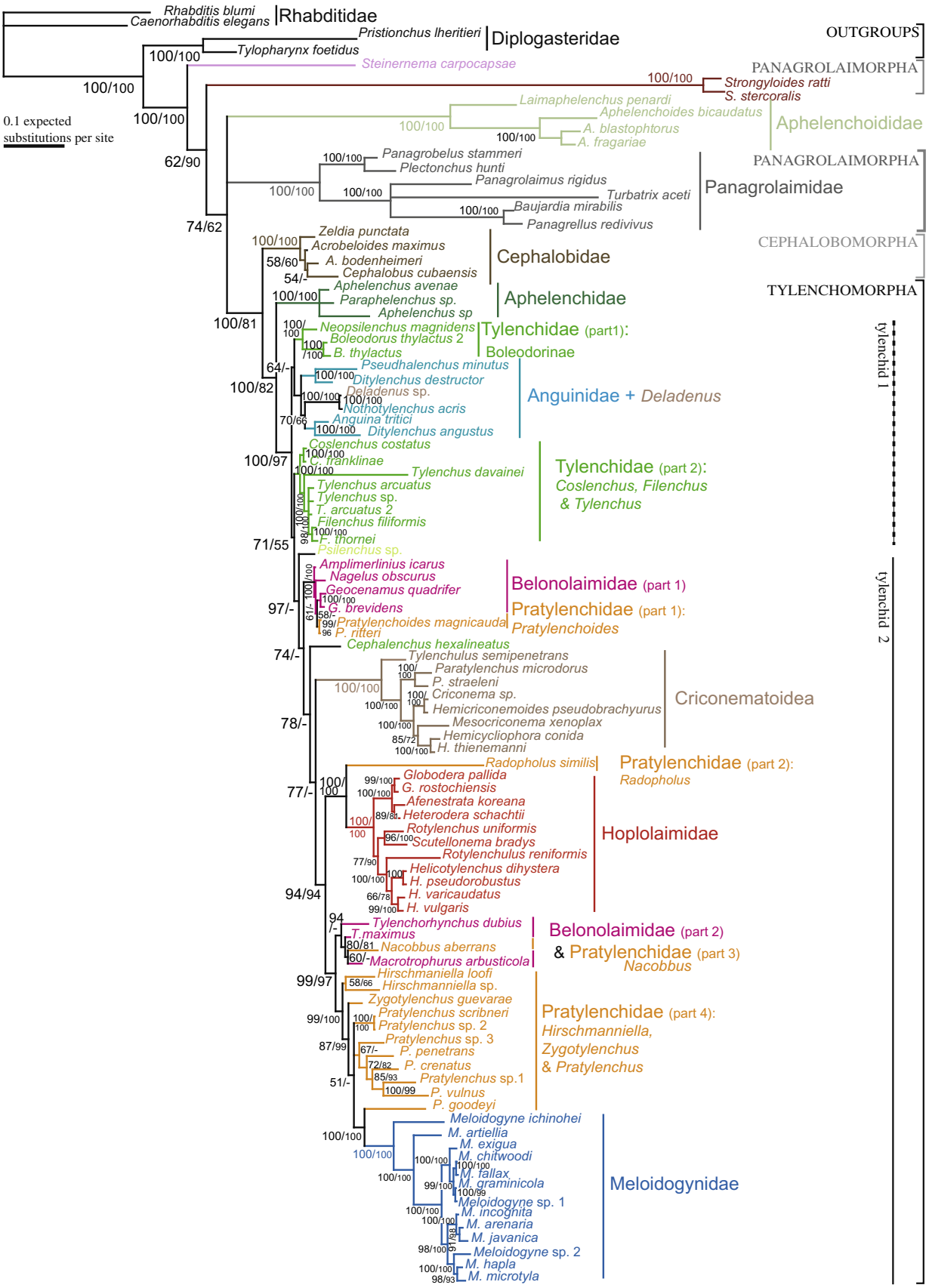

Fig. 1. BI 50% majority rule consensus phylogeny of the Tylenchina based on SSU rDNA data, analyzed with a stem–loop dataset, using a GTR + I + G model for the RNA loop regions and a doublet model for RNA stem regions. Branch support is indicated with PP; in addition, PP values from the BI-unpartitioned analysis (Supplementary data, Appendix 2A) are given (PP BI-partitioned analysis/PP BI-unpartitioned analysis). Species representing the same family are highlighted in the same colour.

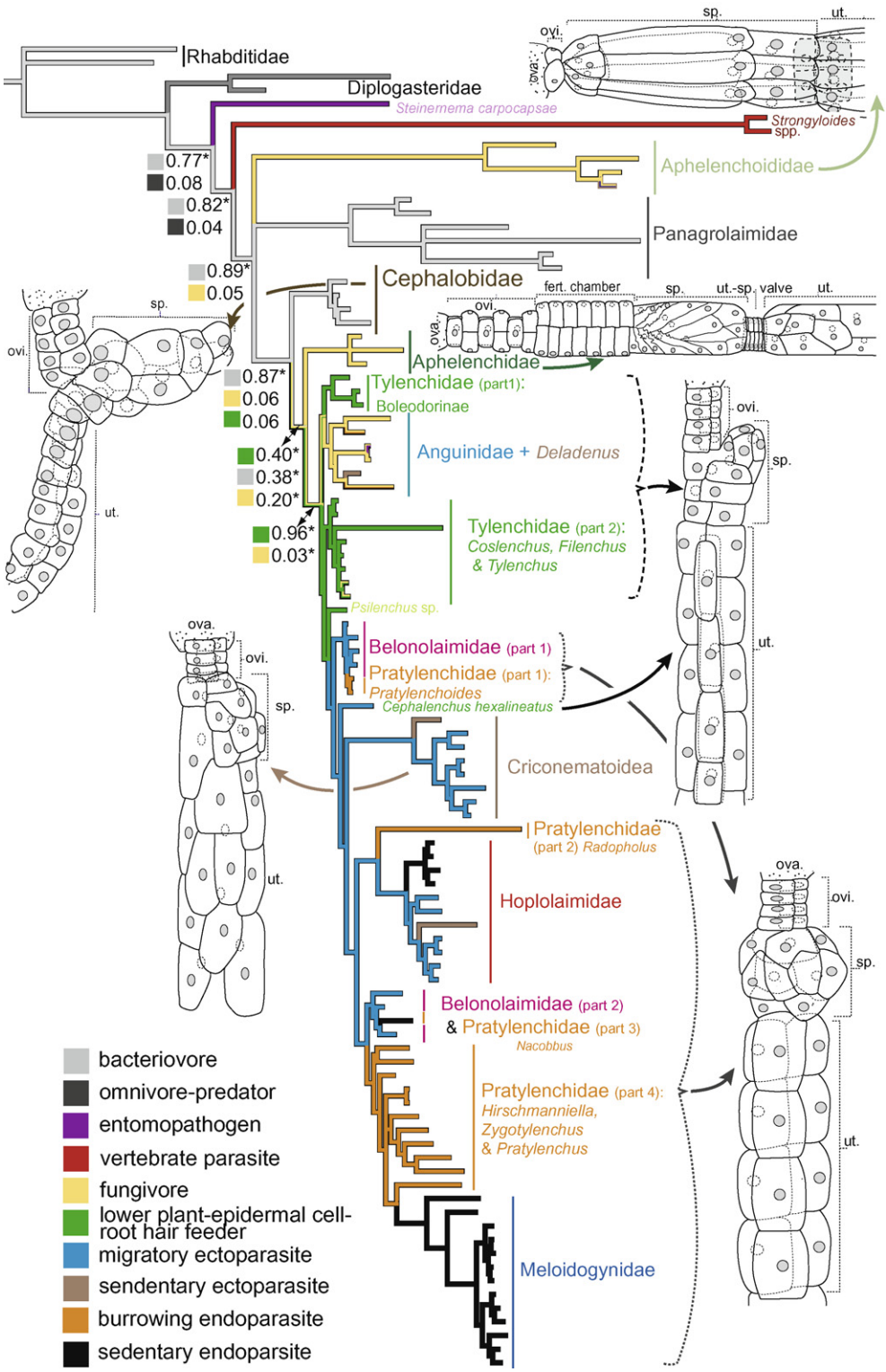

Fig. 2. Parsimony feeding type reconstruction onto the phylogenetic tree (see Fig. 1). Drawings represent generalized graphics of the gonoduct architecture of Aphelenchoididae, Aphelenchidae, Cephalobidae, "basal" tylenchids (≈tylenchid 1 in Fig. 4), Criconematoidea and other "derived" tylenchids (together with Criconematoidea ≈ tylenchid 2 in Fig. 4). Proportional likelihood values of likelihood reconstruction of ancestral states (symmetrical Mk1 model) are given if they are lower than 0.96 or if likelihood reconstructions differ (see ↔) from parsimony reconstructions of ancestral states (=colour at a given node). Significant level (∗): if likelihood value of character state is higher by at least two log units than the likelihood value of the other character state.

ship with *Tylenchorhynchus*, *Macrotrophurus* and *Nacobbus* was strongly supported (PP = 99). Also monophylies of Hoplolaimidae and Meloidogynidae were strongly supported (PP = 100). Cyst-forming (Heteroderinae) and root-knot nematodes (*Meloidogyne*) arose thus independently and the burrowing endoparasitic Pratylenchidae appeared to be polyphyletic.

Table 3
Node resolution and summary of inconsistencies between BI-partitioned, BI-unpartitioned, ML, MP and LogDet-transformed distance analysis of the SSU rDNA alignment

	BI (GTR + I + G & RNA)	BI (GTR + I + G)	ML	MP	LogDet
Node resolution[a]	61/28/6 64/29/6%	60/21/15 63/22/16%	48/18/29 51/19/31%	47/15/33 49/16/35%	57/15/23 60/16/24%
Sister relationship of *Steinernema* and the remaining Tylenchina {and associated support for the clade Tylenchina without *Steinernema*} (in bold); *vs*. sister relationship of *Steinernema carpocapsae* and *Strongyloides* spp.	**100 {62}**	**100 {90}**	**96 {62}**	72	78
Sister relationship of Cephalobidae and Tylenchomorpha (without Aphelenchoididae) {and associated support for Aphelenchidae + tylenchid nematodes} (in bold); *vs.* sister relationship of Aphelenchidae and Cephalobidae	**100 {100}**	**81 {82}**	**67 {60}**	**97 {86}**	62
Cephalenchus hexalineatus sister to the Criconematoidea, Pratylenchidae (without part 1), Hoplolaimidae, Belonolaimidae (part2) and Meloidogynidae (in bold); *vs. C. hexalineatus* unresolved within clade of Tylenchidae, Belonolaimidae (part1), Pratylenchidae (part1), *Psilenchus* and Anguinidae; *vs*. unresolved position(indicated by a ∗)	**78**	**87**	∗	∗	84
Psilenchus sp. sister relation to Belonolaimidae, Criconematoidea, Pratylenchidae, Hoplolaimidae and Meloidogynidae (in bold); *vs Psilenchus* sp. unresolved within clade of Tylenchidae, Belonolaimidae (part1), Pratylenchidae (part1), *Cephalenchus* and Anguinidae; *vs*. unresolved position (indicated by a ∗)	**97**	∗	∗	∗	84

[a] Number and percentages of high (⩾95% BI; ⩾70% ML, distance, MP)/moderate (<95% to ⩾50% BI; <70% to ⩾50% ML, distance, MP)/low (<50% BI, ML, distance, MP) supported branches.

3.3. Evolution of feeding type

The parsimony and likelihood reconstruction of the evolution of feeding types along the Bayesian phylogram is illustrated in Fig. 2. Both analyses reconstructed bacteriovore feeding at the root of Tylenchina with a proportional likelihood (pLh) of 0.77∗, the next best supported character state is omnivore-predator (pLh: 0.08). Following the hypothesis of the polyphyletic state of Aphelenchoidea, fungal-feeding has evolved at least twice: in the Aphelenchoididae and Tylenchomorpha. The ancestral feeding state of the Tylenchomorpha (without Aphelenchoididae) is ambiguous; parsimony reconstruction appointed fungal-feeding as the ancestral state, likelihood reconstruction resulted in lower plant/root-hair feeding (pLh: 0.40∗), bacteriovore (pLh: 0.38∗) or fungal-feeding (pLh: 0.20∗) as the ancestral state. The most parsimonious ancestral feeding state of the tylenchid nematodes was fungal-feeding, but likelihood reconstruction appointed lower plant/root-hair feeding as a better supported state (pLh: 0.96∗) compared to fungal-feeding (pLh: 0.03). For the remaining ancestral state reconstructions, parsimony and likelihood methods yielded consistent results. Migratory ectoparasitic feeding is ancestral for all four major clades of nematodes that exclusively parasitize higher plants. Burrowing endoparasitism has evolved at least six times independently, twice in Anguinidae and four times, always from migratory ectoparasitic ancestors, in the polyphyletic Pratylenchidae. Sedentary endoparasitism has also evolved three times independently; *Nacobbus* (false root-knot nematodes) and the cyst-forming nematodes most likely evolved from migratory ectoparasitic nematodes, while root-knot nematodes appeared to have evolved from burrowing endoparasitic nematodes.

3.4. Morphological results: the cellular architecture of the female gonoduct of Panagrolaimidae, Aphelenchoidea, Anguinidae and Criconematoidea

The oviduct of the panagrolaimid nematode *Panagrobelus stammeri* (Fig. 3A) was clearly differentiated from ovary and spermatheca and comprised two rows of two cells. The spermatheca comprised 10–12 cells and at the transition spermatheca-uterus, a pair of protruding small cells was clearly offset from the remaining gonoduct cells. The uterus consisted distally of two cell rows, each one six to seven cell pairs long, with two or four uterus cells close to the spermatheca clearly enlarged and filled with granulated cytoplasm. The uterus cells were gradually more irregularly arranged in a more proximal position. The oviduct of *Panagrolaimus rigidus* (Fig. 3B) comprised two cell rows, each row 11 to 13 cells long. A spermatheca was not differentiated, sperm was observed in the proximal part of the oviduct and in the distal part of the uterus. The uterus was composed of more than 60 variably arranged cells; up to eight cells were observed at optical cross-sections at the widest part of the uterus.

The gonoduct architecture of Aphelenchoidea (Fig. 3C–E; schematically see Fig. 2) appear to be dissimilar to that of all other nematodes. Hence, homology assessment is problematic and aphelenchid gonoduct structures are designated here as proposed by Geraert (1981) but coded with additional character states in further analysis. As observed in the Aphelenchidae *Aphelenchus avenae* and *A. isomerus* (Fig. 3C), the ovary was proximally followed by the oviduct, fertilisation chamber, spermatheca, uterus-spermatheca valve and uterus. The oviduct comprised five to seven spherical cell pairs, each pair positioned at right angles to the preceding cell pair. The fertilisation chamber consisted of two rows of seven cells; the two cell rows were often slightly helical. The spermatheca was formed by 18–24 elongated cells with strongly meandering cell boundaries, the cells were oriented parallel to the body axis; the distal spermatheca cells had highly crenated cell boundaries. Two rows of five to six cells formed a valve-like structure between spermatheca and uterus. The uterus comprised longitudinally elongated and variably arranged cells that formed a clear lumen. Within the Aphelenchoididae, the oviduct *Aphelenchoides trivialis*, *A. fragariae* and *Laimaphelenchus penardi* consisted of two flattened cells (Fig. 3D and E). The elongate tubular spermatheca comprised three groups of five to eight spermatheca cells, which surrounded a distinct lumen. The distal part of the uterus wall was formed by longitudinally arranged elongated cells.

Within the Anguinidae, the oviduct of the studied *Ditylenchus* species (*D. destructor*, *D. angustus and D. myceliophagus*) comprised two slightly helical rows of five cells (Fig. 3F). The 16-celled spermatheca was axial and oblong. The uterus comprised four rows of four cells and this columnar part was separated from the uterine sac by a constriction that consisted of two rows of three cells.

All examined Criconematoidea (*Criconema* sp., *Mesocriconema xenoplax*, *M. rusticum*, *Hemicycliophora thienemanni*, *H. conida*, *Paratylenchus similis*, *P. microdorus*) displayed a similar gonoduct structure (Fig. 3G–J; schematically see Fig. 2). The oviduct comprised two rows of four cells and was often partly enveloped by the spermatheca or uterus–spermatheca complex. The spermatheca was not clearly differentiated from the uterus; both structures formed an integrated complex, of which the spermatheca constitutes the distal ventral corner. In *Hemicycliophora* the

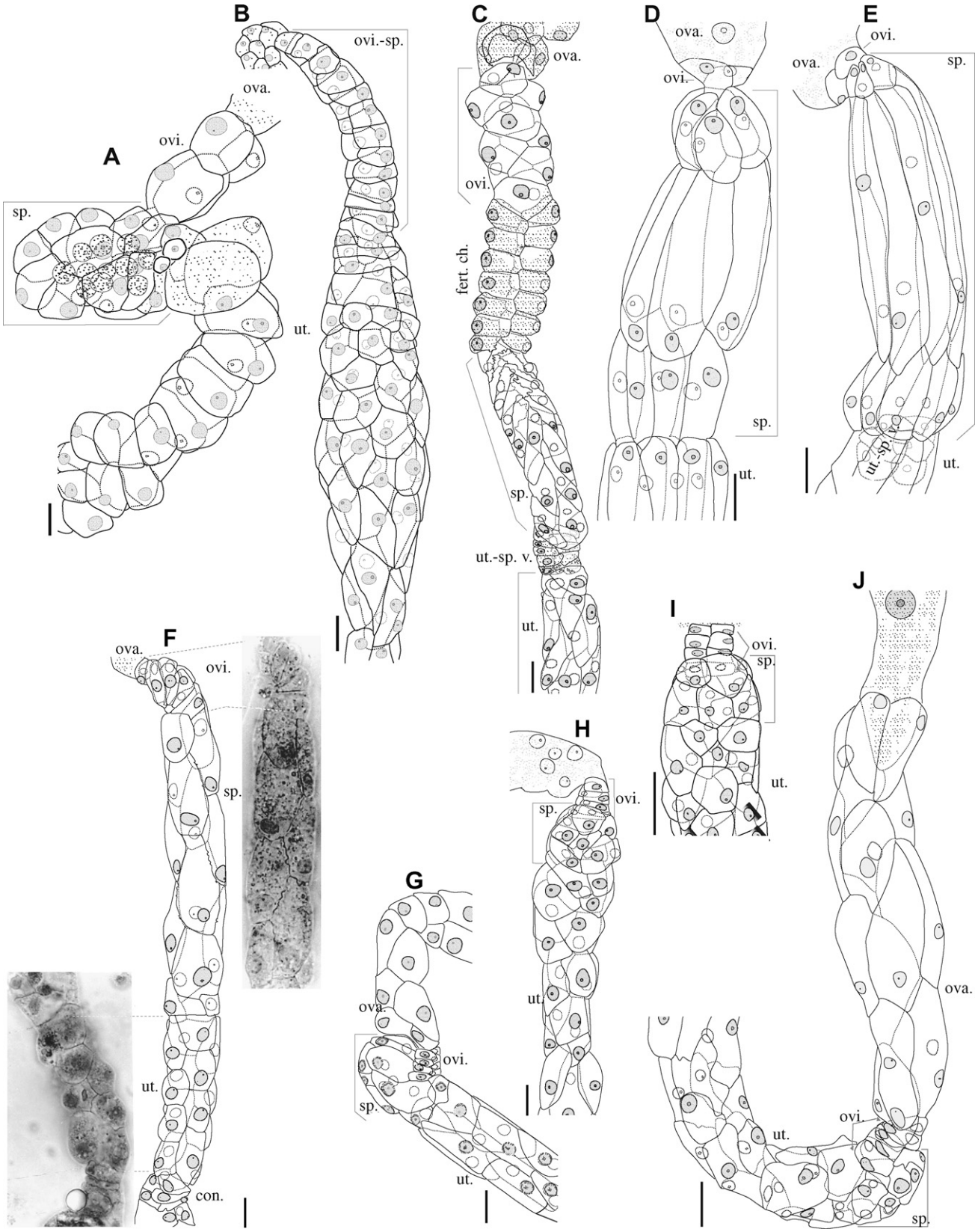

Fig. 3. Line drawings of the cellular composition of oviduct, spermatheca and distal part of uterus of representatives of Panagrolaimidae, Aphelenchoididae, Aphelenchidae, Anguinidae and Criconematoidea. (A) *Panagrobelus stammeri*; (B) *Panagrolaimus rigidus*; (C) *Aphelenchus isomerus*; (D) *Laimaphelenchus penardi*; (E) *Aphelenchoides fragariae*; (F) *Ditylenchus myceliophagus* including LM photographs; G: *Hemicycliophora thienemanni*; (H) *Hemicycliophora conida*; (I) *Paratylenchus similis*; (J) *Mesocriconema rusticum*. Abbreviations: ova., ovary; ovi, oviduct; sp., spermatheca; ut., uterus; fert. ch., fertilization chamber; ut.-sp. v., uterus-spermatheca valve; con., constriction. (Scale bars = 10 μm).

spermatheca was slightly more differentiated and comprised 10–12 (mostly 12) variably arranged cells. The flattened uterine wall cells were not arranged in clear rows and a lumen appeared to be present. The uterus of the studied *Hemicycliophora* species consisted of 16 to 18 cells, the exact uterus cell number of the other Criconematoidea could not be determined.

3.5. Evolution of the gonoduct architecture

Parsimony and likelihood reconstruction of gonoduct characteristics yielded similar results. Fig. 4 illustrates the parsimony reconstruction of the evolution of gonoduct characters, i.e. number of gonoduct branches, the number and arrangement of oviduct, sper-

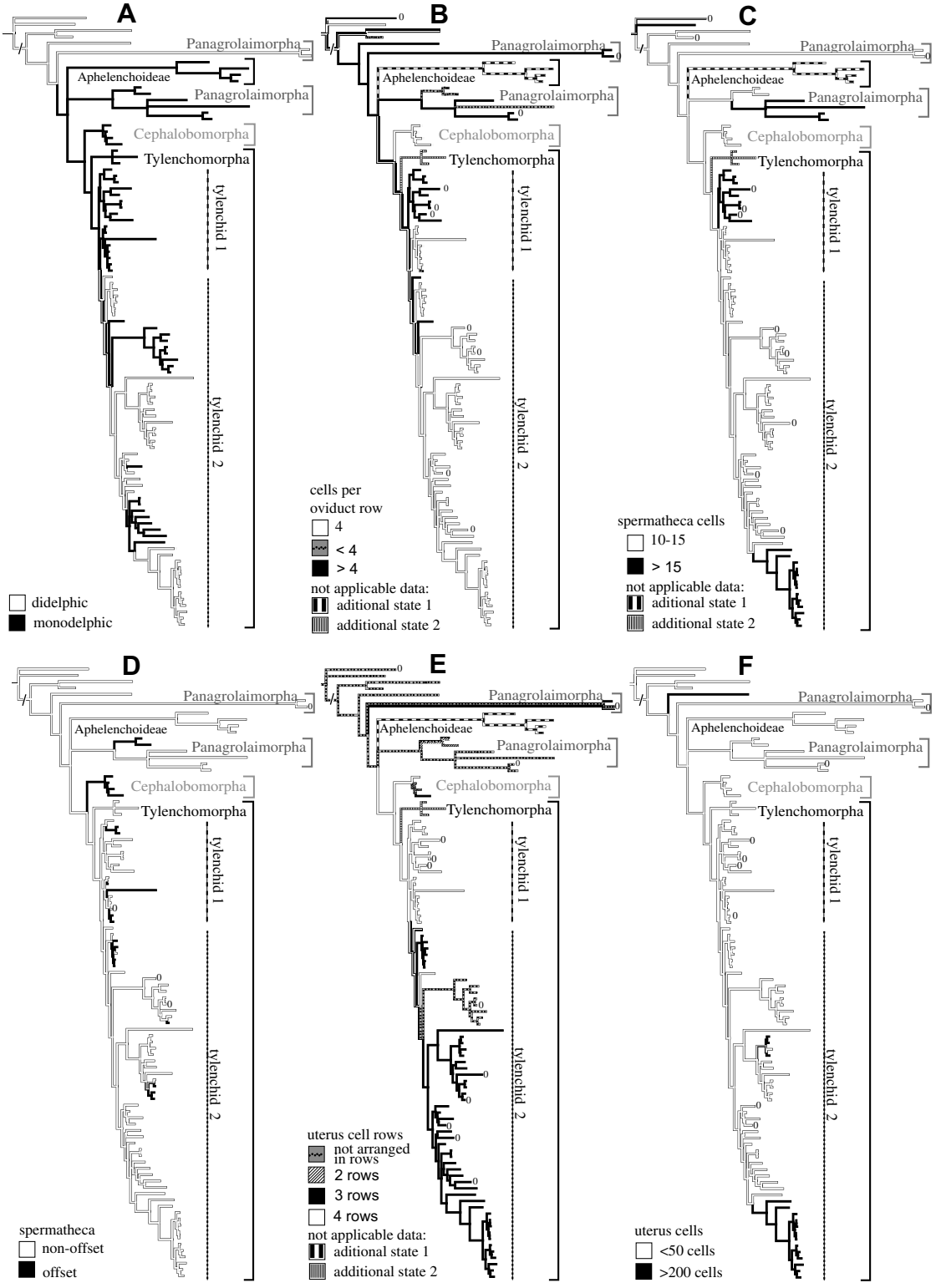

Fig. 4. Parsimony gonoduct character reconstruction on phylogenetic tree (see Fig. 1). (Tylenchid 1) Tylenchid nematodes (=nematodes with tylenchid stylet) predominantly feeding on fungi, lower plants, epidermal cells or root-hairs; (tylenchid 2) tylenchid nematodes exclusively parasitizing higher plants; (0) missing character state in terminal taxa; (/) branch scaled to 10%.

matheca and uterus cells (see Table 1), along the BI-partitioned phylogram (Fig. 1).

Based on the present phylogeny, parsimony and likelihood analyses suggested that a didelphic gonoduct is ancestral for the Tylenchina, but the proportional likelihood of the didelphic character state was not significant (pLh: 0.65) (Fig. 4A). The monodelphic state is ancestral for the Cephalobomorpha–Tylenchomorpha clade (pLh: 0.99*) and for the tylenchid nematodes (pLh: 0.99*). The parsimony reconstruction suggested three shifts from didelphic to monodelphic and only two reversals to the didelphic state.

An oviduct that comprises two rows is ancestral and invariant within Tylenchina except for the genus *Steinernema* (reconstruction not shown). The number of cells forming the oviduct was considerably variable outside the Tylenchomorpha–Cephalobomorpha clade, from two cells per row in *Panagrobelus stammeri* to more than 20 cells in *Steinernema carpocapsae*, and the increase and the reduction of the number of oviduct cells per row evolved several times independently (Fig. 4B). Four cells per oviduct cell row is most likely the ancestral state for the Cephalobomorpha–Tylenchomorpha clade (pLh: 0.76*). A slightly longer oviduct (5–7 cells) has only evolved within this group in the weakly supported Sphaerularioidea–Boleodorinae clade and in the taxa *Psilenchus* and *Cephalenchus* (Fig. 4B). The number of spermatheca cells showed a partly similar pattern, a limited number of spermatheca cells (10–15 cells) appeared to be most likely the ancestral state for the Cephalobomorpha–Tylenchomorpha clade (pLh: 0.99*). An increase of spermatheca cells was observed for the Sphaerularioidea–Boleodorinae clade, *Meloidogyne* spp. clade and *Psilenchus* sp. (Fig. 4C). The spermatheca cells formed an offset spermatheca pouch several times independently in various clades (Fig. 4D).

Fig. 4E illustrates that uterus cells arranged in a variable pattern, not forming distinct cell rows, emerged as the ancestral state (pLh: 0.87*). Uterus cells turned out to be arranged in regular rows in *Strongyloides* spp. (four rows), within Panagrolaimorpha (*Panagrobelus* and *Plectonchus*: 2 rows) and in the Cephalobomorpha–Tylenchomorpha clade (two, three or four rows). The proportional likelihood of four rows as the ancestral state in the latter clade is 0.86* versus 0.001 for three rows. A switch from four to three uterus rows was observed for the clades containing the "higher plant-parasites" (Belonolaimidae, Hoplolaimidae, Pratylenchidae and Meloidogynidae); *Cephalenchus* (Tylenchidae), a taxon that is placed with low support within these clades retained the four rows state. The pattern of uterus cells being arranged in regular rows was shown to be lost in the Criconematoidea. The total number of uterus cells appeared to be limited (about 16–60 cells) and constant for a given species; however, with the notable exception of the entomopathogenic genus *Steinernema* and the sedentary endoparasitic plant-parasites of which the uterus was composed of more than 200 cells (Fig. 4F). Our results indicate that this proliferation of uterus cells has evolved recurrently in entomopathogenic steinernematids, cyst-forming, false root-knot and root-knot endoparasitic nematodes. In the latter group the proliferation is restricted to the elongation of the uterus and the typical arrangement of cells in three rows is kept along the full length of the uterus.

A striking result of current analysis is the apparent similarity of the gonoduct of the otherwise morphologically dissimilar Cephalobomorpha and Tylenchomorpha. Ancestral state analysis could reconstruct a hypothetical gonoduct architecture at the node leading to the Tylenchomorpha and Cephalobomorpha: a monodelphic (pLh: 0.99*) gonoduct system comprising an oviduct composed of two rows (pLh: 0.99*, reconstruction not shown) with four cells (PLh: 0.76*), a spermatheca consisting of 10–15 cells (pLh: 0.99*) and uterus cells arranged in distinct rows (pLh: 0.99*; reconstruction not shown), four uterus rows in particular (pLh: 0.86*). Hypothesising such a cellular architecture in other ancestral nodes outside the Tylenchomorpha–Cephalobomorpha clade was less probable; the proportional likelihoods at the node corresponding with the Tylenchina without *Strongyloides* spp. and *Steinernema* showed that certain ancestral states of the hypothesized "Tylenchomorpha–Cephalobomorpha gonoduct" are likely but their combination is considerably less likely compared to the values at the Tylenchomorpha–Cephalobomorpha node [monodelphic (0.92*), two (0.99*) oviduct rows of four (0.50) cells, a small spermatheca (0.99*), uterus cells arranged in rows (0.80*), four uterus rows (0.51)]. A combination of ancestral character state reconstructions of the hypothesised Tylenchomorpha–Cephalobomorpha gonoduct in ancestral nodes corresponding with more early diverging radiations are very unlikely; the proportional likelihoods of the character states monodelphic, four rows per oviduct row, uterus arranged in rows and four uterus rows at these nodes varied between 0.13 and 0.65.

4. Discussion

4.1. Phylogeny of Tylenchina

4.1.1. Mayor lineages within Tylenchina

Only relatively recently have nematode classifications been guided by molecular phylogenies (De Ley and Blaxter, 2002). The present phylogenetic hypothesis, based on expanded taxon sampling and more adequate phylogenetic analysis, agrees in general with the Tylenchina framework as proposed by these authors. Our analyses indicate a monophyletic grouping of the Tylenchina, including *Steinernema*. The latter was placed within the Rhabditina in a LSU rDNA phylogenetic analysis (Nadler et al., 2006a; see also Section 1). The hypothesis that Cephalobomorpha share most recent common ancestry with the Tylenchomorpha (without the Aphelenchoididae) (Blaxter et al., 1998) is confirmed with maximal support. The placement of the Aphelenchoidea clade outside of the Tylenchomorpha has been appointed to phylogenetic bias, possibly caused by long branch attraction and/or elevated AT-content (De Ley and Blaxter, 2002; Holterman et al., 2006). However, several additional analyses, including removal of long branches did not unite the aphelenchs. On the other hand, the monophyletic grouping of the Aphelenchidae and Aphelenchoididae was not significantly rejected (ML Shimodaira–Hasegawa test). The morphological correspondence of Aphelenchidae and Aphelenchoididae is manifest; the organization of the cephalic region, stylet and pharynx structure, the dorsal gland orifice position and the shape of the anus demonstrate the most apparent similarities. Conversely, the cellular gonoduct architecture of both groups turned out to be strikingly different and does not preclude their polyphyletic origin. Yet, it is impossible to establish the homology of the gonoduct characters of the Aphelenchidae and Aphelenchoididae with respect to each other, to other Tylenchomorpha and even to other nematodes. Consequently, the gonoduct structure does not provide any objective clues to speculate about the position of both aphelenchid groups. Notice that possible phylogenetic bias related to the position of the aphelenchids do not have major influence on further discussion on gonoduct evolution, since both the Aphelenchidae and Aphelenchoididae gonoduct were treated as different to all other gonoducts by coding their respective gonoduct characters with an additional state.

4.1.2. "Primitive" versus "derived" tylenchid nematodes

The tylenchid nematodes, those Tylenchomorpha that are characterized by a tylenchid stylet and sampled most extensively in

this analysis, appeared to be clearly monophyletic. The general picture of their internal relationships in this study does not contradict morphology based concepts that divide tylenchid nematodes in groups with supposedly ancestral morphological characters, including Tylenchidae and Sphaerularioidea and more derived groups that include the remaining tylenchid taxa (Luc et al., 1987; Siddiqi, 2000). The nematodes with derived morphological features, which enable them to attack higher plants are unified in a single clade within the Tylenchomorpha and the plant-parasites with the most sophisticated host parasite relationships are present in the most distal clades. Notably, the genus *Psilenchus* which is the subject of longstanding discussions[1] of its either basal (e.g. Luc et al., 1987) or more derived position (e.g. Siddiqi, 2000) is placed in our BI-partitioned analysis sister to all derived plant-parasites and this is congruent with views that consider *Psilenchus*-like forms as sister to or within the "derived" plant-parasites (Siddiqi, 2000; Subbotin et al., 2006). Nevertheless, despite the general overall coherence of the results of the phylogenetic methods used, differences in the position of *Psilenchus* were found depending on the method (Table 2).

Notably, gonoduct data substantiate the appointment of two major groups within the tylenchid nematodes; the "primitive" Tylenchidae and Sphaerularioidea (including Anguinidae) (*sensu* Siddiqi, 2000) have uterus cells that are arranged in 4 rows (=ancestral uterus rows state of the Tylenchomorpha, pLn: 0.99*) while in the other "derived" taxa the number of uterus rows is reduced or lost (see further discussion on the evolution of the gonoduct). Limited developmental analysis point to the same direction; Dolinski et al. (2001) described for Anguinidae and Tylenchidae (including *Psilenchus*) an embryogenesis that displays an asynchronous division order and a partially linear blastomere arrangement similar to that of the Cephalobidae. The "derived taxa" on the other hand (based on Meloidogynidae, Pratylenchidae, Belonolaimidae, Hoplolaimidae and Criconematoidea) have a synchronous division order and a completely linear blastomere arrangement.

The position of the relations among the early diverging taxa Tylenchidae, Anguinidae and Sphaerularioidea is subject to discussion. Anguinidae are considered inside Sphaerularioidea (e.g. Siddiqi, 1986; Ryss, 1993; De Ley and Blaxter, 2002), while others situate them close to the Tylenchidae (e.g. Maggenti et al., 1987; Brzeski, 1998; Siddiqi, 2000). Our analysis does not support the monophyly of either of these taxa; *Deladenus* (non-anguinid member of the Sphaerularioidea) was retrieved within the Anguinidae, and Anguinidae were placed within the polyphyletic Tylenchidae. This agrees with the barely propagated idea of Raski and Maggenti (1983) to consider a broader concept of the Tylenchidae that includes Anguinidae and at least part of the other Sphaerularioidea. Adding in the analysis, above *Deladenus*, additional insect pathogenic tylenchids (GenBank records of *Bradynema listronotum*, *Fergusobia* sp. and *Howardula* spp. with unknown gonoduct morphology and slightly shorter sequenced SSU) did only have a slight effect on branch support but did not alter the tree topology (Supplementary data, Appendix 3). These additional taxa were grouped in a single clade, sister to *Deladenus* and *Nothotylenchus* (PP = 95).

Current analyses are also largely in agreement with the tylenchid tree topologies obtained by Subbotin et al. (2006) based on phylogenetic analysis of the D2–D3 regions of the LSU rDNA. The very few differences that do not depend on the different choice of sampling include the placement of *Ditylenchus destructor*, which is not placed outside the Anguinidae here, and the relation of the family Tylenchidae and Belonolaimidae that do not form a well supported sister relationship. These present results are more in agreement with traditional views.

The exact feeding habits of the early diverging tylenchs, especially in the family Tylenchidae, have been difficult to determine (Yeates, 2003), and as a consequence the reconstruction of the ancestral feeding states of Tylenchomorpha and tylenchid nematodes remains poorly resolved. In the current study, all types of supposedly lower plant-feeding, epidermal cell and root-hair feeding have been lumped in anticipation of more specific feeding trials. However, future analysis will probably demonstrate for instance that certain Tylenchidae are fungal-feeding instead of lower plant-, epidermal cell or root-hair feeding (Okada and Kadota, 2003).

4.1.3. Criconematoidea

Criconematoidea appeared to be a well-supported monophyletic group and its internal topology is congruent with analysis of a more extended dataset (Subbotin et al., 2005). The Criconematoidea studied here are characterized by a remarkable overall similarity in cellular gonoduct morphology (Fig. 3G–J; schematically see Fig. 2). The combination of absence of a well separate spermatheca and a uterus not arranged in regular rows (Figs. 2 and 3) is a distinct apomorphic characteristic for the Criconematoidea. The homogeneous internal morphology of this group is in contrast with the very diverse general morphology, especially the endless variations in external cuticular ornamentations.

The sister relationship of Criconematoidea with the most devastating plant-parasites (Hoplolaimidae, Pratylenchidae and Meloidogynidae) is relatively weakly supported but this is in agreement with phylogenetic analysis of the D2–D3 regions of the LSU rDNA analysis (Subbotin et al., 2006). However, this position disagrees with a supposed sister relationship with Anguinidae and Tylenchidae (Holterman et al., 2006) or a sister relationship with all remaining tylenchid nematodes (Meldal et al., 2007) based on similar phylogenetic analysis of the SSU rDNA analysis. Since the arrangement of uterus cells in regular rows is lost it cannot be assessed if the number of uterus rows provides a morphological clue for an affinity with the "basal" tylenchs (four uterus cell rows) or with the "advanced" tylenchs (three rows).

4.1.4. Sedentary endoparasitic Heteroderinae and Meloidogynidae

Our molecular analyses add strong support to the existing evidence that sedentary endoparasitism in Heteroderinae (cyst-forming nematodes) and *Meloidogyne* (root-not nematodes) is convergent (De Ley and Blaxter, 2002; Baldwin et al., 2004a; Subbotin et al., 2006). Here we also demonstrate that endoparasitism in both groups have a different origin. Cyst-forming endoparasitic nematodes are sister to Hoplolaiminae and evolved most likely from *migratory ectoparasitic* nematodes, while root-knot nematodes are most closely related to *Pratylenchus* spp. and appear to have evolved from *burrowing endoparasitic* nematodes. The latter is congruent with some morphological data (head patterns) that have shown an affiliation of root-knot nematodes with Pratylenchidae (Geraert, 1997).

4.1.5. Burrowing endoparasitic Pratylenchidae

Our analyses clearly indicate that Pratylenchidae are polyphyletic, the analyzed Pratylenchidae forming four independent lineages. A lineage of *Pratylenchus* spp., *Zygotylenchus* and *Hirschmanniella* spp. that are sister to *Meloidogyne*; the sedentary endoparasitic genus *Nacobbus* is placed within a clade of *Tylenchorhynchus* spp. (Belonolaimidae) and *Macrotrophurus* (Belonolaimidae or Tylenchidae depending on the classification, see Siddiqi, 2000); *Radopholus similis* is sister to the Hoplolaimidae; and *Pratylenchoides* spp. are sister to a clade that comprises the remaining Belonolaimidae. The inclusion of the burrowing

[1] *Psilenchus* combines morphological features of early diverging (e.g.; weak stylet, elongated tail *etc.*) and derived plant parasites (e.g. phasmids on its tail and didelphic genital system) (Sturhan and Rahi, 1996; Subbotin et al., 2006).

endoparasitic nematodes in a single family Pratylenchidae is classically defined by a context of similar morphological character sets that are likely the result of convergent evolution related to similar feeding modes. Close relationship of *Pratylenchoides* with the Belonolaimidae, especially *Amplimerlinius*, was already postulated based on certain morphological similarities such as the shape of the cephalic region, pharyngeal glands sometimes forming a pseudo-bulb, female tail and details of the lateral field (Siddiqi, 2000). Most significant is the placement of *Radopholus* sister to the Hoplolaimidae which include cyst-forming nematodes, a well supported result that is congruent with LSU based phylogenetic results (Subbotin et al., 2006). With developing understanding of plant-nematode interactions in the economically very important genera *Radopholus* and *Pratylenchus*, faulty assumptions of monophyly of burrowing endoparasitic nematodes versus convergence has critical implications when extrapolating insights of one group (i.e. *Pratylenchus* spp.) to another (i.e. *Radopholus* spp.).

4.2. Evolution of the gonoduct architecture

It is generally accepted that convergent evolution of mono- or didelphic genital systems is likely, considering the results of cell lineages analysis which revealed that monodelphy results from programmed cell death of one single cell (Horvitz and Sternberg, 1982) and the many cases in which didelphic and monodelphic species coexist in the same putative family. Our analyses indicate however relatively few switches in the number of gonad arms in Tylenchina, indicating that the monodelphic *vs.* didelphic state has relatively stronger historical and genetic determinants than suggested from traditional classifications. Most remarkably is the position of the didelphic pratylenchid nematodes *Radopholus* and *Pratylenchoides* within didelphic clades and thus away from the monodelphic *Pratylenchus* spp., their putative family members. Present analyses indicate that monodelphy is ancestral for tylenchid nematodes; this undermines the argument that *Psilenchus* is the most primitive tylenchid form (="prototylenchid") because of its supposedly ancestral didelphic genital system (Luc et al., 1987). Considering *Psilenchus* as a non-primitive tylenchid is also consistent with our phylogenetic analysis.

The extended analyses here presented on the cellular architecture essentially confirm previous reports that the nematode gonoduct is composed of a relative small and nearly constant number of cells (Geraert, 1981). Geraert (1981) postulated that the genital system has an invariable number of cells, confirming the widely held belief that nematodes are eutelic (Van Cleave, 1932). However, the entomopathogenic genus *Steinernema* and the three clades of sedentary endoparasitic plant-parasites have significantly more uterus cells (>200 *vs.* usually about 40 cells), and these high cell numbers are correlated with the presence of more than one nucleus per cell and variable cell numbers (Bert et al., 2002; unpublished results). Furthermore, extensive evidence of variable cell number in nematodes was already demonstrated for the epidermis cells (Cunha et al., 1999). Our analyses indicate that a large and likely variable uterus cell number is associated with the derived endoparasitic feeding and associated reproduction modes. It appears that in relatively stable, sedentary conditions (within insect corpus or plant), the gonoduct has escaped eutely at least four times independently to accommodate a huge number of eggs associated with fast reproduction under favourable conditions. Remarkably, despite the proliferation of uterus cells the typically arrangement of cells in three rows (tricolumella) is not lost within certain sedentary endoparasitic nematodes.

The part of the gonoduct that was already described as a remarkably evolutionary stable structure within the Nematoda is the oviduct (Geraert, 1983), and two oviduct cell rows were considered as an apomorphic character state of the order Rhabditida (=Secernentea in Geraert, 1983). With the only exception of *Steinernema carpocapsae*, we obtained identical results. The number of cells per oviduct row on the other hand varies considerably. Within the Tylenchomorpha–Cephalobomorpha clade slight variations of the ancestral number of four cells per row appeared to be correlated with the diversification within these infraorders. A slightly longer oviduct (5–7 cells *vs.* 4 cells per row) is characteristic for the clade containing the Boleodorinae plus Sphaerularioidea and correlates with a slightly increase in the number of spermatheca cells. The spermatheca cell architecture, including the number of cells, generally correlates with the phylogenetic patterns observed for the oviduct length and is also highly variable outside the Cephalobomorpha–Tylenchomorpha clade. Our analyses however strongly suggest that the presence of an offset spermatheca has evolved multiple times. *Plectonchus* and *Panagrellus*, nested within the Panagrolaimidae, were previously thought to be related to the Cephalobomorpha with an offset spermatheca as putative synapomorphy. However, in comparison to the Cephalobomorpha and Tylenchomorpha, differences in spermatheca cell architecture (Stock et al., 2002) particularly in combination with a different oviduct structure (2 *vs.* 4 cells per row) are consistent with an independent evolution (see also Nadler et al., 2006a).

4.3. The apparent similarity of the cephalobid and tylenchid gonoduct

In traditional morphology-based classifications the bacteriovorous cephalobs and the predominantly plant-parasitic tylenchs were thought to be distantly related because of their obvious morphological differences (for a critical review, see De Ley and Blaxter, 2002). However, because molecular evidence strongly supports common ancestry for the two groups (Blaxter et al., 1998; Meldal et al., 2007; Holterman et al., 2006), Tylenchomorpha and Cephalobomorpha have recently been classified in the same suborder Tylenchina, but with the remark that no obvious morphological synapomorphies are known for this suborder (De Ley and Blaxter, 2002). However, our analyses indicate that the tylenchid and cephalobid gonoduct evolved from a common ancestral cellular construction and that it is unlikely that such a gonoduct structure already appeared in other ancestral nodes outside the Tylenchomorpha–Cephalobomorpha clade (combinations of the relative likelihoods of the hypothesized ancestral gonoduct states are low).

We have shown that the architecture of the gonoduct is evolutionary stable in contrast to an apparent ability to adapt to a specific food source, given the multiple convergent evolutions of the feeding types. Thus, it can be assumed that the radically different adult morphology between tylenchid and cephalobid nematodes, with the most significant exception of the reproductive system, reflects the completely different niche segregation of relatively closely related organisms. Few other shared morphological character states include ultrastructural details of the feeding system, such as the position and number of muscle cells of the basal pharyngeal bulb (Baldwin et al., 2001, 2004b). Based on the putative close relationship of Cephalobidae with plant-parasites, which is confirmed in this study, and the fact that they are amenable to culture under laboratory conditions, Cephalobidae were already proposed as suitable model organisms for plant-parasitic nematode research (Blaxter et al., 1998). Furthermore, given the importance of the reproductive system for the study of fundamental problems in cell biology and developmental biology (Hubbard and Greenstein, 2000), we can anticipate that the cephalobid gonad is, in relation to *C. elegans*, valuable for the study of developmental and cell-biological aspects of plant-parasitic nematodes.

5. Conclusions

Tylenchina include an ecologically diverse array of nematode species with repeated evolution of plant-parasitic lifestyles within the Tylenchomorpha. Focussing on relatively easily scorable gonoduct characters has permitted screening of a wide range of representatives, and the resulting broad congruence between the gonoduct characters and the molecular phylogenetic hypothesis strongly suggests that the characters surveyed are phylogenetically informative. The gonoduct "bauplän" appears to be constrained, especially in the infraorders Cephalobomorpha and Tylenchomorpha. Minimal changes in the cellular arrangement appear to be retained independent of shifts in biology. The selective force on morphological characters associated with the gonoduct is clearly different compared to that on feeding-related structures; only a proliferation of uterus cells but not the specific arrangement of cells is associated with specific niche segregation. Our study confirms widely found observations that certain aspects of the adult morphology are more evolutionary constrained than others (Hall, 1996).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.04.011.

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