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An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa

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Abstract

Phylogenetic reconstructions of relations within the phylum Nematoda are inherently difficult but have been advanced with the introduction of large-scale molecular-based techniques. However, the most recent revisions were heavily biased towards terrestrial and parasitic species and greater representation of clades containing marine species (e.g. Araeolaimida, Chromadorida, Desmodorida, Desmoscolecida, Enoplida, and Monhysterida) is needed for accurate coverage of known taxonomic diversity. We now add small subunit ribosomal DNA (SSU rDNA) sequences for 100 previously un-sequenced species of nematodes, including 46 marine taxa. SSU rDNA sequences for >200 taxa have been analysed based on Bayesian inference and LogDet-transformed distances. The resulting phylogenies provide support for (i) the re-classification of the Secernentea as the order Rhabditida that derived from a common ancestor of chromadorean orders Araeolaimida, Chromadorida, Desmodorida, Desmoscolecida, and Monhysterida and (ii) the position of *Bunonema* close to the Diplogasteroidea in the Rhabditina. Other, previously controversial relationships can now be resolved more clearly: (a) *Alaimus*, *Campydora*, and *Trischistoma* belong in the Enoplida, (b) *Isolaimium* is placed basally to a big clade containing the Axonolaimidae, Plectidae, and Rhabditida, (c) *Xyzzors* belongs in the Desmodoridae, (d) Comesomatidae and *Cyartonema* belongs in the Monhysterida, (e) *Globodera* belongs in the Hoplolaimidae and (f) *Paratylenchus dianeae* belongs in the Criconematoidea. However, the SSU gene did not provide significant support for the class Chromadoria or clear evidence for the relationship between the three classes, Enoplia, Dorylaimia, and Chromadoria. Furthermore, across the whole phylum, the phylogenetically informative characters of the SSU gene are not informative in a parsimony analysis, highlighting the short-comings of the parsimony method for large-scale phylogenetic modelling.

Keywords: Nematoda; Adenophorea; Secernentea; Enoplea; Chromadorea; Rhabditida; SSU rDNA; Parsimony; LogDet transformation; Bayesian inference; Phylogenetic reconstruction; Evolution; Systematics; Taxonomy; Marine nematodes

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1. Introduction

The Nematoda are one of the most diverse taxa in the animal kingdom, with estimates ranging from 0.1 to 100 million species (May, 1988; Hammond, 1992; Lambshead, 1993; Coomans, 2000). Free-living species are found in every soil or sedimentary habitat with very few exceptions (e.g. Convey and McInnes, 2005) and are used as indicator species in biodiversity assessments and biomonitoring (reviewed by Lambshead, 2004; Yeates and Boag, 2004; Cook et al., 2005). Nematodes have developed a multitude of parasitic life styles causing numerous human diseases and large financial losses to agriculture and livestock rearing (reviewed by Manzanilla-Lópes et al., 2004). Effective use and control of nematodes requires knowledge of their relationships. Nematodes are also used increasingly as model organisms. *Caenorhabditis elegans* was the first metazoan organism to have its complete genome sequenced (the *C. elegans* sequencing Consortium, 1998) and currently over 30 nematode genome sequencing projects are ongoing (Mitreva et al., 2005). However, no sequencing projects are underway for marine nematodes (e.g. Araeolaimida, Chromadorida, Desmodorida, Desmoscolecida, Enoplida, and Monhysterida), largely because it is difficult to collect enough high-quality, species-specific material.

Although life cycles and relationships of nematodes have been studied intensively for over 350 years, the lack of objective criteria for assessing homology of morphological characters has hampered the reconstruction of the phylogeny of the Nematoda. Rarely have marine and terrestrial, animal and plant parasitic species been studied by the same authors. Even where the whole of the phylum has been investigated authors often shoe-horned those groups together for which they did not have much detailed knowledge. Furthermore, the ontogeny and ultrastructure of nematodes is poorly understood and there is a lack of an informative fossil record (e.g. Poinar, 1977, 1983, 2003). Such difficulties have led to the erection of multiple, at least partially conflicting classifications (De Ley and Blaxter, 2002) that can be grouped into two overall hypotheses.

Chitwood (1933, 1937) and Chitwood and Chitwood, 1950) divided the Nematoda into the Adenophorea ('gland bearers') and Secernentea ('secretors'). The former include virtually all aquatic nematodes (Enoplida and Chromadorida) and selected terrestrial omnivores or plant-feeders (Dorylaimia), the latter group includes almost all parasitic species (Strongylina, Tylenchina, Ascaridina, and Spirurida) and the majority of terrestrial freeliving nematodes (Rhabditina). Lorenzen (1981) followed Chitwood and characterised the classification of the Adenophorea in more detail.

Andrássy (1976) gave each of the two Adenophorean groups, the Torquentia (roughly equivalent to the Chromadorida) and the Penetrantia (roughly equivalent to the Enoplida) the same rank as the Secernentia (≈ Secernentea). However, while ranking them equally, he claimed that the Secernentia evolved from a torquentian ancestor, thus violating the established ranking relationships. Nevertheless, the three-part division found more support overall than the two-part division (Maggenti, 1963, 1970; Gadéa, 1973; Drozdovskii, 1980; Adamson, 1987; Malakhov, 1994).

Advances in molecular-biology techniques allowed an objective, empirical analysis of the evolutionary history of the Nematoda. Blaxter et al. (1998) produced the first molecular phylogenetic framework of the phylum using sequences of the nuclear ribosomal small subunit (SSU). However, their analysis was based primarily on terrestrial and economically important parasitic species such as Dorylaimida, Mermithida, Mononchida, Rhabditida, Trichinellida, and Triplonchida and lacked data from the full range of taxa found in marine habitats (e.g. Araeolaimida, Chromadorida, Desmodorida, Desmoscolecida, Enoplida, and Monhysterida). Further phylum-wide studies (Aleshin et al., 1998; Kampfer et al., 1998; Litvaitis et al., 2000) added more marine species but many major clades remained under-represented (e.g. Enoplida, Chromadorida, Monhysterida, and Desmoscolecida). On the small scale, numerous studies tested molecular markers for the easy identification of pest species. However, these markers, often mitochondrial genes, while able to distinguish between members of the same genus or family, are uninformative for higher level taxonomic studies (e.g. Hyman, 1988; Thomas and Wilson, 1991; Powers et al., 1993; Zarlenga et al., 1998; Hoberg et al., 1999; Watts et al., 1999; Nadler et al., 2000). Recently, De Ley and Blaxter (2002, 2004) updated the classification of the phylum Nematoda using molecular data available from additional species, with morphological data to assist the placement of taxa for which SSU sequences were not yet available. Nevertheless, the system was still based mostly on terrestrial and parasitic taxa.

In this study, we further revise the molecular phylogeny of De Ley and Blaxter (2002) by adding sequences to the nematode SSU data set from previously under-represented marine taxa and from additional terrestrial and parasitic groups. We analysed the phylogeny of 212 nematode taxa and 16 outgroup taxa using two different evolutionary models, Bayesian inference and LogDet-transformed distance analysis. In particular, the addition of sequences from marine taxa was crucial both in resolving the relationships of several major taxa and in affirming the relationships of some previously sequenced species whose phylogenetic positions remained uncertain or controversial.

2. Materials and methods

2.1. Specimen collection

2.1.1. Coastal sampling

Intertidal sediment and macroalgae samples were taken at several locations in the estuary of Southampton Water, UK, and preserved in 99.7% molecular-grade ethanol. Heavy sediment was removed from the sample by decantation

and nematodes were extracted by flotation in Ludox™ 50. Nematodes were mounted individually onto slides for identification (Cook et al., 2005). The first 30 nematodes of seven samples were identified to the lowest taxonomic level possible (Bastian, 1865; Riemann, 1966; Gerlach and Riemann, 1973/1974; Lorenzen, 1977; Platt, 1983; Platt and Warwick, 1983, 1988; Warwick et al., 1998). We found that genomic DNA degraded during storage in glycerol (Meldal, 2004) and so storage in glycerol for desiccation and identification was kept to a maximum of two weeks.

Bathylaimus assimilis, *Metadesmolaimus* sp., and *Theristus acer* originated from intertidal sediments in the polyhaline reach of the Schelde Estuary (SW Netherlands). They were extracted from the sediment by decantation. Individuals of each distinguishable species were sorted under a dissection microscope. One fraction was fixed in hot 4:1 formaldehyde–glycerin solution, transferred to anhydrous glycerol and mounted on permanent aluminium slides with double cover slips for identification (Seinhorst, 1959). Another fraction with similar numbers of animals was stored at −80 °C for molecular analysis.

Diplolaimelloides meyli, *Diplolaimella dievengatensis*, and *Geomonhystera disjuncta* were obtained from monospecific cultures on agar (Moens and Vincx, 1998).

2.1.2. Terrestrial and freshwater sampling

Soil samples were collected in or near the authors' homes in Belgium, around aquatic plants in the Botanic Garden of Ghent University, and in pots with various African crop plants kept in the greenhouse of the former International Institute of Parasitology (St. Albans, UK). Nematodes were extracted either in White trays, using a simple substitute for the Baermann funnel (Schlinder, 1961) or decanted over a 38-μm sieve. From each sample, a putatively single-species population was selected using a dissection microscope. One fraction of the population was killed and fixed in hot 4:1 formaldehyde–glycerin solution, transferred to anhydrous glycerol and mounted on permanent slides for identification. Digital vouchers of morphology of one or more of these fixed specimens were created as described by De Ley and Bert (2002) and are available at http://nematol.unh.edu for downloading. The other fraction was stored at −80 °C or in acetone and only used for molecular analyses when the examined population proved to be a single species.

2.1.3. Parasitic nematodes

Specimens of spirurid and strongylid parasitic nematodes were donated from colleagues worldwide after identification. They were snap-frozen at source, shipped on dry ice and stored at −80 °C.

2.2. DNA extraction, amplification, and sequencing

DNA extraction, PCR and sequencing of marine nematodes from UK waters were performed as described in Cook et al. (2005). PCR amplification and sequencing primers are detailed in Table 1.

Table 1
Amplification and sequencing primers

Primer name	Sequence (5′ → 3′)	Reference
G18S4	GCT TGT CTC AAA GAT TAA GCC	Blaxter et al. (1998)
22R	GCC TGC TGC CTT CCT TGG A	Blaxter et al. (1998)
22F	TCC AAG GAA GGC AGC AGG C	Blaxter et al. (1998)
26R	CAT TCT TGG CAA ATG CTT TCG	Blaxter et al. (1998)
24F	AGR GGT GAA ATY CGT GGA CC	Blaxter et al. (1998)
24F1	AGA GGT GAA ATT CTT GGA TC	Present study
13R	GGG CAT CAC AGA CCT GTT A	Blaxter et al. (1998)
18P	TGA TCC WMC RGC AGG TTC AC	Blaxter et al. (1998)
2FX	GGA AGG GCA CCA CCA GGA GTG G	Present study
23R	TCT CGC TCG TTA TCG GAA T	Blaxter et al. (1998)
23F	ATT CCG ATA ACG AGC GAG A	Blaxter et al. (1998)
9FX	AAG TCT GGT GCC AGC AGC CGC	Present study
9R	AGC TGG AAT TAC CGC GGC TG	Blaxter et al. (1998)

All other specimens were treated as follows: nematodes were transferred into 25 μl worm lysis buffer (50 mM KCl, 10 mM Tris, pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP 40 (Tergitol Sigma), 0.45% Tween 20, and 60 μg/ml Proteinase K), cut into pieces and transferred into a 0.5 ml tube. The tubes were incubated at −80 °C for 10 min, 65 °C for 1 h and 95 °C for 10 min, consecutively. After centrifugation for 1 min at 16,000*g*, 5 μl of the DNA suspension was added to the PCR mixture including primers G18S4 and 18P (Blaxter et al., 1998) (Table 1). The PCR conditions were 30 s at 94 °C, 30 s at 54 °C and 2 min at 72 °C for 40 cycles. Products were stored at −20 °C prior to sequencing.

PCR products were purified for sequencing using shrimp alkaline phosphatase/exonuclease I treatment. This material was then used as template for cycle sequencing without any further purification using primers G18S4, 18P, 2FX, 23R, 13R, 23F, 9FX, 9R, 26R and 22R (Blaxter et al., 1998) (Table 1) and BigDye v2.0 Terminator reagents (Applied Biosystems) according to the manufacturer's instructions. Cycle sequence products were precipitated by adding 25 μl of 95% ethanol and 1 μl 3 M sodium acetate (pH 4.6) to each cycle sequencing reaction (10 μl). The samples were placed at −20 °C for 15 min and centrifuged at 14,000 rpm for 15 min. After precipitation, an additional wash of the pellet was performed with 125 μl of 70% ethanol and centrifuged at 14,000 rpm for 5 min. The pellet was dried in a Speedvac concentrator, re-dissolved in loading buffer and run on a 48 cm 4.25% acrylamide–bisacrylamide (29:1) gel on a Perkin-Elmer ABI Prism 377 automated DNA sequencer. Sequencing was performed in both directions.

2.3. Phylogenetic analysis

Sequence traces were checked for quality and assembled using Autoassembler 1.4 (Applied Biosystems), AssemblyLign (Accelrys) or Chromas version 1.45 (McCarthy, 1997). In order to align the sequences and to take into account the secondary structure of the SSU, a profile of already aligned nematode sequences was obtained from the European Ribosomal RNA Database (http://www.psb.ugent.be/rRNA/). Additional nematode

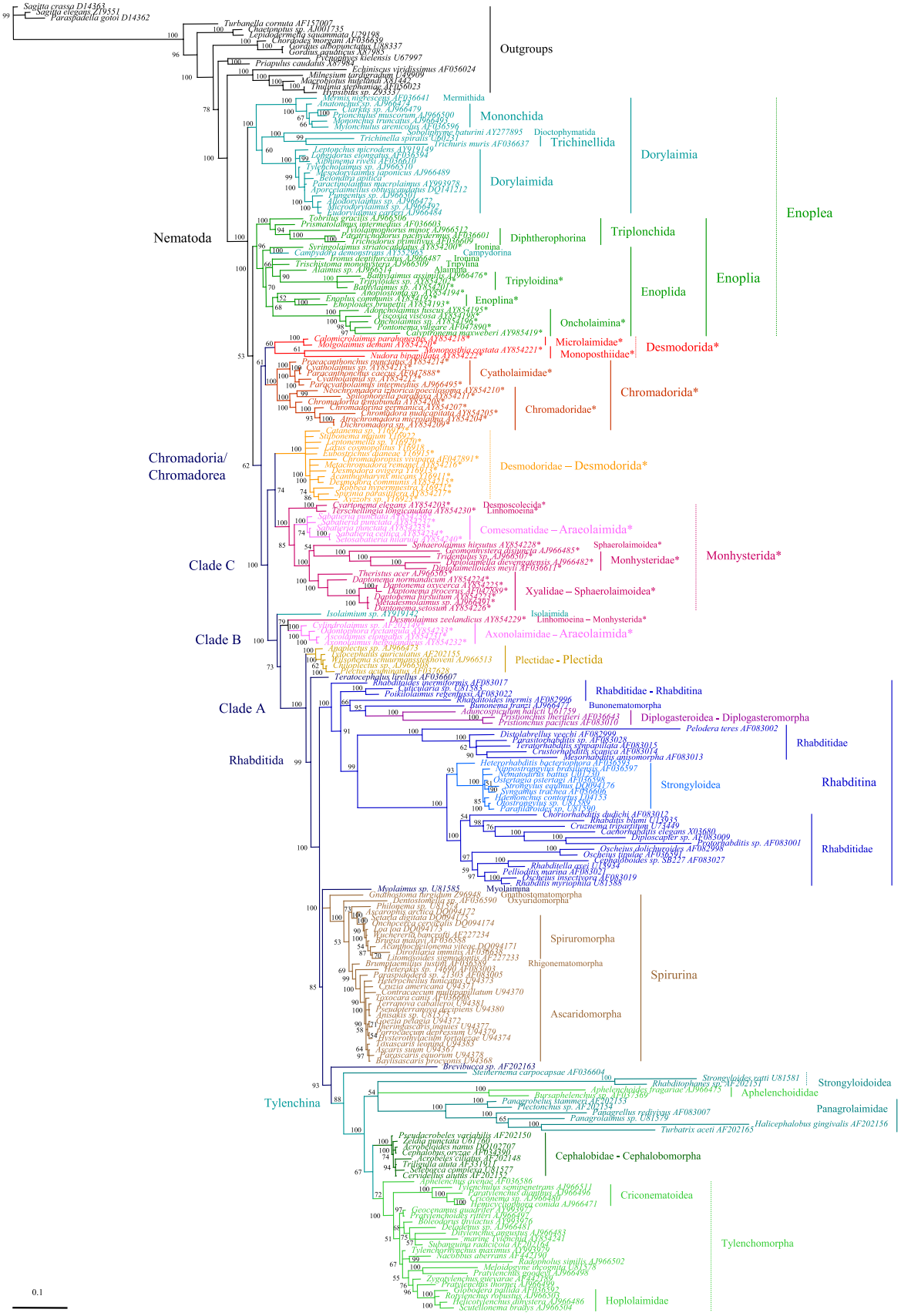


Fig. 1. Majority-rule consensus tree of 2700 phylogenetic trees saved under Bayesian inference after burn-in. Posterior probabilities (in percentage) are given for internal branches if >50%. Among the vertical lines on the right, the dotted lines depict paraphyletic or unresolved taxa. Marine species, and taxa including marine species, are marked by ∗. Species representing major taxa according to De Ley and Blaxter (2004) are highlighted in the same colour.

sequences were downloaded from the EMBL database providing a total of 212 nematode SSU sequences. (Accession numbers are given in Supplement Table 1 and all new sequences are marked.) Outgroups were selected from taxa shown to be part of a superphyletic clade including the Nematoda in Peterson and Eernisse's (2001) phylogenetic reconstruction of the Metazoa; these sequences were also downloaded from the European RNA Database. After outgroup analysis (Meldal, 2004) 16 sequences were retained: Chaetognatha (3 species), Gastrotricha (3), Kinorhyncha (1), Nematomorpha (3), Priapulida (1), and Tardigrada (5). The additional sequences were aligned to the profile from the European RNA database using the programme Clustal_X with default settings (Thompson et al., 1997, version 1.81). Further small-scale editing was carried out by hand using BioEdit (T. Hall, unpublished software, version 5.0.9) but many regions of the SSU gene are highly variable among the Nematoda and therefore remained difficult to align unambiguously. GBlocks v0.91b (Castresana, 2000) was used to eliminate the most ambiguously aligned positions using the following parameters: minimum number of sequences for a conserved position = 116, minimum number of sequences for a flanking position = 155, maximum number of contiguous non-conserved positions = 5, minimum length of a block = 2, and allowed gap positions = half. In addition to the GBlocks exclusions, a highly variable region of 200 bp was eliminated entirely, leaving 1167 out of a total of 1884 aligned characters for phylogenetic analyses.

Analyses were performed under unweighted parsimony, under parsimony after character weighting on the rescaled consistency index, under Bayesian inference and using LogDet-transformed distances. In the latter analysis, proportion of invariant sites was set to 0.21 according to Modeltest (see below). When using parsimony or LogDet optimality criteria, 10 random replicates of stepwise sequence additions were carried out. Branch swapping was performed under TBR. The number of rearrangements per replicate was limited to 2×10^9 under the parsimony criterion and 5×10^6 under LogDet transformations. After analysis under parsimony or LogDet criteria strict consensus trees were constructed of all fundamental trees with equal best scores. To estimate nodal support, 1000 bootstrap replicates under TBR branch swapping were calculated using heuristic search criteria. Per bootstrap replicate a single random stepwise sequence addition run was performed and 100 trees saved.

The general time reversible model assuming a proportion of invariant sites and a Γ distribution for the rate of the remaining sites (GTR + Γ + I) was determined as the best-fit maximum likelihood model for the Bayesian inference using Modeltest under the AIC model selection criterion (Posada and Crandall, 1998, version 3.06). The parameters for base frequencies, substitution rate matrix, Γ rate distribution and shape and proportion of invariant sites were allowed to vary throughout the analysis. Trees were sampled every 1000 generations. The burn-in value was set to 300 trees (i.e. 300,000 generations), which equated to the level at which all variable parameters reached a stable value in a preliminary run. The total number of generations was set to 3 million generations, 10 times higher than the burn-in value. Four parallel chains (one cold and three heated) were used. The analysis was repeated five times. Majority-rule consensus trees were reconstructed after discarding the burn-in.

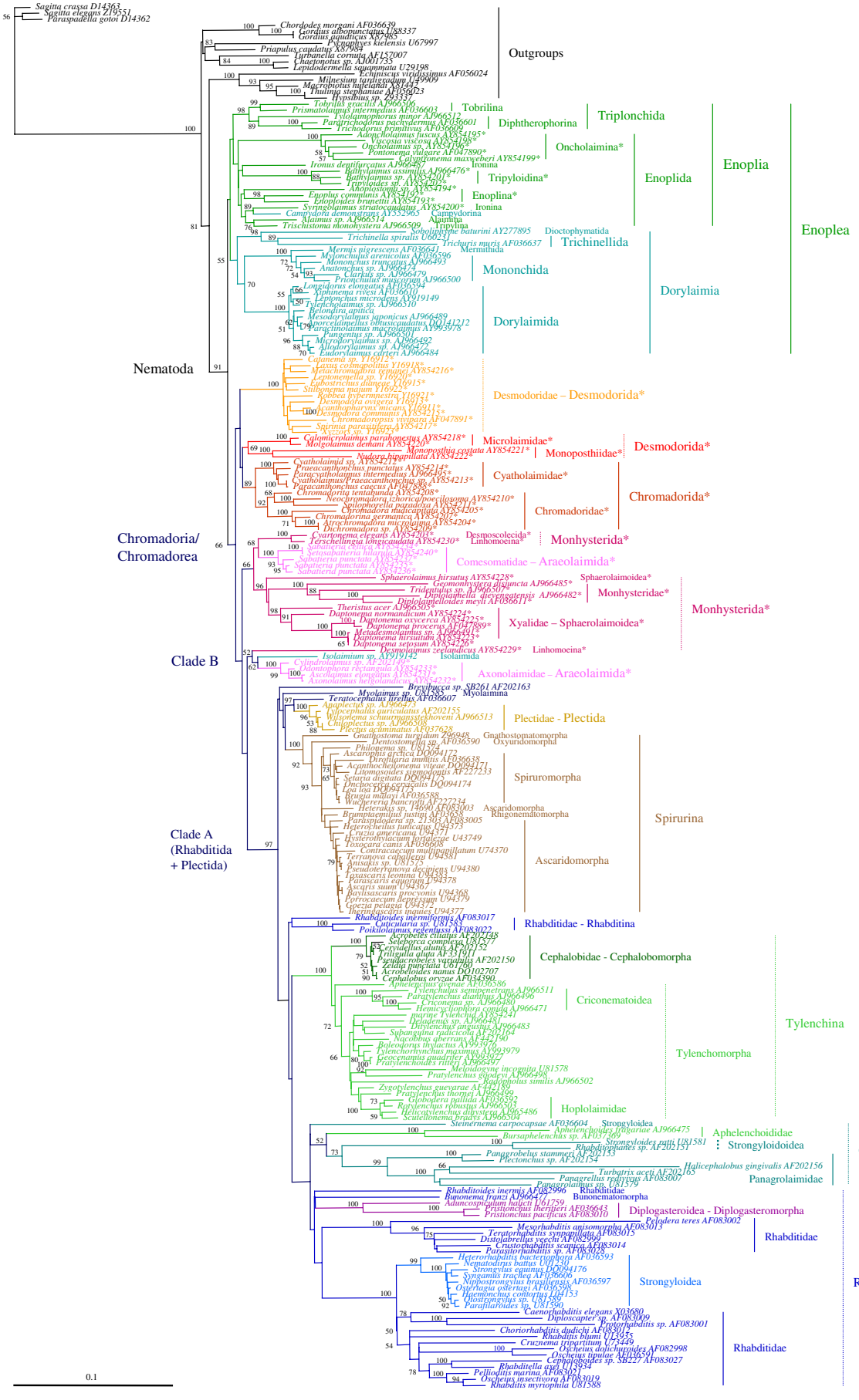
Most analyses were performed with PAUP* version 4.0beta10 (phylogenetic analysis using parsimony; Swofford, 2002) apart from Bayesian inferences which were calculated using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001).

3. Results

3.1. Characteristics of different models

The parsimony analyses resulted in topologies that did not correspond to any credible phylogeny, and thus were likely to be driven by phylogenetically uninformative signals or hindered by long-branch-attraction artefacts, to which parsimony is most sensitive (for this and other possible difficulties with using parsimony on rRNA genes for deep phylogeny, see Mallatt et al., 2004). For the Bayesian analysis, majority consensus trees of the 2700 saved trees after burn-in in each of the five repeats resulted in almost identical topologies. Fig. 1 depicts the majority consensus tree from one Bayesian analysis with a final log likelihood value of −41,851. For the LogDet analysis, Fig. 2 depicts the strict consensus tree of the two saved trees with a score of 7.03169. No single best tree was obtained in the distance analysis because a time constraint had to be employed for reasons of practicality. Support values are only given where $\geqslant$50%. Relationships are only interpreted as significant if they were supported by $\geqslant$70% after LogDet distance analysis and $\geqslant$95% after Bayesian inference. Posterior probabilities from the Bayesian inference were generally higher than bootstrap support from LogDet-transformed data. 67% of nodes had $\geqslant$95% support and 84% of nodes had $\geqslant$70% support in Bayesian inference while the values for LogDet-transformed data were 32% and 50%, respectively. Fig. 3 shows an overview of our current interpretation of the phylogeny of the Nematoda.

Fig. 2. Strict consensus tree of the 2 trees of the phylogenetic analysis under the distance criterion after LogDet transformation. Bootstrap support values (in percentage) are given for internal branches if >50%. Among the vertical lines on the right, the dotted lines depict paraphyletic or unresolved taxa. Marine species, and taxa including marine species, are marked by ∗. Species representing major taxa according to De Ley and Blaxter (2004) are highlighted in the same colour. (For interpretation of the references to colour in the Figure legend, the reader is referred to the web version of this paper).


Outgroups
Nematoda
Chromadoria/
Chromadorea
Clade B
Clade A
(Rhabditida
+ Plectida)
Enoplea
Enoplia
Enoplida
Triplonchida
Tobrilina
Diphtherophorina
Oncholaimina*
Tripyloidina*
Enoplina*
Ironina
Campydorina
Alaimina
Tripylina
Dioctophymatida
Trichinellida
Mermithida
Mononchida
Dorylaimia
Dorylaimida
Desmodoridae – Desmodorida*
Desmodorida*
Microlaimidae*
Monoposthiidae*
Cyatholaimidae*
Chromadorida*
Chromadoridae*
Monhysterida*
Desmoscolecida*
Linhomoeina*
Comesomatidae – Araeolaimida*
Sphaerolaimoidea*
Monhysteridae*
Monhysterida*
Xyalidae – Sphaerolaimoidea*
Isolaimida
Axonolaimidae – Araeolaimida*
Myolaimina
Plectidae - Plectida
Gnathostomatomorpha
Oxyuridomorpha
Spirurina
Spiruromorpha
Rhigonemidomorpha
Ascaridomorpha
Rhabditidae - Rhabditina
Cephalobidae - Cephalobomorpha
Tylenchina
Criconematoidea
Tylenchomorpha
Hoplolaimidae
Strongyloidea
Aphelenchoidea*
Strongyloidoidea
Aphelenchoidea
Panagrolaimidae
Bunonematomorpha
Diplogasteroidea - Diplogasteromorpha
Rhabditidae
Rhabditina
0.1

Observed numbers of transitions and transversions of all 1167 informative and unambiguous characters were plotted against the total number of expected substitutions (Fig. 4). The observed rates of substitutions are lower than the rates expected under the Tamura and Nei (1993) model (as suggested by Modeltest, Posada and Crandall, 1998). Furthermore, transversions show a near-linear substitution rate while that of transitions becomes gradually saturated falling behind the rate of transversions. Therefore, the rate of mutation has reached saturation in transitions.

3.2. Systematic interpretation of phylogenies

The monophyly of the Nematoda is supported at 100% under Bayesian inference and 91% under distance analysis. The relationship between the three classes of the Nematoda (Dorylaimia, Enoplia, and Chromadoria) could not be resolved using SSU data. Topologically, the Bayesian tree showed the Enoplia and Chromadoria as sister clades but the distance tree showed the Dorylaimia and Enoplia as sister clades (the traditional Enoplea) and showed that the root was found somewhere between the Enoplea and Chromadorea. However, most relevant nodes for the root relationships had insignificant support, with the exception of the monophyletic Dorylaimia (100%) and monophyletic Enoplia (96%) that had significant support under Bayesian inference (Fig. 1).

3.2.1. Dorylaimia

The Dorylaimia and its orders were monophyletic and supported by $\geqslant$98% by Bayesian analysis (Fig. 1), under LogDet only the Dorylaimida (100%) and Trichinellida (89%) were significantly supported (Fig. 2). In both analyses, the Mermithida and Mononchida were sister taxa with 100% support and the Dioctophymatida and Trichinellida were sister taxa with $\geqslant$98% support. The relationships between the remaining orders remained variable and the internal relationships of the Dorylaimida and Mononchida could not be resolved.

3.2.2. Enoplia

The Enoplida and Triplonchida were the two sister-orders forming the Enoplia (96% under Bayesian inference). However, the relationships of suborders within the two orders remained unresolved. Three species that were previously not reliably placed in the Enoplea were consistently found in this clade: *Alaimus* (formerly Dorylaimia or Triplonchida), *Campydora demonstrans* (formerly Dorylaimia or Enoplia), and *Trischistoma monohystera* (formerly Triplonchida).

3.2.3. Chromadorea

The Chromadorea were recovered in both analyses but without significant support (Figs. 1 and 2). The monophyletic Cyatholaimidae (100%) and Chromadoridae (>92%) form the Chromadorida (>89%) (Figs. 1 and 2). The monophyletic Microlaimidae (100%) and Monoposthiidae (100% only under LogDet, Fig. 2) always formed a single clade but appear not closely related to the other family of the Desmodorida, the Desmodoridae.

The Comesomatidae were consistently found in a clade with the Monhysterida and Desmoscolecida (only species represented *Cyartonema elegans*) (100% by Bayesian, Fig. 1) and were not closely related to the other family of the Araeolaimida, the Axonolaimidae. However, the relationships between the families of the Monhysterida and the Comesomatidae remain uncertain. Nevertheless, *Cyartonema elegans*, which has been placed into various orders, was consistently found as sister taxon to *Terschellingia longicaudata* (Linhomoeidae) in the Monhysterida (100%, both analyses, Figs. 1 and 2). *Desmolaimus zeelandicus* was found near the base of a large clade, containing the Rhabditida, Plectida, Axonolaimidae, and *Isolaimium* sp. (100% support under Bayesian inference), rather than with the other species of the Linhomoeidae, *T. longicaudata.*

The monophyletic Desmodoridae was well supported (100% in both analyses, Figs. 1 and 2) but the internal relationships of this family varied between analyses. *Xyzzors* sp. was always found within the Desmodoridae rather than the Cyatholaimidae.

The Bayesian analysis resulted in three well supported (100%) clades at the deeper phylogenetic level (Fig. 1): Clade A: Plectida and Rhabditida, Clade B: Clade A plus Axonolaimidae, *Desmolaimus zeelandicus* and *Isolaimium* sp., and Clade C: Clade B plus Desmodoridae and Monhysterida (including Comesomatidae, see above). Hence, *Isolaimium* sp. was not closely related to the Dorylaimia as traditionally placed.

The Plectidae, *Teratocephalus lirellus*, and the Rhabditida always formed a well supported clade (>97% in both analyses, Figs. 1 and 2) although their relationships varied between the two analyses. Under Bayesian inference (Fig. 1), the monophyletic Plectidae (100%) was the sister taxon to the clade *T. lirellus* + Rhabditida (99%). Under distance analysis (Fig. 2), *T. lirellus* was the sister taxon to the Plectidae (97%) and both were found in the Rhabditida (97%). The relationships of the sub- and infra-orders of the Rhabditida varied between the two analyses. The positions for *Brevibucca* sp., *Myolaimus* sp. and *Steinernema carpocapsae* remained unresolved.

The monophyletic Spirurina and monophyletic Spiruromorpha were well supported (both 100% in Bayesian, Fig. 1, 100% and 73%, respectively, in LogDet analysis, Fig. 2). The Ascaridomorpha were only monophyletic and well supported (99%) in the Baysian analysis. The relationships within the infra-orders of the Spirurina remain unresolved.

The monophyly of the Tylenchina could not be established. The Panagrolaimidae, Strongyloidea (without *S. carpocapsae*) and Aphelenchoididae were individually well supported (99–100%, Figs. 1 and 2) but whether they together form a clade remains uncertain as it was insignificantly supported. The Cephalobidae are well supported as a family (100%, Figs. 1 and 2) but the internal relationships

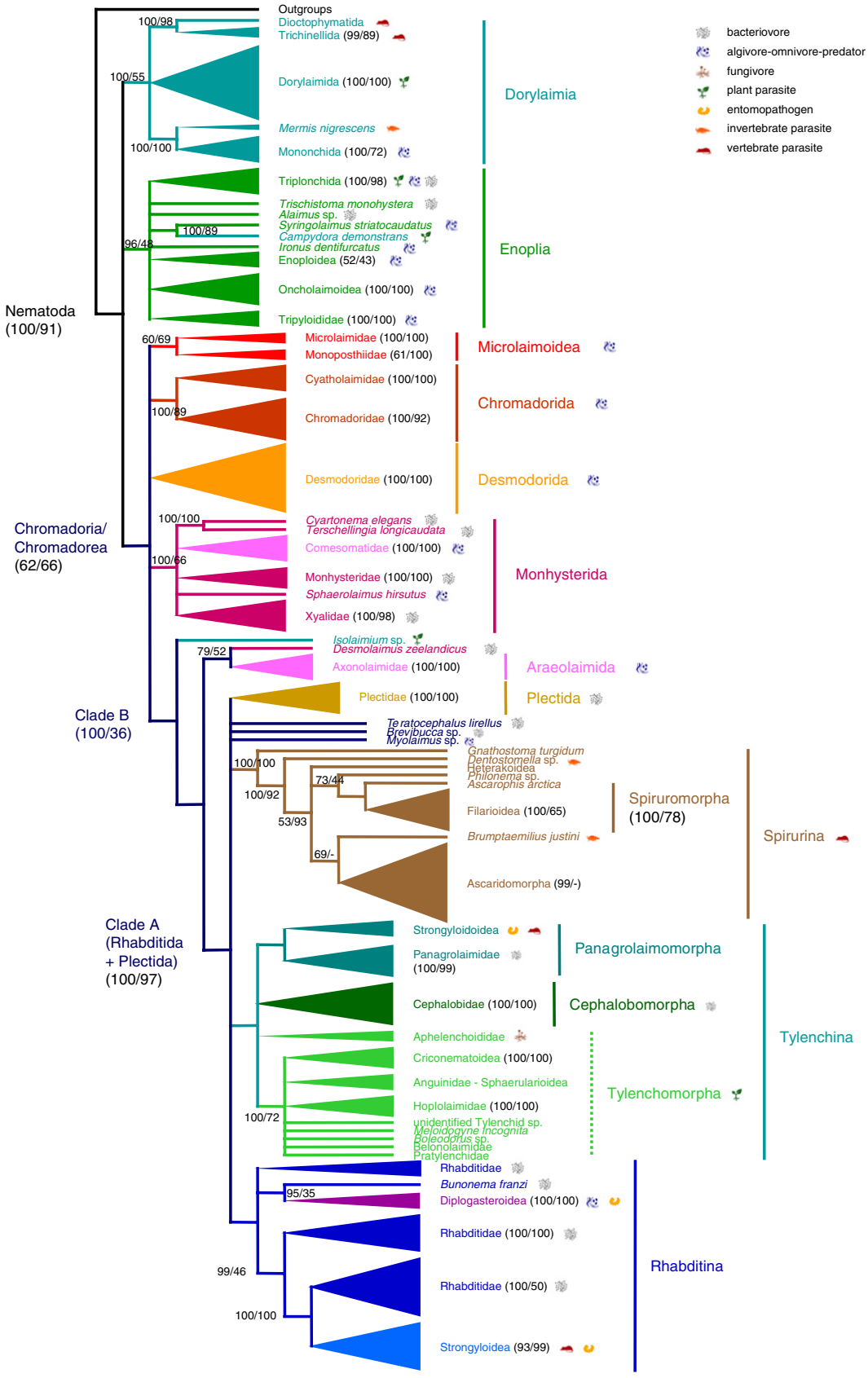


Fig. 3. An overview of the Nematoda based on SSU phylogenetic analyses. Support values (in percentage) from Bayesian inference and LogDet transformation of distances are given for putative monophyletic groups. Marine species, and taxa including marine species, are marked by ∗. Trophic groups are depicted as pictograms. Major taxa according to De Ley and Blaxter (2004) are highlighted in the same colour. (For interpretation of the references to colour in the Figure legend, the reader is referred to the web version of this paper.)

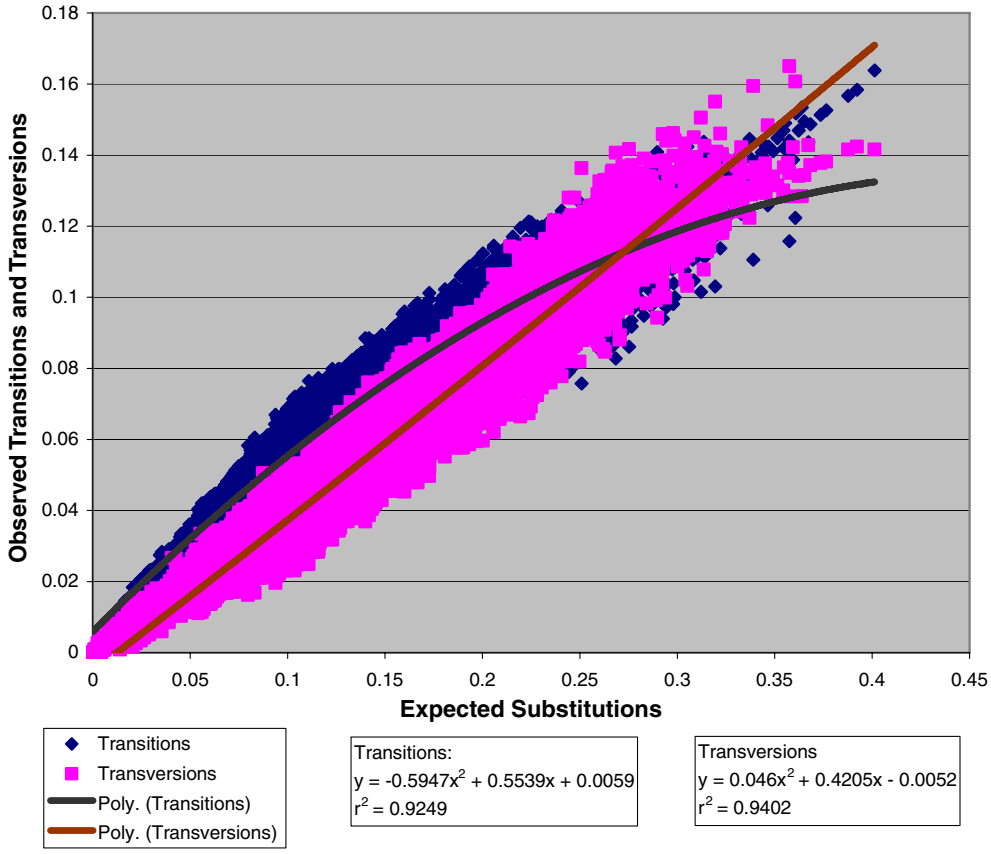


Fig. 4. Saturation analysis: observed transitions and transversions against overall estimated substitution rate.

vary. In the Bayesian analysis (Fig. 1), the Tylenchomorpha without the Aphelenchoidoidea (Aphelenchoididae plus *Aphelenchus avenae*) formed a well supported clade (100%). Within this clade, only the Criconematoidea (100% in both analyses) and the Hoplolaimidae + *Globodera pallida* (100% in both analyses) were well supported. The remaining taxa belong to the Anguinidae (*Ditylenchus*, *Subanguina*), Belonolaimidae (*Geocenamus*, *Tylenchorhynchus*), Meloidogynidae (*Meloidogyne*), Phaenopsitylenchidae (*Deladenus*), Pratylenchidae (*Nacobbus*, *Pratylenchoides*, *Pratylenchus*, *Radopholus*, and *Zygotylenchus*), and Tylenchidae (*Boleodorus*) but all these families were paraphyletic.

The Rhabditina were monophyletic and well supported (100%), but only under Bayesian inference. *Bunonema franzi* was consistently found as sister taxon to the Diplogasteromorpha but only supported under Bayesian inference (95%, Fig. 1). The Strongyloidea seemed supported (although only 93% in Bayesian, Fig. 1, 99% in LogDet analysis, Fig. 2). The Rhaditidae were paraphyletic and split into at least three clades, two of which were well supported (100%) under Bayesian inference (Fig. 1), one in the LogDet analysis (Fig. 2).

4. Discussion

Our data bring new resolution to nematode phylogenetics, but there remain areas of uncertainty. Many of the findings are consistent with those of De Ley and Blaxter (2002, 2004). We will focus here on cases where our results differ or provide improved resolution. The Bayesian posterior probabilities and the LogDet bootstrap support values are high for most clades that are consistently reconstructed but low for those recovered by only one of the two analytical methods. While consistency gives no certainty of a correct phylogeny, it is nevertheless a good indicator and the reconstructions agree to a large extent with recent revisions of the classical systems (Aleshin et al., 1998; Blaxter et al., 1998; De Ley and Blaxter, 2002) and with some interpretations of morphological data (Maggenti, 1963; Andrássy, 1976; Inglis, 1983; Adamson, 1987).

4.1. The phylogeny of the Nematoda

With information from additional marine chromadorids, we can confirm the suggestion by Blaxter et al. (1998) and De Ley and Blaxter (2002) that the former class 'Secernentea' needs to be downgraded because it derived from a common ancestor with the Axonolaimidae, *Desmolaimus zeelandicus* and *Isolaimium* sp. (see Figs. 1 and 2) and not directly from the ancestor of all nematodes. This finding is in agreement with Maggenti (1963, 1970), Inglis (1983), Gadéa (1973), Andrássy (1976), Drozdovskii (1980), Adamson (1987) and Malakhov (1994), as they all suggested synapomorphy of the valves in the posterior oesophageal bulb of the Plectidae and

Rhabditidae. The results do not support Chitwood and Chitwood (1950) and Lorenzen (1981) who adhered to the division of the nematodes into the 'Aphasmidia/Adenophorea' and the 'Phasmidia/Secernentea'.

Although the three major classes of the Nematoda (the Enoplia, Dorylaimia, and Chromadoria) were recovered, the Chromadoria were never significantly supported and the Dorylaimia and Enoplia were only significantly supported under Bayesian inference (100% and 96%, respectively, Fig. 1). The orders within the Enoplia and Dorylaimia, as proposed by De Ley and Blaxter (2002), were generally recovered. However, only two of the orders of the Chromadoria were recovered as monophyletic clades (the Chromadorida and Plectida) matching fully with current classification. Additionally, the Bayesian analysis recovered three clades with 100% support within the unsupported Chromadoria: Clade A: Plectida and Rhabditida, Clade B: Clade A plus Axonolaimidae, *Desmolaimus zeelandicus* and *Isolaimium* sp., and Clade C: Clade B plus Desmodoridae and Monhysterida (including Comesomatidae).

4.1.1. The Enoplia

The Triplonchida were confirmed as an order within the Enoplia, consistent with Siddiqi (1983) but contrary to many earlier classifications that were based on morphological data alone and placed part of this group among the Dorylaimia (e.g. Thorne, 1939; Clark, 1961; Siddiqi, 1961, 1973; De Coninck, 1965; Coomans and Loof, 1970). Within the Triplonchida, the Diphtherophoroidea were monophyletic and well supported. Contrary to morphological classifications, *Trischistoma monohystera* appeared to be more closely related to the Enoplida than to the Triplonchida as the latter orders formed a well supported clade excluding *T. monohystera*.

The internal relationships of the Enoplida were not well resolved. Only the Oncholaimoidea and Tripyloididae were well supported monophylies. The present data suggest that the Tripyloididae were part of the Enoplida, as supported by cephalic morphology (Filipjev, 1918, 1934; Gerlach and Riemann, 1973/1974; Lorenzen, 1981). The Tripyloididae were not associated with the Chromadoria, as has been inferred from the presence of spiral amphids (De Coninck and Schuurmans Stekhoven, 1933; Chitwood and Chitwood, 1950; De Coninck, 1965; Andrássy, 1976). The position of *Campydora demonstrans* as part of the Enoplida has been confirmed (Siddiqi, 1983; Mullin et al., 2003). This taxon was previously classified amongst the Dorylaimia (Thorne, 1939; Jairajpuri and Ahmad, 1992; De Ley and Blaxter, 2002).

The Ironidae appeared to be polyphyletic as its two sequenced representatives, *Ironus dentifurcatus* and *Syringolaimus striatocaudatus*, never formed a sister relationship. Lorenzen (1981) established the monophyly of the Ironidae based on the buccal cavity armature but he also pointed out considerable differences in the members of this family. As is evident from the various types of teeth and feeding stylets in the Nematoda, the evolution of the buccal armature seems largely directed by functionality. So it is quite possible that the three or four movable teeth found in the Ironidae are homoplastic.

The Alaimidae (represented by *Alaimus* sp.) appeared to be part of the Enoplida, in accordance with Chitwood and Chitwood (1950), and were not associated with the Dorylaimia, as was proposed by Filipjev (1934), Thorne (1939) and Lorenzen (1981). This conclusion also casts doubt on the phylogenetic validity of Lorenzen's interpretation of the position of the oesophageal gland outlets as key character to separate Enoplia and Dorylaimia, because the glands are situated posteriorly in both the Dorylaimia and in *Alaimus* (Lorenzen, 1981).

4.1.2. The Dorylaimia

The Dorylaimia were recovered in correspondence with previous studies (De Ley and Blaxter, 2002) with the above mentioned exceptions: *Alaimus*, *Campydora*, and *Trischistoma* were placed in the Enoplida. The Dioctophymatida and Trichinellida were confirmed as a single clade (Rusin et al., 2003) but its position within the Dorylaimia remains uncertain. All three represented species have long branches and more sequences are needed to resolve the placement of this clade in the Dorylaimia.

4.1.3. The marine Chromadorea

The Chromadorida were always monophyletic and well supported. Within this order, the Chromadoridae and Cyatholaimidae were sister families. All major classical authors also reached this conclusion. The Desmodorida were never recovered as a monophyletic group, with the Microlaimidae and Monoposthiidae forming one suggestive clade and the Desmodoridae found in a different part of the trees. The genera of the Desmodoridae were not demonstrably monophyletic and *Xyzzors* sp. was found in the Desmodoridae, as opposed to the Cyatholaimidae (De Coninck, 1965; Lorenzen, 1981).

The content of the order Monhysterida has been extended to include the Comesomatidae (see below) and *Cyartonema elegans*. The latter was always found as sister taxon to *Terschellingia longicaudata*; this placement is in accordance with De Coninck (1965) who put *Cyartonema* Cobb, 1920, into the Siphonolaimidae and in opposition to Lorenzen (1981), who moved this genus into the Chromadorida (*sensu* Lorenzen, 1981). The Linhomoeidae were paraphyletic as *Terschellingia longicaudata* and *Desmolaimus zeelandicus* never form a sister relationship.

Metadesmolaimus sp. (Xyalidae) was included in a clade of *Daptonema* species; this specimen may have been misidentified and may also be a *Daptonema* species. *Theristus acer* is the sister taxon to the genera *Daptonema* and *Metadesmolaimus*. The current data highlight the difficulty of identifying members of the Xyalidae using morphological traits. The genera of this family have been revised and synonymised repeatedly (Wieser, 1956; Lorenzen, 1977) while Nicholas and Trueman (2002) published a cladistic analysis

based on morphology. A further revision, including molecular evidence, appears timely.

The phylogenetic positions of the Axonolaimidae and Comesomatidae have long been debated. The two families were either placed into the Monhysterida (Filipjev, 1934; Chitwood and Chitwood, 1950; Lorenzen, 1981) or Araeolaimida (Malakhov et al., 1982; Inglis, 1983; Maggenti, 1983; De Ley and Blaxter, 2002) because of the outstretched ovaries and presence of oesophageal tubes. Other authors (Wieser, 1954; De Coninck, 1965; Andrássy, 1976; Platt, 1985; Hope and Zhang, 1995) placed the Comesomatidae into the Chromadorida on account of the punctated cuticle, spiral amphids and presence of precloacal supplements in the male. The current molecular data clearly suggested that the Comesomatidae and Axonolaimidae do not form a sister relationship but that the former are members of the Monhysterida and the latter constitute their own clade, possibly with other families of the Araeolaimida (*sensu* De Ley and Blaxter, 2002) and the Isolaimida.

The Isolaimida (a monogeneric order only represented here by *Isolaimium* sp.) were found basally in Clade B, also containing Rhabditida, Plectida, Axonolaimidae, and *D. zeelandicus* (Fig. 1). This proximity to the Axonolaimidae is in agreement with morphological analysis by Filipjev (1934), Goodey (1963) and Gerlach and Riemann (1973/1974). In contrast, many classic systems placed this taxon in the Dorylaimia (De Coninck, 1965; Timm, 1969; Coomans and Loof, 1970; Andrássy, 1976; Lorenzen, 1981) or Enoplia (Maggenti, 1982; Inglis, 1983). Mullin et al. (2003, 2005) put *Isolaimium* in Dorylaimia using molecular analyses but their datasets did not include adequate representation of the Chromadoria to test its relationship with Axonolaimidae; although, their trees are compatible with our results.

4.1.4. The Plectidae as sister taxon to the Rhabditida

Traditionally, the Plectidae and their nearest relatives were placed either in the Monhysterida (Chitwood and Chitwood, 1950) or into the Araeolaimida (De Coninck, 1965; Andrássy, 1976; Inglis, 1983), until Malakhov et al. (1982) proposed separate order status. In the present study, the Plectidae were always monophyletic and placed either as sister taxon to the Rhabditida (Bayesian inference, Fig. 1) or as a clade within the Rhabditida (distance analysis, Fig. 2). Morphologically, the Plectidae could be placed as the intermediate taxon between the marine Chromadorea (Monhysterida, Araeolaimida) and the Rhabditida (Litvaitis et al., 2000; De Ley and Blaxter, 2002).

In the present analyses, *Teratocephalus lirellus* was found either as sister taxon to the Plectidae, when the Plectidae are part of the Rhabditida (distance analysis, Fig. 2), or *T. lirellus* was found to be the most basal of the Rhabditida (Bayesian inference, Fig. 1). *T. lirellus* is morphologically intermediate between the Plectidae and some Cephalobidae. Therefore, it is suggested that the closest sister taxon to *T. lirellus* are the Plectidae.

4.1.5. The Rhabditida

The current data confirmed the findings of Blaxter et al. (1998) that the redefined Rhabditida (*sensu* De Ley and Blaxter, 2002) derived from a common ancestor of Clade B, including the Axonolaimidae and Plectidae (Fig. 1). They do not constitute a sister group to all other nematodes as has been frequently suggested in past classifications where the name Secernentea and the rank of class was given to this group (e.g. Chitwood and Chitwood, 1950; Lorenzen, 1981; Kampfer et al., 1998). The Rhabditida contain a large number of taxa that are highly derived and divergent from their ancestral chromadorean nematodes. The relationships between and within the suborders of the Rhabditida remained uncertain as many taxa are only represented by a single species.

The wholly parasitic suborder Spirurina was always recovered as a monophyletic and relatively well resolved clade. The two infraorders that were represented by more than one taxon, Spiruromorpha and Ascaridomorpha, were monophyletic but none of the families were recovered as monophyletic groups. *Ascarophis arctica* was consistently recovered as the most basal taxon of the Spiruromorpha and *Gnathostoma turgidum* and *Dentostomella* sp. were the most basal taxa of the Spirurina.

The Tylenchina were monophyletic only under Bayesian inference but just insignificantly supported (93%). The Panagrolaimidae were monophyletic and well supported. The status of the Strongyloidoidea remained unresolved because of the uncertain position of *Steinernema carpocapsae*. The Cephalobidae (Cephalobomorpha) were always monophyletic and well supported but their internal relationships remained uncertain. The Tylenchomorpha were monophyletic, with the exception of the position of the Aphelenchoidea. *Aphelenchoides fragariae* and *Bursaphelenchus* sp. were located among the Panagrolaimomorpha, albeit without significant support, and not as sister taxa to the other representative of the Aphelenchoidea, *Aphelenchus avenae*; additionally, there was no support for the clade *A. avenae* plus the remaining Tylenchomorpha. Blaxter et al. (1998) recovered the same topology. Within the remaining Tylenchomorpha, the current data set established two previously uncertain relationships: (i) *Paratylenchus dianthus* was part of the Criconematoidea which conforms with previous morphological classifications and (ii) *Globodera pallida* belonged to the Hoplolaimidae in accordance with De Ley and Blaxter (2002) and in disagreement with Chitwood and Chitwood (1950), Andrássy (1976), Maggenti et al. (1987) and Siddiqi (2000). The position of the unidentified Tylenchid species from macroalgae could not be resolved. The Anguinidae (*Ditylenchus, Subanguina*), Belonolaimidae (*Geocenamus*, *Tylenchorhynchus*), and Pratylenchidae (*Nacobbus*, *Pratylenchoides*, *Pratylenchus*, *Radopholus*, and *Zygotylenchus*) were paraphyletic (see also Blaxter et al., 1998) and need to be revised.

The Rhabditina formed at least three highly derived clades. One clade consisted of the Diplogasteroidea and

Bunonema franzi, a relationship that has also been suggested by Fürst von Lieven (2002) and Dolinski and Baldwin (2003) based on morphological observations of the buccal cavity, the pharynx, the male genital papillae and the female gonads. The other two clades contain taxa of the paraphyletic Rhabditidae, one of which also gave rise to the monophyletic and well supported parasitic Strongyloidea. Further taxa, classically assigned to the Rhabditidae (*Cuticularia* sp., *Poikilolaimus regenfussi*, *Rhabditoides inermiformis*) did not go with other Rhabditidae and remained uncertain in their position within the Rhabditina (Bayesian inference, Fig. 1) and Rhabditida (LogDet analysis, Fig. 2), respectively.

4.2. Resolving the power of the SSU gene in phylogenetic analyses

This study showed the power of molecular data for the interpretation of phylogenies with conflicting morphological evidence. The SSU rDNA gene proved very effective in the recovery of many traditional monophyletic groups within the phylum Nematoda (e.g. Chromadorida, Dorylaimida, Plectida, Rhabditida, and Spirurina) and provided clarification of relationships that were previously uncertain or controversial, such as the affinity of the Isolaimida with Axonolaimidae and the position of the Comesomatidae in the Monhysterida. It provided strong support for the ingroup node, Nematoda (100% under Bayesian inference, 91% under LogDet analysis). However, there were also certain limitations to the use of the SSU. The gene did not appear to retain enough phylogenetic signal to recover the relationships amongst the three main clades, Enoplia, Dorylaimia, and Chromadoria, and the monophyly of the Chromadoria. Also, the SSU gene appears to be too conserved to accurately reconstruct relationships within certain groups with short branches such as the Dorylaimida, Desmodoridae, Ascaridomorpha, Cephalobidae or Tylenchomorpha. Furthermore, no clear phylogenetic position could be determined for some taxa; this was often the case when a species was the single representative of its family or even suborder (e.g. the morphologically unique *Myolaimus* sp.). Despite these caveats, for the reconstruction of intermediate events in the phylogeny the SSU rDNA gene appears to be an appropriate choice as it clearly distinguishes relationships at all levels between orders to most families.

In those cases where more than one representative per genus was included, a clear distinction was also observed in the SSU sequence between species. In contrast, the sequences of *Atrochromadora microlaima*, *Dichromadora* sp., and *Chromadora* sp. are almost identical, while those of *Sabatieria celtica* and *Setosabatieria hilarula* as well as those of *Daptonema hirsutum* and *D. setosum* are, respectively, identical. In all three cases, at least some of the specimens were juveniles and no adults were found among the first 30 animals identified per sample. Identification errors can, therefore, not be excluded, but it may be that evolutionary rates were relatively reduced in these clades or that these species represented conspecific taxa that exhibit morphological polymorphisms. However, only new data can provide further conclusions.

Other genes will need to be used to resolve both the basal relationships and the internal phylogenetic structure of certain families and orders. The large subunit of the nuclear ribosomal RNA gene contains both highly variable and highly conserved regions that may offer a better phylogenetic signal for the placement of the remaining uncertain taxa (Mallatt et al., 2004). Mitochondrial or nuclear protein genes may also offer improved results.

4.3. Ecological evolution in the phylum Nematoda

Assuming that all life originated in the sea and that metazoan phyla evolved during the Precambrian period more than 550 Million years ago (Mya) (Conway Morris, 1993; Valentine et al., 1996,1999; Fedonkin and Waggoner, 1997; Peterson and Davidson, 2000), it is reasonable to assume that the ancestral nematode was also marine. This is in accordance with calculations that the Spirurina (= Clade III *sensu* Blaxter et al., 2000) diverged from the remaining rhabditids around 500 Mya and that the Nematoda diverged from the remaining Metazoa around 1000 Mya (Vanfleteren et al., 1994; Blaxter et al., 2000; Hedges, 2002). Such dates clearly predate colonisation of the land-masses in the Silurian period (443–417 Mya) by phyla with hard parts and a fossil record, making Nematodes presumably also marine in origin. We note however, that nematodes not only lack an informative fossil record (the oldest known fossil, *Cretacimermis libani* Poinar et al., 1994 (Poinar, 2003) dates to around 135 Mya), but also that extrapolations of molecular clock estimates are based on other taxa: if the Nematoda have idiosyncratic evolutionary rates, then these estimates may be inaccurate.

Due to the lack of resolution at the base of the Nematoda, their marine ancestry, as proposed by Filipjev (1929, 1934) and now widely accepted (e.g. Lambshead and Schalk, 2001), has been questioned by De Ley and Blaxter (2002, 2004) and it is true that a case can be made for a terrestrial origin for the Nematoda. It is possible that highly productive terrestrial ecosystems existed in the Precambrian capable of supporting the evolution of a new phylum (Kenny and Knauth, 2001). Also, Schierenberg (2005) has recently shown that the freshwater nematode *Tobrilus* has the most plesiomorphic gastrulation pattern of those that have been studied. Even the addition of over 40 marine taxa has not improved the situation and the placement of the root is still uncertain.

Nonetheless, traces of a marine origin of the Nematoda can possibly be found in some parts of the current phylogeny where taxa that are currently found in terrestrial habitats are nested within marine clades. The strongest evidence for a marine ancestry of the Nematoda comes from the Chromadorea: the basal clades are all predominantly marine (Microlaimoidea, Chromadorida, Desmodorida,

Monhysterida, and Araeolaimida) and the almost exclusively non-marine Rhabditida derive from the ancestor of the Monhysterida or Araeolaimida.

5. Conclusions

The addition of 100 new SSU sequences, 46 of which are marine species, has provided additional insights into the phylogeny of the phylum Nematoda. This study presents additional support for (i) the descent of the order Rhabditida from a common ancestor of chromadorean orders Araeolaimida, Chromadorida, Desmodorida, Desmoscolecida, and Monhysterida and (ii) the position of *Bunonema* close to the Diplogasteroidea in the Rhabditina. The additional data also resolved some previously controversial relationships more clearly: (a) *Alaimus*, *Campydora*, and *Trischistoma* belong in the Enoplida, (b) *Isolaimium* is placed basally to a big clade containing the Axonolaimidae, Plectidae, and Rhabditida, (c) *Xyzzors* belongs in the Desmodoridae, (d) Comesomatidae and *Cyartonema* belongs in the Monhysterida, (e) *Globodera* belongs in the Hoplolaimidae and (f) *Paratylenchus dianeae* belongs in the Criconematoidea. However, the SSU gene did not provide sufficient resolution at the deepest levels of the phylogeny and the ancestry of the Nematoda has to remain uncertain.

Although this study is the first to sequence a wide representation of marine taxa, we must stress that the specimens used all come from northwest European coastal waters. This is, of course, a tiny fraction of the marine benthic environment. Morphological evidence suggests that many marine nematode genera are cosmopolitan (e.g. see the generic lists in Lambshead et al., 2003 for the genera of the central equatorial Pacific). However, this may be misleading, given the questions raised about the reliability of morphological evidence in this study. It is entirely possible that molecular studies of deep sea and tropical and southern coastal nematode populations will reveal unsuspected clades.

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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.2006.08.025.

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