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Evidence from the small and large ribosomal RNA structure suggests that *Anoplostoma rectospiculum* Gal'tsova, 1976 (Nematoda: Anoplostomatidae) is a member of the superfamily Enoploidea, not Oncholaimoidea

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Summary – Analyses of the primary structure of the 18S rRNA gene and D3 segment of the 28S rRNA, as well as evidence from the secondary structure of the SSU rRNA V7 region, suggest that *Anoplostoma rectospiculum* (Anoplostomatidae) has closer relationships to the family Enoplidae than to the Oncholaimidae. In phylogenetic trees derived from full length SSU rRNA gene and partial LSU rRNA gene (D3 expansion segment) sequence analyses, *A. rectospiculum* exhibits long branches. The associated artefacts of long branch attraction (LBA) are circumvented because of the presence of an undoubted molecular synapomorphy – a low homoplastic 1. b.p. insertion in helix 43 of the SSU rRNA which is shared jointly by Anoplostomatidae and Enoplidae. Analysis of low homoplastic apomorphic characters is considered to be a tool for testing phylogenies against LBA artefacts.

Keywords – Enoplia, Enoplida, homoplasy, molecular, phylogeny, Oncholaimidae, SSU rRNA secondary structure.

The genus *Anoplostoma* Bütschli, 1874 includes about 24 free-living marine nematode species belonging to the order Enoplida. They are all small (*ca* 1 mm length) and inhabit the soft sediments of the marine intertidal zone, estuaries and shallow waters throughout the world (Gerlach & Riemann, 1974; Gal'tsova, 1976). *Anoplostoma viviparum* (Bastian, 1865) is characterised by such unusual features as a variable blastomere arrangement in early embryonic development (Malakhov & Cherdantzev, 1974), a feature also found in *Enoplus brevis* Bastian, 1865 (Enoplidae) and *Pontonema vulgare* Bastian, 1865 (Oncholaimidae), whereas egg cleavage of the remaining nematodes are invariant (Voronov, 1999, 2001).

The genus *Anoplostoma* is unique amongst the Enoplida because of a specific combination of unusual morphological features (Belogurov & Alekseev, 1977; Lorenzen, 1981) *i.e.*, copulatory bursa presented in males; a spacious, unarmed, stoma which is not embedded in pharyngeal tissue; a cephalic capsule which is not connected to the pharyngeal muscles; a unique feature of the endocupola with irregular thickness of the vault; presence of sutures between adjacent stomatorhabdions; the body sharply tapering toward both ends, and viviparous reproduction in many species.

Conventionally, the genus has been placed within either the Oncholaiminae or the Oncholaimidae, depending on the systematic scheme proposed (Filipjev, 1921; Chitwood, 1960; Clark, 1961). The primary argument for this placement was the presence of copulatory alae in males of *Anoplostoma*, a character typical for secernentian nematodes, but rare for adenophoreans. There are isolated instances of adenophorean nematodes with alae, including the oncholaimid genus *Oncholaimellus* de Man, 1890, some genera of Monhysterida, and dioctophymids. In addition, both *Anoplostoma* and *Pelagonema* Cobb, 1894 (Oncholaimidae) possess a stoma devoid of onchia. It was for this reason that De Coninck placed the genus *Anoplostoma* within the subfamily Pelagonematinae De Coninck, 1965, when he divided the family Oncholaimidae (De Coninck, 1965). This systematic position for *Anoplostoma* was later accepted by Hope and Murphy (1972). In their checklist, Gerlach and Riemann

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(1974) established the monotypic family Anoplostomatidae for the genus. The close affinities of Anoplostomatidae and Oncholaimidae were later disputed by Belogurov and Alekseev (1977). These authors suggested that in Anoplostomatidae, such characters as the bursa, unarmed stoma and lost connection of the cephalic capsule with pharyngeal tissue originated independently, while other diagnostic features (arrangement of the cephalic setae in three circles, presence of sutures between adjacent stomatorhabdions, and structure of the posterior testis) were plesiomorphic. Lorenzen (1981) affiliated Anoplostomatidae with the superfamily Enoploidea on the basis of both gonads being positioned on the left of the intestine, whilst the positioning of the gonads on the right of the intestine was considered a holapomorphy of the superfamily Oncholaimoidea. Furthermore, subcuticular proprioreceptors in *Anoplostoma*, similar to those in some Enoplidae, are represented by dorsolateral loxometanemes, whereas all Oncholaimoidea are thought to possess dorsolateral and ventrolateral orthometanemes (Lorenzen, 1981). While some recent systems admitted Lorenzen's view (De Ley & Blaxter, 2002), others traditionally continued to place *Anoplostoma* within the Oncholaimoidea (http://www.ncbi.nlm.nih.gov/Taxonomy). Thus, the systematic position of *Anoplostoma* remains problematic.

Phylogenetic analysis of SSU rDNA sequence data has been used to delimit distinct monophyletic groups of Enoplia, Chromadoria, Triplonchida and Dorylaimia (Aleshin *et al*., 1998; Blaxter *et al*., 1998; Rusin *et al*., 2001; De Ley & Blaxter, 2002). Analysis of partial LSU rDNA sequences provided a preliminary scheme of the systematic relationships within the Enoplida (Litvaitis *et al*., 2000). Both phylogenetic markers revealed considerable divergence between representatives of Enoploidea and Oncholaimoidea, suggesting that resolution of the systematic position of *Anoplostoma* within the framework of enoplid relationships could now be possible.

Until recently, sequence data on ribosomal RNA genes from Anoplostomatidae were lacking. A partial (336 b.p.) sequence of mitochondrial LSU rRNA gene of *Anoplostoma viviparum* (GenBank Accession Number AF317083), and several corresponding sequences of oncholaimoid isolates were previously deposited in GenBank by M. Lange, but so far none of the members of Enoploidea have been sequenced for these genes.

In this study, we obtained a nearly complete sequence of SSU rDNA and a partial sequence of LSU rDNA of *Anoplostoma rectospiculum* Gal'tsova, 1976 and used them in phylogenetic analyses.

Material and methods

Specimens of *Anoplostoma rectospiculum* were collected from the high intertidal zone of Kandalaksha Bay, the White Sea, at the A.N. Pertsov White Sea Biological Station of Moscow State University. The species is very close to *A. viviparum* and is also viviparous. It is abundant in soft sediments of the White Sea intertidal zone (Gal'tsova, 1976).

DNA was isolated with a phenol technique (Sambrook *et al*., 1989) and precipitated with ethanol, otherwise the DIAtom DNA isolation kit was used following the protocol suggested by the manufacturer (Biocom, Moscow, Russia). Nearly complete SSU rDNA and partial LSU rDNA (D3 expansion segment) genes were amplified using universal eukaryotic primers for nuclear SSU rRNA coding regions (Medlin *et al*., 1988; Van der Auwera *et al*., 1994). PCR products were separated on an agarose gel, purified using the QiaGen miniprep system (Germany) and directly sequenced using a set of specific internal primers with an automatic sequencer (ABI Prism 3100-Avant Genetic Analyzer). Alignments were obtained manually according to the predicted SSU rRNA secondary structure scaffold (Wuyts *et al*., 2002). For LSU rDNA data we used a slightly modified D3 expansion segment alignment published elsewhere (Litvaitis *et al*., 2000). The corresponding GenBank accession numbers are given in Figures 1 and 3.

Phylogenetic trees were reconstructed with a number of inference methods, including neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). NJ trees were calculated with the *neighbor* program from the PHYLIP 3.6a2.1 package (Felsenstein, 1993) under the assumptions of the F84 model of molecular substitution (Kishino & Hasegawa, 1989). Series of NJ analyses were conducted with Γ correction (Yang, 1994) or under GTR + Γ + I model (shape = 0,497199, pinvar = 0,17095), the latter using the PAUP* 4.0b10 package (Swofford, 1998). MP analyses were inferred with the PAUP* 4.0b10 package and *dnapars* program from the PHYLIP package. ML searches were conducted with PAUP* under likelihood settings corresponding to the GTR + Γ + I model (nst = 6, shape = 0,497199, pinvar = 0,17095). The model was selected to best fit the data according to results of the likelihood ratio test implemented in ModelTest 3.06 (Posada & Crandall, 1998). Statistic support for phylogenetic nodes was estimated with nonparametric bootstrapping (Felsenstein, 1985) (PAUP settings nrep =

100, AddSeq = random, swap = TBR) and calculation of a posterior probabilities in BI analyses implemented in MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). BI analyses were conducted with six simultaneous runs of Markov chain Monte Carlo (MCMC) algorithm with likelihood settings corresponding to the GTR + Γ + I model for 12 rate categories. The chains were run for 1 000 000 generations and states of the chain before reaching stationarity (20 000 generations) were discarded as burn-in.

In order to register putative molecular synapomorphies, MP searches were conducted with the 'Print sequences at all nodes of tree' option in effect (*dnapars*, PHYLIP). The required topologies were constrained using user-defined tree option of the program. Frequencies of homoplasy for a particular synapomorphy was estimated as amount of homoplastic occurrence of the character in a large dataset of metazoan SSU rDNA sequences (Wuyts *et al*., 2002) available at http://oberon.fvms.ugent.be:8080/rRNA/.

Results

The amplified SSU rRNA gene fragment of *A. rectospiculum* was 1728 bp long (excluding primers) and did not contain extensive indels, which would hamper the alignment procedure. The sequence was deposited in GenBank under accession number AY590149. The analytical dataset assembled included all SSU rDNA sequences of the Enoplia deposited in GenBank. Phylogenetic analysis revealed groupings already inferred by other studies, among them monophyletic Dorylaimia, Triplonchida and Enoplida (Blaxter *et al*., 1998; Rusin *et al.*, 2001).

The sequence of *A. rectospiculum* was placed within the clade of marine Enoplida (Fig. 1). Bootstrap support for the clade with *A. rectospiculum* included ranges from 63% in MP to 98% in ML analyses, whereas the posterior probability was 1.00 in BI analysis.

Distance calculation in NJ analyses showed that *A. rectospiculum* has a highly divergent SSU rDNA amongst other Enoplia. Nevertheless, most analyses converged in grouping this lineage with short-branched *Enoplus* Dujardin, 1845, the sole representative of the family Enoplidae in the set (Fig. 1). Such placement was inferred by 50% bootstrap replicates of NJ analysis, by 61% NJ bootstrap replicates after introducing Γ correction, by 86% bootstrap replicates of ML heuristic search (GTR + f + Γ + I model) and had a posterior probability of 0.99 in BI analyses. The only exception was the MP search, which did not find the common clade for *A. rectospiculum* and *Enoplus*. The clade was not inferred in the most parsimonious tree and was not retained in the majority-rule consensus (it occurred in 29% bootstrap replicates with *dnapars*, PHYLIP and in 24% with PAUP*). In MP analyses, *A. rectospiculum* grouped mainly with Oncholaimidae (67 and 73% bootstrap support in PHYLIP and PAUP* searches, respectively).

Thus, there is an apparent disagreement between the results of MP and other analyses. This may be accounted for by the presence of the long branch attraction artefacts (Felsenstein, 1978). Among other enoplian taxa, only *A. rectospiculum* and Oncholaimidae exhibited long branches in phylogenetic trees (Fig. 1), so their artificial grouping would not be unexpected. To test the results of MP analysis more carefully, we constrained the search to find the problematic clade and re-ran it with the 'Print sequences at all nodes of tree' option in effect (*dnapars*, PHYLIP). Putative synapomorphies of the clade were tested against high variability and all 67 such characters were found to fall in variable regions of the gene and contained high levels of homoplasy (data not shown). Most putative synapomorphies of *A. rectospiculum* and *Enoplus* were also situated at variable sites. However, this clade was also supported by a 1-bp insertion in an internal loop of helix 43 of the SSU rRNA secondary structure, which is more conserved with respect to its length than to its nucleotide composition. This insertion transformed a two base internal loop in 5′ branch of helix 43 into a three base loop (Fig. 2). The loop consists of AU, or more rarely CU, sequence in species outside Enoploidea, while it is ACU in *Enoplus* and *A. rectospiculum*. Thus, the insertion C, or less probably A, residue is a synapomorphy of the these taxa compared to representatives of the other families examined (Fig. 2).

Testing the character against its homoplastic occurrence in a large dataset of metazoan SSU rRNA sequences (http://oberon.fvms.ugent.be:8080/rRNA), including 172 available nematodes, revealed that it was exclusive to *Enoplus*, the only representative of the superfamily Enoploidea available so far. Beyond the Nematoda, the same character was detected in 15 metazoan rRNA sequences out of nearly 1500. All of these belonged to cirripedian crustaceans, which likely reflects its acquisition due to a distinct evolutionary event. Thus, in a dataset of more than 1500 sequences this insertion was fixed no more than twice, in Enoploidea and Cirripedia. The frequency of its homoplastic occurrence is thus very low and independent acquisition of this character in *A. rectospiculum* and *Enoplus* therefore seems unlikely.

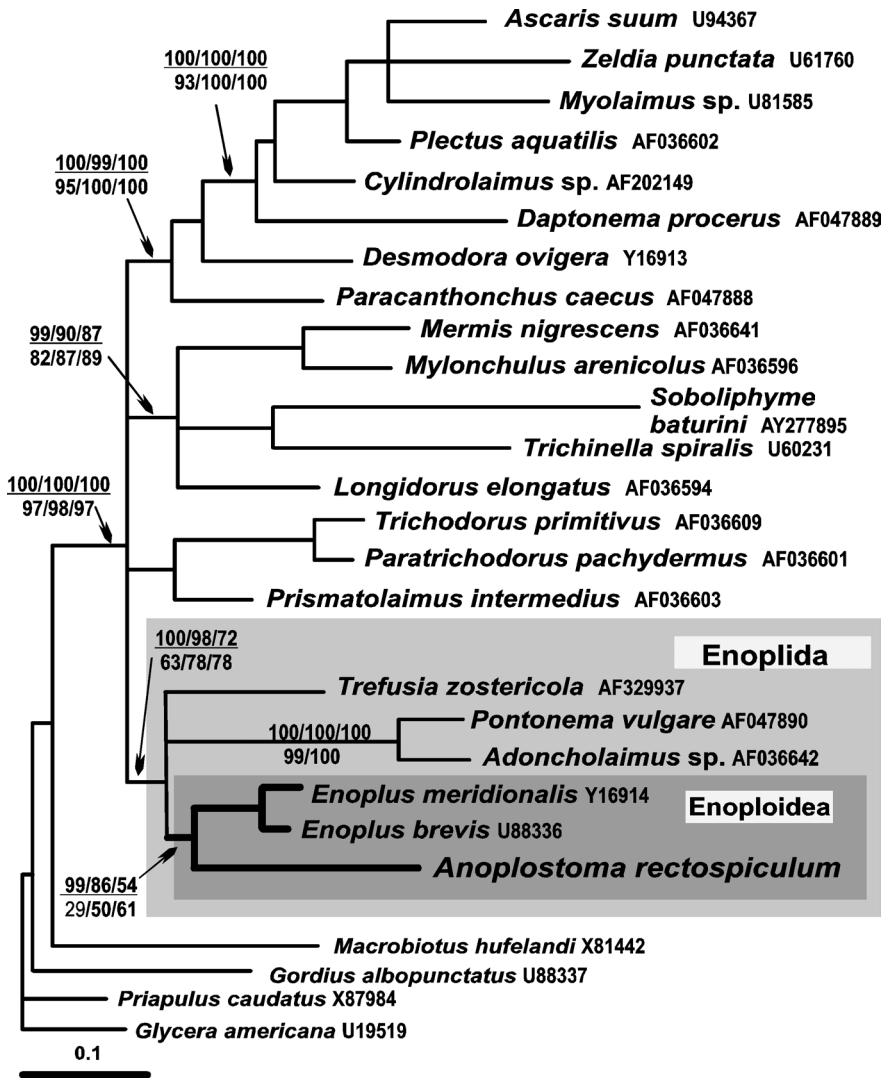

Fig. 1. *Position of the Anoplostomatidae in the SSU rDNA phylogenetic tree of the Nematoda. This is a consensus compiled from outcomes of different inference techniques. Nodes with poor statistical support are collapsed. Branch lengths are ML estimates under GTR + Γ + I model (−Ln L = 18611.96312). Values of statistical support (bootstrap indices and posterior probabilities) are given for selected nodes as follows. Above the bar: BI, ML (GTR + Γ + I), NJ (GTR + Γ + I); below the bar: MP, NJ (F84), NJ (F84 + Γ + I).*

Phylogenies derived from the LSU rDNA D3 segment sequence data were in congruence with those derived from SSU rDNA sequences (Fig. 3). This dataset was more representative with respect to taxonomic sampling of the Enoplida. However, sequences were relatively short, so that after exclusion of ambiguously aligned sites, only 268 residues of the *A. rectospiculum* sequence were processed in the phylogenetic analyses. It is therefore not surprising that many nodes of the tree were poorly resolved in the resulting phylogenies (Fig. 3). This did not apply to the clades of Enoploidea (represented by Enoplidae and Thoracostomopsidae) and Oncholaimoidea (represented by Oncholaimidae and Enchelidiidae), which were strongly supported (*cf.* Litvaitis *et al.*, 2000). These results could not be directly compared with SSU rDNA phylogenies, which did not include Thoracostomopsidae and Enchelidiidae, but provided a good source of alternative evidence. ML, BI, MP and NJ consensus topologies in analyses of the D3 expansion segment sequence data retained *A. rectospiculum* within the superfamily Enoploidea with

outgroup	
Glycera americana U19519	CTCTAGCCTATTAAA-TAGTTCGCCGAT
Priapulus caudatus X87984	CTCTGGCCTACTAAA-TAGTGAGCCGAT
Macrobiotus hufelandi X81442	CTCTAGCCTGCTAAA-TAGCCAACTGAT
Gordius albopunctatus U88337	CTCTAACCTACTTAC-TAGAACGTCGAT
Enoplida	
Alaimus acutus AY146468	CTCTAGCCTATTAAA-TAGTGGCTGAAT
Campydora demonstrans AY146471	CTCTAGCCTATTAAA-TAGAATGCTAAT
Ironus sp. AY146467	CTCTAGCCTACTAAC-TAGTGAATAGAT
Trefusia zostericola AF329937	CTCTrGCCTATTAAA-TAGTCGGyAGAT
Adoncholaimus sp. AF036642	CTCTATCTTGCTAAC-TAGTC-GCAGAT
Pontonema vulgare AF047890	CTCTATCTTGCTAAC-TAGGC-GCAGAT
Enoplus brevis U88336	CTCTAGCCTACTAAAC TAGGCAGCAAAT
Enoplus meridionalis Y16914	CTCTAGCCTACTAAAC TAGGCAGTAGAT
Anoplostoma rectospiculum	CTCTAGCCTATTAAAC TAGACTGATAAT
other Nematoda	
Trichodorus primitivus AF036609	CTsTAGCCTACTAAA-TAGACAGTACTT
Paratrichodorus pachydermus AF036601	CTCTAGCCTACTAAA-TAGACAACACTT
Prismatolaimus intermedius AF036603	CTCTAGCCTGCTAAA-TAGGCAGCGGAT
Longidorus elongatus AF036594	CTCTGGCCTATTAAA-TAGCCGGTATAT
Mermis nigrescens AF036641	CTCTAGCCTATTAAA-TAGACGCGATAT
Mylonchulus arenicolus AF036596	CTCTAGCCTATTAAA-TAGACGATATAT
Trichinella spiralis U60231	CTCTACCCTATTAAA-TAGTGACAGTAT
Soboliphyme baturini	CTCTGCCGTATTAAA-TAGTGAATGTCC
Paracanthonchus caecus AF047888	CTCTAGCCTACTAAC-TAGTGGGTGGAT
Desmodora ovigera Y16913	CTCTAGCCTGCTAAA-TAGTCTACAGAT
Daptonema procerus AF047889	CTCCAACCTACTAAC-TAGTGGGCGAAT
Cylindrolaimus sp. AF202149	CTCTAGCCTACTAAA-TAGTTGGCGGAT
Plectus aquatilis AF036602	CTCTGGCCTACTAAA-TAGTGACAGGAT
Ascaris suum U94367	CTCTAGCCTATTAAA-TAGTCATCGGAT
Myolaimus sp. U81585	CTCTAGCTTATTAAA-TAGATGCCGGAT
Zeldia punctata U61760	CTCTAGCCTACTAAA-TAGTAACGAGAT

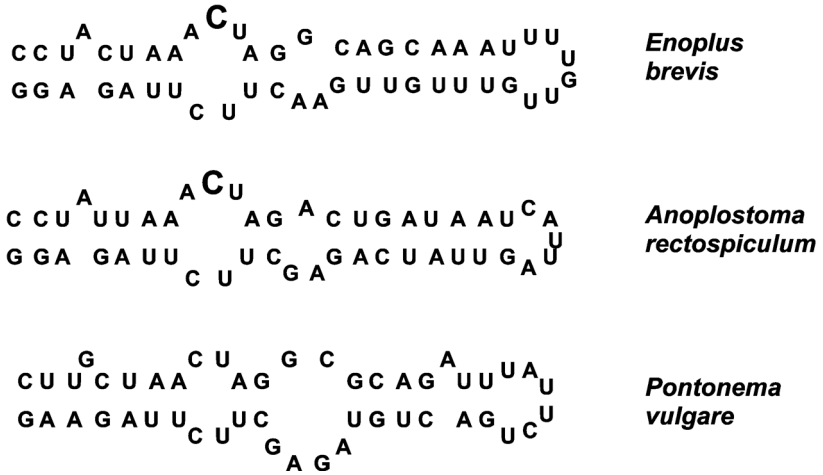

Fig. 2. *Molecular synapomorphy of the superfamily Enoploidea in the region of helix 43 of the SSU rRNA. The single-bp insertion is shaded grey on the alignment fragment and set in large print on the corresponding molecular secondary structure scaffold.*

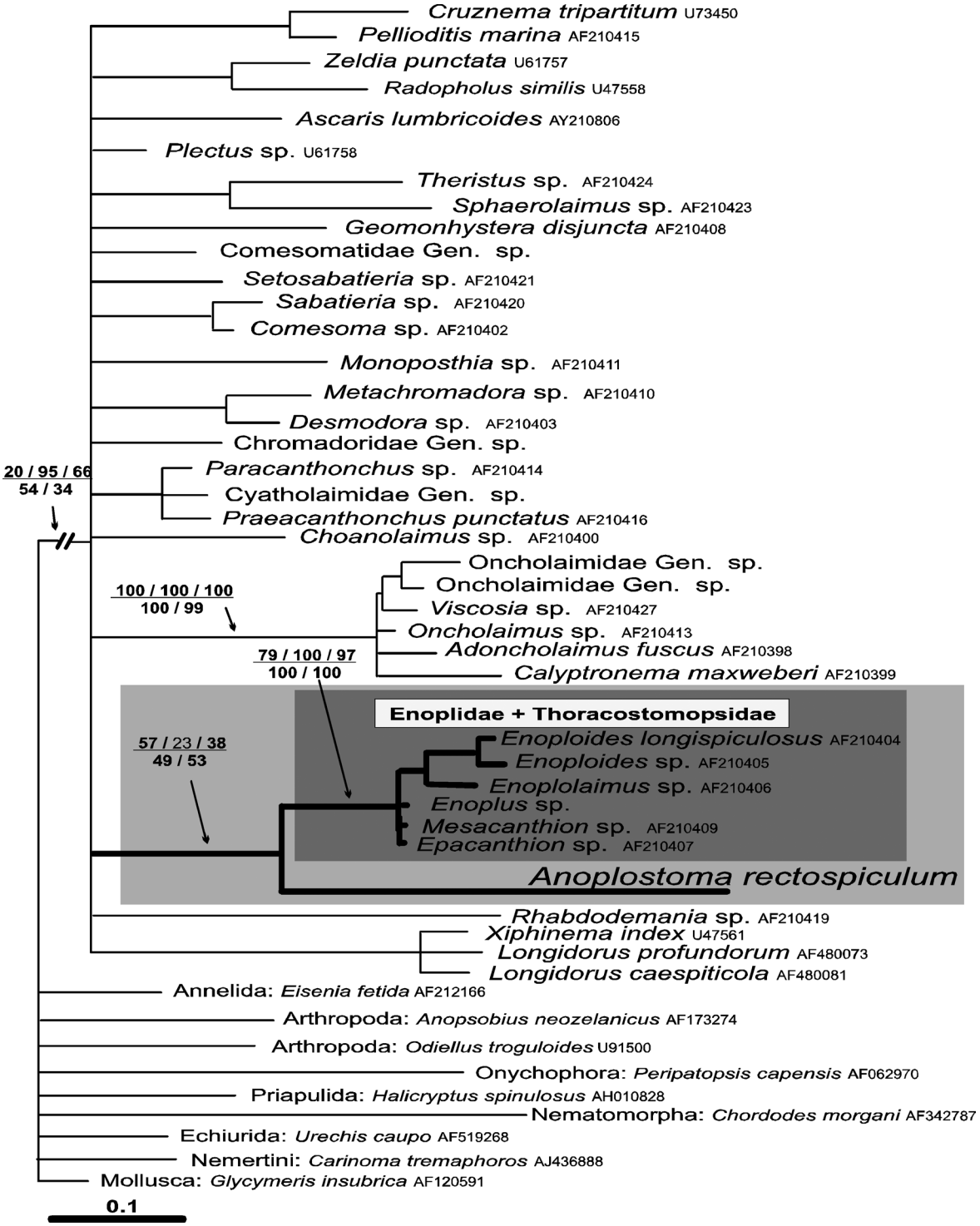

Fig. 3. *Position of the Anoplostomatidae in the LSU rDNA tree of the Nematoda. This topology is a consensus compiled from 50 ML replicates (GTR + Γ + I, AddSeq = random, NReps = 5, Hold = 5). Branch lengths are ML estimates under GTR + Γ + I model (−Ln L = 4116.99516). Values of statistical support (bootstrap indices and posterior probabilities) are given for selected nodes as follows. Above the bar: ML, BI, MP; below the bar: NJ, NJ (Γ + I).*

moderate values of statistical support (Fig. 3). Alternatively, grouping of *A. rectospiculum* with Oncholaimoidea was inferred in 1.0% and 0.1% NJ and MP bootstrapped trees, respectively, producing a posterior probability of 0.01 in Bayesian inference, and was not observed in 50 ML replicates. This suggests that the hypothesis of placing Anoplostomatidae within Oncholaimoidea should be rejected.

Discussion

Distance and ML analyses of the SSU rDNA and the LSU rDNA D3 sequence data, as well as low homoplastic molecular synapomorphies found in conservative sites of the SSU rRNA gene, support the membership of *A. rectospiculum* in the marine Enoplida clade, and, more particularly, support the monophyly of *Anoplostoma*, Enoplidae and Thoracostomopsidae with respect to Oncholaimoidea, Rhabdodemaniidae and *Trefusia* de Man, 1893 (Tripyloididea: Trefusiidae). Introducing Γ approximation to the inference procedure definitely increased the statistical reliability of this hypothesis. Alternative placing of *Anoplostoma* in MP analyses exhibits apparent signs of computational biases caused by the long branch attraction artefact (Felsenstein, 1978) and thus should not be given weight. The hypothesis of closer relationships between *Anoplostoma* and Oncholaimoidea does not, therefore, gain reliable support and should be rejected.

Very little molecular evidence is so far available on representatives of Enoplida. At first sight, it reduces the credibility of the monophyly of Anoplostomatidae, Enoplidae and Thoracostomopsidae within the context of Enoplida, but many other families remain unsampled. However, the presence of an indisputable molecular synapomorphy in the region of helix 43 of the SSU rRNA shared by *Anoplostoma* and *Enoplus* attests to their monophyly with respect to the superfamilies Oncholaimoidea and Tripyloidida noted above, and to the families Alaimidae, Campydoridae, and Ironidae, which retain a plesiomorphic state of the character as judged from partial SSU rDNA sequences (Mullin *et al*., 2003). It provides a strong argument against poor taxon sampling being responsible for the grouping of *Anoplostoma* and *Enoplus*. On the other hand, delimitation of this clade and speculation on its internal systematic structure seems premature.

Molecular evidence supports the conventional view that there are taxa other than Anoplostomatidae which are more closely related to Enoplidae, such as Thoracostomopsidae (Litvaitis *et al*., 2000) and some other groups (unpubl.). Establishing the closest relatives of Anoplostomatidae is a matter for future research. Morphological classifications have united *Anoplostoma* with *Anticoma* Bastian, 1865 (Bütschli, 1874; de Man, 1907; Filipjev, 1918; Belogurov & Alekseev, 1977), *Chaetonema* Filipjev, 1927 (Lorenzen, 1981), or *Pandolaimus* Allgén, 1929 (Jensen, 1976), although molecular evidence on these genera is currently lacking.

Monophyly of morphologically distinct Anoplostomatidae and Enoplidae illustrates the phylogenetic isolation of Oncholaimoidea and Enoploidea and favours the concept of them as being two separate suborders (Lorenzen, 1981; De Ley & Blaxter, 2002). Apart from establishing a set of separate clades within the order Enoplida (corresponding to superfamilies Enoploidea, Oncholaimoidea, as well as the families Alaimidae, Campydoridae, Ironidae, Rhabdodemaniidae, and Trefusiidae), the available data are insufficient to establish their early phylogeny. More extensive taxonomic sampling is required for this purpose. Speculating on patterns of morphological evolution of the order Enoplida is therefore still premature without phylogenetic data based on independent molecular data.

This case study of enoplid relationships shows the remarkable paucity of strong molecular signatures fixed during molecular evolution of ribosomal RNA in large nematode taxa. In previous studies, the order Enoplida was characterised by the presence of only two, low homoplastic, characters in the structure of SSU rDNA, that could be used both as a universal molecular diagnostic and also for the purposes of selective primer and probe design (Rusin *et al*., 2001).

In this study, we identified a molecular signature of the superfamily Enoploidea (Fig. 2). Its high specificity has been verified using a larger set of unpublished SSU rDNA sequences of the Enoplida (L.Yu. Rusin, pers. comm.).

Additional phylogenetic markers supporting the monophyly of a common clade for Anoplostomatidae and Enoplidae also exist in highly variable regions of the 18S rRNA gene. These markers are detected by model-based algorithms of ML analyses, which robustly reconstruct this group. It appears that in the case of nematode 18S rRNA, reliable phylogeny reconstruction requires sophisticated approaches able to detect both multiple weak (high homoplastic) and scattered strong (low homoplastic) molecular synapomorphies in the gene structure.

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