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Evolutionary relationships of Spirurina (Nematoda: Chromadorea: Rhabditida) with special emphasis on dracunculoid nematodes inferred from SSU rRNA gene sequences ☆

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Abstract

The analysis of 26 new small subunit rRNA sequences obtained from helminths that primarily parasitize fishes sampled from five continents provided well-supported trees, allowing us to study the phylogenetic relationships among spirurid nematodes. The analyses have shown that Dracunculoidea is a paraphyletic taxon and Anguillicolidae and Gnathostomatidae constitute the basal branch of the suborder Spirurina. The genera Philometra and Philometroides appear to be paraphyletic, while on the higher taxonomic level, good correlation between the morphology-based system and molecular data was observed. Neither co-evolution of the studied helminths with their hosts, nor phylogeographic pattern, are apparent in our dataset.
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1. Introduction

Nematodes, which are typically considered a phylum, represent an extremely species-rich group. However, from the morphological perspective, they constitute a trait-poor group due to a life-style that seems to preclude appendage evolution and cephalisation in both free-living and parasitic species (Blaxter, 2003). The current nematode taxonomy is thus primarily based on the morphology of the oesophagus, male and female reproductive organs and life-cycle patterns (Chabaud, 1975a,b; Anderson and Bain, 1976). An initial framework for the molecular phylogeny of nematodes, based on the small subunit (SSU) rRNA dataset (Blaxter et al., 1998; Dorris et al., 1999), was for a handful of taxa complemented by the sequencing of other conserved genes, such as the large subunit rRNA, ITS region or elongation factor 1 (Gasser and Newton, 2000; Chilton et al., 2001). Although the recent dataset of nematode SSU rRNA genes comprises more than 300 taxa (Blaxter, 2003), it remains strongly biased towards some groups, while sampling remains poor for several other important groups. One of the underrepresented groups is the spirurine nematodes, an order/suborder of obligatory parasites that includes helminths of cold- and warm-blooded terrestrial and aquatic vertebrates, including humans (Chabaud, 1975a,b; Anderson and Bain, 1976).

Opinions about the phylogenetic relationships among the main groups of Spirurida in Anderson's conception (2000) have been developing gradually, which is reflected in taxonomic systems proposed by different authors (for a recent review see De Ley and Blaxter, 2002). Spirurida was assigned the rank of an order by Chitwood (1933) (subclass Secernentea, class Nematoda in his system), being later subdivided into the suborders Camallanina and Spirurina (Chabaud, 1974). The former suborder is comprised of the superfamilies Camallanoidea and Dracunculoidea,

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whereas the suborder Spirurina represented a group of 10 superfamilies containing hundreds of named species (Chabaud, 1974). Several classifications, all based on morphology and life-cycles, have been created by Chitwood (1933, 1950), Yamaguti (1961), Ivashkin et al. (1971) and Chabaud (1974, 1975a). In the most recent classification systems, based on morphology and biology, Spirurida appeared as an order consisting of 28 families (Moravec et al., 1998; Anderson, 2000). However, these nematodes are classified within a newly defined suborder Spirurina that is placed in Rhabditida, Chromadorea, in the recent classification system based on molecular data (De Ley and Blaxter, 2002). In contrast to previous systems, Spirurina in this conception (subdivided into infraorders Ascaridomorpha, Gnathostomatomorpha, Oxyuridomorpha, Rhigonematomorpha and Spiruromorpha, with Dracunculoidea as *incertae sedis*) is much broader, also including former Ascaridida, Oxyurida and Rhigonematida.

In the SSU rRNA-based trees published so far (Blaxter et al., 1998; Blaxter, 2003), the spirurine clade is robustly supported. This finding is complicated by the lack of any sequences from several key representatives, which obscures the relationships within the clade and with other clades. Therefore, high expectations are placed on molecular phylogeny. Despite the paramount importance of some spirurines for human health, this approach has not been used because of the apparent lack of interest among molecular biologists in these organisms (Lukeš et al., 2005). We have undertaken sequence analysis of the SSU rRNA gene from members of major groups within this zooparasitic taxon. The obtained dataset enabled us not only to examine phylogenetic relationships among spirurines and other nematodes but also to address interesting problems, such as: (i) what is the ancestral invasion strategy of the spirurines? (ii) Did taxa with two-host life-cycles evolve from the base of the group? (iii) What is the pattern of definitive and/or intermediate host usage? (iv) Is there a phylogeographic correlation or co-evolution with the host? Furthermore, we propose the incorporation of the SSU rRNA-based phylogenies into a comprehensive classification.

2. Materials and methods

2.1. Organisms, DNA extraction and PCR

Taxa sampled for phylogenetic analysis are listed in Table 1 and taxa for which sequences have been retrieved from GenBank are listed in Table 2. Most specimens were stored in 70% ethanol for between several days up to a decade prior to DNA isolation (Table 1). Genomic DNA was isolated using the Jetquick Tissue DNA kit (Genomed). About 10–50 ng of genomic DNA was used for PCR amplification of the SSU rRNA gene using oligonucleotide primers D-1F (GCCTATAATGGTGAAACCGCGAAC) and D-1R (CCGGTTCA AGCCACTGCGATTA) and 1 U Taq Purple polymerase (Top-Bio). PCR was performed under the following conditions: 6 cycles of 95 °C for 1 min, 44 °C for 1 min and 72 °C for 2 min followed by 24 cycles with the annealing temperature increased to 48 °C. The amplicons of expected size were gel-purified using the Jetquick gel extraction kit (Genomed) and cloned into the Topo TA Cloning Vector (Invitrogen). Both strands were sequenced using a Beckman Coulter automatic sequencer. Internal oligonucleotides designed to match the conserved regions were used to complete the sequences, which have been assembled with Seqman (DNAStar).

2.2. Phylogenetic analysis

Dataset A was created from 29 SSU rRNA sequences of species listed in Tables 1 and 2. *Plectus aquatilis* (Plectidae) and *Teratocephalus lirellus* (Teratocephalidae) were chosen as outgroups according to De Ley and Blaxter (2002). For datasets B and C, enriched for sequences of Camallanidae and Philometridae, respectively, different outgroups selected based on the analyses of dataset A have been used (see Section 3 for details). In order to include the partial philometrid SSU rRNA sequences available in GenBank for a number of philometrids, an additional ~900 bp-long dataset D was constructed containing 18 specimens of Philometridae and was rooted with *Dracunculus* spp. Multiple alignments of different transition/transversion (Ts/Tv) weights (1:1/2/3/5) were created with Clustal X 1.83 (Thompson et al., 1997) and further edited using BioEdit 7.0.4.1 (Hall, 1999) for all datasets. Ambiguously aligned regions and gaps were removed either with Bioedit or Gblocks software (Castresana, 2000) prior to the analyses. Maximum likelihood trees were calculated from all the datasets and Ts/Tv setting under the GTR+Γ_4+I model of evolution using PHYML 2.4.4 (Guindon and Gascuel, 2003); gamma shape parameter and proportion of invariants (PINVAR) were estimated from the dataset (see figure captions for actual values). The model of evolution was chosen according to the Akaike criterion as implemented in Modeltest 3.7 (Posada and Crandall, 1998). Maximum likelihood (ML) and maximum parsimony (MP) bootstrap support values were calculated by PHYML 2.4.4 and PAUP*4.0b10 (Swofford, 2002) from 1000 replications. Bayesian posterior probabilities were assessed under the above described model with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) software (Bayesian inference—BI), where the Markov chain was set to 2×10^6 generations, every 100th tree was sampled and the first 10^5 generations were omitted from phylogeny reconstruction. Additional LogDet analysis of all alignments of dataset A, run using PAUP*4.0b10, was performed to unmask possible cases of the long-branch attraction phenomenon in topologies obtained with the above-mentioned methods.

3. Results

Twenty-six new SSU rRNA sequences of nematodes (ranging from 1642 to 1787 bp) obtained in this study were complemented with 23 sequences obtained from GenBank.

Table 1
Information on helminths, from which the small subunit rRNA gene was sequenced within the frame of this study

Species name	Host/Family		Locality	Traditional classification
Alinema amazonicum (Travassos, 1960)	*Callophysus macropterus* (Pimelodidae)	Freshwater fish	Peru	Dracunculoidea
Anguillicola crassus (Kuwahara, Niimi, and Itagaki, 1974)	*Anguilla anguilla* (Anguillidae)	Freshwater fish	Czech Republic	Dracunculoidea
Camallanus cotti (Fujita, 1927)	*Awaous guamensis* (Gobiidae)	Freshwater fish	New Caledonia	Camallanoidea
Camallanus lacustris (Zoega, 1776)	*Sander lucioperca* (Percidae)	Freshwater fish	Czech Republic	Camallanoidea
Camallanus carangis (Olsen, 1954)	*Upeneus vittatus* (Mullidae)	Marine fish	New Caledonia	Camallanoidea
Dentiphilometra sp.	*Lutjanus griseus* (Lutjanidae)	Marine fish	Mexico	Dracunculoidea
Dracunculus medinensis (Linnaeus, 1758)	*Homo sapiens* (Hominidae)	Mammal	Ghana	Dracunculoidea
Dracunculus oesophageus (Polonio, 1859)	*Natrix natrix* (Colubridae)	Reptile	Slovakia	Dracunculoidea
Micropleura australiensis (Moravec, Kay et Hobbs, 2004)	*Crocodylus johnsoni* (Crocodylidae)	Reptile	Australia	Dracunculoidea
Molnaria intestinalis (Dogiel et Bychowsky, 1934)	*Scardinius erythrophthalmus* (Cyprinidae)	Freshwater fish	Czech Republic	Dracunculoidea
Neoascarophis macrouri (Moravec, Klimpel et Kara, 2006)	*Macrourus berglax* (Macrouridae)	Marine fish	Greenland	Habronematoidea
Nilonema senticosum (Baylis, 1927)	*Arapaima gigas* (Arapaimidae)	Freshwater fish	Peru	Dracunculoidea
Philometra obturans (Prenant, 1886)	*Esox lucius* (Esocidae)	Freshwater fish	Czech Republic	Dracunculoidea
Philometra cyprinirutili (Creplin, 1825)	*Abramis brama* (Cyprinidae)	Freshwater fish	Czech Republic	Dracunculoidea
Philometra ovata (Zeder, 1803)	*Gobio gobio* (Cyprinidae)	Freshwater fish	Czech Republic	Dracunculoidea
Philometra lateolabracis (Yamaguti, 1935)	*Argyrosomus japonicus* (Sciaenidae)	Marine fish	Australia	Dracunculoidea
Philometroides sanguineus (Rudolphi, 1819)	*Carassius carassius* (Cyprinidae)	Freshwater fish	England	Dracunculoidea
Philonema oncorhynchi (Kuitunen-Ekbaum, 1933)	*Oncorhynchus kisutch* (Salmonidae)	Freshwater fish	Canada	Dracunculoidea
Procamallanus (*Procamallanus*) *pacificus* (Moravec, Justine, Würtz, Taraschewski et Sasal, 2006)	*Anguilla obscura* (Anguillidae)	Freshwater fish	New Caledonia	Camallanoidea
Procamallanus (*Spirocamallanus*) *pintoi* (Kohn et Fernandes, 1988)	*Corydoras atropersonatus* (Callichthyidae)	Freshwater fish	Peru	Camallanoidea
Procamallanus (*Spirocamallanus*) *rarus* (Travassos, Artigas et Pereira, 1928)	*Aguarunichthys* cf. *tocantinsensis* (Pimelodidae)	Freshwater fish	Peru	Camallanoidea
Procamallanus (*Spirocamallanus*) *rebecae* (Andrade-Salas, Pineda-López et García-Magaña, 1994)	*Cichlasoma meeki* (Cichlidae)	Freshwater fish	Mexico	Camallanoidea
Rhabdochona denudata (Dujardin, 1845)	*Leuciscus cephalus* (Cyprinidae)	Freshwater fish	Czech Republic	Thelazioidea
Rondonia rondoni (Travassos, 1920)	*Pterodoras granulosus* (Doradidae)	Freshwater fish	Peru	Oxyuroidea
Skrjabillanus scardinii (Molnár, 1966)	*Scardinius erythrophthalmus* (Cyprinidae)	Freshwater fish	Czech Republic	Dracunculoidea
Terranova scoliodontis (Baylis, 1931)	*Galeocerdo cuvier* (Carcharhinidae)	Shark	New Caledonia	Ascaridoidea

Since we focused on analysis of both inter- and intrafamiliar relationships, the resulting tree suffered from low bootstrap support when all the sequences pooled together (data not shown). Therefore, we have constructed dataset A dedicated to the suprafamiliar analysis with ambiguously aligned parts removed. To infer relationships on a lower taxonomic level, datasets B and C have been constructed, dedicated to analyses confined to Philometridae and Camallanidae, respectively. Sequences were not trimmed in these alignments.

Based on the current view of the nematode phylogeny derived from the SSU rRNA gene (De Ley and Blaxter, 2002), we began the analysis with rooting dataset A with the plectidid *P. aquatilis* and the teratocephalean *T. lirellus*. This combination of outgroups gave us, in comparison with other outgroups, the most parsimonious phylogeny, i.e. the shortest trees (data not shown). The use of alternative taxa for rooting the dataset A, such as the strongylid *Skrjabingylus chitwoodorum* and/or the monhysterid *Daptonema procerus*, did not change the internal branching, indicating a correct choice of the outgroups. Despite some differences in the support values for particular nodes in the respective trees, all four methods used for phylogeny reconstruction (ML, MP, BI and LogDet) returned almost identical and well-supported trees. The tested Ts/Tv weights gave very similar topology that differed mostly in the statistical support of some nodes. The only exception in this respect is the position of *Anguillicola crassus* in the MP and ML trees with Ts/Tv set at 1:1 and 1:5, respectively, where *Gnathostoma* spp. branch off on the base of the tree and *A. crassus* forms a sister group to all the other taxa (data not shown).

The general consensus speaks for a topology shown in Fig. 1a, where Gnathostomatomorpha (represented by *Gnathostoma turgidum* and *Gnathostoma binucleatum*) and the dracunculoid *A. crassus* constitute a common but poorly supported branch as a sister group to the remaining spirurines. As a result of this position of *A. crassus*, an

Table 2
List of taxa, from which the sequence of small subunit rRNA gene was retrieved from GenBank™

Species name	Host	Traditional classification	GenBank accession number
Anisakis sp.	Mammal	Ascaridoidea	**U94365**
Ascaris lumbricoides (Linnaeus, 1758)	Mammal	Ascaridoidea	**U94366**
Ascarophis arctica (Polyansky, 1952)	Fish	Habronematoidea	**DQ094172**
Brugia malayi (Brug, 1927)	Mammal	Filaroidea	**AF036588**
Brumptaemilius justini (Adamson et Anderson, 1985)	Diplopod	Rhigonematoidea	**AF036589**
Clavinema parasiluri (Yamaguti, 1935)	Fish	Dracunculoidea	**DQ076682**
Dentiphilometra monopteri (Moravec et Wang, 2002)	Fish	Dracunculoidea	**DQ076685**
Dracunculus insignis (Leidy, 1858)	Mammal	Dracunculoidea	**AY947719**
Gnathostoma binucleatum (Almeyda-Artigas, 1991)	Mammal	Gnathostomatoidea	**Z96946**
Gnathostoma turgidum (Stossich, 1902)	Mammal	Gnathostomatoidea	**U96948**
Philometra clavaeceps (Dogiel et Akhmerov, 1959)	Fish	Dracunculoidea	**DQ076686**
Philometra (=Clavinema) fujimotoi (Furuyama, 1932)	Fish	Dracunculoidea	**DQ076680**
Philometroides bulbosus (Blaylock et Overstreet, 1999)	Fish	Dracunculoidea	**AB185161**
Philometroides carassii (Ishii, 1933)	Fish	Dracunculoidea	**DQ076683**
Philometroides cyprini (Ishii, 1931)	Fish	Dracunculoidea	**DQ076688**
Philometroides fulvidraconi (Yu, Wu et Wang, 1993)	Fish	Dracunculoidea	**DQ076684**
Philometroides ganzhounensis (Yu, 1998)	Fish	Dracunculoidea	**DQ076681**
Philometroides pseudorasbori (Wang, Yu et Wu, 1995)	Fish	Dracunculoidea	**DQ076687**
Physaloptera alata (Rudolphi, 1819)	Mammal	Physalopteroidea	**AY702703**
Plectus aquatilis (Andrássy, 1985)	Free living	Plectoidea	**AF036602**
Serratospiculum tendo (Nitzsch, 1857)	Bird	Diplotriaenoidea	**AY702704**
Teratocephalus lirellus (Anderson, 1969)	Free living	Araeolaimida	**AF036607**
Wuchereria bancrofti (Cobbold, 1877)	Mammal	Filaroidea	**AF227234**

Bold format denotes taxa used for construction of the dataset D (Fig. 2).

economically important parasite of eels, Dracunculoidea are paraphyletic in all our analyses. *Rondonia rondoni* (family Atractidae), a parasite of neotropical fishes (Moravec, 1998) and the only representative of Oxyuridomorpha in our dataset, branches at the base of all remaining spirurids. The common origin of Rhigonematomorpha (represented by *Brumptaemilius justini*) and Ascaridomorpha (represented by *Ascaris lumbricoides*, *Anisakis* sp. and *Terranova scoliodontis*) is highly supported, with the latter group being monophyletic. This tree returns Spiruromorpha as a paraphyletic assembly, in which the families Physalopteridae and Onchocercidae are grouped together with very robust internal branching. The only clade supported with less than 65% bootstrap is that of *Physaloptera alata* (Fig. 1a). However, the spiruromorphid family Camallanidae (represented by *Camallanus* sp., *Procamallanus pacificus* and *Procamallanus rarus*) appears in a common and well-supported clade with the Dracunculoidea.

This clade brings together the families Philometridae, Dracunculidae, Micropleuridae, Skrjabillanidae and Camallanidae (Fig. 1a). This grouping is in good agreement with the morphology-based taxonomy, since with the above-mentioned exception of Camallanidae, all families belong to the superfamily Dracunculoidea. Moreover, each family appears monophyletic in our dataset. The only exception is *Philonema oncorhynchi*, so far affiliated with the Philometridae, which joins the Skrjabillanidae. The family Micropleuridae, represented by *Micropleura australiensis*, appears either at the base of the Philometridae–Dracunculidae clade (Fig. 1a) or with the Ts:Tv ratio set to 1:1 constitute a sister group of the Philometridae (data not shown).

We have constructed additional datasets B and C and performed detailed analyses of Philometridae and Camallanidae with *Dracunculus* spp. and *P. oncorhynchi* used as the respective outgroups (Figs. 1b and c). Analysis of the Camallanidae dataset revealed a poorly supported tree (Fig. 1c) indicating that a finer structuring within this group is below the resolution power of SSU rRNA. Other, more variable phylogenetic markers have to be employed to solve this problem. In Philometridae, however, we were able to obtain fully resolved topology (Fig. 1b). The South American clade *Alinema–Nilonema* separated from the rest of the family, with high bootstrap support for this basal position. The genera *Philometra* and *Philometroides* represented by four and two species, respectively, became paraphyletic. It should be noted that the genetic distance between, e.g. *Philometra* sp. and *Philometra ovata*, despite their accommodation in the same genus, significantly exceeds the intergeneric distance between *Alinema amazonicum* and *Nilonema senticosum* (Fig. 1b).

In order to better address questions concerning host and geographic specificity of the philometrid helminths, we have trimmed our alignment down to about 900 bp and rooted it with *Dracunculus* spp. (Fig. 2). This enabled us to extend the dataset by the addition of partial SSU rRNA sequences available in the GenBank for philometrids isolated from fishes in China (Wu et al., 2005). Despite the reduction of the informational content, we were still able to obtain quite a robust tree, which further supported conclusions derived from a narrower dataset (Fig. 1b). In particular, all three genera represented by more than one species (*Philometra*, *Dentiphilometra* and *Philometroides*) are paraphyletic. By superimposing the fish hosts of these

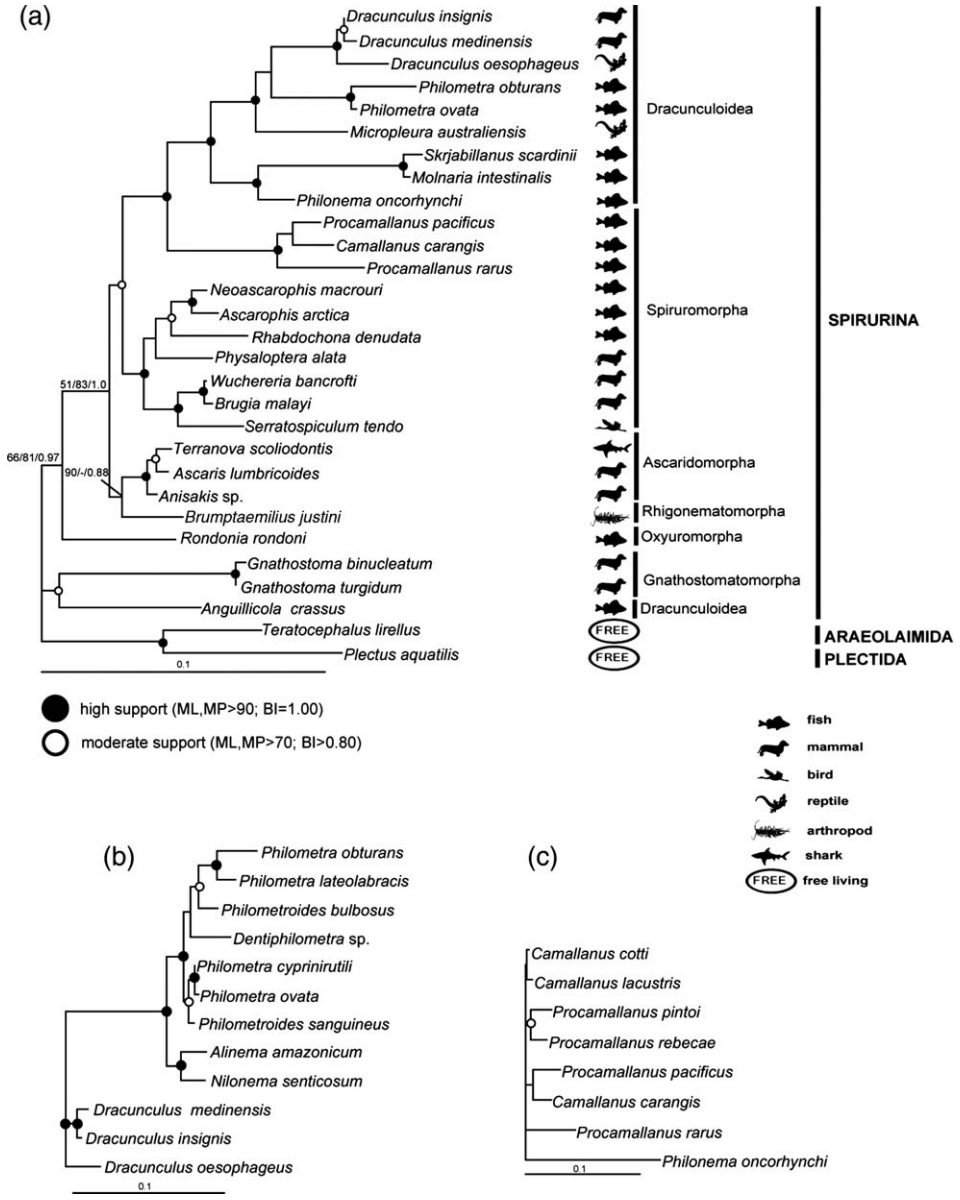

Fig. 1. Phylogeny of Spirurina sensu De Ley and Blaxter (2002) (a), Philometridae (b) and Camallanidae (c) based on phylogenetic analyses of the SSU rRNA sequences. Maximum likelihood gamma corrected trees were calculated under the GTR+Γ_4+I model of evolution as implemented in PHYML 2.4.4. Maximum likelihood and maximum parsimony bootstrap support values were calculated with PHYML 2.4.4 and PAUP*4.0b10 from 1000 replications. Bayesian posterior probabilities were assessed under the above described model with MrBayes 3.0b4 (mcmc=2×10^6 generations, of which the first 10^5 were omitted from tree reconstruction). All values stated bellow apply to the alignments with Ts/Tv set to 1:3. (a) Tree was constructed from dataset A (Ts/Tv 1:3) from 1601 characters (309 parsimony-informative). Loglk=−7872.26900, gamma shape parameter=0.588, PINVAR=0.521. Icons on the right side of the tree represent final hosts (see descriptions below). (b) Strict consensus tree constructed from dataset B (all Ts/Tv weights) from 1654 characters (116 parsimony-informative). Loglk=−4030.08070, gamma shape parameter=0.745, PINVAR=0.622. (c) Strict consensus tree constructed from dataset C (all Ts/Tv weights) from 1690 characters (59 parsimony-informative). Loglk=−3887.47902, gamma shape parameter=0.618, PINVAR=0.709.

philometrids in the tree, we have demonstrated that there is no strong co-evolution of these parasites with their hosts and that no phylogeographic pattern could be retrieved (Fig. 2).

4. Discussion

Our study provides new molecular data for the detailed analysis of relationships within the suborder Spirurina, as defined by De Ley and Blaxter (2002). We have sequenced the SSU rRNA gene of 26 species sampled from five continents. The set was biased towards parasites of fishes and thus centred to the superfamilies Camallanoidea, Dracunculoidea, Thelazioidea, Gnathostomatoidea, Habronematoidea and Physalopteroidea.

In an extensive SSU rRNA-based framework, Spiruromorpha has been linked with Ascaridomorpha and Rhigonematomorpha, whereas Oxyuromorpha represented the

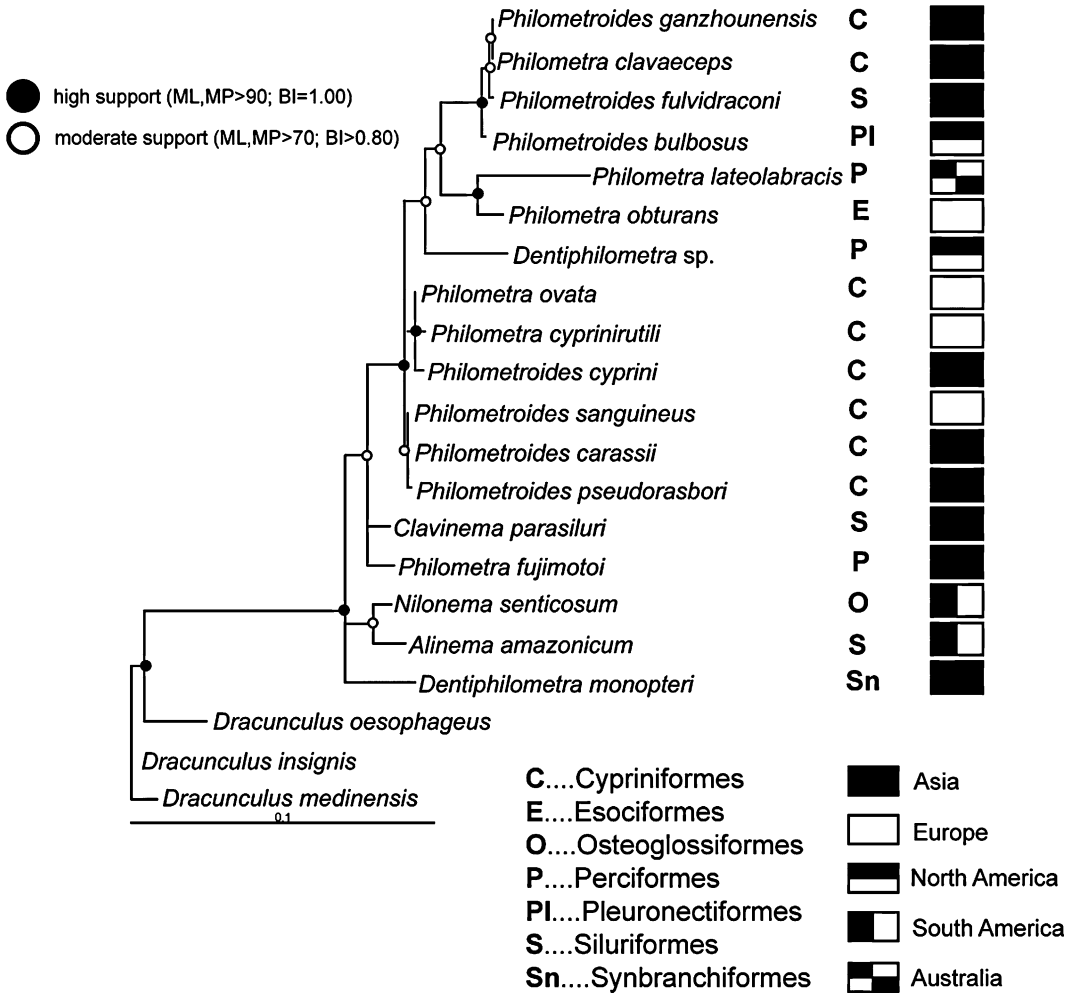

Fig. 2. Phylogeny of Philometridae based on truncated SSU rRNA sequences (dataset D). Strict consensus maximum likelihood gamma corrected tree calculated from dataset D (all Ts/Tv weights) from 853 characters (89 parsimony-informative) under the GTR+Γ_4+I model of evolution as implemented in PHYML 2.4.4. Loglk=−2516.05743, gamma shape parameter=0.686, PINVAR=0.639. All values stated above apply to the alignment with Ts/Tv set to 1:3. See Fig. 1. caption for detailed explanation of statistical support. Letters on the right side of the tree stand for the fish host's taxonomic position and icons depict geographic origins.

earliest branch of this clade (Blaxter et al., 1998; De Ley and Blaxter, 2002). However, a comparative analysis of the mitochondrial genomes provided contrasting results. On the basis of mitochondrial gene arrangement and sequence, a well-supported sister relationship between Ascaridomorpha and Rhabditomorpha (reported as Ascaridida and Rhabditida) has been detected, with the ascarid–spirurid affinity being strongly rejected (Hu et al., 2003; Kim et al., 2006). We can only speculate about reasons behind the striking disparity between the nematode phylogenies based on the nuclear and mitochondrial genes, although there is precedent for this among other eukaryotes (Hey, 1997; Shaw, 2002; Meyer and Zardoya, 2003). Our phylogenetic analysis unambiguously confirmed major relationships previously established from the SSU rRNA dataset by Blaxter and colleagues (Blaxter et al., 1998; De Ley and Blaxter, 2002; Blaxter, 2003). Moreover, based on the resulting extended dataset, we attempted to resolve relationships below the order level.

One of the most surprising results of our study is the distant location of *A. crassus* from the Dracunculoidea and other spirurid superfamilies, showing instead a relationship with Gnathostomatomorpha at the base of the spirurid tree. This reveals that in its current conception, the Dracunculoidea is paraphyletic and that *Anguillicola*, based on our data, should be removed from this superfamily. The genus *Anguillicola* should be evaluated within an independent superfamily Anguillicoloidea, whose position within a higher taxonomic unit remains uncertain at best. Since *Gnathostoma* and *Anguillicola* formed a long branch, one could anticipate that the observed effect is related to the 'long branch attraction' artefact. The LogDet distance method, which is particularly useful for unmasking this type of artefact, confirmed this branching order. Still, with the available set of species and only one gene analyzed, possible influence of the long-branch phenomenon cannot be excluded.

According to Anderson (2000), the most primitive nematodes are members of the order Rhabditida, whose species

are free-living in soil. These organisms gave rise to the three main orders parasitizing vertebrates, Strongylida, Ascaridida and Spirurida, and Rhigonematida, which infect diplopods (representing infraorders Ascaridomorpha, Spiruromorpha, Rhigonematomorpha and the superfamily Dracunculoidea in the conception of De Ley and Blaxter, 2002). The present results indicate that Anguillicolidae and Gnathostomatidae significantly differ from other Spiruromorpha and Dracunculoidea, showing some affinities with Ascaromorpha and Rhigonematomorpha. Although such a relationship can hardly be justified with available morphological traits, it may be reflected in life-cycle patterns. Whereas species of *Anguillicola* and *Gnathostoma* are oviparous and the second-stage larva develops inside the egg-shell (as in some Ascaromorpha), all the other Spiruromorpha and Dracunculidae are either viviparous, producing first-stage larvae or the first-stage larva develops inside the egg shell (Anderson, 2000). All these features indicate the ancient character of these nematode groups, parasitizing mostly (except *Gnathostoma*) archaic hosts such as elasmobranchs, eels and turtles. The basal position of the Gnathostomatoidea fits well with the interpretation based on classic morphology, which presented them as the most archaic Spirurida (Chabaud and Bain, 1994).

With the prominent exclusion of *A. crassus*, the SSU rRNA tree confirmed the monophyletic status of Dracunculoidea and Camallanoidea and their close mutual relationship (Ivashkin et al., 1971; Chabaud, 1974; Anderson, 2000). Dracunculoidea is a coherent group of tissue parasites with a similar life pattern (copepods or branchiurids being intermediate hosts), unified by the absence or reduction of a buccal capsule and frequently exhibiting a pronounced sexual dimorphism associated with an atrophying vulva and anus in gravid females. Moreover, dracunculoids have a highly characteristic way of larval release into the environment (Moravec, 2004). Besides several other families, this superfamily contains the families Dracunculidae and Philometridae, a division based on the presence or absence of the cephalic shield. Their distinction suggested by Ivashkin et al. (1971) and refused by other authors (Vismanis and Nikulina, 1972; Pan et al., 1990), is substantiated by our data.

Moreover, in the SSU rRNA-based tree, several established members of the Dracunculoidea appeared outside the main group. The erection of the family Micropleuridae by Chabaud (1975a) to accommodate *M. australiensis* seems to be correct as it constitutes a basal branch. The same concerns *Molnaria intestinalis* and *Skrjabillanus scardinii*, where the present results support the existence of an independent family Skrjabillanidae within the Dracunculoidea (Ivashkin et al., 1971; Moravec et al., 1998). Such a subdivision is in good correlation with morphology, since in contrast to other dracunculoids, members of Skrjabillanidae have a sclerotized buccal capsule and lack spicules. They can also be distinguished by the transmission of their larvae through ingestion by the haematophagous branchiurid intermediate host. Finally, another member of the Dracunculoidea clade, *P. oncorhynchi*, shows close affinities with the family Skrjabillanidae, which is in contrast with its current ranking within the family Philometridae (Ivashkin et al., 1971; Chabaud, 1975a; Anderson, 2000). Although the genus *Philonema* has a similar life-cycle pattern to other philometrids, it differs from them mainly by the presence of a multinucleate oesophageal gland. This feature, in combination with the sequence data, may warrant a creation of an independent family Philonemidae to accommodate *P. oncorhynchi*. Separation from the other philometrids of the *Alinema–Nilonema* clade, represented by two species isolated from South American freshwater fishes, reflects differences noted in the morphology of the oesophagus. In both genera, its posterior part is expanded and lacks a distinct oesophageal gland. Moreover, the gravid female of *A. amazonicum* retains a caudal vulva, whereas in the other philometrid genera, the same organ is present only in juvenile females (Moravec, 2004; Moravec et al., 2006).

Although Skrjabillanidae are very distant in their morphology and life-cycle pattern, Micropleuridae and Philometridae seem to be related in that the vulva in gravid females is atrophied (except in *Alinema*) and copepods are used as intermediate hosts. Both families differ mainly in the structure of the oesophagus, which is undivided in Philometridae, while in Micropleuridae it is divided into an anterior muscular and a posterior glandular portion. The atrophying vulva and copepods as intermediate hosts are common features for members of Philometridae, Dracunculidae and Micropleuridae, to the exclusion of the other dracunculoid families.

The separate position of the Camallanidae (Camallanoidea) in our tree correlates with the number of unique features known for this group. Species of this family are viviparous, characterized by a large, heavily sclerotized buccal capsule, poorly developed sexual dimorphism and a life cycle involving copepod hosts (Anderson, 2000). The morphological distinction between the species of *Camallanus* and *Procamallanus* rests mainly in a different structure of the buccal capsule, although our results can be used as grounds for abandoning the current genus concept. Members of both genera are intermingled within the camallanid clade and it is unlikely that better sampling or the use of another gene would reverse the present case for monophyly of these genera. The SSU rRNA-based results warrant a new delimitation of genera within this family in the frame of a broad taxonomic revision, supplemented if possible, by additional morphological and molecular characters.

A similar situation emerged in the case of Philometridae. The taxon itself remains robust but as the representatives of this family seem to lack any taxonomic structure and since the species assigned to *Philometroides*, *Dentiphilometra* and *Clavinema* are scattered among members of the genus *Philometra* from a phylogenetic perspective, these genera appear to be invalid and the characters supporting their definitions (Rasheed, 1963; Ivashkin et al., 1971;

Chabaud, 1975a; Moravec and Wang, 2002) have to be re-evaluated. Yet the rich sampling for this group of morphologically and genetically related species enabled us to address whether a phylogeographic pattern or co-evolution with the final host exist for philometrids. Since the species confined to China are intermingled with helminths collected from European, Australian and North American fishes, geographic isolation does not seem to play an important role in speciation of these nematodes. Moreover, since they do not seem to have co-evolved with their final and/or intermediate hosts, multiple host switching and/or lack of host specificity may best explain this situation. However, caution has to be exercised, since some of these conclusions are based just on partial SSU rRNA sequences.

Although results of the present study shed some light on the phylogenetic relationships among representatives of spirurid nematodes, particularly those parasitizing fishes, much work has to be done in this respect, because Spirurida represents a very diverse and species-rich group. As recently pointed out by De Ley and Blaxter (2002), it is necessary to incorporate the new molecular phylogenies into a classification system of nematodes which is being developed based on new morphological, biological and molecular data.

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