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Phylogenetic relationships of some spirurine nematodes (Nematoda: Chromadorea: Rhabditida: Spirurina) parasitic in fishes inferred from SSU rRNA gene sequences

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Abstract: Small subunit rRNA sequences were obtained from 38 representatives mainly of the nematode orders Spirurida (Camallanidae, Cystidicolidae, Daniconematidae, Philometridae, Physalopteridae, Rhabdochonidae, Skrjabillanidae) and, in part, Ascaridida (Anisakidae, Cucullanidae, Quimperiidae). The examined nematodes are predominantly parasites of fishes. Their analyses provided well-supported trees allowing the study of phylogenetic relationships among some spirurine nematodes. The present results support the placement of Cucullanidae at the base of the suborder Spirurina and, based on the position of the genus *Philonema* (subfamily Philoneminae) forming a sister group to Skrjabillanidae (thus Philoneminae should be elevated to Philonemidae), the paraphyly of the Philometridae. Comparison of a large number of sequences of representatives of the latter family supports the paraphyly of the genera *Philometra*, *Philometroides* and *Dentiphilometra*. The validity of the newly included genera *Afrophilometra* and *Caranginema* is not supported. These results indicate geographical isolation has not been the cause of speciation in this parasite group and no coevolution with fish hosts is apparent. On the contrary, the group of South-American species of *Alinema*, *Nilonema* and *Rumai* is placed in an independent branch, thus markedly separated from other family members. Molecular data indicate that the skrjabillanid subfamily Esocineminae (represented by *Esocinema bohemicum*) should be either elevated to the rank of an independent family or Daniconematidae (*Mexiconema africanum*) should be decreased to Daniconematinae and transferred to the family Skrjabillanidae. Camallanid genera *Camallanus* and *Procamallanus*, as well as the subgenera *Procamallanus* and *Spirocamallanus* are confirmed to be paraphyletic. Paraphyly has also been found within Filarioidea, Habronematoidea and Thelazioidea and in Cystidicolidae, Physalopteridae and Thelaziidae. The results of the analyses also show that *Neoascarophis*, *Spinitectus* and *Rhabdochona* are monophyletic, in contrast to the paraphyletic genus *Ascarophis*. They further confirm the independence of two subgenera, *Rhabdochona* and *Globochona*, in the genus *Rhabdochona*. The necessity of further studies of fish-parasitizing representatives of additional nematode families not yet studied by molecular methods, such as Guyanemidae, Lucionematidae or Tetanonematidae, is underscored.

Keywords: Nematoda, Spirurina, SSU rRNA, phylogeny, taxonomy

The present phylum Nematoda (reported as the order Nematoides by Rudolphi 1808) represents a large group of metazoan invertebrates, including animal parasites, free-living nematodes and plant parasites. Traditional classification systems of nematodes including those parasitic in vertebrates (e.g., Chitwood 1933, 1950, Yamaguti 1961, Ivashkin et al. 1971, Anderson et al. 1974–1983, 2009, Gibbons 2010), have solely been based on morphological characters and they often significantly differ from each other.

Chitwood (1933) established the order Spirurida as a part of the subclass Secernentea within his classification system of the class Nematoda. This class is composed of two suborders, Camallanina and Spirurina. Whereas the former contains only the superfamilies Camallanoidea and Dracunculoidea (Anguillicoloidea has recently been singled out from Dracunculoidea as a basal group to Spirurida – see Moravec 2006, Wijová et al. 2006, present data), the latter includes ten superfamilies (Acuarioidea, Aproctoidea, Diplotriaenoidea, Filarioidea, Gnathostomatoidea, Habronematoidea, Physalopteroidea, Rictularioidea, Spiruroidea and Thelazioidea) (Chabaud 1974, Gibbons 2010). According to the present taxonomy based on morphology, the order Spirurida contains 25 (Anderson 2000) or 28 (Moravec et al. 1998) families.

Camallanoidea contains only one family Camallanidae, whereas Dracunculoidea includes eight families of which, except for Dracunculidae and Micropleuridae, all comprise exclusively fish parasites: Daniconematidae, Guyanemidae, Lucionematidae, Philometridae, Skrja-

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billanidae and Tetanonematidae. Camallanina contains parasites of all classes of vertebrates (Chabaud 1974). Whereas members of Camallanoidea are gastrointestinal parasites of cold-blooded vertebrates, those of Dracunculoidea are parasitic in a variety of host tissues and cavities in both cold- and warm-blooded vertebrates (Anderson 2000). Intermediate hosts of Camallanina representatives are aquatic crustaceans, mostly Copepoda, less often Branchiura or Ostracoda (Moravec 2004).

Spirurina contains ten superfamilies (see above), of which members of four (Gnathostomatoidea, Habronematoidea, Physalopteroidea and Thelazioidea) are also parasitic in freshwater, brackish-water and marine fishes, whereas the six remaining superfamilies are represented by parasites of amphibians, reptiles, birds and mammals including man. Parasites of fishes belong to four families: Gnathostomatidae (Gnathostomatoidea), Physalopteridae (Physalopteroidea), Cystidicolidae (Habronematoidea) and Rhabdochonidae (Thelazioidea) (Moravec 2007). The life cycles of spirurine nematodes are noted for considerable variations. Intermediate hosts are invertebrates, in fish parasites mainly crustaceans and aquatic insects (copepods only in Gnathostomatoidea) (Chabaud 1974, Anderson 2000, Moravec 2007). Some species exhibit precocious larval development or even maturation in the body of the intermediate host. Paratenic and postcyclic parasitisms are fairly common in these nematodes (Moravec 1994). Representatives of this suborder are gastrointestinal or tissues parasites of all classes of vertebrates.

At the end of the 1990's, the first extensive molecular-phylogenetic analyses of these parasites were published (Blaxter et al. 1998), which placed spirurid nematodes in a separate branch designated as 'clade III' (Blaxter et al. 1998, Nadler et al. 2007). Later, in their newly proposed classification system, De Ley and Blaxter (2002, 2004) named it the suborder Spirurina, including the groups Ascaridomorpha, Gnathostomatomorpha, Oxyuridomorpha, Rhigonematomorpha, Spiruromorpha and Dracunculoidea (the latter as incertae sedis). These groups mostly correspond to the morphologically-established orders Ascaridida (Ascaridomorpha), Oxyurida (Oxyuromorpha), Rhigonematida (Rhigonematomorpha) and Spirurida (Gnathostomatomorpha, Spiruromorpha, Dracunculoidea). According to De Ley and Blaxter (2004), Spirurina includes 21 superfamilies, 9 of which contain fish parasites (Moravec 2007). However, the following studies suggest that the arrangement within this group will further change as the number of molecular data increases (Wijová et al. 2006, Nadler et al. 2007).

The latest molecular studies including a large number of examined species have revealed the paraphyly of numerous groups within the suborder Spirurina after De Ley and Blaxter (2002) and even inside 'clade III' itself (Nadler et al. 2007). This result is not surprising and, as Wijová et al. (2006) and Nadler et al. (2007) remarked, the solution of phylogenetic interrelationships in this group requires significantly more examined species as well as the sequencing of additional genes to obtain statistically better supported conclusions.

The results of the evaluation of phylogenetic relationships within the nematode suborder Spirurina of De Ley and Blaxter (2002), inferred from SSU rRNA gene sequences mainly of species of the families Cystidicolidae, Philometridae and Rhabdochonidae parasitizing fishes, are presented herein.

MATERIALS AND METHODS

Taxa sampled for phylogenetic analyses are listed in Table 1, whereas those for which sequences have been obtained from GenBank are shown in Table 2. Prior to DNA extraction, collected specimens were stored in 70–99% ethanol. Genomic DNA was isolated using standard proteinase K treatment and phenol-chloroform extraction (Sambrook and Russell 2001). The extraction was stored at −20 °C until further use. About 10 ng of genomic DNA was used for PCR amplification of the SSU rRNA gene using either the primer pair PhilonemaF and PhilPCRr (Table 3) or separately in three overlapping fragments: the first fragment: the forward primer PhilonemaF in combination with the reverse primer ameb620r; the second fragment: the forward primer WF400 and the reverse primer WR800; the third fragment: the forward primer ameb620f and the reverse primer PhilPCRr. PCR cycling parameters included denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 sec, 50 °C for 40 sec, 72 °C for 2 min, followed by a final elongation at 72 °C for 10 min. Most SSU PCR products were used for direct sequencing following enzymatic treatment using exonuclease I and shrimp alkaline phosphatase or some products were cloned before sequencing. For cloning, PCR products were purified from gel using the QIAquick Gel Extraction Kit (QIAGEN) and cloned into the pGEM-T Easy Vector II (Promega). Both strands were sequenced using a Beckman Coulter Automated Sequencer.

The resulting sequences were refined using the Editseq and Seqman programs (Dnastar). The sequence alignments were created using Mafft Multiple alignment program for amino acid or nucleotide sequences) ver. 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2002) and ambiguous sites were manually edited in BioEdit ver. 7.0.5.2 (Hall 1999).

The 'clade III' dataset (results of analysis are shown in Fig.1) was created from 104 SSU rRNA sequences from Table 1 and Table 2 and was rooted by 5 taxa according to Meldal et al. (2007): Areolaimida (*Anaplectus* sp., *Plectus aquatilis*, *Tylocephalus auriculatus*) and Rhabditida (*Brevibucca* sp., *Myolaimus* sp.). To test the monophyly of 'clade III' (recently infirmed by Nadler et al. 2007) we also constructed 'Chromadoria' dataset (Fig. 3), which included 51 representatives of the group Chromadoria (from which 10 taxa represent 'clade III') according to Meldal et al. (2007). The phylogenetic tree was rooted with the sequences of related groups Dorylaimia: Dorylaimida (*Aporcelameillus obtusicaudatus*), Mononchida (*Prionchulus muscorum*), Trichocephalida (*Trichinella spiralis*) and Enoplia: Enoplida (*Alaimus* sp.). The use of the broadest taxon sampling possible is usually recommended in phylogenetics. However, in the case of markers with hyper-variable regions, such as SSU

Table 1. Specimen, taxonomic (traditional classification), host, and locality information for nematodes from which the small subunit rRNA gene was sequenced within the frame of this study.

Species name	Traditional classification	Host		Locality	GenBank accession number
Ascarophis (*Dentiascarophis*) *adioryx*	Cystidicolidae/Habronematoidea	*Sargocentron spiniferum*	Marine fish	New Caledonia	JF803930
Afrophilometra hydrocyoni	Philometridae/Dracunculoidea	*Hydrocynus forskahlii*	Freshwater fish	Kenya	JF803946
Camallanus hypophthalmichthys	Camallanidae/Camallanoidea	*Aristichthys nobilis*	Freshwater fish	China	JF803915
Caranginema americanum	Philometridae/Dracunculoidea	*Caranx hippos*	Marine fish	Florida (USA)	JF803939
Cucullanus baylisi	Cucullanidae/Seuratoidea	*Synodontis* sp.	Freshwater fish	Sudan	JF803935
Cystidicola farionis	Cystidicolidae/Habronematoidea	*Salmo trutta fario* × *Salmo marmorata*	Freshwater fish	Italy	JF803919
Esocinema bohemicum	Skrjabillanidae/Dracunculoidea	*Esox lucius*	Freshwater fish	Russia	JF803917
Goezia spinulosa	Raphidascarididae/Ascaridoidea	*Arapaima gigas*	Freshwater fish	Brazil	JF803924
Heliconema longissimum	Physalopteridae/Physalopteroidea	*Anguilla japonica*	Freshwater fish	Japan	JF803949
Heliconema longissimum	Physalopteridae/Physalopteroidea	*Anguilla* sp.	Freshwater fish	Madagascar	JF803926
Metabronema magnum	Cystidicolidae/Habronematoidea	*Gnathonodon speciosus*	Marine fish	New Caledonia	JF803918
Mexiconema africanum	Daniconematidae/Dracunculoidea	*Auchenoglanis occidentalis*	Freshwater fish	Kenya	JF803947
Neoascarophis longispicula	Cystidicolidae/Habronematoidea	*Coryphaenoides mediterraneus*	Marine fish	Atlantic ocean (Middle Ridge)	JF803921
Paraquimperia africana	Quimperiidae/Seuratoidea	*Anguilla marmorata*	Freshwater fish	South Africa	JF803925
Philometra bagri	Philometridae/Dracunculoidea	*Bagrus bajad*	Freshwater fish	Kenya	JF803948
Philometra brevispicula	Philometridae/Dracunculoidea	*Lutjanus griseus*	Marine fish	Florida (USA)	JF803943
Philometra diplectri	Philometridae/Dracunculoidea	*Diplectrum formosum*	Marine fish	Florida (USA)	JF803942
Philometra floridensis	Philometridae/Dracunculoidea	*Sciaenops ocellatus*	Marine fish	USA	JF803928
Philometra gymnosardae	Philometridae/Dracunculoidea	*Gymnosarda unicolor*	Marine fish	Maldives	JF803916
Philometra lati	Philometridae/Dracunculoidea	*Lates niloticus*	Freshwater fish	Kenya	JF803945
Philometra morii	Philometridae/Dracunculoidea	*Epinephelus morio*	Marine fish	Florida (USA)	JF803933
Philometra ocularis	Philometridae/Dracunculoidea	*Epinephelus areolatus*	Marine fish	New Caledonia	JF803929
Philometra saltatrix	Philometridae/Dracunculoidea	*Pomatomus saltatrix*	Marine fish	South Carolina (USA)	JF803920
Philometra sp.	Philometridae/Dracunculoidea	*Mycteroperca microlepis*	Marine fish	Florida (USA)	JF803940
Philometra spiriformis	Philometridae/Dracunculoidea	*Lates niloticus*	Freshwater fish	Kenya	JF803944
Philometroides grandipapillatus	Philometridae/Dracunculoidea	*Caranx hippos*	Marine fish	Florida (USA)	JF803941
Procamallanus (*Spirocamallanus*) *fulvidraconis*	Camallanidae/Camallanoidea	*Pelteobagrus fulvidraco*	Freshwater fish	China	JF803914
Procamallanus (*Procamallanus*) *laeviconchus*	Camallanidae/Camallanoidea	*Synodontis schall*	Freshwater fish	Sudan	JF803934
Procamallanus (*Procamallanus*) *annulatus*	Camallanidae/Camallanoidea	*Siganus lineatus*	Marine fish	New Caledonia	JF803932
Procamallanus (*Spirocamallanus*) *monotaxis*	Camallanidae/Camallanoidea	*Lethrinus genivittatus*	Marine fish	New Caledonia	JF803931
Procamallanus (*Spirocamallanus*) *rarus*	Camallanidae/Camallanoidea	*Callophysus macropterus*	Freshwater fish	Peru	JF803912
Rhabdochona (*Rhabdochona*) *hellichi hellichi*	Rhabdochonidae/Thelazioidea	*Barbus barbus*	Freshwater fish	Czech Republic	JF803913
Rhabdochona (*Rhabdochona*) *hellichi turkestanica*	Rhabdochonidae/Thelazioidea	*Schizothorax* sp.	Freshwater fish	India	JF803937
Rhabdochona (*Rhabdochona*) *hospeti*	Rhabdochonidae/Thelazioidea	*Tor* sp.	Freshwater fish	India	JF803938
Rhabdochona (*Globochona*) *mazeedi*	Rhabdochonidae/Thelazioidea	*Clupisoma garua*	Freshwater fish	India	JF803936
Rumai rumai	Philometridae/Dracunculoidea	*Arapaima gigas*	Freshwater fish	Brazil	JF803923
Salmonema ephemeridarum	Cystidicolidae/Habronematoidea	*Ephemera danica*	Mayfly	Czech Republic	JF803927
Spinitectus tabascoensis	Cystidicolidae/Habronematoidea	*Ictalurus meridionalis*	Freshwater fish	Mexico	JF803922

rRNA, a considerable amount of data may potentionally be discarded prior to analysis due to their incapacity to be aligned unambiguously. This situation is usually caused by a high divergence between compared sequences. For example, the 'clade III' dataset with broad sampling was reduced from 2452 to 1562 nucleotides. On the other hand, the 'Camallanoidea' dataset (see bellow) contained 1660 nucleotides (of 1802) after the ambiguous-site removal. To get deeper insight into the relationships of some groups of our interest, we also performed analyses on a subset of four 'smaller' datasets. *Camallanus oxycephalus*, *Camallanus hypophthalmichthys*, *Procamallanus pintoi* were used as outgroups in the 'Dracunculoidea' dataset, concentrating on the dracunculoid nematodes (results shown in Fig. 2). *Dracunculus insignis*, *Dracunculus medinensis* and *Dracunculus oesophageus* were used as outgroups in the 'Philometridae' dataset (Fig. 4), where we included also shorter sequences of some philometrids (~ 950 bp) from GenBank. *Philonema oncorhynchi* was used as an outgroup in the 'Camallanoidea' dataset (Fig. 4) and *Philonema oncorhynchi* and *Camallanus cotti* were used as outgroups in the 'Spirurina' dataset (Fig. 5). All datasets are available upon request from the corresponding author.

Maximum likelihood (ML) trees were calculated under the GTR+Γ+I model of evolution using PHYML 2.4.2. This model of evolution was chosen according to the Akaike criterion as

Table 2. List of nematode species from which the sequence of small subunit rRNA gene was retrieved from GenBank, with traditional classification and GenBank accession number.

Species name	Traditional classification	GenBank accession number
Acanthocheilonema viteae	Onchocercidae/Filarioidea	DQ094171
Aduncospiculum halicti	Diplogasteridae/Diplogasterida	U61759
Alaimus sp.	Alaimidae/Oxystominoidea	AJ966514
Alinema amazonicum	Philometridae/Dracunculoidea	DQ442672
Anaplectus sp.	Plectidae/Plectoidea	AJ966473
Anguillicola crassus	Anguillicolidae/Anguillicoloidea	DQ490223
Aphelenchus avenae	Aphelenchidae/Aphelenchoidea	AF036586
Aporcelaimellus obtusicaudatus	Aporcelaimidae/Dorylaimoidea	DQ141212
Ascaris lumbricoides	Ascarididae/Ascaridoidea	U94366
Ascarophis arctica	Cystidicolidae/Habronematoidea	DQ094172
Axonolaimus helgolandicus	Axonolaimidae/Axonolaimoidea	AY854232
Boleodorus thylactus	Tylenchidae/Tylenchoidea	AY993976
Brevibucca sp.	Brevibuccidae/Panagrolaimoidea	AF202163
Brugia malayi	Onchocercidae/Filarioidea	AF036588
Brumptaemilius justini	Rhigonematida	AF036589
Bunonema franzi	Bunonematidae/Bunonematoidea	AJ966477
Caenorhabditis elegans	Rhabditidae/Rhabditoidea	X03680
Calomicrolaimus parahonestus	Microlaimidae/Richtersioidea	AY854218
Camallanus cotti	Camallanidae/Camallanoidea	EF180071
Camallanus lacustris	Camallanidae/Camallanoidea	DQ442663
Camallanus oxycephalus	Camallanidae/Camallanoidea	DQ503463
Camallanus sp.	Camallanidae/Camallanoidea	DQ442664
Catanema sp.	Desmodoridae/Desmodorida	Y16912
Cervidellus alutus	Cephalobidae/Cephaloboidea	AF202152
Chromadoria nudicapitata	Chromadoridae/Chromadorida	AY854205
Clavinema parasiluri	Philometridae/Dracunculoidea	DQ076682
Criconema sp.	Criconematidae/Criconematoidea	AJ966480
Cruzia americana	Kathlaniidae/Cosmocercoidea	U94371
Cyartonema elegans	Cyartonematidae/Monhysterida	AY854203
Cyatholaimus sp.	Cyatholaimidae/Chromadorida	AY854213
Cyrnea mansioni	Habronematidae/Habronematoidea	AY702701
Daptonema procerus	Xyalidae/Monhysterida	AF047889
Dentiphilometra monopteri	Philometridae/Dracunculoidea	DQ076685
Dentiphilometra sp.	Philometridae/Dracunculoidea	DQ442673
Dentostomella sp.	Heteroxynematidae/Oxyuroidea	AF036590
Desmodora ovigera	Desmodoridae/Desmodorida	Y16913
Desmolaimus zeelandicus	Linhomoetidae/Monhysterida	AY854229
Dichromadora sp.	Chromadoridae/Chromadorida	AY854209
Dirofilaria immitis	Onchocercidae/Filarioidea	AF036638
Dracunculus insignis	Dracunculidae/Dracunculoidea	AY947719
Dracunculus medinensis	Dracunculidae/Dracunculoidea	AY852268
Dracunculus oesophageus	Dracunculidae/Dracunculoidea	AY852269
Echinuria borealis	Acuariidae/Acuarioidea	EF180064
Geocenamus quadrifer	Belonolaimidae/Tylenchoidea	AY993977
Gnathostoma binucleatum	Gnathostomatidae/Gnathostomatoidea	Z96946
Gnathostoma turgidum	Gnathostomatidae/Gnathostomatoidea	Z96948
Goezia pelagia	Raphidascarididae/Ascaridoidea	U94372
Heterakis sp.	Heterakidae/Heterakoidea	AF083003
Heterakis gallinarum	Heterakidae/Heterakoidea	DQ503462
Isolaimium sp.	Isolaimidae/Isolaimoidea	AY919142
Loa loa	Onchocercidae/Filarioidea	DQ094173
Margolisianum bulbosum	Philometridae/Dracunculoidea	AB185161
Meloidogyne incognita	Meloidogynidae/Tylenchoidea	U81578
Micropleura australiensis	Micropleuridae/Dracunculoidea	DQ442678
Molnaria intestinalis	Skrjabillanidae/Dracunculoidea	DQ442668
Monoposthia costata	Monoposthiidae/Desmodorida	AY854221
Myolaimus sp.	Myolaimidae/Myolaimoidea	U81585
Neoascarophis macrouri	Cystidicolidae/Habronematoidea	DQ442660
Nilonema senticosum	Philometridae/Dracunculoidea	DQ442671
Nippostrongylus brasiliensis	Heligmonellidae/Trichostrongyloidea	AF036597
Onchocercidae gen. sp.	Onchocercidae/Filarioidea	DQ103704
Oxyuris equi	Oxyuridae/Oxyuroidea	EF180062
Panagrolaimus sp.	Panagrolaimidae/Panagrolaimoidea	U81579
Paraspidodera sp.	Aspidoderidae/Heterakoidea	AF083005
Philometra clavaeceps	Philometridae/Dracunculoidea	DQ076686
Philometra cyprinirutili	Philometridae/Dracunculoidea	DQ442675
Philometra fujimotoi	Philometridae/Dracunculoidea	DQ076680
Philometra lateolabracis	Philometridae/Dracunculoidea	FJ161972
Philometra madai	Philometridae/Dracunculoidea	FJ161974
Philometra nemipteri	Philometridae/Dracunculoidea	FJ161975
Philometra obturans	Philometridae/Dracunculoidea	AY852267
Philometra ovata	Philometridae/Dracunculoidea	DQ442677
Philometra sawara	Philometridae/Dracunculoidea	FJ161973
Philometra sciaenae	Philometridae/Dracunculoidea	FJ161971
Philometra sp.	Philometridae/Dracunculoidea	DQ442674
Philometroides carassii	Philometridae/Dracunculoidea	DQ076683
Philometroides cyprini	Philometridae/Dracunculoidea	DQ076688
Philometroides fulvidraconi	Philometridae/Dracunculoidea	DQ076684
Philometroides ganzhounensis	Philometridae/Dracunculoidea	DQ076681
Philometroides pseudorasbori	Philometridae/Dracunculoidea	DQ076687
Philometroides sanguineus	Philometridae/Dracunculoidea	DQ442676
Philometroides seriolae	Philometridae/Dracunculoidea	FJ155811
Philonema oncorhynchi	Philometridae/Dracunculoidea	DQ442670
Philonema sp.	Philometridae/Dracunculoidea	U81574
Plectus aquatilis	Plectidae/Plectoidea	AF036602
Physaloptera alata	Physalopteridae/Physalopteroidea	AY702703
Physaloptera sp.	Physalopteridae/Physalopteroidea	EF180065
Pratylenchus thornei	Pratylenchidae/Tylenchoidea	AJ966499
Procamallanus pacificus	Camallanidae/Camallanoidea	DQ442665
Procamallanus pintoi	Camallanidae/Camallanoidea	DQ442666
Procamallanus rebecae	Camallanidae/Camallanoidea	DQ442667
Prionchulus muscorum	Mononchidae/Mononchoidea	AJ966500
Raillietnema sp.	Cosmocercidae/Cosmocercoidea	DQ503461
Rhabdochona denudata	Rhabdochonidae/Thelazioidea	DQ442659
Rhabditis myriophila	Rhabditidae/Rhabditoidea	U81588
Rhabditoides inermis	Rhabditidae/Rhabditoidea	AF082996
Rhigonema thysanophora	Rhigonematidae/Rhigonematoidea	EF180067
Rondonia rondoni	Atractidae/Rhabditida	DQ442679
Sabatieria punctata	Comesomatidae/Monhysterida	AY854236
Scutellonema bradys	Hoplolaimidae/Tylenchoidea	AJ966504
Serratospiculum tendo	Diplotriaenidae/Diplotriaenoidea	AY702704
Skrjabillanus scardinii	Skrjabillanidae/Dracunculoidea	DQ442669
Sphaerolaimus hirsutus	Sphaerolaimidae/Monhysterida	AY854228
Spinitectus carolini	Cystidicolidae/Habronematoidea	DQ503464
Spirocamallanus istiblenni	Camallanidae/Camallanoidea	EF180076
Spirocamallanus rarus	Camallanidae/Camallanoidea	DQ494195
Spirocerca lupi	Thelaziidae/Thelazioidea	AY751497
Strongyloides ratti	Strongyloididae/Panagrolaimoidea	U81581
Strongylus equinus	Strongylidae/Strongyloidea	DQ094176
Subanguina radicicola	Anguinidae/Tylenchoidea	AF202164
Synhimantus laticeps	Acuariidae/Acuarioidea	EU004818
Teratocephalus lirellus	Teratocephalidae/Teratocephaloidea	AF036607
Teratorhabditis synpapillata	Rhabditidae/Rhabditoidea	AF083015
Terschellingia longicaudata	Linhomoetidae/Monhysterida	AY854230
Terranova scoliodontis	Anisakidae/Ascaridoidea	DQ442661
Tetrameres fissipina	Tetrameridae/Habronematoidea	EF180077
Thelazia lacrymalis	Thelaziidae/Thelazioidea	DQ503458
Toxocara canis	Ascarididae/Ascaridoidea	AF036608
Toxocara canis	Ascarididae/Ascaridoidea	U94382
Tridentulus sp.	Monhysteridae/Monhysterida	AJ966507
Trichinella spiralis	Trichinellidae/Trichocephalida	U60231
Truttaedacnitis truttae	Cucullanidae/Seuratoidea	EF180063
Turgida torresi	Physalopteridae/Physalopteroidea	EF180069
Turgida turgida	Physalopteridae/Physalopteroidea	DQ503459
Tylenchidae gen. sp.	Tylenchidae/Tylenchoidea	AY854241
Tylocephalus auriculatus	Plectidae/Plectoidea	AF202155
Zeldia punctata	Cephalobidae/Cephaloboidea	U61760

Table 3. List of primers used for PCR and sequencing.

Primer	Primer sequence 5'- 3'	Usage
PhilonemaF	GCC TAT AAT GGT GAA ACC GCG AAC	PCR and sequencing
PhilPCRr	CCG GTT CAA GCC ACT GCG ATT A	PCR and sequencing
WF400	GCG CAA ATT ACC CAC TCT	PCR and sequencing
WR400	CAA CTT CAT GCG GCT GAA	Sequencing
ameb620f	GCC AGC ACC CGC GGT AAT TCC	PCR and sequencing
ameb620r	GGA ATT ACC GCG GGT GCT GGC	PCR and sequencing
WF760	GCC TGA ATA CTC GTG CAT	Sequencing
WR800	GGT ATC GTT TAC GGT CAG	PCR and sequencing
Phil1200F	CCG TGA GGA TTG ACA GAT TGA GAG C	Sequencing
Phil1200R	CCG CGG CTG CTG GCA CCA GAC TT	Sequencing
WF1550	CCT AGT AAG TGT GAG TCA	Sequencing
WR1600	CAG ACT CAC CAA TTG ACG	Sequencing

implemented in Modeltest 3.7 (Posada and Crandall 1998). For Bayesian inference (BI), we ran two chains for a variable number of generations (for details see figure legend) under the GTR+Γ+I and default priors in MrBayes 3.1.2 (Huelsenbeck et al. 2001). After the check for convergence, we discarded the first 25% as a burn-in and the rest was used for topology and posterior probability reconstruction. Branching support was estimated using non-parametric bootstraping in case of ML (number of replications varied in between the datasets, see figure legend for details) or posterior probability (BI). Nodes were considered to be highly supported with the boostrap value higher than 90 and posterior probability equal to 1.0; moderately supported with the bootstrap value 70–90 and posterior probability higher than 0.94.

When our topology was not congruent with conventional classification (i.e. we found some groups to be poly/paraphyletic), we forced monophyly of respective groups, recomputed phylogeny under the constraint using RAxML 7.2.8 (Stamatakis 2006) and performed approximately-unbiased test (au-test, Shimodaira 2004) as implemented in Consel (Shimodaira and Hasegawa 2001).

RESULTS

Thirty-eight new SSU rRNA sequences of nematodes (ranging from 1629 to 1920 bp) were used along with 126 of those from previously published taxa for phylogenetic analyses. The most extensive analysis comprises 109 taxa from which 104 species represent 'clade III' (Fig. 1). The superfamily Seuratoidea appears at the base of the phylogenetic tree, being represented by the family Quimperiidae (*Paraquimperia africana*) on the first branch and the family Cucullanidae (*Cucullanus baylisi* and *Truttaedacnitis truttae*) on the second branch. The position of Quimperiidae, which constitutes a sister group to the well-promoted cluster of the remaining representatives of Spirurina according to De Ley and Blaxter (2002), is supported only by poor posterior probability and low bootstrap. To address possible paraphyly of the whole 'clade III', we constructed the tree into which selected representatives of the group Chromadoria were involved (Fig. 3). In this analysis, 'clade III' appears as monophyletic, but with still a relatively low statistical support.

According to the previous 'clade III' analysis (Fig. 1), Oxyurida is the only monophyletic order, whereas other orders (Ascaridida, Rhigonematida and Spirurida) are paraphyletic. Representatives of Spirurida (except for three species of two superfamilies), Ascaridida (except for four species of three superfamilies), Rhigonematida and Oxyurida form polytomy in the middle part of the tree. Most species of Spirurida rank with a cluster on the upper part of the tree and split in two morphologically and molecularly defined suborders Camallanina and Spirurina, as previously defined by Chitwood (1937). Representatives of the superfamilies Anguillicoloidea (Camallanina) and Gnathostomatoidea (Spirurina) are located on the distant branch forming a well-supported sister group to all the mentioned orders (see above), which, before dataset completion with species of the superfamily Seuratoidea, was situated at the base of the whole 'clade III' (Wijová et al. 2006).

Monophyly of the superfamily Dracunculoidea is well supported after the elevation of Anguillicolidae to the superfamily rank. The superfamily Camallanoidea (family Camallanidae), forming a sister group to Dracunculoidea, is also monophyletic. The Dracunculoidea includes monophyletic families Dracunculidae, Micropleuridae, Daniconematidae and paraphyletic Philometridae and Skrjabillanidae. The well-supported part of Philometridae forms a sister group to Dracunculidae and Micropleuridae with indefinite relationships. Fig. 2 displays the phylogenetic tree constructed from a dataset containing only representatives of Dracunculoidea and brings more detailed view on the relationships within this taxon. Polytomy is eliminated and *Micropleura australiensis* (Micropleuridae) forms a sister group to Dracunculidae. A sister group to the three above-mentioned families is that comprising Skrjabillanidae, Daniconematidae and partly Philometridae (*Philonema* sp.). *Mexiconema africanum* (Daniconematidae) incorporates between skrjabillanids and forms a sister group to the species *Micropleura australiensis* and *Skrjabillanus scardinii*. These three species are situated on the same branch with the part of Philometridae represented two species (*Philonema* sp. and *Philonema oncorhynchi*), which forms a sister group to the two above-mentioned families.

Another two phylogenetic trees illustrate relationships in the families Philometridae and Camallanidae (Fig. 4). The former was examined within the whole superfamily Dracunculoidea in the first analysis (Fig. 2). The second phylogenetic tree in Fig. 4 is focused on only the family Camallanidae. Both these analyses support results of a previous study (Wijová et al. 2006). The genera *Philometra*, *Philometroides* and *Dentiphilometra* are paraphyletic. The well-supported branch with two South

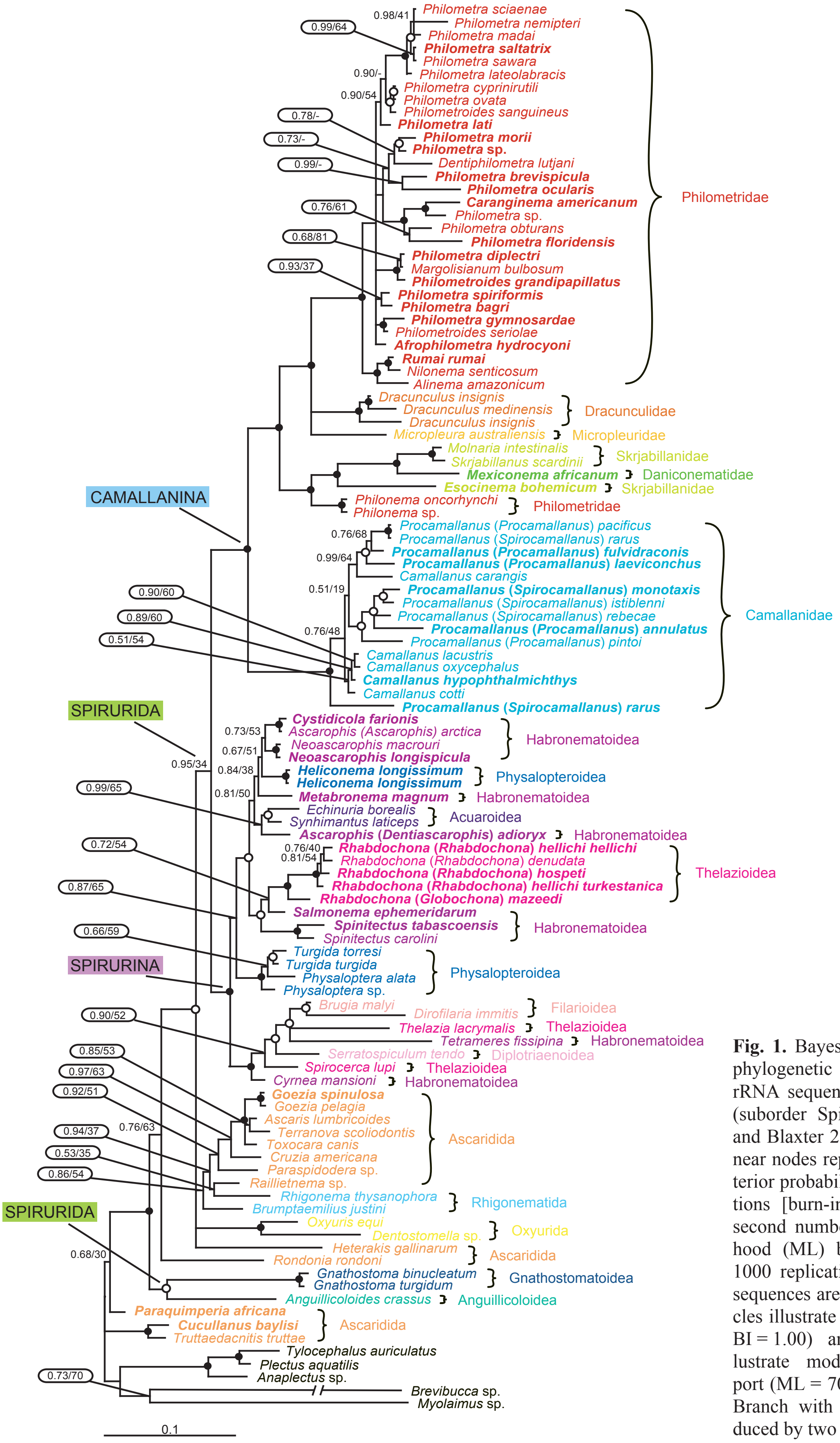

Fig. 1. Bayesian tree inferred from phylogenetic analysis of the SSU rRNA sequences dataset 'clade III' (suborder Spirurina sensu De Ley and Blaxter 2002). The first number near nodes represents Bayesian posterior probability (for 1×10^7 generations [burn-in = 2.5×10^6]) and the second number is maximum likelihood (ML) bootstrap value (from 1000 replications). Newly acquired sequences are marked bold. Full circles illustrate high support (ML>90, BI = 1.00) and empty circles illustrate moderate bootstrap support (ML = 70–90, BI = 0.80–0.99). Branch with *Brevibucca* sp. is reduced by two thirds.

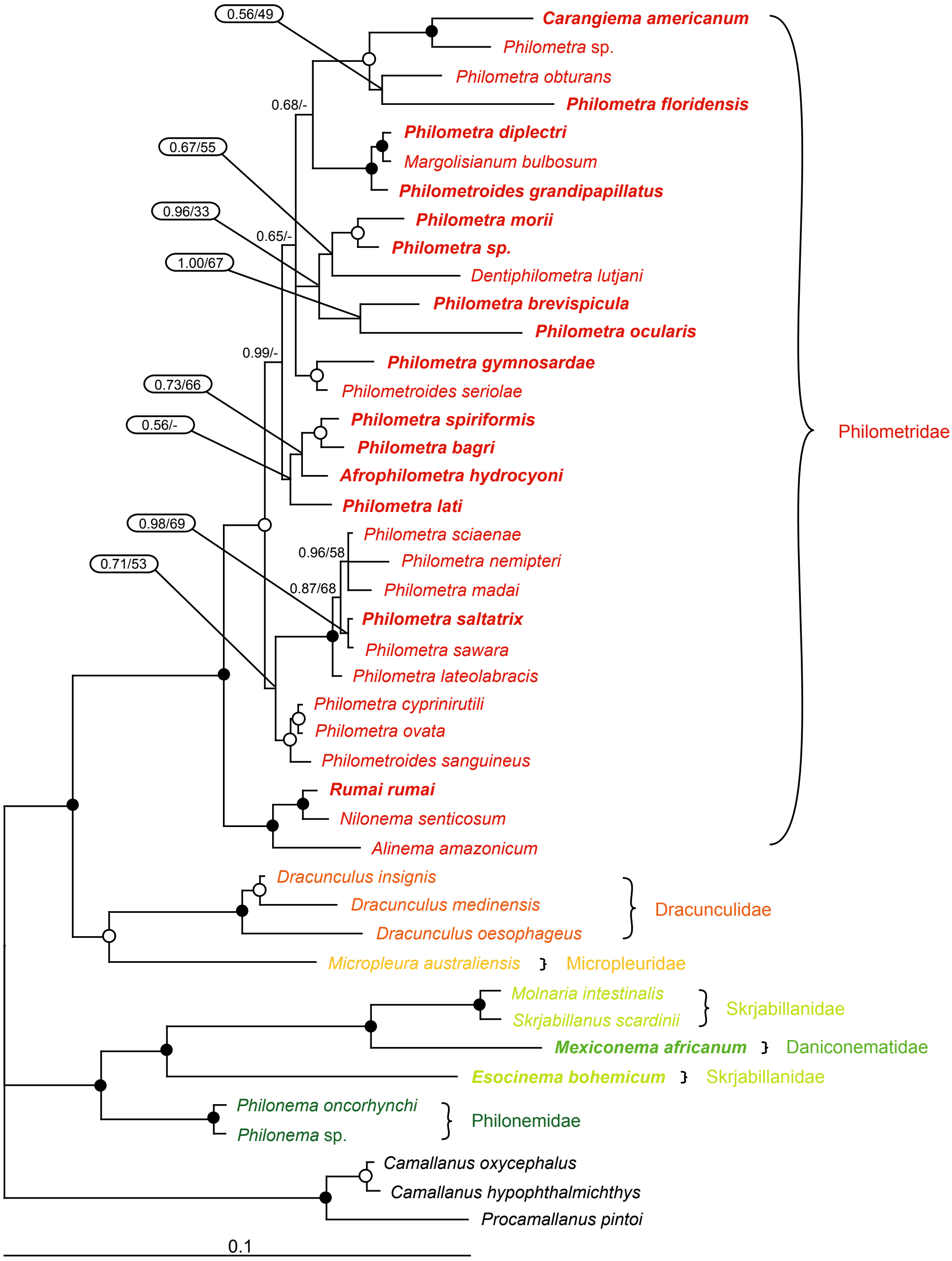

Fig. 2. Bayesian tree inferred from phylogenetic analysis of the SSU rRNA sequences dataset Dracunculoidea. The first number near nodes represents Bayesian posterior probability (for 5×10^6 generations [burn-in = 2×10^6]) and the second number is maximum likelihood (ML) bootstrap value (from 500 replications). Newly acquired sequences are marked bold. Full circles illustrate high support (ML>90, BI = 1.00) and empty circles illustrate moderate bootstrap support (ML = 70–90, BI = 0.80–0.99).

American species (*Nilonema senticosum* and *Alinema amazonicum*) separating from others is completed by the next one, *Rumai rumai*, which is situated on the common branch with *Nilonema*. It is evident from the phylogenetic tree examining relationships within the family Camallanidae that the genera *Camallanus* and *Procamallanus*, as well as the subgenera *Procamallanus* and *Spirocamallanus* are paraphyletic (Fig. 4). A sister group to the suborder Camallanina constitutes the morphologically defined suborder Spirurina Chitwood, 1937. In one analysis, this fission is supported by a high posterior probability (0.95), while the ML analysis shows low bootstrap (34). The suborder Spirurina Chitwood, 1937 divides into two stable branches, of which the first is formed by one monophyletic superfamily Acuarioidea (family Acuariidae) and three paraphyletic superfamilies Habronematoidea,

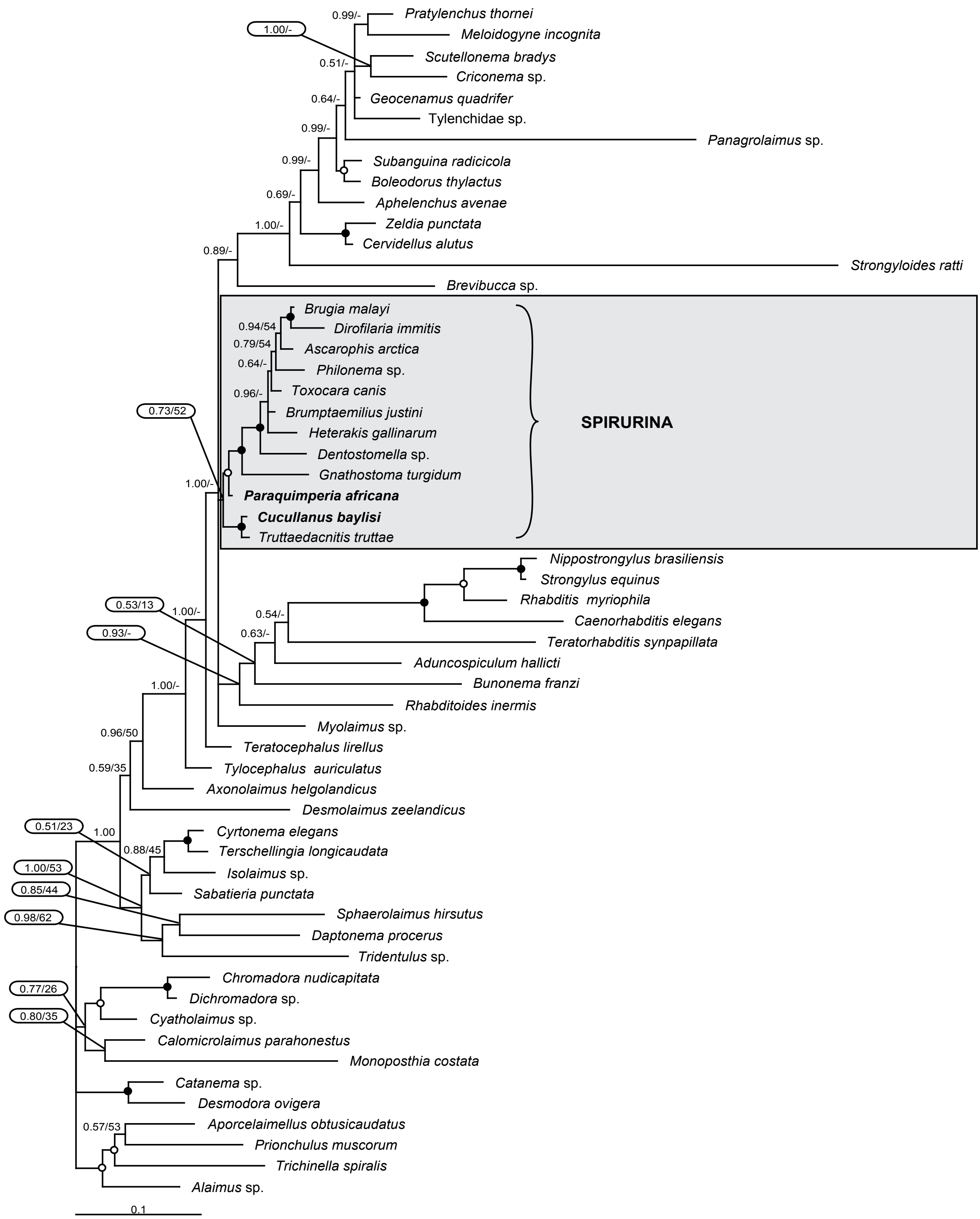

Fig. 3. Bayesian tree inferred from phylogenetic analysis of the SSU rRNA sequences dataset Chromadoria. The first number near nodes represents Bayesian posterior probability (for 5×10^6 generations [burn-in = 1×10^6]) and the second number is maximum likelihood (ML) bootstrap value (from 500 replications). Newly acquired sequences are marked bold. Full circles illustrate high support (ML>90, BI = 1.00) and empty circles illustrate moderate bootstrap support (ML = 70–90, BI = 0.80–0.99).

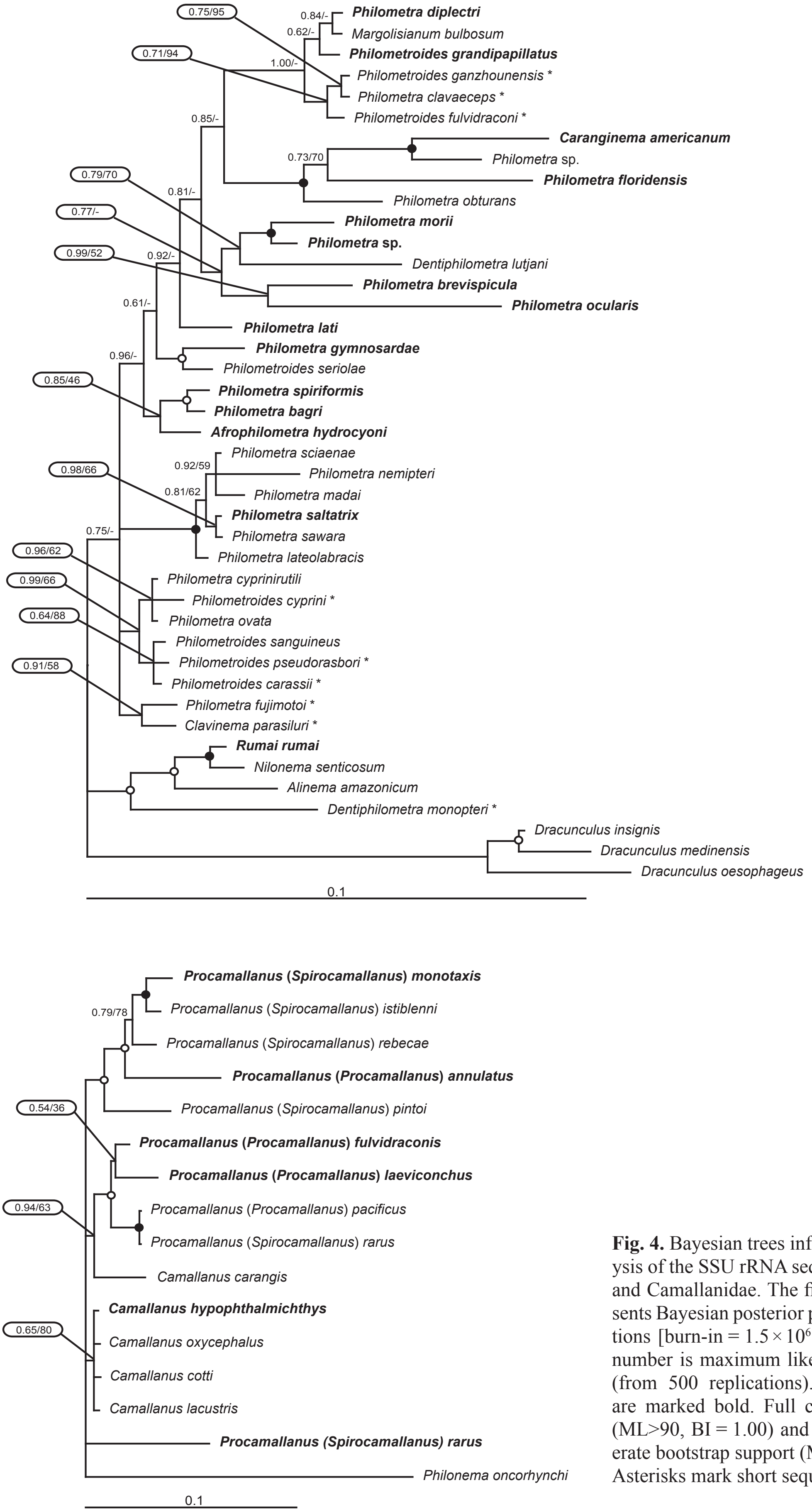

Fig. 4. Bayesian trees inferred from phylogenetic analysis of the SSU rRNA sequences dataset Philometridae and Camallanidae. The first number near nodes represents Bayesian posterior probability (for 5×10^6 generations [burn-in = 1.5×10^6 and 2×10^6]) and the second number is maximum likelihood (ML) bootstrap value (from 500 replications). Newly acquired sequences are marked bold. Full circles illustrate high support (ML>90, BI = 1.00) and empty circles illustrate moderate bootstrap support (ML = 70–90, BI = 0.80–0.99). Asterisks mark short sequences.

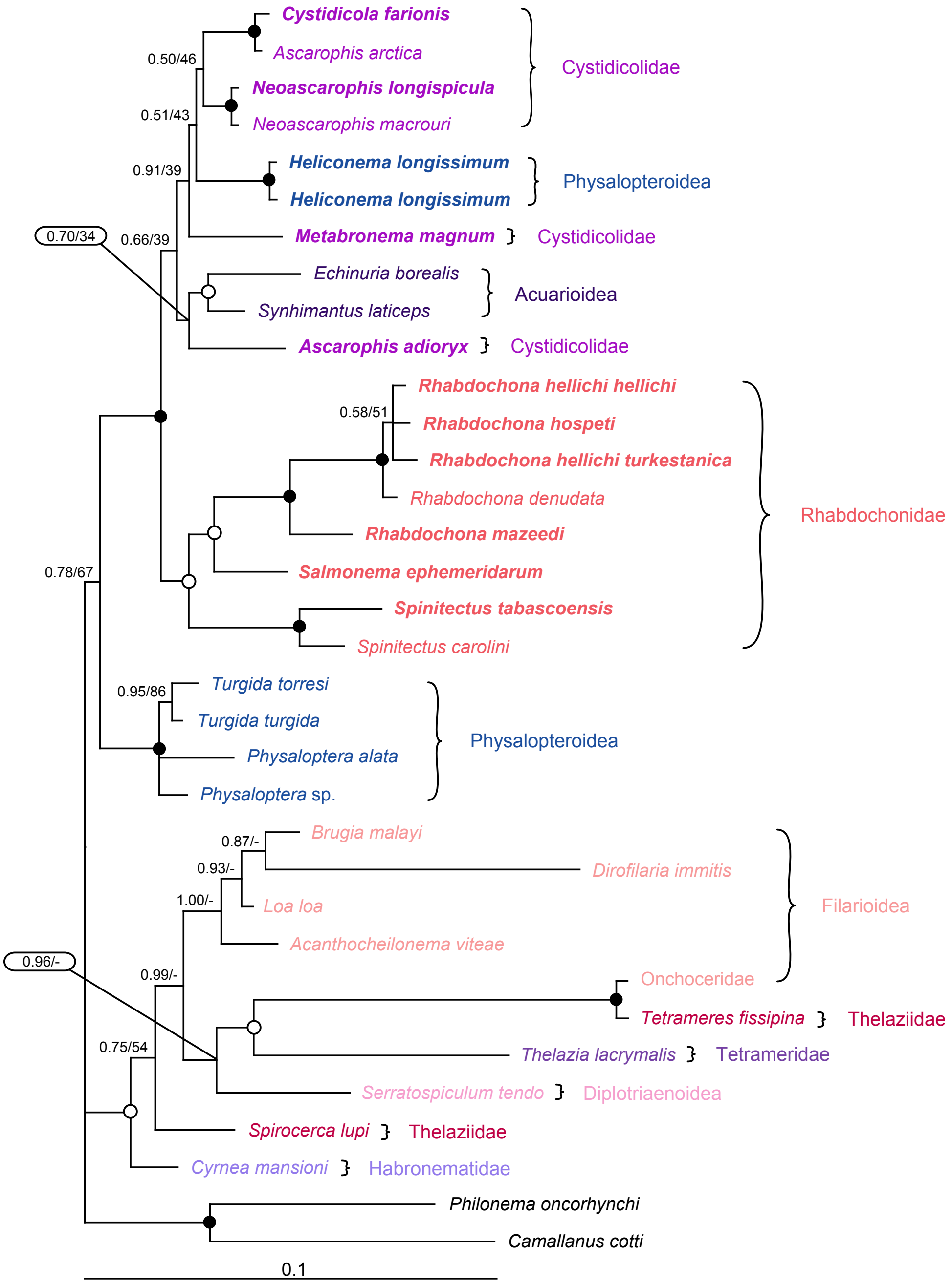

Fig. 5. Bayesian tree inferred from phylogenetic analysis of the SSU rRNA sequences dataset Spirurina. The first number near nodes represents Bayesian posterior probability (for 5×10^6 generations [burn-in = 2×10^6]) and the second number is maximum likelihood (ML) bootstrap value (from 500 replications). Newly acquired sequences are marked bold. Full circles illustrate high support (ML>90, BI = 1.00) and empty circles illustrate moderate bootstrap support (ML = 70–90, BI = 0.80–0.99).

Physalopteroidea (family Physalopteridae) as well as Thelazioidea. An interesting aspect of result is that the part of the superfamily Physalopteroidea (with the species *Heliconema longissimum*) splits off from related genera (*Physaloptera* sp., *Turgida* sp.) occurring on the basis of this branch and integrates between Habronematoidea and Acuarioidea. The second branch includes representatives of paraphyletic superfamilies Filarioidea (family Onchocercidae) (Fig. 5), Thelazioidea, Habronematoidea and Diplotriaenoidea (family Diplotriaenidae), each with only one species studied. Fig. 5 illustrates paraphyly of the families Cystidicolidae and Thelaziidae, monophyly of the family Rhabdochonidae, and families Tetrameridae and Habronematidae represented by only a single species. This conclusion is corroborated by the results of this analysis, in which the genera *Neoascarophis*, *Spinitectus* (Cystidicolidae), *Rhabdochona* (Rhabdochonidae) and the species *Heliconema longissimum* (Physalopteridae) are monophyletic and well supported, in contrast to the genus *Ascarophis*, which is paraphyletic.

DISCUSSION

Present studies on the molecular phylogeny of nematodes are noted for the considerable imbalance of studied samples. Whereas the majority of the included nematodes is represented by groups of free-living species and plant parasites, the number of sequenced nematodes parasitizing vertebrates comprises only a negligible part in recent molecular-phylogenetic studies, such as in the group designated as 'clade III' by Blaxter et al. (1998). This sample bias decreases the utility of these studies for creating new classification systems within the framework of the phylum Nematoda (De Ley and Blaxter 2002, 2004, Holterman et al. 2006, Nadler et al. 2007, van Megen et al. 2009).

The present study substantially increases the number of sequenced nematode species parasitizing vertebrates, mainly fish. In total, sequences were obtained from 36 nominal species and subspecies belonging to 10 families, mostly of the superfamilies Camallanoidea, Dracunculoidea, Habronematoidea and Thelazioidea. Furthemore in this study, representatives of the families Daniconematidae (*Mexiconema africanum*) and Quimperiidae (*Paraquimperia africana*) are included for the first time, as well as species of previously unstudied genera *Cystidicola* (*C. farionis*), *Metabronema* (*M. magnum*) and *Salmonema* (*S. ephemeridarum*) in Cystidicolidae, *Afrophilometra* (*A. hydrocyoni*), *Caranginema* (*C. americanum*) and *Rumai* (*R. rumai*) in Philometridae and *Esocinema* (*E. bohemicum*) in Skrjabillanidae. In many of these cases, rare samples of recently described new nematode species were analysed.

The present molecular-phylogenetic analysis on the basis of SSU rRNA genes made possible a new evaluation of a number of relationships among these parasites. It can be deduced from the obtained results that Cucullanidae (*Cucullanus baylisi* and *Truttaedacnitis truttae* [= *Cucullanus* (*Truttaedacnitis*) *truttae*]) forms a basal group of the Spirurina proposed by De Ley and Blaxter (2002). Anderson et al. (2009) placed Cucullanidae and Quimperiidae in the superfamily Seuratoidea, the representatives of which are noted for some archaic characters. According to the Russian system of nematodes, these groups are ranked within the suborder Cucullanata, being considered an interface between Ascaridata and Spirurata (Ivashkin and Khromova 1976). Species of Cucullanidae and Quimperiidae are intestinal parasites of cold-blooded vertebrates, mainly fishes, as well as amphibians and reptiles (tortoises). *Goezia spinulosa* is located on the same branch as *Goezia pelagia*, thus confirming the placement of the genus in the superfamily Ascaridoidea of the order Ascaridida, as in the conception of De Ley and Blaxter (2004), within the family Raphidascarididae (representetives are intestinal parasites of fish).

Camallanoidea and Dracunculoidea constitute a monophyletic group creating two distinct evolutionary lines, which indicates some remote affinities of both groups. Species of these groups exhibit a similar ontogenetic development, characterised by ovoviviparity, and intermediate hosts are crustaceans mainly of the suborder Copepoda or less often Branchiura (fish lice) (Anderson 2000). A notable result of this study, already indicated by Wijová et al. (2006), is that *Philonema* sp. along with representatives of Skrjabillanidae and Daniconematidae form a separate group splitting off other Dracunculoidea. Chitwood (1937) placed the genus *Philonema* in Philometridae, but noticed that it differs from other genera by the presence of multinucleate oesophageal glands. Ivashkin et al. (1971) established an independent subfamily Philoneminae within Philometridae on the basis of multinucleate oesophageal glands and cone-shaped caudal body end. This classification was followed by Chabaud (1975). Later, however, Moravec (2006) transferred *Philonema* from Philometridae to Micropleuridae (subfamily Micropleurinae), based on the oesophageal structure and general morphology (in addition, Philoneminae was not recognised by him). However, the results of this molecular study show that the genera *Philonema* and *Micropleura* form two different lines (see also Wijová et al. 2006), which could be taken for two different families in the system of dracunculoid nematodes. Similar results were also obtained by Nadler et al. (2007). Therefore, it is possible to raise the subfamily Philoneminae Ivashkin, Sobolev et Khromova, 1971 to the family rank Philonemidae and to consider it valid.

A very important result of the present work is the establishment of the phylogenetic position of *Mexiconema* as the first representative of Daniconematidae. It shows that these nematodes fall into the same evolutionary line as typical representatives of Skrjabillanidae (*Molnaria* [= *Kalmanmolnaria*] and *Skrjabillanus*), which is placed on the same branch with Philoneminae. On the contrary, the position of *Esocinema*, also established within Skrjabillanidae, is more distant from typical skjabillanid genera than *Mexiconema*. Moravec (2006) accommodated *Esocinema* into the separate subfamily Esocineminae, whereas the nominotypical subfamily Skrjabillaninae contains *Kalmanmolnaria* (syn. *Molnaria*), *Sinoichthyonema* and *Skrjabillanus*. All these genera include tissue parasites of fishes, developing via blood-sucking branchiurids, whereas the monophyletic line Philoneminae has representatives also parasitizing freshwater fishes, but their development is with the participation of copepod intermediate hosts.

As evidenced by these results, Philometridae is paraphyletic. Phylogenetic trees contain great numbers of molecular-studied species (32), nearly a half of which (15) is first reported in this study, including some representatives of the previously uncharacterised genera *Afrophilometra*, *Caranginema* and *Rumai*. Species of *Philometra* and *Philometroides* are paraphyletic, which may indicate that these genera are not natural and thus not reflecting true phylogenetic relationships. However, this situation is not the case for *Alinema*, *Nilonema* and *Rumai*, species of which occur in freshwater fishes in South America (*Alinema*, *Rumai*) or South America and Africa (*Nilonema*). In contrast to other philometrids, these species are characterised by a somewhat different structure of the oesophagus, certain specific features in the structure of cephalic end, or even the presence of a functional vulva in *Alinema*, which is otherwise atrophied in all other adult philometrids.

The next line is represented by species of *Philometra* and *Afrophilometra* from freshwater fishes in Africa. Despite their different sites of infection within the host (body cavity in *P. lati*, gill arches and covers in *P. spiriformis*, subcutaneous tissue in *P. bagri*, fins in *A. hydrocyoni* – see Moravec et al. 2009) and phylogenetically distant hosts (Characiformes, Perciformes and Siluriformes), these nematodes constitute a separate evolutionary line, but only in one analysis. This group fragments in the two other trees. Another group is formed by three species from European freshwater cyprinids: *Philometra cyprinirutili* and *Philometra ovata* parasitic in the abdominal cavity, and *Philometroides sanguineus* parasitic in fins. As already indicated by Wijová et al (2006), the phylogenetic tree involving philometrid representatives with short sequences shows that also three Asian species, *Philometroides cyprini*, *Philometroides carassii* and *Philometroides pseudorasbori* from freshwater cyprinids, belong to this group. A sister group to them is formed by two species also from Asia, *Philometra fujimoioi* from freshwater catfishes (Siluriformes) and *Clavinema parasiluri* from freshwater snakeheads (Perciformes).

The cluster comprising *Philometra lateolabracis*, *P. madai*, *P. nemipteri*, *P. saltatrix*, *P. sawara* and *P. sciaenae* represents species from marine perciform fishes, all parasites of gonads, mostly from the Pacific Ocean near Japan: only *P. saltatrix* is found in the eastern and western parts of the Atlantic near North America and Europe (Mediterranean Sea). Here, the clustering appears to be due to their infection of the same location within the host than geographic isolation or dependence on a type of host, even though another congeneric species, *P. floridensis* from the gonads of a brackish-water perciform fish, is genetically different and is a part of the other evolutionary line. Also the other two main lines are represented by species largely from marine perciform fishes, which in contrast parasitize under the skin of head, in the oral cavity, fins or in eye orbits. On the other branch, there are *Caranginema americanum* and *Philometra* sp., whose sister group is constituted of two species, *Philometra floridensis* and *P. obturans* (the latter occurring in the circulatory system of European pikes). A sister group to the above-mentioned branch is formed by the species *Philometra diplectri*, *Philometroides grandipapillatus* and *Margolisianum bulbosum* (gen. et sp. inquirendae – mixed two species, *Philometra overstreeti* + *Philometroides paralichthydis* – see Moravec and de Buron 2006). All these species occur in marine fishes in the West Atlantic. A sister clade to this cluster is formed by a group of freshwater species made up of *Philometra clavaeceps*, *Philometroides fulvidraconi* and *P. ganzhounensis* (= *Philometroides pseudaspii*), all parasites of East and Central Asian freshwater fishes (Russian Far East, China, Mongolia). Whereas the first species is parasitic in the fish abdominal cavity, the other species are parasites of fish subcutaneous tissues. The second line is represented by the species *Philometra morii* and *Philometra* sp. (both forms represented by two morphologically closely related species parasitizing fishes of the family Serranidae) and the phylogenetically rather more distant *Dentiphilometra lutjani*, and a sister group of *Philometra brevispicula* and *Philometra ocularis*. All these species, except for the last, are distributed in the West Atlantic region (Gulf of Mexico, Caribbean Sea), whereas *P. ocularis* is found in the Pacific (Japan, New Caledonia).

The remaining group is formed by two species, both from marine perciform fishes of the family Scombridae, of which *Philometra gymnosardae* parasitizes the body cavity of tuna fishes in the Indian Ocean (Maldive Islands), whereas *Philometroides seriolae* infects the musculature of fishes of the genus *Seriola* in the Pacific Ocean (Japan). Even though the speciation of these parasites in dependence on their hosts and geographical insulation might have been involved in this case, it is more likely with regard to the small number of studied species that a casual relationship of hosts of these nematodes was concerned. It is remarkable that the family Philometridae, along with Dracunculidae and Micropleuridae, forms a monophyletic group. It is possible to judge from the phylogenetic tree and the type of hosts of recent representatives of these families (Moravec 2006) that Micropleuridae (parasites of sharks, amphibians and reptiles, mainly crocodiles) initially split off first from the line leading to Philometridae (parasites of fishes) and Dracunculidae (parasites of warm-blooded vertebrates) only later.

The superfamily Camallanoidea (with only one family Camallanidae) is monophyletic and forms a sister group to Dracunculoidea. Whereas all freshwater species of *Camallanus* comprise one phylogenetic line, *Camallanus carangis* from marine fishes points affinities to the other line leading to freshwater and marine forms of *Procamallanus*. Since representatives of *Procamallanus* (including subgenera *Procamallanus* and *Spirocamallanus*) appear in different phylogenetic lines represented by species from marine and freshwater fishes from different continents, it can be supposed that these genera, i.e., *Camallanus* and *Procamallanus*, are formal taxa not reflecting true phylogenetic relationships. Surprisingly, the nematodes *Procamallanus* (*Spirocamallanus*) *rarus* from two different hosts from Peru appear in different branches. In this case, a wrong species determination of the specimens in question could be the cause.

The presently studied spirurine nematodes belong to three families, Cystidicolidae (Habronematoidea), Rhabdochonidae (Thelazioidea) and Physalopteridae (Physalopteroidea), according to both the current system of nematodes based on morphology (Gibbons 2010) and the molecular system (De Ley and Blaxter 2004). Nevertheless, Nadler et al. (2007) create the new superfamily Spiruroidea for Cystidicolidae. However, it follows from the phylogenetic tree that, even if representatives of these three families from fishes are placed on one branch, other species of the same superfamilies from birds and mammals occur in other phylogenetic lines and are thus paraphyletic. Hence it is evident that Habronematoidea, Thelazioidea and Physalopteroidea, and also the later-established Spiruroidea, are not natural taxa corresponding to mutual relationships. According to the morphological system, all representatives of the hitherto family Physalopteridae from fishes belong to the subfamily Proleptinae (Moravec et al. 2007, Anderson et al. 2009). It seems that in the future it will be necessary to elevate this subfamily to the rank of an independent family, whereas the actual existence of the superfamily Physalopteroidea remains unclear.

It follows from the results of the present analysis that Proleptinae belongs to the same cluster with Cystidicolidae and Rhabdochonidae from fishes, but also with Acuarioidea from birds. The phylogenetic tree confirms that species of Cystidicolidae and Rhabdochonidae from fishes occur in two distinct lines, of which Rhabdochonidae (*Rhabdochona*) is monophyletic and placed only in one of them; however, three species of the genera (*Salmonema* and *Spinitectus*) assigned so far to Cystidicolidae are also found in it. A sister group to this branch is mostly formed by representatives of Cystidicolidae along with those of Acuarioidea and the fish-parasitizing Physalopteroidea. Notable are also the mutual distance of *Ascarophis arctica* and *Ascarophis adioryx* and the location of *Metabronema magnum*. Whereas *Metabronema* and *Cystidicola* include swimbladder parasites of fishes, species of *Ascarophis* and *Neoascarophis* are parasites of the digestive tract (stomach). An interesting finding is also the location of *Salmonema ephemeridarum*, a freshwater parasite of salmonids whose morphology and biology are very similar to those of the marine species of *Ascarophis*. In the second line, a marked separation of *Rhabdochona mazeedi* from the other studied representatives of this genus is observed; this evidently indicates that this species belongs to the subgenus *Globochona*, whereas others to the nominotypical subgenus *Rhabdochona*. Such a position of the representative of *Globochona* suggests that this taxon should be elevated to genus. The phylogenetic tree also shows a genetic difference between the population of *Rhabdochona hellichi* in European barbels (*Barbus*) and that from fishes of the genus *Schizothorax* in the Oriental Region; both populations are now taken for independent subspecies *R. hellichi hellichi* and *R. hellichi turkestanica* (see Moravec et al. 2010), but may actually represent separate species.

In the herein established phylogenetic trees, low-supported branches are found in one or both analyses. Furthermore, topology mutually differs in several cases, as well as the formation of polytomy. A possible solution of this problem could be the involvement of other, more variable genes (e.g., LSU, ITS1 and ITS2) in the analyses, which might bring phylogenetically relevant information.

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