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Short Communication

Molecular phylogeny of slug-parasitic nematodes inferred from 18S rRNA gene sequences

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ABSTRACT

Terrestrial molluscs are diverse and are infected by many nematodes. We propose a phylogeny of slug-parasitic nematodes using 18S rRNA gene sequences from nematodes isolated from slugs collected from six countries. Eight species, representing six families of nematodes were identified and trees inferred placed them within four (I, III, IV and V) out of the five clades of Nematoda, indicating multiple origins of slug parasitism. Five species representing three families formed a monophyletic group in clade V. Although these species are closely related, their morphology has changed greatly, suggesting adaptive radiation to fill different niches within the host.

1. Introduction

The phylum Nematoda is one of the most abundant and diverse invertebrate groups in the world (Megen et al., 2009). Classification within this phylum can be troublesome due to lack of reliable morphological characters; however this has been overcome with the development of molecular phylogenetics. Blaxter et al. (1998) were the first to produce the molecular phylogeny of the phylum using rRNA gene sequences and proposed that the pylum was divided into five separate clades. More recent work has further subdivided the phylum into 12 clades (Holterman et al., 2006). Although the classification of the phylum continues to develop, there remains a lack of data on the phylogenetic positions of certain ecologically important groups. One such group is the nematodes that infect terrestrial molluscs. Lack of such nematodes has been implicated as an important factor in the invasion of European slugs into the USA (Ross et al., in press) and one species, *Phasmarhabditis hermaphrodita*, has been commercialised as a biological molluscicide and is sold throughout Europe (Rae et al., 2007). Current understanding of mollusc/nematode associations is based on surveys of Germany (Mengert, 1953), France (Morand, 1988), Maine, USA (Gleich et al., 1977) and Australia (Charwat and Davies, 1999), along with numerous individual descriptions of mollusc–parasitic nematodes from around the world. Although nematodes are known to parasitize both slugs and snails, slugs are parasitized more frequently and by a greater diversity of parasites than snails (Mengert, 1953) due to their increased exposure to nematodes in the soil. There are a total of seven families of nematodes known to be associated with slugs including, Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (particularly the genus *Phasmarhabditis*). These families are known to form a number of different relationships with slugs, including parasitic (specialist or generalist), phoretic and necromenic associations.

The present paper proposes a phylogeny of nematodes that form parasitic associations with slugs using 18S rRNA gene sequences from nematodes isolated from slugs in Belgium, Chile, Norway, Slovenia, UK and USA. Interpretation of these data will elucidate the relationship between these distinct taxonomic groups of nematodes and attempt to solve the unanswered question regarding the number of times the acquisition of parasitism in slugs has occurred.

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2. Materials and methods

2.1. Specimen collection

Slugs were collected from sites in Belgium, Chile, Norway, Slovenia, UK and USA. All slugs were rinsed to remove surface-dwelling nematodes then dissected and examined for the presence of internal nematodes. Larvae of *Alloionema appendiculatum* living on foot of slugs were picked after an infected slug was dissected and pedal musculature was relaxed allowing the nematodes to recover.

2.2. DNA extraction

DNA extraction and Polymerase Chain Reaction was conducted by two teams using different protocols. The team in Russia (Method A) sequenced specimens collected in Belgium, while the team in Scotland (Method B) sequenced samples collected in Chile, Norway, Slovenia and USA. Samples collected in the UK were analysed by both teams using Methods A and B.

Method A: Live nematodes were picked individually and homogenised in Eppendorf tubes containing 8 μl of worm lysis buffer (100 mM KCl, 20 mM Tris–HCl pH 8.3, 3 mM MgCl2, 2 mM DTT and 0.9% Tween 20), 10 μl double distilled water and 2 μl Proteinase K (600 μg/ml) and incubated at 65°c then heated to 94 °C. 1–3 μl of resulting mixture was used as template in PCR reaction. Nematodes that were fixed in ethanol prior to DNA extraction, were transferred to 5 μl 0.25 M NaOH in Eppendorf tubes, centrifuged and incubated at 99 °C for 3 min. Samples were then cooled to room temperature and 1 μl 1 M HCl, 2 μl 0.5 M Tris–HCl (pH 8.0) and 1.25 μl of 2% Triton X-100 were added. The tubes were re-centrifuged and again incubated at 99 °C for 3 min. 0.4–0.8 μl of resulting mixture was used as DNA template for PCR reactions.

Method B: Individual nematodes stored in 70% ethanol were picked directly into 25 μl of 5% chelex and 5 μl of Proteinase K (600 μg/ml) in 0.2 ml tubes, then incubated at 60 °C for 30 min. The lysate was then heated at 94 °C for 10 min, stored at −20 °C for 20 min then centrifuged (8000 rpm) for 5 min. The supernatant was then removed, and 2 μl was then used as DNA template for PCR reactions.

2.3. Polymerase chain reaction

Method A: PCR used the 18S universal primer pairs: G18S4, 26R, 24F and 18P (Table 1). PCR cycling parameters for amplification of 18S rRNA included primary denaturation at 94 °C for 5 min followed by 34 cycles of 94 °C for 60 s, 54 °C for 90 s, and 72 °C for 1 min, followed by a final 72 °C for 10 min. The PCR reaction products were visualized on 1% agarose gels and bands were excised for DNA extraction with Qiagen® gel extraction and product cleaning kits.

Method B: Nematodes underwent PCR with the same 18S universal primer pairs as Method A, but included an additional primer pair 22F and 1080JR to obtain complete coverage of the 18S region (Table 1). The mermithid nematode (isolated in the UK) underwent PCR with different primers from all other nematodes. These included; 55F, 920DR, 555F and 1165SR, 18s5F and 18s9R (Table 1). PCR cycling parameters for Method B involved primary denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 60 s, 55 °C for 90 s, and 72 °C for 2 min, followed by a final 72 °C for 10 min. The PCR reaction products were visualized in 1% agarose gel. Successful PCR's were cleaned up using Qiagen QIAquick® PCR Purification Kit.

Table 1
18S rRNA gene primers used for PCR and sequencing.

Primer name	Sequence (5′–3′)	Reference
G18S4	GCTTGTCTCAAAGATTAAGCC	Blaxter et al. (1998)
26R	CATTCTTGGCAAATGCTTTCG	Blaxter et al. (1998)
22F	TCCAAGGAAGGCAGCAGGC	Blaxter et al. (1998)
1080JR	TCCTGGTGGTGCCCTTCCGTCAATTTC	Present study
24F	AGRGGTGAAATYCGTGGACC	Blaxter et al. (1998)
18P	TGATCCWKCYGCAGGTTCAC	Blaxter et al. (1998)
55F	GCCGCGAATGGCTCGGTATAAC	Present study
920DR	CTTGGCAAATGCTTTCGCAG	Present study
555F	AGCCGCGGTAATTCCAGCTC	Present study
1165SR	CGTGTTGAGTCAAATTAAGCCGCAGG	Present study
18s-5F	GCGAAAGCATTTGCCAAGAA	Vandergast and Roderick (2003)
18s-9R	GATCCTTCCGCAGGTTCACCT	Vandergast and Roderick (2003)

2.4. DNA sequencing

Sequences of purified PCR products were obtained in both directions using the same primer pairs for PCR (Table 1) by cycle sequencing using ABI PRISM BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) and electrophoresis on a 310 Applied Biosystems Automated Sequencer.

2.5. Phylogenetic analysis

Sequence traces were checked for quality and then assembled automatically using Sequencer 4.1 (Genes Codes Corp. Ann Arbor, Michigan, USA). Sequences were uploaded onto the GenBank Database, at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Accession numbers can be found in Table 2. Additional nematode sequences were downloaded providing a total of 127 nematode 18S rRNA gene sequences for analysis (Table S1).

Five separate datasets were compiled and then aligned manually using BioEdit Sequence Alignment Editor (Hall, 1999) before removing regions of ambiguous alignment. Dataset 1 used the framework proposed by Blaxter et al. (1998) and included taxa sampled from across the phylum Nematoda (Table S1) and used 780 aligned characters. Datasets 2–5 represented clades I, III IV and V respectively (no slug-parasitic nematodes were found in clade II) and these analyses used 1275, 1545, 915 and 1170 aligned characters, respectively.

Phylogenetic analyses of the five datasets were performed on unambiguously aligned positions only. For each individual alignment, maximum-likelihood (ML) (PHYML (Guindon and Gascuel, 2003)), distance (PHYLIP (Felsenstein, 2007)) and maximum parsimony (MP) (PHYLIP) phylogenies were constructed. Alignments were analysed using Modeltest and the general-time reversible (GTR) model was used with among-site rate heterogeneity modelled using an eight-category gamma correction with a fraction of invariant sites calculated from ML analysis. Bootstrap support was calculated for all analyses using 1000 replicates.

3. Results

A total of 56 18S rRNA gene sequences were generated (Table 2). These sequences represented eight species from six families (Table 2). Sequences of the same species were identical across the 18S rRNA gene, so only one representative sequence was submitted for each taxon (Table S1/Table 2). Phylogenetic analysis showed that four (I, III, IV and V) out of the five clades of Nematoda proposed by Blaxter et al. (1998) contained nematodes that were parasitic in terrestrial slugs (Fig. 1).

Table 2
Slug-parasitic nematodes isolated from terrestrial slugs collected in Belgium, Chile, Norway, Slovenia, UK and USA.

Species	Host	Locality	Accession number
Family: Agfidae			
Agfa flexilis	*Limax flavus*	UK	EU573704
	Deroceras reticulatum		
	Arion hortensis		
	Limax flavus	USA	
	Limax maximus		
	Limas marginatus		
Family: Alloionematidae			
Alloionema appendiculatum	*Arion lusitanicus*	Belgium	EU573707
	Arion lusitanicus	Slovenia	
	Arion ater	USA	
	Arion lusitanicus	Norway	
	Arion flagellus	UK	
Family: Angiostomatidae			
Angiostoma limacis	*Arion distinctus*	UK	EU573705
Angiostoma dentifera	*Limax flavus*	USA	FJ516752
	Limax maximus		
	Limas marginatus		
Family: Cosmocercidae			
Cosmocercoides dukae	*Deroceras panormitanum*	USA	FJ516753
	Arion subfuscus		
Family: Mermithidae			
Mermithid sp.	*Deroceras caruanae*	UK	FJ982324
Family: Rhabditidae			
Phasmarhabditis hermaphrodita	*Deroceras reticulatum*	UK	FJ516755
	Arion ater		
	Arion lusitanicus	Norway	
	Deroceras reticulatum	Chile	
Phasmarhabditis neopapillosa	*Deroceras reticulatum*	UK	FJ516754
	Deroceras panormitanum		
	Arion ater		
	Arion dsitinctus		

3.1. The relationship of slug-parasites to other nematodes in clades I, III, IV and V

Trees inferred from maximum-likelihood (ML), maximum parsimony (MP) and distance analysis revealed similar topologies. Therefore only maximum-likelihood results are presented (Fig. 2) and a comparison of the three methods of analysis is discussed.

3.1.1. Clade I

The mermithid nematode clustered with another unclassified Mermithid sp. (97%/98%/98%) in all three topologies (Fig. 2a). These genera were a sister group to *Mermis nigrescens*, Mermis sp., Mermithid sp. and *Isomermis lairdi*. Strong bootstrap support was observed for this clade in distance (99%), MP (100%) and ML (99%) trees. In all topologies, Mermithida were found to cluster with a group of mononchids (algivores, omnivores, predators) including; *Mylonchulus arenicolus*, *Mylonchulus* sp., *Clarkus papillatus*, *Anatonchus tridentatus*, *Mononchus truncates* and *Mononchus tunbridgensis*.

3.1.2. Clade III

Cosmocercoides dukae was found to cluster together with the snail parasite *Nemhelix bakeri,* in all three methods of analysis (Fig. 2b). Ninety-six percent bootstrap support was observed for this clade in distance and ML trees and 97% in MP trees. In all three topologies, these genera (*C. dukae* and *N. bakeri*) were found to cluster with *Raillietnema* sp., however only under weak bootstrap support (57%/58%/58%). In distance, ML and MP analysis, these genera were found to cluster with members of the animal parasitic orders Ascaridida and Spirurida.

3.1.3. Clade IV

Alloionema appendiculatum was the only slug-parasite found within this clade and its relationships with other members of the clade depended on the method of analysis used. In all topologies, *A. appendiculatum* clustered with (100% support) *Strongyloides* and *Rhabditophanes* (Fig. 2c). Under ML (53%) and MP (53%) analysis, these three genera were members of a wider bacterial-feeding panagrolaimid clade, which formed a sister group to the fungivore genus *Bursaphelenchus*. Conversely in distance analysis, the genera *Alloionema, Strongyloides* and *Rhabditophanes* were a sister group to entomopathogenic nematodes of the genus *Steinernema* (52%).

3.1.4. Clade V

Clade V contained more slug-parasites than all other clades combined. Three separate families (Agfidae, Angiostomadiae and Rhabditidae) comprising three genera, represented by five species (*Angiostoma limacis*, *A dentifera, Agfa flexilis, P. hermaphrodita* and *Phasmarahbditis neopapillosa*) were found. Despite their taxonomic diversity, all slug-parasites in this clade clustered in a strongly supported monophyletic clade (93% support for ML, 95% for distance and 96% for MP).

This slug-parasitic group clustered with *Pellioditis typica* in the phylograms obtained in all three methods of analysis (Fig. 2d), however only under weak bootstrap support (42%/41%/43%). While bootstrap support is weak, in light of the work of Kiontke et al. (2007) it is quite likely that *P. typica* does indeed represent the closest free-living relative and thus, is important in determining the origin of parasitism. These authors found greater than 80% support for this relationship when other nuclear genes were included in the analysis. This group clustered together with a set of Rhabditidae genera including *Cephaloboides, Cruznema, Oscheius* and *Rhabditella* (66%/66%/67%). All the aforementioned genera were a sister group to two nematode taxa trophically associated with animals, the entomopathogenic nematode family Heterorhabditidae and the vertebrate-parasitic strongylids (96%/98%/97%).

4. Discussion

A total of six out of the seven families of nematodes known to be associated with terrestrial slugs were included in this study (we found no members of the Diplogasteridae). Analysis of 18S rRNA gene sequences reveal that four (I, III, IV and V) out of the five clades of Nematoda proposed by Blaxter et al. (1998) contain nematodes that are parasitic to slugs, indicating multiple origins of slug parasitism (Fig. 1). In all cases, the slug-parasitic groups cluster closely with other groups of animal parasites (clades I, III, and IV) or nematodes that associate with invertebrates phoretically or necromenically (clade V).

4.1. The relationship of slug-parasites to other nematodes in clades I, III, IV and V

4.1.1. Clade 1

The undetermined mermithid nematode isolated in this study was found to cluster with other members of the Mermithidae family (Fig. 2a). The Mermithidae family are all parasites of invertebrates and can be extremely large nematodes, reaching up to 50 cm in length, but more usually between 1–10 cm (Stock and Hunt, 2005). Mermithidae are usually associated with arthropods, however there have been several noted accounts in molluscs (Théodoridès, 1965). *Hexamermis albicans*, which is a common par-

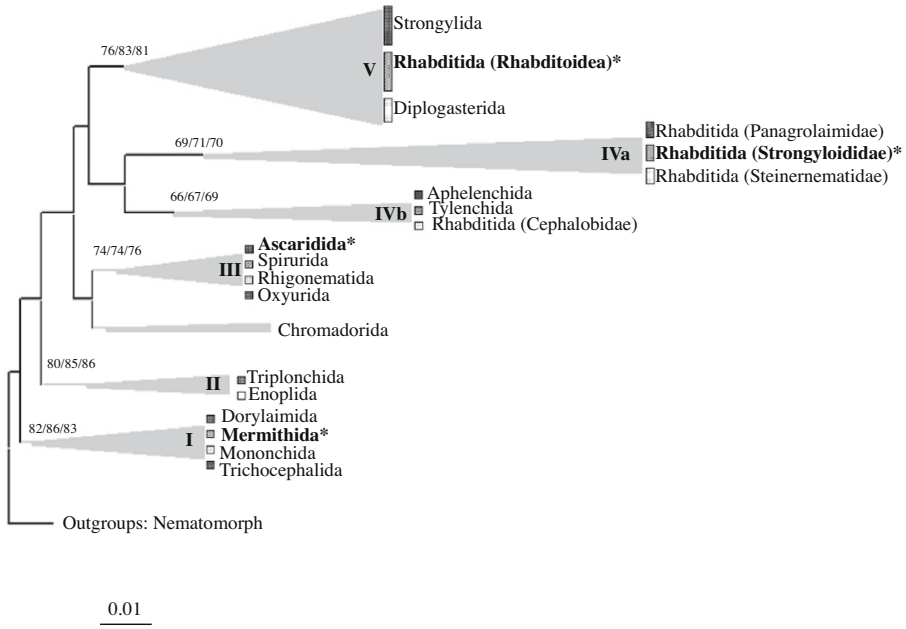

Fig. 1. Maximum-likelihood phylogenetic tree of 57 18S rRNA gene sequences showing the five major clades of nematodes (according to Blaxter et al. (1998)), four of which contain nematodes which are parasitic to slugs (marked by ∗). Phylogenetic analysis of 780 unambiguously aligned nucleotide positions used the GTR correction model with eight gamma-rates and invariable sites. Bootstrap support was calculated using maximum-likelihood, distance and maximum parsimony methods respectively (1000 replicates each). Only values above 65% are shown. Nematomorph was used as the outgroup.

asite of arthropods, has been recovered from the mollusc families Agriolimacidae and Succineidae (Théodoridès, 1965). Phylogenetic analysis showed that the Mermithida formed a sister group with the order Mononchida, which is a group of algivore–omnivore predators. These findings confirm the results of Megen et al. (2009) who studied the phylum using 1200 full-length small subunit ribosomal DNA sequences. However the results of this study disagree with Stock and Hunt (2005) who suggested that the closest evolutionary relatives of the Mermithidae are predacious dorylaimids.

4.1.2. Clade III

Unlike the other nematode clades that are trophically diverse, clade III contains only animal parasites. In this clade, *C. dukae* was found to cluster with the snail nematode *N. bakeri*, suggesting evolution from a single common ancestor (Fig. 2b). Both *C. dukae* and *N. bakeri* are obligate parasites of many terrestrial mollusc species (Anderson, 1960; Morand and Petter, 1986). These two species are atypical of the family Cosmocercidae which are predominantly parasites of amphibians and reptiles. The phylogenetic analysis showed that *C. dukae* and *N. bakeri* clustered with *Raillietnema* sp., however only under weak bootstrap support (49%/51%/49%). The poor resolution of the Cosmocercidae family (*N. bakeri, Raillietnema* sp. and *Cruzia Americana*) was also observed by Nadler et al. (2007). The Cosmocercidae family (*C. dukae*, *N. bakeri, Raillietnema* sp. and *C. Americana*) clustered with the orders Ascaridida and Spirurida, which are groups of vertebrate parasites.

4.1.3. Clade IV

Alloionema appendiculatum (the only slug-parasitic group represented in this clade) was found to cluster with other nematodes of the superfamily Strongyloidoidea that are predominantly parasites of vertebrates (Fig. 2c). These findings confirm the prediction of Dorris et al. (2002) who studied the genus *Strongyloides* and related nematodes but did not have a sequence for *A. appendiculatum*. Dorris et al. (2002) also commented on the close relationship between *Alloionema* and *Rhabditophanes* (the latter being free-living nematodes that are thought to have evolved from parasites). In all three methods of analysis, *Alloionema* was in a basal position to *Rhabditophanes* which clustered with *Strongyloides,* supporting the suggestion of Dorris et al. (2002) that the first parasites in this group used gastropod molluscs as hosts. Our data, thus, do not support the monophyly of the family Alloionematidae (as a group of *Alloionema* and *Rhabditophanes*).

It is not possible from our data to confidently infer the relationship between these three genera (*Alloionema + Strongyloides + Rhabditophanes*) and other members of the clade as our three phylogenetic constructions were inconsistent.

4.1.4. Clade V

The majority of slug-parasitic nematodes occurred within this clade (*A. flexilis, Angiostoma dentifera, A. limacis, P. hermaphrodita, Phasmarhabditis neopapillosa*). These five species formed a monophyletic clade when analysed with all three methods (Fig. 2d).

Although the genera *Agfa, Angiostoma* and *Phasmarhabditis* are molecularly conservative, their morphology has changed dramatically due to their parasitic way of life, and this is reflected by the fact that under morphological classifications, these nematodes were classified into three separate families (Agfidae, Angiostomatidae and Rhabditidae). The morphology of *Angiostoma* and *Agfa* as obligate parasites differs from free-living rhabditids in the development of a spacious stoma in *Angiostoma*, an extremely thin neck region in *Agfa* and an increase in body size in both genera. Conversely the facultative parasites *Phasmarhabditis* have retained features characteristic to other free-living rhabditids,

We believe the rapid morphological evolution within this group reflects adaptive radiation into different niches within host slugs, as the three nematode families all inhabit different anatomical regions within the slug. The slug-parasitic angiostomatids are obligate parasites that are found predominantly in the anterior region of the intestinal lumen (Ivanova and Wilson, 2009). The Agfidae are also obligate parasites that inhabit either the salivary glands or genital tract of the mollusc (Ribas and Casanova, 2002) whereas *Phasmarhabditis* spp. are found within the mantle cavity in close association with the slug's vestigial shell. Unlike *Agfa* and *Angiostoma, Phasmarhabditis* spp. are facultative parasites known to live on slug faeces and also leaf litter (Tan and Grewal,

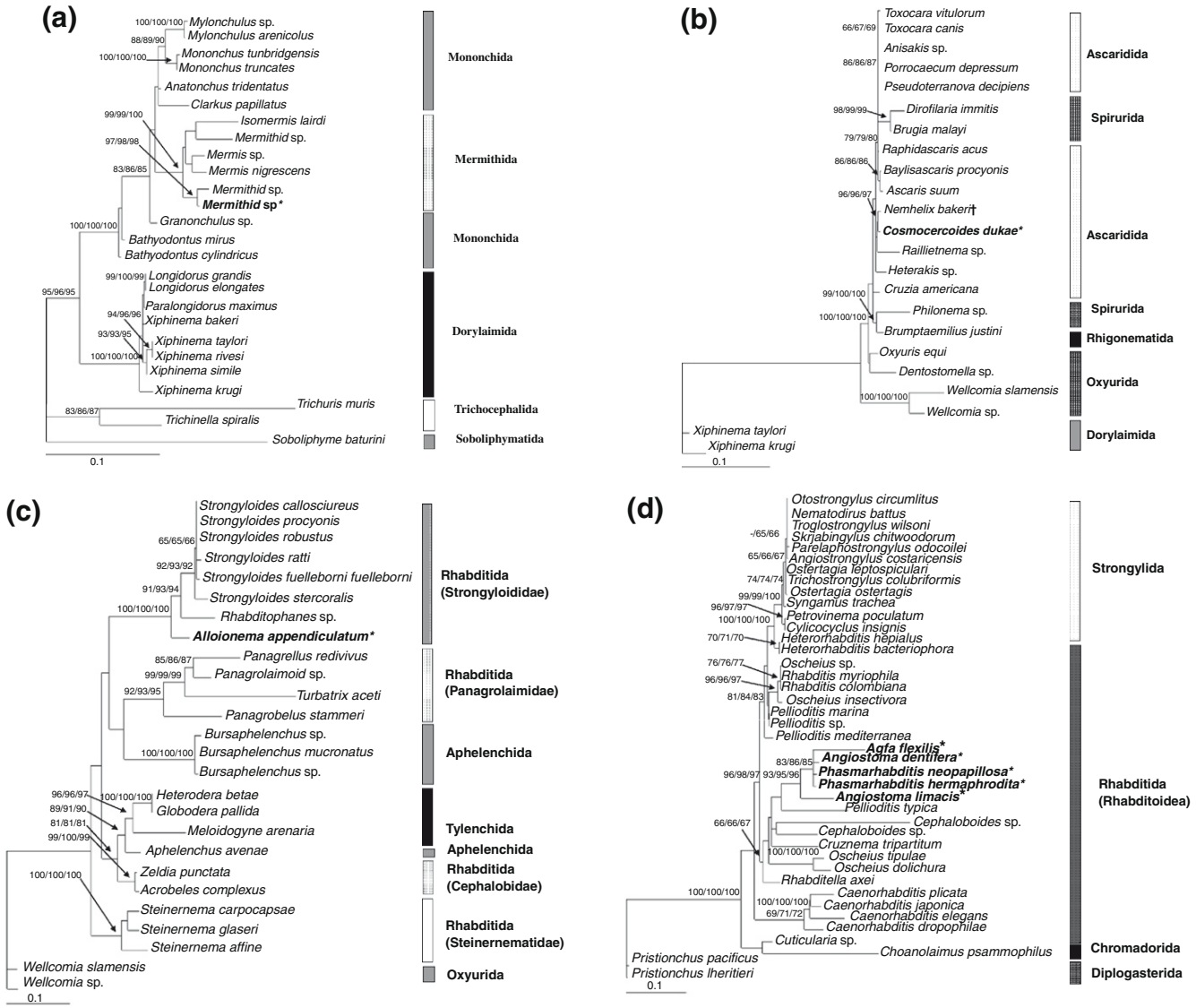

Fig. 2. Maximum-likelihood (ML) phylogenetic analyses of 18S rRNA gene sequences of nematode taxa sampled across clades I (a), III (b), IV (c) and V (d). Analyses were performed as described in Fig. 1, with bootstrap support calculated using maximum-likelihood, distance and maximum parsimony methods respectively (1000 replicates each). Only values above 65% are shown. Slug-parasitic nematodes obtained in this study are marked by ∗ and other mollusc nematode parasites are marked by †. Outgroups are *Soboliphyme baturini* for clade I, *Xiphinema taylori* and *Xiphinema krugi* for clade III, *Wellcomia slamensis* and *Wellcomia* sp. for clade IV *and Pristionchis lheritieri* and *Pristionchus pacificus* for clade V.

2001; MacMillen et al., 2009) and this may be why their morphology has remained more similar to other free-living rhabditid nematodes. The fact that all the well-studied clade V slug-parasites have a broad host range, but still inhabit the same niches irrespective of host, may add further support to our hypothesis.

While most *Angiostoma* spp. are mollusc parasites, four species have been described from the intestine and bronchi of amphibian and reptile hosts (Chitwood, 1933; Bursey and Goldberg, 2000; Bursey and Manire, 2006; Falcon-Ordaz et al., 2008). This has led Grewal et al. (2003) to believe that angiostomatids 'are parasites of amphibians and reptiles and use snails and slugs as obligatory intermediate hosts'. However, due to the large diversity of angiostomatids that have been recovered from mollusc hosts (12 species), we hypothesise that Angiostomatidae are specialized parasites of molluscs. The recovery of fully developed male and female stages from mollusc hosts would also indicate that slugs are not intermediate hosts (López et al., 2005). Further studies on the relationships between angiostomatids, molluscs are vertebrates are needed to confirm our hypothesis.

The slug-parasites of clade V cluster with a group of rhabditid genera including *Cephaloboides, Oscheius* and *Pellioditis* (66%/66%/67%). Based on molecular and morphological data it has been proposed that these genera should be considered subgenera within the '*Eurhabditis*' group of the genus *Rhabditis* (Sudhaus and Fitch, 2001). The *Eurhabditis* group includes many nematodes that associate with invertebrates such as *Oscheius* spp. that associate with insects and millipedes (e.g. *Oscheius insectivora, O. myriophila, O. necromena*) and some *Rhabditis* spp. that associate with earthworms (e.g. *Rhabditis guignardi* Maupas, 1900). Parasitic species that are closely related phylogenetically to the "*Eurhabditis*" group included the entomopathogenic nematodes (family Heterorhabditidae) and the vertebrate-parasitic strongylids.

5. Conclusion

This study presents new 18S rRNA gene sequences of slug-parasitic nematodes and provides an additional insight into the phylogeny and evolution of the phylum Nematoda. Four out of the

five clades of Nematoda include slug-parasitic nematodes, suggesting multiple origins of slug parasitism. However, the majority of slug-parasites were found in clade V where we showed for the first time that the families Agfidae, Angiostomatidae and the genus *Phasmarhabditis* formed a tight monophyletic group. However, while very closely related, these families are morphologically diverse, suggesting rapid evolution. We believe the morphological differences arise from adaptive radiation to fill different niches within the slug's body.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.026.

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