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Molecular Phylogeny Inferred from 18S rRNA Gene Sequences of Nematodes Associated with Cernuella virgata, a Pest Snail in Australia

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Abstract

Pest snails are economically important pests of the grain industry. Nematode based bioagent appears to be a hope for controlling pest snails in an environment friendly way. Based on the dataset of 18S rRNA gene sequences, we propose a molecular phylogeny of nematodes baited with *Cernuella virgata* in soils collected from southern states of Australia. A total of 12 species (representing eight genera of nematodes) were identified and the inferred phylogenetic trees (Neighbor-Joining and Minium Evolution) placed them within three (I, IV and VII) out of the seven clades, indicating the possibility of multiple origins of snail parasitism. In Clade I and Clade VII, nematodes associated with *Cernuella virgata* formed sister group relationships with some slug – parasitic nematodes. We assume that snail – parasitic nematodes and slug - parasitic nematodes might share common ancestors in their evolutionary histories.

Keywords: Phylogeny; 18S rRNA; *Diplogasterida*; *Panagrolaimida*; *Rhabditida*; *Nematode*; *Cernuella virgata*

Introduction

Nematode is one of the most abundant and diverse phylum in the animal kingdom [1]. Due to the lack of objective criteria for assessing homology of morphological characters regarding many nematodes, the systematics of this phylum has been contentious [2]. With the rapid development of molecular phylogeny, the evolutionary history of Nematoda was reassessed and new phylogenetic framework was pointed out [3-5]. Nevertheless, little is known about the phylogeny of nematodes associated with terrestrial gastropods, which are economically important invertebrates.

Ross et al. [2] reported the molecular phylogeny of slug-parasitic nematodes based on 18S rRNA gene sequences. A total of eight slug - parasitic nematode species (*Agfa flexilis, Alloionema appendiculatum, Angiostoma limacis, Angiostoma dentifera, Cosmocercoides dukae, Mermithid sp., Phasmarhabditis Hermaphrodita and Phasmarhabditis neopapillosa*) from six families (*Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Mermithidae and Rhabditidae*) were included in their study. The resulting phylogenetic trees placed eight species within four (I, III, IV and V) out of the five clades of Nematoda, indicating multiple origins of slug parasitism. Five out of the eight nematode species were clustered within Clade V, forming a monophyletic group covering two families (*Agfidae, Angiostomatidae*) and one genus (*Phasmarhabditis*). By considering the morphological diversity among these families, they stated that rapid evolution had occurred during the evolutionary history of slug – parasitic nematodes.

While the phylogeny of slug – parasitic nematodes was studied, the phylogeny of snail – parasitic nematodes remains unclear. One of the reasons is that few scientific data are available regarding the snail – parasitic nematodes around the world. Our understanding for the snail/nematode associations is mostly based on surveys conducted by Mengert [6] in Germany, Morand [7] in France, Gleich et al. [8] in USA and Charwat and Davies [9] in Australia. Currently the confirmed snail – parasitic nematodes is quite limited (e.g. *Angiostoma aspersae* (*Angiostomatidae*), *Phasmarhabditis hermaphroditaI* (*Rhabditidae*) and *Nemhelix bakeri* (*Cosmocercidae*) [10,11].

Terrestrial snails play a big role in agriculture and other industries. For examples, four introduced species of Mediterranean snails [*Cernuella virgata* (Da Costa), *Theba pisana* (Müller), *Cochicella acuta* (Müller) and *Cochicella barbara* (Linnaeus)], cause serious damage to the grain industry in Australia each year [12]. To control these pest snails efficiently and environment friendly, nematode – based biological control method was regarded as a priority among other options [9].

Effective use of nematodes requires knowledge of their relationships. Understanding the diversity of nematodes that are parasitic to terrestrial snails (especially for pest snails) and resolving the phylogeny of these nematodes will be useful to the development of nematode – based bioagent against pest snails.

In present study, we conducted a survey in southern Australia to screen nematode species with potentials as parasites of *C. virgata*. We also aim to solve the phylogenetic relationships of these nematode isolates using data from 18S rRNA gene sequences.

Materials and Methods

Soil sampling and nematode isolation

Samples were collected from 27 locations in South Australia, Victoria and New South Wales in Australia between August 2007 and September 2008 (Table 1). Sites were chosen based on accessibility and habitats. At each site, five to eight subsamples were taken with at least a two meters distance between them. Each subsample was obtained using a hand trowel from the top soil (10-15 cm deep). Approximately 0.5 kg soil was taken from each spot and was placed in separate polyethylene bag to minimize dehydration. Soil samples were stored in an ice box while being transported to the laboratory. Nematodes were isolated from each sample by baiting with nematode-free snails (*C. virgata*) as reported by Charwat and Davies [9], then placed in water for 24 hours to release nematodes.

Order	Family	Closest match in GENBANK	GENBANK accession numbers	Collecting site	Similarity (%)	Isolate number(s)
Diplogasterida	*Neodiplogasteridae*	*Pristionchus americanus* isolate 1373	FJ040445	Portland, Western Victoria	97	4211
				Mt Gambier, South Australia	98	4611
				Naracoorte, South Australia	97	4711
				Myponga, South Australia	98	4712
				Waikerie, South Australia	98	5011
		Pristionchus lheritieri isolate ED2088	AF430477	Cooma, Snowy Mountains NSW	91	3923
		Pristionchus pacificus strain PS312	AF083010	Henty, Riverina NSW	94, 94	2921, 2923
				Culcaim, Riverina NSW	95	3012
				Kiandra, Snowy Mtns NSW	95, 95, 95	3811, 3812, 3813
				Adaminaby, Snowy Mtns NSW	95, 95, 95	3814, 3815, 3816
				Heywood, Western Victoria	94	4013
		Mononchoides striatus strain MonEStr	AY593924	Adaminaby, Snowy Mtns NSW	90, 91, 91, 91	3912, 3826, 3914, 3915
				Cooma, Snowy Mtns NSW	90, 96	3922, 3926
Panagrolaimida	*Cephalobidae*	*Acrobeloides bodenheimeri* strain PS1158	AF202159	Yorketown, Yorke Peninsula SA	97	5512
		Acrobeloides butschlii strain DWF1107	EU543174	Currawarna, Riverina NSW	96, 97	1015, 0823
		Cephalobus persegnis isolate CephPer1	AY284662	Warooka, Yorke Peninsula SA	96	5211
Rhabditida	*Mesorhabditidae*	*Mesorhabditis* sp. JH-2004 isolate MRhaSp2	AY284660	Gobbagombalin, Riverina NSW	96	212
				Miniaton, Yorke Peninsula SA	98	5112
	Rhabditidae	*Oscheius tipulae* strain CEW1	EU196009	Malebo, Riverina NSW	98	431
				Cootamundra, Riverina NSW	97	2611
				Culcaim, Riverina NSW	96, 97	3013, 3021
				Uranquinty, Riverina NSW	97	3111
				Griffith, Riverina NSW	97, 98, 99	3312, 3323, 3312

				Leeton, Riverina NSW	95, 97, 98, 98	3524, 3522, 3521, 3523
				Narradera, Riverina NSW	97, 97	3621, 3623
		Oscheius sp. PS1131	OBU81587	The Rock, Riverina NSW	99	2822
				Albury, Hume Murray NSW	98	2911
		Rhabditis sp. DF5059	EU196007	Adaminaby, Snowy Mtns NSW	98	3821
				Port Fairy Western Victoria	98	4411
Rhabditida	*Heterorhabditidae*	*Heterorhabditis bacteriophora*	AF036593	Euberta, Riverina NSW	91	512

Table 1: Nematode isolates from this study.

DNA extraction

Nematode DNA was extracted from individual nematodes using a modification of the protocol described by Floyd et al. [13]. In brief, individual nematodes (adults or larvae) were transferred to a 0.2 ml Eppendorf tube containing 20 µl of 0.25 M NaOH, incubated at 25°C for 3-5 hours, then heated at 95°C in a Dri-Block heater (DB-2A: Techne Inc., Duxford UK) for 3 min. The resulting lysate was neutralized with 4µl (1 M) HCl and 10µl 0.5 M Tris-HCl (buffered at pH 8.0), then heated for 3 min at 95°C, followed by addition of 5 µl 2% Triton X-100. The final extract was stored at -20°C for later use.

Choice of DNA markers

Both nuclear and mitochondrial genes (18S rRNA, 28S rRNA, Cytochrome C oxidase I and 16S rRNA) were considered for study. 18S rRNA gene was chosen for three reasons. First, in pilot trials, PCR amplifications were obtained more reliably from 18S rRNA gene than from other candidate genes. Second, a large dataset of sequences was available on GENBANK or NemATOL for many species of nematodes across a range of taxonomic groups [3,13-15]. Third, this gene contains both conserved stem and highly divergent loop regions, making it suitable for taxonomic differentiation [13].

DNA amplification and sequencing

PCRs were conducted in 0.2 ml thin-walled Eppendorf PCR tubes. For each extract, 25 µl of reaction solution was prepared, containing 3µl extracted DNA, 5 µl 5x colourless GoTaq® reaction buffer, 2 µl 25 mM MgCl2, 2.5 µl 2 mM *deoxyribonucleotide* triphosphates (dNTPs), 0.04 units GoTaq® DNA Polymerase (*Promega*), 6.5 µl ddH2O, and 3 µl 2.5 µM each of the two primers: SSU18A (AAAGATTAAGCCATGCATG) and SSU26R (CATTCTTGGCAAATGCTTTCG) [3]. The thermocycling was performed on a PC -960C cooled thermal cycler (Corbett Research), with parameters of 94°C for 5 min, 35 cycles of 94°C for 45s, 56°C for 45s and 72°C for 1.5 min, and a final extension of 72°C for 10min, followed by a holding temperature of 15°C. The 3µl PCR products were visualized on agarose gels stained with ethidium bromide.

Sequences of purified PCR products were obtained from both directions using the same primer pairs for PCR. Sequencing reactions were performed with the Applied Biosystems BigDyeTM Terminator Ready Reaction Kit (Version 3.1) (Applied Biosystems Ltd). Final capillary separation was carried out at Australian Genome Research Facility Ltd (AGRF), where the samples were analysed using an AB3730xl (Applied Biosystems).

Phylogenetic analysis

Sequence traces were checked for their quality using the Trace Editor of MEGA v 4.0. [16]. A total of 47 DNA sequences were screened for their statistical similarities (positive matrix scores) with 18S rRNA gene sequences of identified nematodes in GENBANK by performing blast search [17]. Among the 12 identified groups, a single DNA sample was selected from each group to align with other 51 nematode 18S rRNA gene sequences that were downloaded from GenBank (Table 2). These additional nematode taxa were chosen based on their taxonomy positions and their relationships with terrestrial molluscs and other invertebrates. The alignments of these DNA sequences were conducted with Clustal X using the default parameters for gap opening and gap extension penalties [18]. A final 543 aligned characters were applied in the phylogenetic analysis. Neighbour-Joining (NJ) and Minimum Evolution (ME) trees were constructed with MEGA v 4.0 [16] using Kimura 2- parameter model. Gaps were treated as missing data in the analysis. The outgroup of *Tylenchus arcuatus* (*Chromadore*a, *Nematoda*) (Accession number: EU306349) was used to root the trees and for character polarization. Bootstrap support was calculated for all analyses using 1000 replicates.

Counting	Taxon (species name and strain and identification code)	Source material	Trophic ecology	GENBANK
1	*Acrobeles complexus*	GenBank	Bacteriovore	AY284671
2	*Acrobeloides bodenheimeri (5512)*	Current study	Bacteriovore	TBA
3	*Acrobeloides butschlii (0823)*	Current study	Bacteriovore	TBA

4	*Agfa flexilis*	GenBank	Invertebrate parasite	EU573704
5	*Alloionema appendiculatum*	GenBank	Invertebrate parasite	EU573707.
6	*Angiostoma dentifera*	GenBank	Invertebrate parasite	FJ516752
7	*Angiostoma limacis*	GenBank	Invertebrate parasite	EU573705
8	*Bathyodontus cylindricus*	GenBank	Bacteriovore	AY552964
9	*Brumptaemilius justini*	GenBank	Invertebrate parasite	AF036589
10	*Caenorhabditis dropophilae*	GenBank	Bacteriovore	AF083025
11	*Caenorhabditis elegans*	GenBank	Bacteriovore	AY268117
12	*Caenorhabditis japonica*	GenBank	Bacteriovore	AY602182
13	*Caenorhabditis plicata*	GenBank	Bacteriovore	AY602178
14	*Cephaloboides sp.*	GenBank	Bacteriovore	AF083027
15	*Cephalobus persegnis (5211)*	Current study	Bacteriovore	TBA
16	*Cosmocercoides dukae*	GenBank	Invertebrate parasite	FJ516753
17	*Cruznema tripartitum*	GenBank	Bacteriovore	CTU73449
18	*Cuticularia sp.*	GenBank	Bacteriovore	CSU81583
19	*Diploscapter coronatus*	GenBank	Bacteriovore	AY593921
20	*Heterorhabditis bacteriophora*	GenBank	Entomopathogen	FJ040428
21	*Heterorhabditis bacteriophora (0512)*	Current study	Bacteriovore	TBA
22	*Heterorhabditis hepialus*	GenBank	Entomopathogen	AF083004
23	*Isomermis lairdi*	GenBank	Invertebrate parasite	FN400900
24	*Mermis nigrescens*	GenBank	Invertebrate parasite	AF036641
25	*Mermis sp.*	GenBank	Invertebrate parasite	FJ973464
26	*Mermithid sp.*	GenBank	Invertebrate parasite	AY284743
27	*Mermithidae*	GenBank	Invertebrate parasite	FJ982324
28	*Mermithidae*	GenBank	Invertebrate parasite	FJ040480
29	*Mesorhabditis sp. (5112)*	Current study	Bacteriovore	TBA
30	*Mononchoides striatus*	GenBank	Bacteriovore	AY593924
31	*Mononchoides striatus (3912)*	Current study	Bacteriovore	TBA
32	*Nemhelix bakeri*	GenBank	Invertebrate parasite	DQ118537
33	*Oscheius dolichura*	GenBank	Bacteriovore	EU196010
34	*Oscheius insectivora*	GenBank	Bacteriovore	AF083019
35	*Oscheius sp.*	GenBank	Bacteriovore	OBU81587
36	*Oscheius sp. (3623)*	Current study	Bacteriovore	TBA
37	*Oscheius tipulae*	GenBank	Bacteriovore	EU196009
38	*Oscheius tipulae (3524)*	Current study	Bacteriovore	TBA
39	*Panagrellus redivivus*	GenBank	Bacteriovore	AF083007

40	*Panagrobelus stammeri*	GenBank	Bacteriovore	AF202153
41	*Panagrolaimus sp.*	GenBank	Bacteriovore	U81579.1
42	*Pellioditis marina*	GenBank	Bacteriovore	AF083021
43	*Pellioditis mediterranea*	GenBank	Bacteriovore	AF083020
44	*Pellioditis sp.*	GenBank	Bacteriovore	EU196011
45	*Pellioditis typica*	GenBank	Bacteriovore	PTU13933
46	*Phasmarhabditis hermaphrodita*	GenBank	Invertebrate parasite	FJ516755
47	*Phasmarhabditis neopapillosa*	GenBank	Invertebrate parasite	FJ516754
48	*Plectus acuminatus*	GenBank	Bacteriovore	AF037628
49	*Prismatolaimus intermedius*	GenBank	Bacteriovore	AY284729
50	*Pristionchus americanus (4611)*	Current study	Bacteriovore	TBA
51	*Pristionchus lheritieri (3923)*	Current study	Bacteriovore	TBA
52	*Pristionchus pacificus (3812)*	Current study	Bacteriovore	TBA
53	*Rhabditella axei*	GenBank	Bacteriovore	RAU13934
54	*Rhabditis colombiana*	GenBank	Bacteriovore	AY751546
55	*Rhabditis myriophila*	GenBank	Bacteriovore	RMU81588
56	*Rhabditis sp. (4411)*	Current study	Bacteriovore	TBA
57	*Rhabditophanes sp.*	GenBank	Bacteriovore	AF202151
58	*Steinernema affine*	GenBank	Entomopathogen	FJ040425
59	*Steinernema carpocapsae -*	GenBank	Entomopathogen	AF036604
60	*Steinernema glaseri*	GenBank	Entomopathogen	FJ040422
61	*Teratocephalus lirellus*	GenBank	Bacteriovore	AF036607
62	*Turbatrix aceti*	GenBank	Bacteriovore	AF202165
63	*Tylenchus arcuatus*	GenBank	Plant parasite	EU306349
64	*Zeldia punctata*	GenBank	Bacteriovore	ZPU61760

Table 2: Taxa used in present study for NJ and ME analyses.

Results

Nematode isolates

A total of 47 nematode isolates were obtained by baiting C. virgata in soils collected from southern states of Australia. The corresponding 18S rRNA gene sequences of these nematode isolates matched 12 nematode species listed in the GENBANK database (Table 1). Among of them, the most common species was *Oscheius tipulae* (14 isolates from 7 sites), followed by *Pristionchus pacificus* (10 isolates from 5 sites), *Mononchoides striatus* (6 isolates from 2 close sites) and *Pristionchus americanus* (5 isolates from 5 sites). According to the currently accepted classification of nematodes [19], the species fell into eight genera (*Pristionchus, Mononchoides, Acrobeloides, Cephalobus, Mesorhabditis , Oscheius , Rhabditis , Heterorhabditis*), and three orders (*Diplogasterida, Rhabditida* and *Panagrolaimida*) (Table 1).

Phylogenetic analysis

Phylogenetic trees (Neighbour Joining and Minimum Evolution) were constructed via phylogenetic analyses of 18S rRNA gene dataset arising from 64 taxa described above. In these phylogenetic trees, seven Clades were revealed and three out of them (clade I, IV and VII) contained nematodes associated with *C. virgata* (Figure 1, Figure 2).

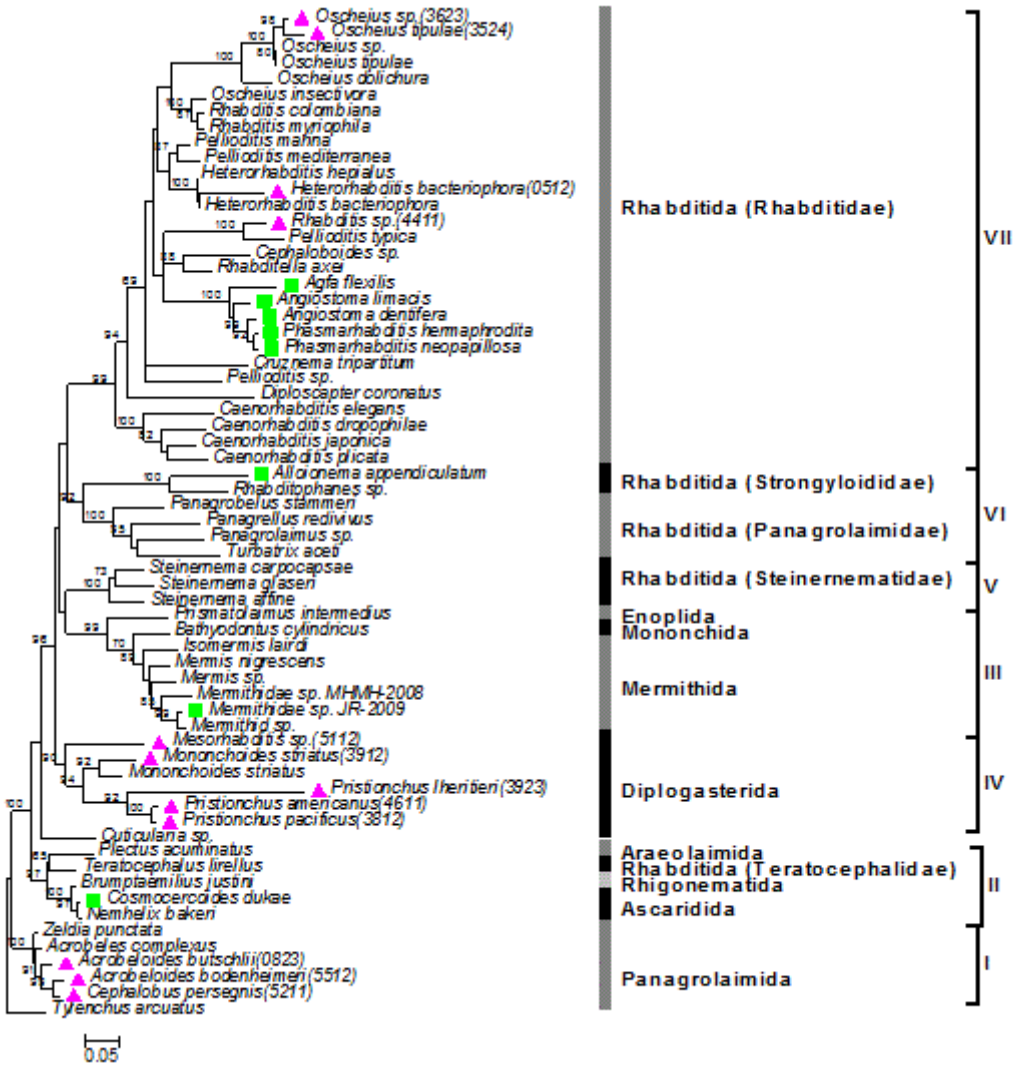

Figure 1: Minimum Evolution phylogenetic tree of 64 18S rRNA gene sequences showing the major seven Clades of nematodes, three of which contain nematodes which are potentially parasitic to *C. virgata*. Bootstrap support was calculated using Minimum Evolution method (1000 replicates each). Only values above 65% are shown. *Tylenchus arcuatus* was used as the outgroup. Pink triangle: potential snail – parasitic nematodes from current study; Green square: slug – parasitic nematodes.

Clade I: Three nematode isolations from the present study, *Acrobeloides butschlii* (0823), *Acrobeloides bodenheimeri* (5512) and *Cephalobus persegnis* (5211) were placed in Clade I (*Panagrolaimida*) in all topologies (Figure 1, Figure 2). Both NJ and ME trees depicted a sister-group relationship between these taxa and the other two members of *Panagrolaimida* (*Acrobeles complexus* and *Zeldia punctata*). This placement received very strong bootstrap support in both phylogenetic trees (100%).

Clade IV: In both NJ and ME trees, four nematode species from current study, *Mononchoides striatus* (3912), *Pristionchus americanus* (4611), *Pristionchus lheritieri* (3923) and *Pristionchus pacificus* (3812), were included in this clade IV (*Diplogasterida*). Among of them, P. *americanus* (4611), P. *lheritieri* (3923), and P. *pacificus* (3812) formed a monophyletic clade with strong bootstrap support (96% in NJ tree and 92% in ME tree). This monophyletic clade is nested within the Clade IV and sister to *Mononchoides striatus* (3912) and *Mononchoides striatus*. This result received very strong bootstrap support (98% in NJ tree and 94% in ME tree).

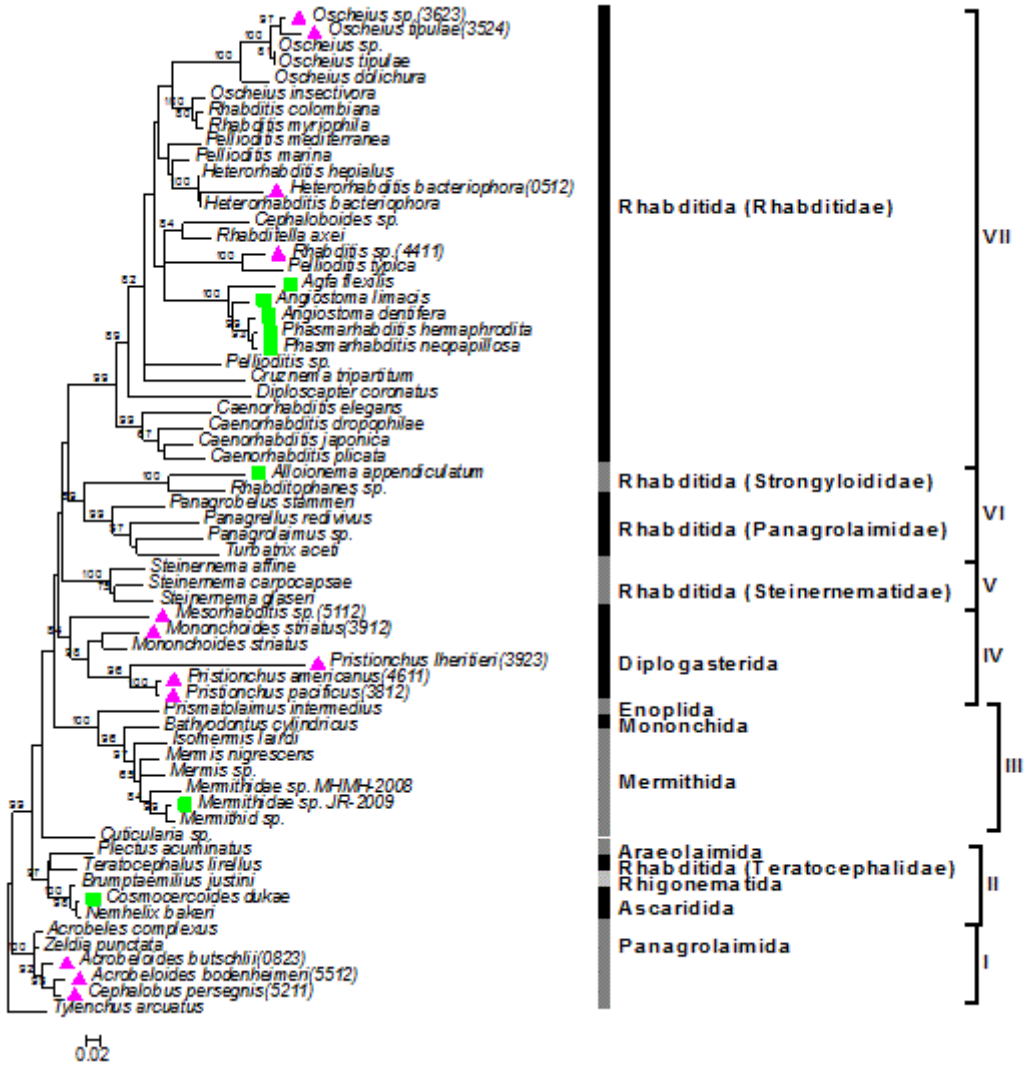

Figure 2: Neighbor: Joining (NJ) phylogenetic tree of 64 18S rRNA gene sequences showing the major seven Clades of nematodes, three of which contain nematodes which are potentially parasitic to *C. virgata*. Bootstrap support was calculated using Neighbor – Joining method (1000 replicates each). Only values above 65% are shown. *Tylenchus arcuatus* was used as the outgroup. Pink triangle: potential snail – parasitic nematodes from current study; Green square: slug – parasitic nematodes.

Clade VII: Four nematode isolations from the present study, *Heterorhabditis bacteriophora* (0512), *Oscheius tipulae* (3524), *Oscheius sp.* (3623) and *Rhabditis sp.* (4411), were placed in this clade (*Rhabditida*), the largest clade across all phylogenetic analyses. Among of them, *Oscheius tipulae* (3524) and *Oscheius sp.* (3623) formed a well-supported clade with three other members of *Oscheius* (*Oscheius sp., Oscheius tipulae* and *Oscheius dolichura*) (100% in both NJ and ME trees). *Heterorhabditis bacteriophora* (0512) was found to cluster with *Heterorhabditis bacteriophora* under weak bootstrap support. They were sister to *Heterorhabditis hepialus* and formed a clade with 100% bootstrap support across both phylogenetic trees. Instead of being clustered with other members of *Rhabditis*, *Rhabditis sp.* (4411) was found to cluster with *Pellioditis typica* in all phylogenetic trees with strong bootstrap support (100%). Both NJ and ME trees also depicted a sister – group relationship between these two species and other five slug – parasites (*Agfa flexilis, Angiostoma limacis, Angiostoma dentifera, Phasmarhabditis Hermaphrodita* and *Phasmarhabditis neopapillosa*).

Discussion

The present study revealed a total of 12 nematode species that are potentially associated with *C. virgata*, a pest snail in Australia. Phylogenetic analyses of 18S rRNA gene sequences placed these nematode species into three large groups: *Panagrolaimida*, *Diplogasterida* and *Rhabditida* (Figure 1, Figure 2), indicating the possible multiple origins of snail parasitism, which is similar to the findings of slug – parasitic nematodes [2].

The relationship of potential snail parasites in relation to other nematodes in clade I, IV and VII

Clade I: In present study, phylogenetic analyses recovered a monophyletic clade I (Panagrolaimida), which includes *Acrobeloides bodenheimeri* (5512), *Acrobeloides butschlii* (0823), *Cephalobus persegnis* (5211), and two other members of *Cephalobidae*: *Zeldia punclata* and *Acrobeles complexus*. This finding is in consistent with the results of Nadler et al. [20], who confirmed the monophyly of cephlobids at superfamily level based on phylogenetic analyses of ribosomal (LSU) sequences data.

Cephalobidae include a diverse array of species ranging from soil dwelling microbivores to parasites of vertebrates and invertebrates [20]. The phylogeny of genera within *Cephalobidae* (such as *Acrobeloides*, *Cephalobus*, *Chiloplacus*, *Eucephalobus* and *Pseudacrobeles*) has been in controversy [20]. Molecular trees did not support traditional genera as natural group [20]. Similarly, morphological characters traditionally applied for distinguishing most genera (e.g. labial variations) were not regarded as diagnostic with the discovery of increasing new species [21]. Such a controversy was reflected in present study. Both NJ and ME trees did not support *Acrobeloides* and *Cephalobus* as monophyletic groups.

The NJ and ME trees also depicted a closely related relationship between Clade I and a group including the slug - parasite (*Cosmocercoides dukae*) and the snail – parasite (*Nemhelix bakeri*) (Figure 1, Figure 2), indicating the possibility of a common ancestor between these nematodes.

Clade IV: The monophyly of clade IV (*Diplogastropoda*), which includes *Pristionchus lheritieri* (3923), *Pristionchus americanus* (4611), *Pristionchus pacificus* (3812), *Mononchoides striatus* (3912) and *Mononchoides striatus*, was resolved through both NJ and ME analyses in present study. Strong bootstrap support was observed for this clade in NJ (98%) and ME (94%) trees (Figure 1, Figure 2). This finding is in consistent with the results reported by Fürst von Lieven [22], who constructed a robust cladogram for *Diplogastropoda* based on morphological data (e.g. the variable structures of the buccal cavity and the function of the stomatal structures).

Traditionally Diplogastropoda was regarded as a sister taxa of *Tylenchina* [23] because the morphology of pharynx between these two groups is very similar. Data from molecular and ultrastructure, however, strongly object the *Diplogasterida*/*Tylenchida* clade [3,24]. The close phylogenetic relationship between *Diplogastropoda* and *Tylenchina* is not supported by our results. Neither the NJ analysis nor the ME analysis indicated that *Diplogastropoda* is a sister taxa of *Tylenchina* (Figure 1, Figure 2).

Surveys conducted by Mengert [6], Morand [7], Gleich et al. [8], Charwat and Davies [9] indicated that some species within the Diplogastropoda might associate with terrestrial molluscs parasitically, phoretically or necromenically. The present study supported their findings but was inconsistent with Ross et al. [2], who found no members of the Diplogasteridae were parasitic to slugs.

Clade VII: Being the largest clade recovered from the present study, Clade VII (*Rhabiditidae*) includes 12 genera (*Agfa, Angiostoma, Caenorhabditis, Cephaloboides, Cruznema, Diploscapter, Heterorhabditis, Oscheius, Pellioditis, Phasmarhabditis, Rhabditella* and *Rhabditis*). While the monophyly of this clade (*Rhabditidae*) was strongly supported (99% in both NJ and ME trees), the monophyly of some genera within *Rhabditidae* was not fully supported. As described previously, four nematode isolates from present study, *Oscheius tipulae* (3524), *Oscheius sp.* (3623), *Rhabditis sp.* (4411) and *Heterorhabditis bacteriophora* (0512), were placed within this clade. *Oscheius tipulae* (3524) and *Oscheius sp.* (3623) were closely related with other members of *Oscheius* but separated from *Oscheius insectivora*; *Rhabditis sp.* (4411) was clustered with *Pellioditis typica* rather than with other members of *Rhabditis*. All these unexpected grouping indicate that additional data are needed to resolve the position of these genera.

Within the Clade VII, *Rhabditis sp.* (4411) formed a sister relationship with *Agfa flexilis, Angiostoma limacis, Angiostoma dentifera, Phasmarhabditis Hermaphrodita and Phasmarhabditis neopapillosa*. The latter five nematodes are all slug – parasites [2]. Such a connection strongly suggests the possibility that snail- parasitic nematodes might share a common ancestor with slug – parasitic nematodes.

The remaining nematode isolate, *Mesorhabditis* sp. (5112), was separated from other nematode isolates in both NJ and ME analyses (Figure 1, Figure 2). As a member of *Mesorhabditidae*, it was expected to cluster with other members of *Rhabditida*. However, it was actually sister to Clade IV (*Diplogasterida*) in all phylogenetic trees (84% in NJ and 90% in ME). Additional research is thus required to resolve the phylogenetic position of this taxon.

Other phylogenetic finding in term of nematode phylogeny incurred from this study

While recovering the phylogenetic positions of our 12 nematode isolates, the resulting NJ and ME trees also presented enlightenments on the phylogeny of other nematode taxa.

Mermithida are a group of insect – parasitic nematodes. They are usually associated with arthropods but were also found to be parasites of Molluscs [25]. Our analyses resolved the monophyly of *Mermithda* (89% in ME tree and 97% in NJ tree). The phylogenetic trees also had moderate to strong support to the sister group relationship between *Mermithida* and *Monochida* (70% in ME tree and 96% in NJ tree). These findings are in consistent with other author's results Megan et al. [1] and Ross et al. [2] but disagree with Stock and Hunt [26], who placed the *Mermithidae* as a sister group to the plant - parasitic Dorylaimids.

Another clade that was proved to be monophyletic is *Sterinernematidae* (100% for both NJ and ME). *Sterinernematidae* is a family of entomopathogenic nematodes (EPN) [27]. It shares similar life history with the other family of entomopathogenic nematodes (*Heterorhabditidae*) (such as killing insects by realising symbiotically associated bacteria into the hemocoel of insects), but has distantly related phylogenetic relationship with *Heterorhabditidae* [28]. This situation was reflected in our phylogenetic analyses: the members of *Heterorhabditidae* (*Heterorhabditis bacteriophora* and *Heterorhabditis hepialus*) were placed in clade VII while the member of *Sterinernematidae* formed a separate clade (clade V) across both NJ and ME trees.

The inferred phylogenetic trees also showed that *Sterinernematidae* was more closely related to a clade including most *Panagrolaimidae* (free-living and insect associates). Both NJ and ME trees strongly supported the monophyly of *Panagrolaimidae* (100% in ME and 99% in NJ). These results are in consistent with the finding reported by Adam et al. [29].

Are these nematodes really snail parasites?

By using *C. virgata* as baiting material, we found that 12 nematodes species *Acrobeloides butschlii* (0823), *Acrobeloides bodenheimeri* (5512), *Cephalobus persegnis* (5211), *Mononchoides striatus* (3912), *Pristionchus americanus* (4611), *Pristionchus lheritieri* (3923), *Pristionchus pacificus* (3812), *Heterorhabditis bacteriophora* (0512), *Oscheius tipulae* (3524), *Oscheius sp.* (3623), *Rhabditis sp.* (4411) and *Mesorhabditis sp.* (5112)] were potentially associated with *C. virgata*, a pest snail in Australia. Although it is hard to seek testable evidence to confirm this finding, the hypothesis of these nematodes (or some of them) as potential parasites of *C. virgata* is justified as below.

Reports about bacterivorous nematodes being developed as bioagent against pest slugs (e.g. P. *hermaphrodita*) have been published [30,31]. From the point of ecological view, all our nematodes isolates fall into the category of free-living bacterivorous nematodes (FLBN). The close relationship between some of our nematodes isolates with some slug parasites were also revealed by the phylogenetic analyses conducted in present study. In this respect, we could not deny the potentiality that bioagents against pest snails such as *C. virgata* can be developed from these nematode isolates.

All parasitic nematodes were originally evolved from free living nematodes [3]. Parasitism of plants and animals has evolved independently at least nine times in the history of the nematodes [14]. The adoption of parasitism in nematodes probably required either the adaptation of genes present in their free-living ancestors or horizontal gene transfer from bacteria and/or fungus in their environment [32-35]. Given the fact that our nematode isolates are bacterivirous, and have been isolated from the cadavers of pest snails (*C. virgata*), it is likely that they could acquire “parasitism genes” from bacteria in their environment, and become parasites of pest snails at some stages of their life cycle.

Identification of some "parasitism genes" by examining the expression pattern of their *C. elegans* orthologs at certain stage of development (e.g. the third larval stage) would be useful in assessing the parasitism of nematodes [32,36]. Further pathogenicity tests are now underway to assess the biocontrol potential of these nematode isolates.

Conclusion

This study presents the molecular phylogeny of nematodes baited from the pest snail of *C. virgata* in Australia. Both NJ and ME trees constructed based on the dataset of 18S rRNA gene sequences placed 12 nematode isolates into three out of seven Clades (I, IV and VII), suggesting the possibility of multiple origins of snail parasitism. In Clade I and Clade VII, nematodes associated with *C. virgata* formed sister group relationships with some slug – parasitic nematodes. We assume that snail – parasitic nematodes and slug – parasitic nematodes might share common ancestors in their evolutionary histories.

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