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 Minimum 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; Diplagasterida; 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 (Agta flexilis, Allionema appendiculatum, Angiostoma limacis, Angiostoma dentifera, Cosmocercoides dukae, Mermithid sp., Phasmharbatiis hermpheodrida and Phasmharbatiis neapoliposa) from six families (Agidae, Allionematidae, 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 (Agidae, Angiostomatidae) and one genus (Phasmharbatiis). 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), Phasmharbatiis hermpheodrida (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     | Neodiplogasterida       | 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, 95         | 3922, 3926       |
| Panagrolaimida     | Cephalobidae            | Acrobateloboides bodenheimeri strain PS1168 | AF202159                  | Yorketown, Yorke Peninsula SA    | 97             | 5512             |
|                    |                         | Acrobateloboides butschilli 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             |
|                    |                         | 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 |
modification of the protocol described by Floyd et al. [13]. In brief, making it suitable for taxonomic differentiation [13].

neutralized with 4µl (1 M) HCl and 10µl 0.5 M Tris-HCl (buffered at (AAAGATTAAGCCATGCATG) and SSU26R

For each extract, 25 µl of reaction solution was prepared, containing (CATTCTTGGCAAATGCTTTCG) [3]. The thermocycling was

amplifications were obtained more reliably from 18S rRNA gene than

Cytochrome C oxidase I and 16S rRNA) were considered for study.

° for 3-5 hours, then heated at 95

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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 arculatus (Chromadorea, 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        | Acrobeles bodenheimeri (5512)                        | Current study  | Bacteriovore   | TBA     |
| 3        | Acrobeles butschlii (0823)                           | Current study  | Bacteriovore   | TBA     |
|   | Species                                | Database | Type                  | Accession |
|---|----------------------------------------|----------|-----------------------|-----------|
| 4 | Agla flexilis                          | GenBank  | Invertebrate parasite | EU573704  |
| 5 | Alioonema 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)            | GenBank  | Invertebrate parasite | TBA       |
| 16| Cosmocercoides dukes                   | GenBank  | Invertebrate parasite | FJ516753  |
| 17| Cruznema tripartitum                   | GenBank  | Bacteriovore          | CTU73449  |
| 18| Cucicularia sp.                        | GenBank  | Bacteriovore          | CSU81583  |
| 19| Diploscapter coronatus                 | GenBank  | Bacteriovore          | AY593921  |
| 20| Heterorhabditis bacteriophora          | GenBank  | Bacteriovore          | FJ040428  |
| 21| Heterorhabditis bacteriophora (0512)   | GenBank  | Bacteriovore          | TBA       |
| 22| Heterorhabditis hepialus              | GenBank  | Entomopathogen        | AF083004  |
| 23| Isomermis lairdi                      | GenBank  | Invertebrate parasite | FN400900  |
| 24| Memis nigrescens                      | GenBank  | Invertebrate parasite | AF036641  |
| 25| Memis 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)              | GenBank  | Bacteriovore          | TBA       |
| 30| Mononchoides striatus                 | GenBank  | Bacteriovore          | AY593924  |
| 31| Mononchoides striatus (3912)           | GenBank  | Invertebrate parasite | TBA       |
| 32| Nemhelix bakeri                       | GenBank  | Bacteriovore          | DQ118537  |
| 33| Oscheius dolichura                    | GenBank  | Bacteriovore          | EU196010  |
| 34| Oscheius insectivora                  | GenBank  | Bacteriovore          | AF083019  |
| 35| Oscheius sp.                          | GenBank  | Bacteriovore          | OBU81587  |
| 36| Oscheius sp. (3623)                   | GenBank  | Current study         | TBA       |
| 37| Oscheius tipulae                      | GenBank  | Bacteriovore          | EU196009  |
| 38| Oscheius tipulae (3524)               | GenBank  | Current study         | 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 hermaphroditais | GenBank | Invertebrate parasite | FJ516755 |
| 47 | Phasmarhabditis neopappillosa | GenBank | Invertebrate parasite | FJ516754 |
| 48 | Plectus acuminatus | GenBank | Bacteriovore | AF037628 |
| 49 | Pristionchus intermedius | GenBank | Bacteriovore | AY284729 |
| 50 | Pristionchus americanus (4611) | Current study | Bacteriovore | TBA |
| 51 | Pristionchus thertlent (3923) | Current study | Bacteriovore | TBA |
| 52 | Pristionchus pacificus (3812) | Current study | Bacteriovore | TBA |
| 53 | Rhabditella axeai | 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 | Tylenchus arcuatus | GenBank | Plant parasite | EU006349 |
| 62 | Tylenchus 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 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 Iheritieri* (3923) and *Pristionchus pacificus* (3812), were included in this clade IV (*Diplogasterida*). Among of them, *P. americanus* (4611), *P. Iheritieri* (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*. This result received very strong bootstrap support (98% in NJ tree and 94% in ME tree).
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 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].

![Image of phylogenetic tree](image-url)
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 punctata 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 phyllogy of genera within Cephalobidae (such as Acrobeloides, Cephalobus, Chiloplacus, Eucephalobus and Pseudacrobelus) 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 duka) 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 hirteri (3923), Pristionchus americanus (4611), Pristionchus pacificus (3812), Mononchoides striatus (3912) and Monochoioides 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 Först 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/Tylenchina 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 (Rhabditidae) includes 12 genera (Agla, Angiostoma, Caenorhabditis, Cephaloboides, Cruznema, Diploscapter, Heterorhabditis, Oscheius, Pelioditis, 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 tipulæ (3524), Oscheius sp. (3623), Rhabditis sp. (4411) and Heterorhabditis bacteriophora (0512), were placed within this clade. Oscheius tipulæ (3524) and Oscheius sp. (3623) were closely related with other members of Oscheius but separated from Oscheius insectivora, Rhabditis sp. (4411) was clustered with Pelioditis 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 Agla flexilis, Angiostoma limacis, Angiostoma dentiæ, Phasmaharbditidis Hermaphroditis and Phasmaharbditidis 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 Rhadinida. However, it was actually sister to Clade IV (Diplogastropoda) 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 Mermithida (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 [16], 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 hepalis) 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 persegna* (5211), *Monchohoides striatus* (3912), *Pristionchus americanus* (4611), *Pristionchus herteri* (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 test extable 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 (FLNB). 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 slugs 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 bacterivorous, 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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