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Phylogenetic relationships of three tribes of cloacinine nematodes (Strongylida: Chabertiidae) from macropodid marsupials

N.B. Chilton[1,2], F. Huby-Chilton[2], A. Koehler[2], R.B. Gasser[2] and I. Beveridge[2]

[1]Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada and [2]Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Victoria, Australia

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Author for correspondence:
I. Beveridge, E-mail: ibeve@unimelb.edu.au

Abstract

The phylogenetic relationships of 42 species of cloacinine nematodes belonging to three tribes (Coronostrongylinea, Macropostrongylinea and Zoniolaiminea) were examined based on sequence data of the first and second internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA. All nematodes examined are parasites of Australian macropodid marsupials. None of the three nematode tribes was monophyletic. Paraphyly was also encountered in three genera: *Papillostrongylus*, *Monilonema* and *Wallabinema*. Species within the genus *Thallostonema* were limited to a single host genus (i.e. *Thylogale*), whereas species within the five principal genera (*Coronostrongylus*, *Macropostrongylus*, *Popovastrongylus*, *Wallabinema* and *Zoniolaimus*) were found to occur in multiple host genera. Potential modes of evolution among these nematodes are discussed.

Introduction

The strongyloid nematode subfamily Cloacininae occurs exclusively in the stomach and oesophagus of macropodid marsupials and is currently represented by 39 genera and 285 species, with significant numbers of species as yet undescribed (Spratt & Beveridge, 2016). This subfamily is possibly the largest strongyloid nematode radiation in mammals (Beveridge & Chilton, 2001). The evolution of complex strongyloid radiations in both eutherian and marsupial herbivores has been a relatively long-standing area of research and speculation (Inglis, 1971; Chabaud & Durette-Desset, 1978; Kennedy & Bush, 1992). However, co-phylogenetic studies of these nematodes and their macropodid marsupials are lacking, as this requires molecular phylogenies for both the parasites and their hosts.

Kangaroos and wallabies represent a speciose group (63 species) that are parasitized by a large number of described nematode species (285) (Spratt & Beveridge, 2016). These hosts therefore provide significant opportunities to test hypotheses concerning the evolution of complex nematode communities, provided that a sound molecular phylogeny exists for the nematodes. Here we examine the phylogenetic relationships of three tribes of cloacinine nematodes from macropodids.

Four tribes were recognized within the Cloacininae by Lichtenfels (1980); namely, the Cloacininea, Macropostrongylinea, Pharyngostrongylinea and Zoniolaiminea. Beveridge (1983) subdivided the Zoniolaiminea, erecting the new tribe Labiostrongylinea, and subsequently (Beveridge, 1986a) subdivided the Macropostrongylinea, erecting another new tribe, Coronostrongylinea, thus resulting in six tribes. The tribes Macropostrongylinea and Coronostrongylinea are each defined by morphological synapomorphies (Beveridge, 1986a). The former tribe is defined by the possession of a poorly sclerotized buccal capsule with prominent surrounding musculature, whereas the latter is defined by a buccal capsule consisting of a reduced outer, sclerotized layer and an inner non-sclerotized layer (Beveridge, 1986a). Explicit morphological arguments have not been advanced for the remaining tribes. In the case of the Zoniolaiminea, Beveridge (1983) indicated that the division between the Pharyngostrongylinea and the Zoniolaiminea was not clear. These two tribes are distinguished by the presence (Pharyngostrongylinea) or absence (Zoniolaiminea) of a transversely striated buccal capsule, and differences in the morphology of the labial structures (Beveridge, 1982, 1983). The monophyly of tribes within the Cloacininae has not been tested using molecular data. Recent molecular studies of the tribe Labiostrongylinea (Chilton *et al.*, 2011), of three genera within the tribe Pharyngostrongylinea (i.e. *Cyclostrongylus*, *Pharyngostrongylus* and *Rugopharynx*) (Chilton *et al.*, 2016a, b, c), and within the genus *Cloacina* (tribe Cloacininea) (Chilton *et al.*, 2017), have demonstrated the utility of the internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA for determining the relationships between nematode species belonging to this subfamily.

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In the present study, we use the same approach for testing the monophyly of three tribes, Coronostrongylinea, Macropostrongylinea and Zoniolaiminea, and the genera within them, as well as comparing the host ranges of the parasites. Due to uncertainties regarding the relationships of the Zoniolaiminea and Pharyngostrongylinea (Beveridge, 1983), representative genera of the latter tribe were also included in the analysis.

Materials and methods

Nematodes were obtained from the stomachs of kangaroos and wallabies that had been shot commercially, collected as fresh road-kills or from road-kills frozen prior to examination. Nematodes were washed in saline and then frozen in liquid nitrogen and stored at −80° prior to examination. Additional nematode samples from each host were fixed in Berland's fluid (glacial acetic acid and formalin) (Gibson, 1979) for morphological examination.

Frozen nematodes were thawed, and the head and tail were removed from individuals, fixed in lactophenol and either mounted permanently in polyvinyl lactophenol or returned to ethanol as voucher specimens, with the mid-body region being used for genetic analyses. Nematodes were identified following Beveridge (1981a, b, 1982, 1983, 1985, 1986b, c, d, 2002), Beveridge & Johnson (1981), Chilton *et al.* (2002) and Huby-Chilton *et al.* (2002). Voucher specimens (hologenophores) were deposited in the South Australian Museum (SAM), Adelaide (table 1). In some instances, the unique specimen used for genetic studies (the hologenophore) was not preserved. In these instances, fixed specimens of the same species from the same host individual (paragenophores) were deposited in SAM (table 1). Some species included in this study have relatively broad host ranges (Spratt & Beveridge, 2016). Only the host species from which the parasite was collected are considered here.

More than one specimen of each nematode species was examined if material was available from different host species, from different host sub-species, or from different geographical regions of the continent, particularly from the island state of Tasmania. Host nomenclature follows van Dyck & Strahan (2008), as the use of subgenera in this classification is potentially more informative in demonstrating host relationships than the more recent classification of Jackson & Groves (2015).

Genomic DNA was isolated from the remaining part of each nematode using a small-scale sodium-dodecyl-sulphate/proteinase K extraction procedure (Gasser *et al.*, 1993), followed by purification using a mini-column (Wizard™ Clean-Up, Promega, Madison, USA). The region of rDNA comprising the ITS-1, 5.8S rRNA gene, ITS-2 and flanking sequences (= ITS+) was amplified by polymerase chain reaction (PCR) using primers NC16 (forward; 5′-AGTTCAATCGCAATGGCTT-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′). PCRs were performed in 50 µl volumes using the following conditions: 30 cycles at 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 30 s (extension), followed by one cycle at 72°C for 5 minutes (final extension). Negative (no-DNA) controls were included in each set of reactions. Amplicons were purified using mini-columns (using Wizard™ PCR-Preps, Promega, Madison, USA), and the ITS+ sequenced in both directions using the primers NC16 and NC2 in separate reactions. The sequences generated in the present study have been deposited in GenBank (table 1). Additional sequences already present in the GenBank database (table 2) were also utilized.

Sequences were initially aligned using Muscle (Edgar, 2004) and alignments were adjusted manually using the program Mesquite v. 3.03 (Maddison & Maddison, 2015). Analyses of the ITS-1 and ITS-2 sequence data (i.e. excluding the 5.8S rRNA gene and other flanking regions) were conducted by Bayesian inference (BI) using Monte Carlo Markov Chain analysis in the program MrBayes v. 3.2.3 (Ronquist & Huelsenbeck, 2003). The likelihood parameters set for the BI analysis of sequence data were based on the Akaike Information Criterion test in jModeltest v. 2.1.7 (Posada, 2008). The number of substitutions was set at six, with a gamma-distribution. For the trees, posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was < 0.01, and the potential scale reduction factor approached one. A 50% majority rule consensus tree was constructed based on the final 75% of trees produced by BI. Analyses were run three times to ensure convergence and insensitivity to priors. The same unpartitioned data were also subjected to the neighbour joining (NJ) distance method (Saitou & Nei, 1987) in MEGA v. 7.0.20 (Kumar *et al.*, 2016). Evolutionary distances were computed using the number of differences (Nei & Kumar, 2000), including transitions and transversions among nematode species. Rates among sites were considered uniform and gaps were treated using pairwise deletion. A total of 2000 bootstrap replicates were performed and are reported as bootstrap (bs) values. The ITS-1 and ITS-2 sequences of *Arundelia dissimilis*, a species within a related genus in a related tribe, Cloacininea, was used as the outgroup in the phylogenetic analysis. The BI and NJ trees had similar topologies and, when in agreement, both pp and bs values are indicated (fig. 1).

The host associations of the parasites were examined by comparison with a molecular phylogeny of the hosts based primarily on Meredith *et al.* (2008), with additions for the genus *Thylogale* based on Macqueen *et al.* (2010). As there is no comprehensive molecular phylogeny for the Macropodidae, any taxa missing from the above studies (i.e. *M.* (*N.*) *dorsalis*) were interpolated based on the comprehensive dataset of Cardillo *et al.* (2004) and the resultant tree is therefore presented as a cladogram (fig. 2), as are the parasite data. Some of the nematode species are known to occur in more than one host species (Spratt & Beveridge, 2016). In the cladogram, only the host from which the nematode was sequenced is shown.

Results

This molecular study included 41 of the 63 known species of the tribes Coronostrongylinea (12/23), Macropostrongylinea (12/16) and Zoniolaiminea (18/24). The parasite genera included in this study have never previously been tested for monophyly using molecular data. However, of the nematode genera included in the study, the monophyly of *Alocostoma*, *Coronostrongylus*, *Macroponema*, *Thallostonema* (with novel additions), *Thylonema* and *Zoniolaimus* (to the exclusion of *Z. dendrolagi*) was highly supported with BI values of > 0.97 (fig. 1). Species of *Macropostrongylus* and *Popovastrongylus* each formed a cluster in the phylogenetic analyses but with low nodal support. *Macropostrongylus* species are parasites of *M.* (*Notamacropus*) and *Petrogale*, thus being widely distributed among host species, while *Popovastrongylus* is distributed among species of *Macropus* (*Macropus*), *Macropus* (*Osphranter*), *Petrogale* and *Thylogale*, thus also exhibiting a broad host range (fig. 2).

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Table 1. New sequences of ITS-1 and ITS-2 of cloacinine nematodes included in the current study, with host, locality, coordinates, SAM registration numbers of voucher specimens and GenBank registration numbers for sequences. Abbreviations of host generic names: *D.*, *Dendrolagus*, *M.*, *Macropus*; *P.*, *Petrogale*; *T.*, *Thylogale*; *W.*, *Wallabia*. Australian state names: NSW, New South Wales; Qld, Queensland; SA, South Australia; Tas, Tasmania; Vic, Victoria.

Nematode	Code	Host	Locality	Coordinates	SAM no.	GenBank no.
Alocostoma clelandi	F912 (1P2)	*M. robustus*	Barcaldine, Qld	23°33'S 147°17'E	36421	MG865615
Alocostoma clelandi	G91 (XD7)	*M. giganteus*	Taroom, Qld	25°39'S 149°48'E	36422	MG865616
Alocostoma propinquum	RG 22(BB11)	*M. giganteus*	Sutton Grange, Vic	36°49'S 144°10'E	36423	MG865618
Alocostoma propinquum	F899 (14J7)	*M. giganteus*	Warraweena Stn via Bourke, NSW	29°56'S 146°14'E	48175	MG865617
Cassunema exiguum	RG 12 (7I11)	*T. stigmatica*	Mt Glorious, Qld	27°20'S 152°46'E	36424	MG865619
Coronostrongylus coronatus	RG2 (XN)	*M. rufogriseus*	38 km N of Miles, Qld	26°40'S 150°11'E	36425	MG865623
Coronostrongylus coronatus	F117 (BM1)	*M. rufogriseus*	Launceston, Tas	41°26'S 147°8'E	36426	MG865622
Coronostrongylus johnsoni	F517 (22P1)	*M. dorsalis*	Bowen, Qld	20°1'S 148°15'E	36427	MG865624
Coronostrongylus barkeri	F915 (22E2)	*M. rufus*	Pentland, Qld	20°32'S 145°24'E	48191	MG865620
Coronostrongylus closei	F917 (PA6)	*P. persephone*	Shute Harbour, Qld	20°16'S 148°43'E	36428	MG865621
Foliostoma macropodis	RG 20 (6R9)	*T. stigmatica*	Broken River, Eungella, Qld	21°6'S 148°26'E	36429	MG865625
Macroponema beveridgei	F893 (AU5)	*M. dorsalis*	Charters Towers, Qld	20°05'S 146° 16'E	48213	MG957433
Macropostrongylus macropostrongylus	G 75 (AX8)	*M. agilis*	Ingham, Qld	18°39'S 146°09'E	36430	MG865626
Macropostrongylus macrostoma	G106 (3V11)	*M. parryi*	Keppel Sands, Qld	23°20'S 150°48'E	48190	MG865627
Macropostrongylus petrogale	F908 (3F6)	*P. assimilis*	Mt Stuart, Qld	19°22'S 146°46'E	36431	MG865628
Macropostrongylus yorkei	F746 (XL1)	*M. agilis*	Gumlu, Qld	19°54'S 147°35'E	45457	MG865629
Monilonema lacunosum	RG 7 (6A1)	*T. stigmatica*	Julatten, Qld	16°37'S 145°20'E	36432	MG865630
Parapharyngostrongylus dentatus	RG 15 (8R28)	*W. bicolor*	Mt Julia, Proserpine, Qld	20°24'S 148°33'E	36121	MG865631
Pararugopharynx protemnotontis	F87 (U11)	*M. rufogriseus*	Emu Flat, Bondo State Forest, NSW	34°35'S 149°42'E	36433	MG865632
Popovastrongylus macropodis	F895 (1Q3)	*M. rufus*	Barcaldine, Qld	23°33'S 147°17'E	48192	MG865633
Popovastrongylus pearsoni	G93m(J1)	*M. fuliginosus fuliginosus*	Kangaroo Island, SA	35° 47'S 137°16'E	36435	MG865634
Popovastrongylus pearsoni	RG3 (D3)	*M. fuliginosus ocydromus*	Kersbrook, SA	34°46'S 138°51'E	36434	MG865635
Popovastrongylus tasmaniensis	G90 (BD34)	*T. billardierii*	Launceston, Tas	41°26'S 147°8'E	36436	MG865636
Popovastrongylus thylogale	G57 (WB8)	*T. stigmatica*	Rex Range, Qld	16°37'S 145°20'E	36437	MG865638
Popovastrongylus thylogale	F902 (2A4)	*P. persephone*	Proserpine, Qld	20°24'S 148°33'E	48174	MG865637
Popovastrongylus tasmaniensis	G90 (BD 34)	*T. billardierii*	Launceston, Tas	41°26'S 147°8'E	36436	MG865636
Rugostrongylus labiatus	RG 35 (3V16)	*M. parryi*	Keppel Sands, Qld	23°20'S 150°48'E	36438	MG865639
Thallostonema kirkpatricki	F307 (9N18)	*T. stigmatica*	Green Mtn, Lamington Nat. Park, Qld	28°15'S 153°8'E	36439	MG865640
Thallostonema lichtenfelsi	F321 (6A2)	*T. stigmatica*	Julatten, Qld	16°37'S 145°20'E	36440	MG865641
Thallostonema queenslandense	F167 (PG2)	*P. persephone*	Mount Lucas, Strathdickie, Qld	20°19'S 148°35'E	36441	MG865642
Thallostonema rarum	F160 (XN 11)	*M. rufogriseus*	38 km N of Miles, Qld	26°40'S 150°11'E	36442	MG865643
Thallostonema setiferum	F153 (U2)	*M. rufogriseus*	Emu Flat, Bondo State Forest, NSW	34°35'S 149°42'E	36443	MG86564
Thallostonema setiferum	F157(XN 10)	*M. rufogriseus*	38 km N of Miles, Qld	26°40'S 150°11'E	36444	MG865645
Thallostonema setiferum	RG 11(BL4)	*M. rufogriseus*	Launceston,,Tas	41°26'S 147°8'E	36445	MG865646
Thallostonema thylogalarum	F334 (7E12)	*T .thetis*	Green Mtn, Lamington Nat. Park, Qld	28°15'S 153°8'E	48176	MG865647
Thylonema barkeri	RG 17 (7I21)	*T. stigmatica*	Mt Glorious, Qld	27°20'S 152°46'E	36446	MG865649
Thylonema barkeri	F304 (8Z25)	*T. stigmatica*	Julatten, Qld	16°37'S 145°20'E	48193	MG865648

(*Continued*)

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Table 1. (*Continued.*)

Nematode	Code	Host	Locality	Coordinates	SAM no.	GenBank no.
Thylonema thylonema	F 309 (8Z25)	*T. stigmatica*	Julatten, Qld	16°37'S 145°20'E	36447	MG865650
Thylostrongylus tasmaniensis	F119 (BF4)	*T. billardierii*	Launceston, Tas	41°26'S 147°8'E	36448	MG865651
Trigonostonema trigonostoma	RG 23 (7J4)	*T. stigmatica*	Mt Glorious, Qld	27°20'S 152°46'E	48195	MG865652
Wallabinema australe	RG 79 (AH11)	*M. rufogriseus*	20 km S of Grafton, NSW	30°5'S 152°24'E	36449	MG865653
Wallabinema cobbi	RG 10 (AC8)	*M. rufus*	Wallerberdina Stn, Pt Augusta, SA	31°43'S 138°7'E	36450	MG865654
Wallabinema labiatum	RG 77 (AH11)	*M. rufogriseus*	20 km S of Grafton, NSW	30°5'S 152°24'E	36451	MG865655
Wallabinema tasmaniense	F173 (BF4)	*T. billardierii*	Launceston, Tas	41°26'S 147°8'E	36452	MG865656
Woodwardostrongylus obendorfi	RG 16 (CB4)	*M. parryi*	Dawes, Qld	24°40'S 151°15'E	36453	MG865657
Zoniolaimus buccalis	RG 73 (AO4)	*M. dorsalis*	Rockhampton, Qld	23°23'S 150°30'E	36454	MG865658
Zoniolaimus dendrolagi	RG 85 (PP3)	*D. lumholtzi*	Mt Baldy State Forest, Qld	17°17'S 145°27'E	36455	MG865659
Zoniolaimus elegans	RG 72 (CC4)	*M. parryi*	Dawes, Qld	24°40'S 151°15'E	48194	MG865660
Zoniolaimus mawsonae	RG106 (1J15)	*M. rufus*	46 km N of Cunnamulla, Qld	28°04'S 145°41'E	28369	MG865661
Zoniolaimus petrogale	RG 80 (PG1)	*P. persephone*	Mount Lucas, Strathdickie, Qld	20°19'S 148°35'E	36456	MG865662
Zoniolaimus setifera	RG 89 (XN6)	*M. rufogriseus*	38 km N of Miles, Qld	26°40'S 150°11'E	36457	MG865663

Three genera, *Monilonema*, *Wallabinema* and *Papillostrongylus*, appeared to be paraphyletic in this analysis. *Monilonema ochetocephalum* from *Wallabia bicolor* clustered with species of *Macropostrongylus*, although with low nodal support, within the tribe Macropostrongylinea. Two species, *M. lacunosum* and *Foliostoma macropodis*, both found in *Thylogale stigmatica,* clustered with species of *Thallostonema*, which are also primarily parasites of *Thylogale* species. *Wallabinema* appeared to be paraphyletic. There was high nodal support for a sister taxa relationship between *W. australe*, with a host range including *M.* (*Notamacropus*), *Wallabia* and *Thylogale*, and *W. cobbi* from *M.* (*Osphranter*). These two species belonged to a group, with total nodal support, that included two species of *Thylonema*. This group did not include *W. labiatum* or *W. tasmaniense*. There was total nodal support for *W. labiatum*, found primarily in *M.* (*N.*) *rufogriseus* and *W. bicolor*, and *W. tasmaniense*, a parasite of *T. billardieri*, representing sister taxa.

Paraphyly was also indicated within *Papillostrongylus*, with *P. papillatus*, from *M.* (*N.*) *dorsalis*, clustering, but with low nodal support from *P. barbatus*, which is a parasite of *M.* (*O.*) *rufus*.

Table 2. Sequences of nematodes used from GenBank.

Nematode species	Host	GenBank no.
Arundelia dissimilis	*Wallabia bicolor*	MF284673
Macroponema beveridgei	*Macropus robustus*	HE775534-5
Macroponema comani	*Macropus giganteus*	HE775536
Monilonema ochetocephalum	*Wallabia bicolor*	HE775537
Papillostrongylus barbatus	*Macropus rufus*	AJ309961
Papillostrongylus labiatus	*Macropus dorsalis*	AJ309960
Zoniolaimus latebrosus	*Macropus rufus*	KJ851996

Tribes

The current analysis provides only limited support for the composition of the three cloacinine tribes as currently defined. *Monilonema lacunosum* and *Foliostoma macropodis*, both currently placed within the Macropostrongylinea, clustered with species of *Thallostonema* (Zoniolaiminea) (fig. 1). *Popovastrongylus*, currently placed within the Coronostrongylinea, clustered strongly with the genera of the Macropostrongylinea, as did *Papillostrongylus*. Current members of the Zoniolaiminea were split between two highly supported clades. The first included *Thallostonema*, but with the addition of *Monilonema lacunosum* and *Foliostoma*. The second clade included *Zoniolaimus*, *Cassunema* and *Wallabinema*, but also included *Thylonema* (currently included in the Coronostrongylinea), as well as three genera currently included in the Pharyngostrongylinea (*Pararugopharynx*, *Thylostrongylus* and *Woodwardostrongylus*). *Parapharyngostrongylus* and *Rugostrongylus* (Pharyngostrongylinea) clustered together to the exclusion of all other genera and to the exclusion of other genera of the Pharyngostrongylinea (*Pararugopharynx*, *Thylostrongylus* and *Woodwardostrongylus*).

Species

The opportunity was taken, where possible, to include representatives of the same nematode species from different host species or from widely different geographical localities. *Alocostoma clelandi* from two different host species in Queensland were identical genetically, as were *A. propinquum* from Victoria and northern New South Wales. Similarly identical or near identical DNA sequences were obtained for *Thylonema barkeri* from northern and southern Queensland, and *Popovastrongylus pearsoni* from mainland and island sub-species of *Macropus fuliginosus* (*M. f. fuliginosus* and *M. f. ocydromus*).

Slight genetic differences were detected between *Popovastrongylus thylogale* from two different host species, *Petrogale persephone* and *Thylogale stigmatica* in northern Queensland, and between specimens of *Thallostonema setiferum* from Queensland, New South Wales and

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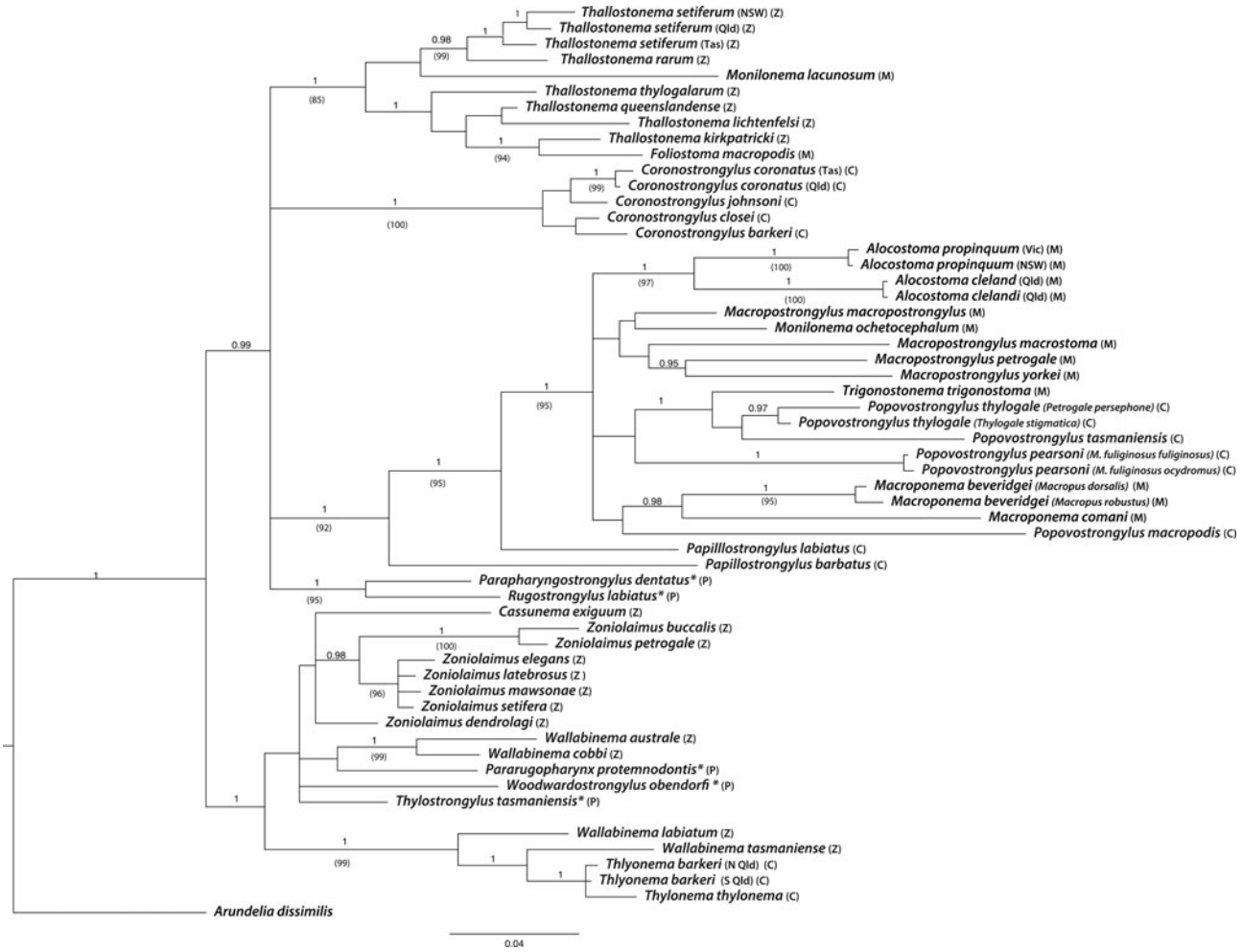

Fig. 1. Phylogram of the genetic interrelationships of genera of the Coronostrongylinea (C), Macropostrongylinea (M) and Zoniolaiminea (Z). Numbers above branches indicate posterior probabilities obtained in the Bayesian analysis; those below branches indicate bootstrap values from the Neighbour Joining method. Branch lengths indicate genetic distances. * indicates members of the Pharyngostrongylinea (P).

Tasmania, in *Coronostrongylus coronatus* from Queensland and Tasmania, and in *Macroponema beveridgei* from two different host species from the same locality in Queensland. In each instance, sequences of the same nematode species formed a highly supported clade.

Discussion

Cloacinine nematodes belonging to the tribes Coronostrongylinea, Macropostrongylinea, Zoniolaiminea and Pharyngostrongylinea are common parasites of the gastrointestinal tracts of Australian macropodid marsupials (Beveridge & Spratt, 2016). These tribes and the genera within them are defined primarily on a phenetic, morphological basis. There are proposed morphological synapomorphies for the Coronostrongylinea and Macropostrongylinea, but not for the Zoniolaiminea and Pharyngostrongylinea (Beveridge, 1983). The primary aim of this study was to test current morphologically based hypotheses of the phylogenetic relationships of cloacinine nematodes within the Coronostrongylinea, Macropostrongylinea and Zoniolaiminea using a molecular approach. Some representatives of the Pharyngostrongylinea were also included in this study. The nematode genera (including the three principal tribes) included in this study have not previously been tested for monophyly based on analyses of ITS-1 and ITS-2 sequence data.

The results showed that there was strong support for the monophyly of the genera *Coronostrongylus*, *Thylonema*, *Macroponema*, *Alocostoma*, *Thallostonema* (with the unexpected addition of two genera) and *Zoniolaimus* (with the exclusion of a single species). Two other nematode genera, *Macropostrongylus* and *Popovastrongylus*, each formed a monophyletic clade; however, there was low nodal support for such groups, even though each genus is well defined morphologically (Beveridge, 1983, 1985). In contrast, three genera, *Wallabinema*, *Monilonema* and *Papillostrongylus*, were paraphyletic (figs 1 & 2).

Host ranges of the various genera varied significantly. *Macropostrongylus*, *Monilonema*, *Papillostrongylus*, *Popovastrongylus*, *Coronostrongylus*, *Thallostonema*, *Wallabinema* and *Zoniolaimus* occur in a wide range of hosts, including *Macropus* (subgenera: *Macropus*, *Osphranter* and *Notamacropus*), *Petrogale* and *Thylogale*. By contrast, species within the genus *Thylonema* are restricted to the host genus (*Thylogale*), whereas species of *Macroponema* and *Alocostoma* are found only in hosts of two closely related subgenera of *Macropus* (i.e. *Macropus* and *Osphranter*).

A formal co-phylogenetic analysis is not possible at this time because of a lack of a definitive molecular phylogeny of the

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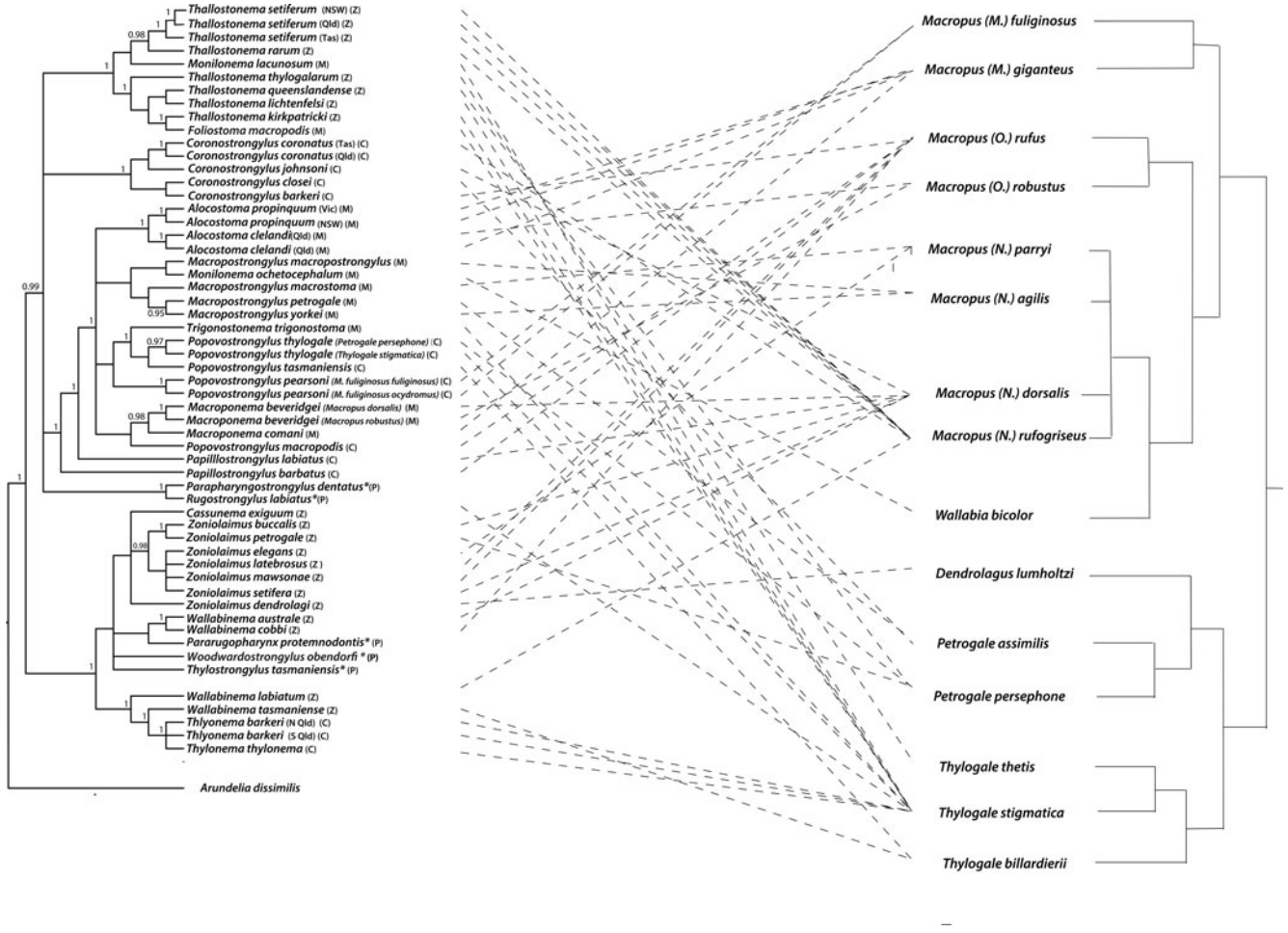

Fig. 2. Comparison of the phylogenetic relationships of genera and species of the Coronostrongylinea (C), Macropostrongylinea (M) and Zoniolaiminea (Z), presented as a cladogram, with a composite cladogram of their macropodid hosts. Numbers above branches in the nematode tree indicate posterior probabilities obtained in the Bayesian analysis; those below branches indicate bootstrap values from the Neighbour Joining method. Branch lengths indicate genetic distances. * indicates members of the Pharyngostrongylinea (P), for which host associations are not shown. Abbreviations: M, *Macropus*; N, *Notamacropus*; O, *Osphranter.*

Macropodidae (see Materials and methods). However, visual inspection of the host–parasite relationships (fig. 2) provides little evidence of co-speciation. These host–parasite associations are potentially best explained in evolutionary terms by a process of host colonization, as proposed in previous molecular studies of related tribes of the Cloacininae (Chilton *et al.*, 2011, 2016a, b, c, 2017), but this should be considered as a preliminary hypothesis pending the possibility of more formal analyses. In several instances, genetically identical (based on ITS-1 and ITS-2 sequences) individuals of a nematode species (e.g. *Alocostoma clelandi*) were found in different host species. This clearly indicates the possibility of nematodes switching hosts without undergoing genetic differentiation. The suggestion made here of the significance of host switching accords with a recent overview of the processes of speciation in parasites that gives overwhelming support to the priority of host switching over co-speciation (Nylin *et al.*, 2017).

Genera such as *Thylonema*, *Thallostonema* and one clade of *Wallabinema* have diversified primarily in the host genus *Thylogale*. In the case of species of *Wallabinema*, *W. australe* and *W. cobbi* (which form a clade in this molecular study) differ distinctively in oesophageal morphology from *W. labiatum* and *W. tasmaniense* (also included in this study) (Beveridge, 1983) and may therefore warrant generic recognition. The latter species, *W. labiatum* and *W. tasmaniense*, represent species present in *M.* (*N.*) *rufogriseus* (*W. labiatum*) as well as a series of morphologically similar species (*W. tasmaniense*, *W. gallardi*, *W. parvispiculare*) parasitic in the genus *Thylogale* (Beveridge, 1983). The host distribution of this clade is thus similar to that of *Thallostonema*, with most species occurring in hosts belonging to the genus *Thylogale*, but with additional species in *M.* (*N.*) *rufogriseus*. Within the genus *Thylogale*, parasite species associations are complicated as there are distinctive sub-species of *T. stigmatica* present in Queensland (Macqueen *et al.*, 2010), although there is some evidence of the sub-species interbreeding in central Queensland (Eldridge *et al.*, 2011). In addition, *T. stigmatica* occurs in sympatry with *T. thetis* in south-eastern Queensland, with some evidence of introgression between these two species (Eldridge *et al.*, 2011). Consequently, additional analyses are required to determine the evolutionary relationships between nematodes parasitic in species of *Thylogale*. *Thylogale*, together with *Petrogale* and *Dendrolagus*, belong to a clade that separated from *Macropus* and *Wallabia c.* 10 mya (Meredith *et al.*, 2008). This host genus also has a remarkable number of nematode genera that are either entirely specific to it or occur primarily within it (i.e. *Cassunema*, *Foliostoma*, *Thylonema*, *Trigonostonema*, *Thylostrongylus*, *Thallostonema*, *Tethystrongylus*

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and *Wallabinema*) suggesting that an investigation into their relationships might provide critical insights into the evolution of the Cloacininae.

Although the present study has highlighted the radiation of some nematode genera within a particular host genus (e.g. *Thylonema*, *Trigonostonema* and *Thallostonema* in species of *Thylogale*, and *Alocostoma and Zoniolaimus* in species of *Macropus*), relatively few instances of within-host speciation were identified, a phenomenon occurring in the related genus *Cloacina* (Chilton *et al.*, 2017). As indicated above, associations within *Thylogale* spp. are difficult to interpret. *Zoniolaimus mawsonae* and *Z. latebrosus* co-occur commonly in the stomach of *M.* (*O.*) *rufus* (see Huby-Chilton *et al.*, 2002), but the current analysis does not provide any evidence for within-host speciation in this case.

The data presented here also provide a basis for future studies. The tribes of the Cloacininae are currently phenetically based (Lichtenfels, 1980), with only two (i.e. Macropostrongylinea and Coronostrongylinea) proposed on the basis of morphological phylogenetic hypotheses (Beveridge, 1986a). Clearly, all of these taxonomic hypotheses warrant testing using molecular methods, as the current study did not support the tribal associations examined here.

Apart from the current molecular analyses raising concerns as to the validity of the current distribution of cloacinine genera among tribes, in particular the genera of the Pharyngostrongylinea, the apparent paraphyly of *Wallabinema* and *Monilonema* also needs to be addressed. The present study did, however, identify five highly supported clades that could warrant recognition at tribal level. The first is the Coronostrongylinea, which includes a single genus, *Coronostrongylus*. The second is the Macropostrongylinea, which includes *Alocostoma*, *Macroponema*, *Macropostrongylus*, *Papillostrongylus*, *Popovastrongylus*, *Trigonostonema* and *Monilonema* (in part). The third is the Pharyngostrongylinea, currently restricted to *Parapharyngostrongylus* and *Rugostrongylus* but with additional genera potentially to be added. The fourth clade is the Zoniolaiminea, with the current genera *Zoniolaimus*, *Wallabinema* and *Cassunema*, but would also include *Thylonema* as well as three currently pharyngostrongylidean genera, *Pararugopharynx*, *Thylostrongylus* and *Woodwardostrongylus*. A fifth, novel clade includes species of *Thallostonema* together with *Monilonema lacunosum* and *Foliostoma*, for which there is no current taxonomic identity.

Therefore, the current molecular-based study has contributed to our understanding of the phylogenetic relationships of nematodes within genera from four nominal tribes of the Cloacininae. It has provided insights into inadequacies in the current taxonomy of these nematodes at the generic and tribal levels, which will need to be addressed.

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