



Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw



Detection of cryptic species of *Rugopharynx* (Nematoda: Strongylida) from the stomachs of Australian macropodid marsupials



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ARTICLE INFO

Article history:

Received 30 January 2016
Received in revised form
12 April 2016
Accepted 14 April 2016

Keywords:

Rugopharynx
Strongylida
Cloacinae
Macropodidae
Phylogeny
Cryptic species

ABSTRACT

Sequences of the internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) were determined for species of the genus *Rugopharynx* and *Rugonema labiatum*, nematodes from the stomachs of macropodid marsupials. Phylogenetic analyses of the aligned sequence data were conducted. The relationships provided molecular support for all species currently recognised, some of which are based on minor morphological differences and on multilocus enzyme electrophoretic data, but also indicated that additional, cryptic species exist within the genus. In addition, the genus *Rugonema* is placed as a synonym of *Rugopharynx*, its sole species becoming *Rugopharynx labiatum* n. comb. The molecular data provided some insights into the evolution of complex buccal capsule morphologies within the genus, but there was no evidence of co-evolution between the macropodid hosts and their parasites.

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1. Introduction

The sacculated forestomachs of kangaroos and wallabies (family Macropodidae) commonly contain large numbers of nematodes belonging to the strongylid sub-family Cloacinae. For example, [Vendl and Beveridge \(2014\)](#) reported means and ranges in numbers of cloacine nematodes in the stomachs of the red-necked wallaby, *Macropus rufogriseus*, the eastern grey kangaroo, *Macropus giganteus* and the swamp wallaby, *Wallabia bicolor*, as 60,800 (2000–210,000), 20,500 (7000–79,000) and 20,000 (3000–58,000), respectively. These data support earlier published figures for high intensities of infection in the western grey kangaroo, *Macropus fuliginosus*, the red kangaroo, *Macropus rufus*, and *M. giganteus* ([Arundel et al., 1979; Beveridge and Arundel, 1979](#)).

Although there is considerable species diversity in the stomach-inhabiting cloacine nematodes ([Spratt et al., 1991](#)), members of the genus *Rugopharynx* are prominent representatives of these nematode communities. For instance, *Rugopharynx australis* dominates the gastric helminth community of *M. rufus*, with mean and

maximum burdens of 47,000 and 266,000 nematodes, respectively ([Arundel et al., 1979](#)). In spite of the numerical significance of this genus in the gastric helminth communities of macropodids, continuing taxonomic studies are needed, as it is unlikely that all species have yet been described and the presence of cryptic species could potentially complicate the interpretation of ecological data published to date. The genus *Rugopharynx* was revised by [Beveridge \(1982\)](#), who recognised nine species. However, [Beveridge \(1982\)](#) noted that *R. australis* was potentially a complex of a number of species, differentiable only by very minor and often overlapping morphological characteristics. Multilocus enzyme electrophoretic (MEE) studies, combined with morphological evidence, indicated the existence of two new species, *R. sigma* and *Rugopharynx mawsonae*, both formerly confused with *Rugopharynx zeta* ([Chilton et al., 1993; Beveridge et al., 1994](#)), while an additional MEE investigation of *R. australis* by [Chilton et al. \(1996\)](#) provided evidence for at least seven species within this taxon. Subsequently, [Beveridge and Chilton \(1999\)](#) split *R. australis* into 10 species and resurrected *R. alpha* as a valid species. The latter revision was based on extrapolating the minor morphological differences identified in samples included in the earlier electrophoretic study across the entire species complex. In spite of this progress, there has been no attempt to independently verify the validity of the species erected to date using molecular methods. A closely related genus,

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Rugonema was erected by Beveridge (1999) for specimens formerly referred to as *R. australis* occurring in the stomach of the black-gloved wallaby, *Macropus irma*, from Western Australia, based on morphological differences in the labial collar. Because of this close association with *Rugopharynx*, *Rugonema labiatum* was also included in the present study.

Given the prevalence and abundance of this nematode genus in the stomachs of kangaroos and wallabies, and the diversity of species currently recognised based on minor morphological criteria, this study was undertaken to attempt to establish species boundaries within the genus based on sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA. These molecular target regions have proved to be highly informative for the specific identification of a range of strongylid nematodes, including taxa within the Cloacinae (Chilton, 2004), the subfamily to which *Rugopharynx* belongs.

2. Materials and methods

2.1. Collection, storage and preparation of nematodes

Nematodes were obtained from the stomachs of a range of kangaroos and wallabies (Fig. 1; Table 1), which had been collected as fresh road-kills or from road-kills frozen prior to examination. Host nomenclature follows Van Dyck and Strahan (2008). In instances where a nematode species occurred across a large geographical area, an attempt was made to include samples from

different geographical regions of Australia, particularly any occurring on the island of Tasmania (Fig. 1). Australian state names are abbreviated as: NSW, New South Wales; Qld, Queensland; SA, South Australia; Tas, Tasmania; Vic, Victoria; WA, Western Australia. Nematodes were washed in saline, frozen in liquid nitrogen and stored at -80 °C until morphological and molecular studies were undertaken. Nematodes were then thawed, the head and tail of each worm removed, fixed in lactophenol and mounted permanently in polyvinyl lactophenol as voucher specimens. Nematodes were identified according to previous descriptions (Beveridge, 1982; Beveridge et al., 1994; Chilton et al., 1993; Beveridge and Chilton, 1999; Appan et al., 2004). Voucher specimens have been deposited in the South Australian Museum (SAM), Adelaide (Table 1). The mid-body region was used for genetic analyses.

2.2. Morphological methods

Species within the genus *Rugopharynx* were divided into three groups based on the morphology of the buccal capsule (Table 1). Nematodes with a simple, cylindrical buccal capsule were designated as type I (Fig. 2), while those with a buccal capsule divided into two sections were designated as type II. These buccal capsules were further subdivided into species with a buccal capsule divided in the mid region (*R. epsilon*) (type IIA) and those in which the division occurred in the anterior quarter (*R. rufogrisea*) (type IIB). Species with a buccal capsule divided into three segments were



Fig. 1. Localities within Australia at which specimens of *Rugopharynx* used in this study were collected. Coordinates for each locality are provided in Table 1. 1, Lake Clifton; 2, Warooна; 3, Collie, Wellington Dam; 4, Perup River; 5, Kalgoorlie; 6, Wallerberdina Station; 7, Port Augusta; 8, Ashbourne; 9, Kangaroo Island; 10, Naracoorte; 11, Hattah Lakes National Park; 12, Yan Yean; 13, The Gurdies; 14, Launceston; 15, Emu Flat, Bondo State Forest; 16, Trangie; 17, Grafton; 18, Lamington National Park; 19, Miles; 20, Dawes; 21, Mt Sebastopol; 22, Rockhampton; 23, Winton; 24, Proserpine; 25, Bowen; 26, Magnetic Island; 27, Lake Barrine.

Table 1

Specimens of *Rugopharynx* and *Rugonema labiatum* used in molecular analyses with collection data, deposition of morphological voucher specimens, and GenBank registration numbers for sequences.

Parasite species	Host species ^a	Specimen Code	Locality ^b	Coordinates	SAM voucher no.	GenBank no.	Buccal Capsule Type
<i>R. alpha</i>	<i>P. purpureicollis</i>	F886	Winton, Qld	22° 23'S 143° 3'E	36137	LN906946	I
<i>R. australis</i>	<i>M. rufus</i>	F739	Wallerberdina Stn via Pt Augusta, SA	31° 43'S 138° 7'E	36138	LN906947	I
		G97	Kalgoorlie, WA	30° 44'S 121° 27'E	36209	LN906948	
	<i>M. giganteus</i>	F751	Trangie, NSW	32° 2'S 147° 59'E	36139	LN906949	
		F754	Trangie, NSW	32° 2'S 147° 59'E	36140	LN906950	
	<i>M. dorsalis</i>	F758	Miles, Qld	26° 40'S 150° 11'E	36141	LN906951	
	<i>M. robustus</i>	F704	Pt Augusta, SA	32° 30'S 137° 46'E	36142	LN906952	
	<i>M. fuliginosus</i>	F784	Hattah Lakes, National Park, Vic	34° 42'S 142° 17'E	36143	LN906953	
<i>R. chi</i>	<i>T. billardierii</i>	F716	Launceston, Tas	41° 26'S 147° 8'E	36144	LN906954	I
<i>R. delta</i>	<i>M. dorsalis</i>	F97	Rockhampton, Qld	23° 16'S 150° 49'E	36145	LN906955	III
<i>R. epsilon</i>	<i>M. rufogriseus</i>	F72	Naracoorte, SA	36° 57'S 140° 44'E	36146	LN906956	IIA
		F372	Launceston, Tas	41° 26'S 147° 8'E	36147	LN906957	
		F76 & F77	Emu Flat, NSW	34° 35'S 149° 42'E	36148	LN906958	
						LN906959	
<i>R. longibursaris</i>	<i>W. bicolor</i>	F80	Emu Flat, NSW	34° 35'S 149° 42'E	36149	LN906960	
	<i>M. rufogriseus</i>	F373	Launceston, Tas	41° 26'S 147° 8'E	36150	LN906961	III
		F100	Naracoorte, SA	36° 57'S 140° 44'E	36151	LN906962	
<i>R. macropodis</i>	<i>M. fuliginosus</i>	F731	Naracoorte, SA	36° 57'S 140° 44'E	36152	LN906963	I
		F736	Kangaroo Island, SA	35° 49'S 137° 12'E	36153	LN906964	
	<i>M. giganteus</i>	F727	Emu Flat, NSW	34° 35'S 149° 42'E	36212	LN906965	
<i>R. mawsonae</i>	<i>M. dorsalis</i>	F108-F110	Rockhampton, Qld	23° 23'S 150° 30'E	36154	LN906966	III
						LN906967	
						LN906968	
<i>R. mu</i>	<i>W. bicolor</i>	YE1	The Gurdies, Vic	38° 23'S 145° 34'E	30903	LN906969	I
		F510	Emu Flat, NSW	34° 35'S 149° 42'E	36155	LN906970	
<i>R. omega</i>	<i>M. rufogriseus</i>	F107	Grafton, NSW	29° 41'S 152° 56'E	36156	LN906971	
<i>R. pi</i>	<i>M. rufogriseus</i>	F710	Grafton, NSW	29° 41'S 152° 56'E	36157	LN906972	I
		F712	Emu Flat, NSW	34° 35'S 149° 42'E	36158	LN906973	
<i>R. rho</i>	<i>M. parryi</i>	F750	Dawes, Qld	24° 40'S 151° 15'E	36159	LN906974	
	<i>M. eugenii</i>	F724	Kangaroo Island, SA	35° 49'S 137° 12'E	36160	LN906975	I
		F905	Perup River, WA	34° 24'S 116° 26'E	36161	LN906976	
	<i>M. fuliginosus</i>	F780	Ashbourne, SA	35° 17'S 138° 46'E	36162	LN906977	
		F910 & F911	Waroona, WA	32° 51'S 115° 59'E	36163	LN906978	
<i>R. rosemariae</i>	<i>M. irma</i>	G114	Lake Clifton, WA	32° 47'S 115° 40'E	36164	LN906979	
<i>R. rufogrisea</i>	<i>M. giganteus</i>	F889	Yan Yean, Vic	37° 33'S 145° 10'E	36165	LN906980	I
	<i>M. rufogriseus</i>	F84	Emu Flat, NSW	34° 35'S 149° 42'E	36166	LN906981	IIIB
		F720	Launceston, Tas	41° 26'S 147° 8'E	36167	LN906982	
	<i>M. parryi</i>	F381	Dawes, Qld	24° 40'S 151° 15'E	36168	LN906983	
<i>R. setonicis</i>	<i>S. brachyurus</i>	F872	Wellington Dam, WA	30° 16'S 152° 52'E	36169	LN906984	I
<i>R. sigma</i>	<i>T. thetis</i>	F794	Lamington National Park, Qld	28° 15'S 153° 8'E	36170	LN906985	III
	<i>T. stigmatica</i>	G384	Lamington National Park, Qld	28° 15'S 153° 8'E	36210	LN906986	
		F92	Lake Barrine, Qld	17° 15'S 145° 38'E	36171	LN906987	
<i>R. spratti</i>	<i>M. rufogriseus</i>	F375	Launceston, Tas	41° 26'S 147° 8'E	36172	LN906988	I
		F770	Emu Flat, NSW	34° 35'S 149° 42'E	36173	LN906989	
<i>R. tau</i>	<i>T. thetis</i>	F793	Lamington National Park, Qld	28° 15'S 153° 8'E	36174	LN906990	I
<i>R. theta</i>	<i>M. dorsalis</i>	F112	Bowen, Qld	20° 1'S 148° 15'E	36175	LN906991	I
<i>R. zeta</i>	<i>P. assimilis</i>	F88	Magnetic Island, Qld	19° 8'S 146° 50'E	36176	LN906992	III
	<i>P. inornata</i>	G110	Proserpine, Qld	20° 24'S 148° 35'E	36177	LN906993	
	<i>P. herberti</i>	G152	Mt Sebastopol, Qld	20° 43'S 146° 42'E	36178	LN906994	
<i>Rugonema labiatum</i>	<i>M. irma</i>	F875	Collie, WA	33° 22'S 116° 9'E	36179	LN906995	I

^a Abbreviations of host generic names: *M.*, *Macropus*; *P.*, *Petrogale*; *S.*, *Setonix*; *T.*, *Thylagale*; *W.*, *Wallabia*.

^b Abbreviations and contractions of Australian state names: NSW, New South Wales; Qld, Queensland; SA, South Australia; Tas, Tasmania; Vic, Victoria; WA, Western Australia.

^c Specimen deposited was F87.

designated as type III. In some species, such as *R. spratti* and *R. tau*, the potential division of the buccal capsule is subtle; hence, in these cases, species were classified as type I.

2.3. Molecular methods and phylogenetic analysis

Genomic DNA was isolated from the mid-body part of each nematode using a small-scale sodium-dodecyl-sulphate/proteinase K extraction method (Gasser et al., 1993), followed by purification using a mini-column (Wizard™ Clean-Up, Promega). The region of rDNA comprising the ITS-1, 5.8S rRNA gene, ITS-2 and flanking sequences (= ITS+) was amplified by the PCR using primers NC16 (forward; 5'-AGTTCAATCGCAATGGCTT-3') and NC2 (reverse; 5'-TTAGTTCTTCCCTCCGCT-3'). PCR was performed in a 50 µl volume

for 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 min (final extension). Negative (no-DNA) controls were included in each set of reactions. Amplicons were purified using mini-columns (using Wizard™ PCR-Preps, Promega), and the ITS+ sequenced in both directions using the same primers (separately) as used for PCR. The sequences generated in the present study have been deposited in the GenBank database (accession numbers LN906946-LN906995; Table 1). Sequences were initially aligned using the program Muscle (Edgar, 2004), and alignments adjusted manually using the program Mesquite v.2.75 (Maddison and Maddison, 2011).

Phylogenetic analyses of the aligned sequence data were conducted by Bayesian inference (BI) using Monte Carlo Markov Chain

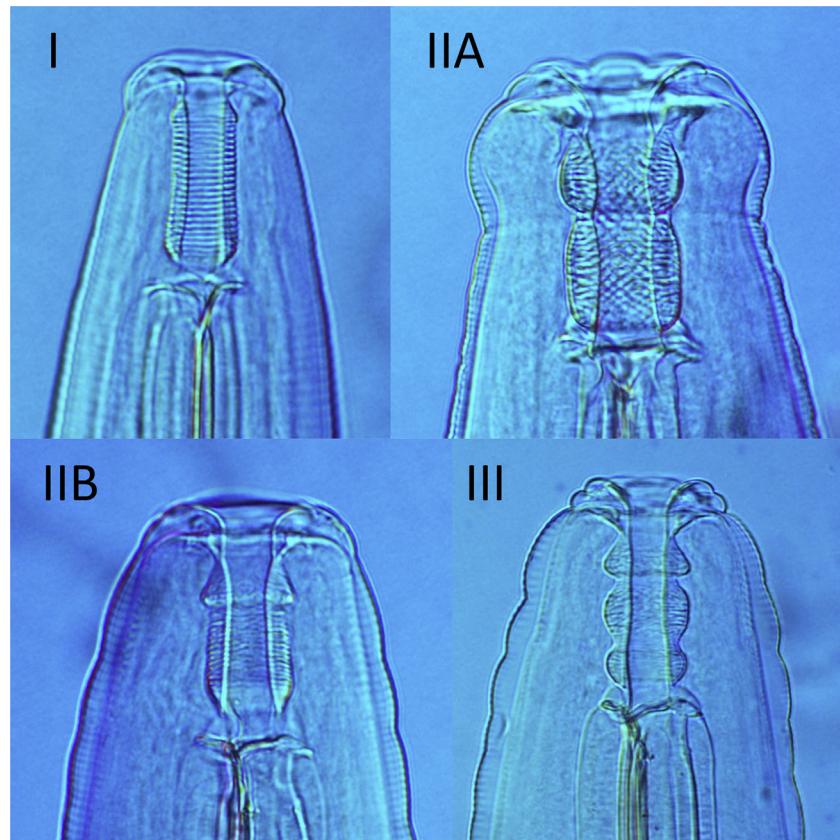


Fig. 2. Morphological buccal capsule types in the genus *Rugopharynx*. I, simple cylindrical buccal capsule, *R. macropodis*; II A, bilobed buccal capsule with subequal divisions, *R. epsilon*; II B, bilobed buccal capsule with anterior lobe shorter, *R. rufogrisea*; III, trilobed buccal capsule, *R. longibursaris*.

analysis in the program MrBayes v.3.2.2 (Ronquist and Huelsenbeck, 2003). The likelihood parameters set for the BI analysis of sequence data were based on the Akaike Information Criteria test in jModeltest v.2.1.5 (Posada, 2008). The number of substitutions was set at 6, with a gamma-distribution. For the tree, 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. For each analysis, 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. Phylogenetic analyses of the ITS+ sequence data were also conducted using the neighbor-joining (NJ) and maximum parsimony (MP) methods in PAUP* 4.0b10 (Swofford, 1999). For the MP analyses, heuristic searches were carried out with random addition of sequences ($n = 100$), tree-bisection-reconstruction (TBR) branch swapping, the MulTrees option in effect, MaxTrees set at 2000 and saving all equally parsimonious trees. Characters were treated as unordered and were weighted equally. Alignment gaps were treated as a fifth character state in the MP analyses. The tree length (L), consistency index, excluding uninformative characters (CI) and the retention index (RI) were recorded. Bootstrap analyses (1000 replicates) were conducted to determine the relative support for clades in the consensus trees; nodal support was expressed as a percentage. The ITS+ sequences of *Labiostrongylus australis* (GenBank accession numbers AJ308403-AJ308411) were used as the outgroup in the phylogenetic analyses, because this nematode species belongs to a related tribe within the same subfamily (i.e. Cloacinae) (see

Lichtenfels, 1980) to which *Rugopharynx* also belongs (Beveridge, 1982; Chilton et al., 1997) and because it has been relatively well studied genetically (Chilton et al., 2009b).

A consensus tree depicting the phylogenetic relationships of the nematodes was compared with a composite tree of the hosts. The host phylogenetic tree was based primarily on the molecular analyses of Meredith et al. (2008); however, as some species (e.g., *Thylogale billardierii*, *Macropus dorsalis*, *Petrogale herberti*, *Petrogale assimilis* and *Petrogale inornata*) were not included in that study, the tree was modified to incorporate these hosts based on the studies of Campeau-Péloquin et al. (2001), Cardillo et al. (2004) and Eldridge et al. (2011). The host phylogenetic tree also includes three species, *Macropus (Notamacropus) agilis*, *M. (Osphranter) bernardus* and *M. (O.) antilopinus*, from which no species of *Rugopharynx* have been reported. Not all species of *Petrogale* and *Thylogale* are included in the phylogenetic tree, only those included in the present study.

3. Results

The length of the ITS+ region, excluding flanking regions was 744–777 bp for all taxa within the genus *Rugopharynx* and *Rugonema labiatum*. The length of the ITS-1 sequences ranged from 373 bp (*Rugopharynx mawsonae*, *R. sigma* from *Thylogale thetis* and *Rugonema labiatum*) to 387 bp (*R. mu*, *R. rufogrisea* and some individuals of *R. australis*), whereas the ITS-2 sequences were much shorter, ranging from 216 bp (*R. longibursaris*, *R. omega* and *R. zeta*) to 313 bp (*R. alpha* and *R. mawsonae*) (Supplementary Table 1). The length (153 bp) and nucleotide sequence of the 5.8S rRNA gene was the same for all morphospecies within the genus *Rugopharynx* and *Rugonema labiatum*.

Each morphospecies had a unique set of ITS+ sequences. *Rugonema labiatum* was most genetically similar to *R. pi*. There were five fixed differences (i.e. where there are no shared nucleotides at an alignment position) between these two taxa; two in the ITS-1 and three in the ITS-2. The magnitude of fixed differences in ITS+ sequence among morphospecies within the genus *Rugopharynx* ranged from eight (i.e. between *R. omega* and *R. longibursaris*) to 84 (i.e. between *R. alpha* and *R. rufogrisea*). Only one fixed difference in ITS-1 sequence was detected between *R. setonicis* and *Rugopharynx rho* compared with 10 fixed differences between these two morphospecies for the ITS-2, while the lowest number of fixed differences in ITS-2 sequence among morphospecies (i.e. five) was between *R. omega* and *R. longibursaris*.

For some morphospecies, there was variation among individuals in the DNA sequences of the ITS-1 and/or ITS-2 (see *Supplementary Tables 2–8*). Three variable positions (two in the ITS-1 and one in the ITS-2) were detected in the aligned sequences of *R. mu* individuals from hosts (*W. bicolor*) collected in New South Wales and Victoria; however, none of these mutations represented a fixed difference (*Supplementary Table 2*). Similarly, there were no fixed differences in ITS+ sequence among three specimens of *R. mawsonae* from the same host (*M. dorsalis*), or among two specimens of *R. pi* from two host species (*M. rufogriseus* and *M. parryi*), even though variations were detected at nine (four in the ITS-1 and five in the ITS-2) and three (two in ITS-1 and one in the ITS-2) alignment positions, respectively. A similar pattern of intraspecific variation was found for three specimens of *R. macropodis* from two host species (*M. giganteus* and *M. fuliginosus*), except for a single nucleotide insertion in the ITS-1 sequence for one of two individuals (i.e. F736) collected from *M. giganteus*. Specimens of *R. longibursaris* from *M. rufogriseus* collected from Launceston (Tasmania) and Emu Flat (New South Wales) differed in ITS+ sequence at eight alignment positions (five in the ITS-1 and three in the ITS-2), whereas no genetic variation occurred in the DNA sequence of *R. spratti*, specimens of which were collected from the same host species and localities as *R. longibursaris*. Similarly, there were no fixed differences in ITS+ sequence of five *R. epsilon* individuals collected on the mainland (i.e. from *M. rufogriseus* and *W. bicolor*) and in Tasmania (from *M. rufogriseus*), except for a single insertion in the ITS-1 sequence of specimen F77 collected in New South Wales (*Supplementary Table 3*).

In contrast, fixed differences in both the ITS-1 and ITS-2 sequences were detected among individuals of four morphospecies, *R. rufogrisea*, *R. australis*, *R. zeta* and *R. rho*, collected from different host species and/or geographical regions (see *Supplementary Tables 4–8*, respectively). In the case of *R. rufogrisea*, although there were no fixed nucleotide differences in ITS+ sequences between specimens from *M. rufogriseus* collected in New South Wales (F84) and Tasmania (F720), there were seven (of 12) variable nucleotide positions (two in the ITS-1 and five in the ITS-2) when compared to a specimen from *M. parryi* collected in Queensland. Similarly, there were 52 variable nucleotide positions in ITS+ between *R. sigma* specimens from *Thylagale stigmatica* and those from *T. thetis*, most (25 in ITS-1 and 24 in ITS-2) representing putative fixed differences between nematodes from the two host species; 20 of these differences (five in ITS-1 and 15 in ITS-2) represented 1–10 bp nucleotides. In addition, nucleotide variation was detected at 20 positions in ITS+ sequences among individuals of *R. zeta* collected from different species of *Petrogale* in Queensland. There was only one fixed nucleotide difference in ITS-1 sequence between specimens of *R. zeta* and those from *P. herberti* and *P. inornata*, whereas these nematodes differed unequivocally at 13 (11 in ITS-1 and two in ITS-2) of 20 variable positions when compared with the ITS+ sequence from a specimen of *R. zeta* from *P. assimilis*. Genetic

variation was also detected at 52 nucleotide positions in the ITS+ sequences among seven individuals of *R. australis*. This morphospecies could be separated into two groups (clades) based on their ITS+ sequences. Nematodes in clade 1 had fixed differences at 38 (14 in ITS-1 and 24 in ITS-2) alignment positions when compared to those in clade 2. One eastern grey kangaroo (*M. giganteus*) collected from New South Wales contained taxa from both clades 1 and 2 (i.e. specimens F751 and F754). Likewise, in the case of *R. rho*, individuals from *Macropus eugenii* from South Australia (F724) and Western Australia (F905) had almost identical ITS+ sequences, which differed at 16 positions (eight in ITS-1 and eight in ITS-2) from specimens from *M. fuliginosus* from South Australia (F780) and Western Australia (F910), and from *M. irma* (G114) from Western Australia. Eighteen (five in ITS-1 and 13 in ITS-2) of these differences represented 1–4 nucleotides.

Phylogenetic analyses were conducted to determine whether individual morphospecies from different host species and/or geographical regions represented monophyletic assemblages. The ITS+ sequences were aligned over 824 positions (404 for ITS-1, 153 for 5.8S rDNA and 267 for ITS-2), 175 of which were informative for the MP analysis. The topology of the strict consensus tree of the MP analysis (not shown), based on 1217 equally most parsimonious trees ($L = 579$, $CI = 0.58$, and $RI = 0.83$), was very similar to that produced from the BI (*Fig. 3*) and the NJ analyses (*Fig. 4*). In all phylogenetic analyses, there was no support for all specimens of *R. australis* representing a monophyletic assemblage. However, there was absolute support ($pp = 1.0$ in the BI analysis and $bs = 100\%$ in both the NJ and MP trees) for the separation of *R. australis* into two clades. Similarly, there was no support for *R. sigma* from the two host species (*T. stigmatica* and *T. thetis*) forming a monophyletic assemblage. There was strong support ($pp = 0.999$ and 1, and $bs = 95\text{--}100\%$) for the separation of *R. rho* into two clades, one containing individuals from *M. eugenii* and the other containing individuals from *M. fuliginosus* and *M. irma*. However, there was support in the BI analysis ($pp = 0.994$) for a sister taxa relationship for the two clades of *R. rho*, and absolute support ($pp = 1$) for a sister relationship of the two clades with *R. setonicis*. In contrast, in the MP and NJ analyses, there was no support for a sister taxon relationship between the two clades of *R. rho*. However, there was strong support ($bs = 90\%$) in the NJ analysis for an assemblage comprising *R. setonicis* and the two clades of *R. rho*. There was also strong support ($pp = 1.0$ in the BI analysis and $bs = 96\text{--}97\%$ in the MP and NJ analyses) for *R. zeta* from the three host species, with support ($bs = 70\text{--}95\%$, $pp = 0.831$) for *R. zeta* from *P. inornata* and *P. herberti* forming a clade to the exclusion of *R. zeta* from *P. assimilis*. Similarly, there was absolute support ($pp = 1.0$; $bs = 100\%$) for *R. rufogrisea* representing a monophyletic clade, and support ($bs = 85\text{--}100\%$; $pp = 0.882$) for *R. rufogrisea* collected from *M. rufogriseus* in Tasmania and New South Wales forming a clade to the exclusion of *R. rufogrisea* collected from *M. parryi* in Queensland.

In the phylogenetic analysis of ITS+ sequence data for the morphospecies, *R. alpha* was the sister species to the remaining congeners. There was support in the BI and MP analyses (i.e. $pp = 0.973$; $bs = 86\%$) for a clade consisting of *R. macropodis*, *R. rosemariae*, *R. rufogrisea* and *R. theta* forming a clade with respect to the other species. *Rugonema labiatum* nested in a clade with *R. pi* and *R. mu*. There was also some support (i.e. $pp = 0.995$; $bs = 71\text{--}75\%$) for a clade containing *R. longibursaris*, *R. omega*, *R. tau* and *R. sprattii* (*Figs. 3 and 4*).

Comparison of the phylogeny of the nematodes, derived from the analysis of ITS+ sequence data, with that currently available for the host species (*Fig. 5*) provides little evidence for co-speciation between nematodes and hosts. Also included on the nematode tree were the different buccal capsule types. This comparison

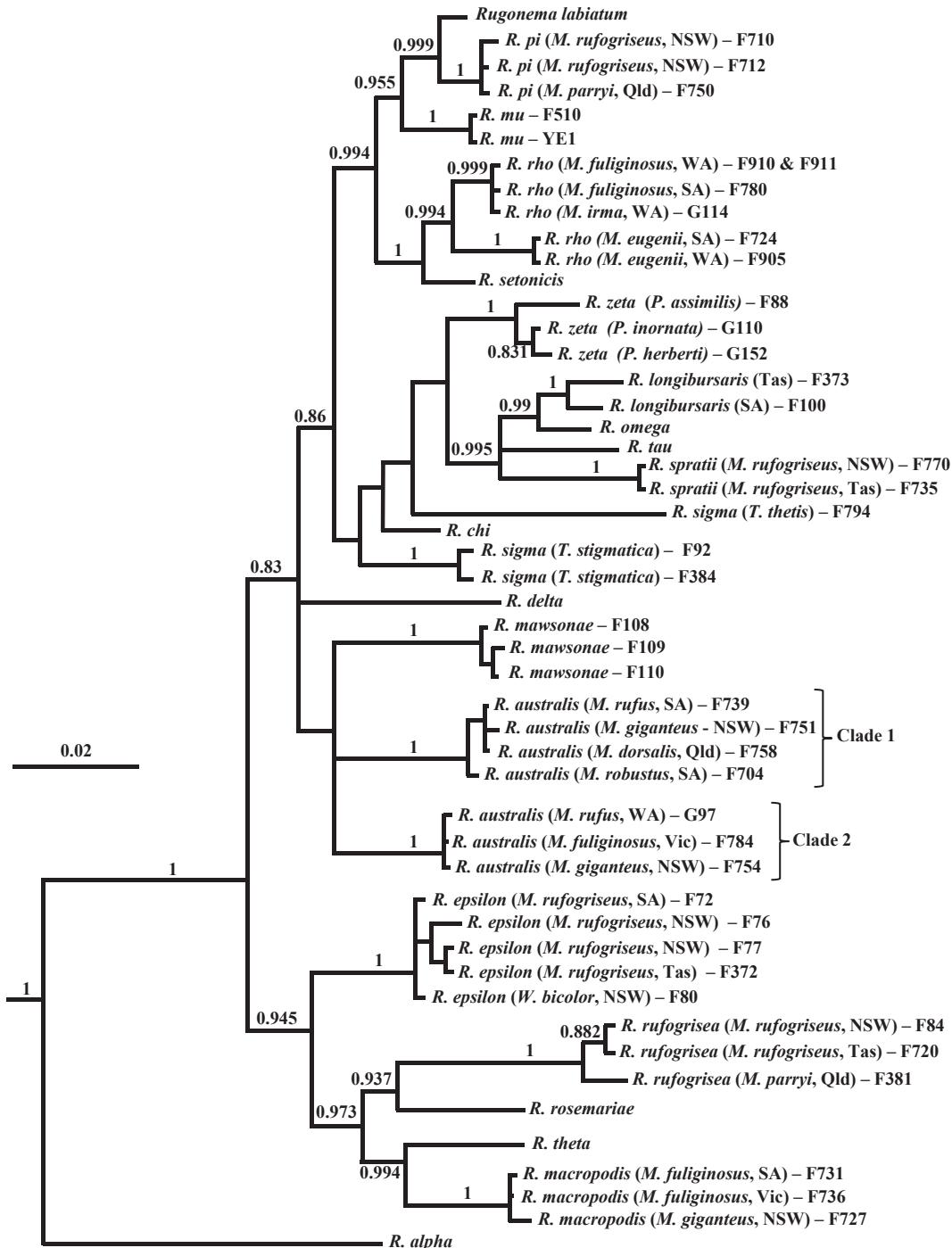


Fig. 3. Phylogenetic relationships of species of *Rugopharynx* and *Rugonema labiatum* based on a Bayesian analysis of the sequence data of the ITS+ nuclear ribosomal DNA. Values above branches indicate posterior probabilities that were greater than 0.8. Abbreviations of Australian state names are provided in Table 1.

revealed that a simple buccal capsule (I) appears to be the plesiomorphic state (as for *R. alpha*). Of the three principal clades depicted in Fig. 5, type I buccal capsules occurred in all clades, being the exclusive buccal capsule type in one clade, but was mixed with type II capsules in a second clade and mixed with type III capsules in the third clade.

4. Discussion

To date, species of the genus *Rugopharynx* found in the stomachs

of macropodid marsupials have been identified solely on the basis of morphological criteria (Beveridge, 1982) or the combination of morphological and MEE data (Chilton et al., 1993; Beveridge et al., 1994). In describing new species within the *R. australis* complex, Beveridge and Chilton (1999) relied on limited MEE data (Chilton et al., 1996) and extrapolated from this base in describing nine new species based on small but potentially significant morphological characters.

The present study represents the first detailed examination of the genus using DNA sequence data and therefore represents the

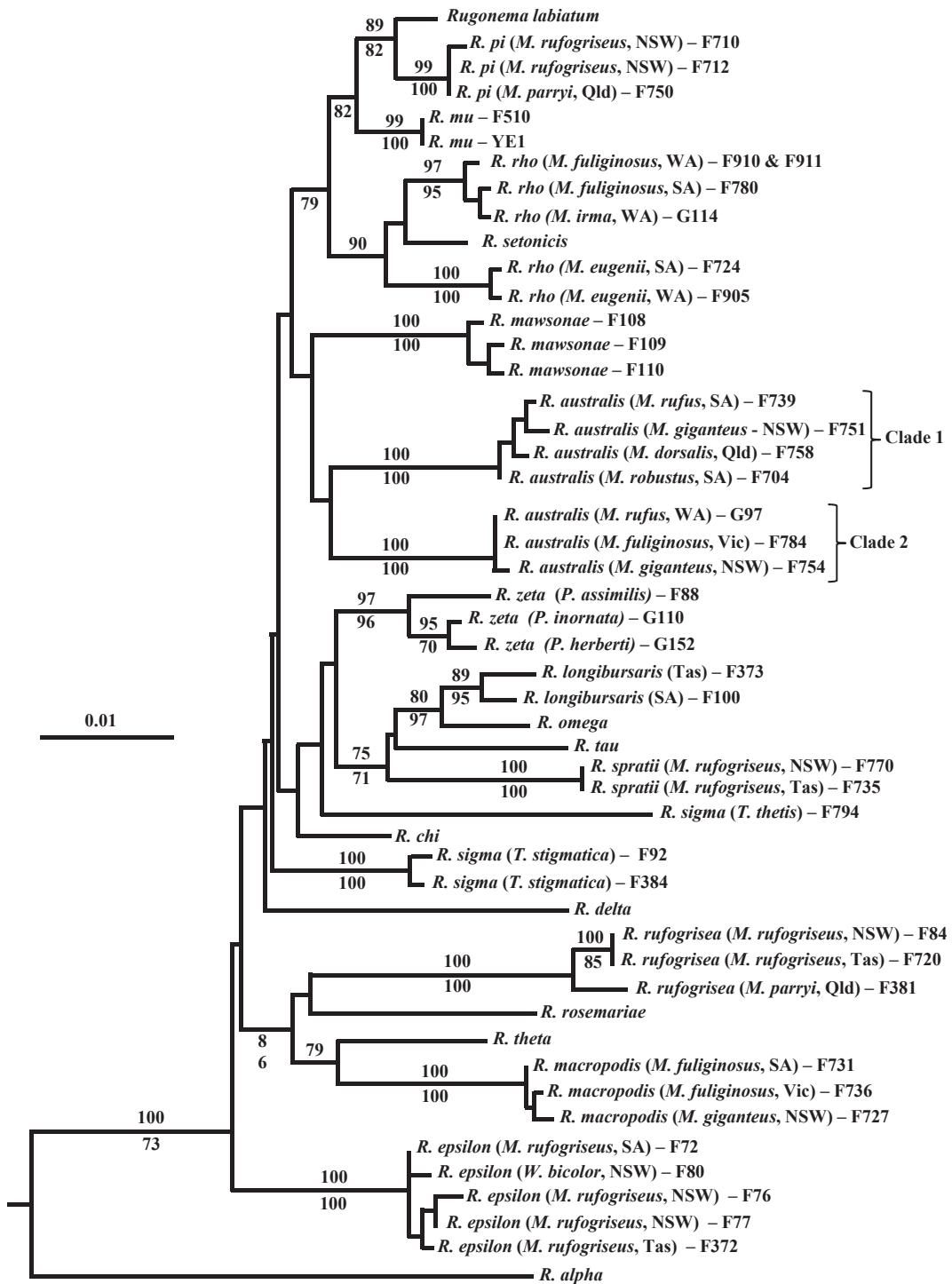


Fig. 4. Phylogenetic relationships of species of *Rugopharynx* and *Rugonema labiatum* based on a neighbor-joining analysis of the sequence data of the ITS+ nuclear ribosomal DNA. Values above and below branches represent the NJ and MP bootstrap values (respectively) that were greater than 70%.

first test of the validity of the range of species currently recognised either exclusively on morphological grounds or on the basis of morphological and MEE data. The current molecular analyses included most of the currently known species, apart from *R. longispicularis* and *R. petrogale*. Both of these species occur in hosts, such as the parma wallaby, *Macropus parma*, and the brush-tailed rock wallaby, *Petrogale penicillata*, which are currently considered to be rare or vulnerable species (Maynes, 2008; Eldridge

and Close, 2008); therefore, it is challenging to obtain parasite material from these host species for molecular studies. No species of *Rugopharynx* are known to occur in *M. (N.) agilis*, *M.(O.) antilopinus* and *M. (O.) bernardus* (Spratt et al., 1991).

In the current study, all of the species of *Rugopharynx* presently recognised based on morphological differences (i.e. morphospecies), in some cases with supporting MEE data, had a unique set of ITS+ sequences, thereby providing additional evidence in support

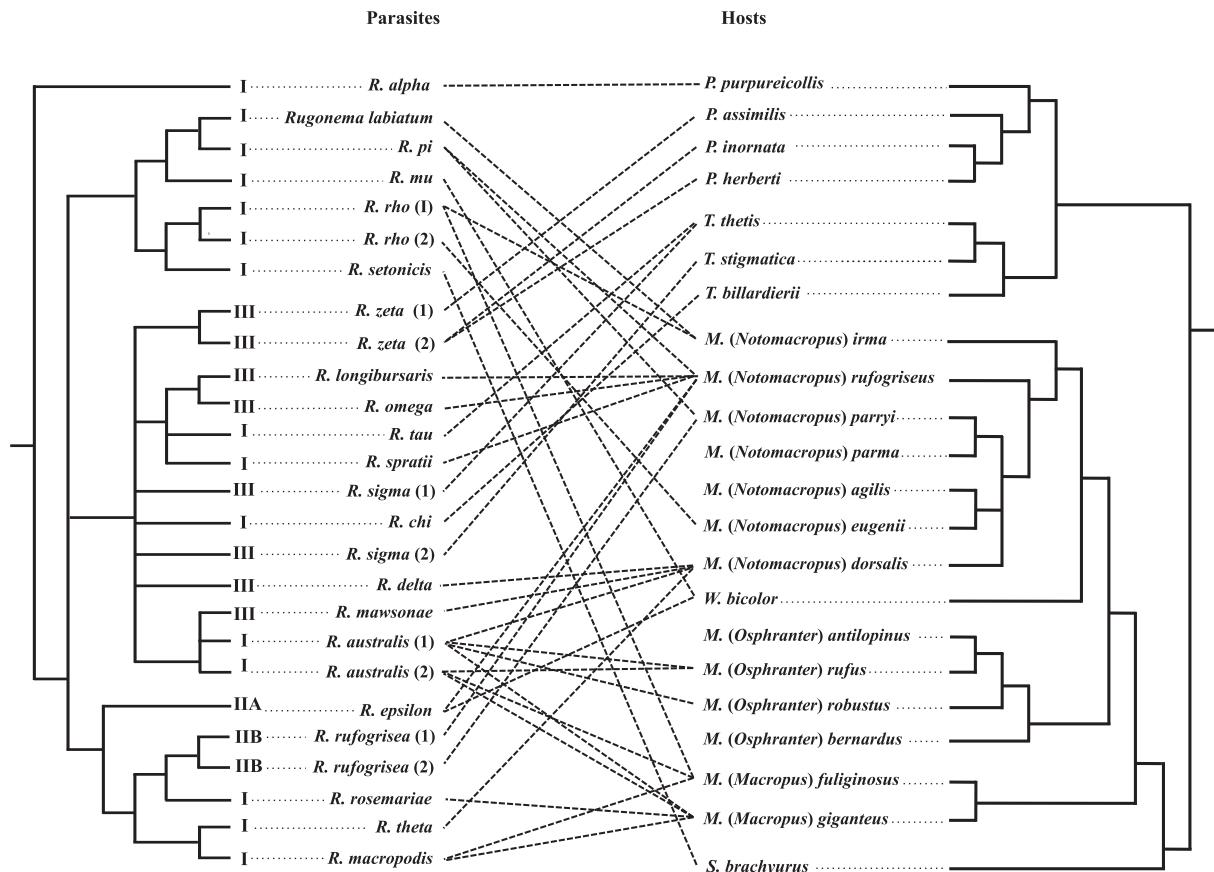


Fig. 5. Molecular phylogeny of species of *Rugopharynx* and *Rugonema labiatum* based on a consensus of the BI, NJ and MP trees (Figs. 3 and 4), and the relationships of their hosts. This figure includes species of *Macropus* (*M. agilis*, *M. antilopinus*, *M. bernardus*) which are not hosts to species of *Rugopharynx*, as well as *M. parma*, from which no material could be obtained for genetic studies. Only those species of *Petrogale* included in this study are shown on the host tree. The morphology of the buccal capsule for each nematode taxon is also shown.

of their validity. Fixed differences in the ITS-1 and ITS-2 sequences between or among morphospecies were limited (e.g., 0.3% between *R. setonicis* and *R. rho*, and 2.3% between *R. omega* and *R. longibursaris*, for the ITS-1 and ITS-2, respectively), and data from a single pair of ribosomal DNA spacers may not always provide unequivocal support for specific status (Nadler and Pérez-Ponce de León, 2011). However, given the reliability of ITS-1 and ITS-2 sequences for identifying and distinguishing nematode species from macropodids to date (e.g., Chilton et al., 2009a, 2012), the evidence presented here is relatively strong. In some instances, the morphological and genetic differentiation is supported by the occurrence of formerly cryptic species in the same host individual. In the case of *R. australis* and *R. macropodis* (i.e. previously included within *R. australis*), the former occurring in kangaroos in arid environments and the latter in areas of higher rainfall (Beveridge and Chilton, 1999), both species were found at one intermediate location in Victoria (Pine Plains Station) (Beveridge and Chilton, 1999), indicating genetic isolation and providing further support for the validity of the two species.

The data presented here suggest that additional cryptic (i.e. genetically distinct but morphologically similar) species of *Rugopharynx* remain to be described. Specimens of *R. australis* occurred in two quite distinct clades, although some specimens from the two clades were collected from the same individual host (*M. giganteus*). The magnitude of fixed differences in ITS-1 and ITS-2 sequences between members of the two *R. australis* clades (3.6% and 10.4%, respectively) was greater than that between related morphospecies

(e.g., 0.8% and 2.3%, respectively, between *R. omega* and *R. longibursaris*). Furthermore, there was no support for the two *R. australis* clades forming a monophyletic assemblage. Examination of the voucher specimens involved in this study suggested that the two specimens were differentiable based on spicule lengths, with those from *M. giganteus* from Trangie, NSW (F751) being 2.0 mm, and those of the additional specimen from the same individual host (F754) being only 1.28 mm long; the spicules of a similar specimen from *M. fuliginosus* from Hattah Lakes, Victoria (F784), were 1.36 mm long. Beveridge and Chilton (1999) gave the spicule lengths of *R. australis* as 1.44–1.95 mm, which virtually encompasses the range of the specimens used in this study. The spicule lengths of the most (morphologically) similar species to *R. australis*, (i.e. *R. macropodis*) are 1.14–1.23 mm (Beveridge and Chilton, 1999). In addition, Beveridge and Chilton (1999) noted significant differences in bursal morphology within this species. Therefore, it appears that *R. australis*, as currently defined, is a composite of at least two species.

In the case of *R. sigma*, there was limited variation (i.e. two fixed differences in ITS-2 but none in ITS-1) in the ITS+ sequences of two specimens from *T. stigmatica*, collected 1200 km from one another in Queensland, whereas they had 49 fixed differences (25 in ITS-1 and 24 in ITS-2) when compared with *R. sigma* from *T. thetis*. This magnitude of sequence difference (6.5% and 10.3% for ITS-1 and ITS-2, respectively) exceeded that among many morphospecies within the genus. Furthermore, specimens F794 from *T. thetis* and G384 from *T. stigmatica* were collected at the same locality (Lamington

National Park, Qld), thus being in sympatry. The phylogenetic analyses also showed that *R. sigma* from the two host species did not form a monophyletic clade, providing additional support that they represent cryptic species. Examination of the female voucher specimen of *R. sigma* from *T. thetis* (F794) indicates a tail length of 0.21 mm compared with 0.39–0.45 mm for specimens from *T. stigmatica* and the distance of the vulva from the posterior end as 0.30 mm compared with 0.60–0.70 mm in specimens from *T. stigmatica* (see Chilton et al., 1993). Hence, there appear to be morphological features supporting the molecular differences for these specimens. Griffith et al. (2000) concluded that the helminth communities of these two host species in southern Queensland, where they are sympatric, were essentially similar. The current genetic studies suggest that these conclusions may need to be revised.

Specimens of *R. zeta* from *P. inornata* and *P. herberti* formed a strongly supported clade to the exclusion of specimens from *P. assimilis*. These three closely related species of rock wallabies have parapatric distributions along the eastern coast of Queensland (Potter et al., 2012). Chilton et al. (2009a) examined three species of cloacine nematodes, *Cloacina caenis*, *C. pearsoni* and *C. robertsi*, which occur in these related rock wallaby species and demonstrated genetic differences between them, suggesting the existence of cryptic species. Current data also suggest the possible existence of cryptic species within *R. zeta*. There was one fixed difference in ITS+ between specimens of *R. zeta* from *P. inornata* and *P. herberti* (but none in the ITS-2), whereas they had 13 fixed differences (11 in ITS-1 and 2 in ITS-1) when compared to the *R. zeta* from *P. assimilis*. The magnitude of sequence difference in ITS-1 (2.8%) exceeds that detected between *R. omega* and *R. longibursaris*, whereas the 0.9% sequence differences in the ITS-2 is less than between these two morphospecies. Therefore, additional molecular investigations are required to test the hypothesis that *R. zeta* represents a species complex. The current data suggest the existence of one species in *P. assimilis* and a second species in both *P. inornata* and *P. herberti*. *Rugopharynx zeta* also occurs in a number of related species of rock wallabies (*Petrogale mareeba*, *Petrogale sharmani* and *P. penicillata*) (Spratt et al., 1991) and specimens from these additional hosts would need to be included in future studies.

Rugopharynx rho from *M. fuliginosus* from both South Australia and Western Australia, together with *M. irma* from Western Australia, formed a clade distinct from the same morphospecies obtained from *M. eugenii* in South Australia and Western Australia. There was also no support in the MP and NJ analyses for these two clades forming a monophyletic assemblage. As *M. fuliginosus* and *M. eugenii* are sympatric at both localities (Van Dyck and Strahan, 2008), the data suggest that cryptic species may also exist within this taxon. In Western Australia, *M. irma* and *M. fuliginosus* are sympatric (Van Dyck and Strahan, 2008) and the occurrence of this species in *M. irma* may have resulted from host switching.

Consequently the data presented here suggest that additional cryptic species may exist within *R. australis*, *R. rho*, *R. sigma* and *R. zeta*. There is also a possibility of cryptic species within *R. rufogrisea*. There were no fixed differences in ITS+ sequence between specimens of *R. rufogrisea* from *M. rufogriseus* collected on the mainland of Australia and the island state of Tasmania, which is consistent with the findings for other morphospecies (e.g., *R. epsilon* and *R. spratti*) that parasitise *M. rufogriseus*. However, the magnitude of the fixed sequence differences between *R. rufogrisea* from *M. rufogriseus* and *M. parryi* (0.5% and 2.1% in ITS-1 and ITS-2, respectively) is very similar to that between *R. omega* and *R. longibursaris*, two other species that parasitise *M. rufogriseus* (Beveridge, 1982). This aspect needs to be explored further using multiple specimens from both host species.

The current analysis also provides some insight into host

specificity within the genus. Although many of the species examined in this study appear to be moderately host specific, occurring in one or two host species (i.e. *R. alpha*, *R. chi*, *R. delta*, *R. longibursaris*, *R. mawsonae*, *R. mu*, *R. omega*, *R. pi*, *R. spratti*, *R. tau* and *R. theta*), other species included in this study appear to have a wide host range. Thus, *R. australis* was identified in *M. rufus* (the type host for the species), as well as in *M. fuliginosus*, *M. giganteus*, *Macropus robustus* and *M. dorsalis*. Specimens from the first four host species were collected in arid or semiarid regions of Australia (Table 1) where these host species are sympatric and *R. australis* is a common parasite in each of them (Arundel et al., 1979; Beveridge et al., 1998). However, *R. australis* is an uncommon parasite of *M. dorsalis* (see Beveridge et al., 1998) and the specimen collected here was in an area in which *M. dorsalis* is sympatric with kangaroo species commonly parasitized by this nematode.

Analyses of ITS+ sequence data of *R. epsilon* specimens from different host species (i.e. *M. rufogriseus* and *W. bicolor*) suggest that it represents a single species with a broad host range, given that *R. epsilon* parasitizes a variety of macropodid hosts (Spratt et al., 1991). Therefore, examination of additional specimens of *R. epsilon* from other host species needs to be studied to test this proposal.

The genus *Rugonema* was erected (Beveridge, 1999) for a single species of nematode, *Ru. labiatum*, from the stomach of *M. irma* which resembled *Rugopharynx* but in which the labial collar, instead of forming an annulus, was prominently four-lobed, similar to the genus *Wallabinema*. However, *Wallabinema* lacks a striated buccal capsule and differs in the morphology of the oesophagus. The genetic data presented here show that *Ru. labiatum* is clearly nested within the genus *Rugopharynx*. The sole distinguishing morphological feature of *Rugonema*, that is the four lip-like lobes of the labial collar, is an autapomorphy within the tribe Pharyngostrixylinea. Based on the molecular data and a reconsideration of its morphological differentiation, *Rugonema* is here made a synonym of *Rugopharynx* with its sole species becoming *Rugopharynx labiatum* (Beveridge, 1999) n. comb. This species was most genetically similar to *R. pi* and belonged to a clade that also included *R. mu*.

Beveridge (1982) divided *Rugopharynx* into three groups based on the morphology of the buccal capsule, with either a simple cylindrical buccal capsule, a bilobed or a trilobed buccal capsule. The group with bilobed buccal capsules consisted of *R. epsilon* and *R. rufogrisea*, with the indentation occurring in the mid region in *R. epsilon* and in the anterior quarter in *R. rufogrisea*. However, among the new species described by Beveridge and Chilton (1999), some were difficult to allocate to a particular group (i.e., *R. tau* and *R. petrogale*) because the division of the buccal capsule was subtle (*R. tau*) or variable (*R. petrogale*). The mapping of the morphological data onto the consensus nematode phylogenetic tree (Fig. 5) suggest that the simple buccal capsule is the plesiomorphic state within the genus and that bilobed buccal capsules have evolved independently. The findings of the present study also suggest that the trilobed buccal capsules are a derived character. The lack of resolution in the cladogram prevents more detailed conclusions from being drawn on the evolution of buccal capsule shapes within the genus.

There appears to be no co-evolutionary relationship between nematodes and hosts. Based on molecular evidence, *Setonix* diverged within the macropodine lineage about 10 million years ago, while *Thylogale* and *Petrogale* are sister taxa to the clade that contains *Macropus* and *Wallabia*, their estimated time of divergence being about eight million years (Meredith et al., 2008). The largest clade of the molecular phylogenetic tree of the nematodes contains species from each of these macropodine genera apart from *Setonix*. Beveridge and Chilton (2001) undertook a morphological

phylogenetic analysis of the *R. australis* complex, which produced a completely unresolved tree, and consequently these authors concluded that there was no obvious co-evolutionary association with hosts. Comparable studies of other cloacine genera also supported the hypothesis that evolution within this group of nematodes was primarily by host switching (Beveridge and Chilton, 2001), a hypothesis concordant with the data presented above. Chilton et al. (2016) have provided molecular evidence for host switching in the related cloacine genus *Cyclostrongylus*.

In summary, the molecular data presented here support the earlier morphological studies of the genus *Rugopharynx* and provide additional evidence that the species of *Rugopharynx* currently established, many of them based on minor morphological differences and MEE data, are indeed valid. The study has also revealed the existence of additional cryptic species within the genus that need to be characterised morphologically. Comparisons of buccal capsule morphology with the phylogenetic tree provided some insights into the evolution of more complex buccal capsules, but there were no obvious co-evolutionary associations with hosts, the data instead suggesting a pattern of host switching in the evolution of the genus.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

This study was funded largely by grants from the Australian Research Council and the Australian Biological Resources Study. Specimens were collected under the following permits: Victorian Department of Sustainability and Environment 90-053, 91-095, 93-016, 10000434, 100003649), Queensland National Parks and Wildlife Service T00436, T1131), New South Wales National Parks and Wildlife Service A68.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijppaw.2016.04.003>.

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