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Speciation in the genus *Cloacina* (Nematoda: Strongylida): species flocks and intra-host speciation

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SUMMARY

Sequences of the first and second internal transcribed spacers (ITS1 + ITS2) of nuclear ribosomal DNA were employed to determine whether the congeneric assemblages of species of the strongyloid nematode genus *Cloacina*, found in the forestomachs of individual species of kangaroos and wallabies (Marsupialia: Macropodidae), considered to represent species flocks, were monophyletic. Nematode assemblages examined in the black-striped wallaby, *Macropus* (*Notamacropus*) *dorsalis*, the wallaroos, *Macropus* (*Osphranter*) *antilopinus*/*robustus*, rock wallabies, *Petrogale* spp., the quokka, *Setonix brachyurus,* and the swamp wallaby, *Wallabia bicolor,* were not monophyletic and appeared to have arisen by host colonization. However, a number of instances of within-host speciation were detected, suggesting that a variety of methods of speciation have contributed to the evolution of the complex assemblages of species present in this genus.

Key words: *Cloacina*, Nematoda, Strongylida, speciation, species flocks, internal transcribed spacers.

INTRODUCTION

The phenomenon of 'species flocks', that is the occurrence of numerous species of congeneric (or confamilial) parasites in the same host species, has been the focus of a number of studies, particularly of parasitic nematodes. One of the best-known examples of the phenomenon is that of the oxyuroid nematodes found in the colon of tortoises (Schad, 1963; Petter, 1966). In this instance, each species of tortoise harbours large numbers of congeneric or confamilial nematode species, all belonging to the family Pharyngodonidae (Petter, 1966). However, there are differences in the longitudinal and radial distributions of these nematode species within the colon of the host (Schad, 1963). Other examples of species flocks of parasitic nematodes are those found in the large intestines of equids, elephants and rhinoceroses, as well as those in the sacculated forestomachs of kangaroos and wallabies (Inglis, 1971). All of these nematodes have direct life-cycles with the ingestion of eggs or third-stage larvae from the environment (Anderson, 2000).

In the case of equids (horses, donkeys and zebras), 14 genera and 50 species of strongyloid nematodes belonging to the tribe Cyathostominea are currently recognized (Lichtenfels *et al*. 2008), with the common co-occurrence of many species (Bucknell *et al*. 1996; Anjos and Rodrigues, 2003; Bu *et al*. 2009; Kuzmina *et al*. 2009), but again with differences in the distribution of species within the gastro-intestinal tract (Ogbourne, 1976; Mfitilodze and Hutchinson, 1985; Bucknell *et al*. 1995; Stancampiano *et al*. 2010). Comparably detailed studies on the strongyloid nematodes of elephants and rhinoceroces have not been conducted, but four genera and 49 species belonging to the related strongyloid tribes Kiluluminea, Murshidinea and Quiloninea are known to occur in their large intestines (Chabaud, 1957; Round, 1968; Canaris and Gardner, 2003; Beveridge and Jabbar, 2013). In the case of kangaroos and wallabies (family Macropodidae) some 36 genera and 256 species of nematodes belonging to the sub-family Cloacininae occur in the sacculated forestomach (Beveridge and Chilton, 2001), frequently in large numbers (Beveridge and Arundel, 1979), with again, some degree of spatial separation within the stomach (Mykytowycz, 1964; Arundel *et al*. 1979; Pamment *et al*. 1994).

In a critical review of the phenomenon of species flocks in parasitic helminths, Kennedy and Bush (1992) indicated several difficulties in the application of this appellation to the examples cited above. First of all, these authors noted that species flocks, according to classical definitions, could be defined either by ecological parameters such as co-occurrence (Mayr, 1984) and endemism (Ribbink, 1984) or could be circumscribed phylogenetically, with a species flock being a monophyletic assemblage (Greenwood, 1984). In the former case, such associations of multiple congeners could develop through a number of host colonization events, while in the latter case, the communities could evolve through intra-host

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speciation. Intra-host speciation (Price, 1980; Poulin, 2007) or even sympatric speciation (Kunz, 2002) are considered to be potentially common modes of evolution in parasites.

The phylogenetic definition of species flocks has been successfully applied to cichlid fishes (Seehausen, 2006) and to rock fishes (Alesandrini and Bernardi, 1999), but the paucity of rigorous phylogenetic studies of parasitic nematodes means that this definition is often not applicable. In the case of the cyathostomin nematodes of equids (*Equus* spp.), available molecular evidence suggests that they do indeed form a monophyletic assemblage (Hung *et al*. 2000; McDonnell *et al*. 2000). The same situation may apply to the strongyloid nematodes of elephants as most species belong to two related tribes, the Murshidiinea and Quiloninea (Lichtenfels, 1980). This may well also apply to the Cloacininae in macropodids. However, appropriate morphological and molecular phylogenetic studies are lacking. A similar situation pertains, in the case of molecular studies, to the oxyuroid nematodes in tortoises for which molecular data are lacking. As a consequence, among nematodes, it appears that the phylogenetic definition of a species flock may only be applicable currently to the cyathostomin nematodes of equids. A molecular study of species of *Onchocerca* occurring in cattle also suggests within-host speciation in this nematode genus, but the result is dependent upon the inclusion of remaining congeners (Morales-Hojas *et al*. 2006).

In considering the ecological definition of a species flock, Kennedy and Bush (1992) pointed out that the criterion of 'co-occurrence' was also difficult to apply since, for several parasite groups, relevant data were not available on the co-occurrence of congeneric or confamilial parasites within an individual host; rather published data described the meta-community. This ecological difference is also reflected in phylogenetic studies in which the criterion of sympatry in potential examples of intra-host speciation can be problematical (McCoy, 2003). Data on the co-occurrence of species of nematodes are however available for the cyathostomins of equids (Bucknell *et al*. 1996; Anjos and Rodrigues, 2003; Bu *et al*. 2009; Kuzmina *et al*. 2009) as well as for the cloacinine nematodes of macropodids (Beveridge *et al*. 2002), but not for the remaining nematode communities cited.

In spite of these potential difficulties, the phenomenon of species flocks in parasites clearly warrants further study. The genus *Cloacina*, found in the stomachs of macropodid marsupials may represent a suitable model for additional studies as it currently contains 116 described species (Beveridge, 1998, 1999, 2002, 2014; Beveridge and Speare, 1999; Beveridge *et al*. 2014*a*, *b*) with several additional species as yet undescribed (Chilton *et al*. 2009). These nematodes also have a relatively high degree of host specificity (Beveridge *et al*. 2002). In addition, the study of a single genus (although the monophyly of *Cloacina* has not yet been investigated using molecular methods) overcomes the difficulty of deciding whether species flocks should be considered as being composed of congeners or whether the concept should expand to con-sub- familiar or confamiliar taxa (see Kennedy and Bush, 1992), a potential complication in the studies of the nematode assemblages of equids. Furthermore, studies to date of different species of kangaroos and wallabies (seven species of the currently recognized 54 host species were studied by Beveridge *et al*. 2002) have shown that assemblages of nematode species range across a continuum, from three to 12. This spectrum of hosts with varying numbers of co-occurring congeners is not represented in any other host-parasite system as there remain only five extant species of equids (all with a very similar parasite fauna), three species of elephants and four species of rhinoceros, all relicts of formerly more diverse faunas (Franzen, 2010). In addition a number of the latter host species are endangered thereby imposing a limitation on parasitological studies.

In an attempt to address the question of whether the species flocks of *Cloacina* seen in macropodids are monophyletic, Beveridge *et al*. (2002) undertook a phylogenetic analysis based on morphological characters. However, given that only a limited number of morphological characters was available, a common phenomenon for parasitic nematodes, the resulting phylogeny exhibited a relatively low consistency index. In spite of this reservation, it was suggested that while several nematode species pairs could be identified in individual host species, there was no strong evidence for the existence of monophyletic species flocks (Beveridge *et al*. 2002). It was therefore tentatively suggested that the assemblages might have arisen through host switching.

Given the difficulties encountered in the use of morphological characters to establish phylogenetic relationships among nematodes, it was decided to re-examine the problem using molecular methods. The combined first and second internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA represent ideal markers as they have previously been used successfully to establish the phylogenetic relationships among some strongylid nematodes (Hung *et al*. 2000; Gouÿ de Bellocq *et al*. 2001).

In the present study, we focus on macropodid host species which harbour large numbers of co-occurring nematode species. Based on the preliminary work of Beveridge *et al*. (2002), these hosts were the swamp wallaby, *Wallabia bicolor*, the black-stripe wallaby, *Macropus* (*Notamacropus*) *dorsalis*, the wallaroo, *M*. (*Osphranter*) *robustus* (and its close, sympatric relative *M*. (*O*.) *antilopinus*), the quokka, *Setonix brachyurus* and the rock wallabies,

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Petrogale spp. Other host species such as the red kangaroo, *Macropus* (*O.*) *rufus*, the red-legged pademelon, *Thylogale stigmatica*, the whiptail wallaby, *M.* (*N.*) *parryi*, the agile wallaby, *M.* (*N.*) *agilis* and the tammar wallaby, *M.* (*N.*) *eugenii*, were also included as sequence data were also available for species of *Cloacina* found in them.

MATERIALS AND METHODS

Nematodes were obtained from the stomachs of kangaroos and wallabies which 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 samples of nematodes from each host were fixed in Berland's fluid (glacial acetic acid and formalin; Gibson, 1979) for morphological examination.

Frozen nematodes were thawed, the head and tail were removed from individuals, fixed in lactophenol and mounted permanently in polyvinyl lactophenol as voucher specimens, with the mid-body region being used for genetic analyses. Nematodes were identified following Beveridge (1998, 1999) and Beveridge *et al.* (2014*a*, *b*). Voucher specimens (hologenophores) have been 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) have been deposited in SAM (Table 1). Where possible, registration data for both hologenophores and paragenophores have been included. Codes for slide numbers and/or host identifications included in Table 1 correspond to entries in the SAM database.

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). The region of rDNA comprising the ITS-1, 5·8S rRNA gene, ITS-2 and flanking sequences (=ITS+) was amplified by the polymerase chain reaction (PCR) using primers NC16 (forward; 5′-AGTTCAATC GCAATGGCTT-3′) and NC2 (reverse; 5′-TTAG TTTCTTTTCCTCCGCT-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 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 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 were utilized and are indicated in Table 1. In the case of published studies of the genetic variability within species of *Cloacina* (Shuttleworth *et al.* 2014, 2016*a*, *b*), a representative sequence from each species was selected. In these studies, although sequence variability was found within each species of *Cloacina*, individuals representing each species formed a distinctive clade. On this basis, additional species have been added as a single sequence. In instances in which the sequences available were from different host species (*Cloacina parva* from *Macropus* (*O.*) *robustus* and *Petrogale purpureicollis*; *Cloacina phaedra* from *Macropus* (*N.*) *agilis* and *M.* (*N.*) *parryi*), both sequences were included in the analyses. Due to the occurrence of hybridization between the related phascolostrongyline nematodes *Paramacropostrongylus typicus* and *Paramacropostrongylus iugalis* (Chilton *et al.* 1997*b*), all chromatograms were examined for the possible occurrence of hybrids within the genus.

Sequences were initially aligned using Muscle (Edgar, 2004) and alignments adjusted manually using the program Mesquite v.3.03 (Maddison and Maddison, 2015). Analyses of sequence data were conducted by Bayesian inference (BI) using Monte Carlo Markov Chain analysis in the program MrBayes v.3.2.3 (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.2.1.7 (Posada, 2008). The alignment was partitioned into two datasets (ITS1 and ITS2). The number of substitutions was set at 6 (Nst = 6), with a gamma-distribution and a proportion of invariable sites. 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. The ITS+ sequence of *Arundelia dissimilis*, a species within a related genus in the same tribe, Cloacininea, was used as the outgroup.

The parasite phylogeny was compared with a molecular phylogeny of the hosts based primarily on Meredith *et al.* (2008). As there is no comprehensive molecular phylogeny for the Macropodidae, any taxa missing from the latter study were interpolated based on the comprehensive dataset of Cardillo *et al.* (2004) and the resulting tree is presented as a cladogram. For comparison with the host phylogeny, the parasite tree was also converted to a cladogram.

Macropus (*O.*) *antilopinus* and *M.* (*O.*) *robustus* share most of their parasites (Beveridge *et al.*

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Table 1. Species of *Cloacina* and *Arundelia* included in this study with collection details, morphological voucher numbers (paragenophores in parentheses) and molecular sequence registration numbers

Nematode species	Code (slide no./ host no.)	Host species	Collection locality	Coordinates°, ′S °, ′E	SAM voucher	Genbank number
A. dissimilis	RG1(4J10)	*W. bicolor*	Phillip Is., Vic.	38°28′ 145°13′	36284 (24651)	MF284673
C. antigone	G40/42(2N3)	*W. bicolor*	Shute Harbour, Qld	20°17′ 148°47′	36283 (24392)	MF284674
C. anthea	G108 (WD8)	*W. bicolor*	Dixon's Creek, Vic	37°34′ 145°25′	35789 (46736)	MF284699
C. annulata	(-) 8R33·32	*W. bicolor*	Proserpine, Qld	20°22′ 148°17′	46764 (24226)	KM219839 (Shuttleworth *et al.* 2014)
C. atthis		*M. robustus*	Fortescue River Roadhouse, WA	21°17′ 116°08′	46963 (45937, 45945, 45965)	KM016823 (Beveridge *et al.* 2014*b*)
C. bancroftorum	86G (7A1·10)	*M. dorsalis*	Bowen, Qld	20°01′ 148°15′	36276 (27162)	MF284675
C. burnettiana	72G (3S41·5)	*M. dorsalis*	Marlborough, Qld	22°49′ 149°53′	36279 (2608)	MF284676
C. cadmus	G216 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36268 (31932)	MF284677
C. caenis	G303 (-)	*P. assimilis*	Mt Louisa, Qld	19°17′ 146°44′	28750 (28749)	FM992588 (Chilton *et al.* 2009)
C. castor	(-) 39R1·3	*W. bicolor*	Healesville, Vic	37°39′ 145°31′	46765 (-)	KM219841 (Shuttleworth *et al.* 2014)
C. cloelia	G279	*P. persephone*	Airlie Beach, Qld	20°16′ 148°43′	36282 (23712)	FM992612 (Chilton *et al.* 2009)
C. ceres	G210 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36269 (32967)	MF284678
C. chiron	G207 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36265 (32969)	MF284679
C. circe	G206 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36266 (32970)	MF284680
C. clymene	G68 (7M49)	*M. robustus*	Warwick, Qld	28°13′ 152°02′	46965 (25321)	KMO16822 (Beveridge *et al.* 2014*b*)
C. communis	G4 (-)	*M. robustus*	Wollomombi, NSW	30°30′ 152°03′	47957 (44360)	KU853896, KU853908 (Shuttleworth *et al.* 2016*b*)
C. dahli	F695 (XK1)	*T. stigmatica*	Sarina, Qld	21°26′ 149°13′	47857 (27210)	MF284681
C. daveyi	G11 (-)	*M. robustus*	Mulga Park Stn, NT	25°59′ 131°35′	47600 (46219)	KU853840, KU853909, (Shuttleworth *et al.* 2016*b*)
C. dindymene	161G (8K41)	*M. antilopinus*	Mt Surprise, Qld	18°09′ 144°19′	36281 (25909)	MF284682
C. dirce	139G (8K 5·1)	*M. antilopinus*	Mt Surprise, Qld	18°09′ 144°19′	36280 (25928)	MF284683
C. dryope	68G (3U4·1)	*M. dorsalis*	Yeppoon, Qld	23°08′ 150°44′	36272 (-)	MF284684
C. echidne	G12 (-)	*M. robustus*	Fortescue River Roadhouse, WA	21°17′ 116°08′	47601 (45941)	KU853841, KU853924 (Shuttleworth *et al.* 2016*b*)
C. edwardsi	(-) WX3·1	*W. bicolor*	Miles, Qld	26°39′ 150°11′	36286 (36287-9)	MF284685
C. eos	(-) 4B8·4	*W. bicolor*	Miles, Qld	26°39′ 150°11′	46769 (24143)	KM219917 (Shuttleworth *et al.* 2014)
C. epona	G14 (-)	*M. robustus*	Cloncurry, Qld	20°42′ 140°31′	47602 (32394)	KU853844, KU853914 (Shuttleworth *et al.* 2016*b*)
C. ernabella	G174 (-)	*P. purpureicollis*	Winton, Qld	22°27′ 143°09′	28733 (28733)	FM992598 (Chilton *et al.* 2009)
C. feronia	G19 (-)	*M. robustus*	Kalgoorlie, WA	30°44′ 121°27′	- (31800)	KU853839, KU853951 (Shuttleworth *et al.* 2016*b*)
C. frequens	G27 (-)	*M. robustus*	Kangaroo Hills Stn, Qld	18°55′ 145°40′	47610 (32738)	KU853830, KU853907 (Shuttleworth *et al.* 2016*b*)
C. galatea	(-) 8R33·24	*W. bicolor*	Proserpine, Qld	20°24′ 148°34′	46741(46740)	MF284700
C. gallardi	(-) 8Y22	*W. bicolor*	Proserpine, Qld	20°24′ 148°34′	46775 (26604)	KM219854 (Shuttleworth *et al.* 2014)
C. hebe	G75 (3U 27·2)	*M. dorsalis*	Yeppoon, Qld	23°08′ 150°44′	36273 (25548)	MF284686

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Table 1. (*Cont.*)

Nematode species	Code (slide no./ host no.)	Host species	Collection locality	Coordinates°, ′S °, ′E	SAM voucher	Genbank number
C. hecuba	G66, G257-8 (3U28)	*M. dorsalis*	Yeppoon, Qld	23°08′ 150°44′	36272 (25558)	MF284687
C. hydriformis	(-) 39G1·3	*M. rufus*	Mulga Park Stn, NT	25°54′ 131°40′	46717 (46203)	KJ776347 (Lott *et al*. 2014)
C. io	(-) 2N4·1	*W. bicolor*	Shute Harbour, Qld	20°17′ 148°47′	- (26765)	KM219856 (Shuttleworth *et al*. 2014)
C. ixion	G31 (-)	*M. robustus*	Roebourne, WA	20°46′ 117°08′	47612 (45930)	KU853863, KU853905 (Shuttleworth *et al*. 2016*b*)
C. johnstoni	G83 (-)	*M. robustus*	Menzies, WA	29°41′ 121°02′	47643 (47637)	KU853837, KU853921 (Shuttleworth *et al*. 2016*a*)
C. liebigi	(-) WP13·8	*M. rufus*	Bourke, NSW	30°05′ 145°56′	46713 (24306)	KJ776350 (Lott *et al*. 2014)
C. linstowi	G77 (7A1·1)	*M. dorsalis*	Bowen, Qld	20°01′ 148°15′	37277 (27165)	MF284688
C. longelabiata	G37 (-)	*M. robustus*	Mulga Park Stn, NT	25°54′ 131°40′	47614 (46217)	KU853838, KU835926 (Shuttleworth *et al*. 2016*b*)
C. macropodis	G37 (-)	*M. robustus*	Wollomombi, NSW	30°30′ 152°03′	47616 (44361)	KU853834, KU853953 (Shuttleworth *et al*. 2016*b*)
C. mawsonae	(-) AN8·4	*W. bicolor*	Rockhampton, Qld	23°24′ 150°30′	46786 (23471)	KM219858 (Shuttleworth *et al*. 2014)
C. papillata	(-) 4B7·5, 4C18·1	*W. bicolor*	Miles, Qld	26°39′ 150°11′	46780, 46784 (24172)	KM219868, KM219869 (Shuttleworth *et al*. 2014)
C. papillatissima	(-) 4J15·1	*W. bicolor*	Phillip Island, Vic	38°29′ 145°12′	46877 (24559)	KM219861 (Shuttleworth *et al*. 2014)
C. parva 1	(-) 15H1	*P. purpureicollis*	Mt Isa, Qld	20°44′ 139°29′	46729 (25941)	KJ776351 (Lott *et al*. 2014)
C. parva 2	G51 (-)	*M. robustus*	Kingstown, NSW	30°26′ 151°01′	47626 (-)	KU853858, KU853902 (Shuttleworth *et al*. 2016*b*)
C. pearsoni	G177 (-)	*P. purpureicollis*	Winton, Qld	22°27′ 143°09′	28735 (32612)	FM992604 (Chilton *et al*. 2009)
C. petrogale	G192 (5Y3)	*P. purpureicollis*	Mary Kathleen, Qld	20°47′ 139°59′	36285 (27199)	MF284689
C. petronius	G135 (3G12)	*P. assimilis*	Townsville, Qld	19°15′ 146°48′	- (26547)	MF284690
C. phaeax	G131 (G2)	*M. eugenii*	Kangaroo Island, SA	35°48′ 137°12′	- (26785)	MF284691
C. phaedra 1	G133 (CN9)	*M. parryi*	Darling Plains Stn, Qld	24°30′ 150°07′	- (27187)	MF284693
C. phaedra 2	G134 (AM2)	*M. agilis*	Rockhampton. Qld	23°24′ 150°30′	- (26521)	MF284692
C. phaethon	G37 (-)	*M. robustus*	Kangaroo Hills Stn, Qld	18°55′ 145°40′	47630 (32736)	KU853833, KU853861 (Shuttleworth *et al*. 2016*b*)
C. pollux	(-) 39R1·1	*W. bicolor*	Healesville, Vic	37°39′ 145°32′	46788 (30551)	KM219872 (Shuttleworth *et al*. 2014)
C. polyxo	G116 (XJ2·2)	*M. dorsalis*	Yeppoon, Qld	23°08′ 150°44′	36271 (25575)	MF284694
C. robertsi	G229 (-)	*P. persephone*	Airlie Beach, Qld	20°16′ 148°43′	28748 (31011)	FM992610 (Chilton *et al*. 2009)
C. setonicis	G202 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36267 (31935)	MF284695
C. similis	(-) 22W5·1	*P. herberti*	Mt Sebastopol, Qld	23°43′ 146°41′	46726 (32448)	KJ776377 (Lott *et al*. 2014)
C. telemachus	G 215 (24H1)	*S. brachyurus*	Wellington Dam, WA	33°23′ 115°59′	36270 (31936)	MF284696
C. thetidis	G70G (3U4·3, 4·4)	*M. dorsalis*	Yeppoon, Qld	23°08′ 150°44′	36275 (25592)	MF284697
C. tyche	G63 (3S43·2)	*M. dorsalis*	Marlborough, Qld	22°49′ 149°53′	36278 (26803)	MF284698
C. typhon	G80 (-)	*M. robustus*	Wollomombi, NSW	30°30′ 152°03′	47636 (44358)	KU853836, KU853913 (Shuttleworth *et al*. 2016*b*)

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1998). *Cloacina dirce* and *Cloacina dindymene* from *M.* (*O.*) *antilopinus*, included in this study, are also both common parasites of *M.* (*O.*) *robustus* (Beveridge, 1998). Similarly, the four species of *Petrogale* included in this study (*Petrogale assimilis*, *Petrogale herberti*, *Petrogale persephone*, *Petrogale purpureicollis*) have extremely similar helminth communities (Beveridge *et al.* 1989; Begg *et al.* 1995) and the host genus is treated as a single entity in the results section. *Cloacina robertsi* occurs in all four species of rock wallaby, while *Cloacina caenis* and *Cloacina pearsoni* occur in all but *P. persephone*. *Cloacina parva* occurs in *P. purpureicollis*, but not in the remaining rock wallaby species (Beveridge, 1998; Chilton *et al.* 2009). Host nomenclature and distributions (in Fig. 1) follow van Dyck and Strahan (2008).

In instances where sister species of *Cloacina* were identified within the same macropodid host species in the molecular phylogeny, additional data were sought to confirm whether the occurrences of the sister species were sympatric in order to provide evidence for or against the hypothesis that these were instances of within host speciation.

The ITS+ molecular tree was compared with the phenetic arrangement of taxa proposed by Beveridge (1998) in an attempt to correlate principal morphological features with the molecular phylogenetic data.

RESULTS

ITS+ sequence data were available for 59 species of *Cloacina* (Table 1). Of these, 25 are novel sequences while the remainder have been published previously and deposited on GenBank. Given that the Neighbour-Joining and BI trees constructed were similar in topology to one another, only the BI tree is presented here (Fig. 2). In the BI trees, multiple clades were identified with high posterior probabilities (>0·92).

No evidence of hybrids was found among the nematodes included in the study.

The small basal clades in the phylogenetic tree consisted of a mixture of species found in *W. bicolor* (*Cloacina annulata*, *Cloacina papillatissima* and *Cloacina galatea*), *M.* (*N.*) *dorsalis* (*Cloacina burnettiana* and *Cloacina polyxo*) and *T. stigmatica* (*Cloacina cloelia*) (Fig. 2). In the remaining tree, three major clades were evident. One clade was relatively small containing five species from *S. brachyurus* (*Cloacina cadmus*, *Cloacina ceres*, *Cloacina circe*, *Cloacina setonicis* and *Cloacina telemachus*) together with single species (in a sister species relationship) from *W. bicolor* (*Cloacina castor*). A second clade consisted of 16 nematode species from multiple host species. Members of this clade characteristically exhibited long branches. A third clade consisted of 31 species, again from multiple host

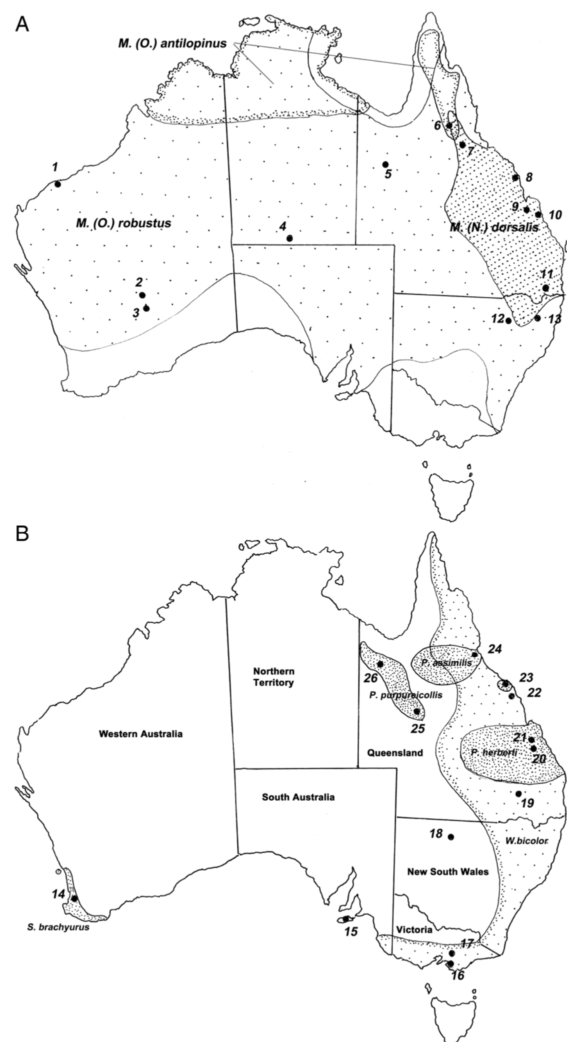

Fig. 1. Collecting localities for all host species examined and geographical ranges of the principal macropodid hosts included in this study: *Macropus* (*O.*) *antilopinus*, *M.* (*N.*) *dorsalis*, *M.* (*O.*) *robustus*, *Petrogale* spp., *Setonix brachyurus* and *Wallabia bicolor*. Collecting localities close to one another have been combined on the maps. The host species collected are indicated for each locality. **A.** Collecting localities: 1, Roebourne, Fortescue River Roadhouse (*M.* (*O.*) *robustus*); 2, Menzies (*M.* (*O.*) *robustus*); 3, Kalgoorlie (*M.* (*O.*) *robustus*); 4, Mulga Park Station (*M.* (*O.*) *robustus, M.* (*O.*) *rufus*); 5, Cloncurry (*M.* (*O.*) *robustus*); 6, Mount Surprise (*M.* (*O.*) *antilopinus*); 7, Kangaroo Hills Station (*M.* (*O.*) *robustus*); 8, Bowen (*M.* (*N.*) *dorsalis*); 9, Marlborough (*M.* (*N.*) *dorsalis*); 10, Rockhampton, Yeppoon (*M.* (*N.*) *agilis, M.* (*N.*) *dorsalis, W. bicolor*); 11, Warwick (*M.* (*O.*) *robustus*); 12, Kingstown (*M.* (*O.*) *robustus*); 13, Wollomombi (*M.* (*O.*) *robustus*). **B**. Collecting localities: 14, Wellington Dam (*S. brachyurus*); 15. Kangaroo Island (*M.* (*N.*) *eugenii*); 16, The Gurdies, Phillip Island (*W. bicolor*); 17, Healesville, Dixon's Creek (*W. bicolor*); 18, Bourke (*M.* (*O.*) *rufus*); 19 Miles (*W. bicolor*); 20, Darling Plains Station (*M.* (*N.*) *parryi*); 21, Mount Sebastopol (*Petrogale herberti*); 22, Sarina (*Thylogale stigmatica*); 23, Proserpine, Shute Harbour, Airlie Beach (*P. persephone*, *W. bicolor*); 24, Mount Louisa (*P. assimilis*); 25, Winton (*P. purpureicollis*); 26, Mount Isa, Mary Kathleen (*P. purpureicollis*).

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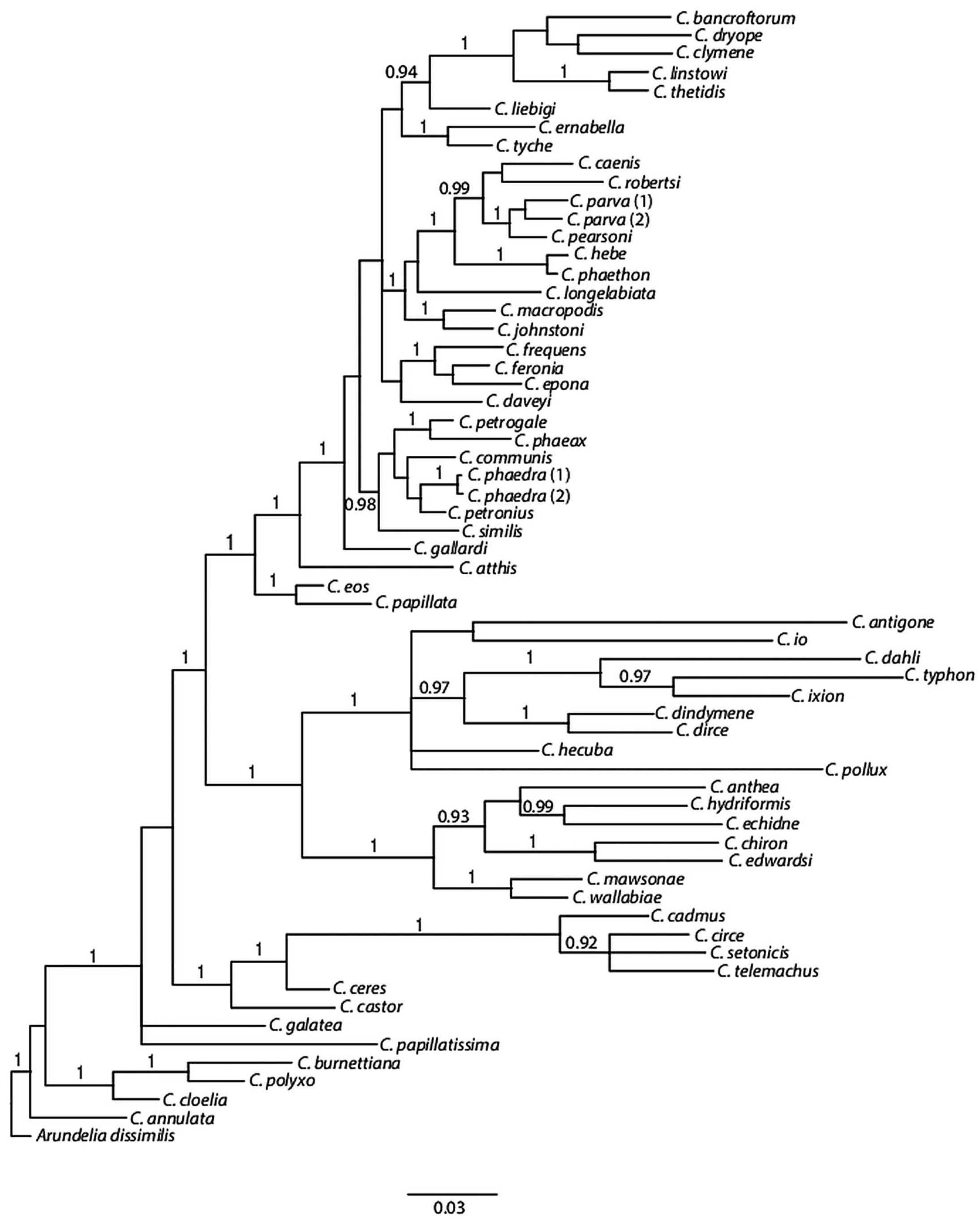

Fig. 2. Bayesian Inference (BI) phylogenetic tree of associations between species of *Cloacina*. Posterior probabilities >0·90 are shown at nodes. Clades identified are discussed in the text.

species but generally with shorter branch lengths than those seen in the second clade.

Of the principal host species included in the analysis (*M.* (*N.*) *dorsalis*, *M.* (*O.*) *robustus* (together with *M.* (*O.*) *antilopinus*), *Petrogale* spp., *W. bicolor*, *S. brachyurus*), that is those host species parasitised by several species of *Cloacina*, their nematode species were spread across multiple clades (Fig. 3). The 17 species of *Cloacina* from *M.* (*O.*) *robustus* (including *M.* (*O.*) *antilopinus*) were distributed across 11 clades, the nine species from *M.* (*N.*) *dorsalis* were distributed across four clades, the 14 species from *W. bicolor* were distributed across 11 clades, the eight species from *S. brachyurus* were distributed across two clades and the six species from *Petrogale* spp. were distributed across three clades (Fig. 4). Thus, none of the assemblages of *Cloacina* spp. in these macropodid hosts was monophyletic. Other host species included were represented by fewer parasite taxa, but in the case of species occurring in *M.* (*O.*) *rufus* and *T. stigmatica*, the two species of nematodes occurred in different clades (Fig. 2).

Sister species occurring in the same macropodid host species were identified in a number of instances

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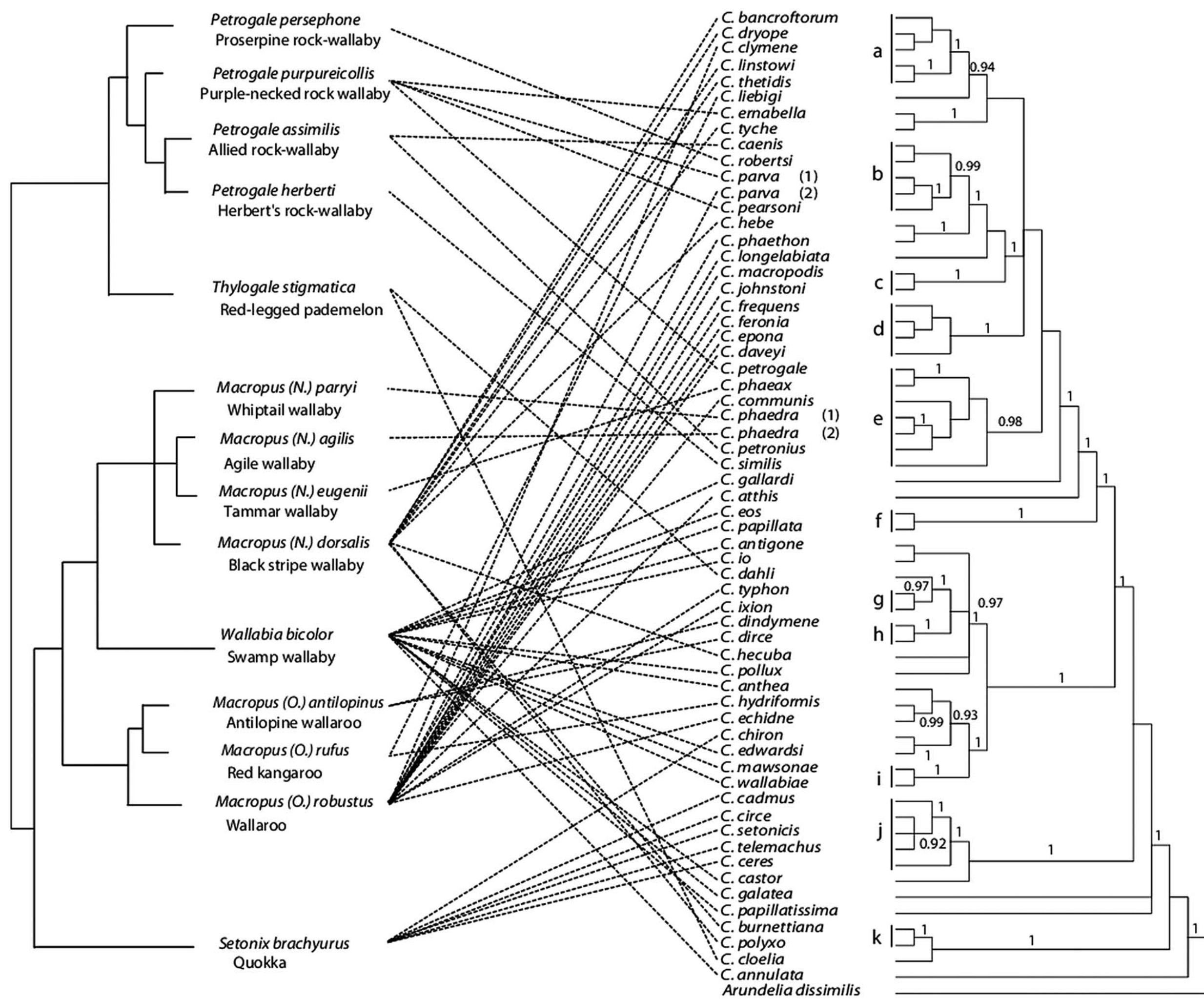

Fig. 3. Cladogram of associations between species of *Cloacina*, with posterior probabilities >0·90 shown at nodes, on left, with the corresponding phylogram of host relationships, based on Meredith *et al*. (2008) but with interpolations of missing taxa based on Cardillo *et al*. (2004) on the right. Clades a–d, f–k, indicate sister species occurring in the same macropodid host species; clade e indicates a series of morphologically related species.

(Figs 3 and 4). In *M.* (*O.*) *robustus* (included with (*M.* (*O.*) *antilopinus*), four groups of sister species were identified (clades c, d, g, h); in *M.* (*N.*) *dorsalis*, two groups of sister species were identified (clades a, k); in *W. bicolor*, two pairs of sister species were identified (clades f, i); *Cloacina antigone* and *Cloacina io* also formed a clade (Fig. 2) but with low statistical support; in *S. brachyurus*, one group of five sister species was identified (clade j) while in *Petrogale* spp., a single clade of four sister species was identified (clade b). Of the remaining host species included in the analysis, *M.* (*O.*) *rufus* had two species of *Cloacina* in different clades, while *T. stigmatica* also had two species of *Cloacina* in different clades. Other host species included were parasitised by a single species of *Cloacina* and comments on sister species relationships are consequently not relevant.

Specimens of *Cloacina phaedra* were included from two host species, *M.* (*N*). *agilis* and *M.* (*N*). *parryi*. However, no genetic differences were detected between these nematodes. Specimens of *C. parva* from *P. purpureicollis* and *M.* (*O.*) *robustus* showed some sequence differences but were closely related.

DISCUSSION

The principal question posed at the outset of this study was whether the assemblages of *Cloacina* species found in the different species of macropodid hosts represented monophyletic groups. Although the molecular tree presented herein for the 59 species of *Cloacina* is not considered to be a definitive phylogeny of the genus, it does provide insight into genetic associations among currently known taxa. The study of the nematodes of several host species harbouring multiple species of *Cloacina* presented here (*M.* (*N.*) *dorsalis*, *M.* (*O.*) *robustus*, *Petrogale* spp., *W. bicolor*) provides strong evidence that they do not each represent a monophyletic assemblage and that representatives of each

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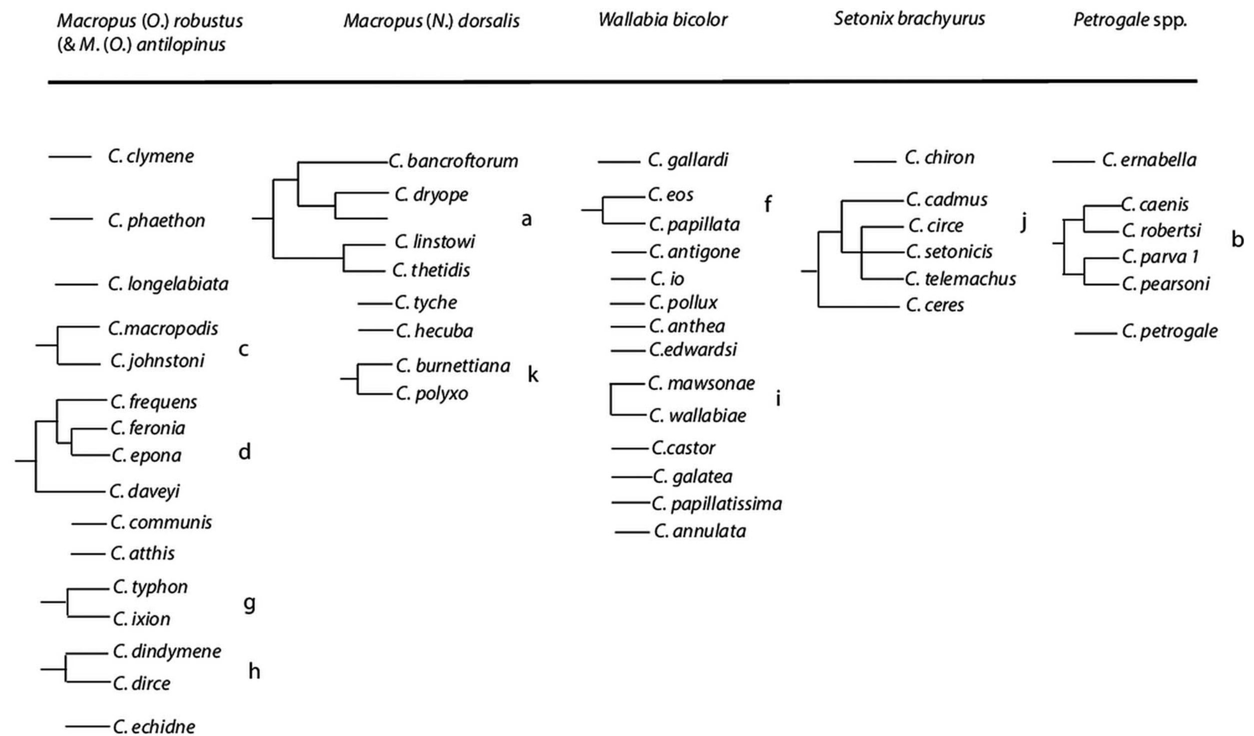

Fig. 4. Species of *Cloacina* from the principal hosts utilized in this study, the wallaroo, *Macropus* (*O.*) *robustus* (combined with *M.* (*O.*) *antilopinus*), the black-striped wallaby, *M.* (*N.*) *dorsalis*, the swamp wallaby, *Wallabia bicolor*, the quokka, *Setonix brachyurus*, and rock wallabies, *Petrogale* spp., showing the distribution of species from each host or host group by clade from Fig. 2. Clades identified by letters are the same as those shown in Fig. 3.

assemblage belong to different clades within the genus. These data confirm the tentative suggestions of a previous morphological study that also suggested the assemblages of *Cloacina* spp. in macropodid hosts were not monophyletic (Beveridge *et al.* 2002). Some caution may be needed in accepting this conclusion as only approximately 50% of species of this large nematode genus were included and extensive assemblages in the grey kangaroos (*M.* (*M.*) *fuliginosus* and *M.* (*M.*) *giganteus*) (subgenus *Macropus*) as well as those from the New Guinea scrub wallabies (*Dorcopsis* spp.) (tribe Dorcopsini of Prideaux and Warburton, 2010), some of which clustered on a within-host basis in the preliminary study of Beveridge *et al.* (2002), remain to be examined.

As a consequence, based on the examples presented here, the assemblages of *Cloacina* spp. in kangaroos and wallabies do not appear to comply with the definition of a species flock based on monophyly, but, by contrast appear to represent assemblages in which host colonization has played a significant role in their evolution (Fig. 3). This outcome is consistent with previously published morphological studies (Beveridge and Chilton, 2001; Beveridge *et al.* 2002) as well as recent molecular studies of three related cloacinine genera *Cyclostrongylus*, *Rugopharynx* and *Pharyngostrongylus* in which host colonization appears also to have played a major role in parasite evolution (Chilton *et al.* 2016*a*, *b*, *c*).

In a number of instances, pairs of sister species or multiple sister species were found in the same kangaroo or wallaby host species suggesting the possible occurrence of within-host speciation (Fig. 4). Within-host speciation needs to be distinguished from sympatric speciation as parasites may have diverged within allopatric populations of the same host species (McCoy, 2003). Consequently, in examining the sister-groups of species of *Cloacina* apparently exhibiting within-host speciation, their known geographical distribution and co-occurrence in individual hosts also need to be taken into consideration. Such data are available for the species of *Cloacina* (clade d) from *M.* (*O.*) *robustus* containing *Cloacina epona*, *Cloacina feronia* and *Cloacina frequens* (Shuttleworth *et al.* 2016*b*). *M.* (*O.*) *robustus* is distributed across virtually the entire Australian continent (Clancy and Croft, 2008) (Fig. 1). In this instance, *C. frequens* exhibits a northern and western distribution while *C. feronia* exhibits a southern and eastern distribution, with overlap in central and Western Australia. *Cloacina daveyi* occurs in south and central Australia, overlapping with *C. feronia* and *C. frequens*. *Cloacina epona*, however, is restricted to north-western Queensland (Shuttleworth *et al.* 2016*b*). In the case of these species, it is possible that past allopatry of host populations has been involved in parasite speciation, although there are no phylogeographic studies of the host species currently available to test this

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hypothesis. By contrast, *Cloacina johnstoni* and *Cloacina macropodis* commonly co-occur in *M.* (*O*). *r. erubescens*, although *C. macropodis* has a wider geographical distribution than that of *C. johnstoni*, occurring as well in *M. r. robustus*, providing the possibility that they may have co-evolved in sympatry (Shuttleworth *et al*. 2016*a*).

The sister species pair *C. dindymene* and *C. dirce* are parasites of both *M.* (*O.*) *robustus* and *M.* (*O.*) *antilopinus* in northern Australia (Beveridge, 1998). Their co-occurrence has been studied in only one part of the ranges of the two host species (i.e. in north-eastern Queensland) (Beveridge *et al*. 1998) but limited records indicate co-occurrence of these nematode species in the Northern Territory and the Kimberley region of Western Australia as well (Beveridge, 1998). However, since two host species are involved, their interrelationships require additional investigation. Likewise, in clade g, *C. typhon* is a common parasite of *M.* (*M.*) *giganteus* as well as occurring at lower prevalences in other sympatric macropodids (Beveridge *et al*. 1998). Consequently, interpretations of the evolution of the *C. typhon* – *C. ixion* species pair may have involved host colonization.

Macropus (*N.*) *dorsalis* has a more restricted distribution in north-eastern Australia (Johnson, 2008) (Fig. 1), but is host to two clades (a, k) of host-specific species, which have apparently evolved within the same host species. One additional species in clade a (*C. clymene*) is a parasite of *M.* (*O.*) *robustus*, suggesting a case of host colonisation. Studies of the co-occurrence of species of *Cloacina* in *M.* (*N.*) *dorsalis* are currently limited to the more northern parts of its range (Beveridge *et al*. 1998) with few additional records from more southern regions (Beveridge, 1998). The current data suggest that these species have evolved within the host in sympatry, but additional collections are required to confirm this hypothesis.

In *W. bicolor*, two pairs of sister species (clades f, i) were identified. All four of these species occur throughout the geographical range of the host (Beveridge, 2016) and consequently the possibility of allopatric differentiation of these species seems unlikely. They may represent instances of both within-host and sympatric speciation. However, the helminth community of *W. bicolor* exhibits distinct regional differentiation (Beveridge, 2016), so that the possibility of allopatric speciation cannot be excluded.

The quokka, *S. brachyurus*, has an extremely limited distribution in Western Australia (de Torres, 2008) (Fig. 1) and is phylogenetically basal to the remaining macropodid genera (Meredith *et al*. 2008). In spite of this, it is host to five sister species (Clade j) suggesting instances of within-host and possibly sympatric speciation. The original descriptions of these species were from island populations (Beveridge, 1999). The current molecular data are from mainland populations of the host in which the same nematode species were recovered. It seems unlikely that there are significant differences between island and mainland populations of these nematodes, an hypothesis supported by similar molecular studies of species of the related genus *Rugopharynx* from island and mainland populations of its hosts (Chilton *et al*. 2016*c*).

The rock wallabies, *Petrogale* spp., represent a distinctive clade within the Macropodidae (Meredith *et al*. 2008) and their principal species of *Cloacina*, *C. caenis*, *C. pearsoni* and *C. robertsi* (Beveridge *et al*. 1989) form a distinctive clade (clade b) suggesting a degree of co-evolution with the host genus. However, because of the complexity of taxonomic relationships between species of *Petrogale* (Potter *et al*. 2012) and the possibility that each of the nematode species included in this study represents a species complex, with a different genetic form in each wallaby host species (Chilton *et al*. 2009), any conclusions need to be guarded.

Attempts to correlate principal clades identified in the BI analysis with defining, autapomorphic characters as utilized by Beveridge (1998) were largely unsuccessful, suggesting that many of the morphological characters currently utilized for identification are homoplasious. This topic however is potentially the focus of an additional study. One clade (clade e) did however conform to the current phenetic classification with couplet 7 in the key to the genus (Beveridge, 1998), which identified transverse folds in the lining of the anterior oesophagus as a taxonomic feature. This feature separated *C. similis*, *C. communis*, *C. petronius*, *C. petrogale*, *C. phaeax* and *C. phaedra* from congeners. All of these species are members of clade e, but occur in a wide range of host species, consistent with the proposal for speciation being primarily by host colonisation. *Cloacina petrogale* was shown to be a species complex using multilocus enzyme electrophoretic data (Chilton *et al*. 1997*a*, *b*). The species was subsequently subdivided based on minor morphological differences by Beveridge (1998). The current molecular sequence data support both the electrophoretic data and the taxonomic decisions made on this basis. In this study, *C. phaedra* collected from both *M.* (*N.*) *agilis* and *M.* (*N.*) *parryi* was shown to be identical and therefore to be the same species. This species was found to occur in *M.* (*N.*) *parryi* at a prevalence of 36% but was found in only a single individual of *M.* (*N.*) *agilis* (3%) (Beveridge *et al*. 1998), suggesting that *M.* (*N.*) *parryi* was the primary host but that transfer to a sympatric host, *M.* (*N.*) *agilis*, was possible.

Cloacina parva was obtained from *M.* (*O.*) *robustus* and *P. purpureicollis*, with slight differences in the sequence data. Although originally described from *M.* (*O.*) *robustus* by Johnston and Mawson

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(1938) and is a common parasite of this host species (prevalence 51%, Beveridge *et al*. 1998), it is also common in *Petrogale* spp. (Beveridge *et al*. 1989; Bradley *et al*. 2000). The data presented here suggest that it originated in a clade of *Petrogale* specific taxa and therefore its occurrence in *M*. (*O*.) *robustus* is a colonization event. This hypothesis requires more detailed examination.

Speciation within *Cloacina* appears to have been primarily by host colonization but with additional examples of within-host speciation. The proposition of host colonization as a principal mode of speciation is not novel within this subfamily (Beveridge and Chilton, 2001 (morphological analyses); Chilton *et al*. 2016*a*, *b*, *c* (molecular analyses)). The reasons for this mode of evolution probably relate to the life-cycles of the parasites and the ecology of their hosts (Beveridge and Spratt, 1996). The life-cycles of the parasites are presumed to be direct based primarily on the study of *Labiosimplex eugenii* by Smales (1977). Third stage larvae develop in the external environment and are ingested on herbage. As multiple species of macropodids may graze in the same environment (Jarman and Phillips, 1989) (Fig. 1), ingestion of larvae deposited by a related species of macropodids is likely. An instance of this phenomenon in the present study is the occurrence of *C*. *phaedra* in its principal host *M*. (*N*.) *parryi* and its occurrence in a sympatric host species, *M*. (*N*.) *agilis*, but at a much lower prevalence. Such host transfers provide the basis for colonization of a new host species. A second factor identified by Beveridge and Spratt (1996) was the voluminous forestomachs of macropodids, the site which these nematodes inhabit and therefore the lack of potential competition in occupying a novel niche. Hoste and Beveridge (1993) were unable to establish any evidence of competition between the nematode species present in the forestomachs of the macropodid species, which they studied. Consequently, the system of multiple macropodid species grazing in the same environment, the direct life cycle of the parasites and the apparent lack of competition in the site of establishment in the host are likely to facilitate host colonization.

The examples presented here are of within-host speciation warrant additional scrutiny as possible examples of sympatric speciation. However, in order to establish that sympatric speciation has occurred, it is necessary to show that the initial stages of divergence occurred in sympatry (McCoy, 2003). Thus the possibility that a past host transfer from a now extinct host species is difficult to exclude. In the case of equids and rhinoceroses, the extant species represent relics of a much more diverse fauna present during the Pleistocene (2 million years ago) (Franzen, 2010) and similarly, the extant macropodids represent a fraction of the species that existed previously (Prideaux and Warburton, 2010). Consequently, establishing that macropodid nematode species evolved in sympatry represents a challenge. In addition, the phylogeographical history of most extant macropodids remains unknown.

Molecular phylogeographic data exist for some macropodid species (*M*. (*O*.) *rufus*, *M*. (*M*.) *fuliginosus* and *M*. (*M*.) *giganteus* (Clegg *et al*. 1998; Neaves *et al*. 2009; Coghlan *et al*. 2015)) but not for the host species included in this study. Evidence which may potentially support a hypothesis of sympatric speciation could be the demonstration of niche separation within the macropodid forestomach. While some degree of niche separation has been demonstrated among *Cloacina* spp. occurring in the forestomach of *M*. (*M*.) *fuliginosus* (Pamment *et al*. 1994) there are no comparable studies on the parasites of the hosts included in the current molecular study.

The data presented here on host- switching and within-host speciation in the evolution of complex parasite communities of nematodes are mirrored in comparable studies on the monogenean parasites of fish (Šimková *et al*. 2000, 2001, 2004; Huyse and Volckaert, 2005) suggesting that these patterns may be common among the various groups of parasitic helminths.

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