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Genetic variation within the genus *Macropostrongyloides* (Nematoda: Strongyloidea) from Australian macropodid and vombatid marsupials

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Research Article

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Abstract

The genetic variation and taxonomic status of the four morphologically-defined species of *Macropostrongyloides* in Australian macropodid and vombatid marsupials were examined using sequence data of the ITS+ region (=first and second internal transcribed spacers, and the 5.8S rRNA gene) of the nuclear ribosomal DNA. The results of the phylogenetic analyses revealed that *Ma. baylisi* was a species complex consisting of four genetically distinct groups, some of which are host-specific. In addition, *Ma. lasiorhini* in the common wombat (*Vombatus ursinus*) did not form a monophyletic clade with *Ma. lasiorhini* from the southern hairy-nosed wombat (*Lasiorhinus latifrons*), suggesting the possibility of cryptic (genetically distinct but morphologically similar) species. There was also some genetic divergence between *Ma. dissimilis* in swamp wallabies (*Wallabia bicolor*) from different geographical regions. In contrast, there was no genetic divergence among specimens of *Ma. yamagutii* across its broad geographical range or between host species (i.e. *Macropus fuliginosus* and *M. giganteus*). *Macropostrongyloides dissimilis* represented the sister taxon to *Ma. baylisi*, *Ma. yamagutii* and *Ma. lasiorhini*. Further morphological and molecular studies are required to assess the species complex of *Ma. baylisi*.

Introduction

Strongyloid nematodes of the gastrointestinal tracts of Australian macropodid and vombatid marsupials have undergone extensive diversification (Beveridge *et al.*, 2010). Currently, there are over 300 described species within 45 genera (Spratt and Beveridge, 2016), with significant numbers of species awaiting formal taxonomic description. Many of these are cryptic (i.e. genetically distinct but morphologically similar) species (e.g. Chilton *et al.*, 1993; Beveridge *et al.*, 1994; Chilton *et al.*, 1995, 1996), whereby more than one species has been included under the same specific name (Bickford *et al.*, 2007). For instance, *Hypodontus macropi*, which occurs in the caecum and colon of macropodid marsupials, represents a species complex of at least 10 species based on multilocus enzyme electrophoresis (MEE) and DNA sequence data (Chilton *et al.*, 1992, 1995, 2012). However, none of the species in this complex can currently be distinguished from one another using morphological characters.

The genus *Macropostrongyloides* belongs within the sub-family Phascolostrongylinae (Lichtenfels, 1980). Species within this genus are found in macropodid (e.g. kangaroos and wallabies) and vombatid marsupials (i.e. wombats) (Beveridge and Mawson, 1978). Currently, there are five morphologically defined species of *Macropostrongyloides,* most of which inhabit the caecum and/ or colon of their hosts. The sole exception is *Ma. dissimilis* which occurs exclusively in the stomach of the swamp wallaby (*Wallabia bicolor*) (Beveridge and Mawson, 1978). The other species in the genus are *Ma. dendrolagi*, a parasite of tree kangaroos in Indonesia (Beveridge, 1997), *Ma. lasiorhini* which occurs in wombats, *Ma. yamagutii*, a parasite of western grey kangaroos (*Macropus fuliginosus*) and occasionally eastern grey kangaroos (*Macropus giganteus*), and *Ma. baylisi*, a generalist species that occurs in several macropodid marsupials (Beveridge and Mawson, 1978). However, host usage by *Ma. baylisi* varies geographically. In northeastern Australia (i.e. Queensland), *Ma. baylisi* occurs at a high prevalence in the whiptail wallaby, *Notamacropus parryi,* and the eastern wallaroo, *Osphranter robustus robustus* (Beveridge *et al.*, 1998), and has been reported from other sympatric hosts, including the antilopine wallaroo, *Osphranter antilopinus*, the black-stripe wallaby, *Notamacropus dorsalis, W. bicolor,* and the spectacled hare wallaby, *Lagorchestes conspicillatus* (Beveridge *et al.*, 1992, 1998). In southern Australia, *Ma. baylisi* occurs primarily in *M. giganteus*. Although the geographical range of *M. giganteus* overlaps with other hosts such as the red-necked wallaby, *Notamacropus rufogriseus, M. fuliginosus, W. bicolor* and the red kangaroo, *O. rufus*, only limited host switching has been recorded (Arundel *et al.*, 1979; Beveridge *et al.*, 1993; Aussavy *et al.*, 2011).

Beveridge and Mawson (1978) hypothesised that *Ma. baylisi* from *M. giganteus* may be distinct from *Ma. baylisi* in *O. robustus* and *N. parryi* based on a comparison of morphological characters. This hypothesis was supported by data from a MEE study (Beveridge *et al.*, 1993) in

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which *Ma. baylisi* from *M. giganteus* had fixed genetic differences at 33% of loci from *Ma. baylisi* in *O. robustus* and *N. parryi*. However, a formal description of a new species was not undertaken due to insufficient evidence of morphological differences (Beveridge *et al*., 1993). The MEE study also examined the genetic relationships between *Ma. baylisi* and the morphologically distinct species *Ma. yamagutii*. The results indicated that *Ma. baylisi* in *M. giganteus* was genetically more similar to *Ma. yamagutii* in *M. fuliginosus* than to *Ma. baylisi* in *O. robustus* and *N. parryi* (Beveridge *et al*., 1993). The phylogenetic relationships of *Ma. baylisi* and *Ma. yamagutii*, and the other species that occur in Australia, *Ma. lasiorhini* in wombats, and *Ma. dissimilis* in *W. bicolor*, have yet to be examined using DNA sequence data.

In the present study, we investigated the genetic variability based on the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) and the 5.8S gene of the nuclear ribosomal DNA of *Ma. baylisi, Ma. yamagutii, Ma. dissimilis* and *Ma. lasiorhini* collected from various hosts throughout Australia. Prevalence data for *Ma. baylisi* were also examined to better understand its pattern of host-specificity and geographic distribution.

Materials and methods

Sample collection

Adult specimens of *Ma. baylisi* ($n = 68$), *Ma. yamagutii* ($n = 19$), *Ma. dissimilis* ($n = 5$) and *Ma. lasiorhini* ($n = 3$) were sourced from the frozen parasite collection at the School of Veterinary Science, The University of Melbourne. These specimens had been collected from culled or road-killed hosts from various localities in Australia (Table 1). Additional specimens of *Ma. baylisi* from road-killed *M. giganteus* ($n = 1$), *O. robustus* ($n = 3$), *N. parryi* ($n = 1$) and *N. rufogriseus* ($n = 1$) were collected in Queensland during September 2018. These nematodes were preserved in 70% ethanol and stored at −80 °C until required for DNA extraction. Individual nematodes were thawed and cut into three segments. The anterior and posterior extremities of each nematode were cleared in lactophenol and mounted on slides for morphological identification. The mid-body segments of each nematode were rinsed in H_2O prior to DNA extraction. Voucher morphological specimens representing each ITS+ (= ITS-1 and ITS-2) sequence genotype have been deposited in the South Australian Museum, Adelaide (SAM 48599–48626). Host nomenclature follows Jackson and Groves (2015).

Molecular methods

Total genomic DNA (gDNA) was isolated from individual nematodes using the Wizard SV Genomic DNA Purification kit (Promega, Madison, WI, USA). The concentration and purity of each DNA sample were determined spectrophotometrically (ND-1000 UV-VIS spectrophotometer v.3.2.1; NanoDrop Technologies, USA). The ITS+ was amplified by PCR using primers NC16 (5′–AGTTCAATCGCAATGGCTT–3′) and NC2 (5′– TTAGTTTCTTTTCCTCCGCT–3′) (Gasser *et al*., 1993; Chilton *et al*., 2003). PCRs were conducted in 50 μL volumes containing 2 μL of DNA template, 10 mM Tris-HCl (pH 8.4), 50 mM KCl (Promega), 3.5 mM $MgCl_2$, 250 μM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each primer, and 1 U of GoTaq polymerase (Promega). The PCR conditions used were: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 20 s, and 72 °C for 20 s, followed by 72 °C for 5 min. Negative (no DNA template) and positive controls (*Haemonchus contortus* gDNA) were included in the PCR analyses. An aliquot (5 μL) of each amplicon was subjected to agarose gel electrophoresis. Gels (1.5% gels in 0.5 TAE buffer containing 20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA) were stained using GelRed Nucleic Acid Gel Stain (Biotium GelRed stain, Fisher Scientific, Waltham, Massachusetts, USA) and photographed using a gel documenting system (Kodak Gel Logic 1500 Imaging System, Eastman Kodak Company, Rochester, NY, USA).

Amplicons were purified using shrimp alkaline phosphate and exonuclease I prior to automated Sanger DNA sequencing using a 96-capillary 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA) at Macrogen Incorporation, South Korea. The ITS+ was sequenced using the primers NC16 and NC2 in separate reactions. The quality of the sequences was appraised using the Geneious R10 software (Biomatters Ltd., Auckland, New Zealand). Polymorphic sites were designated using the International Union of Pure and Applied Chemistry (IUPAC) codes. DNA sequences have been submitted to the GenBank database under the accession numbers MK842122 – MK842147 (Table 1).

Phylogenetic analyses

The ITS+ and 5.8S gene sequences were aligned using the log-expectation (MUSCLE) algorithm in the software MEGA 7.0.26 (Kumar *et al*., 2016). Pairwise comparisons of genetic similarity among sequences were determined using Geneious. Given the lack of variability on the 5.8S gene, this fragment was removed from the alignment and excluded from subsequent analysis. Phylogenetic analyses were performed on the aligned ITS+ sequence data using Neighbour-Joining (NJ) and Bayesian Inference (BI) methods. The NJ analysis was conducted using MEGA and nodal support was estimated from 10 000 bootstrap replicates. Bayesian inference analysis was conducted using the Markov Chain Monte Carlo (MCMC) method in the program MrBayes (Ronquist and Huelsenbeck, 2003). The likelihood parameters set for the BI analysis were based on the Akaike Information Criteria test in jModeltest v.2.1.7 (Guindon and Gascuel, 2003; Darriba *et al*., 2012). The general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR + G + I), was utilised for the BI analysis of the sequence data, with nst = 6. Posterior probability (pp) values were calculated by running 10 000 000 generations with four simultaneous tree-building chains (three heated and one cold). Trees were saved every 100th generation. At the end of each run, the standard deviation of split frequencies was <0·01, and the PSRF (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 *Labiostrongylus grandis* (GenBank accession no. FR854199), a species within a related sub-family (Cloacininae) was used as the outgroup for the analyses. Tree topology was checked for consensus between NJ and BI analyses using the software Figtree (Rambaut, 2012).

Results

Molecular characterisation of Macropostrongyloides species

The ITS+ of 93 specimens of *Macropostrongyloides* was sequenced. Nineteen distinct genotypes were identified in *Ma. baylisi*, four in *Ma. dissimilis*, two in *Ma. lasiorhini* and one in *Ma. yamagutii*. The lengths of the ITS+ ranged from 773–802 base pairs (bp) in *Ma. baylisi*, 773 bp in *Ma. yamagutii*, 782–786 bp in *Ma. dissimilis* and 775–786 bp in *Ma. lasiorhini* (Table 2). The ITS-1 sequences were longer than ITS-2 sequences in all species and varied between 383 to 398 bp in *Ma. baylisi*, 383–385 bp in *Ma. lasiorhini*, 383 bp in *Ma. yamagutii* and 392 bp in *Ma. dissimilis* (Table 2). The length of the ITS-2 varied in *Ma. baylisi* between 237 to 251 bp but was

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Table 1. Specimens of *Macropostrongyloides* spp. used in this study. The details of host species, geographic location and GenBank accession numbers of specimens are also provided

Host species	Collection locality	Coordinates: Lat (S), Long (E)	Specimen ID	SAM registration no.	GenBank accession no.
Macropostrongyloides yamagutii					
M. fuliginosus	Ashbourne, SA	35° 17′, 138° 45′	DS1.1	48 616	MK842122
	Waroona, WA	32° 50′, 115° 55′	24D5.1	–	MK842122
	Kalgoorlie, WA	30° 44′, 121° 28′	23J1	–	MK842122
	Hattah Lakes National Park, Vic	34° 45′, 142° 20′	DC3	–	MK842122
M. giganteus	Warrawena Stn *via* Bourke, NSW	30° 15′, 146° 07′	14R8	–	MK842122
Macropostrongyloides lasiorhini					
V. ursinus	Gippsland, Vic	37° 30′, 147° 51′	41R1	48 622	MK842123
V. ursinus	Bullengarook, Vic	37° 29′, 144° 28′	F19	48 623	MK842123
L. latifrons	Blanchetown, SA	34° 34′, 139° 36′	F516	48 624	MK842124
Macropostrongyloides dissimilis					
W. bicolor	Buangor, Vic	37° 22′, 143° 09′	16L8	48 609	MK842125
	Kamarooka, Vic	36° 28′, 144° 22′	10W2, 10W7	48 600	MK842126 MK 842127
	Miles, Qld	26° 39′, 150° 11′	4C14	48 618	MK842128
Macropostrongyloides baylisi					
O. rufus	Kalgoorlie, WA	30° 44′, 121° 28′	23M1, 23R1.2	48 615, 48 626	MK842129 MK842130
	Werribee Park Zoo, Vic	37° 55′, 144° 40′	8M10	–	MK842140
O. r. robustus	142 km N of Charters Towers, Qld	19°14′, 145° 27′	50S1	48 607	MK842131
	Gore, Qld	28° 18′, 151° 28′	49J	48 606	MK842132
	20 km S of Coonabarabran, NSW	31° 22′, 149° 15′	XY6.1, XY6.2	48 611	MK842133
	Kingstown, NSW	30° 31′, 151° 06′	35R1.5	–	MK842133
O. r. woodwardii	Edith River, NT	14° 15′, 131° 53′	27G5	–	MK842134
O. r. erubescens	Mulga Park Stn *via* Alice Springs, NT	25° 54′, 131° 40′	39N1.1	48 610	MK842133
	Kalgoorlie, WA	30° 44′, 121° 28′	23Q1.1	–	MK842135
	Yalgoo, WA	28° 22′, 116° 19′	23E1	–	MK842135
	Menzies, WA	29° 49′, 121° 05′	35U2	48 603	MK842135
	Fortescue River Roadhouse, WA	21° 18′, 116° 07′	38F1.1	–	MK842135
	Devoncourt Stn *via* Cloncurry, Qld	21° 10′, 140° 28′	21W1.1	–	MK842136
	Broken Hill, NSW	31° 54′, 141° 35′	44H	–	MK842136
	Port Augusta, SA	32° 30′, 137° 47′	42B2.1	48 612	MK842136
	70 km W of Cloncurry, Qld	20° 46′, 139° 53′	21P1, 21T1, 21V1.1	48 614	MK842145
	Jumba Stn *via* Charters Towers, Qld	21° 80′, 146° 26′	50J1	48 608	MK842147
	Warrawee Stn *via* Charters Towers, Qld	20° 18′, 146° 38′	AT12.1	48 613	MK842147
	Kangaroo Hills Stn *via* Greenvale, Qld	19° 38′, 145° 47′	27L2.2	48 625	MK842147
N. parryi	Dawes, Qld	24° 31′, 151° 16′	CD14, CA8	48 602	MK842137
	30 km S of Wowan, Qld	24° 16′, 150° 60′	49Z	48 617	MK842138
	Mount Fox, Qld	18° 17′, 145° 48′	12P13.1	48 604	MK842139
M. giganteus	Portland, Vic	38° 22′, 141° 36′	25G5	–	MK842140
	Pomonal, Vic	37° 12′, 142° 37′	19E.1	48 599	MK842140
	10 km N of Bacchus Marsh, Vic	37° 37′, 144° 47′	13M18.1	–	MK842140
	Craigieburn, Vic	37° 35′, 144° 53′	W685	–	MK842140
	Heathcote, Vic	36° 54′, 144° 43′	W689	–	MK842140

(*Continued*)

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Table 1. (*Continued.*)

Host species	Collection locality	Coordinates: Lat (S), Long (E)	Specimen ID	SAM registration no.	GenBank accession no.
	Wilson's Promontory, Vic	38° 58′, 146° 22′	45R1	–	MK842140
	Sutton Grange, Vic	36° 57′, 144° 20′	BB4.1	–	MK842140
	Avalon, Vic	38° 03′, 144° 25′	WA1	–	MK842140
	Nagambie, Vic	36° 45′, 145° 09′	YC22.1	–	MK842140
	13 km N of Jerilderie, NSW	35° 15′, 145° 47′	14X9	48 601	MK842140
	Warrawee Stn *via* Bourke, NSW	29° 44′, 145° 57′	14G2	–	MK842140
	4 km E of Omanama, Qld	28° 23′, 151° 19′	49T	48 605	MK842141
	Cape Bridgewater, Vic	38° 17′, 141° 23′	41X2.1	–	MK842142
	Lara, Vic	38° 00′, 144° 24′	41N1.1	–	MK842146
M. fuliginosus	Nyngan, NSW	31° 13′, 146° 53′	14R2	48 619	MK842143
P. purpureicollis	Leichhardt River, Qld	20° 43′, 139° 47′	8V7	–	MK842144
M. rufogriseus	Greymare, Qld	28° 10′, 151° 44′	7N15	–	MK842133
	Omanama, Qld	28° 20′, 151° 22′	49L1	–	MK842140
M. dorsalis	Jericho, Qld	23° 36′, 146° 08′	1T1	48 621	MK842147
O. antilopinus	Mount Surprise, Qld	18° 06′, 144° 31′	8K3	48 620	MK842147

Qld, Queensland; NSW, New South Wales; WA, Western Australia; NT, the Northern Territory; SA, South Australia; Vic, Victoria; SAM, South Australian Museum.

uniform in *M. dissimilis* (241 bp), *Ma. yamagutii* and *Ma. lasiorhini* (237 bp). The 5.8S gene was 153 bp in length for all specimens and contained no sequence variation except for a transversion (A↔T) at the alignment position 153 in all specimens of *Ma. dissimilis* (see Supplementary Fig. 1).

Macropostrongyloides dissimilis was genetically the most distinct species in the genus based on ITS+ sequence variation of 20% between *Ma. dissimilis* and *Ma. baylisi* from *O. rufus* (Table 2). The ITS+ sequences of *Ma. yamagutii* were more similar to those of *Ma. baylisi* from *O. robustus* (sequence variation: 3.3%) than to *Ma. baylisi* from *M. giganteus* (2.6%). Specimens of *Ma. baylisi* showed the highest level of intraspecific variation (Table 2). The ITS+ sequence variation ranged from 0.6 to 9.2%, and the specimens from *O. robustus* showed the highest variation while those from *M. giganteus* revealed the least variation (see Supplementary File 1). Intraspecific variation among *Ma. dissimilis* was slightly greater (2.0%) in specimens from Miles (Queensland) and Kamarooka (Victoria) than that of specimens (0.3%) from Buangor (Victoria). Specimens of *Ma. lasiorhini* from *V. ursinus* differed from those collected from *L. latifrons* by 3.6%. By contrast, specimens of *Ma. yamagutii* from *M. fuliginosus* were identical across all localities in South Australia, Victoria and Western Australia, including the specimen from a single *M. giganteus* from Bourke, New South Wales.

Phylogenetic relationships

The NJ and BI analyses of the ITS+ sequence data resulted in similar tree topologies, hence, only the majority rule consensus tree of the BI analysis of the ITS+ sequences aligned over 824 bp is presented (Fig. 1). The topology of the phylogenetic tree showed that *Ma. dissimilis* in *W. bicolor* formed a monophyletic assemblage with total nodal support (i.e. NJ = 100% and BI = 1), and represented the sister taxon to a monophyletic assemblage (with total nodal support) that included all specimens of *Ma. baylisi, Ma. yamagutii* and *Ma. lasiorhini*. Specimens of *Ma. dissimilis* from *W. bicolor* collected at three localities in Victoria formed a monophlyetic group with strong nodal support (NJ = 99% and BI = 0.8) to the exclusion of the *Ma. dissimilis* from Miles in Queensland (Fig. 1). In the assemblage containing the three morphologically defined species of *Macropostrongyloides*, there was no statistical support for *Ma. lasiorhini* from *V. ursinus* and *L. latifrons* representing a monophyletic assemblage. There was no genetic divergence among specimens of *Ma. yamagutii* from *M. fuliginosus* in Western Australia, South Australia and Victoria, and *M. giganteus* in New South Wales. In the phylogenetic tree, *Ma. yamagutii* represented a sister taxon to *Ma. baylisi*, in *O. robustus* and *N. parryi*; however, there was no statistical support for this relationship.

Macropostrongyloides baylisi was not a monophyletic assemblage, but represented four genetically distinct clades, each with total statistical support (NJ = 100% and BI = 1.0) (Fig. 1). The first clade comprised specimens of *Ma. baylisi* collected from *O. r. woodwardi, O. r. erubescens* and the purple-necked rock wallaby, *Petrogale purpureicollis*. The second clade primarily contained specimens of *Ma. baylisi* from *M. giganteus* from multiple localities in eastern Australia (Victoria, New South Wales and Queensland), *M. fuliginosus* from Nyngan (New South Wales), *O. rufus* from Werribee Zoo (Victoria) and *N. rufogriseus* from Omanama (Queensland). The third clade contained exclusively specimens of *Ma. baylisi* from *O. rufus* collected from Kalgoorlie (Western Australia). The fourth and largest clade consisted of specimens of *Ma. baylisi* in *O. r. erubescens*, *O. r. robustus*, *O. antilopinus*, *N. parryi* and *N. dorsalis* (Fig. 1). This clade is divided into three groups, which are partially based on host species or subspecies, and the localities from which they were collected.

The geographical distribution of the four clades of *Ma. baylisi* is shown in Fig. 2. Clade 4 was the most widely distributed with genotypes occurring in all states except Victoria and Tasmania. Clade 2, containing specimens from *M. giganteus*, was restricted to Victoria and New South Wales, except for one specimen from Omanama, in southeastern Queensland. There were several localities at which more than one genotypes of *Ma. baylisi* was present and these included Kalgoorlie (Western Australia), Greymare and Cloncurry (Queensland).

Prevalence and distribution of Ma. baylisi

The prevalence of *Ma. baylisi* in different hosts and geographic regions was compiled from unpublished and published records

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Table 2. Characteristics (lengths, G + C contents and variation) of the ITS-1, ITS-2 and ITS+ sequences of *Macropostrongyloides* specimens from different hosts

		ITS-1			ITS-2			ITS+[a]		
Nematode species	Host species	size (bp)	G + C content	Variation (%)	size (bp)	G + C content	Variation (%)	size (bp)	G + C content	Variation (%)
Ma. baylisi	*M. giganteus*	383	42.6–43.3	0.4–0.8	237	40.5	0	733	43.6–43.7	0.3–0.5
	O. robustus	385–398	42.7–43.6	0.87–9.4	237–251	40.4–41.4	0.21–12.4	755–802	43.3–44.1	0.1–8.8
	N. parryi	385	43.6	0–0.4	237	40.5–41.4	0–0.7	755–773	43.9–44.1	0–0.5
	M. rufogriseus	383–385	43.1–43.6	3.6	237	40.5–40.9	3	773–775	34.6–44.0	2.7
	O. rufus	384	43.2–43.5	0.26	237	40.9	0	774	43.8–43.9	0.1
	N. dorsalis	385	43.6	0	237	41.4	0	775	44.1	0
	O. antilopinus	385	43.6	0	237	41.4	0	775	44.1	0
	P. purpureicollis	398	42.7	0	251	40.6	0	802	43.4	0
	M. fuliginosus	383	43.1	0	237	40.5	0	773	43.6	0
Ma. yamagutii	*M. fuliginosus*	383	43.1	0	237	41.4	0	773	43.9	0
	M. giganteus	383	43.1	0	237	41.1	0	773	43.9	0
Ma. dissimilis	*W. bicolor*	392	42.1–43	0.3–2	241	38.6	0	782–786	42.3–42.9	0.3–1.9
Ma. lasiorhini	*V. ursinus*	383	43.1	0	237	38.4	0	773	42.9	0
	L. latifrons	385	43.1	0	237	40.9	0	775	43.7	0

[a]The ITS+ comprises the ITS-1, the 5.8S rRNA gene (153 bp) and the ITS-2

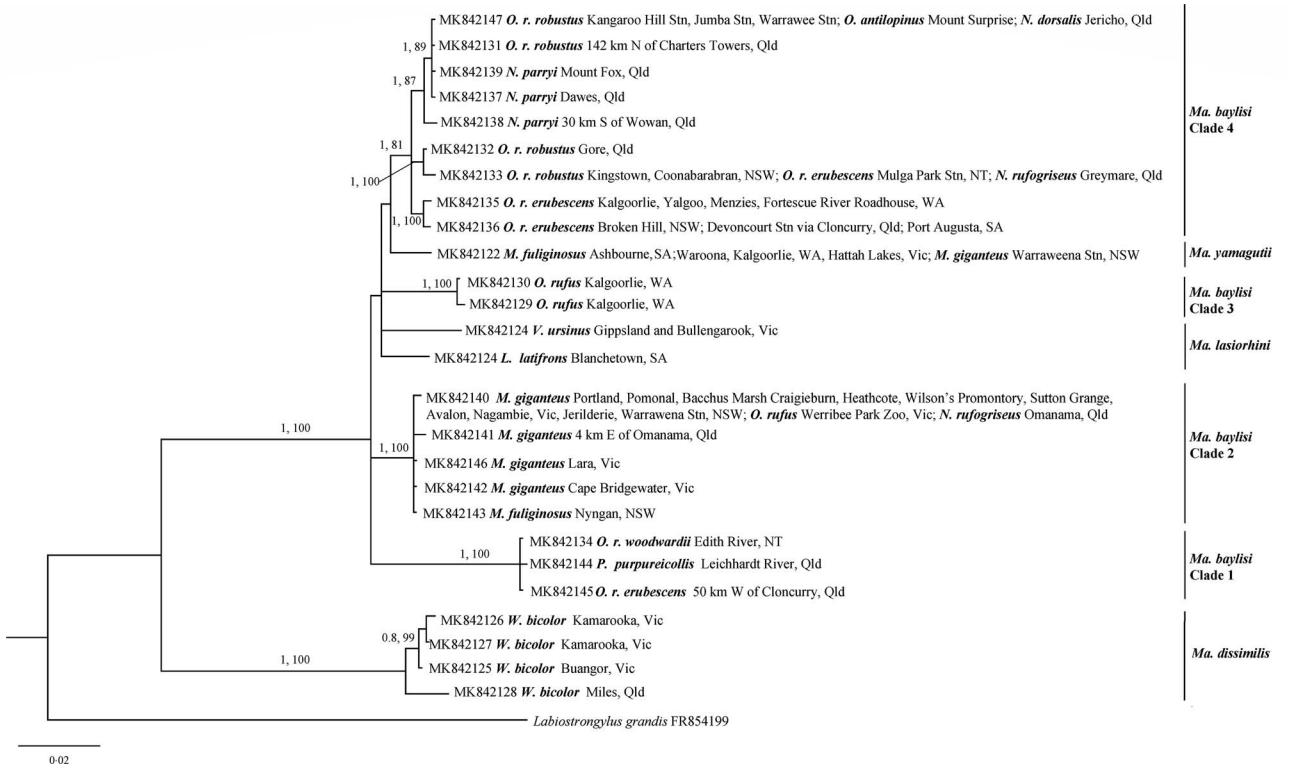

Fig. 1. Phylogenetic relationships of *Macropostrongyloides baylisi, Ma. yamagutii*, *Ma. lasiorhini* and *Ma. dissimilis* from different hosts and localities. The relationships were inferred based on Bayesian Inference (BI) and Neighbour Joining (NJ) analyses of the concatenated sequences of the first and second internal transcribed spacers. *Labiostrongylus grandis* was used as the outgroup. Nodal support is indicated by the BI posterior probabilities (pp) followed by NJ bootstrap values. BI pp values below 0.5 and bootstrap values 75% are not shown. The scale bar indicates the number of substitutions per nucleotide site. Qld, Queensland; NSW, New South Wales; WA, Western Australia; NT, the Northern Territory; SA, South Australia; Vic, Victoria; Stn, station; N, north; S, south; E, east; W, west.

dating back to 1979 (Table 3). The host range of *Ma. baylisi* is limited to the family Macropodidae; it has not been recorded from members of the Potoroidae, Hypsiprymnodontidae or Vombatidae (Beveridge *et al.*, 1992). The prevalence of *Ma. baylisi* within macropodid marsupials varied between different host genera and geographic regions. In Queensland, the highest prevalence of *Ma. baylisi* recorded was in *N. parryi* (76%) and *O. antilopinus* (64%). Other sympatric hosts examined, including *N. dorsalis*, the bridled nail-tail wallaby, *Onychogalea fraenata* and *L. conspicillatus*, were infected by *Ma. baylisi*, but at a lower prevalence (Table 3). Among the rock wallabies examined (genus *Petrogale*), *P. purpureicollis* was the only species in which one animal was infected with *Ma. baylisi*. *Macropostrongyloides baylisi* was present throughout the distribution of *O. robustus* (Fig. 2) and the prevalence was highest in New South Wales (Table 3). Although *M. giganteus* is distributed throughout most of eastern Queensland (Fig. 2), the prevalence of *Ma. baylisi* in Queensland was only 19% (unpublished data). The prevalence of 38% recorded by Beveridge and Arundel (1979) was based on data from New South Wales and Victoria combined, and therefore it was not possible to determine the prevalence in each state. *Macropostrongyloides baylisi* has been encountered most commonly in *M. giganteus* in Victoria and New South Wales (Table 3).

Discussion

Analyses of the ITS+ sequences of four morphologically defined species of *Macropostrongyloides* in Australia revealed considerable genetic variation within three species: *Ma. baylisi*, *Ma. lasiorhini* and *Ma. dissimilis*. However, there was no genetic variation in ITS + sequences in *Ma. yamagutii* collected from hosts across its broad geographical range. The primary host of *Ma. yamagutii* is *M. fuliginosus*. However, this nematode is also capable of infecting *M. giganteus* sharing the same habitat (Aussavy *et al.*, 2011). Furthermore, *M. fuliginosus* is a genetically variable host, with four genetically distinct populations inhabiting different geographical regions (Neaves *et al.*, 2009). Therefore, it is surprising that no genetic divergence was detected in *Ma. yamagutii* from different host populations.

In contrast, there was extensive genetic variation within *Ma. baylisi*. The phylogenetic analyses showed that this nematode species did not represent a monophyletic assemblage. There were four well-supported clades within *Ma. baylisi*. This finding provides further support to previous studies proposing the hypothesis that *Ma. baylisi* represents a species complex (Beveridge and Mawson, 1978; Beveridge *et al.*, 1993). The genetic differences detected between *Ma. baylisi* from *M. giganteus* and those in *O. robustus* and *N. parryi* were consistent with previous MEE data (Beveridge *et al.*, 1993). Clade 1, which comprised specimens of *Ma. baylisi* from *O. r. erubescens*, *O. r. woodwardi* and *P. purpureicollis*, was genetically the most distinct from the other three clades of *Ma. baylisi*. The presence of *Ma. baylisi* from *O. r. woodwardi* in clade 1 is consistent with the distinctive morphological variation detected in *Ma. baylisi* from the same host in the Kimberley Region of Western Australia (Beveridge *et al.*, 1993). However, additional material from *O. r. woodwardi* is required to determine whether there is genetic variation among *Ma. baylisi* in this host. The specimens of *Ma. baylisi* from *O. r. erubescens* collected near Cloncurry, Queensland, appear to represent a case of host switching from *O. r. woodwardi* since other sequences from *O. r. erubescens* in other locations were placed in two subgroups within clade 4 (Fig. 1). This could be due to the lack of clear geographical separation between

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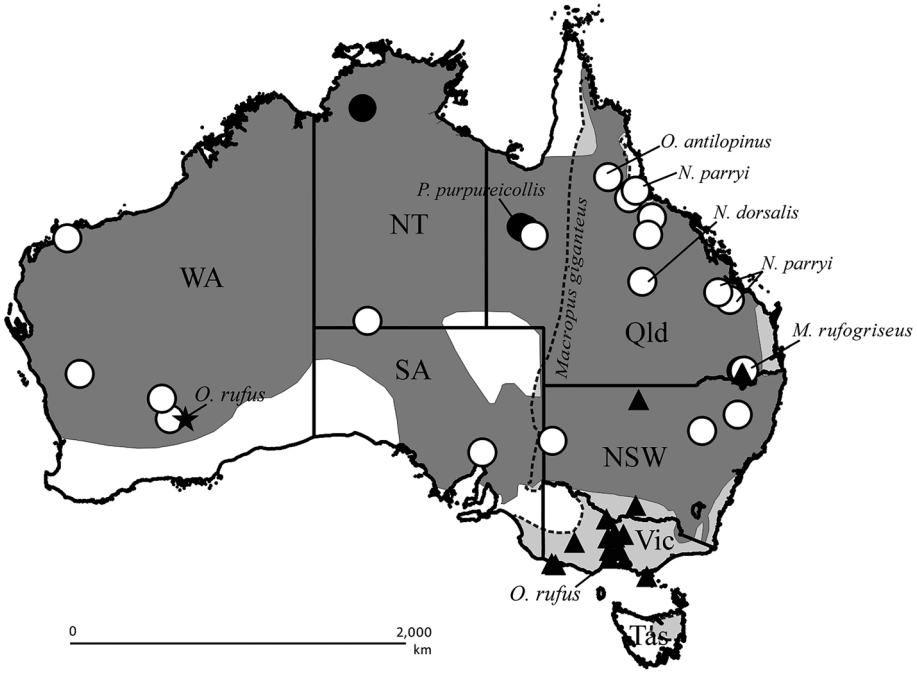

Fig. 2. Map showing the distribution of *Macropostrongyloides baylisi* and each of the four *Ma. baylisi* clades based on the phylogenetic analysis. Clade 1 is represented by closed circles, clade 2 is indicated by closed triangles, clade 3 is depicted by a closed star, and clade 4 is shown by open circles. The distribution of *O. robustus* is shaded in dark grey and the distribution of *M. giganteus* is shaded in light grey bordered by a dotted line.

the two subspecies of wallaroo in northeastern Queensland (Clancy and Croft, 2008) therefore allowing genetically distinct populations of nematodes to switch between these hosts. The inclusion of the specimen from *P. purpureicollis* in this clade is most likely due to host switching from *O. r. erubescens*. *Petrogale purpureicollis* is not a usual host for *Ma. baylisi* as sampling has found only one specimen in *P. purpureicollis*, and no specimens have been found in any of the other *Petrogale* species (Beveridge *et al.*, 1989). It is known that the habitats used by *O. r. erubescens* and *P. purpureicollis* in north-western Queensland overlap and as a consequence these two host species share many parasitic helminths (Bradley *et al.*, 2000).

Host switching was also present within the second clade of *M. baylisi* which contained primarily specimens from *M. giganteus*. Also included in this clade were specimens of *M. baylisi* from three host species (i.e. *O. rufus*, *M. fuliginosus* and *N. rufogriseus*,) that have overlapping ranges with *M. giganteus* (Van Dyck and Strahan, 2008). The specimen of *Ma. baylisi* from *O. rufus* at Werribee Park Zoo had the same ITS+ sequence as the majority of specimens from *M. giganteus* indicating the nematode's capacity to infect distantly related hosts under captive conditions. The specimens of *Ma. baylisi* found in *N. rufogriseus* at two collection localities in south-east Queensland near Greymare represent a new host record. Previous studies have not detected the presence of *Ma. baylisi* in *N. rufogriseus* despite extensive sampling of this host species at localities where *Ma. baylisi* occurs in *M. giganteus* at a high prevalence (Aussavy *et al.*, 2011). Of significance, one nematode in *N. rufogriseus* belonged to clade 2 (= *Ma. baylisi* in *M. giganteus*), while the second nematode had an ITS+ sequence identical to that of some *Ma. baylisi* in *O. r. robustus* and *O. r. erubescens* (= Clade 4).

The third clade consisted of *Ma. baylisi* found only in *O. rufus* from Kalgoorlie, Western Australia. There are records of *Ma. baylisi* infecting *O. rufus* in South Australia and New South Wales (Table 3); however, no material was available from these geographical areas for molecular analysis. Additional sampling of *Ma. baylisi* from *O. rufus* in other localities, particularly in areas of sympatry with other hosts species is required to define the true geographic distribution of this genotype in *O. rufus*. Nonetheless, the nematodes in *O. rufus* (clade 3) represent a genetically distinct group within the *Ma. baylisi* complex and they were collected in sympatry (i.e. at Kalgoorlie in Western Australia) with *Ma. baylisi* in *O. r. erubescens* (i.e. clade 4).

Clade 4 comprised *Ma. baylisi* collected from *O. r. erubescens*, *O. r. robustus*, *O. antilopinus*, *N. parryi* and *N. dorsalis*. This clade contains four groups of specimens which correspond to some extent to different host species or subspecies of *O. robustus*, and geographical locality. The clustering of specimens from *O. r. erubescens* from various localities into one group distinct from those in *O. r. robustus*, together with the occurrence of specimens from *O. r. woodwardi* in a completely separate clade suggests that host subspecies may have some influence over genetic divergence in *Ma. baylisi*. However, specimens from *O. r. erubescens* in Alice Springs share the same ITS+ sequence as those from *O. r. robustus* in Queensland and New South Wales which complicates this hypothesis. Additional samples are required to determine whether two genetically distinct populations of *Ma. baylisi* are present in *O. r. erubescens*. The group comprising specimens from *O. r. robustus, O. antilopinus, N. parryi* and *N. dorsalis* was consistent with the MEE findings from Beveridge *et al.* (1993). The similarities in the genetic sequences of *Ma. baylisi* shared by these different hosts is most likely due to the overlapping of habitats and instances of host switching (Beveridge *et al.*, 1998).

The genetic distinction between *Ma. lasiorhini* from two species of vombatid marsupials, *V. ursinus* and *L. latifrons*, suggests that this nematode may also represent two host-specific species. However, this requires further examination. Nonetheless, it is interesting to note that the prevalence of *Ma. lasiorhini* differs between the two hosts, with a significantly higher prevalence in *L. latifrons* than in *V. ursinus* (Beveridge and Mawson, 1978 and unpublished observations).

Genetic variation in ITS+ sequences was detected between specimens of *Ma. dissimilis* from Queensland and Victoria, located 1,600 km apart. Although the distribution of *W. bicolor* is continuous along the east coast of Australia (Merchant, 2008), the results of the present study indicate genetic differences between the northern and southern population of *Ma. dissimilis*. This geographical pattern of genetic divergence is consistent with that detected previously for two other parasites of *W. bicolor*; the

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Table 3. Prevalence of *Macropostrongyloides baylisi* in macropodid hosts reported in published and unpublished surveys

Host	Animals examined	Prevalence	Location	Reference
Macropus giganteus	36	38	Vic, NSW, Qld	Beveridge and Arundel (1979)
	28	11	Qld	Beveridge *et al.* (1998)
	18	50	Grampians, Vic	Aussavy *et al.* (2011)
	16	6	Portland, Vic	Vendl and Beveridge (2014)
	28	32	Serendip, Vic	Cripps *et al.* (2015)
	10	40	Anglesea, Vic	Cripps *et al.* (2015)
	16	68	Vic	Unpublished data
	28	57	NSW	Unpublished data
	21	19	Qld	Unpublished data
Macropus fuliginosus	52	2	Vic, NSW, Qld	Unpublished data
	40	0	Vic, NSW, Qld	Beveridge and Arundel (1979)
	25	0	Kangaroo Island, SA	Webley *et al.* (2004)
Osphranter rufus	115	3	Kinchega, NSW	Arundel *et al.* (1979)
	16	6	SA	Beveridge *et al.* (1993)
	52	31	SA, WA, NSW, Qld, NT	Unpublished data
Notamacropus dorsalis	38	10	Qld	Beveridge *et al.* (1998)
Notamacropus parryi	29	76	Qld	Beveridge *et al.* (1998)
	5	60	Qld and NSW	Unpublished data
Wallabia bicolor	100	2	Qld	Beveridge (2016)[a]
Osphranter robustus	30	48	Qld	Beveridge *et al.* (1998)
	10	45	Qld	Unpublished data
	26	69	NSW	Unpublished data
	20	40	NT	Unpublished data
	24	37.5	WA	Unpublished data
	22	22.7	SA	Unpublished data
Osphranter antilopinus	12	64	Qld	Beveridge *et al.* (1998)
Lagorchestes conspicillatus	8	25	Qld	Beveridge *et al.* (1992)
Petrogale purpureicollis	12	8	Qld	Bradley *et al.* (2000)
Onychogalea fraenata	4	20[b]	Qld	Turni and Smales (2001)

Qld, Queensland; NSW, New South Wales; WA, Western Australia; NT, the Northern Territory; SA, South Australia; Vic, Victoria; SAM, South Australian Museum.
[a]Includes data from earlier publications.
[b]Erroneously cited as 25% in paper.

intestinal nematode, *H. macropi* (Chilton *et al.*, 2012), and a bile-duct cestode *Progamotaenia festiva* (Beveridge *et al.*, 2007). However, population genetic inferences cannot be made without additional sampling from this host in the intermediate localities.

The results of the phylogenetic analyses of ITS+ data showed that *Ma. dissimilis* is the sister taxon to all other Australian species of *Macropostrongyloides*. Specimens of the fifth species, *Ma. dendrolagi*, a parasite that occurs in the colon of tree kangaroos in Indonesia (Beveridge, 1997), were not available for inclusion in this study. The phylogenetic relationship of *Ma. dissimilis* is interesting because it is the only member of the genus that occurs in the stomach of its hosts (Beveridge and Mawson, 1978). Moreover, the morphology of the vagina of *Ma. dissimilis* is distinct from congeners. The vagina in this nematode species is *J*-shaped or Type-2 of Lichtenfels (1980), whereas the vagina in *Ma. baylisi, Ma. yamagutii*, *Ma. lasiorhini* and *Ma. dendrolagi* is *Y*-shaped or Type-1 (Beveridge and Mawson, 1978; Beveridge, 1997). This morphological difference between female nematodes suggests that *Ma. dissimilis* may be more closely related to the cloacinine stomach-inhabiting nematodes, which also have a Type-2 ovejector, and may have more recently colonised the stomach (Beveridge, 1987). However, this hypothesis requires testing using morphological and molecular characterisation of a larger number of specimens. Furthermore, phylogenetic inferences based on a single molecular marker such as ITS represent molecular prospecting and are insufficient to delineate a species or fully explain speciation processes (Nadler and Pérez-Ponce de León, 2011). In addition, the ITS region has shown contrasting evolutionary patterns in some plant parasitic nematodes (Pereira and Baldwin *et al.*, 2016). Such questions could be addressed by the characterisation of the mitochondrial genome which has been found to accumulate mutations more rapidly compared to the nuclear genome. Mitochondrial genome sequence data have been used to investigate the population genetics and systematics of nematodes (Hu *et al.*, 2003). Molecular investigations involving characterisation of the ITS nuclear DNA sequence (Chilton *et al.*, 2012) and the amino acid sequence of the mitochondrial genome to validate the presence of three

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genetically distinct groups within *H. macropi* (Jabbar *et al.*, 2013) suggests that such an approach might also be applied to *Macropostrongyloides* spp., thereby helping to resolve discrepancies found between electrophoretic and ITS datasets.

In conclusion, the separation of *Ma. baylisi* into four genetically distinct clades based on phylogenetic analyses of ITS+ sequence data provides additional support for the hypothesis that *Ma. baylisi* represents a species complex. *Macropostrongyloides lasiorhini* may also represent two genetically distinct species, while there is genetic divergence between *Ma. dissimilis* from different geographical areas. These findings represent an important contribution to document the diversity of Australian parasitic nematodes. They also highlight the need for future studies into the comprehensive molecular analysis of these nematodes to better understand the evolutionary processes leading to their existence.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019001008.

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Conflict of interest. The authors declare that they have no competing interests.

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