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Isospora anthochaerae n. sp. (Apicomplexa: Eimeriidae) from a Red wattlebird (*Anthochaera carunculata*) (Passeriformes: Meliphagidae) in Western Australia

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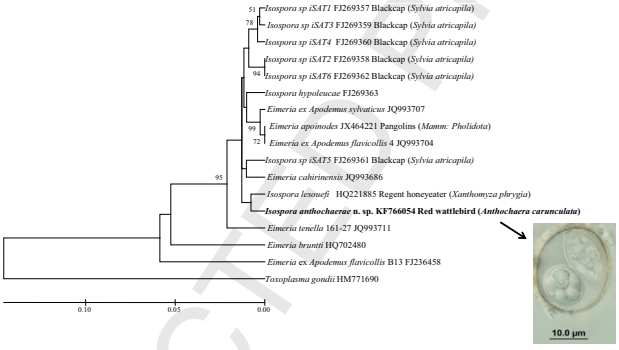
HIGHLIGHTS

- Description of a new species of *Isospora* in Red wattlebirds.
- Morphological characterisation.
- Molecular characterisation at 4 loci.

GRAPHICAL ABSTRACT



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ABSTRACT

A new species, *Isospora anthochaerae* n. sp. is described from a Red wattlebird (*Anthochaera carunculata*). Sporulated oocysts ($n = 37$) are subspherical, with smooth colourless to pale brown bilayered oocyst wall, 0.8 µm thick (outer layer 0·6 µm, inner 0.2 µm thick). Oocyst with 2 spheroidal to subspheroidal sporocysts. Oocyst length, 23.4 µm (20.0–26.0); oocyst width, 20.7 µm (19.0–22.0); oocyst length/width (L/W) ratio, 1.1. Micropyle, oocyst residuum and polar granule are absent. Sporocysts with compact sporocyst residuum and 4 sporozoites. Sporocyst length, 14.5 µm; sporocyst width, 10.1 µm sporocyst L/W ratio, 1.4. Molecular analysis was conducted at four loci; the ribosomal internal transcribed spacer (ITS), the 18S and 28S ribosomal RNA and the mitochondrial cytochrome oxidase gene (COI). At the COI locus, *I. anthochaerae* n. sp. exhibited 98.5% similarity to *Isospora lesouefi* from a Regent honeyeater (*Xanthomyza phrygia*) and 98% similarity with an *Isospora* sp. (iSAT5) from a blackcap (*Sylvia atricapilla*). Based on morphological and molecular data, this isolate is a new species of coccidian parasite that to date has only been found in Red wattlebirds.

1. Introduction

The genus *Isospora* was first described by Schneider in 1881 and its taxonomy has been a source of controversy since due to morphological differences and paraphylogeny of the genus. In 1977, the genus *Cystoisospora* was proposed because of the presence of unizoic tissue cysts in lymphoid tissues in rodents which function as intermediate hosts of *Cystoisospora felis* and *Cystoisospora rivolta* of cats (Frenkel, 1977). In 2005, Barta et al. assigned all tetrasporozoic, diplosporocystic oocysts from mammals without Stieda bodies in their sporocysts, to the genus *Cystoisospora*

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(*Sarcocystidade*), and all such oocysts from birds with Stieda bodies in their sporocysts to the genus *Isospora*. *Atoxoplasma* was determined to be a junior objective synonym for *Isospora* (Barta et al., 2005).

The avian order Passeriformes includes 5000 species worldwide and accounts for 50% of all avian species and *Isospora* are the most common coccidian parasites infecting passerine birds (Duszynski et al., 1999). While numerous species of *Isospora* infecting birds have been described (cf. Berto et al., 2011), relatively few species have been characterised genetically (Olson et al., 1998; Carreno et al., 1999; Schrenzel et al., 2005; Dolnik et al., 2009; Morin-Adeline et al., 2011)

The Red wattlebird (*Anthochaera carunculata*) (also known as Barkingbird or Gillbird) is a passerine species that belongs to the family Meliphagidae (honeyeaters), a group of birds found mainly in Australia and New Guinea. It is amongst the largest of the Australian honeyeaters and has a wide range of habitats, which include woodlands, eucalypt forests, scrubs, heaths, orchards and parks. The Red wattlebird has a fleshy reddish wattle on the side of the neck and its plumage is grey–brown on its body, whilst the middle of the belly is lemon-yellow in colour. The tail is long and is tipped in white. It uses its thin curved beak to probe flowers for nectar on which it feeds, supplemented with insects and berries (Pizzey and Knight, 2007). To date, *Isospora lesouefi* has been characterised from the endangered Regent honeyeater (*Xanthomyza phrygia*), which is endemic to south-eastern Australia (Morin-Adeline et al., 2011) and *Isospora samoaensis* has been described morphologically from the Wattled honeyeater (*Foulehaio carunculata*) from American Samoa (Adamczyk et al., 2004). In the present study, we characterized a new species of *Isospora* from a Red wattlebird (*A. carunculata*) in Western Australia, both morphologically and genetically, and propose the species name *Isospora anthochaerae*.

2. Materials and methods

2.1. Sample collection

A survey was conducted over a 7-month period (September 2012–March 2013), to determine the incidence of coccidian parasites in a population of Red wattlebirds (*A. carunculata*) that had been admitted to the Kanyana Wildlife Rehabilitation Centre (KWRC) in Western Australia. All birds were wild and came into care either as a result of cat attacks or as nestlings that had fallen out of their nests. A total of 13 faecal samples were collected from 13 different Red wattlebirds at KWRC under the KWRC permit. Samples were stored at 4 °C until parasitological examination and DNA extraction.

2.2. Morphological analysis

The presence of oocysts was determined by direct microscopic examination of a faecal suspension in saline, as well as faecal flotation analysis using a saturated sodium chloride and 50% sucrose (w/v) solution. If any sample was found to contain coccidian oocysts, a portion of faeces was placed in 2% (w/v) potassium dichromate solution ($K_2Cr_2O_7$), mixed well and poured into petri dishes to a depth of less than 1 cm and kept at room temperature in the dark to facilitate sporulation. Sporulated oocysts were observed using the 100× oil immersion objective of an Olympus CH-2 binocular microscope, in combination with an ocular micrometre.

2.3. DNA isolation

Total DNA was extracted from 200 mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California) with some modifications. Briefly, the faeces for DNA extraction were subjected to four cycles of freeze/thaw by liquid nitrogen and boiling water to ensure efficient lysis of oocysts before being processed using the manufacturer's protocol. A negative control (no faecal sample) was used in each extraction group.

2.4. PCR amplification of ITS, 18S, 28S and COI loci

Amplification of a 404 bp region of the ribosomal *internal* transcribed *spacer* (ITS) locus from samples was conducted as described by Johnson et al. (2008). Samples were then amplified at the 18S locus for *Isospora* using a nested PCR with the primers and PCR conditions as described by Pieniazek et al. (1996), which produced a 497 bp product. Samples were also amplified at the *Isospora* 28S ribosomal RNA (28S rRNA) locus using a nested PCR with the external primers: 28SExF: 5′-TAC CCG CTG AAC TTA AGC and 28SExR: 5′-CMA CCA AGA TCT GCA CTA G as previously described (Schrenzel et al., 2005), which produced a PCR product size of ~1495 bp. Internal primers were designed for the present study using Primer 3 (http://frodo.wi.mit.edu/). The internal primers 28SInF: 5′-ACT ATG TTC CCT AGT AAC G and 28SInR 5′-AAC GCT TCG CCA CGA TCC produced a PCR product size of 1420 bp. The 25 µl PCR reaction contained 2.5 µl of 10× Kapa PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 nM dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 µl of DNA and 16.9 µl of H_2O. Both primary and secondary PCR's were conducted with the same cycling conditions; 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s and a final extension of 72 °C for 5 min. Finally, samples were screened at the COI locus for *Isospora* using primers and conditions described by Dolnik et al. (2009).

2.5. Sequence and cloning analysis

Secondary PCR products were gel purified using an in house filter tip method without any further purification for down stream sequencing as previously described (Yang et al., 2013)

Gel-purified PCR amplicons from the 28S rRNA and ITS loci were cloned in the pGEM-T Easy Vector System II (Promega, USA) due to the low PCR product yield. After transformation into JM109 competent cells, plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) from cultured clones grown overnight and 10 colonies were sequenced with the T7 (5′ TAA TAC GAC TCA CTA TAG GG) and SP6 (5′ ATT TAG GTG ACA CTA TAG) primers in both directions, using v3.1 BigDye® Terminator chemistry at 1/16 × dilution (Life Technologies, Foster City, California).

The results of the sequencing reactions were analysed and edited using Finch *TV*® v1.4.0. (http://seq.mc.vanderbilt.edu/dna/html/SoftDetail.html). Sequences were compared to existing *Isospora* sp. ITS, 18S and 28S rDNA and COI sequences on GenBank using BLAST searches and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp).

2.6. Phylogenetic analysis

Phylogenetic trees were constructed for *Isospora* sp. at the ITS, 18S, 28S and COI loci with additional isolates from GenBank. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Tamura–Nei model and grouped using Neighbour-Joining. Parsimony analyses were conducted using MEGA version 5.1 (MEGA5.1: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA). Bootstrap analyses were conducted using 1000 replicates to assess the reliability of inferred tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper et al., 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006).

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2.7. Statistical analysis

Measurements of 37 sporulated oocysts were analysed using Statistical Package for the Social Sciences (SPSS v21) and results are presented in micrometres as the mean, with the observed range in parentheses.

3. Results

3.1. Description

3.1.1. I. anthochaerae n. sp. (Alveolata Cavalier-Smith, 1991, Apicomplexa Levine, 1970, Eimeriidae Minchin, 1903)

Diagnosis: Sporulated oocysts ($n = 37$) are spherical to subspherical with colourless to pale brown bilayered oocyst wall, 0.8 (0.6–0.9) thick (outer layer 0·6 µm, inner 0.2 µm) and measure 23.4 (20–26) × 20.7 (19–22) µm in size with a width to length ratio of 1.1 (1.0–1.4). Oocysts with 2 spherical to subspherical sporocysts. Micropyle, oocyst residuum and polar granule are absent. Sporocysts with compact sporocyst residuum and 4 sporozoites. Sporocyst length, 14.5 (11–17); sporocyst width, 10.1 (9–11); sporocyst L/W ratio, 1.4 (1.1–1.8). The stieda body is broad, hemi-dome-like with a rather rectangular-shaped substieda body. Parasteidia body is absent (Fig. 1).

Type hosts: Red wattlebird (*A. carunculata*).

Type locality: Leeming, Perth, Western Australia.

Prevalence: Isospora sp. was detected in 2/13 samples screened, an estimated prevalence of 15.4% (95% CI 0–35).

Other hosts: Unknown.

Prepatent period: Unknown.

Patent period: Unknown.

Site of infection: Unknown.

Sporulation time: Unknown but assumed to be less than 24 h as some of the oocysts were already sporulated in the fresh faecal samples.

Material deposited: DNA sequences have been deposited in GenBank under accession numbers KF766052, KF766053, KF766051 and KF766054 for the 18S, 28S, ITS and COI loci, respectively.

Etymology: This species is named *I. anthochaerae* n. sp. after its host *A. carunculata* (Red wattlebird).

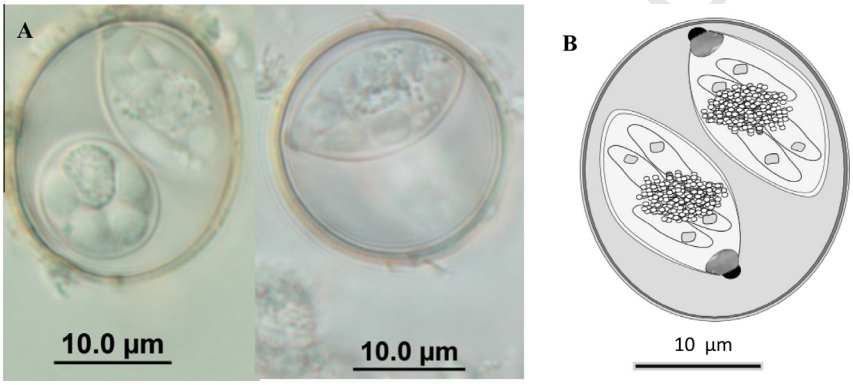

Fig. 1. (A) Nomarski interference-contrast photomicrographs of *Isospora anthochaerae* n. sp. showing sporulated oocysts. Scale bar = 10 µm. (B) Composite line drawing of *Isospora anthochaerae* n. sp. sporulated oocyst. Scale bar = 10 µm.

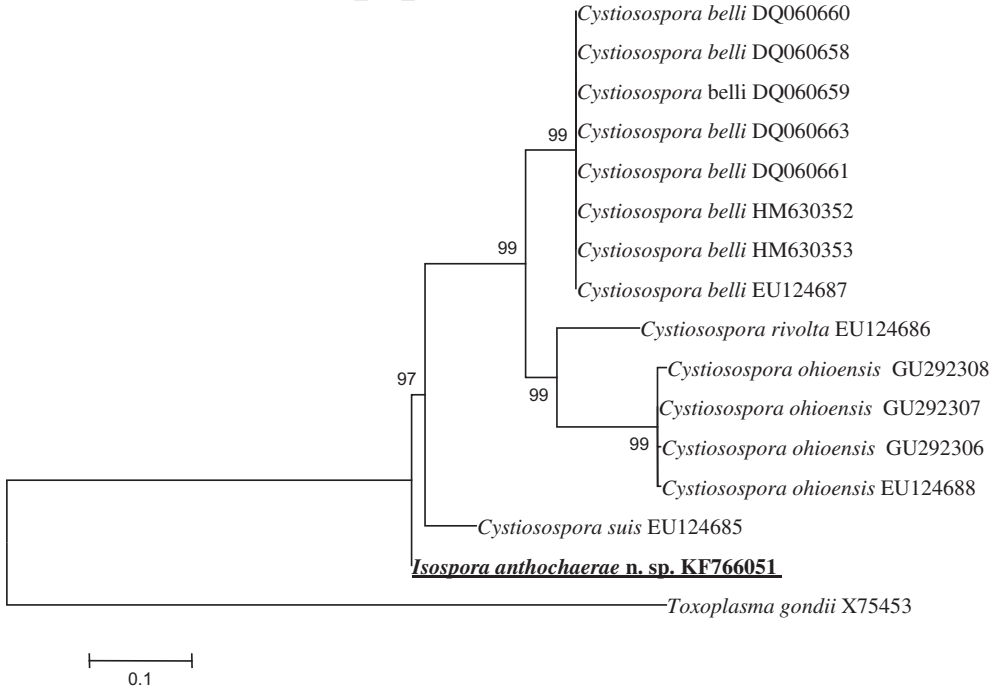

Fig. 2. Evolutionary relationships of *Isospora anthochaerae* n. sp. inferred by distance analysis of ITS rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from Maximum Likelihood (ML) analyses is indicated at the left of the supported node.

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3.2. Phylogenetic analysis of *I. anthochaerae* n. sp. at the ITS locus

Two partial ITS sequences (404 bp) were obtained from cloned PCR products of *I. anthochaerae* n. sp., which exhibited 1 single nucleotide polymorphism (SNP), when compared to each other. Phylogenetic analyses of the partial nucleotide sequences from *I. anthochaerae* n. sp. at the ITS locus using Distance, Parsimony and ML analyses produced similar results (Fig. 2 – ML tree shown). Unfortunately bird-derived *Isospora* sequences were not available at the ITS locus and phylogenetic analysis placed *I. anthochaerae* n. sp. in a clade by itself but grouping closest (93% similarity) with *Cystioisospora suis*. The two sequences from *I. anthochaerae* n. sp. were 99.9% similar to each other.

3.3. Phylogenetic analysis of *I. anthochaerae* n. sp. at the 18S locus

Two identical 18S *Isospora* sequences were obtained from the Red wattlebirds faecal samples and were aligned with 3 other *Isospora* sp. sequences from passerine birds; *Isospora gryphoni* – GenBank accession number: AF080613 (Olson et al., 1998), *Isospora* sp. *MS-2003* – GenBank accession number: AY331571 (Schrenzel et al., 2005) and *Isospora robini* – GenBank accession number: AF080612 (Carreno and Barta, 1999) and 3 mammalian *Cystiosospora* sp. (*C. suis* – GenBank accession number: U97523, *C. felis* – GenBank accession number: L76471 and *Cystiosospora belli* – GenBank accession number: U94787), as well as other apicomplexan 18S rRNA sequences. *Cryptosporidium muris* was used as an outgroup.

Phylogenetic analysis using distance, parsimony and ML revealed that *I. anthochaerae* n. sp. exhibited 98% similarity with *I. gryphoni* and *Isospora* sp. *MS-2003*, which were identified from American goldfinches (*Carduelis tristis* L.) and Southern cape sparrows (*Passer melanurus melanurus*), respectively (Fig. 3).

3.4. Phylogenetic analysis of *I. anthochaerae* n. sp. at the 28S locus

Four identical 28S cloned PCR amplicons from *I. anthochaerae* were obtained. In Genbank, 32 28S *Isospora* sequences were available, of which 31 were sequences and genotypes from a unique species of *Isospora* (*MS-2003*), from passerine birds (Schrenzel et al., 2005), as well as a 28S sequence of *C. felis* from a cat. Phylogenetic analysis at this locus showed that *I. anthochaerae* was most

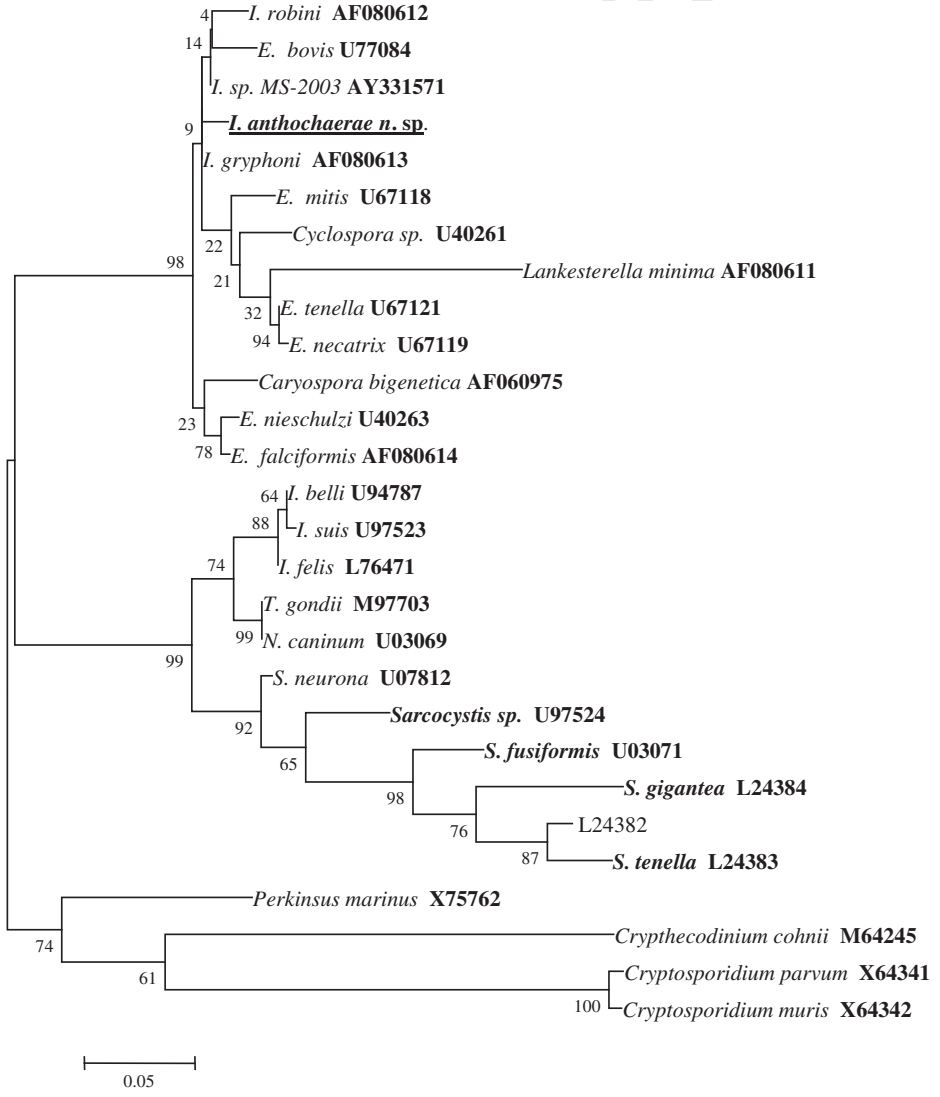

Fig. 3. Evolutionary relationships of *Isospora anthochaerae* n. sp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from maximum likelihood analyses is indicated at the left of the supported node.

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closely related to *Isospora* sp. *MS-2003* isolated from a Grosbeak starling (*Scissirostrum dubium*) (95.3% similarity) (Fig. 4).

3.5. Phylogenetic analysis of *I. anthochaerae* n. sp. at the COI locus

Direct sequencing of the COI gene fragment from 2 isolates produced clean and identical chromatograms, indicating that only one sequence was present. These were then aligned with 8 other *Isospora* sp. sequences from passerine birds (*Isospora hypoleucae* – GenBank accession number: FJ269363, *I. lesouefi* – GenBank accession number: HQ221885, *Isospora* sp. *iSAT1* – GenBank accession number: FJ269357, *Isospora* sp. *iSAT2* – GenBank accession number: FJ269358, *Isospora* sp. *iSAT3* – GenBank accession number: FJ269359, *Isospora* sp. *iSAT4* – GenBank accession number: FJ269360, *Isospora* sp. *iSAT5* – GenBank accession number: FJ269361, *Isospora* sp. *iSAT6* – GenBank accession number: FJ269362), as well as 7 *Eimeria* COI gene sequences. *Toxoplasma gondii* was used as the outgroup. *I. anthochaerae* n. sp. exhibited 98.5% similarity with *I. lesouefi* and 98% similarity with *Isospora* sp. *iSAT5*, which were identified from a Regent honeyeater (*X. phrygia*) and a blackcap warbler (*Sylvia atricapilla*), respectively (Fig. 5).

4. Discussion

In the present study, the prevalence of *I. anthochaerae* n. sp. in Red wattlebirds was 15.4% (2/13). The prevalence rate of coccidian oocysts in our samples is lower than the 91% reported by Morin-Adeline et al., 2011 for *I. lesouefi* in the Regent honeyeater (*X. phrygia*). In that study, the authors compared the rate of *I. lesouefi* oocyst shedding in faeces in the morning (AM) to the afternoon (PM). Significant diurnal periodicity was revealed in oocyst shedding, as the AM prevalence was 21% (18/84) (mean = 499 oocysts/g^{-1}) compared to a PM prevalence of 91% (82/90) (mean = 129,723 oocysts/g^{-1}). In the present study, all 13 samples were collected in the morning and the prevalence of 15.4% identified for *I. anthochaerae* is similar to the morning prevalence for *I. lesouefi* (21%). The Regent honeyeaters tested in the study by Morin-Adeline et al. (2011) were captive birds whereas the Red wattlebirds in the present study were wild-caught and had only been in care for a few days.

Sporulated oocysts of *I. anthochaerae* n. sp. were spherical to subspherical and measured 23.4 (20.0–26.0) × 20.7 (19.0–22.0) μm in size with a width to length ratio of 1.12. Oocysts of *I. lesouefi*

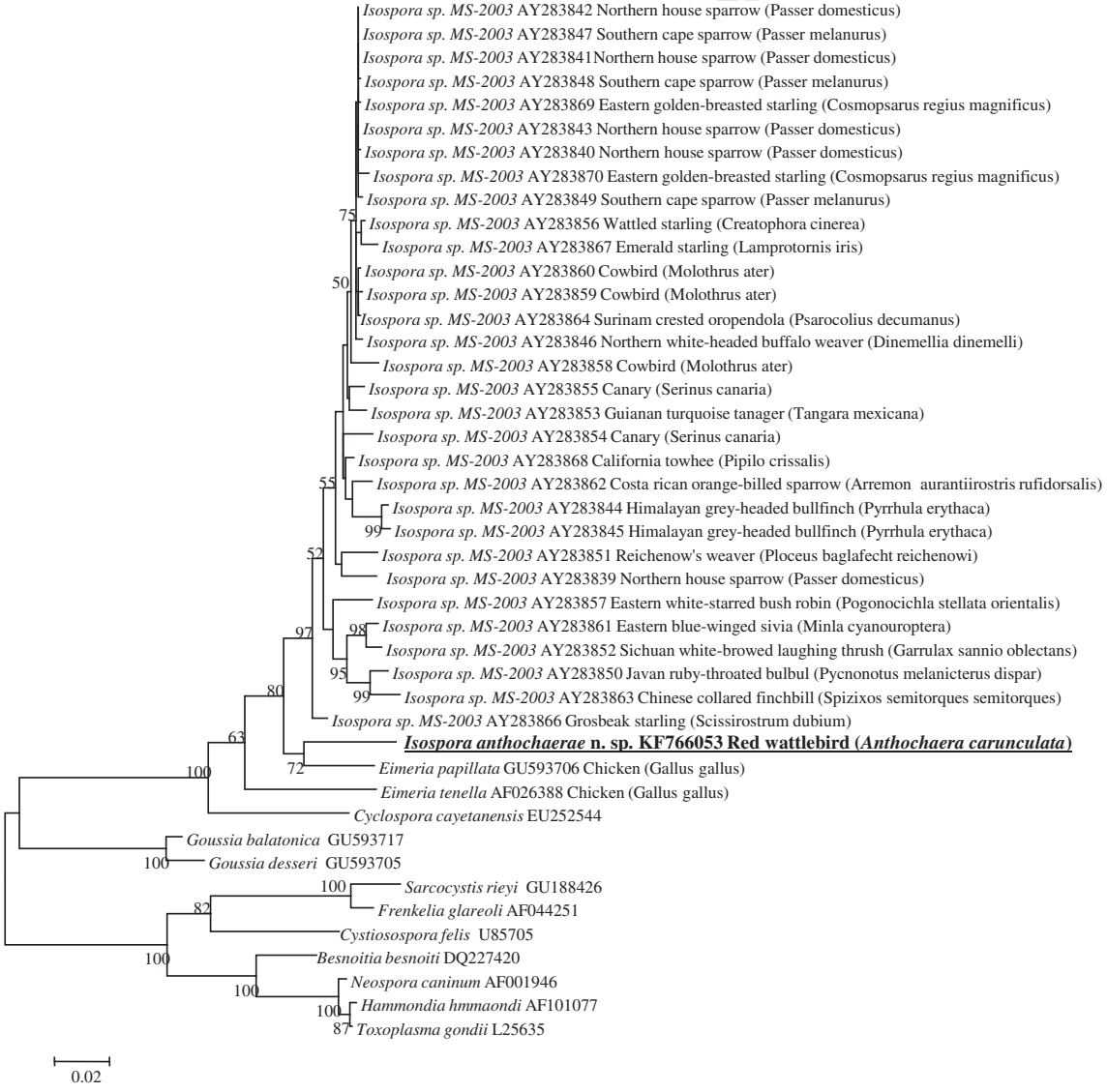

Fig. 4. Evolutionary relationships of *Isospora anthochaerae* n. sp. inferred by distance analysis of 28S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from maximum likelihood analyses is indicated at the left of the supported node.

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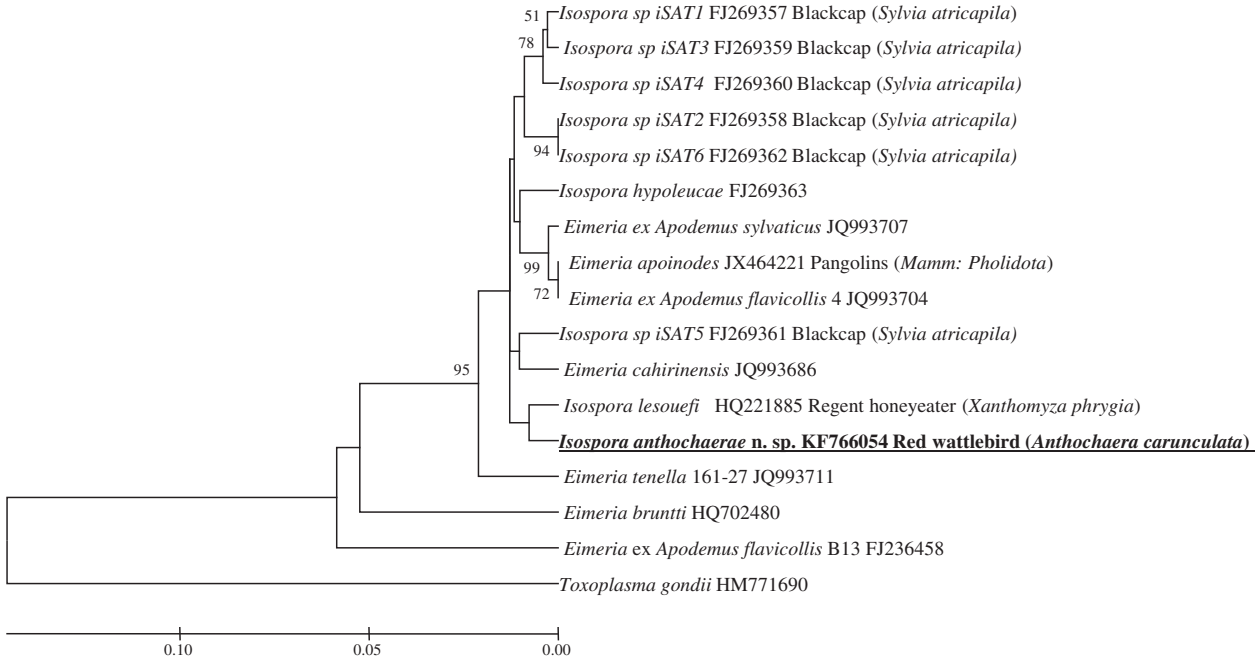

Fig. 5. Evolutionary relationships of *Isospora anthochaerae* n. sp. inferred by distance analysis of mitochrondial cytochrome oxidase gene (COI). Percentage support (>50%) Q6 from 1000 pseudoreplicates from maximum likelihood analyses is indicated at the left of the supported node.

Q4 are also spherical and measured 25.8 (22.5–28.7) × 23.8 (20–26.2) µm with a width to length ratio of 1.08 (Morin-Adeline et al., 2011) (Table 1). Oocysts of *I. samoaensis* measured 28.9 × 26.1 (25–32 × 23–30) µm with a width to length ratio of 1.1 (Adamczyk et al., 2004). All three species have two ovoid shaped sporocysts, but sporocysts of *I. anthochaerae* n. sp. measured 14.5 (11.0–17.0) × 10.1 (9.0–11.0) µm, sporocysts of *I. lesouefi* measured 18.7 (17–19) × 9.5 (9–10) µm and sporocysts of *I. samoaensis* measured 17.1 × 10.9 (16–18 × 10–11) µm. A polar granule is present in *I. lesouefi* and *I. samoaensis* but is absent in *I. anthochaerae* n. sp. In addition, the morphological dimensions of *I. anthochaerae* n. did not match any other existing *Isospora* species from Passeriformes listed online (http://biology.unm.edu/biology/coccidia/passer1.html. Accessed 21 Oct 2013).

Delimiting avian species of *Isospora* is problematic due to (i) ambiguities in the morphology and (ii) unknown host specificity (Grulet et al., 1982; Levine, 1982). Molecular data are therefore essential to accurately delimit species. In the present study, a comprehensive molecular characterization of *I. anthochaerae* n. sp. was conducted at 4 different loci; the ITS, 18S, 28S and COI loci. Due to the very limited availability of sequences for avian *Isospora* species at the 4 loci, the phylogenetic trees were generated with different data sets. Initial characterisation at the ITS locus was only able to group *I. anthochaerae* n. sp. with *C. suis* (93% similarity) as no reference sequence from avian *Isospora* species were available at the ITS locus. The 18S rRNA is the most common locus for phylogenetic analysis of coccidia and is widely used for *Eimeria* and *Isospora* phylogenetic analysis. At this locus, distance, ML and parsimony analysis grouped *I. anthochaerae* n. sp. most closely (98% similarity) with *I. gryphoni* from American goldfinches (*C. tristis* L.), whose oocysts are considerably larger (29.2–30.7 µm) than *I. anthochaerae* n. sp. (Olson et al., 1998) and *Isospora* sp. MS-2003 from a Southern cape sparrow (*P. melanurus melanurus*) (Schrenzel et al., 2005). At the 28S rRNA locus, *I. anthochaerae* n. sp. exhibited 95.3% similarity with *Isospora* sp. *MS-2003* (GenBank accession number – AY283866) from a Grosbeak starling (*S. dubium*). Interestingly, *I. anthochaerae* n. sp. also grouped with *Eimeria papillata* (GenBank accession number – GU593706), which was isolated from a chicken (*Gallus gallus*) but exhibited 94.9% similarity with this isolate. At the COI locus, *I. anthochaerae* n. sp. exhibited 98.5% similarity to *I. lesouefi* (Morin-Adeline et al., 2011) and 98% similarity with an *Isospora* sp. (iSAT5) from a blackcap (*S. atricapilla*) (Dolnik et al., 2009). The genetic differences at the COI locus are phylogenetically significant as the COI gene is highly conserved (Barta, 2001) and has

Table 1
Comparative morphology of *Isospora anthochaerae* n. sp. and *Isospora* sp. recorded from the family Meliphagidae (honeyeaters).

Species	Hosts	References	Oocysts					Sporocysts				
			Shape	Measurements (µm)	Shape index	Wall (µm)	Polar granule	Shape	Measurements	Stieda body	Substida body	Residuum
Isospora samoaensis	Wattled honeyeater (*Foulehaio carunculata*)	Adamczyk et al. (2004)	Ovoid	28.9 × 26.1 (25–32 × 23–30)	1.1	Bi-layered c. 0.8	Present	Ovoid	17.1 × 10.9 (16–18 × 10–11)	Broad	Dome-like	Compact
Isospora lesouefi	Regent Honeyeater (*Xanthomyza phrygia*)	Morin-Adeline et al. (2011)	Spherical	25.8 × 23.8 (22.5–28.7 × 20–26.2)	1.08	Bi-layered c.1.0	Present	Ovoid	18.67 × 9.45 (17–19 × 9–10)	Flat	Spherical	Compact
Isospora anthochaerae	Red wattlebird (*Anthochaera carunculata*)	Current study	Subspherical	23.4 × 20.7 (20.0–26.0 × 19.0–22.0)	1.1	Bi-layered c. 0.8	Absent	Ovoid	14.5 × 10.1 (11–17 × 9–11)	Hemi-dome	Rectangular-shaped	Compact

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been shown to have a higher resolving power than the 18S gene in delineating recent speciation events (Ogedengbe et al., 2011). COI has become the target gene for the "Barcode of Life" project that uses the marker for rapid identification of a range of species including parasites (Ratnasingham and Hebert, 2007). One drawback of using this gene is the paucity of avian *Isospora* sequences at this locus but for example the genetic similarity between the accepted *Eimeria* species, *E. tenella* and *E. necatrix* at this locus is 98.4% which is very similar to the genetic similarity between *I. anthochaerae* n. sp. and *I. lesouefi* (98.5%). Based on the morphological and molecular differences, *I. anthochaerae* n. sp. is a separate species.

In the present study, morphological and molecular data were used to describe *I. anthochaerae* n. sp. found in the faeces of Red wattlebirds in Western Australia. Future studies need to concentrate on obtaining afternoon faecal samples from a variety of wattlebird species and conducting morphological and genetic characterisation to understand the extent of diversity within *Isospora* sp. in wattlebirds.

5. Uncited reference

Q5 Duszynski and Wilber (1997).

Acknowledgments

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