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Phylogenetic analysis of *Isospora manorinae* . *n.* sp. at 18S rRNA locus

a.

b.
Morphological and molecular characterization of *Isospora manorinae* n. sp. in a yellow-throated miner (*Manorina flavigula wayensis*) (Gould, 1840).

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Abstract

A new *Isospora* (Apicomplexa:Eimeriidae) species is described from a single yellow-throated miner bird (*Manorina flavigula*) (subspecies *M. f. wayensis*) in Western Australia. Sporulated oocysts (n=32) of this isolate are spherical to subspherical, 22.8 (20.3-23.8) × 18.3 (17.7-18.7) µm, with a shape index (length/width) of 1.25 (1.2 – 1.3); and a smooth and bilayered oocyst wall, 1.3 µm thick (outer layer 0.9 µm, inner 0.4 µm). A polar granule is present, but the micropyle and oocyst residuum are absent. The sporocysts are lemon-shaped, 15.5 (14.6-15.8) × 9.5 (9.5-10.2) µm, with a shape index of 1.6. Stieda and substieda bodies are present, the Stieda body being knob-like and the substieda body being subspherical-shaped. A sporocyst residuum is present and composed of numerous granules of different size scattered among the sporozoites, a spheroid or subspheroid refractile body is present in the sporozoite. Morphologically, the oocysts from this isolate are different from those of all known valid *Isospora* spp. Molecular analysis was conducted at 3 loci; the 18S and 28S ribosomal RNA and the mitochondrial cytochrome oxidase (COI) gene. At the 18S locus, this new isolate exhibited 99.2% similarity to *Isospora gryphoni* and three other *Isospora* spp. Further analysis of a subgroup of 300 bp long 18S sequences (8), including *Isospora anthochaerae* was conducted. This new isolate grouped in a clade with *I. anthochaerae* and exhibited 99.3% similarity. At the 28S locus, this new isolate grouped with *I. anthochaerae* with which it shared 99.1% similarity. At the COI locus, this new isolate exhibited 96.8% similarity to *Isospora* sp. JCI-2015 from a spectacled warbler (*Sylvia conspicillata*) in Spain. Further analysis from a subgroup of shorter COI sequences (n=13) was performed and this new isolate exhibited 99.1% similarity to *I. anthochaerae*. Based on morphological and molecular data, this isolate is a new species of *Isospora*, which is named *Isospora manorinae* n. sp. after its host, the yellow-throated miner (*Manorina flavigula wayensis*).

Keywords: *Isospora*; yellow-throated miner; morphology; phylogeny; 18S rRNA; 28S rRNA; COI.
1. Introduction

The yellow-throated miner (Manorina flavigula) is a species of honeyeater, native to Australia in the family of Meliphagidae. It is also known as the yellow-throated minah, the white-rumped miner or the dusky miner (Fraser and Gray, 2013). The yellow-throated miner is a medium-sized bird, reaching a length of 26 to 28 centimetres. The dorsal surface is a dark grey and the ventral surface almost white. The beak, skin around the eye, side of the throat, legs, feet and parts of the wing and tail are yellow. The feathers surrounding the eye are black and the rump is white (Surhone et al., 2010). There are four subspecies of the yellow-throated miner; Manorina flavigula flavigula, Manorina flavigula lutea, Manorina flavigula obscura and Manorina flavigula wayensis. Manorina f. flavigula is the most eastern species, found in Queensland, New South Wales, north western Victoria and the eastern half of South Australia (http://en.inforapid.org/index.php?search=Noisy%20Miner). Manorina f. lutea is native to the Northern Territory, the western half of South Australia, and eastern Western Australia, while M. f. obscura is native to south-western Western Australia and M. f. wayensis is native to the rest of Western Australia. It has recently been reported that the presence of yellow-throated miners in an area is associated with a significant reduction in the number of different bird species and also results in fewer small birds being present in the area (O’Loughlin et al., 2015).

Isospora spp. from passerine birds have been reported worldwide (Duszynski et al., 1999), and in recent years especially, several species of Isospora have been characterised (Schrenzel et al., 2005; Berto, et al., 2011; Berto, et al., 2013; Schoener et al., 2013, Yang et al., 2014, Yang et al., 2015a). In Australia, two species of Isospora from honeyeaters (Meliphagidae) have been described; I. lesouefi from the endangered regent honeyeater (Xanthomyza phrygia), which is endemic to south-eastern Australia (Morin-Adeline et al., 2011) and I. anthochaerae from a red wattlebird (Anthochaera carunculata) in Western Australia (Yang et al., 2014). Another Isospora species, I. streperae has been described from a grey currawong (Strepera versicolour plumbea) in Western
australia (yang et al., 2015a). to date, no species of isospora has been characterized from the yellow-throated miner. in the present study, we investigated the coccidia of the yellow-throated miner, another species of the passerine birds.

2. materials and methods

2.1 sample collection

a juvenile yellow-throated miner was admitted to the kanyana wildlife rehabilitation centre (kwrc), perth from a mine-site located in the pilbara region (approximately 150km north of newman) in western australia. the bird was unable to fly as it was being weighed down by dried clay, which was coating its feathers. a faecal sample was collected on the day of admittance to kwrc. microscopy was performed and the sample was found to contain large numbers of unsporulated coccidian oocysts. treatment was implemented and the bird was released back to the location where it had been found 3 weeks later.

2.2 morphological analysis

the presence of oocysts was determined by direct microscopic examination of a faecal suspension in saline. unsporulated coccidian oocysts were allowed to sporulate by mixing a portion of faeces with a 2% (w/v) potassium dichromate solution (k₂cr₂o₇). this was then poured into a petri dish to a depth of less than 1cm and kept at room temperature (20-22°C) in the dark. 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 oocyst purification
The faecal sample was resuspended in cold PBS and mixed, then strained through pre-wetted surgical gauze. It was then made up to 50 ml with cold PBS and washed by centrifuging at 2000 x g for 8 mins at 4°C. The supernatant was removed and the resulting pellet was then washed with cold PBS until the supernatant became clear. The pellet was resuspended in 2-3 ml cold PBS, and carefully layered on the top of a cold Ficoll gradient, centrifuged for 20 min at 2000 x g at room temperature (Hijjawi et al., 2001). The 0.5%-1.0% phase was transferred to a 50 ml tube, made up to 45 ml with BPS and centrifuged at 2000 g for 5 mins at 4°C and washed twice with PBS, resuspended in 1.5 ml cold PBS and stored in 4°C.

2.4 DNA extraction

DNA was extracted from the purified oocysts using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Chadstone, VIC. Australia) according to the manufacturer's instructions. In order to fully lyse the oocysts, four freeze-thaw cycles were applied prior to the DNA extraction.

2.5 PCR amplification of 18S and 28S ribosomal sequences and the COI gene

A one step PCR using the primers EiF1 5’- GCT TGT CTC AAA GAT TAA GCC (Power et al., 2012) and EIR3 5’- ATG CAT ACT CAA AAG ATT ACC (Yang et al., 2012) were used for the amplification of the 18S rRNA gene. The expected PCR product was ~1,510 bp. The PCR reaction contained 2.5 µL of 10 × Kapa PCR buffer, 2 µl of 25 mM MgCl₂, 1.0 µL of 10nM dNTP’s, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 µL of DNA (~50 ng) and 16.4 µL of H₂O. PCR cycling conditions were 1 cycle of 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 2 min and a final extension of 72°C for 5 min.
The PCR for the 28S rRNA locus was carried out using a one-step PCR using the primers:

28SExF: 5’-TAC CCG CTG AAC TTA AGC and 28SExR: 5’- CMA CCA AGA TCT GCA CTA

as previously described (Schrenzel et al., 2005), which produced a PCR product size of ~1,420 bp.

The PCR amplification for the COI locus as described by Ogedengbe et al. (2011).

2.6 Sequence analysis

The amplicons from the second round PCRs were gel purified using an in house filter tip method as previously described (Yang et al., 2013). All the PCR products were sequenced using forward and reverse primers in duplicate using amplicons from different PCR runs. Any mismatches were confirmed by re-amplifying with pfu (Promega, Sydney, Australia) and repeating the sequencing. An ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) was used for Sanger sequencing according to the manufacturer’s instructions.

The results of the sequencing reactions were analysed and edited using Finch TV® v1.4.0. (http://www.geospiza.com/Products/finchtv.shtml). Sequences were compared to existing Isospora and other coccidian parasite sequences available on GenBank using BLAST searches and aligned with reference sequences with BioEditor (http://bioeditor.sdsc.edu/download.shtml).

2.7 Phylogenetic analysis

Phylogenetic trees were constructed for Isospora spp. at the 18S, 28S and COI loci with additional isolates from GenBank. Parsimony analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis software, version 6, Arizona State University, Tempe, Arizona, USA). Neighbor-joining (NJ) and maximum likelihood (ML) analyses were conducted Tamura-Nei
based on the most appropriate model selection using ModelTest in MEGA 6. Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies.

2.8 Line drawing

Oocyst line drawings were conducted using Inkscape (http://www.inkscape.org/en/).

3. Results

3.1 Description of I. manorinae n. sp.

Sporulated oocysts (n=32) of Isospora manorinae n. sp. are spherical to subspherical, 22.8 (20.3-23.8) × 18.3 (17.7-18.7) µm, with a shape index (length/width) of 1.25 (1.2 – 1.3); and a smooth and bilayered oocyst wall, 1.3 µm thick (outer layer 0.9 µm, inner 0.4 µm). A polar granule is present, but the micropyle and oocyst residuum are absent. The sporocysts are lemon-shaped, 15.5 (14.6-15.8) × 9.5 (9.5-10.2) µm, with a shape index of 1.6. Stieda and substieda bodies are present, the Stieda body being knob-like shaped and the substieda body being subspherical-shaped. A sporocyst residuum is present and composed of numerous granules of different size scattered among the sporozoites and a spheroid or subspheroid refractile body is present in the sporozoite (Fig. 1 and Table 1).

Type hosts: the yellow-throated miner (Manorina flavigula wayensis) (Gould, 1840)

Type locality: Cloudbreak mine (22.3240° S, 119.3969° E), Western Australia.

Prevalence: Unknown

Other hosts: Unknown.

Prepatent period: Unknown.


Site of infection: Unknown

Sporulation time: 48-72 hours.

Material deposited: DNA sequences have been deposited in GenBank under the accession numbers KT224379, KT224381 and KT224377 for the 18S, 28S and COI loci respectively.

Etymology: This species is named Isospora manorinae n. sp. after its host (Manorina flavigula wayensis) (yellow-throated miner).
3.2 Phylogenetic analysis of *I. manorinae* n. sp. at the 18S locus

A 1,338 bp sequence of 18S rRNA from *I. manorinae* n. sp was aligned with eight other *Isospora* spp. sequences from passerine birds; *I. gryphoni* (AF080613) (Olson et al., 1998), *I. robini* (AF080612) (Carreno and Barta, 1999), *I*. sp. MS-2003 (JX984668), *I*. sp. MS-2003 (JX984668) (AY331569), *I*. sp. MS-2003 (AY331571) (Schrenzel et al., 2005), *I. serinuse* (KR477877) (Yang et al., 2015c), *I*. sp. MAH-2013a (KF648870) and *I*. sp. MAH-2013b (KF648871) (unpublished), two *Isospora* spp. sequences from domestic pigeons (*Isospora* sp. Tokyo - AB757860 and AB757862), as well as 16 *Eimeria* 18S rRNA sequences from GenBank. *Toxoplasma gondii* was used as the outgroup.

Phylogenetic analysis using distance, parsimony and ML revealed that at the 18S locus, *I. manorinae* n. sp. exhibited 99.2% similarity with *I*. sp. Tokyo from a domestic pigeon (*Columba livia domestica*), *I. gryphoni* from an American goldfinch (*Carduelis tristis*), sp. MAH-2013a from a superb glossy starling (*Lamprotornis superbus*) and *I*. sp. MS-2003 (KF648871) from a Southern cape sparrow (*Passer melanurus melanurus*), respectively (Fig. 2a). *Isospora anthochaerae* was not included in the 18S tree because only a 300 bp sequence was available from this species. However, a separate phylogenetic analysis that included this species revealed that both species grouped together with a genetic similarity of 99.3% (Fig. 2b).

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

A 1,329 bp amplicon from *I. manorinae* n. sp. was obtained at the 28S rRNA locus. Phylogenetic analysis included thirty-one *Isospora* sequences from passerine birds from a single report by Schrenzel et al., (2005), *I. anthochaerae* (KF766053) from a red wattlebird (Yang et al., 2014) and *I. serinuse* (KR477878) from a domestic canary (*Serinus canaria forma domestica*)
(Yang et al., 2015c). Analysis revealed that I. manorinae n. sp. grouped with I. anthochaerae (Fig. 3) with a similarity of 99.1%.

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

A 770 bp amplicon at the COI locus from I. manorinae n. sp. was obtained. Phylogenetic analysis included 18 sequences from avian Isospora species available in GenBank and 16 Eimeria COI gene sequences. Toxoplasma gondii (HM771690) was used as the outgroup (Fig. 4a). Isospora manorinae n. sp. sat in a separate group with the highest similarity of 95.9% with two Isospora isolates (KP688316 and KP688318) from spectacled warblers (Sylvia conspicillata) (Illera et al., 2015). A subset of 215 bp long COI gene sequences including I. anthochaerae and another 5 isolates from Eurasian blackcaps (Sylvia atricapilla) in Germany, were used for further phylogenetic analysis. In this analysis, I. manorinae n. sp. grouped with I. anthochaerae (KF766054) with a similarity of 99.1% (Fig. 4b).

4. Discussion

Sporulated oocysts of I. manorinae n. sp. are morphologically distinct from other characterized Isospora species and did not match any other existing documented Isospora species from Passeriformes (http://biology.unm.edu/biology/coccidia/passr.html (Accessed on 18 June 2015) with the exception of I. anthochaerae. Both species have similar oocyst dimensions but are not morphologically identical. Oocysts of I. manorinae n. sp. measured 22.8 (20.3-23.8) × 18.3 (17.7-18.7) µm in size with a L/W ratio = 1.25 and oocysts of I. anthochaerae measured 23.4 x 20.7 (20.0-26.0 x 19.0-22.0) µm, with L/W ratio = 1.1. The polar granule was present in I. manorinae n. sp. whilst it was absent in I. anthochaerae (Table 1).
Molecular characterization of the oocysts of *I. manorinae* n. sp. also showed that this *Isospora* was closest to *I. anthochaerae*. Phylogenetic analysis of 18S rRNA, 28S rRNA and COI loci revealed that *I. manorinae* n. sp. exhibited 99.3%, 99.1% and 99.1% similarity respectively with *I. anthochaerae*. While *I. manorinae* n. sp is genetically very closely related to *I. anthochaerae* (> 99% at all three gene loci), the morphological / morphometric differences observed are sufficient to consider *I. manorinae* n. sp as a new species. The avian hosts of both these species are also closely related. A recent study of the phylogeny and evolution of the Australo-Papuan honeyeaters (Passeriformes, Meliphagidae) at four loci from 75 species in the family Meliphagidae reported that the red wattlebird and the yellow-throated miner are genetically similar and were in parallel sister clades (Driskell and Christidis, 2014).

18S rRNA sequences are widely used for coccidian phylogenetic analysis (Carreno et al., 1998; Morrison et al., 2004; Castellanos-Martínez et al., 2013). In the present study, phylogenetic analysis at the 18S locus showed that *Isospora* is polyphyletic, as the avian isosporans were interwoven with various *Eimeria* spp., This has also been reported by Morrison et al. (2004) and clearly a major re-assessment of *Eimeria* taxonomy is necessary for future studies.

In the present study, morphological and molecular data were used to describe *I. manorinae* n. sp. found in the faeces of a yellow-throated miner in Western Australia. Future studies are needed to examine if this species has any pathogenic effects and more isolates need to be genetically characterized at multiple loci to better understand the epidemiology of *Isospora* sp. infecting the yellow-throated miner.
Acknowledgments

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References


of Cryptosporidium hominis and C. parvum in clinical and environmental samples. Exp. Parasitol. 135, 142-147.


Fig. 1. Nomarski interference-contrast photomicrographs of *I. manorinae* n. sp. (1-3) to show the oocysts at different development stages (1-2) and a crushed oocyst to show a free sporocyst (3) and composite line drawing of *I. manorinae* n. sp. sporulated oocyst (4). Scale bar = 20 µm.

Fig. 2.a. Evolutionary relationships of *I. manorinae* n. sp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (‘_’ = Not available). b. Phylogenetic relationships of *I. manorinae* n. sp., and 12 other *Isospora* sequences including *I. anthochaerae* from a honeyeater in Western Australia (based on 300 bp 18S rRNA sequence only).

Fig. 3. Evolutionary relationships of *I. manorinae* n. sp. inferred by distance analysis of 28S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (‘_’ = Not available).

Fig. 4.a. Evolutionary relationships of *I. manorinae* n. sp. inferred by distance analysis of COI sequences. Percentage support (>50%) from 1000 pseudoreplicates from distance, ML and parsimony analysis, respectively, is indicated at the left of the support node (‘_’ = Not available). b. Phylogenetic relationships of *I. manorinae* n. sp., and 11 other *Isospora* sequences including *I. anthochaerae* from a honeyeater in Western Australia (based on 215 bp COI sequence only).
Table 1. Morphological comparison of *I. manorinae* n. sp. with other *Isospora* species.

<table>
<thead>
<tr>
<th>Coccidia</th>
<th>Hosts</th>
<th>References</th>
<th>Oocysts</th>
<th>Sporocysts</th>
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<td>Oocysts</td>
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<td>oocyst residuum</td>
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<td>Residuum</td>
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<td><em>I. anthochaerae</em></td>
<td>red wattlebird (<em>Anthochaera carunculata</em>) canary (<em>Serinus canaria Linnaeus</em>) American goldfinch (<em>Carduelis tristis L.</em>) yellow-throated miner (<em>Manorina flavigula obscura</em>)</td>
<td>Yang et al., 2014  Box 1975; Berto et al., 2013 Olson et al., 1998  This study</td>
<td>subspherical 23.4 x 20.7 (20.0-26.0 x 19.0-22.0) 1.12 bi-layered c. 0.8 absent absent ovoid 14.5 x 10.1 (11.0-17.0 x 9.0-11.0) hemi-dome rectangular-shaped compact</td>
<td>subspherical to ellipsoidal 24.6 x 21.8 (17-30 x 17-30) 1.13 tri-layered c. 1.2 present absent lemon 18.1 x 11.5 (17.0-22.0 x 1.00-13.0) nipple-like 2.0 x3.0 compact</td>
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<td><em>I. canaria</em></td>
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<td>spherical 29.2 x 30.7 (25.0-33.0 x 28.0-34.0) 1.13 bi-layered c. 0.8 present absent ovoid 22.2 x 13.4 (15-25.0 x 12.0-14.5) small indistinct prominent</td>
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<td><em>I. gryphoni</em></td>
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<td>spherical to subspherical 22.8x18.3 (20.3-23.8x17.7-18.7) 1.25 bi-layered c. 1.3 present absent lemon 15.5x9.7 (14.6-15.7x9.5-9.7) hemi-dome rectangular-shaped compact</td>
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<td><em>I. manorinae</em> n. sp.</td>
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<td>spherical to subspherical 20.1 x 19.2 (13.0-23.0 x13.0-23.0) 2.5 x 23.5 (24.4-27.0 x 22.0-24.8) 1.05 tri-layered c. 1.2 bi-layered c. 1.2 present absent ellipsoidal 15.2 x 9.4 (13.0-16.0 x 8.0-11.0) 18.9 x 11.8 (17.8-20.2 x 10.6-13.0) 2.0x0.6 5.0 x3.0 scattered granules indistinct compact</td>
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<td><em>I. serini</em></td>
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<td>spherical to subspherical 23.8 x 22.5 (22.45 x 21.8 x 24.5) 1.06 bi-layered c. 1.0 absent present ovoid 14.4 x 11.2 (11.5-15.8 x 10.4-12.5) hemi-dome rectangular-shaped compact</td>
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Phylogenetic analysis of *Isospora manorinae*. n. sp. at 18S locus

a.

b.
Highlights

• A new *Isospora* species (*I. manorinae*. n. sp.) in a yellow-throated miner
• Morphology study distinct to other validated *Isospora* species.
• Genetic study: Most close to *I. anthochaerae* n. sp. at 18S, 28S and COI loci.