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Morphological and molecular characterization of a new species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) from the western wattlebird *Anthochaera lunulata* Gould in Western Australia

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ABSTRACT

A new coccidian species, *Isospora lunulatae* n. sp., from the western wattlebird *Anthochaera lunulata* Gould in Western Australia is described and characterised molecularly. Microscopic analysis of a faecal sample identified subspheroidal oöcysts measuring 27–34 × 26–31 (30.6 × 29.4) μm ($n = 20$), with a length/width (L/W) ratio of 1.0–1.1 (1.0). Oöcysts have a bi-layered wall, 0.9–1.2 (1.0) μm thick; the outer layer is smooth, representing *c.*2/3 of total thickness. Micropyle and oöcyst residuum are both absent, but a polar granule is present. Sporocysts are ovoidal, 17–19 × 10–12 (18.3 × 10.7) μm, with a L/W ratio of 1.6–1.8 (1.7) and occupying about 21% of the area (each one) within the oöcyst. Stieda body is flattened to rounded, measuring on average 0.9 × 1.8 μm; sub-Stieda body is rounded to rectangular, measuring on average 1.5 × 2.6 μm; para-Stieda body is absent. Sporocyst residuum has an irregular shape consisting of numerous granules and appears membrane-bound. Sporozoites are vermiform 12.8 × 3.0 μm on average, with prominent striations at the more pointed end and two refractile bodies below striations. Segments of three gene loci (18S rRNA, 28S rRNA and *cox*1) were sequenced and *I. lunulatae* n. sp. exhibited 99.6% genetic similarity to *Isospora phylidonyrisae* Yang, Brice, Berto & Ryan, 2021 at the 18S rRNA gene locus, 99.8% genetic similarity to *Isospora anthochaerae* Yang, Brice & Ryan, 2014 and shared a 98.1% genetic similarity with *Isospora manorinae* Yang, Brice, Jian & Ryan, 2016 at the *cox*1 gene locus. Morphological and molecular data support the distinct species status of the new species.

1. Introduction

The western wattlebird *Anthochaera lunulata* Gould, also known as the brush wattlebird, is a passerine bird endemic to Australia. It is a member of the honeyeater family (Meliphagidae) and is most frequently found along coastal and subcoastal south-western Australia, roughly south of a line from the north Gairdner Range to Hopetoun and east to the Cape Arid National Park (Higgins et al., 2020). These honeyeaters inhabit forests, woodlands, heath, urban gardens and Mallee (Pizzey and Knight, 2007).

Coccidia of the genus *Isospora* Schneider, 1881 are the most common in passerine birds (Duszynski et al., 1999). Many species of *Isospora* have been described from passerine birds worldwide (Schrenzel et al., 2005; Berto et al., 2011; Yang et al., 2014, 2015a, b, 2016a, b, 2018; Liu et al., 2020; Yang et al., 2021), including three species from birds in the honeyeater family: *Isospora lesouefi* Morin-Adeline, Vogelnest, Dhand, Shiels, Angus & Šlapeta, 2011 from the endangered regent honeyeater *Anthochaera phrygia* Shaw, which is endemic to south-eastern Australia (Morin-Adeline et al., 2011), *Isospora anthochaerae* Yang, Brice & Ryan, 2014 from the red wattlebird *Anthochaera carunculata* Shaw (see Yang et al., 2014) and recently, *Isospora phylidonyrisae* Yang, Brice, Berto & Ryan, 2021 from the New Holland honeyeater *Phylidonyris novaehollandiae* Latham in Australia (see Yang et al., 2021). In the present study, we describe morphological and molecular characteristics of a new species of *Isospora* from the western wattlebird in Western Australia.

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2. Materials and methods

2.1. Sample collection and storage

A wild, western wattlebird juvenile was admitted to the Kanyana Wildlife Rehabilitation Centre (KWRC), Perth, Australia, in October 2014 after it had been attacked by a domestic cat. A faecal sample was collected from the bird on admission and screened by microscopy (wet mounts) for parasites. Numerous unsporulated coccidian oöcysts were observed. Faecal flotation was performed using a saturated sodium chloride and 50% sucrose (w/v) solution. A portion of faeces was also placed in 2% (w/v) potassium dichromate solution ($K_2Cr_2O_7$). The resulting dichromate/oöcyst suspension was poured into a thin layer at the bottom of a Petri dish. Unsporulated oöcysts were kept in the Petri dish in dark conditions at room temperature (20–22 °C) to sporulate. Samples were regularly checked for oöcyst sporulation under the light microscope. Sporulated oöcysts were collected within 48 h and shipped to Murdoch University, Australia, for oöcyst measurement, imaging and molecular analysis.

2.2. Morphological analysis

Sporulated coccidian oöcysts were observed using an Olympus BX50 microscope. Images were taken using a Nomarski contrast imaging system with a 100× oil immersion objective in combination with an ocular micrometer. All measurements are presented in micrometres with the means in parentheses following the ranges.

Line drawings were edited using two software applications from CorelDRAW® (Corel Draw Graphics Suite, Version 2020, Corel Corporation, Canada), i.e. Corel DRAW and Corel PHOTO-PAINT (Yang et al., 2021).

2.3. DNA extraction from faeces, PCR, sequencing and phylogenetic analysis

Total DNA from a 250 mg of faecal sample was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) as described by Yang et al. (2014).

Partial fragments of 18S rRNA, 28S rRNA and *cox*1 genes were amplified by performing nested PCRs as previously described (see Yang et al., 2016a). PCR products at all three loci were purified and sequenced in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions (Yang et al., 2013).

Phylogenetic trees were constructed for *Isospora* spp. using partial 18S rDNA, 28S rDNA sequences and partial *cox*1 sequences aligned with additional isolates from GenBank. Distance analyses and phylogenies were conducted using MEGA X (Kumar et al., 2018). Briefly, Sanger sequencing chromatogram files were imported into MEGA X and the nucleotide sequences of each gene was curated, analysed, and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Maximum likelihood (ML) trees were constructed, after first identification of the most appropriate nucleotide substitution model (TN93+G+I for 18S and 28S rRNA genes, and GTR+G+I for the *cox*1 gene). Bootstrap support was estimated from 1000 replicates. Genetic similarities were calculated with MEGA X.

3. Results

3.1. Isospora lunulatae n. sp.

3.1.1. Taxonomic summary

Type-host: *Anthochaera lunulata* Gould (Passeriformes: Meliphagidae), the western wattlebird.

Type-locality: 31.953512S, 115.857048E, Perth, Western Australia, Australia.

Type-material: Oöcysts fixed in 10% formalin and oöcyst photosyntypes were deposited in the Western Australian Museum under the reference number WAM Z100500. Photovouchers of the host specimens are deposited in the same collection.

Prevalence: 100% (1/1).

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:BB3BAA55-54AE-47BE-B942-5809858381A4. The Life Science Identifier (LSID) of the new name *I. lunulatae* is urn:lsid:zoobank.org:act:DA53DDC0-2673-4770-AF3D-4A5DA6209736.

Representative DNA sequences: DNA sequences have been deposited in the GenBank database under the accession numbers MW771609 (18S rRNA gene), MW776413 (28S rRNA gene) and MW720599 (*cox*1 gene).

Etymology: The species name of the parasite is derived from the host species name.

3.1.2. Description

[Based on 20 oöcysts and sporocysts; Figs. 1 and 2.] Oöcysts subspheroidal, measuring 27–34 × 26–31 (30.6 × 29.4); length/width (L/W) ratio 1.0–1.1 (1.0). Oöcyst wall bi-layered, 0.9–1.2 (1.0) thick; outer layer smooth; *c.*2/3 of total thickness. Micropyle and oöcyst residuum absent, but one polar granule present. Sporocysts ovoidal, measuring 17–19 × 10–12 (18.3 × 10.7); L/W ratio 1.6–1.8 (1.7), occupying about 21% of the area (each one) within the oöcyst. Stieda body present, flattened to rounded, 0.7–1.1 × 1.7–1.9 (0.9 × 1.8); sub-Stieda body present, rounded to rectangular, 1.2–1.8 × 1.9–3.1 (1.5 × 2.6); para-Stieda body absent. Sporocyst residuum present, with irregular shape, consisting of numerous small granules that appear to be membrane-bound. Sporozoites 4, vermiform, 12.1–13.3 × 2.8–3.2 (12.8 × 3.0), with prominent striations at the more pointed end and two refractile bodies below striations (Figs. 1 and 2).

3.1.3. Differential diagnosis

Following the host family specificity criterion, which is widely accepted for passerine coccidia and compiled in the papers by Duszynski and Wilber (1997) and Berto et al. (2011), the oöcysts recovered from *A. lunulata* in this study were compared with the coccidian species recorded in birds of the family Meliphagidae, and from other close families in the order Passeriformes (Table 1). As shown in Table 1, *I. lunulatae* n. sp. has larger oöcysts than all previously described coccidians from passerine birds, except for *Isospora samoaensis* Adamczyk,

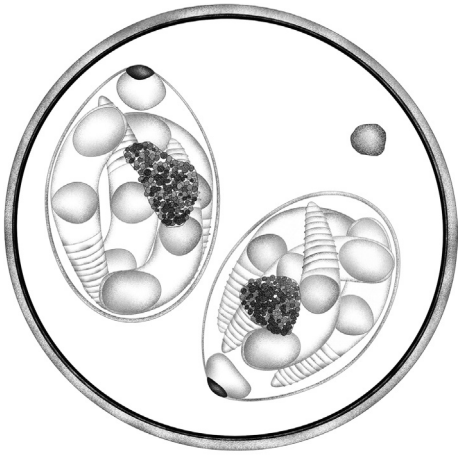

Fig. 1. Line drawing of the sporulated oöcyst of *I. lunulatae* n. sp. *Scale-bar*: 10 μm.

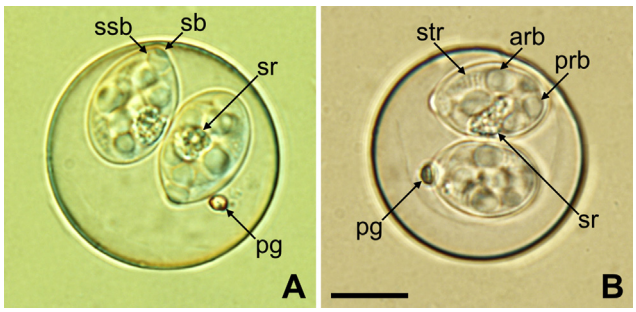

Fig. 2. Nomarski interference-contrast photomicrographs of sporulated oöcysts of *I. lunulatae* n. sp. Note the polar granule (pg); Stieda (sb) and sub-Stieda bodies (ssb); sporocyst residuum (sr); anterior (arb) and posterior (prb) refractile bodies; and striations (str). *Scale-bar*: 10 μm.

McQuistion & LaPointe, 2004, *Isospora gryphoni* Olson, Gissing, Barta & Middleton, 1998 and *I. phylidonyrisae*, which have morphometric ranges close to *I. lunulatae* n. sp. *Isospora samoaensis* was described from endemic birds in the American Samoa, which are unlikely to share the same coccidian species with *A. lunulata*, as they inhabit distinct and distant island environments. Further, *I. lunulatae* differs from *I. samoaensis* in having oöcysts with a single polar granule (*vs* 1–2 polar granules) and sporozoites with 2 refractile bodies (*vs* a single posterior refractile body). Although the oöcyst dimensions of *I. lunulatae* n. sp. were similar to those of *I. gryphoni* (30.6 × 29.4 *vs* 30.7 × 29.2 μm) (Table 1), the sporocysts of *I. lunulatae* n. sp. were on average smaller than those of *I. gryphoni* (18.3 × 10.7 *vs* 22.2 × 13.4 μm) (Table 2). *Isospora phylidonyrisae* was described from New Holland honeyeaters which are sympatric with western wattlebirds in south-western Australia; therefore, it becomes possible for these honeyeaters to share their coccidian parasites.

Table 1
Comparative morphological data for oöcysts of *Isospora lunulatae* n. sp. and *Isospora* spp. recorded from birds in the order Passeriformes.

Species	Host	Distribution	Shape	Measurements (μm)	Shape index	Wall (μm)	Polar granule	Oöcyst residuum	Reference
Isospora lunulatae n. sp.	Western wattlebird (*Anthochaera lunulata* (Gould)) (Meliphagidae)	Australia	Subspheroidal	27–34 × 26–31 (30.6 × 29.4)	1.04	Bi-layered (*c.*1.0)	Present	Absent	This study
Isospora anthochaerae Yang, Brice & Ryan, 2014	Red wattlebird (*Anthochaera carunculata* (Shaw)) (Meliphagidae)	Australia	Subspheroidal	20–26 × 19–22 (23.4 × 20.7)	1.12	Bi-layered (*c.*0.8)	Absent	Absent	Yang et al. (2014)
Isospora butcherae Yang, Brice, Jian & Ryan, 2018	Silvereye (*Zosterops lateralis* (Latham)) (Zosteropidae)	Australia; Fiji; New Caledonia; New Zealand; Vanuatu	Spheroidal to subspheroidal	23–25 × 23–24 (24.2 × 23.3)	1.02	Bi-layered (*c.*1.2)	Present	Absent	Yang et al. (2018)
Isospora gryphoni Olson, Gissing, Barta & Middleton, 1998	American goldfinch (*Spinus tristis* (L.)) (Fringillidae)	Bahamas; Canada; Mexico; Saint Pierre and Miquelon; Turks and Caicos Islands; USA	Spheroidal	25–33 × 28–34 (29.2 × 30.7)	1.05	Bi-layered (*c.*0.8)	Present	Absent	Olson et al. (1998)
Isospora coronoideae Liu, Brice, Elliot, Ryan & Yang, 2019	Australian raven (*Corvus coronoides* (Vigors & Horsfield)) (Corvidae)	Australia	Subspheroidal	18–24 × 17–21 (21.2 × 18.8)	1.13	Bi-layered (*c.*1.2)	Present	Present	Liu et al. (2020)
Isospora lesouefi Morin-Adeline, Vogelnest, Dhand, Shiels, Angus & Šlapeta, 2011	Regent honeyeater (*Anthochaera phrygia* (Shaw)) (Meliphagidae)	Australia	Spheroidal	23–29 × 20–26 (25.8 × 23.8)	1.08	Bi-layered (*c.*1.0)	Present	Absent	Morin-Adeline et al. (2011)
Isospora manorinae Yang, Brice, Jian & Ryan, 2016	Yellow-throated miner (*Manorina flavigula obscura* (Gould)) (Meliphagidae)	Australia	Spheroidal to subspheroidal	20–24 × 18–19 (22.8 × 18.3)	1.25	Bi-layered (*c.*1.3)	Present	Absent	Yang et al. (2016a)
Isospora neochmiae Yang, Brice & Ryan, 2016	Red browed finch (*Neochmia temporalis* (Latham)) (Estrildidae)	Australia	Spheroidal	18–19 × 18–19 (18.3 × 18.2)	1.01	Bi-layered (*c.*1.2)	Present	Absent	Yang et al. (2016b)
Isospora phylidonyrisae Yang, Brice, Berto & Ryan, 2021	New Holland honeyeater (*Phylidonyris novaehollandiae* (Latham)) (Meliphagidae)	Australia	Subspheroidal	29–32 × 28–31 (29.8 × 29.4)	1.01	Bi-layered (*c.*1.5)	Present	Absent	Yang et al. (2021)
Isospora samoaensis Adamczyk, McQuistion & LaPointe, 2004	Polynesian wattled honeyeater (*Foulehaio carunculatus* (Gmelin)) (Meliphagidae)	American Samoa; Fiji; Samoa; Tonga; Wallis and Futuna	Ovoidal	25–32 × 23–30 (28.9 × 26.1)	1.10	Bi-layered	Present	Absent	Adamczyk et al. (2004)
Isospora serinuse Yang, Brice, Elliot & Ryan, 2015	Canary (*Serinus canaria* (L.)) (Fringillidae)	Australia (type-locality)	Spheroidal to subspheroidal	24–27 × 22–25 (25.5 × 23.5)	1.09	Bi-layered (*c.*1.2)	Present	Absent	Yang et al. (2015b)
Isospora streperae Yang, Brice, Al Habsi, Elliot & Ryan, 2015	Grey currawong (*Strepera versicolor* (Latham)) (Artamidae)	Australia	Spheroidal	22–25 × 22–25 (23.8 × 22.5)	1.06	Bi-layered (*c.*1.0)	Absent	Present	Yang et al. (2015a)

Table 2
Comparative morphological data for sporocysts of *Isospora lunulatae* n. sp. and *Isospora* spp. recorded from birds in the order Passeriformes.

Species	Measurements (μm)	Stieda body	Sub-Stieda body	Residuum	Reference
Isospora lunulatae n. sp.	17–19 × 10–12 (18.3 × 10.7)	Flattened to rounded	Rounded to rectangular	Scattered granules	This study
Isospora anthochaerae Yang, Brice & Ryan, 2014	11–17 × 9–11 (14.5 × 10.1)	Hemi-dome-shaped	Rectangular	Compact	Yang et al. (2014)
Isospora butcherae Yang, Brice, Jian & Ryan, 2018	16–17 × 10–12 (16.1 × 10.5)	Hemi-dome-shaped	Rectangular	Scattered granules	Yang et al. (2018)
Isospora gryphoni Olson, Gissing, Barta & Middleton, 1998	15–25 × 12–15 (22.2 × 13.4)	Small	Indistinct	Prominent	Olson et al. (1998)
Isospora coronoideae Liu, Brice, Elliot, Ryan & Yang, 2019	14–19 × 8–13 (16.3 × 10.7)	Hemi-dome-shaped	Indistinct	Scattered granules	Liu et al. (2020)
Isospora lesouefi Morin-Adeline, Vogelnest, Dhand, Shiels, Angus & Šlapeta, 2011	17–19 × 9–10 (18·7 × 9.5)	Flattened	Spheroidal	Scattered granules	Morin-Adeline et al. (2011)
Isospora manorinae Yang, Brice, Jian & Ryan, 2016	15–16 × 9–10 (15.5 × 9.5)	Knob-like	Subspherical	Scattered granules	Yang et al. (2016a)
Isospora neochmiae Yang, Brice & Ryan, 2016	10–16 × 7–10 (13.3 × 8.6)	Indistinct	Absent	Compact	Yang et al. (2016b)
Isospora phylidonyrisae Yang, Brice, Berto & Ryan, 2021	18–19 × 12–14 (18.4 × 12.3)	Flattened	Rounded	Compact	Yang et al. (2021)
Isospora samoaensis Adamczyk, McQuistion & LaPointe, 2004	16–18 × 10–11 (17.1 × 10.9)	Broad, dome-like	Rectangular	Compact	Adamczyk et al. (2004)
Isospora serinuse Yang, Brice, Elliot & Ryan, 2015	18–20 × 11–13 (18.9 × 11.8)	Small	Indistinct	Compact	Yang et al. (2015b)
Isospora streperae Yang, Brice, Al Habsi, Elliot & Ryan, 2015	12–16 × 10–13 (14.4 × 11.2)	Hemi-dome-shaped	Rectangular	Compact	Yang et al. (2015a)

Furthermore, the 18S rDNA sequence for the new species exhibited the greatest similarity (99.6%; Table 3) to a sequence for *I. phylidonyrisae*. However, *I. phylidonyrisae* differs from *I. lunulatae* n. sp. in having oöcysts with two polar granules (*vs* one) and wider sporocysts (12–14 *vs* 10–12 μm) with flattened Stieda body (*vs* flattened to rounded), uniformly rounded sub-Stieda body (*vs* rounded to rectangular), and barely discernible striations in sporozoites (*vs* prominent). In addition, it is worth noting that sporocysts of *I. lunulatae* n. sp. are smaller in relation to oöcyst size, occupying about 21% (*vs* 27%) of the area within the oöcyst (Supplementary Fig. S1).

Comparative sequence analysis also revealed that *I. lunulatae* n. sp. shared the highest sequence similarities with *I. anthochaerae* (99.8%; 28S rRNA gene) and *Isospora manorinae* (98.1%; *cox*1 gene) (Table 3). These two species differ from *I. lunulatae* n. sp. in possessing smaller oöcysts. Additionally, *I. anthochaerae* lacks polar granules (*vs* one in the new species) and *I. manorinae* possesses a scattered sporocyst residuum (*vs* scattered granules in the new species).

3.2. Phylogenetic analyses

3.2.1. 18S rRNA gene

Three identical 1214 bp 18S rDNA sequences were obtained from three individual oöcysts from the faecal sample of *A. lunulata*; these were aligned with 11 other *Isospora* spp. sequences from birds, 17 *Eimeria* spp., two *Caryospora* spp. and one *Lankesterella* spp. The justification for the selection of the reference sequences was based on the NCBI BLAST similarities (one sequence per species) and covered all sequences for *Isospora* spp. A sequence of *Toxoplasma gondii* (Nicolle & Manceaux, 1908) (L24381) was used as the outgroup. *Isospora lunulatae* n. sp. shared 99.6% and 99.1% homology with *I. phylidonyrisae* (GenBank: MW422271) and *Isospora coronoideae* Liu, Brice, Elliot, Ryan & Yang, 2019 (GenBank: MK530653), respectively. As shown in Fig. 3, *Isospora* spp. were grouped in a separate clade albeit with no support, except for *Isospora wiegmanniana* Megía-Palma, Martínez, Nasri, Cuervo, Martín, Acevedo, Belliure, Ortega, García-Roa, Selmi & Merino, 2016 (GenBank: KU180242) which was recovered in the *Caryospora* clade and *Isospora lugensae* Yang, Brice, Liu, Berto, Austen & Ryan, 2021 (GenBank: MW287753) which grouped in the seabird *Eimeria* clade. *Isospora lunulatae* n. sp. grouped in a strongly supported clade with *I. phylidonyrisae* (MW422271; genetic similarity of 99.6%), isolated from the New Holland honeyeater *P. novaehollandiae* and *I. coronoideae* (GenBank: MK530653; genetic similarity of 99.1%) isolated from the Australian raven *Corvus coronoides* Vigors & Horsfield along with other two species identified from Western Australian passerine birds (*Isospora serinuse* Yang, Brice, Elliot & Ryan, 2015 from *Serinus canaria* (L.) and *I. manorinae* from *Manorina flavigula obscura* (Gould)) plus an isolate (*Isospora* sp. Tokyo 1) from a domestic pigeon in Japan. The second clade of *Isospora* spp. included three species identified from North American

Table 3
Genetic similarity (in %) between *I. lunulatae* n. sp. and related *Isospora* spp. sequences at the 18S and 28S ribosomal RNA and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) loci.

Species	Host	18S rRNA gene	28S rRNA gene	*cox*1 gene	Reference
I. gryphoni	*Spinus tristis* (Fringillidae)	99.0 (1213 bp)	na	97.6 (399 bp)	Olson et al. (1998)
I. lesouefi	*Anthochaera phrygia* (Meliphagidae)	na	na	95.2; 96.1; 95.7; 96.1; 97.8 (230 bp)[a]	Morin-Adeline et al. (2011)
I. anthochaerae	*Anthochaera carunculata* (Meliphagidae)	100 (300 bp)	99.8 (1339 bp)	99.0 (206 bp)	Yang et al. (2014)
I. streperae	*Strepera versicolor* (Artamidae)	99.3 (739 bp)	94.1 (923 bp)	na	Yang et al. (2015a)
I. serinuse	*Serinus canaria f. domestica* (Fringillidae)	97.0 (1214 bp)	94.9 (1339 bp)	94.8 (633 bp)	Yang et al. (2015b)
I. manorinae	*Manorina flavigula obscura* (Meliphagidae)	99.0 (1214 bp)	98.9 (1327 bp)	98.1 (633 bp)	Yang et al. (2016a)
I. neochmiae	*Neochmia temporalis* (Estrildidae)	98.9 (1214 bp)	93.0 (1338 bp)	95.7 (633 bp)	Yang et al. (2016b)
I. butcherae	*Zosterops lateralis* (Zosteropidae)	98.1 (1214 bp)	92.9 (1327 bp)	95.9 (633 bp)	Yang et al. (2018)
I. coronoideae	*Corvus coronoideae* (Corvidae)	99.1 (1214 bp)	95.0 (1339 bp)	95.7 (633 bp)	Liu et al. (2020)
I. phylidonyrisae	*Phylidonyris novaehollandiae* (Meliphagidae)	99.6 (1214 bp)	98.3 (1327 bp)	96.4 (633 bp)	Yang et al. (2021)

Abbreviation: na, not available.
[a] Five isolates.

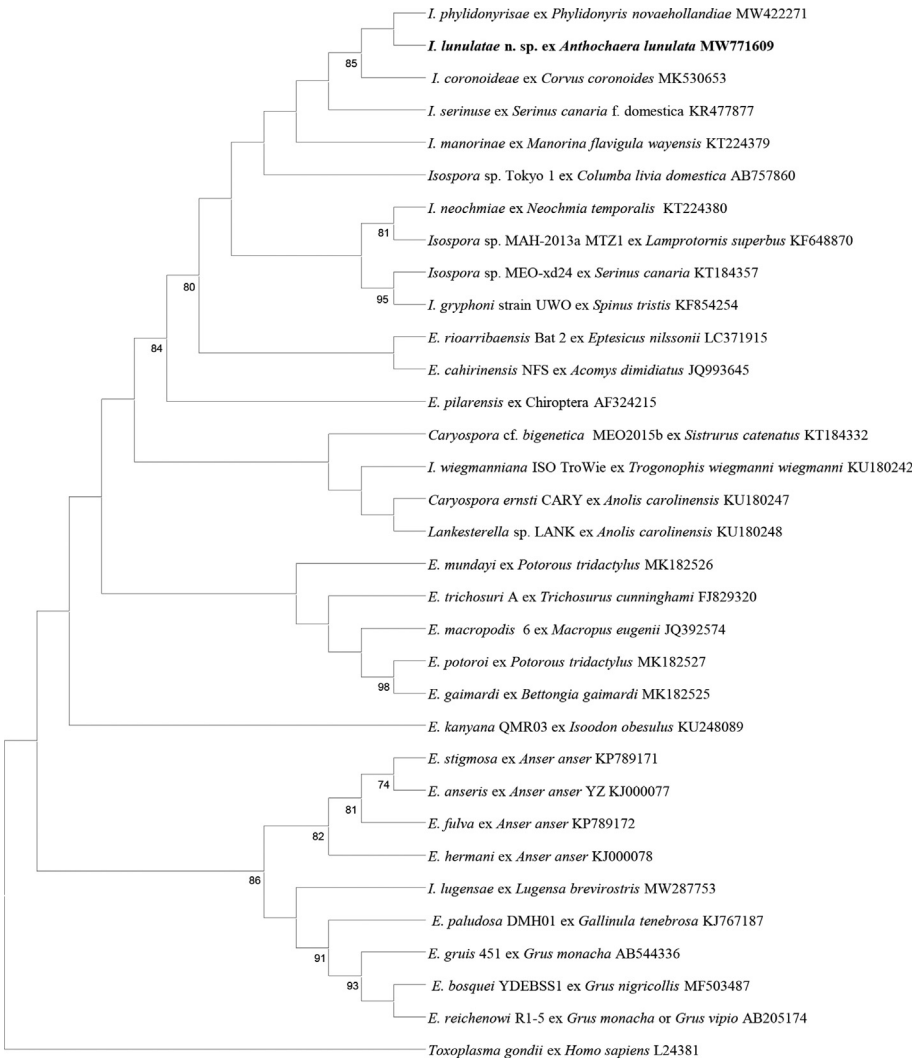

Fig. 3. Evolutionary relationships of *I. lunulatae* n. sp. inferred by maximum likelihood analysis (ML) of 18S rDNA sequences (1214 bp). Percentage support (> 70%) from 1000 pseudoreplicates from the ML analysis is indicated at the nodes.

passerine birds and one (*Isospora neochmiae* Yang, Brice & Ryan, 2016) from a Western Australian passerine bird (the red-browed finch *Neochmia temporalis* (Latham)) (Fig. 3).

3.2.2. 28S rRNA gene

Three identical 28S rDNA sequences (1218 bp) from three individual oöcysts were aligned with 28 sequences for *Isospora* spp. (some of the *Isospora* spp. 28S rRNA sequences deposited in the GenBank database were named as *Atoxoplasma* Garnham, 1950 in the early days) from birds and one sequence for *Eimeria* spp. Similar to the 18S rRNA gene analysis, the selection of the 28S rDNA reference sequences were based on the NCBI BLAST similarities (one sequence per species) and covered all of the *Isospora* spp. sequences. *Toxoplasma gondii* was used as the outgroup. Phylogenetic analysis showed that *I. lunulatae* n. sp. grouped together with *I. anthochaerae* (GenBank: KF766053; genetic similarity of 99.8%) from *A. carunculata* in a separate clade, which was a sister clade to the clade containing *I. phylidonyrisae* (GenBank: MW422270) and *I. manorinae* (GenBank: KT224381) isolated from the yellow-throated miner *M. flavigula obscura*. As shown in Fig. 4, *I. coronoideae* (GenBank: MK530654), *I. serinuse* (GenBank: KR477878), as well as the four *Isospora* spp. mentioned above (including the new species of *Isospora*) formed a strongly supported clade in the phylogenetic tree. All six *Isospora* spp. were identified from passerine birds in Western Australia (see Fig. 4).

3.2.3. cox1 gene

One partial *cox*1 sequence (633 bp) was obtained from *I. lunulatae* n. sp. and aligned with another 9 sequences for *Isospora* spp. from birds, 19 for *Eimeria* spp., 2 for *Cyclospora* spp. and one for *Choleoeimeria* spp. All *cox*1 reference sequences were selected based on the NCBI BLAST similarities and covered all *Isospora* spp. in the database. A sequence for *Lankesterella* sp. (GenBank: KT369006) was used as the outgroup. *Isospora lunulatae* n. sp. exhibited the highest similarity (98.1%) with *I. manorinae* (GenBank: KT224377) isolated from the yellow-throated miner *M. flavigula obscura*. In the phylogenetic tree, *I. lunulatae* n. sp. was most close to *I. phylidonyrisae* (Fig. 5). Only a 206 bp *cox*1 sequence was available for *I. anthochaerae* and *I. lesouefi* (five isolates), therefore they were not included in this phylogenetic analysis. *Isospora gryphoni* Olson, Gissing, Barta & Middleton, 1998, identified from the American goldfinch *Spinus tristis* (L.) in Canada, exhibited similar oöcyst morphological features. The 399 bp of the overlapping *cox*1 sequence (GenBank: KC346355) of *I. gryphoni* and *I. lunulatae* n. sp. (GenBank: MW720599) showed a genetic similarity of 97.6%.

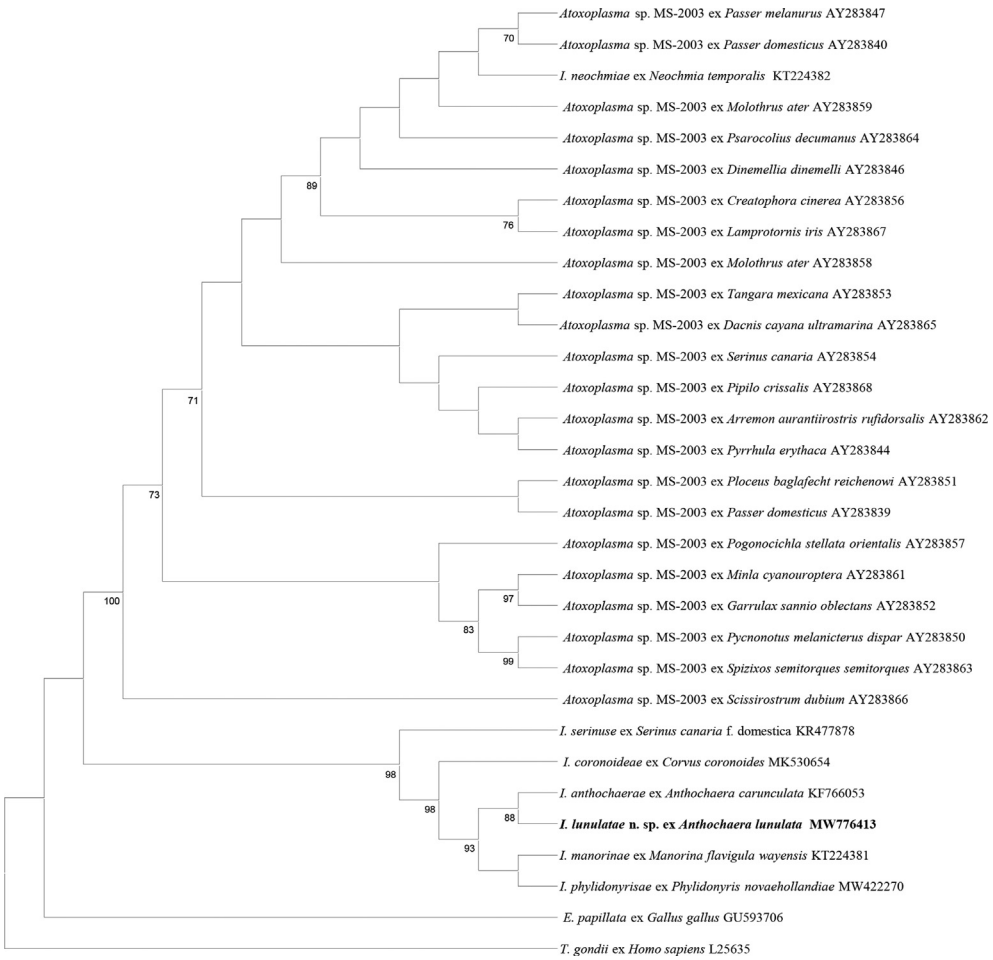

Fig. 4. Evolutionary relationships of *I. lunulatae* n. sp. inferred by maximum likelihood analysis (ML) of 28S rDNA sequences (1218 bp). Percentage support (> 70%) from 1000 pseudoreplicates from the ML analysis is indicated at the nodes.

4. Discussion

O'Donoghue and Adlard (2000) catalogued protozoan parasites that had been recorded in wattlebirds in Australia. They listed four species, namely *Haemoproteus danilewskyi* Kruse, 1890, *Leucocytozoon anellobiae* Cleland & Johnston, 1911, *Trypanosoma anellobiae* Cleland & Johnston, 1910 and *Trypanosoma* sp., that had been detected in the blood of the little wattlebird *Anthochaera chrysoptera* (Latham). To date, no coccidian species have been characterized from *A. lunulata* in Australia. Species of *Isospora* discovered in honeyeaters so far include *I. lesouefi* from the endangered regent honeyeater *A. phrygia* (see Morin-Adeline et al., 2011), *I. anthochaerae* from the red wattlebird *A. carunculata* (see Yang et al., 2014) and *I. samoaensis* from the Polynesian wattled honeyeater *Foulehaio carunculatus* (Gmelin) in America (see Adamczyk et al., 2004). Recently, *I. phylidonyrisae* was characterized from the New Holland honeyeater *P. novaehollandiae* in Western Australia (see Yang et al., 2021).

In the present study, we characterized *I. lunulatae* n. sp. from *A. lunulata* morphologically and molecularly. A comparison of oöcyst morphology revealed that the oöcyst dimensions of *I. lunulatae* n. sp. are most similar to those of *I. samoaensis* and *I. phylidonyrisae*; however, the differences of the oöcyst features are notable (Table 1).

At the molecular level, the 18S rDNA sequence for *I. lunulatae* n. sp. was most similar to that of *I. phylidonyrisae*, while the 28S rDNA sequence shared the highest similarity with *I. anthochaerae* from the red wattlebird *A. carunculata*, and the *cox*1 sequence was most similar to that of *I. manorinae* from the yellow-throated miner *M. flavigula obscura* (Gould) (Table 3).

The results of the phylogeny reconstructed for the three loci, but mainly for the 18S and 28S rRNA genes, showed the monophyly of *Isospora* spp. of Australian passerines, which must be related to the morphological and ecological proximity between coccidian species and their hosts, respectively, as it occurs between *I. phylidonyrisae* and *I. lunulatae* n. sp. These two coccidians parasitize hosts of the same family, with close ecological niches and which are sympatric in Australia. Therefore, it is assumed that these two species have a common ancestor reasonably close in the evolutionary tree and that they can possibly parasite both meliphagid hosts in Australia. However, we consider the morphological and molecular differences observed in oöcysts of *I. lunulatae* n. sp. and highlighted in this study sufficient to justify the distinct species status of the new species.

The molecular phylogenetic analysis in this study, based on the three loci, demonstrated that the intraspecific genetic divergence in *Isospora* spp. is lower than interspecific genetic divergence (sequences from the same species were always grouped together, therefore, only one sequence per species was selected for the phylogenetic analysis). It further confirmed that not only could the sequencing data be used in coccidia molecular taxonomy, but they can also serve as a tool to source the origin of the disease. For example, 18S and 28S sequences of *I. neochmiae* identified from a red-browed finch *N. temporalis* (subspecies *N. t. temporalis*), that was part of a captive population in Western Australia (Yang et al., 2016b) were similar to *Isospora* spp. from North America (Figs. 3 and 4).

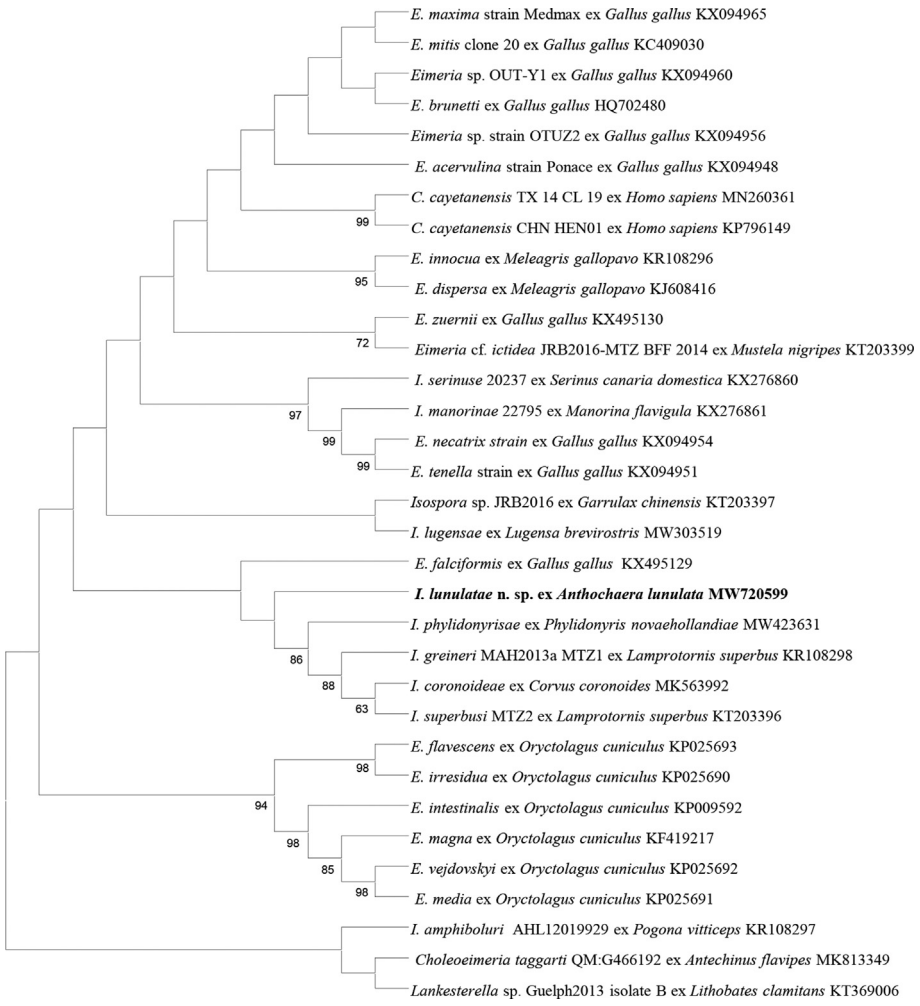

Fig. 5. Evolutionary relationships of *I. lunulatae* n. sp. inferred by maximum likelihood analysis (ML) of partial *cox*1 gene sequences (633 bp). Percentage support (> 70%) from 1000 pseudoreplicates from the ML analysis is indicated at the nodes.

5. Conclusion

Isospora lunulatae n. sp. from *A. lunulata* is described based on consideration of the morphological and molecular differences.

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Ethical approval

Not applicable.

CRediT author statement

Rongchang Yang: Sampling, imaging, PCR and sequencing, writing - review & editing. Belinda Brice: Sample collection, coccidian primary screening and identification, writing - original draft and paper reviewing. Bruno P. Berto: Morphological identification of the new species, preparation of line drawings and paper reviewing. Alireza Zahedi: Phylogenetic analysis and paper reviewing.

Data availability

The type-material is deposited in the Western Australian Museum, Perth, Australia, under the reference number WAM Z100500. The newly generated sequences are deposited in the GenBank database under the accession numbers MW771609 (18S), MW776413 (28S) and MW720599 (*cox*1).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2021.100050.

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