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

Rongchang Yang a, Belinda Brice b, Bruno P. Berto c, Alireza Zahedi d

a Australian National Phenome Centre, Health Futures Institute, Murdoch University, Harry Perkins Building, Perth, WA 6150, Australia
b Kanyana Wildlife Rehabilitation Centre, 120 Gilchrist Road, Lesmurdie, WA 6076, Australia
c Departamento de Biología Animal, Instituto de Ciencias Biológicas e da Saúde, Universidade Federal Rural do Rio de Janeiro, BR-465 km 7, Seropédica, RJ, 23897-000, Brazil
d The Centre of Biosecurity and One Health, Harry Butler Institute, Murdoch University, Perth, WA 6150, Australia

**A R T I C L E   I N F O**

**Keywords:**
- Coccidia
- *Isospora*
- Western wattlebird
- 18S rRNA gene
- 28S rRNA gene
- *cox1* gene

**A B S T R A C T**

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 oocysts 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). Oocysts 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 oocyst 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 oocyst. 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 *cox1*) 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 *cox1* 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 (Pizey 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; Schneider, 1881 (Apicomplexa: Eimeriidae) from the western wattlebird *Anthochaera lunulata* Gould in Western Australia is described and characterised molecularly. Microscopic analysis of a faecal sample identified subspheroidal oocysts 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). Oocysts 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 oocyst 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 oocyst. 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 *cox1*) 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 *cox1* gene locus. Morphological and molecular data support the distinct species status of the new species.

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 & Slapeta, 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.

* Corresponding author. Australian National Phenome Centre, Health Futures Institute, Murdoch University, Harry Perkins Building, Perth, Western Australia 6150, Australia.

E-mail address: R.Yang@murdoch.edu.au (R. Yang).

https://doi.org/10.1016/j.crpvbd.2021.100050
Received 12 July 2021; Received in revised form 24 August 2021; Accepted 17 September 2021
2667-114X/Crown Copyright © 2021 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 oocysts were observed. Faecal flotation was performed using a saturated sodium chloride and 50% sucrose (w/v) solution. A portion of faeces was also observed. Faecal sample was collected from a wild, western wattlebird juvenile admitted to the Kanyana Wildlife Rehabilitation Centre (KWRC), Perth, Australia, in October 2014 following its attack 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 oocysts were observed. Faecal flotation was performed using a saturated sodium chloride and 50% sucrose (w/v) solution. A portion of faeces was also observed.

2.2. Morphological analysis

Sporulated coccidian oocysts 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 were 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 cox1 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 cox1 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:1 for 18S and 28S rRNA genes, and GTR+G+I for the cox1 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: Oocysts fixed in 10% formalin and oocyst phototypes 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:BB3BAASS-54AE-47BE-B942-5809858381A4. The Life Science Identifier (LSID) of the new name I. lunulatae is urn:lsid:zoobank.org:act:DA533DDC-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 (cox1 gene).

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

3.1.2. Description

[Based on 20 oocysts and sporocysts; Figs. 1 and 2.] Oocysts sub-spheroidal, measuring 27–34 × 26–31 (30.6 × 29.4); length/width (L/W) ratio 1.0–1.1 (1.0). Oocyst wall bi-layered, 0.9–1.2 (1.0) thick; outer layer smooth; c.2/3 of total thickness. Micropyle and oocyst residuum absent, but one polar granule present. Sporocysts ovoidal, measuring 31 (30.6) × 26–31 (30.6); L/W ratio 1.0–1.1 (1.0). Oocyst residuum present, with irregular shape, consisting of numerous small granules that appear to be membrane-bound. Sporozoites 4, vermiform, 12.1 × 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 oocysts recovered from A. lunulatae 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 oocysts than all previously described coccidians from passerine birds, except for Isospora samoensis Adamczyk,
Comparative morphological data for oocysts of *Isospora* spp. recorded from birds in the order Passeriformes.

| Species                      | Host                                      | Distribution                | Shape                | Measurements (μm) | Shape index | Wall granule | Oocyst residuum | Reference                                   |
|------------------------------|-------------------------------------------|----------------------------|----------------------|-------------------|-------------|--------------|-----------------|---------------------------------------------|
| *Isospora luminata* n. sp.   | Western wattlebird (Anthus novaehollandiae) | Australia                  | Subspheroidal        | 27.34 × 26.31     | 1.04        | Bi-layered   | Present         | Absent Yang et al. (2014)                   |
| *Isospora anthochaera*       | Red wattlebird (Anthus novaehollandiae)    | Australia                  | Subspheroidal        | 20.26 × 19.22     | 1.12        | Bi-layered   | Absent Yang et al. (2014)                   |
|                             |                                           |                            |                      |                   |             |              |                 |                                             |
| *Isospora butcheri*          | Silvereye (Zosterops lateralis)            | Australia; Fiji; New Caledonia; New Zealand; Vanuatu | Spheroidal to subspheroidal | 23.25 × 23.24 | 1.02        | Bi-layered   | Present Yang et al. (2018)                   |
| *Isospora gryphoni*          | American goldfinch (Spinus tristis)        | Bahamas; Canada; Mexico; Saint Pierre and Miquelon; Turks and Caicos Islands; USA | Spheroidal           | 25.33 × 28.34     | 1.05        | Bi-layered   | Present Olson et al. (1998)                  |
| *Isospora coroneoides*       | Australian raven (Corvus coroneoides)      | Australia                  | Subspheroidal        | 18.24 × 17.21     | 1.13        | Bi-layered   | Present Liu et al. (2020)                    |
| *Isospora leucops*           | Regent honeyeater (Anthus novaehollandiae) | Australia                  | Spheroidal           | 23.29 × 20.26     | 1.08        | Bi-layered   | Present Morin-Adeline et al. (2011)         |
| *Isospora manorinae*         | Yellow-throated miner (Manorina flavagua obscura) | Australia                  | Spheroidal to subspheroidal | 20.24 × 18.19     | 1.25        | Bi-layered   | Present Yang et al. (2016a)                  |
| *Isospora neochmiae*         | Red browed finch (Neochmia temporealis)    | Australia                  | Spheroidal           | 18.19 × 18.19     | 1.01        | Bi-layered   | Present Yang et al. (2016b)                  |
| *Isospora phylidonyrisae*    | New Holland honeyeater (Phylidonyris novaehollandiae) | Australia                  | Subspheroidal        | 29.32 × 28.31     | 1.01        | Bi-layered   | Present Yang et al. (2021)                   |
| *Isospora samoaisensis*      | Polynesian wattle honeyeater (Foudia maga) | American Samoa; Fiji; Samoa; Tonga; Wallis and Futuna | Ovoidal              | 25.32 × 23.30     | 1.10        | Bi-layered   | Present Adamiczyk et al. (2004)             |
| *Isospora serinus*           | Canary (Serinus canaria)                   | Australia (type-locality)   | Spheroidal to subspheroidal | 24.27 × 22.25     | 1.09        | Bi-layered   | Present Yang et al. (2015b)                  |
| *Isospora striperosa*        | Grey currawong (Strepera versicolor)       | Australia                  | Spheroidal           | 22.25 × 22.25     | 1.06        | Bi-layered   | Present Yang et al. (2015a)                  |
Furthermore, the 18S rDNA sequence for the new species exhibited the greatest similarity (99.6%) to a sequence for *I. phylidonyrisae*. However, *I. phylidonyrisae* differs from *I. lunulatae* n. sp. in having oocysts 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 oocyst size, occupying about 21% (vs 27%) of the area within the oocyst (Supplementary Fig. S1).

Comparative sequence analysis also revealed that *I. lunulatae* n. sp. shared the highest sequence similarities with *I. anthochaerae* (98.8%; 28S rRNA gene) and *Isospora manorinae* (98.1%; cox1 gene) (Table 3). These two species differ from *I. lunulatae* n. sp. in possessing smaller oocysts. 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 oocysts 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* species from the Order Passeriformes. The second clade of *Isospora* spp. included three species identified from North American

#### 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 | Flattened to rounded | Rounded to rectangular | Scattered granules | This study |
| *I. anthochaerae* Yang, Brice & Ryan, 2014 | 11–17 × 9–11 | Hemi-dome-shaped | Rectangular | Compact | Yang et al. (2014) |
| *I. butcherae* Yang, Brice, Jian & Ryan, 2018 | 16–17 × 10–12 | Hemi-dome-shaped | Rectangular | Scattered granules | Yang et al. (2018) |
| *I. gypophoni* Olson, Gissing, Barta & Middleton, 1998 | 15–25 × 12–15 | Small | Indistinct | Prominent | Olson et al. (1998) |
| *Isospora coronoideae* Liu, Brice, Elliot, Ryan & Yang, 2019 | 14–19 × 8–13 | Hemi-dome-shaped | Rectangular | Scattered granules | Liu et al. (2020) |
| *I. lesoue* Yang, Brice, Liu, Berto, Austen, 2016 | 17–19 × 8–10 | Flattened | Spheroidal | Scattered granules | Morin-Adeline et al. (2011) |
| *I. manorinae* Yang, Brice, Jian & Ryan, 2016 | 15–16 × 9–10 | Knob-like | Subspherical | Scattered granules | Yang et al. (2016a) |
| *Isospora neochmiae* Yang, Brice & Ryan, 2016 | 10–16 × 7–10 | Indistinct | Absent | Compact | Yang et al. (2016b) |
| *Isospora phylidonyrisae* Yang, Brice, Berto & Ryan, 2021 | 18–19 × 12–14 | Flattened | Rounded | Compact | Yang et al. (2021) |
| *I. serinuse* Yang, Brice, Elliot & Ryan, 2015 | 18–19 × 10–11 | Broad, dome-like | Rectangular | Compact | Adamczyk et al. (2004) |
| *Isospora streperae* Yang, Brice, Al Habsi, Elliot & Ryan, 2015 | 12–16 × 10–13 | Small | Indistinct | Compact | Yang et al. (2015b) |

**Abbreviation:** na, not available.

### 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 (cox1) loci.

| Species | Host | 18S rRNA gene | 28S rRNA gene | cox1 gene | Reference |
|---------|------|---------------|---------------|-----------|-----------|
| *I. gypophoni* | Spinus tristis (Fringillidae) | 99.0 (1213 bp) | na | 97.6 (399 bp) | Olson et al. (1998) |
| *I. lesoue* | Anthochaera phrygia (Meliphagidae) | na | na | 95.2; 96.1; 95.7; 96.1; 97.8 (230 bp) | 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 (799 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. coronoidae* | Corvus coronoideae (Corvidae) | 99.1 (1214 bp) | 95.0 (1338 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.
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 oocysts 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. anthochaeræ* (GenBank: KF766053; genetic similarity of 99.8%) from *A. carunculata* in a separate clade, which was a sister clade to the clade containing *I. philidionyræae* (GenBank: MW422270) and *I. manorinae* (GenBank: KT224381) isolated from the yellow-throated miner *M. flavigula obscura*. As shown in Fig. 4, *I. coronoidæae* (GenBank: MK530654), *I. serinæae* (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 *cox1* 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 *Cholecocoeimeria* spp. All *cox1* reference sequences were selected based on the NCBI BLAST similarities and covered all *Isospora* spp. in the database. *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. phylidonyræae* (Fig. 5). Only a 206 bp *cox1* sequence was available for *I. anthochaeræae* 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 oocyst morphological features. The 399 bp of the overlapping *cox1* sequence (GenBank: KC346355) of *I. gryphoni* and *I. lunulatae* n. sp. (GenBank: MW720599) showed a genetic similarity of 97.6%.

**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.
4. Discussion

O’Donoghue and Adlard (2000) listed four species, namely Haemoproteus danilewskyi Kruse, 1890, Leucocytozoon 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. samoensis from the Polynesian wattled honeyeater Foule-haito carunculatus (Gmelin) in America (see Adamczyk et al., 2004). Recently, I. phylydonyriae 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 oocyst morphology revealed that the oocyst dimensions of I. lunulatae n. sp. are most similar to those of I. samoensis and I. phylydonyriae, however, the differences of the oocyst 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. phylydonyriae, while the 28S rDNA sequence shared the highest similarity with I. anthochaerae from the red wattlebird A. carunculata, and the coxl sequence was most similar to that of I. manorinae from the yellow-throated miner M. flavivaga 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. phylydonyriae 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 oocysts 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 coccidium 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).
Isospora lunulatae n. sp. from A. lunulata is described based on consideration of the morphological and molecular differences.

Funding

Official funding for this study was not available.

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 (cox1).

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.

Acknowledgements

The authors wish to thank Helen Riley and the volunteers at the Kanyana Wildlife Rehabilitation Centre for their commitment and dedication in caring for all the animals admitted to the centre. We are also most grateful to the veterinarians and staff at both the Wattle Grove and Kalamunda Veterinary Hospitals for their expert treatment of the wildlife admitted to their practises. BPB thanks fellowships from CNPq (Grant/Award Number: 303899/2019-0) and FAPERJ (Grant/Award Number: E-26/202.797/2019).

Appendix A. Supplementary data

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

Adamczyk, K.J., McQuistion, T.E., LaPointe, D., 2004. A new coccidian parasite, Isospora samoaensis, from the wattled honeyeater (Foudia longicauda) from American Samoa. Acta Protozool. 43, 179–181.

Berto, B.P., Flausino, W., McIntosh, D., Teixeira-Filho, W.L., Lopes, C.W.G., 2011. Coccidia of new World passerine birds (Aves: Passeriformes): a review of Eimeria Schneider, 1875 and Isospora Schneider, 1881 (Apicomplexa: Eimeriidae). Syst. Parasitol. 80, 159–204.

Duszynski, D.W., Wilber, P.G., 1997. A guideline for the preparation of species descriptions in the Eimeriidae. J. Parasitol. 83, 533–536.

Duszynski, D.W., Upton, S.J., Couch, L., 1999. The Coccidia of Passeriformes (Apicomplexa: Eimeriidae). http://biology.unm.edu/coccidia/home.html. (Accessed 27 January 2021).

Donoghue, P.J., Adlard, R.D., 2000. Catalogue of protozoan parasites recorded in Australia. Memoir. Queensl. Mus. 45, 1–164.

Higgins, P.J., Christidis, L., Ford, H., 2020. Little wattlebird (Anthochaera chrysoptera). Birds of the World. Cornell Laboratory of Ornithology, Ithaca, NY, USA. https://www.birds.cornell.edu/birds-of-the-world.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.

Liu, D., Berto, B., Elliot, A., Ryan, U., Yang, R., 2020. Isospora coronoideae n. sp. (Apicomplexa: Eimeriidae) from a grey currawong (Strepera versicolor plumbea) (Passeriformes: Artamidae) in Western Australia. Exp. Parasitol. 151, 45–53.

Yang, R., Brice, B., Ryan, U., 2015a. Isospora streperae n. sp. (Apicomplexa: Eimeriidae) from a captive-bred red-browed finch (Neochmia temporalis) (Aves: Fringillidae) in Western Australia. Exp. Parasitol. 151, 146–152.

Yang, R., Brice, B., Ryan, U., 2016a. Isospora anthochaerae n. sp. (Apicomplexa: Eimeriidae) from a red wattlebird (Anthochaera carunculata) (Passeriformes: Meliphagidae) in Western Australia. Exp. Parasitol. 154, 1–7.

Yang, R., Brice, B., Ryan, U., 2018a. Molecular characterization of Isospora butcherae n. sp. in a silveryeye (Zosterops lateralis Latham, 1801) (Aves: Estrildidae) from Ontario, Canada. J. Parasitol. 84, 153–156.

Putzey, G., Knight, F., 2007. The Field Guide to the Birds of Australia. Harper Collins Publishers Pty Limited, Sydney.

Schrenzel, M.D., Maalouf, G.A., Gaffney, P.M., Tokarz, D., Keener, L.L., McClure, D., et al., 2005. Molecular characterization of isosporid coccidia (Isospora and Axosphaera spp.) in passerine birds. J. Parasitol. 91, 635–647.

Yang, R., Brice, B., Ryan, U., 2016b. Morphological and molecular characterization of Isospora butcherae n. sp. in a silveryeye (Zosterops lateralis Latham, 1801). Parasitol. Res. 115, 1381–1388.

Yang, R., Brice, B., Ryan, U., 2015b. Isospora streperae n. sp. (Apicomplexa: Eimeriidae) from a captive-bred red-browed finch (Neochmia temporalis) (Aves: Fringillidae) in Western Australia. Exp. Parasitol. 151, 45–53.

Yang, R., Brice, B., Ryan, U., 2016a. Isospora anthochaerae n. sp. (Apicomplexa: Eimeriidae) from a red wattlebird (Anthochaera carunculata) (Passeriformes: Meliphagidae) in Western Australia. Exp. Parasitol. 151, 146–152.

Yang, R., Brice, B., Ryan, U., 2018a. Molecular characterization of Isospora butcherae n. sp. in a silveryeye (Zosterops lateralis Latham, 1801) (Aves: Estrildidae) from Ontario, Canada. J. Parasitol. 84, 153–156.