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

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.
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 samoensis has been described morphologically from the Wattled honeyeater (Fousleha 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/w) solution. If any sample was found to contain coccidian oocysts, a portion of faeces was placed in 2% (w/v) potassium dichromate solution (K2Cr2O7), 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 CGG CTA ACG AGT AC 3′; 28SExR: 5’-CAA GAT CCG AGC TCC GC 3′ (no faecal sample) was used in each extraction group. Amplification of a 1420 bp. The 25 μl PCR reaction contained 2.5 μl of 10× Kapa PCR buffer, 2 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP’s, 10 μl of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 μl of DNA and 16.9 μl of H2O. Both primary and secondary PCRs 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 downstream 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′ TAC 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 16 × dilution (Life Technologies, Foster City, California).

The results of the sequencing reactions were analysed and edited using Finch TV® v4.1.0. Sequences were compared to existing Isospora sp. ITS, 18S and 28S rDNA and COI sequences on GenBank using BLAST search 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 using 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).
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


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. Para-stieda 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.


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.

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 *Cystiospora* sp. (C. suis – GenBank accession number: U97523, C. felis – GenBank accession number: L76471 and *Cystiospora bellii* – 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*) 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 closely related to *Isospora robini* (AF080612), *E. bovis* (U77084), *I. gryphoni* (AF080613), *E. mitis* (U67118), *Cyclospora sp.* (U40261), *Lankesterella minima* (AF080611), *E. tenella* (U67121), *E. necatrix* (U67119), *Caryospora bigenetica* (AF060975), *E. nieschulzi* (U40263), *C. gondii* (M97703), *N. caninum* (U03069), *S. neurona* (U07812), *Sarcocystis sp.* (U97524), *S. fusiformis* (U03071), *S. gigantea* (L24384), *S. tenella* (L24383), *Perkinsus marinus* (X75762), *Cryptocodinium cohnii* (M64245), *Cryptosporidium parvum* (X64341) and *Cryptosporidium muris* (X64342).

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.
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: HQ211885, *Isospora* sp. iSAT1 – GenBank accession number: JF269357, *Isospora* sp. iSAT2 – GenBank accession number: JF269358, *Isospora* sp. iSAT3 – GenBank accession number: JF269359, *Isospora* sp. iSAT4 – GenBank accession number: JF269360, *Isospora* sp. iSAT5 – GenBank accession number: JF269361, *Isospora* sp. iSAT6 – GenBank accession number: JF269362), 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. iSAT7, 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 coccidial 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* 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* were more oval in shape than those of *I. anthochaerae* n. sp. (Fig. 5).
Due to the very limited availability of sequences for avian species at the 4 loci, the phylogenetic trees were generated with maximum likelihood analyses is indicated at the left of the supported node.

Comparative morphology of *Isospora* species was problematic due to (i) ambiguities in the morphology and (ii) unknown host specificity. 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. The genetic differences at the COI locus are phylogenetically significant as the COI gene is highly conserved (Barta, 2001) and has been used for phylogenetic analysis of coccidia and is widely used for species-level identification (Dolnik et al., 2011).}

Delimiting avian species of *Isospora* is problematic due to (i) ambiguities in the morphology and (ii) unknown host specificity. 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. lesouefi* from American goldfinches (*C. tristis*), 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. dumium*). 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

<table>
<thead>
<tr>
<th>Species</th>
<th>Hosts</th>
<th>References</th>
<th>Shape</th>
<th>Measurements (μm)</th>
<th>Shape index</th>
<th>Wall (μm)</th>
<th>Polar granule</th>
<th>Shape Measurements (μm)</th>
<th>Stieda body</th>
<th>Substida body</th>
<th>Residuum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isospora samoensis</em></td>
<td>Wattled honeyeater</td>
<td>Adamiczky et al. (2004)</td>
<td>Ovoid</td>
<td>28.9 × 26.1</td>
<td>1.1</td>
<td>Bi-layered c. 0.8</td>
<td>Present Ovoid 17.1 × 10.9 (16–18 × 10–11)</td>
<td>Broad</td>
<td>Dome-like</td>
<td>Compact</td>
<td></td>
</tr>
<tr>
<td><em>Isospora lesouefi</em></td>
<td>Regent Honeyeater</td>
<td>Morin-Adeline et al. (2011)</td>
<td>Spherical</td>
<td>25.8 × 23.8</td>
<td>1.08</td>
<td>Bi-layered c. 1.0</td>
<td>Present Ovoid 18.67 × 9.45 (17–19 × 9–10)</td>
<td>Flat</td>
<td>Spherical</td>
<td>Compact</td>
<td></td>
</tr>
<tr>
<td><em>Isospora anthochaerae</em></td>
<td>Red wattlebird</td>
<td>Current study</td>
<td>Subspherical</td>
<td>23.4 × 20.7</td>
<td>1.1</td>
<td>Bi-layered c. 0.8</td>
<td>Absent Ovoid 14.5 × 10.1 (11–17 × 9–11)</td>
<td>Hemi-dome</td>
<td>Rectangular-Compact shaped</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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

The authors wish to thank June Butcher and the volunteers at the Kanyana Wildlife Rehabilitation Centre for their commitment and dedication in caring for all the animals admitted to the centre.

References


