Isospora basileuterusi n. sp. (Apicomplexa: Eimeriidae) from the golden-crowned warbler Basileuterus culicivorus (Deppe) (Passeriformes: Parulidae) in South America

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1. Introduction

Brazil is the second country in the Neotropical region with the highest number of bird species, with about 1971 species listed by the Brazilian Ornithological Records Committee (Pacheco et al., 2021), which corresponds to half of the diversity of the Neotropical avifauna (Dias, 1992). In relation to research on Neotropical birds, the study of their parasites has been highlighted for their association with ecology, biology and species conservation. Among their parasites, coccidian protozoans are important as a cause of morbidity and mortality, especially in captive birds or impacted environments, thus acting as ecological biomarkers (Berto & Lopes, 2020).

The golden-crowned warbler Basileuterus culicivorus (Deppe) is a passerine bird of the family Parulidae with a wide distribution in the Neotropical region (Sick, 1997; Pacheco et al., 2021). It has insectivorous eating habits and occupies the middle stratum of the ombrophilous forests (Marini & Cavalcanti, 1993; Lima & Manhães, 2009). The present study provides a description and molecular characterization of a new...
species of *Isospora* from golden-crowned warblers *B. culicivorus* captured in the Itatiaia National Park (Parque Nacional do Itatiaia), a conservation unit in south-eastern Brazil.

2. Materials and methods

2.1. Sample collection

A total of 9 expeditions were conducted between 2014 and 2019 in the Itatiaia National Park, a protected area with a high degree of vulnerability, located in the Serra da Mantiqueira on the border of the States of Rio de Janeiro, Minas Gerais and São Paulo (ICMBIO, 2021), in August (22°26′19″S, 44°37′23″W) and November (22°26′57″S, 44°36′25″W) 2014; March (22°27′38″S, 44°35′34″W) 2015; March (22°19′46″S, 44°32′11″W) and October (22°27′38″S, 44°35′34″W) 2016; July (22°26′15″S, 44°18′33″W) and November (22°26′57″S, 44°36′25″W) 2017; August (22°26′57″S, 44°36′25″W) 2018; March (22°26′17″S; 44°37′33″W) 2019. A total of 19 B. culicivorus were captured with mist nets. The birds were kept in individual boxes and faeces collected immediately after defecation. After identification to the species level, the birds was photographed and released, and stool samples were placed in centrifuge tubes containing 2.5% potassium dichromate (K$_2$Cr$_2$O$_7$) solution at 1:6 (v/v).

2.2. Morphological analyses

Samples were examined at the Laboratório de Biologia de Cocóidios, Universidade Federal Rural do Rio de Janeiro (UFRJR). All samples were incubated at room temperature (25 °C) for 10 days or until c.70% of the oocysts were sporulated. Oocysts were isolated by flotation in Sheather’s sugar saturated solution (specific gravity: 1.20) and examined microscopically using the technique described by Duszynski & Wilber (1997) and Berto et al. (2014a). Morphological observations, line drawings, photomicrographs and measurements were made using an Olympus BX binocular microscope (Olympus Optical, Tokyo, Japan) equipped with a digital camera Eurekam 5.0 (BEL Photonics, Monza, Italy). Line drawings were edited using two software applications (Corel DRAW and Corel PHOTO-PAINT) from CorelDRAW® (Corel Draw Graphics Suite, Version, 2020; Corel Corporation, Canada). All measurements are in micrometres and are given as the range followed by the mean in parentheses.

2.3. Molecular data generation

An individual oocyst was isolated from serial dilutions of the oocysts in drops on a microscope slide using a sterile micropipette. This isolated oocyst was resuspended in PBS and washed by centrifuging until the supernatant became clear (Dolnik et al., 2009). DNA was extracted from the oocyst using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, S, 44μm). Oocysts were placed in centrifuge tubes containing 2.5% potassium dichromate (K$_2$Cr$_2$O$_7$) solution at 1:6 (v/v).

2.4. DNA sequence analyses

All PCR amplicons were sequenced using the PCR forward and reverse primers by Ludwig Biotechnology, where an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Foster City, California) was used for Sanger sequencing. The results of the sequencing reactions were analyzed and edited using the program Chromas 2.6. The newly generated sequence was compared to those for *Isospora* spp. and other coccidian parasites available in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed using the newly generated cox1 sequence aligned with sequences for 18 species of *Isospora* available on 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 were curated, analyzed, and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Maximum likelihood (ML) and Neighbor-Joining (NJ) trees were constructed, and the distances computed using the Tamura-Nei method based on model selection using ModelTest in MEGA X. Bootstrap analyses were conducted using 1000 replicates to assess the reliability of inferred tree topologies.

3. Results

Nineteen *B. culicivorus* were examined and four (21%) were positive for coccidian oocysts of a morphotype not reported in the scientific literature. These positive warblers were captured in November 2014 and August 2018 on a trail named “Trilha das Borboletas” (Trail of the Butterflies) (22°26′57″S, 44°36′25″W), and in March 2019 at the starting point of the “Travesia Ruy Braga” (Ruy Braga Crossing) (22°26′17″S; 44°37′33″W) in the Itatiaia National Park. This material is described below.

3.1. *Isospora basileuterausi* Mello & Berto n. sp.

3.1.1. Taxonomic summary

**Type-host:** *Basileuterus culicivorus* (Deppe) (Passeriformes: Parulidae), warbler.

**Type-locality:** Parque Nacional do Itatiaia (22°26′57″S, 44°36′25″W), Brazil.

**Type-material:** Photosyntypes, line drawing and oocysts in 2.5% K$_2$Cr$_2$O$_7$ solution (Williams et al., 2010) are deposited and available (http://r1.ufrjr.org/labiocic/colecao.html) in the Parasitology Collection of the Laboratório de Biologia de Cocóidios, at UFRJR, under the repository number P-124/2021. Photographs of the type-host specimen (symbiotype) are deposited in the same collection.

**Site in host:** Unknown.

**Prevalence:** 21% (4 out of 19 birds examined).

**Representative DNA sequence:** One representative cox1 sequence was deposited in the GenBank database under the accession number OM255014.

**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:DC2F625C-B798-46DE-84F6-F683A90E4A5. The LSID for the new name *Isospora basileuterausi* Mello & Berto n. sp. is urn:lsid:zoobank.org:act:26689BA2-AA09-4E41-B90E-E40CE593413B.

**Etymology:** The specific epithet is derived from the genus name of the type-host.
3.1.2. Description

[Based on 25 oocytes and 25 sporocysts; Figs. 1 and 2.] Oocytes ellipsoidal to ovoidal, 22–28 × 17–23 (25.2 × 21.1); L/W ratio 1.1–1.3 (1.2). Wall bi-layered, 1.5–1.9 (1.6) thick, outer layer smooth. Micropyle and oocyst residuum both absent, but one to three (usually one) polar granules present, 2.4–3.0 × 1.7–2.4 (2.7 × 2.0). Sporocysts 2, ellipsoidal to lemon-shaped, 14–17 × 8–11 (15.3 × 9.5); L/W ratio 1.4–1.8 (1.61). Stieda body present, knob-like, 0.9–1.1 × 1.7–2.1 (1.0 × 1.8); sub-Stieda present, trapezoidal, 1.1–1.7 × 2.5–2.9 (1.4 × 2.7); para-Stieda body absent; sporocyst residuum present, usually a distinctly ellipsoidal body consisting of numerous small granules that appear to be membrane-bound, 4.3–5.2 × 3.5–4.3 (3.9 × 4.8). Sporozoites 4, vermiform, with anterior and posterior refractile bodies and indiscernible nucleus.

3.1.3. Remarks

To date, four *Isospora* spp. are recorded from warblers (Tables 1 and 2). The sizes of the oocytes of all these species are reasonably compatible with *I. basileuterusi* n. sp.; however, these can be easily distinguished by a few characteristic features: the new species is the only one with ellipsoidal to ovoidal, small sub-Stieda body and membrane-bound sporocyst residuum. Additionally, *I. basileuterusi* n. sp. does not have the typical characteristics of the other species, such as the absence of polar granules in *Isospora cardellinae* Salgado-Miranda, Medina, Zepeda-Velázquez, García-Conjee, Galindo-Sánchez, Janczur & Soriano-Vargas, 2016 and *Isospora celata* Berto, Medina, Salgado-Miranda, García-Conjee, Janczur, Lopes & Soriano-Vargas, 2014 (see Berto et al., 2014a;...
Sporidial to lemon-shaped sporocysts, small sub-Stieda body and membra-

Berto, 2020. The same characteristics typical of the new species, i.e. ellip-
molecularly most closely related

Yang, Brice, Elliot

band of

Lopes, 2010 (see Berto et al. (2009); Keeler et al., 2014).

Salgado-Miranda et al., 2016), the presence of oocyst residuum in I. celata,

the compartmentalized sub-Stieda body in Isospora orbisreinitas Keeler,

Yabsley, Adams & Hernandez, 2014 and the large and trapezoidal

sub-Stieda body in Isospora piacabrai Berto, Flausino, Luz, Ferreira & Lopes, 2010 (see Berto et al. (2009); Keeler et al., 2014).

Isospora basileuterusi n. sp. also differs morphologically from the

molecularly most closely related Isospora spp. (Fig. 3). Isospora serinuse

Yang, Brice, Elliot & Ryan, 2015 and Isospora oliveirai Ortúzar-Ferreira &

Berto, 2020. The same characteristics typical of the new species, i.e. ellip-
soidal to lemon-shaped sporocysts, small sub-Stieda body and membrane-
bound sporocyst residuum, are not observed in I. serinuse and I. oliveirai.

3.2. Phylogenetic analysis

DNA amplification of the oocyst of I. basileuterusi n. sp. showed a clear

band of c.250 bp. Phylogenetic analysis included 18 sequences for avian

Isospora spp. available on GenBank (Fig. 3). Toxoplasma gondii (Nicolle &

Manceaux, 1908) was used as the outgroup. Isospora basileuterusi n. sp.

was recovered in a clade with the highest similarity of 99.5% with I.

serinuse from island canaries Serinus canaria (L.) in Western Australia

(Yang et al., 2015). Furthermore, I. basileuterusi n. sp. was closely related

(95–97%) to I. oliveirai from the greenish schiffornis Schiffornis virens

(Lafresnaye) in south-eastern Brazil and Isospora spp. recovered from

thrushes (Turdidae) and tits (Paridae) in Czech Republic (Trefancová &

Kvíčerová, 2019; Ortúzar-Ferreira et al., 2020).

4. Discussion

Duszynski & Wilber (1997) compiled almost all taxonomic studies of
coccidia of passerines and advised that new coccidian identifications
should be based on comparative morphology between coccidian species
recorded in the same host family. In this sense, the morphotype observed

Table 2

Comparative morphological data for sporocysts of Isospora spp. recorded from warblers (Parulidae)

| Species                        | Host                        | Shape                | Size (μm) | Shape index | Stieda body (μm) | Sub-Stieda body (μm) | Sporocyst residuum | Reference               |
|-------------------------------|-----------------------------|----------------------|-----------|-------------|------------------|---------------------|---------------------|------------------------|
| Isospora basileuterusi        |                              | Ellipsoidal to lemon-shaped | 14–17 × 8–11 (15.3 × 9.5) | 1.4–1.8 (1.61) | Present, knob-like, 0.9–1.1 × 1.7–2.1 (1.0 × 1.8) | Present, trapezoidal, 1.1–1.7 × 2.5–2.9 (1.4 × 2.7) | Granules membrane-bound | Present study            |
| Mello & Berto n. sp.          |                              |                      |           |             |                  |                     |                     |                        |
| Isospora cardellinae          | Cardellina rubra (Swinson)   | Ovoidal              | 18–20 × 11–13 (19.0 × 12.0) | 1.6–1.8 (1.7) | Present, knob-like, 1.1–2.4 | Present, trapezoidal to rounded, sometimes with irregular base, (1.8 × 4.5) | Scattered spherules | Salgado-Miranda et al. (2016) |
| Salgado-Miranda, Medina, Zepeda-Velázquez, García-Congeo, Galindo-Sánchez, Janczur & Soriano-Vargas, 2016 | | | | | | | | |
| Isospora celata Berto, Medina, Salgado-Miranda, García-Congeo, Janczur, Lopes & Soriano-Vargas, 2016 | Leiothlypis celata (Say) | Ovoidal | 15–20 × 11–14 (18 × 13) | 1.4–1.5 (1.4) | Present, knob-like, 1.0 × 2.5 | Present, irregular, barely discernible, (1.5 × 4.0) | Scattered spherules | Berto et al. (2014) |
| Isospora orbisreinitas        | Basileuterus rufifrons       | Ovoidal              | 12–19 × 10–14 (16.0 × 11.8) | 1.0–1.9 (1.4) | Present, knob-like | Present, prominent, trapezoidal and compartmentalized | Many diffuse granules | Keeler et al. (2014) |
| Keeler, Yabsley, Adams & Hernandez, 2014 | | | | | | | | |
| Isospora piacabrai            | Geothlypis aquacoclonialis (Gmelin) | Ovoidal | 15–17 × 9–12 (15.8 × 10.5) | 1.4–1.6 (1.5) | Present, knob-like and prominent, 1.0 × 1.7 | Present, large, trapezoidal and homogenous, (2.3 × 4.8) | Granules of different sizes | Berto et al. (2010) |
| Berto, Flausino, Luz, Ferreira & Lopes, 2010 | | | | | | | | |
from the golden-crowned warblers in this study, *I. basileuterusi* n. sp., was compared with the four recorded coccidian species of Parulidae, as shown in Tables 1 and 2. *Isospora basileuterusi* n. sp. differs in several characteristic features, but can be mainly differentiated from the others by the typical lemon-shape of its sporocysts.

The host of the new coccidian species described here, the golden-crowned warbler *B. culicivora*, has a wide distribution in the Neotropical region, from Mexico to southern South America (Pacheco et al., 2021). However, according to BirdLife International (2021) this species is the stripe-crowned warbler, which is restricted to Mexico and Central America, not occurring in Brazil. This misinformation is due to species/subspecies status within the genus *Basileuterus* Cabanis. Birdlife have reclassified some subspecies of *B. culicivora* to the species level, such as *Basileuterus culicivorus auricapilla* (Swainson, 1838) which has been reclassified to the level of species, as *Basileuterus auricapilla* Swainson, 1838. Therefore, this study followed the name listed by the Brazilian Ornithological Records Committee (Pacheco et al., 2021); however, it is noteworthy that the bird specimen in this study is identified as *B. auricapilla* by BirdLife International (2021). Anyway, regardless of the specific identification within *Basileuterus*, due to the wide geographical distributions of warblers (Parulidae) in the Americas, their coccidian species must be equally distributed throughout the Neotropical region.

In the present study, the molecular identification of *I. basileuterusi* n. sp. was performed using the *cox1* gene, which is considered to be the gene with the highest resolution in detecting recent speciation events (Barta, 2001; Ogedengbe et al., 2011). In fact, the 250 bp *cox1* gene sequence was not 100% similar to any other deposited on GenBank, contrary to what occurs with ribosomal gene sequences that are more conserved and more suitable for phylogenetic studies of families and orders (Genovez-Oliveira et al., 2020). On the other hand, the region of the *cox1* sequenced for *I. basileuterusi* n. sp. did not provide conclusive results related to ancestry, as linked to host family, biogeographical region, morphology/biology of the coccidian species, etc. (Fig. 3). This is also observed when *Isospora* spp. amplified and sequenced with the primers (Dolnik et al., 2009) used in the present study are exclusively included in the phylogeny, as in the studies of Yang et al. (2015) and Silva-Carvalho et al. (2018). Perhaps the short sequence of only 250 bp did not allow greater resolution in the phylogenetic study; in this case, sequences with more than 600 bp from other regions of the *cox1* gene, such as those generated by the JAV primer (Genovez-Oliveira et al., 2020), would show better phylogenetic estimations in the future. Fatally, in the present study these JAV primers were not successful in amplifying the samples; however, it has been shown in any case that mitochondrial genes, such as *cox1*, are better suited to work with individual oocysts, as the number of copies of mitochondrial DNA is far greater than the number of copies of nuclear DNA, thus favoring the amplification of mitochondrial genes (Dolnik et al., 2009).

5. Conclusion

The comparison of *I. basileuterusi* n. sp. with *Isospora* spp. described from Neotropical warblers clearly supports the designation as a unique species. Therefore, *I. basileuterusi* is considered as new to science, which is the fifth species described in a host of the family Parulidae and the first molecularly characterized via sequencing the *cox1* gene.

**Funding**

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro. MSO has a post-doctoral scholarship from FAPERJ (Grant/Award Number: E–26/204.228/2021). LASA has a scholarship from CAPES (Grant/Award Number: 001). BPB has fellowships from CNPq (Grant/Award Number: 303899/2019-0) and from FAPERJ (Grant/Award Number: E–26/202.797/2019).

**Ethical approval**

Field-collecting permits were issued by SISBIO/ICMBio (licenses 45,200; 49,605; 54,951; 70,132), CEUA/UFRRJ (protocols IV-036/2014; ICBS-008/2015; IV-666250616) and CEUA/UNIGRANRIO (protocol 021/2019). All applicable institutional, national and international guidelines for the care and use of animals were followed.

**CRediT author statement**

The study was designed by SVC, VML and BPB. Field work was performed by MSO, LASA and BPB. Laboratory procedures for maintenance, recovery, measurements, photomicrographs and isolation of oocysts were performed by MSO and LASA. DNA extraction, amplification and sequencing were performed by ERM, AAO and VML. BPB analyzed the data and drew the coccidian oocyst. The manuscript was written by ERM and BPB and subsequently revised by all other authors. All authors read and approved the final manuscript.

**Data availability**

Photosyntypes, line drawing, and oocysts in 70% ethanol are deposited and available (http://r1.ufrrj.br/labicoc/colecao.html) in the Parasitology Collection of the Laboratório de Biologia de Coccídios, at UFRJ, under the repository number P-124/2021, along with the photographs of the type-host specimen (symbiotype). The generated sequence for *I. basileuterusi* n. sp. is deposited in the GenBank database under the accession number OM025014.

**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**

We are thankful to staff at the Parque Nacional do Itatiaia, mainly to the research coordinator Dr Léo Nascimento, that allowed us to access and use some facilities during the expeditions.

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