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Redescription and molecular identification of *Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009 (Eimeriidae) from tyrant-flycatchers (Tyrannoidea) in South America

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Abstract In the present study *Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009 is redescribed from the photosyntypes and from new samples from a short-crested flycatcher *Myiarchus ferox* (Gmelin), which is the type-host in the type-locality, the Marambaia Island in Southeastern Brazil. In addition, the yellow-olive flycatcher *Tolmomyias sulphurescens* Spix is recorded as a new host for this species, in a new locality, the Itatiaia National Park, in the interior of Southeastern Brazil, providing a preliminary genotypic characterization via sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene. Micropyle and rough oöcyst wall are added to the description of *I. feroxis*, in addition to other details. This is the sixth species identified from suboscine birds (Tyranni) to have a COI gene sequence deposited in GenBank and, although it is not yet possible to make conclusions on the phylogeny

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of *Isospora* spp. from Passeriformes by the COI gene, the molecular analysis confirmed the differences between coccidian species from tyrant-flycatchers.

Introduction

Biodiversity in the Neotropical Region is very relevant for researchers around the world, among various aspects, due to its potential and recurring new discoveries (Tundisi & Matsumura-Tundisi, 2008). In this context, the class Aves stands out for its great diversity and exuberance, attracting not only scientists, but also ecotourists, especially birdwatchers (Stotz et al., 1996). Brazil is the second country in the Neotropical region with the largest number of bird species; where, currently, there are 1919 species listed by the Ornithological Records Committee of Brazil (Piacentini et al., 2015). In the context of research on Neotropical birds, there is the study of their parasites, which has been increasingly related to ecology, physiology and conservation of wild species. Among bird parasites, coccidian protozoa stand out as the cause of morbidity and mortality in unfavorable conditions of host and environment, to a commensalism-like in bird populations in conserved and balanced environments (Berto & Lopes, 2020).

Tyrant-flycatchers represent a superfamily, Tyrannoidea (Piacentini et al., 2015), or simply the family Tyrannidae, according to BirdLife International (Del Hoyo & Collar, 2016), which distribution extends across North, Central and South America, being more concentrated in the Neotropical Region. It is currently the largest superfamily/family of the class Aves worldwide, with 450 species recorded. In Brazil, 218 species are listed (Piacentini et al., 2015; Del Hoyo & Collar, 2016). Even with this great diversity, there are few reports of coccidian species from tyrant-flycatchers, when compared to other bird families. In this context, the current study aimed to redescribe *Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009 from the photosyntypes and from samples of a short-crested flycatcher *Myiarchus ferox* (Gmelin), which is the type-host in the type-locality, the Marambaia Island in Southeastern Brazil. In addition, the yellow-olive flycatcher *Tolmomyias sulphurescens* Spix is recorded as a new host for this species, in a new locality, the Itatiaia National Park, in the interior of Southeastern Brazil, providing a preliminary genotypic characterization via sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene.

Materials and methods

Sample collection

Between August 2014 and August 2018, 19 expeditions were conducted in different locations in the Itatiaia National Park (22°27′S, 44°36′W), 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, 2016), to capture wild birds with mist nets and collect faecal samples. A total of seven yellow-olive flycatchers *T. sulphurescens* were captured. In addition to the Itatiaia National Park, an expedition in September 2014 was conducted on Maramabia Island (23°3′38.86”S, 43°58′47.56”W), on the coast of the state of Rio de Janeiro, Southeastern Brazil, where one short-crested flycatcher *M. ferox* was captured. The birds were kept in individual boxes and faeces collected immediately after defecation. After identification of the species, the bird was photographed and released and faecal samples were placed in centrifuge tubes containing a potassium dichromate 2.5% ($K_2Cr_2O_7$) solution at 1:6 (v/v).

Morphological analyses

Samples were taken to the Laboratório de Biologia de Coccídios, Universidade Federal Rural do Rio de Janeiro (UFRRJ). Samples were incubated at room temperature (25°C) for 10 days or until ~70% of the oöcysts were sporulated. Oöcysts 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. (2014). Morphological observations, line drawings, photomicrographs and measurements were made using an Olympus BX binocular microscope (Olympus Optical, Tokyo, Japan) coupled to a digital camera Eurekam 5.0 (BEL Photonics, Monza, Italy). Line drawings were edited using Corel DRAW® and Corel PHOTO-PAINT (Corel Draw Graphics Suite, Version 11.0, Corel Corporation, Canada). All measurements are in micrometres and

are given as the range followed by the mean in parentheses.

Molecular analyses

An individual oöcyst from a fecal sample of *T. sulphurescens* was isolated from serial dilutions of the oöcysts in drops on a microscope slide using a sterile micropipette. This isolated oöcyst was resuspended in PBS and washed by centrifuging until the supernatant became clear (Dolnik et al., 2009). DNA was extracted from the purified oöcysts using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, São Paulo, Brazil) according to the manufacturer's instructions. In order to fully lyse the oöcyst, four freeze-thaw cycles were applied prior to the DNA extraction. The PCR amplification for the COI gene was carried out using a nested PCR, as previously described by Dolnik et al. (2009) and Yang et al. (2015). The external primers COIbF1 (5′-GWT CAT TAG TAT GGG CAC ATC A-3′) and COIbR1 (5′-CCA AGA GAT AAT ACR AAR TGG AA-3′) produced a PCR product of 302 bp in size. The internal primes COIbF2 (5′-GGG CAC ATC ATA TGA TGA C-3′) and COIbR2 (5′-ATA GTA TGT ATC ATG TAR WGC AA-3′) produced an amplicon of 257 bp in size. The PCR reaction contained 10 µl of 5× Green GoTaq® Flexi Buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 0.4 µM of each primer, 1.25 units of GoTaq® DNA polymerase, 3 µl of DNA (for primary reaction) or 3µl primary PCR product (for the secondary reaction). Both primary and secondary PCR were conducted using the same cycling conditions: 1 cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 47°C for 45 s, and 72°C for 1 min and a final extension of 72°C for 5 min. The amplicons from the second round of PCR were purified using the Qiagen MinElute PCR Purification (Qiagen, São Paulo, Brazil). All PCR products were sequenced using the PCR forward and reverse primers by Ludwig Biotechnology, were 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 (Technelysium Pty Ltd, Queensland Australia).

DNA sequence analyses

The newly generated sequence was compared to those for *Isospora* spp. and other coccidian parasites available on the GenBank database using the Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were constructed for *Isospora* spp. at the COI sequences for additional isolates from GenBank. Alignment and parsimony analyses were conducted using MEGA version 7, using ClustalW as alignment algorithm (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods and the distances were computed using the Tamura-Nei method based on model selection using ModelTest in MEGA 7. Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies.

Obtaining the photosyntypes of Isospora feroxis *Berto, Luz, Flausino, Ferreira & Lopes, 2009*

The photosyntypes of *I. feroxis* from *M. ferox* identified in Berto et al. (2009a), which were deposited in the Parasitology Collection of the Laboratório de Biologia de Coccídios (http://r1.ufrrj.br/labicoc/colecao.html) at UFRRJ under repository number P-30/2009, were required for morphological comparison.

Results

Seven *T. sulphurescens* were examined and five of them (71%), which were captured in the same location in the Itatiaia National Park, known as "Trilha das Borboletas" or "Butterfly Trail" (22°26′57.00"S, 44°36′25.00"W), were positive for coccidia. The short-crested flycatcher *M. ferox* captured in Maramabia Island was also positive for coccidia. All observed oöcysts were morphologically identified as *I. feroxis*. This material is described below.

Eimeriidae Minchin, 1903

***Isospora* Schneider, 1881**

***Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009**

Type-host: *Myiarchus ferox* (Gmelin) (Aves: Passeriformes: Tyranni: Tyrannoidea: Tyrannidae) short-crested flycatcher.
Other host: Tolmomyias sulphurescens Spix (Aves: Passeriformes: Tyranni: Tyrannoidea: Rhynchocyclidae) yellow-olive flycatcher (present study).
Type-locality: Marambaia Island (23°3′38.86”S, 43°58′47.56”W), Southeastern Brazil
Other locality: Parque Nacional do Itatiaia - Itatiaia National Park (22°26′57.00”S, 44°36′25.00”W), Southeastern Brazil.
Type-specimens: Photosyntypes and line drawing are deposited and available (http://r1.ufrrj.br/labicoc/colecao.html) in the Parasitology Collection of the Laboratório de Biologia de Coccídios, at UFRRJ, under the repository number P-30/2009.
Other specimens (present study): Photomicrographs, line drawing, and oöcysts in 2.5% $K_2Cr_2O_7$ solution (Williams et al., 2010) from *T. sulphurescens* are deposited at the Museu de Zoologia at the Universidade Federal Rural do Rio de Janeiro, Brazil, under accession number MZURPTZ2020025. Photomicrographs are also deposited and available (http://r1.ufrrj.br/labicoc/colecao.html) in the Parasitology Collection of the Laboratório de Biologia de Coccídios, at UFRRJ, under repository numbers 108/2020 (*T. sulphurescens*) and 109/2020 (*M. ferox*). Photovouchers of the host specimens are deposited in the same collection.
Site in host: Unknown; oöcysts recovered from faeces.
Prevalence: 75% (6/8) overall; 71% (5/7) for *T. sulphurescens*; and 100% (1/1) for *M. ferox*.
Representative DNA sequence: Representative COI sequences of the oöcysts from *T. sulphurescens* were deposited in the GenBank database under the accession number MT563402.

Description (Figs. 1, 2)
Sporulated oöcyst

Oöcysts (n = 81) subspheroidal, 18–23 × 18–23 (20.7 × 20.0); length/width (L/W) ratio 1.0-1.1 (1.04). Wall bi-layered, 1.3-2.0 (1.7) thick, outer layer with minimal to moderate roughness, *c.*2/3 of total thickness. Micropyle present, 4.4-9.2 (7.4) wide. Oöcyst residuum absent, but 1-3 (usually 2 bonded) polar granules are present.
Sporocyst and sporozoites
Sporocysts (n = 70) 2, ovoidal to ellipsoidal, 11–15 × 8–10 (13.4 × 9.2); L/W ratio 1.3-1.6 (1.45). Stieda body present, flattened to half-moon-shaped, 0.5–0.7 × 1.3–2.2 (0.6 × 1.7); substieda body present, rounded to trapezoidal, 1.0–1.8 × 2.4–3.3 (1.3 × 2.9), infrequently with prominence resembling a compartmentalized substieda; parastieda body absent; sporocyst residuum present, composed of spherules of different sizes. Sporozoites 4, vermiform, 10–11 × 3–4 (10.9 × 3.6), with posterior refractile body and centrally located nucleus.

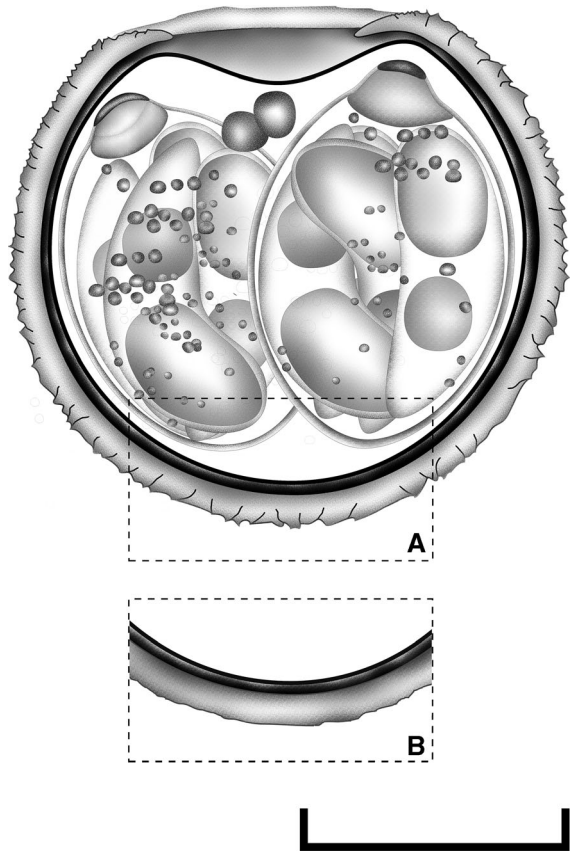

Fig. 1 Composite line drawing of the sporulated oöcyst for redescription of *Isospora feroxis* from tyrant-flycatchers, highlighting the outer layer of the oöcyst wall with moderate (A) or minimal (B) roughness. Scale-bar: 10 μm.

Remarks
Four *Isospora* spp. are recorded from New World tyrant-flycatchers (Table 1). To date, *I. feroxis* had the smallest oöcysts among these *Isospora* spp.; however, in the present study, the wide addition of oöcyst measurements from two hosts increased the range of oöcysts measurements, making them compatible with *Isospora attilae* Rodrigues, Silva, Lopes, Berto, Luz, Ferreira & Lopes, 2015 and *Isospora lopesi* Silva-Carvalho & Berto, 2018. However, *I. feroxis* oöcysts

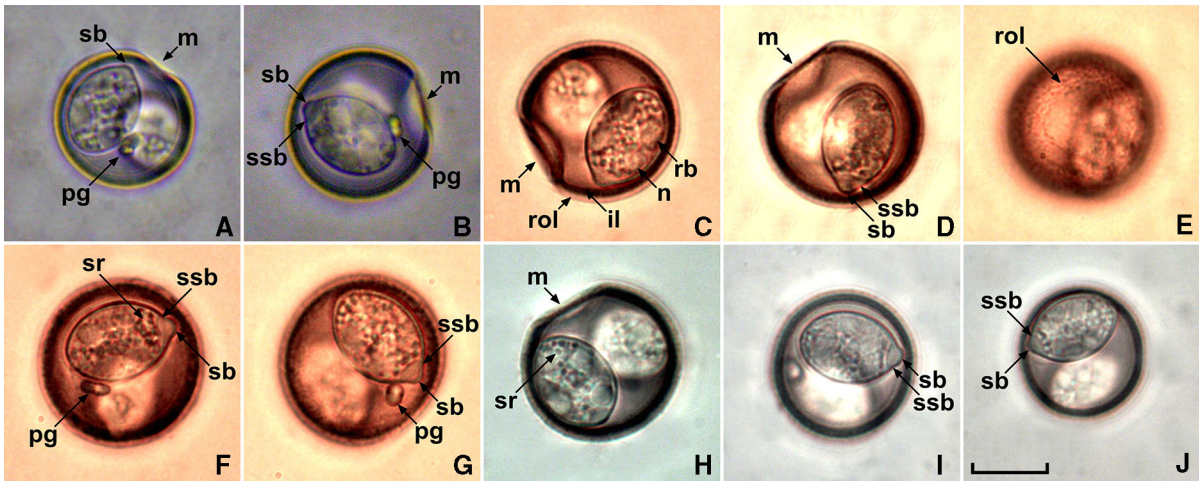

Fig. 2 Sporulated oöcysts of *Isospora feroxis* from the photosyntypes (A, B) and from new samples of a short-crested flycatcher *Myiarchus ferox* (C–G) and of yellow-olive flycatchers *Tolmomyias sulphurescens* (H–J). Note the inner (il) and rough outer (rol) layers of the oöcyst wall; micropyle (m); nucleus (n); polar granule (pg); Stieda (sb) and sub-Stieda bodies (ssb); sporocyst residuum (sr); refractile body (rb). Scale-bar: 10 μm.

are differentiated from these *Isospora* spp. by the less elongated shape of the sporocysts, smaller and more delicate sub-Stieda body and, mainly, by the presence of the micropyle and rough oöcyst wall which were added in the current redescription.

Phylogenetic analysis

DNA amplification of the oöcyst of *I. feroxis* showed a clear band of *c.*250 bp. Phylogenetic analysis included 20 sequences for avian *Isospora* spp. available on GenBank (Fig. 3). *Toxoplasma gondii* (Nicolle & Manceaux, 1908) was used as the outgroup. *Isospora feroxis* sat apart into a large clade containing *Isospora* spp. from thrushes, warblers, honeyeaters, buntings, longspurs, starlings, white-eyes, but also containing *Isospora* spp. from suboscine hosts (Tyranni), which is the Suborder of *T. sulphurescens* and *M. ferox*. *Isospora feroxis* had the highest similarities of 97% with *Isospora massardi* Lopes, Berto, Luz, Galvão, Ferreira & Lopes, 2014 from the yellow-legged thrush *Turdus flavipes* (Vieillot) and *Isospora manorinae* Yang, Brice, Jian & Ryan, 2016 from the yellow-throated miner *Manorina flavigula* (Gould).

Discussion

The description of coccidian species is fundamentally based on the morphology of the oöcysts; although ecological, biogeographical, pathological and molecular complementations are important for the characterisation of a species (Duszynski & Wilber, 1997; Tenter et al., 2002; Berto et al., 2014). In this context, Duszynski & Wilber (1997) established that the identifications must be based on the comparative morphology between the coccidian species recorded in the same family as the host. Since then, the most complete and reliable studies on coccidian taxonomy from wild birds have demonstrated specificity at the host family level (Berto et al., 2013); however, sometimes the number of coccidian species recorded in a host-family is very low or non-existent (e.g., Cardinalidae), making it necessary to compare with coccidian species described from higher taxonomic levels of the host, such as Superfamily, Parvorder or Infraorder. In addition, the divergences in the systems of classification of Aves make it difficult to identify coccidians based on host-family specificity. The classifications of Del Hoyo & Collar (2016) and Piacentini et al. (2015) that supported the present study classify the tyrant-flycatchers as a Family and Superfamily, respectively; therefore, despite this divergence in level and classifications in different families by Piacentini et al. (2015), there were no

Table 1 Comparative morphology of *Isospora* spp. recorded from New World tyrant-flycatchers (Tyrannoidea)

Species	Host	Reference	Oöcyst						Sporocyst					
			Shape	Size (µm)	Shape index	Polar granule	Wall	Micropyle	Shape	Size (µm)	Shape index	Stieda body	Sub-Stieda body	Sporocyst residuum
***Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009**	*Myiarchus ferox* (Gmelin) (Tyrannidae)	Berto et al. (2009a)	subspheroidal	18–20 × 17–20 (18.7 × 18.0)	1.0–1.1 (1.1)	usually 2	**low roughness**	**present**	ovoidal	11–13 × 8–10 (11.7 × 8.5)	1.0–1.5 (1.4)	flattened, (0.3 × 1.2)	prominent, (1.2 × 2.5)	diffuse
	M. ferox	**present study**	**subspheroidal**	**21–23 × 20–23 (21.9 × 21.4)**	**1.0–1.1 (1.03)**	**1-3 (usually 2 bonded)**	**low to moderate roughness**	**present**	**ovoidal to ellipsoidal**	**14–15 × 9–10 (14.8 × 10.1)**	**1.4–1.6 (1.47)**	**flattened to half-moon-shaped, 0.5–0.7 × 1.3–2.2 (0.6 × 1.7)**	**rounded to trapezoidal, 1.0–1.8 × 2.4–3.3 (1.3 × 2.9)**	**diffuse**
	***Tolmomyias sulphurescens* Spix (Rhynchocyclidae)**			**18–23 × 18–22 (20.5 × 19.8)**	**1.0–1.1 (1.04)**					**11–15 × 8–10 (13.1 × 9.1)**	**1.3–1.6 (1.45)**			
Isospora mionectesi Berto, Flausino, Luz, Ferreira & Lopes, 2009	*Mionectes rufiventris* Cabanis (Rhynchocyclidae)	Berto et al. (2009b)	ellipsoidal	23–31 × 19–23 (28.3 × 21.2)	1.2–1.4 (1.3)	1-2	smooth	absent	elongate-ellipsoidal	17–22 × 10–13 (19.7 × 11.7)	1.6–1.8 (1.7)	rounded, (0.8 × 1.1)	prominent, (1.4 × 2.1)	compact, subspherical
Isospora attilae Rodrigues, Silva, Lopes, Berto, Luz, Ferreira & Lopes, 2015	*Attila rufus* (Vieillot) (Tyrannidae)	Rodrigues et al. (2015)	subspheroidal to elipsoidal	18–22 × 18–21 (20.3 × 19.0)	1.0–1.2 (1.07)	1-2	smooth	absent	ellipsoidal	12–15 × 7–9 (13.5 × 7.9)	1.6–1.9 (1.7)	knob like, (1.0 × 2.0)	rounded to trapezoidal, (2.5 × 4.0)	diffuse
Isospora lopesi Silva-Carvalho & Berto, 2018	*Platyrinchus mystaceus* Vieillot (Platyrinchidae)	Silva-Carvalho et al. (2018)	subspheroidal to ovoidal	18–24 × 18–22 (20.6 × 19.7)	1.0–1.2 (1.05)	1	smooth	absent	ellipsoidal	12–16 × 8–11 (14.4 × 8.6)	1.5–1.9 (1.7)	flattened to half-moon-shaped, (1.0 × 2.5)	rounded, (2.0 × 2.5)	diffuse

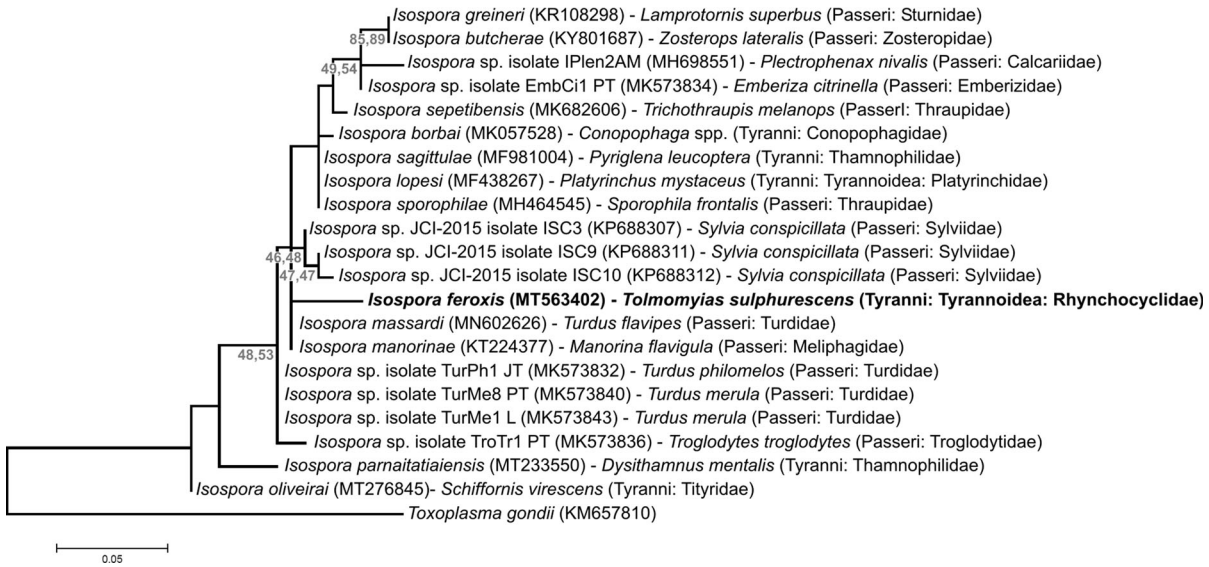

Fig. 3 Maximum likelihood tree estimated from the COI sequences. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 40% shown) for Neighbor-Joining and Maximum Likelihood, respectively. The scale-bar represents the number of nucleotide substitutions per site.

differences in the organization and comparison of coccidians from tyrant-flycatchers that are traditionally related (Berto et al., 2011).

The oöcysts of the original description of *I. feroxis* and those of *M. ferox* and *T. sulphurescens* of the present study differed in measures. These morphometric differences were mainly observed from the low number of oöcysts measured from single hosts *M. ferox*, both in Berto et al. (2009a) and in present study (Table 1). In contrast, the 71 measured oöcysts from 5 *T. sulphurescens* reached a wide range of measures, which is compatible with the largest and smallest oöcysts of *M. ferox*. Thus, it can be concluded that *I. feroxis* has a wide range of measurements detected when observing oöcysts from several hosts. These differences in the size of oöcysts shed from different hosts are natural and already established in the scientific literature as a result of biological and ecological factors (Duszynski, 1971; Fayer, 1981; Berto & Lopes, 2020).

The redescription from the photosyntypes of *I. ferox* proposed in the present study is based on the observation of oöcysts with micropyle (Fig. 2a, b), which were not identified in the original description by Berto et al. (2009a). Probably, the low number of 10 oöcysts observed in Berto et al. (2009a) must have favored the non-observance of the micropyle, since this characteristic feature is observed only in certain positions of the oöcysts (Fig. 2e–g, i, j), especially when they are subspherical (Berto et al., 2014). Thus, this redescription highlights the importance of prioritizing the description of coccidian species from a large number of oöcysts, preventing that certain characteristic features are not observed for the description. In this same sense, the original description by Berto et al. (2009a), did not identify oöcysts with a rough wall. In fact, the oöcysts observed in the current study had a low to moderate roughness, and in one of the hosts *T. sulphurescens*, only oöcysts with very low roughness were predominantly observed, similar to the photosyntypes of *I. ferox*. Therefore, these results reinforce that the description of new coccidian species from a single host specimen should be avoided, ensuring that all possible details of a coccidian species are observed.

Isospora feroxis is the sixth species identified from suboscine birds to have a deposition of COI gene sequence on the GenBank. Even so, few phylogenetic conclusions are observed from the cladogram shown in Fig. 3. *Isospora feroxis* was closer to *Isospora* spp. from thrushes, honeyeaters and warblers than from species of the same parvorder, such as *Isospora oliveirai* Ortúzar-Ferreira & Berto, 2020, and Superfamily/Family, such as *I. lopesi*. At the same time,

Isospora feroxis was as close to coccidians from the Neotropical region, such as *I. massardi*, as well as to coccidian species related to endemic birds in Oceania, such as *I. manorinae*. In fact, this 257 bp fragment of the COI gene has not been totally suitable for the delimitation of coccidian species of passerines, despite that it was pioneered in the work of Dolnik et al. (2009), it has the largest number of deposits at GenBank and, until recently, it has been recommended for phylogenetic studies (Yang et al., 2015). In this sense, the latest works on molecular characterisation of coccidians of Passeriformes has shown that longer sequences and multiple genes are more conclusive in the phylogenetic analyses (Yang et al., 2021). In any case, the molecular analysis confirmed the differences already observed in the morphology of the oöcysts of *I. feroxis* and *I. lopesi*, since these species were genotypically different in 9 base pairs (4.4%) by the COI sequences.

Finally, based on the morphological and molecular features described above, *I. feroxis* is redescribed in the present study, documenting a new host, *T. sulphurescens*, and a new locality, the Itatiaia National Park, in addition to the type-host *M. ferox* in the Marambaia Island, southeastern Brazil.

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Data availability All data generated or analyzed during this study are included in the article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Field-collecting permits were issued by SISBIO/ICMBio (licenses 45200-1; 49605-1; 54951-1) and CEUA/UFRRJ (protocols IV-036/2014; ICBS-008/2015; IV-6606250616). All applicable institutional, national and international guidelines for the care and use of animals were followed.

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