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Isospora lopesi n. sp. (Apicomplexa: Eimeriidae) from the eastern white-throated spadebill *Platyrinchus mystaceus* Vieillot (Passeriformes: Tyranni: Tyrannidae) in South America

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Abstract A species of *Isospora* Schneider, 1881 (Protozoa: Apicomplexa: Eimeriidae) considered as new to science is described and characterised molecularly from the eastern white-throated spadebill *Platyrinchus mystaceus* Vieillot in the Parque Nacional do Itatiaia, southeastern Brazil. *Isospora lopesi* n. sp. has oöcysts that are subspheroidal to ovoidal, 18–24 × 18–22 (20.6 × 19.7) µm, with smooth, bilayered wall, *c.*1.5 µm thick. Micropyle and oöcyst residuum are absent, but one polar granule is present. Sporocysts are ellipsoidal, 12–16 × 8–11 (14.4 × 8.6) µm. The Stieda body is flattened to half-moon-shaped and sub-Stieda body is rounded. Sporocyst residuum is present, consisting of numerous spherules of different sizes. Sporozoites are vermiform with anterior and posterior refractile bodies and nucleus. Molecular analysis was conducted at the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene. This new isolate exhibited similarity greater than 98% with *Isospora* spp. isolates from spectacled warblers *Sylvia conspicillata* Temminck, 1820. This is the fourth isosporoid coccidian described from New World tyrannid birds, but is the first to have a complementary molecular characterisation.

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Introduction

The Brazilian territory has a rich diversity in its avifauna. In the latest listing of bird species records, 29 species were added reaching 1,872 species occurring in Brazil (CBRO, 2014). The eastern white-throated spadebill *Platyrinchus mystaceus* Vieillot is one of the smallest species that inhabits the interior of conserved forests. It has a wide geographical range from Mexico to Bolivia and Argentina. In Brazil, it is distributed from the northeast to the south (Sick, 1997; BirdLife International, 2016).

According to the Brazilian Committee of Ornithological Records (CBRO, 2014), *P. mystaceus* is classified in the order Passeriformes, suborder

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Tyranni, parvorder Tyrannida, superfamily Tyrannoidea and family Platyrinchidae, which includes four other *Platyrinchus* spp., *Neopipo cinnamomea* (Lawrence) and *Calyptura cristata* (Vieillot) in Brazil. In contrast, the BirdLife International (2016), which is currently based on the checklist of del Hoyo et al. (2016), and Brands (2017), classifies *P. mystaceus* in the family Tyrannidae, which is much more comprehensive with 450 species worldwide.

Similar to other passerines, spadebills, tyrants, flycatchers and other tyrannid birds can be parasitised by coccidia. Boughton et al. (1938) and Kawazoe et al. (1989) reported *Isospora* spp. from the eastern phoebe *Sayornis phoebe* (Latham) and the blue-billed black-tyrant *Knipolegus cyanirostris* (Vieillot); however, these coccidian species were not described or named. Since the 2000s, *Isospora* spp. were described from New World tyrannid birds, i.e. *Isospora feroxis* Berto, Luz, Flausino, Ferreira & Lopes, 2009, *Isospora mionectesi* Berto, Flausino, Luz, Ferreira & Lopes, 2009 and *Isospora attilae* Rodrigues, Silva, Lopes, Berto, Luz, Ferreira & Lopes, 2015, originally described from the short-crested flycatcher *Myiarchus ferox* (Gmelin), the greyhooded flycatcher *Mionectes rufiventris* Cabanis, and the grey-hooded attila *Attila rufus* (Vieillot), respectively. The hosts of these three species inhabited the same locality, the Marambaia Island in the southeastern Brazil (Berto et al., 2009a, b; Rodrigues et al., 2015).

The aim of this study was to examine the faeces from eastern white-throated spadebills *P. mystaceus* to determine what coccidian parasites were present. These *P. mystaceus* specimens were captured in the Parque Nacional do Itatiaia (PNI), a protected area with a high degree of vulnerability located in the Serra da Mantiqueira on the border of three states in southeastern Brazil, Rio de Janeiro, Minas Gerais and São Paulo.

Materials and methods

Sample collection

Five expeditions were conducted in the PNI boundaries between March 2015 and October 2016. Sampling occurred in March 2015 (22°27′38″S, 44°35′34″W); May 2015 (22°26′17″S, 44°37′33″W); March 2016 (22°19′46″S, 44°32′11″W); July 2016 (22°26′16″S, 44°18′33″W) and October 2016 (22°27′38″S, 44°35′34″W). A total of 14 *P. mystaceus* were captured with mist nets. The birds were kept in individual boxes and faeces collected immediately after defecation. After identification of the species, the birds were photographed and released and stool 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 two software applications from CorelDRAW® (Corel Draw Graphics Suite, Version 11.0, Corel Corporation, Canada), i.e. Corel DRAW and Corel PHOTO-PAINT. All measurements are in micrometres and are given as the range followed by the mean in parentheses.

Molecular analyses

The oöcysts identified with the characteristic features of the new species under light microscopy, were isolated, 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 oöcysts, four freeze-thaw cycles were applied prior to the DNA extraction. The PCR amplification for the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) 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 *c*.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 *c.*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 3 µl 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 Biotecnology, were an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Foster City, California) was used for Sanger sequencing. The results of the sequencing reactions were analysed and edited using the program Chromas 2.6.

DNA sequence analyses

The newly generated sequences were 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 *cox*1 sequences for additional isolates from GenBank. Alignment and parsimony analyses were conducted using MEGA version 7 (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) 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 1000 replicates to assess the reliability of inferred tree topologies.

Results

Fourteen *P. mystaceus* were examined and three were positive for coccidia. All observed oöcysts were characteristic of *Isospora*. This material is described below.

Family Eimeriidae Minchin, 1903
Genus *Isospora* Schneider, 1881

Isospora lopesi Silva-Carvalho & Berto n. sp.

Type-host: *Platyrinchus mystaceus* Vieillot (Aves: Passeriformes: Tyranni: Tyrannidae), eastern white-throated spadebill.

Type-locality: Parque Nacional do Itatiaia (22°27′S, 44°35′W), southeastern Brazil.

Type-specimens: Photosyntypes, line drawing, and oöcysts in 70% ethanol are deposited at the Museu de Zoologia at the Universidade Federal Rural do Rio de Janeiro, Brazil, under the accession number MZURPTZ2017003. Phototypes and line drawings 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 the repository number P-79/2017. Photographs of the type-host specimen (symbiotype) are deposited in the same collection.

Site in host: Unknown.

Prevalence: 21% (3 out of 14 birds infected).

Representative DNA sequence: One representative *cox*1 sequence was deposited in the GenBank database under the accession number MF438267.

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) for *Isospora lopesi* is urn:lsid:zoobank.org:act:ED3F1187-FB31-439E-B5E2-9F3C0A050BE0.

Etymology: The specific name is derived from the family name of the Brazilian parasitologist Dr Carlos Wilson Gomes Lopes, given in his honor for his contribution to the study of Protozoa.

Description (Figs. 1, 2)

Sporulated oöcyst

Oöcysts (n = 25) subspheroidal to ovoidal, 18–24 × 18–22 (20.6 × 19.7); length/width (L/W) ratio 1.0–1.2 (1.05). Wall bi-layered, 1.3–1.6 (1.5) thick, outer layer smooth, *c.*2/3 of total thickness. Micropyle and oöcyst residuum both absent, but one polar granule is present.

Sporocyst and sporozoites

Sporocysts (n = 24) 2, ellipsoidal, 12–16 × 8–11 (14.4 × 8.6); L/W ratio 1.5–1.9 (1.7). Stieda body present, flattened to half-moon-shaped, 1.0 × 2.5; sub-Stieda present, rounded, 2.0 × 2.5; para-Stieda body absent; sporocyst residuum present, composed of scattered spherules of different sizes. Sporozoites 4, vermiform, with anterior and posterior refractile bodies and centrally located nucleus.

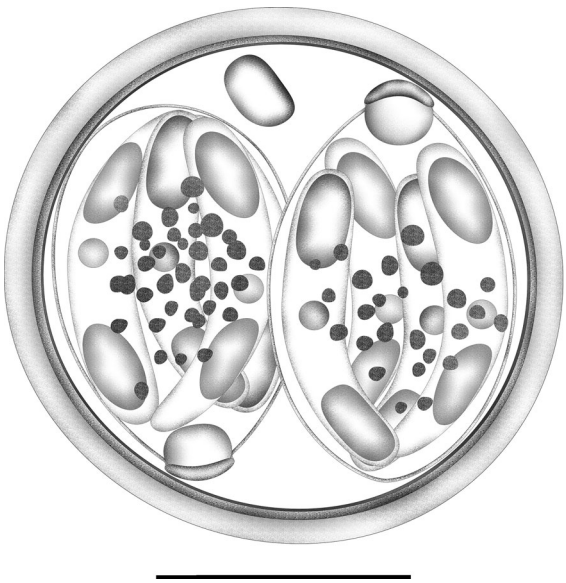

Fig. 1 Composite line drawing of the sporulated oöcyst of *Isospora lopesi* n. sp. ex *Platyrinchus mystaceus*. *Scale-bar*: 10 μm

Remarks

Following the evidence that demonstrate specificity at the familial level reported in papers on the taxonomy of coccidians of Passeriformes (Duszynski & Wilber, 1997; Berto et al., 2011; Silva et al., 2016), *I. lopesi* n. sp. was compared in detail with coccidian species that are morphologically similar and parasitise birds belonging to the same host family. To date, three *Isospora* spp. are recorded from hosts of the Tyrannidae (Table 1). *Isospora mionectesi* has oöcysts and sporocysts larger than *I. lopesi*. The oöcysts of *I. attilae* and *I. feroxis* have similar size and shape to *I. lopesi* n. sp.; however, it can be easily distinguished by the flattened to half-moon-shaped Stieda body. In addition, sporocysts of *I. lopesi* n. sp. are considerably more elongated than those of *I. feroxis* which is morphometrically demonstrated by the shape index (1.7 *vs* 1.4). *Isospora attilae* possesses sporocysts overlapping the size range for *I. lopesi* n. sp. However, the sporocyst in *I. attilae* is more tapered at the end of the Stieda body and has a smaller sporocyst residuum (Berto et al., 2009a, b; Rodrigues et al., 2015).

Phylogenetic analysis

DNA amplification of the oöcysts of *I. lopesi* n. sp. showed a clear band of *c.*250 bp. Phylogenetic analysis included 30 sequences for avian *Isospora* spp. and five sequences for *Eimeria* spp. available on GenBank (Fig. 3). *Toxoplasma gondii* (Nicolle & Manceaux, 1908) was used as the outgroup. *Isospora lopesi* n. sp.

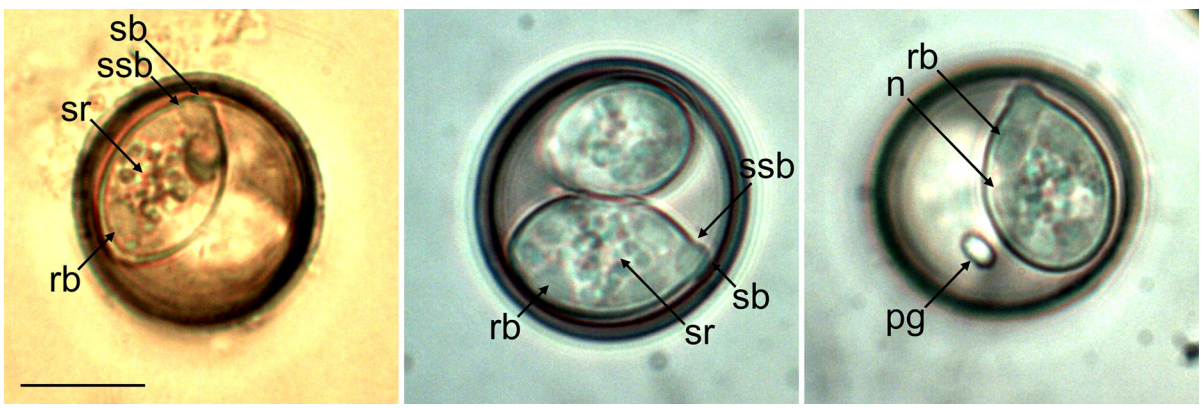

Fig. 2 Photomicrographs of sporulated oöcysts of *Isospora lopesi* n. sp. ex *Platyrinchus mystaceus*. *Abbreviations*: pg, polar granule; sb, Stieda body; ssb, sub-Stieda body; sr, sporocyst residuum; rb, refractile body; n, nucleus. All to same scale. *Scale-bar*: 10 μm

Table 1 Comparative morphology of *Isospora* spp. recorded from New World tyrannid birds (Tyrannidae)

Species	Host	Oöcyst				Sporocyst						Reference
		Shape	Size (μm)	Shape index	Polar granule	Shape	Size (μm)	Shape index	Stieda body	Sub-stieda body	Sporocyst residuum	
Isospora feroxis Berto, Luz, Flausino, Ferreira & Lopes, 2009	*Myiarchus ferox* (Gmelin)	Subspheroidal	18–20 × 7–20 (18.7 × 18.0)	1.0–1.1 (1.1)	Usually 2	Ovoidal	11–13 × 8–10 (11.7 × 8.5)	1.0–1.5 (1.4)	Ovoidal, flattened	Prominent	Diffuse	Berto et al. (2009a)
Isospora mionectesi Berto, Flausino, Luz, Ferreira & Lopes, 2009	*Mionectes rufiventris* Cabanis	Ellipsoidal	23–31 × 9–23 (28.3 × 21.2)	1.2–1.4 (1.3)	1 or 2	Elongate-ellipsoidal	17–22 × 0–13 (19.7 × 11.7)	1.6–1.8 (1.7)	Rounded	Prominent	Subspherical, compact	Berto et al. (2009b)
Isospora attilae Rodrigues, Silva, Lopes, Berto, Luz, Ferreira & Lopes, 2015	*Attila rufus* (Vieillot)	Subspheroidal to elipsoidal	18–22 × 18–21 (20.3 × 19.0)	1.0–1.2 (1.07)	1 or 2	Ellipsoidal	12–15 × 7–9 (13.5 × 7.9)	1.6–1.9 (1.7)	Knob-like	Rounded to trapezoidal	Diffuse	Rodrigues et al. (2015)
Isospora lopesi n. sp.	*Platyrinchus mystaceus* Vieillot, 1818	Subspheroidal to ovoidal	18–24 × 8–22 (20.6 × 19.7)	1.0–1.2 (1.05)	1	Ellipsoidal	12–16 × 8–11 (14.4 × 8.6)	1.5–1.9 (1.7)	Flattened to half-moon-shaped	Rounded	Diffuse	Present study

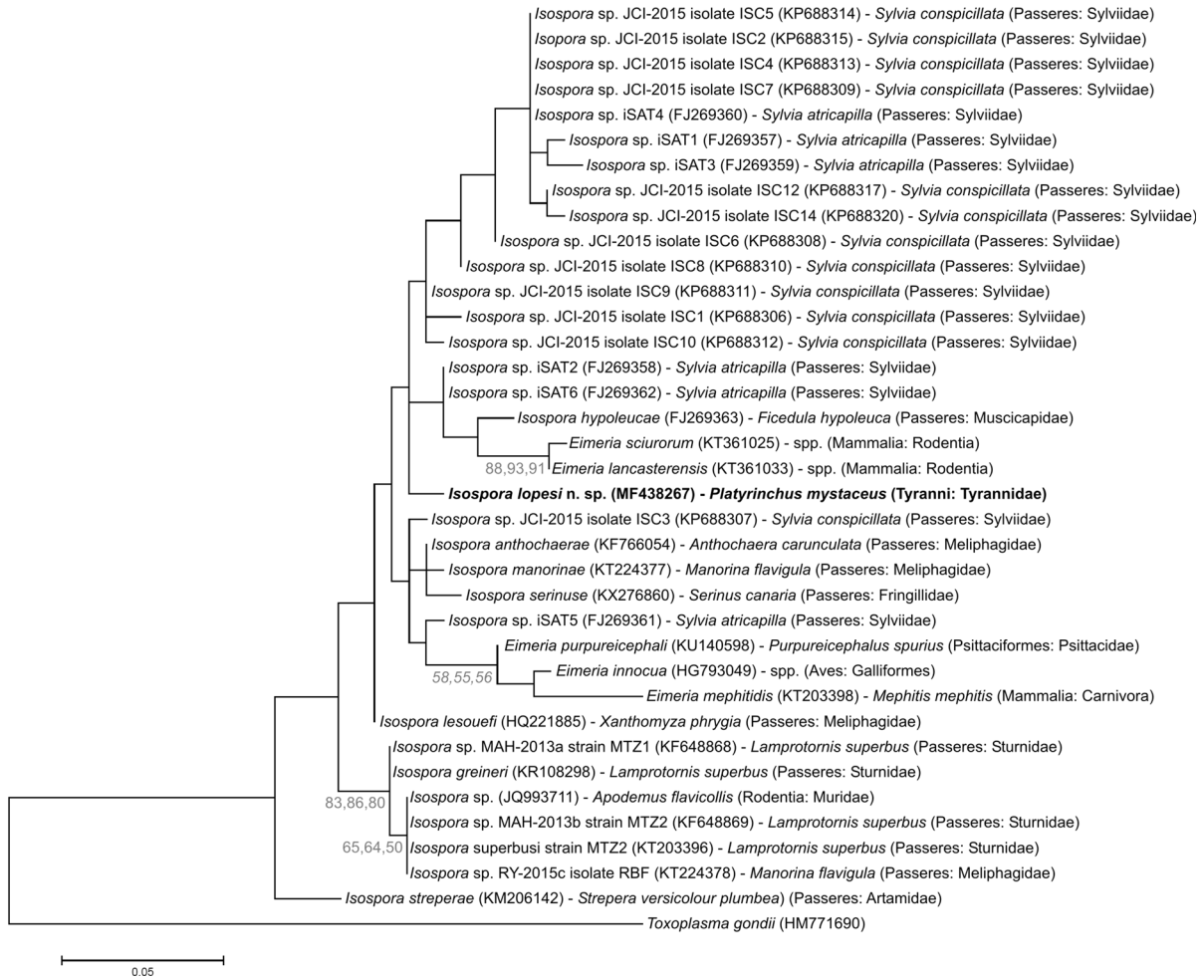

Fig. 3 Maximum likelihood tree estimated from the *cox*1 sequences. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining, Maximum Likelihood and Maximum Parsimony, respectively. The scale-bar represents the number of nucleotide substitutions per site

grouped with 16 *Isospora* spp. isolates from *Sylvia* spp., *Isospora hypoleucae* Dolnik, Rönn & Bensch, 2009 and two *Eimeria* spp. from rodents but with no statistical support. Among these, the new species showed the highest similarity of 98.5% and 98.0% with *Isospora* sp. isolates from the spectacled warbler *Sylvia conspicillata* Temminck (GenBank: KP688311 and KP688312; see Illera et al., 2015). In a second analysis, a subset of 215 bp long *cox*1 gene sequences for 10 *Isospora* spp. was used (Fig. 4). In this analysis, *I. lopesi* n. sp. was resolved as a basal sister taxon to a clade comprising *Isospora hypoleucae* Dolnik, Rönn & Bensch, 2009 and five *Isospora* sp. isolates from the Eurasian blackcaps *Sylvia atricapilla* (Linnaeus) with similarities of 96.1% and 95.1–97.5%, respectively.

Discussion

Isospora lopesi n. sp. is the first coccidian parasite of the Tyrannidae to have its *cox*1 sequence deposited in the GenBank database. As shown in the tree in Fig. 3, most of the available *cox*1 sequences for *Isospora* spp. from hosts of the Passeriformes are from the families Sylviidae, Meliphagidae and Sturnidae. All these families belongs to the suborder Passeres, while *P. mystaceus* belongs to the suborder Tyranni (suboscines), which contains families of primitive passerines that are phylogenetically distant from families of Passeres. Despite this, *I. lopesi* n. sp. exhibited high similarity with isolates of *Isospora* spp. from hosts of the Sylviidae (KP688311 and KP688312; 98.5% and

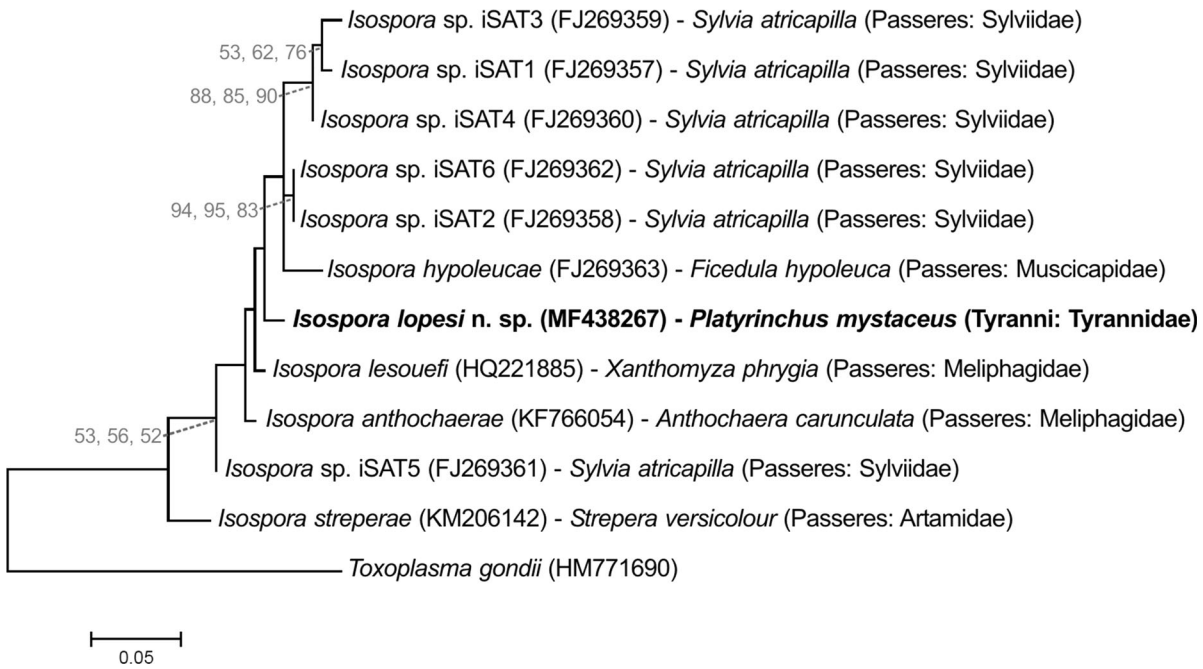

Fig. 4 Maximum likelihood tree estimated from the 215 bp long *cox*1 sequence dataset for *Isospora* spp. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining, Maximum Likelihood and Maximum Parsimony, respectively. The scale-bar represents the number of nucleotide substitutions per site

98.0%). We predict that when more *cox*1 sequences of *Isospora* spp. from hosts in the Tyranni are deposited in the GenBank database, this tree would rearrange to form one or more clades of *Isospora* spp. from suboscines, where *I. lopesi* n. sp. would be placed.

The 28S and 18S rRNA genes have been recognized as useful loci to differentiate *Isospora* spp. from passerines and to estimate their interspecific relationships (Yang et al., 2015, 2016; Tokiwa et al., 2017). Furthermore, the 18S rRNA is the most common locus used for molecular characterisation of coccidian parasites as evidenced by the large number of 18S rRNA sequences from coccidia on GenBank. However, in the present study the *cox*1 gene was chosen for genotyping because it is indicated as the most suitable for phylogenetic studies by having a higher resolving power than the 18S rRNA gene in delineating recent speciation events (Yang et al., 2015; Ogedengbe et al., 2011). In addition, in recent studies of Yang et al. (2015, 2016) with genotyping at 18S, 28S and *cox*1 loci of *Isospora* spp. from passerines, it was emphasised that the *cox*1 PCR primers originally used by Dolnik et al. (2009), which generate 215 bp amplicons, may be more reliable for species delimitation of *Isospora* spp. In this sense, the primers of Dolnik et al. (2009) were chosen for genotyping of *I. lopesi* in the present study. Following Yang et al. (2015, 2016), phylogenetic analyzes were initially constructed comparing *I. lopesi* n. sp. with the most similar *cox*1 sequences of varying lengths deposited in GenBank (Fig. 3) and, subsequently, only with *Isospora* sequences with a length of *c.*215 bp (Fig. 4).

The Tyrannidae is one of the largest families of New World passerine birds (BirdLife International, 2016); however, a few coccidian species are reported from this family (Berto & Lopes, 2013). The study of coccidia from hosts of the Tyrannidae becomes more relevant because two species of *Eimeria*, which is an uncommon coccidian parasite genus of Passeriformes, are described from birds of this family (Berto et al., 2008, 2009a). Allied to this, it is also important to know which of *Isospora* spp. have an extra-intestinal cycle, as species with this cycle are recognized to be more pathogenic causing the so-called atoxoplasmosis (Berto et al., 2011). These extra-intestinal *Isospora* spp. were previously identified and/or named as *Lankesterella* Labbé, 1899 or *Atoxoplasma* Garnham, 1950, until Box (1981) associated the extra-intestinal merogony in canaries with an *Isospora* infection. In this way, all blood forms

described as *Atoxoplasma* and *Lankesterella* in passerines were redescribed as *Isospora* (see Berto et al., 2011). Currently, with the molecular study and with the increasing number of *Isospora* spp. sequences on GenBank, it is possible to identify if a species with morphological and molecular description possesses an extra-intestinal cycle through the molecular detection by PCR/sequencing in samples of blood from passerines (Dolnik et al., 2009; Hafeez et al., 2014). The identification of extra-intestinal species which may have the potential to cause severe coccidiosis (atoxoplasmosis) may help classify hosts and/or families more or less susceptible to the disease, and therefore, guide decision-making for conservation, principally in protected areas.

Based on the morphological features described above, *I. lopesi* is considered as new to science and the fourth isosporoid coccidian species reported from a New World tyrannid bird. In addition, this is the first coccidian parasite of a host of the Tyranni to have a molecular characterisation of the *cox*1 gene.

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Compliance with ethical standards

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

Ethical approval Field collecting permits were issued to BPB by SISBIO/ICMBio (license No. 49605–1) and CEUA/ICBS/UFRRJ (protocol No. 008/2015). All applicable institutional, national and international guidelines for the care and use of animals were followed.

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