Isospora sagittulae *McQuistion & Capparella, 1992 (Apicomplexa: Eimeriidae) from antbirds (Passeriformes: Thamnophilidae) in the Amazon and Atlantic Forest of Brazil: with notes on its distribution and dispersion in the Neotropical region*

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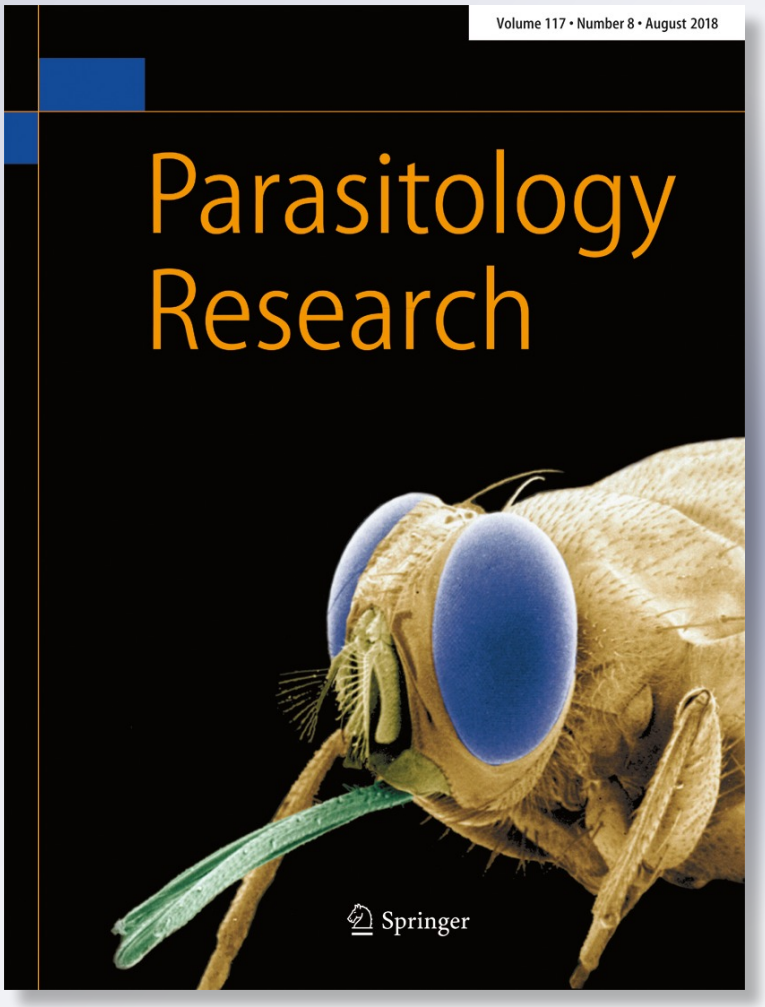

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ORIGINAL PAPER

Isospora sagittulae McQuistion & Capparella, 1992 (Apicomplexa: Eimeriidae) from antbirds (Passeriformes: Thamnophilidae) in the Amazon and Atlantic Forest of Brazil: with notes on its distribution and dispersion in the Neotropical region

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Abstract

In the current study, *Isospora sagittulae* McQuistion and Capparella, 1992 (Protozoa: Apicomplexa: Eimeriidae) is reported from white-shouldered fire-eyes *Pyriglena leucoptera* (Vieillot, 1818) in the Atlantic Forest in southeastern Brazil. To date, this coccidian species was described from antbirds in Ecuador and Brazilian Amazon. In this sense, oocysts and measurements of the description of *I. sagittulae* from Amazonian antbirds were required from the deposit for comparison between samples from the Amazon and Atlantic Forest. The morphology was similar in all aspects, despite the polymorphism associated with the oocyst shape. DNA sequences for the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) locus of the oocysts had similarity of 100%. Therefore, these strong morphological, molecular, and ecological equivalences ensure the unique identification of *I. sagittulae*. Finally, this finding reveals the wide distribution of *I. sagittulae* in the Neotropical region and indicates that other antbirds in the Brazilian Cerrado should disperse *I. sagittulae* to the Amazon and Atlantic Forest.

Keywords Taxonomy · Morphology · Phylogeny · Oocysts · Geographic ranges · Parque Nacional do Itatiaia

Introduction

Thamnophilidae is a family of insectivorous passerine birds that comprises 241 species restricted to the plains and low-altitude forests of the Caribbean Islands, Mesoamerica, and South America. Evidently, due to the large number of species, it is a highly polymorphic family, but it is predominantly composed of wild birds with insectivorous feeding habits (Sick 1997; CBRO 2014; BirdLife International 2016).

Despite this apparent restriction at low altitudes and the limitation of their distributions to geographical barriers in the Neotropical region, some antbirds appear to cross strong geographical barriers, or at least they have populations that are not limited or blocked by these geographical barriers (Sick 1997; BirdLife International 2016).

The possibility of antbirds crossing strong geographical barriers was assumed by Berto et al. (2014a), which evidenced a coccidial dispersion across *trans*- and *cis*-Andean regions by antbirds that inhabit both regions and possibly cross the Andes mountains. The eimeriid coccidia of birds has predominantly oral-fecal transmission; i.e., it occurs through the ingestion of oocysts, which are the infective stages of the coccidia shed in the feces of the host. Therefore, the transmission of coccidia necessarily occurs when susceptible hosts inhabit the same environment. In Berto et al. (2014a), the coccidian parasite

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Isospora sagittulae McQuistion & Capparella, 1992 was reported from white-throated antbirds *Oneillornis salvini* (Berlepsch, 1901) (syn. *Gymnopithys salvini*) and from scale-backed antbirds *Willisornis poecilinotus* (Cabanis, 1847) in Brazilian Amazon, in the *cis*-Andean region; however, this parasite had been originally described from *Hylophylax naevioides* Lafresnaye, 1847, a thamnophilid bird that has a *trans*-Andean distribution in Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, and Panama (McQuiston and Capparella 1992). In this sense, it was presumed that the transmission occurred through antbirds with both *trans*- and *cis*-Andean distributions, as *Cercomacra tyrannina* Sclater, 1855 and *Formicivora grisea* (Boddaert, 1783), which could have maintained and transmitted *I. sagittulae* across the Andes.

In this context, the current work aims to contribute to the knowledge on the identification, distribution, and dispersion of *I. sagittulae* in thamnophilid birds in the Neotropical region, reporting the white-shouldered fire-eye *Pyriglena leucoptera* (Vieillot, 1818) as a new host in the Atlantic Forest of southeastern Brazil and thus demonstrating the wide dispersion of *I. sagittulae*, in addition to morphologically and molecularly comparing the samples of the Amazon and Atlantic Forest.

Materials and methods

Sample collection

A total of 12 expeditions were conducted in 2 different localities in southeastern Brazil: (1) Parque Nacional do Itatiaia, 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), and (2) Cacaria at the Municipality of Piraí in the State of the Rio de Janeiro. A total of 20 white-shouldered fire-eye *P. leucoptera* were captured with mist nets. The birds were kept in individual boxes with clean ground paper. After identification of the species, the bird was photographed and released. A fresh droplet of feces from each individual bird was placed in an individually centrifuge tube with a potassium dichromate 2.5% ($K_2Cr_2O_7$) solution.

Obtaining the Amazon samples

Oocysts in 70% ethanol and the original measurements of *I. sagittulae* from *O. salvini* and *W. poecilinotus* identified in Berto et al. (2014a), 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 52/2014, were required for morphological and molecular comparison.

Morphological analyses

Samples were carried to the Laboratório de Biologia de Coccídios, Universidade Federal Rural do Rio de Janeiro (UFRRJ). Samples were incubated at room temperature for 10 days or until ~70% of the oocysts were sporulated. Oocysts were isolated by flotation in Sheather's sugar solution (specific gravity 1.20) and examined microscopically using the technique described by Duszynski and Wilber (1997) and Berto et al. (2014b). 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). All measurements are in micrometers and are given as the range followed by the mean in parentheses.

Statistical evaluation

Two parametric statistical methods were employed after previous evaluation of the data by D'Agostino's test of normality: (1) analysis of variance (ANOVA) was used to compare measurements of the length, width, and shape index of the oocysts and sporocysts recovered from *O. salvini*, *W. poecilinotus*, and *P. leucoptera*. The statistical package Bioestat 5.0 (Ayres et al. 2007) was used to calculate the mean, variance, degree of freedom, and p value (Sampaio 2002; Berto et al. 2014b) and (2) linear regression to determine the distribution of oocysts recovered from *O. salvini* and *W. poecilinotus* in Amazon and *P. leucoptera* in Atlantic Forest using methods proposed by Norton and Joyner (1981) and subsequently modified by Berto et al. (2014b). The graphs and coefficient of regression line were obtained using the software Microsoft Excel 2007® (Microsoft, Redmond, WA).

Molecular analyses

The oocysts of *I. sagittulae* from *O. salvini*, *W. poecilinotus*, and *P. leucoptera* were isolated on the microscopic slide, resuspended in PBS, and washed by centrifuging until the supernatant become clear (Dolnik et al. 2009). DNA was extracted from the purified oocysts using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, São Paulo, Brazil) according to the manufacturer's instructions. In order to fully lyse the oocysts, four freeze-thaw cycles were applied prior to the DNA extraction. The PCR amplification for the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) 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 and COIbR1 5′-CCA AGA GAT AAT ACR AAR TGG AA produced a PCR product size of ~302 pb. The internal primes COIbF2 5′-GGG CAC ATC ATA TGA TGA C and COIbR2 5′-ATA GTA

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TGT ATC ATG TAR WGC AA produced an amplicon size of ~257 pb. 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, and 3 μL of DNA (for primary reaction) or 3 μL primary PCR product (for secondary reaction). Both primary and secondary PCRs 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 PCRs were purified using the Qiagen MinElute PCR Purification (Qiagen, São Paulo, Brazil). All the PCR products were sequenced using forward and reverse primers by Ludwig Biotechnology, where an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) was used for Sanger sequencing. The results of the sequencing reactions were analyzed and edited using the program Chromas 2.6.

DNA sequence analyses

The newly generated sequences were compared to each other and with other coccidian parasite sequences available on GenBank using the "Basic Local Alignment Search Tool" (BLAST). Phylogenetic trees were constructed for *Isospora* spp. at the *cox1* locus with additional isolates from GenBank/EMBL/DDBJ. Alignment and parsimony analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis software, version 7; Arizona State University, Tempe, AZ, USA). The evolutionary history was inferred using the maximum likelihood (ML) and neighbor-joining (NJ) methods, and the distances were computed using the Tamura-Nei method based on model selection using ModelTest in MEGA. Bootstrap analyses were conducted using 1000 replicates to assess the reliability of inferred tree topologies.

Results

Prevalence and morphology

Nine of 18 *P. leucoptera* captured in the Parque Nacional do Itatiaia were positive for *I. sagittulae*. The two *P. leucoptera* captured in Cacaria were negative. Its oocysts (Fig. 1c, d) were irregularly ovoidal to ellipsoidal, 30.8 (29–33) × 24.4 (22–26) μm, with shape index of 1.3 (1.2–1.5). Oocyst wall bilayered and smooth, 1.1 μm. Micropyle, oocyst residuum is absent, but 1–3 (usually 2) polar granules are present. Sporocysts subspherical to ovoidal, 15.9 (14–17) × 13.4 (12–

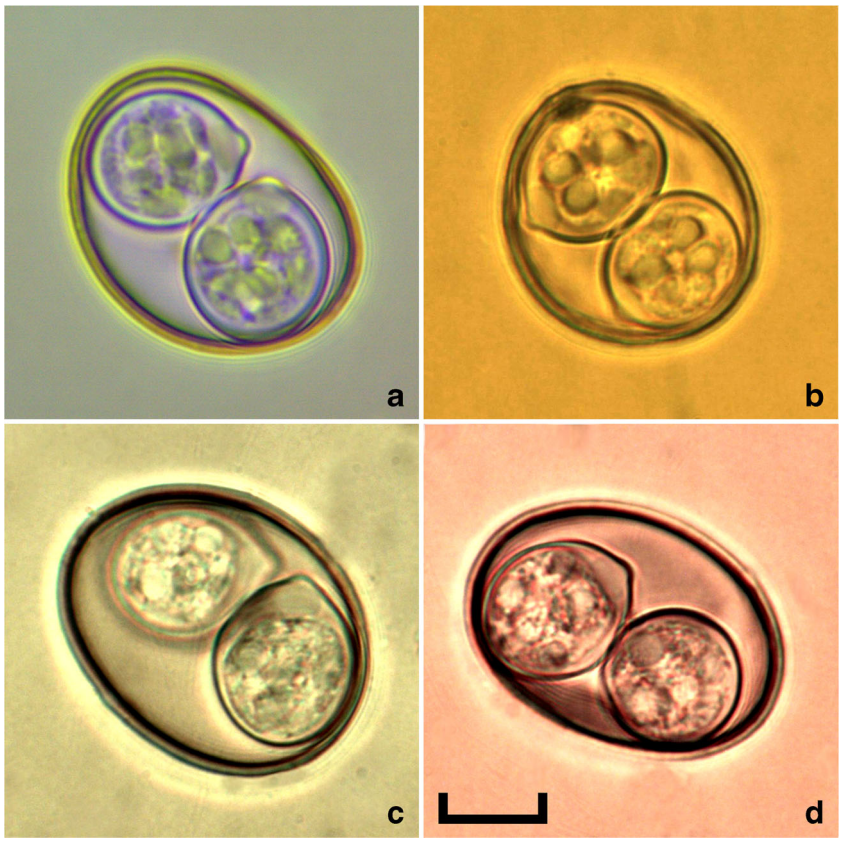

Fig. 1 Photomicrographs of *Isospora sagittulae* recovered from white-throated antbirds *Oneillornis salvini* (**a**), common scale-backed antbirds *Willisornis poecilinotus* (**b**), and white-shouldered fire-eyes *Pyriglena leucoptera* (**c**, **d**). Scale bar: 10 μm

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Table 1 Morphometry of *Isospora sagittulae* oocysts recovered from antbirds (Thamnophilidae)

Host	Locality	n^a	Oocysts			Sporocysts		
			Length (μm)	Width (μm)	Shape index[b]	Length (μm)	Width (μm)	Shape index[b]
Hylophylax naevioides (Lafresnaye, 1847)	Northwestern Ecuador	21	25–30 (27.5)	21–24 (21.8)	1.27	13–16 (14.8)	12–13 (12.4)	1.1–1.3 (1.19)
Oneillornis salvini (Berlepsch, 1901)	Brazilian Amazon	8	27–31 (29.5) a	21–24 (22.8) a,b	1.2–1.3 (1.29) a	14–17 (15.5) a	12–14 (12.9) a	1.1–1.3 (1.20) a
Willisornis poecilinotus (Cabanis, 1847)	Brazilian Amazon	7	27–28 (27.2) b	20–25 (22.0) a	1.1–1.4 (1.25) a	13–16 (14.6) a	12–13 (12.3) a	1.1–1.2 (1.19) a
Pyriglena leucoptera (Vieillot, 1818)	Brazilian Atlantic Forest	27	29–33 (30.8) a	22–26 (24.4) b	1.2–1.5 (1.27) a	14–17 (15.9) a	12–15 (13.4) a	1.1–1.3 (1.19) a

Different letters in each column denote statistically significant differences ($P < 0.01$) by ANOVA

[a] Total number of oocysts measured

[b] Length/width ratio

15) μm, with shape index of 1.2 (1.1–1.3). Stieda body thin and flattened, 0.5 high × 2.0 wide. Substieda body triangular to rounded, 2.5 high × 5.0 wide. Parastieda body absent. Sporocyst residuum composed of scattered granules. Sporozoites with a prominent posterior refractile body and a small nucleus.

Material deposited

Photovouchers and oocysts in 70% ethanol are deposited at the Museu de Zoologia at the Universidade Federal Rural do Rio de Janeiro, Brazil, under accession number MZURPTZ2017005. Photovouchers 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 number 86/2017. Photographs of the host specimens are deposited in the same collection.

Comparative morphometry

Comparative analyses by ANOVA between the oocysts and sporocysts of *I. sagittulae* recovered from *O. salvini*, *W. poecilinotus*, and *P. leucoptera* revealed some significant differences in the size of the oocysts (Table 1). These significant differences and the high irregularity of the shape of the oocysts are also observed in the distribution of measurements in linear regression (Fig. 2).

DNA sequences

DNA amplification of the oocysts of *I. sagittulae* showed a clear band around ~250 bp. DNA sequences of the oocysts recovered from *P. leucoptera* and *W. poecilinotus* had similarity of 100%, and have been deposited in GenBank under the accession numbers MF981004 and MF981005, respectively.

Phylogenetic analysis

Phylogenetic analysis included 32 sequences from *Isospora* spp. available in GenBank (Fig. 3). *Eimeria tenella* (Railliet and Lucet, 1891) was used as the outgroup. *Isospora sagittulae* from *W. poecilinotus* and *P. leucoptera* sat in a clade with the highest similarity of 99.5% with *Isospora lopesi* Silva-Carvalho and Berto, 2018 (Silva-Carvalho et al. 2018) deposited under accession number MF438267.

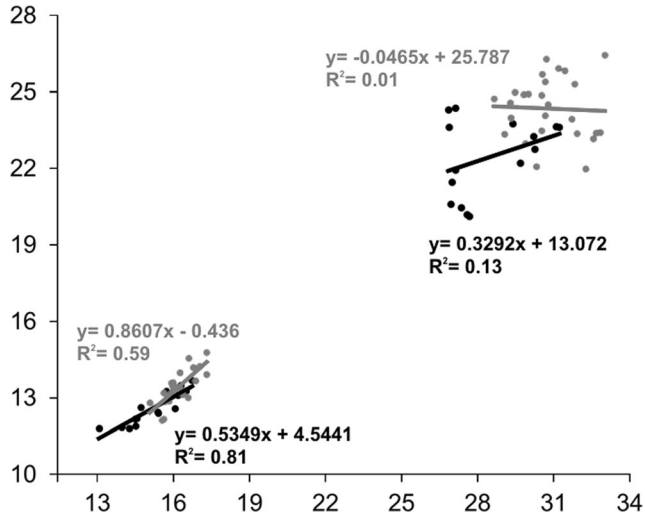

Fig. 2 Comparative linear regressions of oocysts (*above*) and sporocysts (*below*) of *Isospora sagittulae* recovered from *O. salvini* and *W. poecilinotus* in Amazon (black) and *P. leucoptera* in Atlantic Forest (gray)

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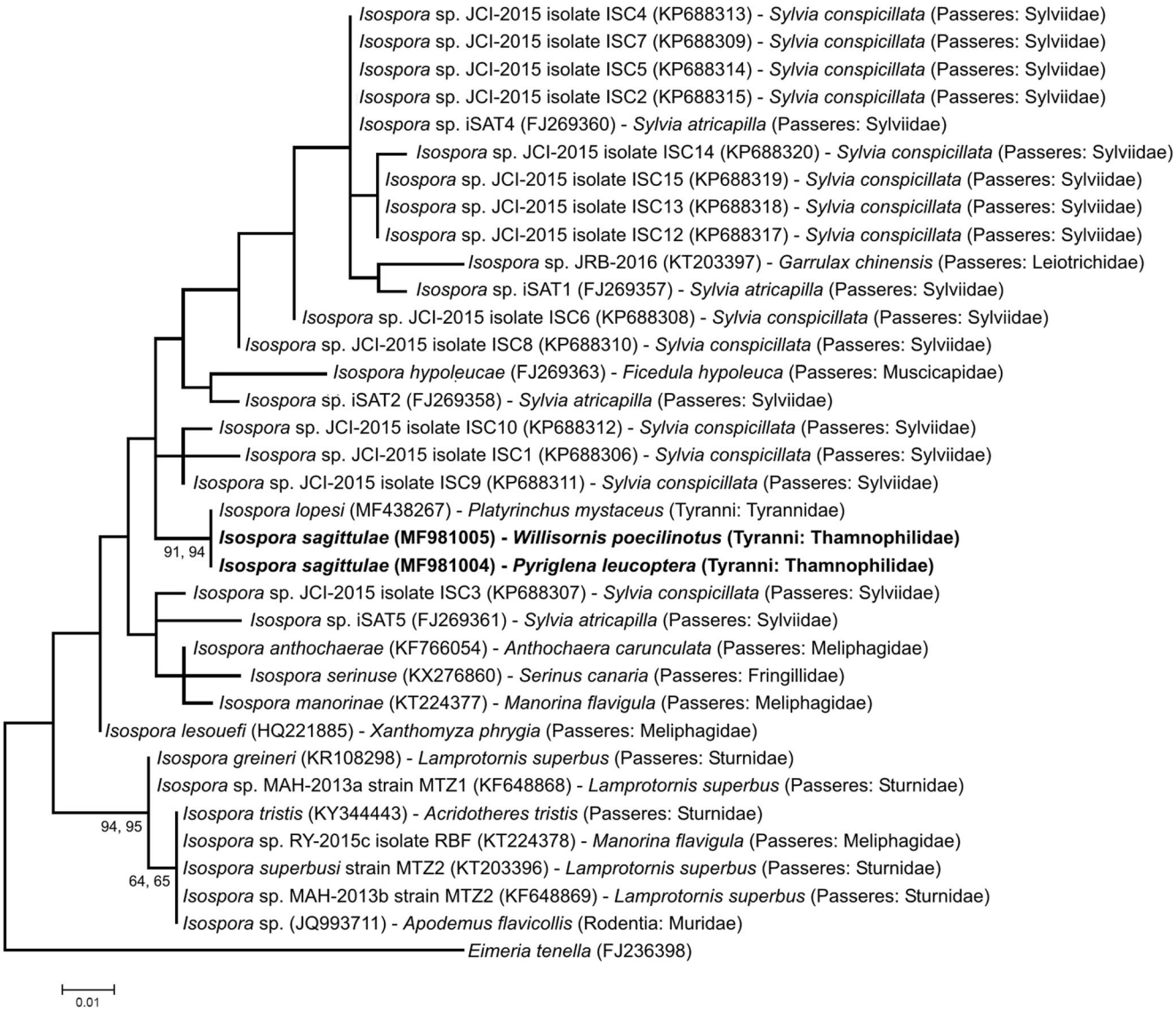

Fig. 3 Maximum likelihood tree estimated from the *cox1* gene sequences of *Isospora* spp. Numbers at nodes represent bootstrap support 1000 replicates (> 50%) for neighbor-joining (NJ) and maximum likelihood (ML), respectively. Scale bar represents the number of nucleotide substitutions per site

Discussion

Morphological and morphometric characterization of the oocysts from the Amazon and Atlantic Forest

In the current work, a larger number of oocysts of *I. sagittulae* was observed (Table 1), which allowed to verify the irregularity in the size and shape of their oocysts. Observing the linear regression in Fig. 2, it is verified that the data points are distant from the regression line, indicating irregularity in the distribution of measurements. In addition, the very low R^2 values obtained from both samples from Amazon and Atlantic Forest of Brazil clearly reveal the polymorphism of the oocysts of *I. sagittulae* (Berto et al. 2014b). This irregular shape was also morphologically observed in some oocysts, mainly in some with boomerang-like shape (Fig. 1a, c) or other irregular shapes.

This polymorphism has already been related in the literature as being intrinsic to a coccidian species, derived from the different positions of the oocysts during the measurements and/or related to several environmental and host factors, such as stress, nutrition, immunity of the host, the infecting dose, the time of oocysts discharge during the patent period, and phenotypic plasticity (Fayer 1980; Parker and Duszynski 1986; Gardner and Duszynski 1990; Berto et al. 2014b).

Molecular identification and species delimitation

The genotypic similarity of 100% for the *cox1* locus observed between the samples from *W. poecilinotus* and *P. leucoptera* corroborates with the morphological similarity between these

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same samples; therefore, these results emphasize that the oocysts recovered from the hosts of Amazon and Atlantic Forest in Brazil belong to the same species, *I. sagittulae*. The *cox1* gene was chosen for genotyping in the current study because it has been 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 *cox1* loci of *Isospora* spp. from passerines, it was emphasized that the *cox1* PCR primers originally used by Dolnik et al. (2009), which generate 215-bp amplicons, may be more reliable for species delimitation of *Isospora* spp.

The delimitation of coccidian species has been overestimated or underestimated in some works. This discussion was raised by Silva et al. (2016), who highlighted studies where about 1% of genotypic difference resulted in separation in two species, and others where about 3% of genotypic difference did not result in species separation. We agree with Silva et al. (2016), who based the species identification in the guidelines of Duszynski and Wilber (1997), where oocysts should be compared with coccidian species that are feature-similar and belong to the same host family; furthermore, we understand that a species must be identified and classified according to all its morphological, biological, ecological, and molecular characteristics. In other words, according to Kunz (2002), the criterion for identification of new species cannot be just on the basis of a certain number of base exchanges within DNA sequence. Anyway, the strong morphological, molecular, and ecological (host specificity) equivalences of the oocysts observed in the present work ensure the unique identification of *I. sagittulae*.

Phylogenetic analysis

Phylogenetic analysis (Fig. 3) revealed that *I. sagittulae* is closer (about 99.5%) to *I. lopesi*, and more distant (about ~97%) from *Isospora* spp. isolated from an Old World warbler, *S. conspicillata* (Illera et al. 2015), which is phylogenetically and geographically distant from the thamnophilid hosts of *I. sagittulae* (delHoyo et al. 2016). Together with the results of Silva-Carvalho et al. (2018), this result evidences the tendency of the formation of a clade containing *Isospora* spp. of primitive passerines (suborder Tyranni), which should be confirmed while more *Isospora* spp. from Tyranni are sequenced by *cox1* gene. In this way, this observation strengthens the coevolution concept of the coccidian parasites and its hosts (Odum 1998).

Distribution and dispersion of *I. sagittulae* in the Neotropical region

Additionally, the results of the current study reinforce the assumption of dispersion of *I. sagittulae* across the Andes, introduced by Berto et al. (2014a). The morphological and molecular identification of *I. sagittulae* in southeastern Brazil reveals the wide dispersion of *I. sagittulae* in Brazil; therefore, it is assumed that *I. sagittulae* is distributed throughout the Neotropical region where antbirds occur.

Just as in Berto et al. (2014a), the hosts for *I. sagittulae* in the current work are not sympatric. *Oneillornis salvini* and *W. poecilinotus* are endemic to the Amazon, while *P. leucoptera* is endemic to the Atlantic Forest. As shown in Fig. 4, the Cerrado biome is located between these two biomes and, consequently, between the geographic ranges of these species. Thus, it is assumed that antbirds in the Cerrado must also be parasitized by *I. sagittulae* and thus they can transmit and disperse *I. sagittulae* to antbirds in the Amazon and Atlantic Forest. Examples of antbirds that are distributed in the Cerrado and could fulfill this function would be the plain antvireo *Dysithamnus mentalis* (Temminck, 1823), the black-bellied antwren *Formicivora melanogaster* (Pelzeln, 1868), the rusty-backed antwren *Formicivora rufa* (Wied, 1831), the black-capped antwren *Herpsilochmus atricapillus* (Pelzeln, 1868), the great antshrike *Taraba major* (Vieillot, 1816), the barred antshrike *Thamnophilus doliatus*

Fig. 4 Geographic range of the thamnophilid hosts of *Isospora sagittulae* in the Neotropical region (based on data from BirdLife International 2016. Only *Oneillornis salvini* and *Willisornis poecilinotus* are sympatric with each other in the Amazon. *Hylophylax naevioides* is separated by the Andes. The new host *Pyriglena leucoptera* has geographic range in the Altlantic Forest in southeastern Brazil, and is separated from the populations of the Amazonian hosts by the Cerrado biome

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(Linnaeus, 1764), the planalto slaty antshrike *Thamnophilus pelzelni* (Hellmayr, 1924), and the rufous-winged antshrike *Thamnophilus torquatus* (Swainson, 1825) (BirdLife International 2016). Among these species, *D. mentalis*, *T. major*, and *T. doliatus* can be highlighted because they have *trans*-Andean and *cis*-Andean distributions, including the Brazilian biomes of Atlantic Forest, Cerrado, and Amazon (BirdLife International 2016); therefore, these species are potential dispersers of *I. sagittulae* in the Neotropical region.

Conclusions

In conclusion, based on all the results reported in the current study, *P. leucoptera* is recorded as a new host for *I. sagittulae* in the Atlantic Forest in southeastern Brazil, revealing the wide distribution of this coccidian species in the Neotropical region; in addition, *I. sagittulae* is molecularly identified from *P. leucoptera* and *W. poecilinotus*, in Atlantic Forest and Amazon, respectively, corroborating with the previous morphological identifications.

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