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The vulnerable *Sporophila frontalis* (Verreaux) and *Haplospiza unicolor* Cabanis as new hosts for *Isospora sporophilae* Carvalho-Filho, Meireles, Ribeiro & Lopes, 2005 (Eimeriidae) in Brazil

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Abstract *Isospora sporophilae* Carvalho-Filho, Meireles, Ribeiro & Lopes, 2005 was morphologically and molecularly identified from the double-collared seedeater *Sporophila frontalis* (Verreaux), which is categorised as 'vulnerable' by the International Union for Conservation of Nature and Natural Resources (IUCN), and from the uniform finch *Haplospiza unicolor* Cabanis in conserved and anthropomorphic/fragmented areas of Atlantic Forest in the southeastern Brazil. The oöcysts recovered from *S. frontalis* and *H. unicolor* had small morphological and genotypic differences that were not considered sufficient for the description of new species, but only different genotypes of *I. sporophilae* related to each host. This coccidian species was originally described from double-collared seedeaters *Sporophila caerulescens* (Vieillot) in a center screening of wild animals; therefore, this new report emphasises a potential occurrence of anthropomorphic dispersion of coccidia through illegal trade, seizures and reintroductions in the wild.

Introduction

Thraupidae Cabanis comprises the second largest family of the order Passeriformes with 408 species distributed predominantly in the Neotropical region

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(BirdLife International, 2016). This family comprises passerines with varied colors, vocalizations, foraging behaviors, ecotypes and habitat preferences (Sick, 1997; Burns et al., 2014).

Recently there was a redistribution of genera in the families of Passeriformes, which considerably expanded the number of genera/species in the Thraupidae. In this redistribution, seedeaters of the genus *Sporophila* and several other genera previously classified in the families Emberizidae and Cardinalidae were included in the Thraupidae (Burns et al., 2014; CBRO, 2014; BirdLife International, 2016; del Hoyo et al., 2016; Brands, 2018).

The beauty and vocal repertoire of the tanagers and seedeaters makes them valuable as companion animals, thereby stimulating captive breeding for legal trade, but also illegal wildlife trade. For this reason, the thraupids are one of the main wild birds, victims of the illegal trade in countries of the Neotropical region. This can be observed in the centers screening of wild animals, which has as one of the functions the rehabilitation and release of wild animals seized from the illegal trade. In these centers, the thraupids are often the most abundant animals (Carvalho-Filho et al., 2005; Berto & Lopes, 2013; Lopes et al., 2013).

In addition to the direct impact of the illegal trade on the wild birds, this activity enables and/or enhances the dispersion of parasites in an unnatural (anthropomorphic) way. Coccidia are one of the principal parasite groups of thraupids, which can be easily transmitted *via* the oral-faecal route, i.e. a thraupid transported from one environment to the other carries its coccidia which could be transmitted to another susceptible thraupid, in both natural and captive environments. This understanding makes the function of the centers screening of wild animals more important, since the failure to identify a parasite of a thraupid seized, followed by its release in the wild, different from its original locality, would provide the introduction of a new parasite to susceptible hosts in a new locality (Berto & Lopes, 2013; Lopes et al., 2013).

In the present study *Isospora sporophilae* Carvalho-Filho, Meireles, Ribeiro & Lopes, 2005, which was originally described from double-collared seedeaters *Sporophila caerulescens* (Vieillot) in a center screening of wild animals (Carvalho-Filho et al., 2005), was morphologically and molecularly identified from two new hosts: (i) the buffy-fronted seedeater *Sporophila frontalis* (Verreaux), which is categorised as 'vulnerable' by the International Union for Conservation of Nature and Natural Resources (IUCN) (BirdLife International, 2016), in a preserved Atlantic Forest area corresponding to the Itatiaia National Park (Parque Nacional do Itatiaia); and (ii) the uniform finch *Haplospiza unicolor* Cabanis in a fragmented Atlantic Forest area in the district of Cacaria, Piraí, all in the southeastern Brazil.

Materials and methods

Sample collection

A total of eight expeditions were conducted in two different localities in southeastern Brazil: (i) 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, 2016), in August and December 2014, April and May 2015, July 2017, and April and July 2017; and (ii) Cacaria, a district of the Municipality of Piraí in the State of the Rio de Janeiro, in September 2016. A total of eight *S. frontalis* (all from Itatiaia National Park) and nine *H. unicolor* (eight from Itatiaia National Park and one from Cacaria) were captured with mist nets. 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 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 *c*.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.

Morphometric analyses

Two parametric statistical methods were employed in the morphometric data of the oöcysts after previous evaluation of the data by D'Agostinos test of normality. Analysis of variance (ANOVA) was used to compare measurements of the length, width and length/width (L/W) ratio of the oöcysts and sporocysts recovered from *S. frontalis* and *H. unicolor*. The statistical package Bioestat 5.0 (Ayres et al., 2007) was used to calculate the mean, variance, degrees of freedom and P-value (Sampaio, 2002; Berto et al., 2014). Linear regression was used to determine the distribution of oöcysts recovered from *S. frontalis* and *H. unicolor* using methods proposed by Norton & Joyner (1981) and subsequently modified by Berto et al. (2014). The graphs and coefficient of regression line were obtained using the software Microsoft Excel 2007® (Microsoft, Redmond, Washington).

Molecular analyses

The oöcysts identified with the same characteristic features 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 manufacturers 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 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 Purfication (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

Sequences were compared to each other and with 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) 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.

Results

Four *S. frontalis* from the Itatiaia National Park and one *H. unicolor* from Cacaria 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 sporophilae* Carvalho-Filho, Meireles, Ribeiro & Lopes, 2005**

Hosts: *Sporophila frontalis* (Verreaux) (Aves: Passeriformes: Thraupidae: Sporophilinae), buffy-fronted

seedeater; *Haplospiza unicolor* Cabanis (Aves: Passeriformes: Thraupidae: Diglossinae), uniform finch.
Localities: Parque Nacional do Itatiaia (22°27′S, 44°35′W) and Cacaria (22°42′S, 43°50′W), both in southeastern Brazil.
Specimens: Photomicrographs, line drawing and oöcysts recovered from *S. frontalis* in 2.5% $K_2Cr_2O_7$ solution (Williams et al., 2010) are deposited at the Museu de Zoologia at the Universidade Federal Rural do Rio de Janeiro, Brazil, under accession number MZURPTZ2018006. Photomicrographs and line drawing 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 88/2018 (*S. frontalis*) and 89/2018 (*H. unicolor*). Photographs of the host specimens are deposited in the same collection.
Site in host: Unknown.
Prevalence: 29% (5 out of 17 birds infected).
Representative DNA sequence: Representative *cox*1 sequences were deposited in the GenBank database under the accession numbers MH464545 (from *S. frontalis*) and MH464544 (from *H. unicolor*).

Description (Figs. 1, 2)

Sporulated oöcyst
Oöcysts (n = 40) subspheroidal, 20−25 × 20−24 (22.7 × 21.7); L/W ratio 1.0−1.1 (1.05). Wall bilayered, 1.0−1.2 (1.1) thick, outer layer smooth, *c.*2/3 of total thickness. Micropyle and oöcyst residuum both absent, but splinter-like or comma-like polar granules are usually present.

Sporocyst and sporozoites
Sporocysts (n = 37) 2, ovoidal, 13−17 × 9−10 (15.4 × 9.7); L/W ratio 1.3−1.9 (1.6). Stieda body present, thin and flattened, 0.5 × 2.0; sub-Stieda present, barely discernible or wide to rounded, 1.0 × 2.5; para-Stieda body absent; sporocyst residuum present, composed of few scattered granules. Sporozoites 4, vermiform, with a prominent posterior refractile body and barely discernible nucleus and striations.

Remarks

The oöcysts recovered and observed in the present study were morphologically and morphometrically equivalent with the original description of Carvalho-Filho et al. (2005) from *S. caerulescens*, which belongs to the same family of *S. frontalis* and *H. unicolor*. The only exception would be the presence of sub-Stieda body, which is not in the original description, but can be seen in the photomicrographs provided in Carvalho-Filho et al. (2005). Added to this, comparative analyses by ANOVA between the oöcysts and sporocysts of *I. sporophilae* recovered from *S. frontalis* and *H. unicolor* showed equivalent means among all morphometric data (Table 1). This equivalence and the regularity of the shape of the oöcysts are also observed in the distribution of measurements in linear regression (Fig. 3).

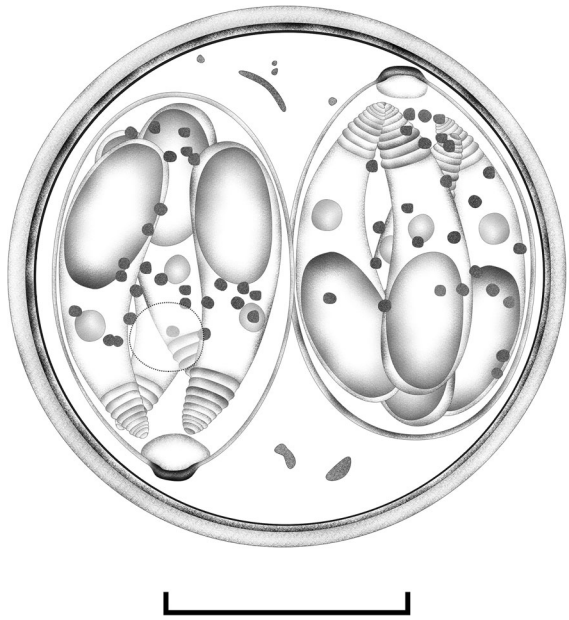

Fig. 1 Composite line drawing of the sporulated oöcyst of *Isospora sporophilae* recovered from buffy-fronted seedeaters *Sporophila frontalis* and uniform finches *Haplospiza unicolor*. *Scale-bar*: 10 μm

Phylogenetic analysis

DNA amplification of the oöcysts of *I. sporophilae* showed a clear band of *c.*250 bp. DNA sequences of the oöcysts recovered from *S. frontalis* and *H. unicolor* had similarity of 99.5%. Phylogenetic analysis included 34 sequences for avian *Isospora* spp. available on GenBank (Fig. 4). *Eimeria tenella* (Railliet & Lucet, 1891) was used as the outgroup. *Isospora sporophilae* from *S. frontalis* and *H. unicolor* was

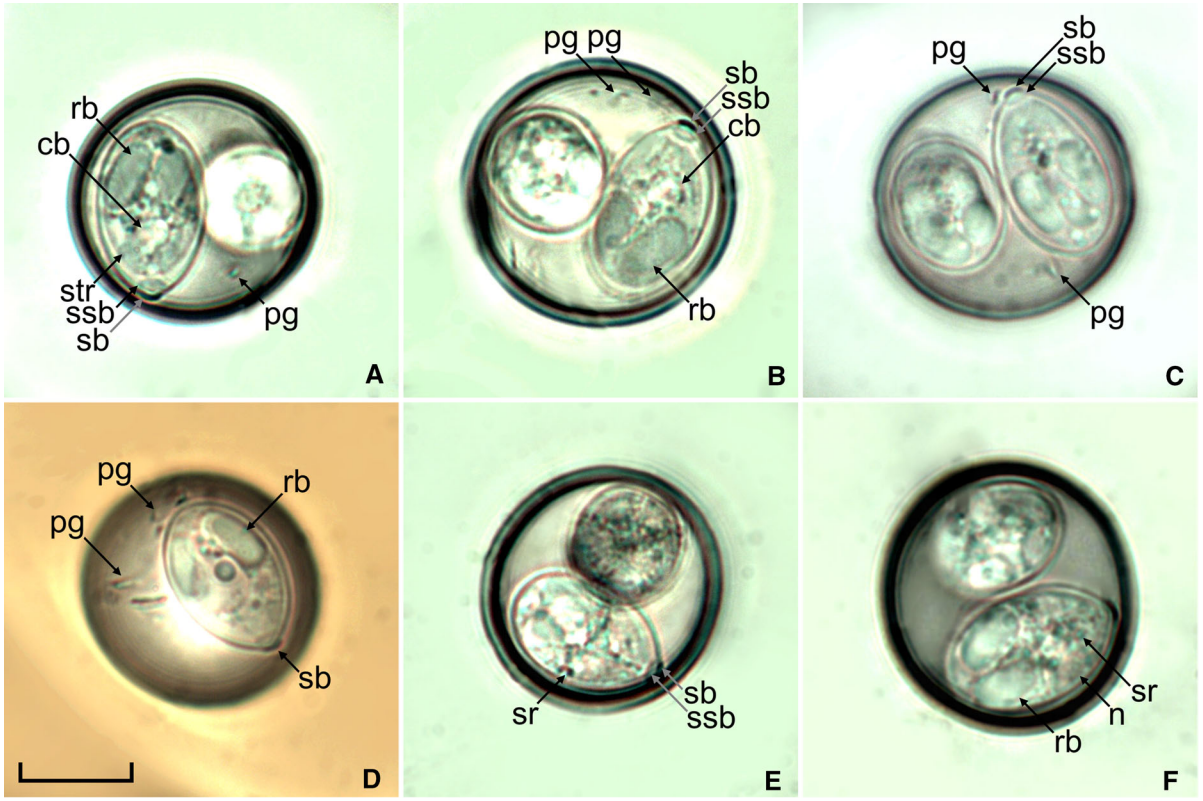

Fig. 2 Photomicrographs of sporulated oöcysts of *Isospora sporophilae* recovered from buffy-fronted seedeaters *Sporophila frontalis* (A–D) and uniform finches *Haplospiza unicolor* (E, F). *Abbreviations*: cb, crystaloid body; n, nucleus; pg, polar granule; rb, refractile body; sb, Stieda body; ssb, sub-Stieda body; sr, sporocyst residuum; str, striations. All to same scale. *Scale-bar*: 10 µm

Table 1 Morphometry of *Isospora sporophilae* oocysts recovered from Thraupidae in southeastern Brazil

Host	Locality	Reference	n	Oöcyst			Sporocyst		
				Length (µm)	Width (µm)	L/W ratio	Length (µm)	Width (µm)	L/W ratio
Sporophila caerulescens (Vieillot, 1823)	Wildlife Screening Center in Seropédica	Carvalho-Filho et al. (2005)	50	19–23 (21.6)	18–23 (20.9)	1.0–1.1 (1.03)	13–17 (15.1)	8–13 (10.6)	1.2–1.8 (1.43)
Sporophila frontalis (Verreaux, 1869)	Federal Conservation Unit of the Itatiaia National Park	Present study	30	20–25 (22.5)[a]	20–24 (21.5)[a]	1.0–1.1 (1.05)[a]	13–17 (15.5)[a]	9–10 (9.7)[a]	1.3–1.9 (1.60)[a]
Haplospiza unicolor Cabanis, 1851	Fragmented Atlantic Forest area in Cacaria	Present study	10	21–25 (23.2)[a]	20–23 (22.0)[a]	1.0–1.1 (1.06)[a]	14–16 (15.0)[a]	9–10 (9.8)[a]	1.4–1.6 (1.53)[a]

[a]Means with the same letters in each column are not significantly different ($P < 0.01$) by ANOVA

Abbreviations: n, total number of oocysts measured; L/W ratio, length/width ratio

recovered in a clade with the highest similarity of 99−100% with *Isospora lopesi* Silva-Carvalho & Berto, 2018 (see Silva-Carvalho et al., 2018a) and *Isospora sagittulae* McQuistion & Capparella, 1992 from the common scale-backed antbird *Willisornis poecilinotus* (Cabanis) and from the white-shouldered fire-eye *Pyriglena leucoptera* (Vieillot) (see Silva-Carvalho et al., 2018b). In a second analysis, a subset

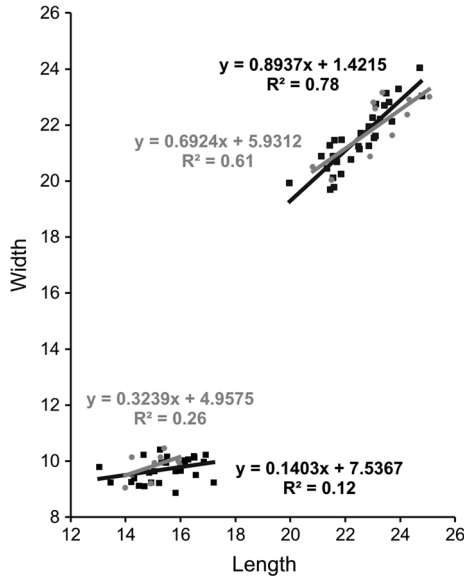

Fig. 3 Comparative linear regressions of oöcysts (above) and sporocysts (below) of *Isospora sporophilae* recovered from *Sporophila frontalis* (black) and *Haplospiza unicolor* (grey)

of 215 bp long *cox*1 gene sequences for 12 *Isospora* spp. was used (Fig. 5). In this analysis, *Isospora sporophilae* was again grouped with *I. lopesi* and *I. sagittulae*, next to the other clade with *Isospora hypoleucae* Dolnik, Rönn & Bensch, 2009 and *Isospora* isolates from Eurasian blackcaps *Sylvia atricapilla* (Linnaeus) with similarities of 96% and 95−97%, respectively.

Discussion

The oöcysts recovered from *S. frontalis* and *H. unicolor* in the present study were morphometrically equivalent in all evaluated aspects (Table 1). These measurements were also very similar to those observed from *S. caerulescens.* The linear regression (Fig. 3) confirms this similarity of the morphometric results when observing the proximity and overlap of the regression lines and the datapoints. In addition, high regularity was observed in the proportion of the width in relation to the length of the oöcysts, as verified by the high values of R^2 from the two regression lines. The sharp slope of the regression line also supports the spherical shape of the oöcysts (Berto et al., 2014). Therefore, comparative morphometry supports the identification of this *I. sporophilae* in these three distinct hosts.

In contrast, it is noteworthy that the morphology of the oöcysts from the two hosts was not identical. Despite the great similarity, the splinter-like or comma-like polar granules were not observed in the oöcysts recovered from *H. unicolor*, whereas these were constantly observed in the oöcysts recovered from *S. frontalis*. The polar granules and residuum of the oöcyst are important characteristic features for the identification of coccidian species, but should not be used as a unique differentiating characteristic, mainly due to having been observed in only one sample from *H. unicolor*. Anyway, some species have already been described as having residual granules in some oöcysts, and in others not (Balthazar et al., 2009; Pereira et al., 2011); therefore, this morphological difference was not considered sufficient to separate the present material into two species.

The sequences obtained from *S. frontalis* and *H. unicolor* were similar in 99.5% (204/205 identities). The present study did not consider substitution of one nucleotide as sufficient to identify distinct coccidian species. This discussion on the delimitation of coccidian species has been raised since Silva et al. (2016), who highlighted some studies separate species with less than 1% of genotype difference (Hafeez et al., 2014); while others, consider samples with 3% of genotype difference being of the same species (Khan et al., 2014). The pioneering study of Duszynski & Wilber (1997) considers that the oöcysts should be compared with coccidian species that are feature-similar and belong to the same host family. Similarly, Kunz (2002) considers that the criterion for identification of new species can not be just on the basis of a certain number of base exchanges within DNA sequence. Therefore, the present study determined the unique identification of *I. sporophilae* based on all its morphological, biological, ecological and molecular characteristics, having a haplotype from *S. frontalis* and another haplotype from *H. unicolor*.

It is suspected that the small morphological and molecular differences observed in the present study can be a consequence of the process of speciation/adaptation to new hosts. As cross-transmission between these hosts should occur because they have similar ecological niches, the recombination between the populations of *I. sporophilae* from these hosts can

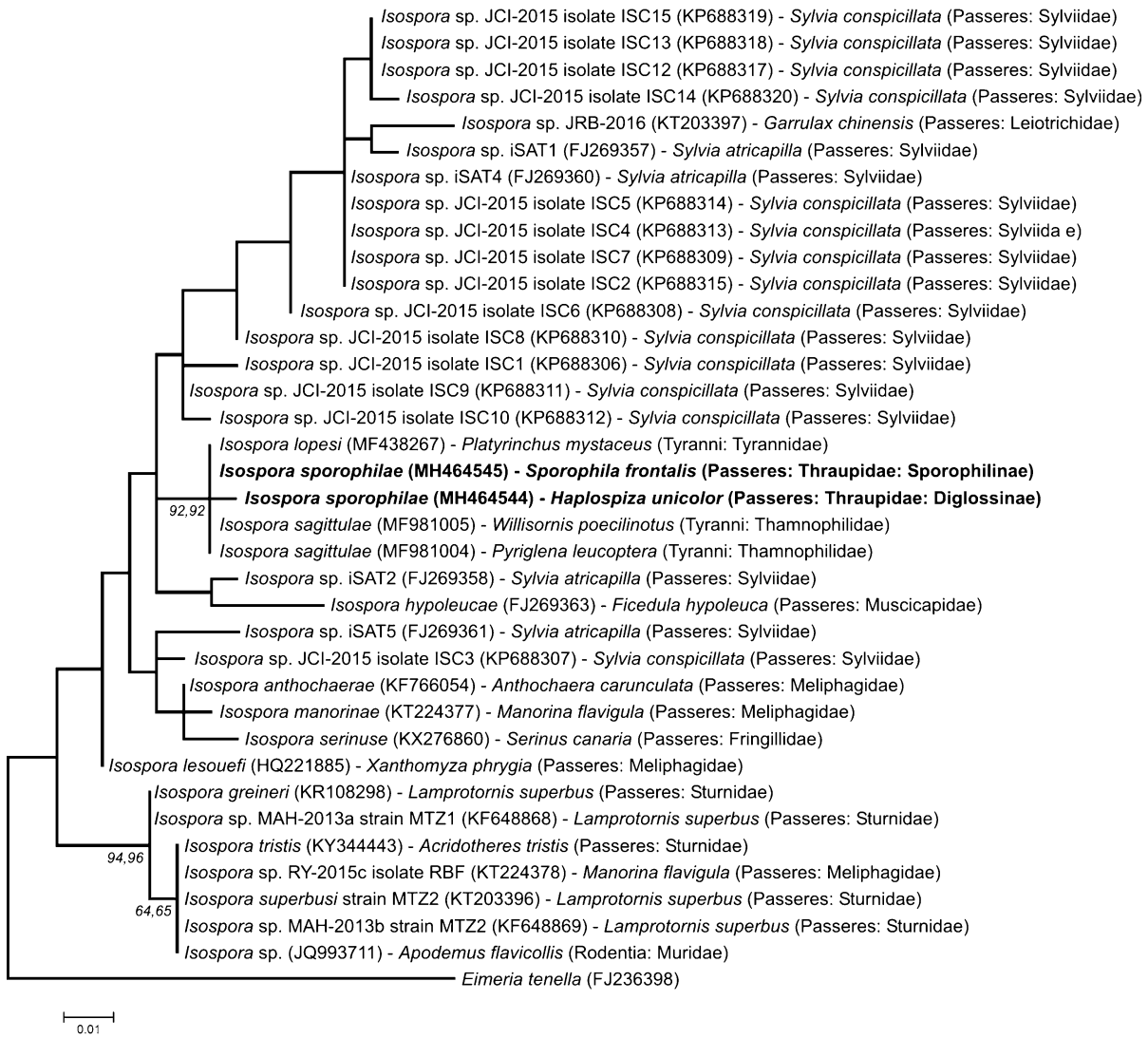

Fig. 4 Maximum likelihood tree estimated from the *cox*1 sequences. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining and Maximum Likelihood, respectively. The scale-bar represents the number of nucleotide substitutions per site

be delaying the speciation process generating irregular morphological characteristics and different genotypes.

Phylogenetic analysis (Fig. 4) revealed that *I. sporophilae* is closer to *I. lopesi* (99−100%) and *I. sagittulae* (99%), and more distant (95−98%) from *Isospora* spp. isolated from an Old World warbler, *S. conspicillata* (see Illera et al., 2015), which is phylogenetically and geographically distant from the hosts of *I. sporophilae* (see del Hoyo et al., 2016; Brands, 2018).

Until the studies of Silva-Carvalho et al. (2018a, b) and others, who deposited *cox*1 sequences for *Isospora* spp. from primitive passerines (suborder Tyranni), there was the hypothesis that a monophyletic group would be forming with the *Isospora* spp. from the Tyranni, evidencing the process of coevolution of parasites and hosts, related to the host phylogeny. However, the present study reported the *cox*1 sequences for *I. sporophilae* from the evolved Thaupidae (suborder Passeri), having a high similarity to the

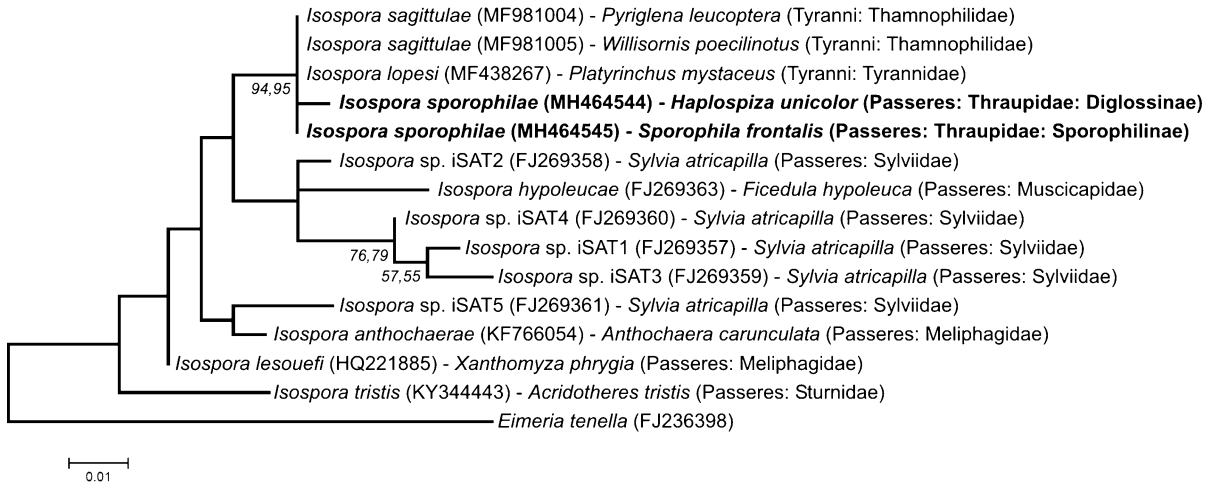

Fig. 5 Maximum likelihood tree estimated from the 215 bp long *cox*1 sequence dataset for *Isospora* spp. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining and Maximum Likelihood, respectively. The scale-bar represents the number of nucleotide substitutions per site

Isospora spp. of the Tyranni, invalidating this initial hypothesis.

It seems that the monophyletic group comprising *I. lopesi*, *I. sagittulae* and *I. sporophilae* differ from the others because they are parasites of Neotropical birds, while the others *Isospora* spp. were reported from passerines of the North America or the Old World. In this sense, the genotypes of this monophyletic group would be characterised as *Isospora* spp. of the Neotropical region, this time, evidencing geographically the process of coevolution of parasites and hosts. Anyway, more definitive conclusions will be reached only when more *Isospora* spp. have been sequenced.

Based on all the results reported in the present study, the vulnerable *S. frontalis* and *H. unicolor* are recorded as new hosts for *I. sporophilae* in the conserved area of the Itatiaia National Park and in the anthropomorphic/fragmented area of Cacaria, respectively. Given the original description of *I. sporophilae* based on material from a bird in a rehabilitation center, it is also concluded that the activities of illegal trade, seizures and reintroductions, should have favored the dispersal of *I. sporophilae* in southeastern Brazil. Finally, *I. sporophilae* from *S. frontalis* and *H. unicolor* had small morphological and genotypic differences that were not considered sufficient for the description of separate species, but only different genotypes related to each host.

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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 by SISBIO/ICMBio (licenses 42798-1; 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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