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Morphological and Molecular Identification of *Isospora sepetibensis* (Chromista: Miozoa: Eimeriidae) from a New Host, *Trichothraupis melanops* (Passeriformes: Thraupidae: Tachyphoninae) in South America

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Abstract. *Isospora sepetibensis* Berto, Flausino, Luz, Ferreira and Lopes, 2008 is a protozoan coccidian parasite (Chromista: Miozoa: Coccidiomorphea: Coccidia) that was originally described from Brazilian tanagers *Ramphocelus bresilius* (Linnaeus, 1766) in the Marambaia Island in the Coast of the State of Rio de Janeiro. In the current work, this species was identified from black-goggled tanagers *Trichothraupis melanops* (Vieillot, 1818) in the Itatiaia National Park, which is a protected area with a high degree of vulnerability in the interior of the State of Rio de Janeiro, distant in more than 100 km of the type-locality. Its oocysts are sub-spherical to elongate ovoidal, 25.9 × 20.7 µm with smooth, bi-layered wall, ~ 1.3 µm and length/width ratio of 1.1–1.4 (1.26). Micropyle and oocyst residuum absent, but one or two polar granules are present. Sporocysts are ellipsoidal, 16.8 × 10.3 µm, with both Stieda and sub-Stieda bodies. Sporocyst residuum present and sporozoites with refractile body and nucleus. Molecular analysis was conducted at the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. This new isolate exhibited similarity greater than 98% with *Isospora* spp. isolates from other Neotropical passerines and with an *Isospora* sp. pseudoparasite of voles of Eurasia. This is the first coccidian parasite from a New World tanager to have a molecular identification of the *cox1* gene.

Key words: morphology, molecular biology, taxonomy, phylogeny, Coccidia, oocysts, Neotropical birds, Thraupidae, Parque Nacional do Itatiaia.

INTRODUCTION

The subclass Coccidia (Chromista: Miozoa: Coccidiomorphea) brings together various genera of parasites of all classes of vertebrates, which may be associated with enteritis and death (Fayer 1980; Ruggiero *et al.* 2015). In Passeriformes, the main genera are *Isospora* Schneider, 1881 and, infrequently, *Eimeria* Schneider, 1875 (Berto *et al.* 2011a).

The black-goggled tanager *Trichothraupis melanops* (Vieillot, 1818) is characterized by presenting the yellowish-colored pile contrasting with a black face.

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The back is gray, with black wings and tail (Sick 1997; Ridgely and Tudor 1989). This species is a very common tanager in Southeastern Brazil. When inhabiting forest undergrowth, these tanagers occur in pairs or in small groups of up to four individuals, generally foraging with mixed flocks and following army ants (Isler and Isler 1987).

Tanagers are frequently parasitized by Coccidia, mainly because of their frugivorous eating habit, which favors oral-fecal transmission (Dolnik *et al.* 2009). *Isospora sepetibensis* Berto, Flausino, Luz, Ferreira and Lopes, 2008 was morphologically described from Brazilian tanagers *Ramphocelus bresilius* (Linnaeus, 1766) of the Marambaia Island (Berto *et al.* 2008); and in the current work, this same coccidian species is identified from the new host *T. melanops* in the interior of the State of the Rio de Janeiro, providing a preliminary genotypic characterization via sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

MATERIAL AND METHODS

Sample collection: Four expeditions were conducted in the Itatiaia National Park (Parque Nacional do Itatiaia), a protected area with a high degree of vulnerability, located in the Serra da Mantiqueira (ICMBIO 2019). Sampling occurred in April 2017 (22°26'17.00"S, 44°37'33.00"W and 22°27'20.58"S, 44°36'28.58"O), June 2017 (22°27'4.00"S, 44°36'51.00"O) and July 2017 (22°26'17.00"S, 44°37'33.00"O). A total of fifteen *T. melanops* were captured with mist nets. The birds were kept in individual boxes and feces 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 oocysts were sporulated. Oocysts were isolated by flotation in Sheather's sugar saturated solution (specific gravity: 1.20) and examined microscopically using the technique described by Duszynski and Wilber (1997) and Berto *et al.* (2014). Morphological observations, 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 micrometres and are given as the range followed by the mean in parentheses.

Molecular analyses: Fifteen oocysts carefully identified with the same characteristics features under light microscopy was isolated and resuspended in PBS (Dolnik *et al.* 2009). DNA was extracted from the 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 *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-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 257 bp in size. The PCR reaction contained 10 µL of 5× Green GoTaq® Flexi Buffer, 3 µL of 25 mM MgCl2, 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) and 30,8 µL of H_2O. 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: 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 with *Isospora* spp. at the *cox1* 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

Fifteen black-goggled tanagers were examined and ten of them shed oocysts in the feces. All observed oocysts were morphologically identified as *I. sepetibensis*. This material is described below.

Isospora sepetibensis Berto, Flausino, Luz, Ferreira, Lopes, 2008 (Fig. 1)

Description of sporulated oocyst: Oocyst shape (n = 18) sub-spherical to elongate ovoidal; 22–29 × 19–22 (25.9 × 20.7) length/width (L/W) ratio 1.1–1.4 (1.26). Wall bi-layered, 1.1–2.0 (1.3) thick, outer layer smooth, *c.*2/3 of total thickness. Micropyle and oocyst residuum absent, but one or two polar granules are present.

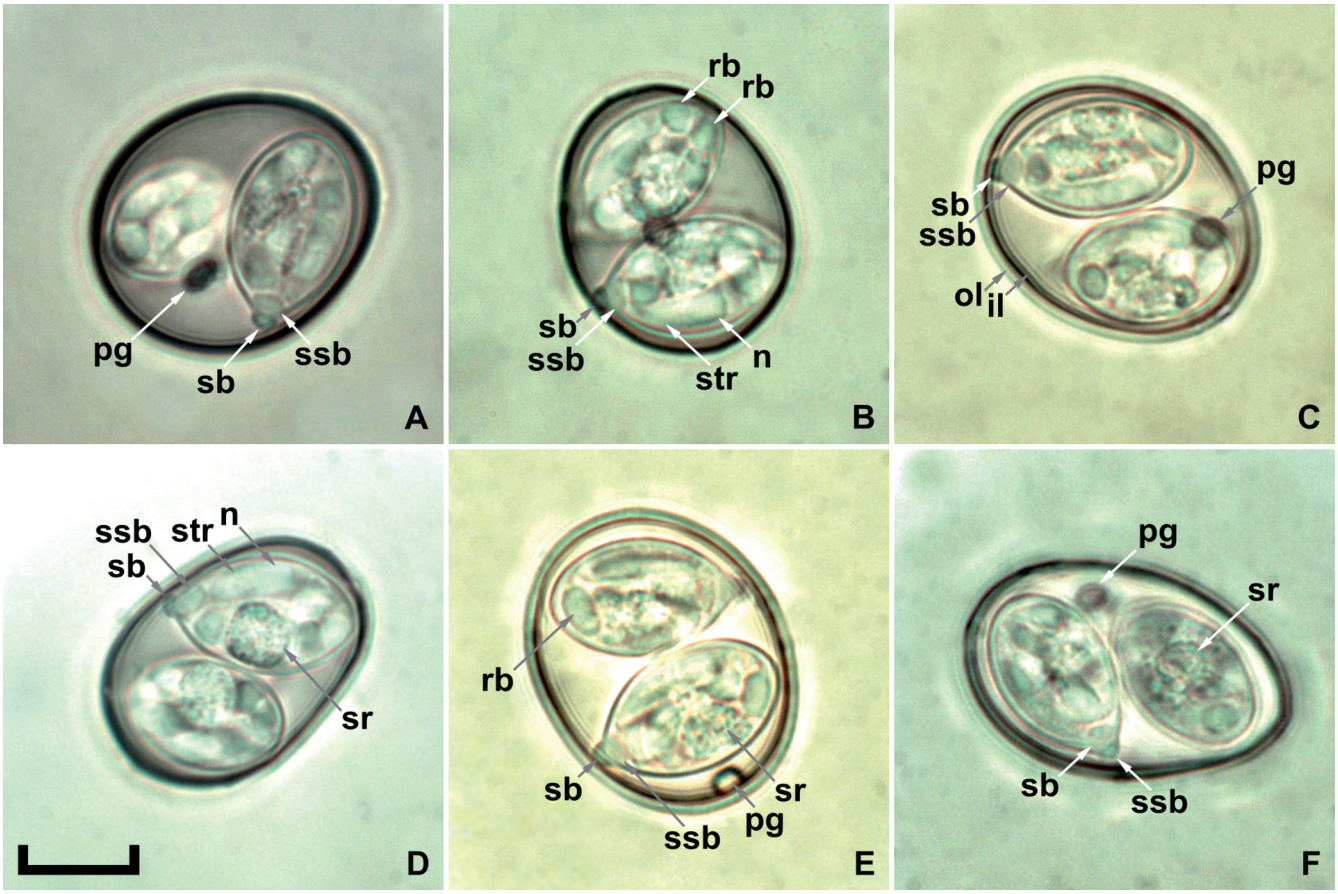

Fig. 1. Photomicrographs of sporulated oocysts of *Isospora sepetibensis*, a coccidium species recovered from the black-goggled tanager *Trichothraupis melanops*. Note the inner (il) and outer (ol) layer of the oocyst wall, nucleus (n), polar granule (pg), Stieda body (sb), sub-Stieda body (ssb), sporocyst residuum (sr), striations (str) and the refractile body (rb). Sheather's sugar solution. Scale-bar: 10 µm.

Description of sporocyst and sporozoites: Sporocysts (n = 18), ellipsoidal, 15–18 × 9–11 (16.8 × 10.3); L/W ratio 1.5–1.8 (1.65). Stieda body present, knob-like, 1.1–1.3 (1.2) high, 2.0–2.2 (2.1) wide; sub-Stieda body present, large to trapezoidal or, occasionally, irregular, 1.2–2.3 (1.7) high, 2.7–3.5 (3.1) wide; para-Stieda body absent. Sporocyst residuum present, consisting of numerous small granules dispersed between the sporozoites or as a distinctly sub-spherical body that appear to be membrane-bounded, 5.5–6.7 (6.1). Sporozoite vermiform with one posterior refractile body, centrally located nucleus and striations.

Host: *Trichothraupis melanops* (Vieillot, 1818) (Passeriformes: Thraupidae: Tachyphoninae).

Locality: Itatiaia National Park (22°26'17.00"S, 44°37'33.00"W), Southeastern Brazil.

Specimens: Photomicrographs and oocysts 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 MZURPTZ2019014. 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 number 92/2019. Photographs of the host specimens are deposited in the same collection.

Site in host: Unknown**.**

Prevalence: 67% (15 out of 10 birds infected).

Representative DNA sequence: Representative *cox1* sequence is deposited in the GenBank database under the accession number MK682606.

Phylogenetic analysis: The amplification of the DNA from the fifteen oocysts of *I. sepetibensis* recov-

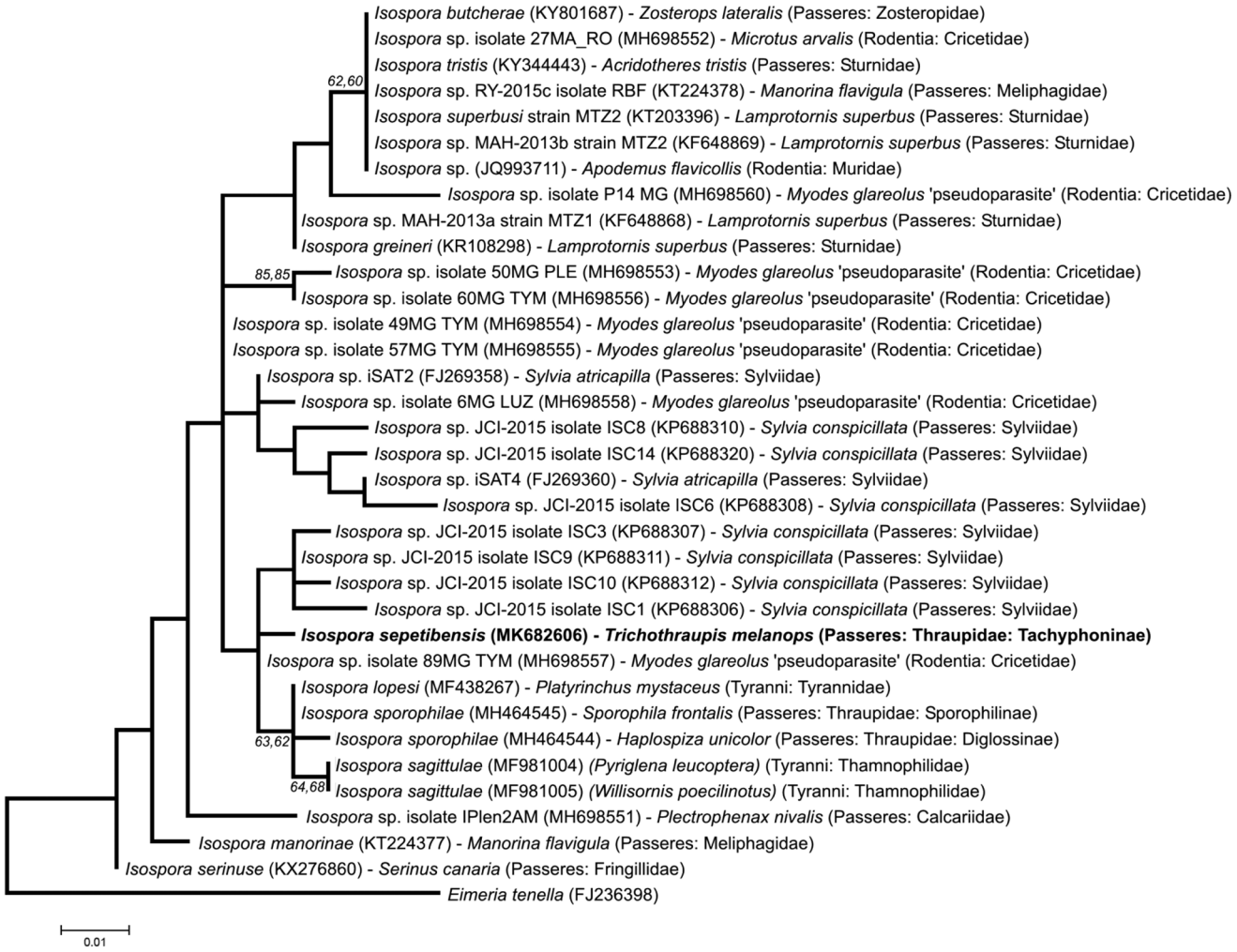

Fig. 2. Maximum likelihood tree estimated from the *cox1* sequences. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining and Maximum Likelihood, respectively. The scale-bar represents the number of nucleotide substitutions per site.

ered from *T. melanops* showed a clear band of *c.* 250 bp. The phylogenetic analysis was constructed with 33 sequences of *Isospora* spp. closest to *I. sepetibensis* available on GenBank (Fig. 2). *Eimeria tenella* (Railliet, Lucet, 1891) was used as the outgroup. *Isospora sepetibensis* sat in a large group with the highest similarity of 98.1% with an *Isospora* sp. reported as pseudoparasite of bank voles *Myodes glareolus* (Schreber, 1780) in Czech Republic (Trefancová *et al.* 2019). It was close to the clade with *Isospora* isolates from the spectacled warbler *Sylvia conspicillata* Temminck, 1820 in Macaronesia (Illera *et al.* 2015); and also was close to the clade with *Isospora* spp. recently sequenced from Neotropical passerines (Silva-Carvalho *et al.* 2018a, 2018b; Rodrigues *et al.* 2019). Subsequently, a subset with only 215 bp long *cox1* gene sequences was constructed (Fig. 3). In this analysis, *Isospora sepetibensis* sat in the same clade of *Isospora* spp. from Neotropical passerines, with the highest similarity of 98.5% with *Isospora lopesi* Silva-Carvalho & Berto, 2018 (Silva-Carvalho *et al.* 2018a).

DISCUSSION

The morphological data reported in the current work showed a high degree of similarity to the descriptions from the Brazilian tanager *R. bresilius* and from the

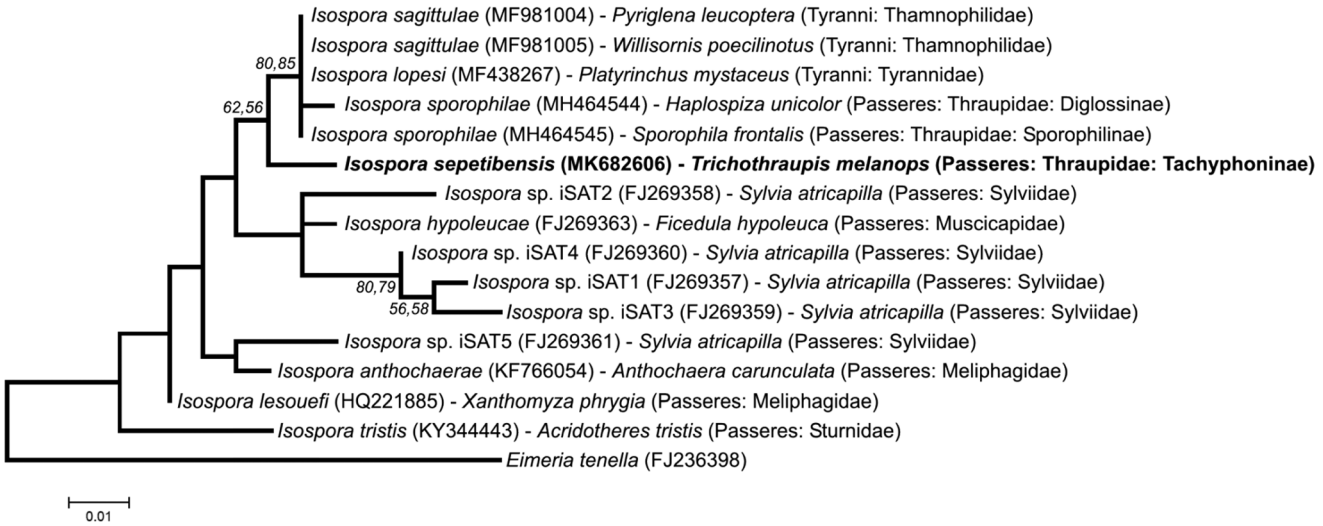

Fig. 3. Maximum likelihood tree estimated from the 215 bp long *cox1* sequences. Numbers at nodes represent bootstrap support (1,000 replicates; only values > 50% shown) for Neighbor-Joining and Maximum Likelihood, respectively. The scale-bar represents the number of nucleotide substitutions per site.

blue dacnis *Dacnis cayana* (Linnaeus, 1766) (Table 1) (Berto *et al.* 2008, 2011b). Despite the polymorphism already characterized for this coccidian species (Berto *et al.* 2011b), patterns of shape/size may be related to each of the hosts and/or localities. The oocysts from *R. bresilius* were smaller and more rounded, as can be confirmed by the lower L/W ratio (Berto *et al.* 2014); whereas, the oocysts from *D. cayana* were smaller; and the oocysts from *T. melanops* were quite elongated and larger than those from *D. cayana* (Table 1). In addition, some specimens from *T. melanops* had an irregular sub-Stieda (Fig. 1c), which was not observed in the previous works (Berto *et al.* 2008, 2011b). Possibly, these patterns/differences may be associated to the process of speciation and adaptation to new hosts and/or localities, or other associated factors (Fayer 1980, Gardner and Duszynski 1990).

The new locality of parasitism of *I. sepetibensis* recorded in the current work is noteworthy. The Marambaia Island, where the two previous reports occurred, is more than 100 km away and more than 1,000 m of altitude in the Itatiaia National Park, on the opposite border of the State of Rio de Janeiro. This observation highlights the great dispersion of this coccidian species in the State of Rio de Janeiro and its adaptation to considerably different environments (Berto and Lopes 2013).

Traditionally, characterization of avian species of *Isospora* has mainly been based on morphological features and host specificity; however, this can be problematic whenever there are morphological equivalents from different hosts, environments or from localities that disfavor the transmissions (Gardner and Duszynski 1990, Berto *et al.* 2011a, Hafeez *et al.* 2014). In this sense, molecular studies become relevant. Molecular methods, as amplification and sequencing of specific genes, complement the morphological studies with new information about genetic diversity and can also address questions about phylogenetic relationships and phylogeographic, which are hard to answer only with morphological data (Perkins and Shall 2002, Adkesson *et al.* 2005, Schrenzel *et al.* 2005, Berto *et al.* 2011a).

In the last 10 years, molecular methods have increasingly been used in studies of diversity, distribution, specificity, ecology, and different aspects of evolutionary biology of avian parasites (Bensch *et al.* 2000, Waldenström *et al.* 2002). In this context, the *cox1* gene was chosen for genotyping in the current work 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, Silva-Carvalho *et al.* 2018a).

Isospora sepetibensis is the first coccidian parasite of a New World tanager to have its *cox1* sequence de-

Table 1. Comparative morphology of *Isospora sepetibensis* recorded from different thraupid hosts and localities in southeastern Brazil.

Hosts	Localities	Reference(s)	Oocysts			Wall (µm)	Polar granule	Sporocysts			Stieda body	Sub-Stieda body	Residuum
			Shape	Measurements (µm)	L/W ratio			Shape	Measurements (µm)	L/W ratio			
Ramphocelus bresilius (Linnaeus, 1766)	Marambaia Island, Coast of the State of Rio de Janeiro	Berto et al. (2008)	sub-spherical to ellipsoidal	24–29 22–26 (25.5 × 23.8)	1.0–1.2 (1.1)	1.3–1.4 (1.4)	1 or 2	ellipsoidal	16–18 × 10–12 (16.9 × 11.0)	–	knob-like	prominent	granular, dislocated laterally
Dacnis cayana (Linnaeus, 1766)	Marambaia Island, Coast of the State of Rio de Janeiro	Berto et al. (2011b)	sub-spherical to ovoidal	22–27 × 20–23 (24.8 × 21.7)	1.0–1.3 (1.14)	1.3	1 or 2	ellipsoidal	16–18 × 10–12 (17.4 × 10.9)	1.5–1.7 (1.59)	knob-like or bubble-shaped, 1.5 × 2.0	prominent, 2.0 × 4.0	granular, scattered or forming a mass
Trichothraupis melanops (Vieillot, 1818)	Parque Nacional do Itatiaia, interior of the State of Rio de Janeiro	current work	sub-spherical to elongate ovoidal	22–29 × 19–22 (25.9 20.7)	1.1–1.4 (1.26)	1.1–2.0 (1.3)	1 or 2	ellipsoidal	15–18 × 9–11 (16.8 × 10.3)	1.5–1.8 (1.65)	knob-like, 1.1–1.3 (1.2) × 2.0–2.2 (2.1)	large to trapezoidal, or irregular, 1.2–2.3 (1.7) × 2.7–3.5 (3.1)	granular, diffuse or membrane-bounded, 5.5–6.7 (6.1)

posited in the GenBank database. The phylogenetic proximity of this coccidian species to the pseudoparasite isolate from the bank voles in Czech Republic weakens the theory of the formation of a monophyletic group with *Isospora* spp. from Neotropical passerines (Rodrigues *et al.* 2019, Trefancová *et al.* 2019). Although it is not known which is the true host of this *Isospora* sp. isolated from the feces of the bank voles, it is logically known that it is not a Neotropical passerine. Anyway, with the current frequency of sequences of *Isospora* spp. from passerines being deposited at Genbank, phylogenetic studies will be more complete and conclusive, especially with the 215 bp long *cox1* gene sequences that have already been considered more useful for specific phylogenetic studies (Yang *et al.* 2015, 2016). In this analysis, *I. sepetibensis* sat in a monophyletic group with *Isospora* spp. from Neotropical passerines, but greater inferences are not possible due to the low number of 215 bp long *cox1* gene sequences deposited in the GenBank database.

Finally, based on all the results reported in the current work, *T. melanops* is recorded as a new host for *I. sepetibensis* in the Itatiaia National Park in Southeastern Brazil; and, in addition, this is the first coccidian parasite from a New World tanager to have a molecular identification of the *cox1* gene.

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