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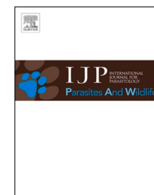

Molecular screening for Sarcocystidae in muscles of wild birds from Brazil suggests a plethora of intermediate hosts for *Sarcocystis falcatula*

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A B S T R A C T

The genus *Sarcocystis* and the species *Toxoplasma gondii* are the most prevalent sarcocystid organisms found in birds. Molecular phylogenies based on the first internal transcribed spacer of the ribosomal coding DNA (ITS1) have been widely used to identify them. Here, pectoral muscles from 400 wild birds from Brazil were screened by means of molecular methods using nested PCR, and Sanger sequencing yielded amplicons. A pan-sarcocystid ITS1-directed nested PCR revealed 28 birds infected by *Sarcocystis falcatula* (ten Piciformes, eight Psittaciformes, five Columbiformes, two Accipitriformes, one Anseriformes, one Passeriformes and one Strigiformes); one infected by *Sarcocystis halieti* (one Accipitriformes); nine infected by unknown or undescribed *Sarcocystis* (six Passeriformes, one Piciformes, one Cathartiformes and one Cuculiformes); and six harboring *Toxoplasma gondii* DNA (three Pelecaniformes, two Falconiformes and one Columbiformes). Samples harboring *S. falcatula*-related ITS1 sequences were further characterized by means of PCR and sequencing of genetic sequences of three surface antigen coding genes (SAGs). From this, 10 new allelic combinations of SAGs (SAG2, SAG3 and SAG4) were identified, in addition to 11 SAG allelic combinations already found in Brazil. Samples with *S. falcatula*-unrelated ITS1 sequences were further characterized by means of PCR and sequencing of cytochrome *c* oxidase subunit I coding sequences (CO1) and 18S ribosomal DNA gene (18S rDNA). This study was the first extensive survey of wild birds in Brazil for Sarcocystidae species. It provides the first molecular evidence of natural *S. falcatula* infection in 14 species, including in the order Piciformes, and shows the high genetic diversity of *S. falcatula* in intermediate hosts in South America. Evidence of occurrence of at least three non-described species of *Sarcocystis* was also presented in this study. This survey corroborated the ubiquity of *T. gondii* infection but revealed surprisingly low prevalence of this parasite (1.5%).

1. Introduction

Sarcocystis is a genus of coccidian parasites characterized by an obligate two-host life cycle. Asexual stages (sarcocysts) develop in the muscles of the intermediate host (prey), while sexual multiplication occurs in the small intestine of the definitive host (predator), with formation of oocysts. While intermediate hosts become infected after ingestion of sporocysts that are available in the environment, the definitive hosts are infected exclusively through carnivorous ingestion of mature sarcocysts (Dubey et al., 2015).

Birds serve as intermediate and definitive hosts for numerous *Sarcocystis* species, and some of these are pathogenic. More than 25 *Sarcocystis* species are known to form sarcocysts in the muscles of birds (Dubey et al., 2015). *Sarcocystis falcatula*, one of the most prevalent *Sarcocystis* species of birds in the Americas, can use a large variety of bird species as intermediate hosts, including the avian orders Accipitriformes (Wünschmann et al., 2010), Charadriiformes (Acosta et al., 2021), Columbiformes (Ecco et al., 2008; Suedmeyer et al., 2001),

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Passeriformes (Box and Duszynski, 1978; Dubey et al., 2001), Pelecaniformes (Konradt et al., 2017), Psittaciformes (Ecco et al., 2008; Godoy et al., 2009; Hillyer et al., 1991; Siegal-Willott et al., 2005; Verma et al., 2018; Villar et al., 2008), Sphenisciformes (Acosta et al., 2018), and Strigiformes (Wünschmann et al., 2009).

Two genetic lineages of *S. falcatula* have been described in the Americas. One of them has been described in a few samples from North America (Tanhauser et al., 1999; Marsh et al., 1999) and the other, apparently much more frequently, has been found in birds on all American continents (Acosta et al., 2018; Origlia et al., 2022; Verma et al., 2018). For this reason, *S. falcatula* have been considered a complex of species since its original description and further reports have assigned the term *Sarcocystis falcatula*-like to those organisms that fit to the molecular and morphological shape of *S. falcatula* (Gondim et al., 2021).

S. falcatula is endemic in the Americas, because the definitive hosts for this parasite are opossums of the genus *Didelphis*, which is exclusive to the Americas. In Brazil, the opossum species *Didelphis albiventris*, *D. marsupialis* and *D. aurita* have been implicated as definitive hosts of *S. falcatula*, *S. neurona*, *S. speeri* and *S. lindsayi* (Dubey et al., 1999, 2000a, 2000b, 2000c, 2001b, 2001c, 2001d; Gallo et al., 2018). In contrast, only one species of opossum found in North America (*Didelphis virginiana*) is the final host of *S. falcatula*, *S. neurona* and *S. speeri* (Dubey et al., 2000d; Elsheikha et al., 2004; Fenger et al., 1995).

When infected by *S. falcatula*, birds that are non-endemic in the Americas tend to suffer from severe infection, with high mortality rates, in contrast to birds that are native to these continents (Acosta et al., 2018). Old World birds, which generally only occur in captivity in the Americas, manifest hyperacute pulmonary and encephalic forms of sarcocystosis, while birds of the New World seem to be resistant in most cases to these types of infection, probably because they co-evolved environmentally with opossums (McCormick-Rantze et al., 2003).

Currently, PCR assays and sequencing are considered to be much more practical, accurate and reliable methods for delineation and identification of *Sarcocystis* species than traditional methods based on morphological characteristics (Gjerde, 2013; Pan et al., 2020). Most avian *Sarcocystis* spp. have been characterized at three genetic loci: the 18S small subunit rDNA gene (18S rDNA); the cytochrome *c* oxidase subunit 1 gene (CO1); and the internal transcribed spacer 1 gene (ITS1) (Dubey et al., 2015). However, the 18S rDNA and CO1 genes have not appeared to be variable enough to discriminate some *Sarcocystis* species that use birds as intermediate hosts (Gjerde et al., 2018; Prakas et al., 2018a). Conversely, the ITS1 gene has been demonstrated to be the best marker for species delimitation within this group (Gjerde et al., 2018; Prakas et al., 2014).

Recently, molecular characterization of *S. falcatula* using surface antigen gene (SAGs) was found to show high diversity of alleles in sporocysts and in experimentally infected birds in Brazil. Among 50 samples of sporocysts from *Didelphis* spp., 10 variants for SAG2, 15 for SAG3 and 11 for SAG4 were encountered (Monteiro et al., 2013; Valadas et al., 2016). In bioassays, budgerigars (*Melopsittacus undulatus*) that had been inoculated orally with sporocysts presented four variants for SAG2, five for SAG3, and four for SAG4, which were found in seven allelic combinations (SAG genotypes) (Cesar et al., 2018; Gondim et al., 2017, 2019).

However, among naturally infected birds, the variability of *S. falcatula* in SAGs is poorly understood. In the carcasses of 16 penguins (*Spheniscus magellanicus*) rescued on the coast of Brazil, all the samples were identical to each other for each allele (Acosta et al., 2018). In the only known case of death attributed to *S. falcatula* in a bare-faced ibis (*Phimosus infuscatus*) from the south of Brazil, molecular analysis demonstrated an unprecedented combination of SAG alleles (Cesar et al., 2018; Konradt et al., 2017). These findings of *Sarcocystis falcatula* in penguins and in a bare-faced ibis in Brazil were both unprecedented SAG genotypes.

Thus, it is relevant to know whether the high genetic diversity of *S. falcatula* derived from definitive hosts so far demonstrated can be correlated with the diversity in the intermediate hosts. Given that detection of *Sarcocystis* spp. DNA in muscle tissue of birds enables reasonable inference about these birds' role as intermediate hosts, this present study was conducted with the aim of screening muscle samples from wild birds that were naturally infected with sarcocystid organisms in the state of São Paulo, Brazil. Considering that all the muscle samples from these birds were screened by means of a pan-sarcocystid nested-PCR technique, it became possible to screen for several sarcocystid species, other than *S. falcatula*.

2. Material and methods

2.1. Ethical considerations

These experiments performed with animals were carried out under the instructions from and approval of the Ethics Committee for Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science, University of São Paulo (under permit number: 9077070416).

2.2. Sampling of wild birds

Between 2016 and 2018, a total of 400 free-living wild birds of 103 species (Table S1, in supplementary file) were screened for *Sarcocystis* spp. Muscle tissues from wild birds that had died through different causes were received from the wildlife rehabilitation center DEPAVE-3 (The Fauna Division of the Municipal Secretariat for Green and Environment of the Municipality of São Paulo, SP, Brazil). The tissue samples were kept frozen (−20 °C) until a molecular analysis was conducted.

2.3. Molecular analyses

About 25–50 mg of pectoral muscle tissue from each bird was homogenized mechanically using a mortar and pestle before total DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations, except for final elution of the product into 50 μL of elution buffer. The DNA samples thus obtained were screened by using a pan-sarcocystid nested-PCR technique based on two primer pairs that flanked the complete ITS1 of the rRNA gene (nPCR-ITS1). After amplification, the nPCR-ITS1 products were viewed under UV light after electrophoresis on 2% agarose gel and staining with ethidium bromide. ITS1 amplicons were excised from the gel and were directly sequenced by means of the Sanger method, as described previously (Hammerschmitt et al., 2020). Chromatograms were scored and assembled with the help of phred-prap programs built in the software Codon code Aligner v.4.2.7. After genetic sequences had been obtained, they were subjected to Blast analysis in order to identify the most similar sequences available in GenBank, through the tool http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Samples closely related to *S. falcatula* at ITS1 (>90% similarity) were also identified through nested PCR and sequencing of surface antigen 2, 3 and 4 coding genes (nPCR-SAG2, nPCR-SAG3 and nPCR-SAG4, respectively). Samples that were not identified as *S. falcatula* but were identified within the genus *Sarcocystis* were further tested by means of nested PCRs directed to 18S rRNA (nPCR-18S) and cytochrome *c* oxidase subunit 1 (nPCR-CO1). Samples identified as *T. gondii* were not further characterized. The oligonucleotides used to amplify genetic sequences of *Sarcocystis* spp., using nested PCR, are depicted in Table S2 (supplementary file).

To make phylogenetic inferences, the genetic sequences were aligned using the program ClustalW in the BioEdit Sequence Alignment Editor (Hall, 1999), with homologous sequences that are available in the GenBank database (National Institutes of Health) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ITS1 phylogeny was reconstructed using MEGA-X, through the maximum likelihood (ML) method and the model for evolutionary distances, which were calculated by means of MEGA-X and varied according to the data set (Kumar et al., 2018). The robustness

of the ML tree was statically evaluated by means of bootstrap analysis with 1,000 bootstrap samples. The software PopART (Population Analysis with Reticulate Trees) (Leigh and Bryant, 2015) was used to infer evolutionary relationships for *S. falcatula* based on SAG loci, by using Integer NJ networks inference method. The genetic sequences were submitted to GenBank with accession numbers: OL335256-OL335364 (SAG2, SAG3, SAG4 and CO1), OL323060-OL323066 (18SrRNA) and OL323067-OL323108 (ITS1).

3. Results

Screening of all the DNA samples using nPCR-ITS1 revealed 36 genetic sequences related to *S. falcatula*, two ITS1 sequences related to other species within the genus *Sarcocystis* (*Sarcocystis halieti* and *Sarcocystis lari*) and six sequences that were almost identical to *T. gondii* (Table 1). The sizes of the amplicons yielded by nPCR-ITS1, from samples relating to *S. falcatula*, were larger than 1000 base pairs (bp); those from samples relating to other species within the genus *Sarcocystis* were around 900 bp; and those from samples identified as *T. gondii* were around 500 bp (not shown).

The ITS1 amplicons were not entirely sequenced because the 5′ and 3′ ends were missing in fragments larger than 1000 bp from the *S. falcatula*-related samples. In some of these sequences, ambiguous peaks in nucleotide chromatograms were typically registered after either of the nucleotide positions 419 or 654, which had also impaired the entire sequencing of the largest ITS1 segments. ITS1 fragments from *T. gondii* were also only partially sequenced (5′ end was missing) because they were sequenced only by using the forward primer.

The phylogenies based on ITS1 were reconstructed using the genetic sequences detected in this study, along with the most similar sequences obtained after Blast analysis on these sequences. Two ITS1 phylogenies were inferred: one included 36 *S. falcatula*-related sequences and the other included the two ITS1 sequences that were related to other species within the genus.

The first ITS1 tree (Fig. 1) showed three well-supported clades: clade A, formed by *S. falcatula* and *S. falcatula*-like parasites (28 sequences from this study was placed in this clade); clade B, formed by seven sequences exclusively detected in the present study; and clade C, formed by a single sequence detected in this study and by *S. lindsayi*. At CO1, the sequences of the clades B and C were identical to each other and to sequences of *S. speeri* and *S. falcatula* (KT207461 and MH665257, respectively). At the 18S locus, clades B and C differed at one SNP from *S. falcatula* (MH626537) and were identical to *S. speeri* (KT207459).

Through SAG genotyping of the 36 *S. falcatula*-related samples, seven alleles were found at SAG2, 11 alleles at SAG3 and 6 alleles at SAG4 (Fig. 2). Among these, 24 samples were genotyped by the 3 SAG locus, and 15 SAG genotypes (SAG) were assigned to the samples (Table S3, supplementary file). Twelve SAG genotypes corresponded to *S. falcatula*-like *parasites* (#1 to #12), whereas genotypes #13 to #15 corresponded to *Sarcocystis* sp. from clade B. *Sarcocystis* sp. from clade C was not fully SAG genotyped. The *Sarcocystis* species from clade B were named *Sarcocystis* sp. ex *Cacicus haemorrhous* and the *Sarcocystis* species from clade C were named *Sarcocystis* sp. ex *Guira guira*.

The second ITS1 tree showed that one of the sequences was related to *Sarcocystis halieti* (#213, *Sarcocystis* sp. ex *Accipiter striatus*), whereas the other was related to *Sarcocystis lari* (#471, *Sarcocystis* sp. ex *Coragyps atratus*) (Fig. 3). Based on 18S rRNA analysis, *Sarcocystis* sp. ex *Accipiter striatus* was 100% identical to *S. halieti* (MH130211, MF946587), as well to various unnamed species of *Sarcocystis* from *Accipiter cooperii* (KY348753, EU810398), *Phalacrocorax carbo* (JQ733511), *Columba livia* (GQ245670) and *Anser albifrons* (EU502869). Concerning CO1, the *Sarcocystis* sp. ex *Accipiter striatus* haplotype was 100% identical to *S. halieti* (MH138308, MH138309, MF946583), *S. corvusi* (MH138314) and *S. columbae* (MH138312). Regarding the 18S rRNA gene, *Sarcocystis* sp. ex *Coragyps atratus* shared the highest similarity (99.23%) with S. *halieti* (MH130211, MF946587) and various unnamed species of

Table 1
Molecular identification of sarcocystids in muscle samples from wild birds in Brazil, based on *n*PCR-ITS1 sequence analysis.

Animal ID- common name (*scientific name*)	Sex[a]	Positives/ total (%)	Sequenced product (bp)	Sequence similarity (%) to closest in GenBank
AMPLICON SIZE > 1000 BP (N = 36)				
Psittaciformes: Psittacidae				
238- plain parakeet (*Brotogeris tirica*)	M	4/14 (20.5)	419	99.8% *S. falcatula* is. Lorikeet (MH626538)
189- plain parakeet (*Brotogeris tirica*)	M		651	99.7% *S. falcatula* is. Lorikeet (MH626538)
210- plain parakeet (*Brotogeris tirica*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
231- plain parakeet (*Brotogeris tirica*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
206- scaly-headed parrot (*Pionus maximiliani*)	M	1/2 (50)	1012	99.8% *S. falcatula* is. Lorikeet (MH626538)
504- turquoise-fronted amazon (*Amazona aestiva*)	M	3/9 (33.3)	1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
519- turquoise-fronted amazon (*Amazona aestiva*)	F		1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
349- turquoise-fronted amazon (*Amazona aestiva*)	M		652	99.7% *S. falcatula* is. Lorikeet (MH626538)
Piciformes: Picidae				
227- blond-crested woodpecker (*Celeus flavescens*)	F	3/9 (33.3)	419	99.8% *S. falcatula* is. Lorikeet (MH626538)
387- blond-crested woodpecker (*Celeus flavescens*)	F		419	99.5% *S. falcatula* is. Lorikeet (MH626538)
103- blond-crested woodpecker (*Celeus flavescens*)	M		1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
208- lineated woodpecker (*Dryocopus lineatus*)	F	1/6 (16.6)	419	99.8% *S. falcatula* is. Lorikeet (MH626538)
Piciformes: Ramphastidae				
197- red-breasted toucan (*Ramphastos dicolorus*)	M	6/8 (75)	419	99.8% *S. falcatula* is. Lorikeet (MH626538)
222- red-breasted toucan (*Ramphastos dicolorus*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
230- red-breasted toucan (*Ramphastos dicolorus*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
233- red-breasted toucan (*Ramphastos dicolorus*)	M		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
244- red-breasted toucan (*Ramphastos dicolorus*)	M		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
258- red-breasted toucan (*Ramphastos dicolorus*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)

(continued on next page)

Table 1 (*continued*)

Animal ID- common name (*scientific name*)	Sex[a]	Positives/ total (%)	Sequenced product (bp)	Sequence similarity (%) to closest in GenBank
138- saffron toucanet (*Pteroglossus bailloni*)	M	1/2 (50)	1021	93.39% *S. falcatula* is. Lorikeet (MH626538)
Columbiformes: Columbidae				
181- picazuro pigeon (*Patagioenas picazuro*)	M	2/13 (15.3)	1012	99.8% *S. falcatula* is. Lorikeet (MH626538)
262- picazuro pigeon (*Patagioenas picazuro*)	M		1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
264- grey-fronted dove (*Leptotila rufaxilla*)	M	1/2 (50)	419	99.8% *S. falcatula* is. Lorikeet (MH626538)
326- eared dove (*Zenaida auriculata*)	F	2/6 (33.3)	654	99.5% *S. falcatula* is. Lorikeet (MH626538)
330- eared dove (*Zenaida auriculata*)	F		419	99.8% *S. falcatula* is. Lorikeet (MH626538)
Passeriformes: Vireonidae				
283- rufous-browed peppershrike (*Cyclarhis gujanensis*)	F	1/1 (100)	1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
Passeriformes: Thraupidae				
137- ruby-crowned tanager (*Tachyphonus coronatus*)	M	1/1 (100)	1021	93.19% *S. falcatula* is. Lorikeet (MH626538)
452- Brazilian tanager (*Ramphocelus bresilius*)	F	1/1 (100)	1021	93.39% *S. falcatula* is. Lorikeet (MH626538)
Passeriformes: Icteridae				
431- red-rumped cacique (*Cacicus haemorrhous*)	M	4/6 (66.6)	1021	93.19% *S. falcatula* is. Lorikeet (MH626538)
282- red-rumped cacique (*Cacicus haemorrhous*)	M		1021	93.00% *S. falcatula* is. Lorikeet (MH626538)
444- red-rumped cacique (*Cacicus haemorrhous*)	M		1021	93.00% *S. falcatula* is. Lorikeet (MH626538)
453- red-rumped cacique (*Cacicus haemorrhous*)	F		1021	93.00% *S. falcatula* is. Lorikeet (MH626538)
Accipitriformes: Accipitridae				
174- Harris's hawk (*Parabuteo unicinctus*)	F	1/4 (25)	651	99.7% *S. falcatula* is. Lorikeet (MH626538)
149- roadside hawk (*Rupornis magnirostris*)	F	1/5 (20)	1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
Strigiformes: Strigidae				
407- tropical screech-owl (*Megascops choliba*)	M	1/9 (11.1)	1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
Anseriformes: Anatidae				
425- duck (*Anas* sp.)	M	1/2 (50)	1013	99.9% *S. falcatula* is. Lorikeet (MH626538)
Cuculiformes: Cuculidae				
163- guira cuckoo (*Guira guira*)	M	1/2 (50)	1008	92.65% *S. falcatula* is. Lorikeet (MH626538) 93.55% *S. lindsayi* (AF387164)
AMPLICON SIZE BETWEEN 500–1000 BP (N = 2)				
Accipitriformes: Accipitridae				
213- sharp-shinned hawk (*Accipiter striatus*)	F	1/5 (20)	842	99.17% *Sarcocystis halieti* is. Ha 1.6 (MF946589)
Cathartiformes: Cathartidae				
471- American black vulture (*Coragyps atratus*)	M	1/11 (9.1)	800	90.04% *Sarcocystis lari* is. Ha. 1.8 (MF946599)
AMPLICON SIZE ABOUT 500 BP (N = 6)				
Columbiformes: Columbidae				
260- picazuro pigeon (*Patagioenas picazuro*)	M	1/13 (7.7)	411	100.00% *Toxoplasma gondii* (MH793505)
Falconiformes: Falconidae				
187- American kestrel (*Falco sparverius*)	M	2/6 (33.3)	411	100.00% *Toxoplasma gondii* (MH793505)
493- American kestrel (*Falco sparverius*)	F		411	100.00% *Toxoplasma gondii* (MH793505)
Pelecaniformes: Ardeidae				
426- snowy egret (*Egretta thula*)	F	2/2 (100)	411	100.00% *Toxoplasma gondii* (MH793505)
293- snowy egret (*Egretta thula*)	M		411	97.76% *Toxoplasma gondii* (MH793505)
433- black-crowned night-heron (*Nycticorax nycticorax*)	M	1/2 (50)	411	97.76% *Toxoplasma gondii* (MH793505)

[a] No significant differences in infection were found between the sexes (p = 0.873, Fisher's test.

Sarcocystis from *Accipiter cooperii* (KY348753, EU810402, EU810398), *Phalacrocorax carbo* (JQ733511), *Columba livia* (GQ245670) and *Anser albifrons* (EU502869). Regarding CO1, the sequence demonstrated 100% similarity with various sequence of *S. lutrae* (MT036250, MT036254, MG273661-MG273670, MF596284-MF596285, MG372106-MG372107, KM657808, KF601326) and S. lari (MF596283, MF596284).

4. Discussion

In this survey, *S. falcatula*-like *parasites* were the most prevalent species of *Sarcocystis* in birds, given that among the 38 samples in which *Sarcocystis* spp. were molecularly identified, 28 were *S. falcatula*. All of these 28 samples were closely related to *S. falcatula* is. Lorikeet, which caused the death of parrots (*Trichoglossus moluccanus*) in a zoo in the United States (Verma et al., 2018); and to unnamed species of *Sarcocystis* that were found in naturally infected Magellanic penguins (*Spheniscus magellanicus*) in Brazil (Acosta et al., 2018).

As previously pointed out, molecular studies have shown that *S. falcatula* consists of a heterogeneous population formed by at least two lineages (Cesar et al., 2018; Dubey et al., 2000c, 2001a, 2001c; Gondim et al., 2017, 2019; Marsh et al., 1999; Valadas et al., 2016). In fact, all bird-derived *S. falcatula*-like of the present survey belonged to the same lineage, along with other isolates that had already been detected in Brazil, e.g. *S. falcatula*-like characterized from cysts in penguins (*S. magellanicus*), *S. falcatula*-like detected in neural tissues from naturally infected ibis (*P. infuscatus*) and *S. falcatula*-like from budgerigars (*M. undulatus*) that were experimentally infected with oocysts derived from Brazilian opossums (Acosta et al., 2018; Gondim et al., 2019; Konradt et al., 2017).

Although the seven sequences of clade B (#137, 138, 282, 431, 444, 452 and 453) showed phylogenetic relatedness to *S. falcatula*-like

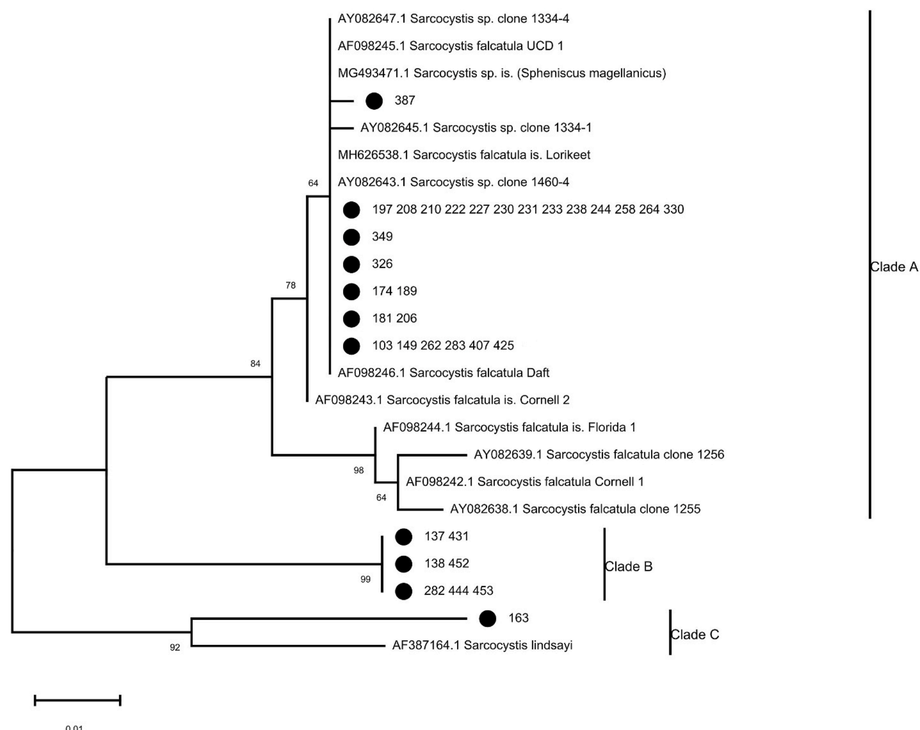

Fig. 1. Phylogenetic tree of *Sarcocystis* spp. based on ITS1 sequences. The tree was constructed through the maximum likelihood method, using the best-fit model K2P + G. The final alignment contained 24 sequences and 389 aligned nucleotide positions. All positions containing gaps and missing data were eliminated (complete deletion option). Numbers on branches represent bootstrap values after 1000 replicates. The black dots identify the sequences obtained in this study.

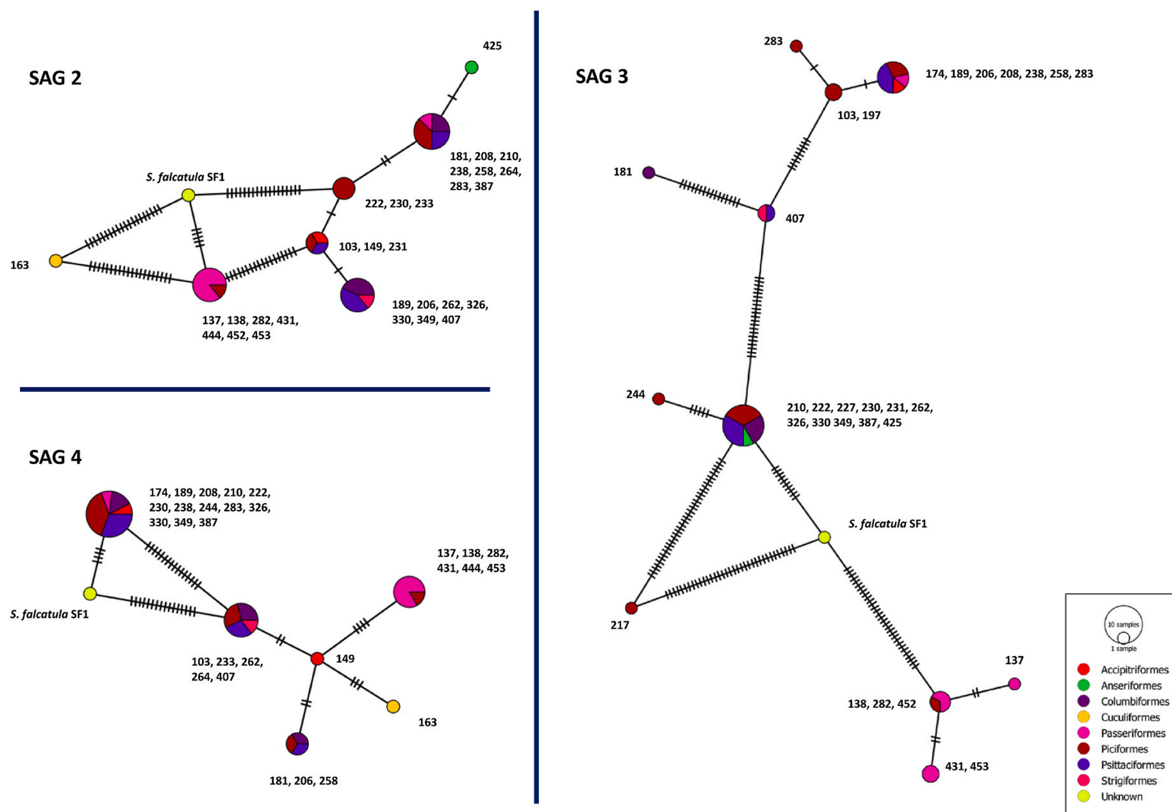

Fig. 2. SAG1 (a), SAG2 (b) and SAG3 (c) haplotype networks for *Sarcocystis falcatula* and other closely related species obtained in this study. Perpendicular bars along the branches refer to mutation changes. The sizes of the circles are proportional to the numbers of haplotypes, and colors indicate the different orders of birds found. The numbers correspond to the sample IDs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

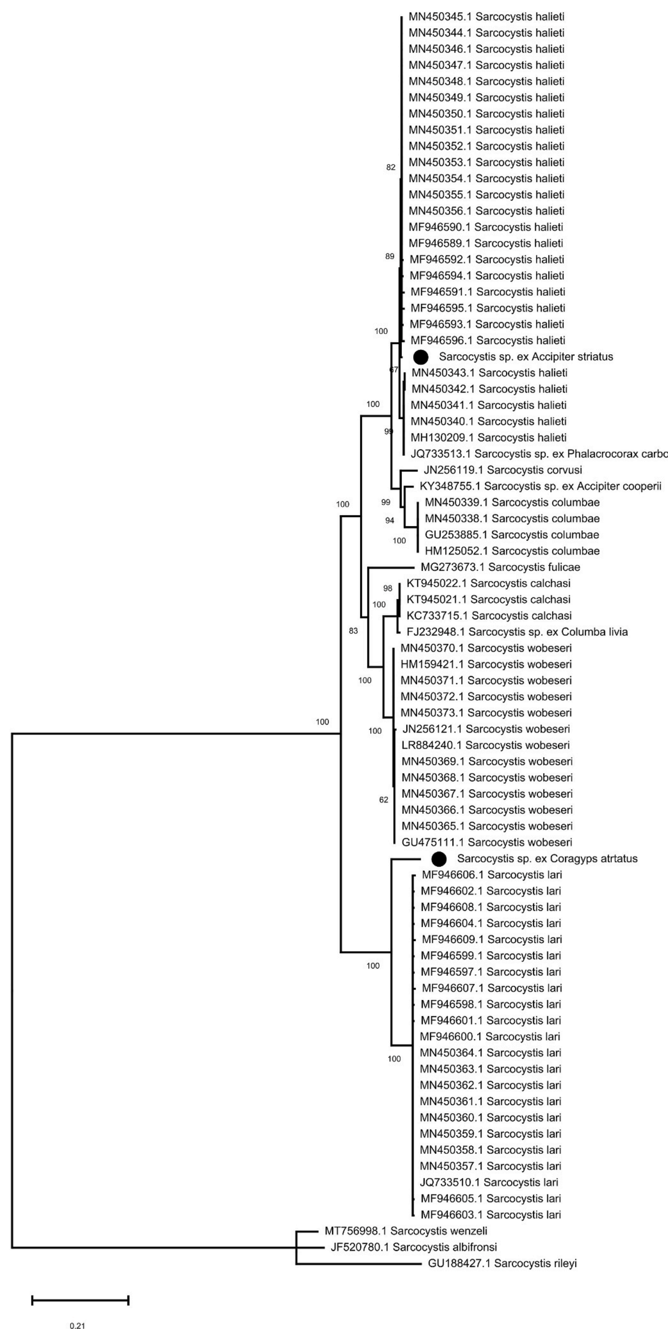

Fig. 3. Phylogenetic tree of *Sarcocystis* spp. based on ITS1 sequences. The tree was constructed through the maximum likelihood method, using the best-fit model HKY + I. The final alignment contained 78 sequences and 661 aligned nucleotide positions. All positions containing gaps and missing data were eliminated (complete deletion option). Numbers on branches represent bootstrap values after 1000 replicates. The black dots identify the sequences obtained in this study.

parasites, a robust evolutionary divergence was revealed between clades A and B, which strongly suggested that the samples in the latter branch belong to a species that has not yet been described. At the ITS1 locus, the clade B samples, which were detected in the birds red-rumped cacique (*Cacicus haemorrhous*), ruby-crowned tanager (*Tachyphonus coronatus*), Brazilian tanager (*Ramphocelus bresilius*) and saffron toucanet (*Pteroglossus bailloni*), had less than 93.50% similarity to both *S. falcatula* is. Lorikeet (MH626538) and *S. falcatula* is. Cornell 1 (AF098242).

Likewise, sample #163 (within clade C, which includes *S. lindsayi*) probably referred to another undescribed species of *Sarcocystis*, given that the ITS1-based phylogeny also showed robust evolutionary divergence from clade C to A and B. A Blast search using #163-ITS1 as the query revealed that this sequence was 93.55% similar to *S. lindsayi* (AF387164), which shows that sample #163 should not be identified as *S. lindsayi*.

As expected, CO1 and 18S were well conserved to allow for differentiation between samples from clades A, B and C. It is well known that these markers do not differentiate between other closely related *Sarcocystis* species that use birds as intermediate hosts (Gjerde et al., 2018; Prakas et al., 2018a). Nevertheless, samples from the clades B and C may correspond to novel species through *Sarcocystis* related to *S. falcatula*. Although our study did not focus on diagnosing sarcocystosis by histopathological assessments because all the samples were frozen and/or lysed, two pectoral muscle samples that were molecularly identified in this study as *S. falcatula* and *Sarcocystis* sp. ex *Cacicus haemorrhous* were thawed, fixed in 10% buffered formalin and stained with hematoxylin and eosin (HE). Surprisingly, despite being frozen for about 2 years, the morphology of the cyst remained intact (Fig. S1, and Fig. S2 in supplementary file).

The allele variants of SAG2, SAG3 and SAG4 that were obtained from samples from clades A, B, and C were compared with homologous material that is available in GenBank. Most of them were 100% identical to the homologous alleles described for *Sarcocystis* spp. that were obtained from opossum-derived sporocysts (Monteiro et al., 2013; Valadas et al., 2016), from isolates in bioassays with parakeets (Cesar et al., 2018; Gondim et al., 2017) and from natural infections in wild birds (Acosta et al., 2018; Konradt et al., 2017) in Brazil.

Nevertheless, 10 undescribed SAG alleles were detected, which corroborates the findings of the aforementioned studies, in which it was claimed that high diversity within *S. falcatula* complex exists in Brazil. In addition, regarding the samples identified as *S. falcatula*-like, twelve SAG genotypes were detected among 19 individuals, of which 10 were unique. This raises the number of SAG genotypes so far described in Brazil from 11 to 21. For *Sarcocystis* sp. ex *Cacicus haemorrhous,* three genotypes were detected in five birds. It was not possible to determine the SAG genotypes for all 36 samples for one of the following two possible reasons: presence of a mixture of sequences (more than one genotype in the same sample) or unsuccessful amplification. Mixed sarcocystosis infections in birds have already been described by Dubey et al. (2004).

Sexual recombination might be an event that shapes the genetic structure of the *S. falcatula* complex population, such that the admixture of highly variable alleles would form a plethora of SAG genotypes. In our sample, the chances of finding infected birds with different genotypes were high. Among the 19 *S. falcatula*-like that were fully SAG genotyped, there were 12 SAG genotypes, which means that the probability that two species selected at random would belong to different genotypes was 88.6%. Unfortunately, it was not possible to compare studies on SAG diversity in *S. falcatula* complex between the southern hemisphere and the northern hemisphere, because no studies have yet been conducted in the northern hemisphere.

Birds of different species can be infected by the same genotype. Orders such as Psittaciformes (*B. tirica*), Piciformes (*C. flavescens*), Passeriformes (*C. gujanensis*) and Accipitriformes (*P. unicinctus*) share the same genotype, thus indicating that the different *S. falcatula*-like SAG genotypes are not host-specific. On the other hand, the same species of bird can be infected by more than one genotype: three genotypes (G1, G2, G10) were identified in toucans (*R. dicolorus*). This contrasts with the observations of Acosta et al. (2018), who described only one genotype in 16 individuals of the Magellanic penguin species. In addition, most of the genotypes found in the birds surveyed here had already been described in Didelphid opossums in Brazil, demonstrating their plausible role as the final host not only for *S. falcatula* complex but also for *Sarcocystis* sp. of clade B.

None of the *S. falcatula*-like positive-birds had combinations of SAG alleles identical to what was described in neurologically affected bare-

faced ibis (*P. infuscatus*), in which *S. falcatula* was incriminated as a causal agent for their death in Brazil. It remains unknown whether any *S. falcatula*-like genotype is especially pathogenic to birds, as is the case of certain variants in *S. neurona*, such as genotypes I and XIII, which were associated with high mortality among aquatic mammals (Barbosa et al., 2015; Miller et al., 2010; Wendte et al., 2010). Further studies are needed in order to support the hypothesis that certain genotypes of *S. falcatula* might be associated with mortality among birds.

To our knowledge, this was the first report on natural infection by *S. falcatula* and related species in 19 species of wild birds from Brazil. This high number of novel species can be explained because birds of the New World live with subclinical infection caused by this agent without presenting symptoms, while birds of the Old World are susceptible to this infection, which has been found to often cause outbreaks with high mortality, when the etiological agent has been investigated.

The order that presented the largest number of bird species infected with *S. falcatula*-like was Piciformes. This was the first time that *S. falcatula complex* had been detected in toucans and woodpeckers. On the other hand, in 11/25 (44%) samples, out of 146 Passeriformes analyzed, only three birds (2%) showed *S. falcatula* DNA. We speculate that the characteristic common to some species of birds, such as those of the families Ramphastidae and Picidae, of nesting in tree holes may favor contact with didelphid feces. Opossums frequently invade nests during the day, in search of food (Smith, 2007).

In the present study, there was high statistical support to show that the sequence of *Sarcocystis* sp. ex *Accipiter striatus* (#213) clustered in a single clade together with 27 sequences of *S. halieti* from other parts of the world (17 *S. halieti* sequences from Norway and 10 from Lithuania). Along with them, there was a sequence derived from *Sarcocystis* sp. that was detected in skuas in Chile. The *S. halieti* clade is a sister group of a clade comprising sequences of *S. corvusi*, *S. columbae* and an unnamed species of *Sarcocystis* that uses *A. cooperii* as its definitive host.

Recent molecular studies identified two species of seabirds from Lithuania, the great cormorant (*Phalacrocorax carbo*) and the herring gull (*Larus argentatus)* (Prakas et al., 2018b; 2020), as intermediate hosts of *S. halieti*. Consequently, the findings from our study suggest that the range of intermediate hosts available for *S. halieti* is much wider and can include small species of Accipitriformes. These hosts include the sharp-shinned hawk, which has never been reported outside of the Americas. This species is considered to have uncertain migratory behavior and seems to be sedentary (Bildstein and Myer, 2000; Eduardo et al., 2007). The white-tailed eagle (*Haliaeetus albicilla*) from Norway (Gjerde et al., 2018) and the Eurasian sparrow-hawk (*Accipiter nisus*) from Germany (Mayr et al., 2016) have been confirmed as definitive hosts for *S. halieti*, with distribution between Europe and Asia (BirdLife International, 2016, 2020). Therefore, the definitive host for *S. halieti* in the Americas must be a similar species of raptor. For the sharp-shinned hawk, some birds of prey such as bald eagles (*Haliaeetus leucocephalus*) and peregrine falcons (*Falco peregrinus*) have been described as predators (Bildstein and Meyer, 2000). Further studies should be conducted to elucidate the life cycle of *S. halieti* in birds in the Americas.

Regarding *Sarcocystis* sp. ex *Coragyps atratus* (#471)*,* the present study suggested that it belongs to species that have not yet been classified, but which are closely related to *S. lari.* The latter has two species of seagulls (*Larus marinus* and *Larus argentatus*) as intermediate hosts and the white-tailed sea-eagle (*Haliaeetus albicilla*) as the definitive host (Gjerde et al., 2018; Prakas et al., 2014, 2020). Numerous published phylogenetic analyses have shown that *Sarcocystis* spp. generally cluster according to their definitive hosts, and the phylogenetic placement of a species may therefore be used to predict its most likely final host (Gjerde, 2014). The phylogenetic relationships of *Sarcocystis* sp. ex *Coragyps atratus* with sequences of *Sarcocystis* spp., using birds of prey as proven or presumed definitive hosts, suggests that the definitive host of this unclassified species is probably also a raptor.

In the case of *Sarcocystis* parasitizing vultures, few investigations have been conducted, and these were limited to assessments of infection prevalence and morphological analysis on cysts by mean of optical microscopy. In the United States, Lindsay and Blagburn (1999) observed bradyzoites in 1/2 (50%) of the black vultures (*C. atratus*), by means of the acid-pepsin digestion technique. In contrast, in the same country, sarcocysts were not detected through histological analysis on three black vultures, although 2/2 (100%) turkey vultures (*Cathartes aura*) were infected with *Sarcocystis* (Dohlen et al., 2019).

Sarcocystosis in raptors is being increasingly reported from North America and Europe, and in some cases it has been associated with clinical disease (Olson et al., 2007; Parmentier et al., 2018; Wünschmann et al., 2009, 2010). However, little is known about protozoan infections of raptors in South America. Here, we reported two species of *Sarcocystis* from birds of prey in Brazil, based on DNA investigations. Further research on *Sarcocystis* epidemiology among birds of prey in South America is needed. Our sequence analysis on three genetic loci showed that *Sarcocystis* sp. ex *Coragyps atratus* is a species of *Sarcocystis* that has not yet been described, but that *Sarcocystis* sp. ex *Accipiter striatus* found in sharp-shinned hawks from Brazil is *S. halieti.*

Toxoplasma gondii DNA was detected in 1.5% (6/400) of the birds examined. Analysis on the 411 bp effectively sequenced from the Picazuro pigeon (*P. picazuro*), American kestrel (*F. sparverius*) and snowy egret (*E. thula*) revealed that this sequence was 100% identical to *T. gondii* (MH793505). For the black-crowned night-heron (*N. nycticorax*) and snowy egret (*E. thula*), only one substitution of the C-T nucleotide was detected at position 62 (taking MH793505 as reference), reaching similarity of 99.76% with *T. gondii.*

Studies in Brazil have reported *T. gondii* DNA from several species of birds, such as the eared dove (*Zenaida auriculata*), crested caracara (*Caracara plancus*), tropical screech-owl (*Megascops choliba*), roadside hawk (*Rupornis magnirostris*), lineated woodpecker (*Dryocopus lineatus*), campo flicker (*Colaptes campestris*), American kestrel (*Falco sparverius*) and toco toucan (*Ramphastos toco*) (Barros et al., 2014; Rêgo et al., 2018; Silva et al., 2018; Vitaliano et al., 2014).

The role of wild birds in the transmission of *T. gondii* has not yet been fully elucidated (Lindsay et al., 1991). We found that two types of herons (snowy egret and black-crowned night-heron) were naturally infected with *T. gondii*, thus providing evidence of contamination of shallow water with oocysts in the state of São Paulo. Occurrences of *T. gondii* antibodies in various species of seabirds, such as the masked booby, brown booby, red-billed tropicbird and white-tailed tropicbird, indicate that *T. gondii* infection is common in waterbirds in Brazil (Gennari et al., 2016). Moreover, among six American kestrels (birds of prey) that were used for direct diagnosis by means of PCR, two (33.3%) were positive for *T. gondii*. The fact that this species has carnivorous habits suggests that the transmission route probably consisted of infection through ingestion of prey that was chronically infected with *T. gondii*. Thus, it is possible to infer that other wild animal species may also be infected by *T. gondii*, thus increasing the number of likely *T. gondii* intermediate hosts. Our study contributes towards expanding the list of birds that possibly participate in the epidemiological chain of *T. gondii*. Nonetheless, further studies are needed in this regard.

The findings reported here put a spotlight on the diversity of the Sarcocystidae in wild birds from South America. Otherwise they might represent an underestimation of the actual prevalence of Sarcocystidae infection, mainly because of the small fragment of tissue examined and to the fact that these parasites may have tropism in different organs, e.g. the nervous system.

5. Conclusions

In summary, the present study extends the range of species of wild birds that have DNA from Sarcocystidae and indicates that there is widespread exposure to *Sarcocystis* species among various orders of wild birds in Brazil. Interestingly, Piciformes and Psittaciformes showed the highest numbers of birds positive for *S. falcatula*. Surface antigen gene (SAG) sequences of *S. falcatula* from 19 bird samples revealed fairly high

haplotype richness that coincided with the extensive diversity of SAG allele variants of sporocysts from South American opossums. This high genetic diversity in species of *S. falcatula* may be explained by processes of gametogony in the definitive host, combined with a high transmission rate in the wild. We also presented evidence of two species of *Sarcocystis* related to *S. falcatula* that have not yet been described. *Sarcocystis* sp. ex *Cacicus haemorrhous* and *Sarcocystis* sp. ex *Guira guira* were detected, from the Passeriformes and Cuculiformes orders, respectively. SAG analysis on one of these species confirmed that opossums can be definitive hosts for new species, in addition to *S. falcatula*, *S. neurona*, *S. lindsayi* and *S. speeri*. Further studies using methods that combine morphological, morphometric, epidemiological and molecular characterization are needed in order to better characterize these species that have not yet been described. To the best of our knowledge, this study provides the first report of *S. halieti* in a species of Accipitriformes in the Americas. Birds of prey that act as final hosts for these *Sarcocystis* species should be present in South America. Therefore, efforts to help clarify their epidemiological cycle need to be conducted. Additionally, we presented evidence for the existence of species of *Sarcocystis* that have not yet been described, which was detected in the American black vulture.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.03.002.

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