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

Horwald A.B. Llano a, b, *, Heloíza Zavatieri Polato b, Lara Borges Keid c, Trícia Maria Ferreira de Souza Oliveira c, Ticiana Zwarg d, Alice S. de Oliveira d, Thaís C. Sanches d, Adriana M. Joppert d, Luís F.P. Gondim e, Rodrigo Martins Soares b

a Investigation Group (GINVER), School of Veterinary Medicine, Corporación Universitaria Remington, Medellín, Colombia
b Department of Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Science, University of São Paulo (USP), São Paulo, SP, Brazil
c Department of Veterinary Medicine, School of Animal Science and Food Engineering, University of São Paulo (USP), Pirassununga, SP, Brazil
d The Fauna Division of the Municipal Secretariat for Green and Environment of the Municipality of São Paulo, SP, Brazil
e Department of Anatomy, Pathology and Clinics, School of Veterinary Medicine and Animal Science, Federal University of Bahia (UFBA), Salvador, BA, Brazil

**ARTICLE INFO**

**Keywords:**
18S
Genetic diversity
ITS1
Molecular characterization
Sarcocystis
Toxoplasma gondii

**ABSTRACT**

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 falcata*una (ten Piciformes, eight Psittaciformes, five Columbiformes, five Accipitriformes, one Anseriformes, one Psittaciformes, one Stigmasteraformes); 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. falcata*una-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. falcata*una-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. falcata*una infection in 14 species, including in the order Piciformes, and shows the high genetic diversity of *S. falcata*una in intermediate hosts in South America. Evidence of occurrence 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 falcata*una, 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 (Écco et al., 2008; Suedmeyer et al., 2001),...
...S. falcatula...
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. falcata. two ITS1 sequences related to other species within the genus Sarcocystis (Sarcocystis halieiti 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 other species within the genus Sarcocystis were around 900 bp; and those from samples identified as being from S. falcata, 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 being from 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. falcata-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. falcata-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. falcata and S. falcata-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. falcata (KT207461 and MH665257, respectively). At the 18S locus, clades B and C differed at one SNP from S. falcata (MH662537) and were identical to S. speeri (KT207459).

Through SAG typing of the 36 S. falcata-related samples, seven alleles were found at SAG2, 11 alleles at SAG3 and 6 alleles at SAG4 (Fig. 2). Among these, 24 sequences 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. falcata-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 halieiti (#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. halieiti (MH130211, MF946587), as well to various unnamed species of Sarcocystis from Accipiter cooperii (KY348753, EU810398), Phalacrocorax carbo (JQ733511), Columba livia (GQ246570) and Anser albifrons (EU502869). Concerning CO1, the Sarcocystis sp. ex Accipiter striatus haplotype was 100% identical to S. halieiti (MH138308, MH138309, MF946583), S. corvus (MH138314) and S. columbae (MH138312). Regarding the 18S rRNA gene, Sarcocystis sp. ex Coragyps atratus shared the highest similarity (99.23%) with S. halieiti (MH130211, MF946587) and various unnamed species of

### Table 1

Molecular identification of sarcocystids in muscle samples from wild birds in Brazil, based on nPCR-ITS1 sequence analysis.

| Animal ID- | common name (scientific name) | Sex | Positives/ total (%) | Sequenced product (bp) | Sequence similarity (%) to closest in GenBank |
|-----------|--------------------------------|-----|----------------------|------------------------|---------------------------------------------|
| Ampligen size > 1000 BP (N = 36) |
| **Psittacidae** |
| 238- plain parakeet (Brotogeris tirica) | M | 4/14 (20.5) | 419 | 99.8% S. falcata is. Lorikeet (MH626538) |
| 189- plain parakeet (Brotogeris tirica) | M | 651 | | 99.7% S. falcata is. Lorikeet (MH662538) |
| 210- plain parakeet (Brotogeris tirica) | F | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 231- plain parakeet (Brotogeris tirica) | F | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 206- scaly-headed parrot (Pionus maximiliani) | M | 1/2 (50) | 1012 | 99.8% S. falcata is. Lorikeet (MH662538) |
| 504- turquoise-fronted amazon (Amazona aestiva) | M | 3/9 (33.3) | 1013 | 99.9% S. falcata is. Lorikeet (MH662538) |
| 519- turquoise-fronted amazon (Amazona aestiva) | F | 1013 | | 99.9% S. falcata is. Lorikeet (MH662538) |
| 349- turquoise-fronted amazon (Amazona aestiva) | M | 652 | | 99.7% S. falcata is. Lorikeet (MH662538) |
| **Piciformes: Picidae** |
| 227- blond-crested woodpecker (Celus flavescens) | F | 3/9 (33.3) | 419 | 99.9% S. falcata is. Lorikeet (MH662538) |
| 387- blond-crested woodpecker (Celus flavescens) | F | 419 | | 99.5% S. falcata is. Lorikeet (MH662538) |
| 103- blond-crested woodpecker (Celus flavescens) | M | 652 | | 99.9% S. falcata is. Lorikeet (MH662538) |
| 208- lined woodpecker (Dryocopus lineatus) | F | 1/6 (16.6) | 419 | 99.8% S. falcata is. Lorikeet (MH662538) |
| **Piciformes: Ramphastidae** |
| 197- red-toucan (Rhamphastos dicolorus) | M | 6/8 (75) | 419 | 99.8% S. falcata is. Lorikeet (MH662538) |
| 222- red-toucan (Rhamphastos dicolorus) | F | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 230- red-toucan (Rhamphastos dicolorus) | M | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 233- red-toucan (Rhamphastos dicolorus) | M | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 244- red-toucan (Rhamphastos dicolorus) | M | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |
| 258- red-toucan (Rhamphastos dicolorus) | F | 419 | | 99.8% S. falcata is. Lorikeet (MH662538) |

(continued on next page)
| Animal ID- common name (scientific name) | Sex¹ | 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 (MH662538) |
| Columbiformes: Columbidae | | | | |
| 181- picazouro pigeon (Patagioenas picazouro) | M | 2/13 (15.3) | 1012 | 99.8% S. falcatula is. Lorikeet (MH662538) |
| Cuculiformes: Cuculidae | | | | |
| 262- picazouro pigeon (Patagioenas picazouro) | M | 1/13 (7.7) | 411 | 100.00% Toxoplasma gondii (MH793505) |
| Passeriformes: Vireonidae | | | | |
| 283- rufous-browed peppershrike (Cyclarhis gujanensis) | M | 1/2 (50) | 419 | 99.8% S. falcatula is. Lorikeet (MH662538) |
| Passeriformes: Fringillidae | | | | |
| 137- ruby-crowned tanager (Tachyphonusoron) | M | 1/1 (100) | 1021 | 93.19% S. falcatula is. Lorikeet (MH662538) |
| Passeriformes: Icteridae | | | | |
| 452- Brazilian tanager (Ramphocelus bresilius) | M | 4/6 (66.6) | 1021 | 93.39% S. falcatula is. Lorikeet (MH662538) |
| Acipitriformes: Accipitridae | | | | |
| 174- Harris’s hawk (Parabuteo unicinctus) | F | 1/4 (25) | 651 | 99.7% S. falcatula is. Lorikeet (MH662538) |
| 317- black-shouldered kite (Milvus lineatus) | M | 1/13 (7.7) | 411 | 100.00% Toxoplasma gondii (MH793505) |
| Strigiformes: Strigidae | | | | |
| 410- screech-owl (Megascops choliba) | M | 1/9 (11.1) | 1013 | 99.9% S. falcatula is. Lorikeet (MH662538) |
| Anseriformes: Anatidae | | | | |
| 163- guia cuckoo (Guira guira) | M | 1/2 (50) | 1008 | 92.65% S. falcatula is. Lorikeet (MH662538) 93.55% S. lindsayi (AP387164) |

**Table 1 (continued)**

| Animal ID- common name (scientific name) | Sex¹ | Positives/ total (%) | Sequenced product (bp) | Sequence similarity (%) to closest in GenBank |
|----------------------------------------|------|----------------------|------------------------|-----------------------------------------------|
| 213- sharp-shinned hawk (Accipiter harrisii) | F | 1/5 (20) | 842 | 99.17% Sarcocystis haemorrhous |
| Cathartiformes: Cathartidae | | | | |
| 471- American black vulture (Coragyps atratus) | M | 1/11 (9.1) | 800 | 90.04% Sarcocystis lari is. Ha. 1.8 |
| Columbiformes: Columbidae | | | | |
| 433- black-shouldered kite (Milvus lineatus) | M | 1/13 (7.7) | 411 | 100.00% Toxoplasma gondii (MH793505) |
| Falconiformes: Falconidae | | | | |
| 452- Brazilian tanager (Ramphocelus bresilius) | M | 1/1 (100) | 1021 | 93.39% S. falcatula is. Lorikeet (MH662538) |
| Pelecaniformes: Ardeidae | | | | |
| 433- black-crowned night-heron (Nycticorax ncticorax) | M | 1/2 (50) | 511 | 97.76% Toxoplasma gondii (MH793505) |

¹ 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 (JQ533511), Columba livia (GQ2145670) and Anser albifrons (EU502869). Regarding CO1, the sequence demonstrated 100% similarity with various sequence of S. lutea (MT037698, MT037699, MG237361-MG237360, MF296284-MF296285, MG237210-MG237217, 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.)
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 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; Konrad 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. falcata complex exists in Brazil. In addition, regarding the samples identified as S. falcata-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 spp. ex Cacus 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. falcata 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. falcata-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. falcata 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. tiraica), Piciformes (C. flavescens), Passeriformes (C. guajanus) and Accipitriformes (P. uncinctus) share the same genotype, thus indicating that the different S. falcata-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, G3) 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. falcata complex but also for Sarcocystis sp. of clade B.

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

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.

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 (Jerde 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. falcata. 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. falcata and Sarcocystis sp. ex Cacus 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; Konrad 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. falcata complex exists in Brazil. In addition, regarding the samples identified as S. falcata-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 Cacus 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. falcata 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. falcata-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. falcata 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. tiraica), Piciformes (C. flavescens), Passeriformes (C. guajanus) and Accipitriformes (P. uncinctus) share the same genotype, thus indicating that the different S. falcata-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, G3) 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. falcata complex but also for Sarcocystis sp. of clade B.

None of the S. falcata-like positive-birds had combinations of SAG alleles identical to what was described in neurologically affected bare-
faced ibis (P. infuscatus), in which S. falcataula was incriminated as a causal agent for their death in Brazil. It remains unknown whether any S. falcataula-like genotype is especially pathogenic to birds, as is the case of certain variants in S. neurola, 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. falcataula might be associated with mortality among birds.

To our knowledge, this was the first report on natural infection by S. falcataula 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. falcataula-like was Piciformes. This was the first time that S. falcataula 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. falcataula 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) 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 skusas in Chile. The S. halieti clade is a sister group of a clade comprising sequences of S. corvus, 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 Accipitridae. 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 (May 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., 2016; 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 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 Pic-azuro pigeon (P. picazuro), American kestrel (S. sparverius) and snowy egret (E. thula) revealed that this sequence was 100% identical to T. gondii (MH793505). For the black-crowned night-heron (N. nicticorax) 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), linedated woodpecker (Dryocopus lineatus), campo flicker (Colaptes campestris), American kestrel (Falco sparverius) and toco toucan (Ramphastos toco) (Barros et al., 2014; Rego 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 Pelecaniformes showed the highest numbers of birds positive for S. falcataula. Surface antigen gene (SAG) sequences of S. falcataula 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. falcatta 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. falcatta 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. falcatta, S. neurona, S. lindsayi and S. speerti. 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 Sarcocystis species 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.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development -Brazil (Process n. 420219/2016-1-CNPNq). Horvold A.B. Llano received a doctoral scholarship (Process n. 161046/2015-0-CNPNq), Lara Borges Keid, Luís F.P. Gondim and Rodrigo Martins Soares are recipients of productivity fellowships from CNPq.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.03.002.

References

Acotta, I.C.L., Gennari, S.M., Llano, H.A.B., Muñoz-Leal, S., Soares, R.M., 2021. Molecular characterization of new haplotypes of genus sarcocystis in seabirds from Magdalena island, southern Chile. Animals 11, 245. https://doi.org/10.3390/ani11020245.

Acotta, I.C.L., Soares, R.M., Mayorga, L.F.S.P., Alves, B.F., Soares, H.S., Gennari, S.M., 2018. Occurrence of tissue cyst forming coccidia in Magellanic penguins (Spheniscus magellanicus) rescued on the coast of Brazil. PLoS One 13, e0209007. https://doi.org/10.1371/journal.pone.0209007.

Barbosa, L., Johnson, C.K., Lambourn, D.M., Gibson, A.K., Haman, K.H., Huggins, J.L., Sweeney, A.R., Sundar, N., Revata, S.A., Grigg, M.E., 2015. A novel Sarcocystis neurona genotype XIII is associated with severe encephalitis in an unexpectedly broad range of marine mammals from the northeastern Pacific Ocean. Int. J. Parasitol. 45, 607–613. https://doi.org/10.1016/j.ijpara.2015.02.013.

Gjerde, B., 2014. Molecular characterization of Sarcocystis rileyi from a common eider (Somateria mollissima) in Norway. Parasitol. Res. 113, 3501–3509. https://doi.org/10.1007/s00436-014-4062-y.

Gjerde, B., 2013. Phylogenetic relationships among Sarcocystis species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. J. Parasitol. 99, 437–539. https://doi.org/10.1645/13-004.1.

Gjerde, B., Vikoren, T., Haines, I.S., 2018. Molecular identification of Sarcocystis halieti sp. nov. Sarcocystis lari and Sarcocystis trunci in the intestine of a white-tailed sea-eagle (Haliaeetus albicilla) in Norway. Int. J. Parasitol. 48, 7–11. https://doi.org/10.1016/j.ijpara.2017.12.001.

Gjerde, B., 2013. Seroprevalence of Sarcocystis falcatus in captive psittacine birds in Brazil. J. Avian Med. Surg. 23, 18–23. https://doi.org/10.1647/11288.1.
