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Original Article

Molecular diversity of *Sarcocystis* spp. in opossums (*Didelphis* spp.) from Southeastern and Midwestern Brazil

Diversidade molecular de *Sarcocystis* spp. em gambás (*Didelphis* spp.) do Sudeste e Centro-oeste do Brasil

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Abstract

South American opossums (*Didelphis* spp.) are definitive hosts of *Sarcocystis neurona, Sarcocystis speeri, Sarcocystis lindsayi* and *Sarcocystis falcatula*. In Brazil, diverse studies have demonstrated a high frequency of *Sarcocystis falcatula*-like in sporocysts derived from opossums, and high genetic diversity has been observed in surface antigen-encoding genes (*SAGs*). In this study, genetic diversity of *Sarcocystis* spp. derived from *Didelphis albiventris* and *Didelphis aurita* from the cities of Campo Grande and São Paulo, was accessed by sequencing *SAG2*, *SAG3*, *SAG4,* the first internal transcribed spacer (ITS-1) and cytochrome c oxidase subunit I (*cox1*). Molecular identification was performed for 16 DNA samples obtained from sporocyst or culture-derived merozoites. The ITS-1, *cox1*, and *SAG3* fragments were cloned, whereas *SAG2* and *SAG4* were sequenced directly from PCR products. Four alleles variants were found for *SAG2*, 13 for *SAG3* and seven for *SAG4,* from which four, 13 and four, respectively, were novel. Twenty-seven allele variants were found for ITS-1, all phylogenetically related to *S. falcatula*-like previously described in Brazil. *Sarcocystis* sp. phylogenetically related to *Sarcocystis rileyi* was evidenced by *cox1* in three opossums. Further studies are needed to clarify the role of *Didelphis* spp. as definitive hosts of *Sarcocystis* spp. other than that previous described.

Keywords: *Sarcocystis* spp., opossums, molecular characterization, ITS-1, *cox1, SAGs*.

Resumo

Gambás sul-americanos (*Didelphis* spp.) são hospedeiros definitivos de *Sarcocystis neurona, Sarcocystis speeri, Sarcocystis lindsayi* e *Sarcocystis falcatula*. No Brasil, diversos estudos têm demonstrado alta frequência de *Sarcocystis falcatula*-like em esporocistos derivados de gambás, com grande diversidade nos genes que codificam antígenos de superfície (*SAGs*). Neste estudo, a diversidade genética de *Sarcocystis* spp., oriundos de *Didelphis albiventris* e *Didelphis aurita,* dos municípios de Campo Grande e São Paulo, foi acessada por meio do sequenciamento de *SAG2*, *SAG3* e *SAG4,* da primeira região espaçadora interna transcrita (ITS-1) e citocromo c oxidase subunidade I (*cox1*). Identificação molecular foi realizada em 16 amostras de DNA, obtidas de esporocistos ou merozoítos derivados de cultivo. Os fragmentos de ITS-1, *cox1* e *SAG3* foram clonados, enquanto *SAG2* e *SAG4* foram sequenciados diretamente dos produtos de PCR. Quatro alelos foram observados em *SAG2*, 13 em *SAG3* e sete em *SAG4,* sendo novos quatro, 13 e quatro, respectivamente. Em ITS-1, 27 alelos foram observados, todos filogeneticamente relacionados à *S. falcatula*-like, previamente detectados no Brasil. *Sarcocystis* sp. filogeneticamente relacionado à *Sarcocystis rileyi* foi evidenciado por *cox1* em três gambás. Mais estudos são necessários para entender o papel de *Didelphis* spp. como hospedeiro definitivo de *Sarcocystis* spp. diferentes daqueles previamente descritos.

Palavras-chave: *Sarcocystis* spp., gambás, caracterização molecular, ITS-1, *cox1, SAGs*.

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Introduction

Sarcocystis spp. Lankester 1882, are obligate intracellular protozoa belonging to the phylum Apicomplexa Levine 1979. Species of the genus *Sarcocystis* have an obligatory prey-predator two-host life cycle. Opossums (*Didelphis* spp. Linnaeus, 1758), which exclusively inhabit the American continents, act as definitive hosts for *Sarcocystis neurona* Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir, Cummings, and Suter 1991, *Sarcocystis speeri* Dubey and Lindsay 1999, *Sarcocystis falcatula* (Stiles 1893) Box, Meier, and Smith 1984, and *Sarcocystis lindsayi* Dubey, Rosenthal, and Speer 2001 (Box et al., 1984; Dubey et al., 1991, 2001a; Dubey & Lindsay, 1999).

Sarcocystis neurona is the chief etiological agent of equine protozoal myeloencephalitis (EPM) (Dubey et al., 2001b). *Sarcocystis falcatula* has been associated with numerous cases of pulmonary sarcocystosis in free-living and captive birds (Smith et al., 1990; Hillyer et al., 1991; Clubb & Frenkel, 1992; Page et al., 1992; Dubey et al., 2001c; Suedmeyer et al., 2001; Villar et al., 2008; Wünschmann et al., 2009, 2010; Verma et al., 2018). *Sarcocystis speeri* and *S. lindsayi* are respectively, experimentally infective to mice and budgerigars (*Melopsittacus undulatus*), but their natural intermediate hosts are unknown (Dubey & Lindsay, 1999; Dubey et al., 2001a).

Several genetic markers have been used to molecularly characterize *Sarcocystis* spp. shed by opossums in the Americas. Genome annotation of the North American *S. neurona* SO SN1 (Blazejewski et al., 2015) and *S. neurona* SN3 provided important insights into the molecular biology of the parasite. In Brazil, various studies have demonstrated the presence of *Sarcocystis* spp. in sporocysts derived from opossums (Casagrande et al., 2009; Monteiro et al., 2013; Gallo et al., 2018; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018). The majority of the *Sarcocystis* identified in the country have been classified as *Sarcocystis falcatula*-like, due to genetic characteristics and/or experimental infectivity to budgerigars (Gondim et al., 2017, 2019; Acosta et al., 2018; Cesar et al., 2018). Extensive variability has been observed in surface antigen-encoding genes (*SAGs*) of *Sarcocystis* spp. derived from opossums in Brazil (Monteiro et al., 2013; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018). It has been suggested that the diversity of *Sarcocystis* species in the intestine of opossums could enable allele exchange through sexual recombination, contributing to their allelic variability (Monteiro et al., 2013).

Considering the widespread occurrence of *Sarcocystis* spp. in opossums in Brazil and the wide genetic variation observed in previous studies, this study sought to assess the genetic diversity of *Sarcocystis* spp. in *D. albiventris* and *D. aurita* sampled in the cities of Campo Grande (midwestern) and São Paulo (southeastern), Brazil. The detection and molecular characterization of these agents in the opossums of these regions contribute to increasing the knowledge related to the genetic diversity of *Sarcocystis* spp. in Brazil.

Material and Methods

Sampling

Between July 2019 and April 2021, five expeditions were performed for capturing free-ranging opossums. Four expeditions were performed in the city of Campo Grande, Mato Grosso do Sul state (midwestern) and one expedition was performed in the city São Paulo, São Paulo state (southeastern), Brazil. The animals were caught in six locations in the urban region of Campo Grande (1–20°41'37.51" S, 54°61'54.65" O; 2– 20°44'88.11" S, 54°57'95.99" O; 3–20°43'95.15" S, 54°57'43.24" O; 4–20°49'96.79" S, 54°61'35.94" O; 5–20°47'17.08" S, 54°65'60.08" O; 6–20°49'32.15" S, 54°58'09.15" O) and in an equestrian club in the city of São Paulo (23°38'31.3" S, 46°42'35.0" O), using Tomahawk and Sherman live traps baited with a mix of bananas, peanut butter, tinned sardines, and bacon. Together, these five expeditions resulted in the capture of 37 opossums: 26 *Didelphis albiventris* from Campo Grande and 11 *Didelphis aurita* from São Paulo). Trapped opossums were transported to the laboratory, where they were chemically restrained with a combination of cetamina and xilazina (30 mg/Kg and 5 mg/Kg, respectively, intramuscular), followed by euthanasia with T-61 (MSD) (0.3 mL/Kg, intravenously). Necropsy was performed. The small intestine was separated, longitudinally sectioned, and the internal surface was scraped and processed as previously described (Dubey et al., 2016; Gondim et al., 2019). Briefly, intestinal scraping was homogenized with a mixture of 30 mL of sodium hypochlorite (2.5% active chlorine) and 70 ml of distilled water, to disrupt cell clumps and release *Sarcocystis* spp. sporocysts. The solution was filtered through gauze and centrifuged at 2000 x *g* at 4 °C for 10 min. A small drop of the sediment was examined using light microscopy (Olympus BX-51, 400x magnification) for the presence of *Sarcocystis* spp. sporocysts. For positive samples, sodium hypochlorite was removed by two additional washes with PBS (pH 7.2). Sporocysts were purified by sucrose gradient flotation, washed in distilled

water, and stored in a commercial 100x concentrated antibiotic/antimycotic solution (10.000 units/mL of penicillin, 10.000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B- Gibco) at 4 °C.

In vitro growth of *Sarcocystis* spp.

Sporocysts of *Sarcocystis* spp. were processed as previously described (Gondim et al., 2019). The antibiotic/antimycotic solution containing sporocysts was adjusted to a minimum of 1×10^4 sporocysts/mL and 1 mL of the resulting solution was treated with 2.5% sodium hypochlorite for 30 min. Simultaneously, ~0.5 mL glass beads (400–600 µm in diameter, Sigma-Aldrich) were treated with 2.5% sodium hypochlorite. Sporocysts and glass beads were washed thrice with Iscove's modified Dulbecco medium (IMDM) (Invitrogen/Gibco, Carlsbad, USA) to remove hypochlorite. The sporocysts were resuspended in 700 µL of IMDM and added to a microtube containing glass beads. The mixture was vortexed at maximum speed for 3 min. A drop of the solution was observed under a light microscope (Olympus BX-51, 400x magnification) to examine the released sporozoites. The solution was filtered using a sterile 5 µm-pore size filter and subsequently inoculated into T25 flasks containing confluent monolayers of Vero cells (BCRJ: 0245). Cultures were maintained in IMDM supplemented with 1% penicillin/streptomycin/amphotericin B and 5% inactivated bovine calf serum, in a 37 °C incubator with 5% CO_2 (Nuare, NU-4750E, Plymouth, MN, USA). Cultures without parasite propagation were discarded 60 d post-infection.

DNA extraction

DNA was extracted from culture-derived merozoites using the DNeasy blood and tissue kit (Qiagen, Valencia, California, USA), and from sporocysts using the QIAamp DNA stool mini kit (Qiagen, Valencia, California, USA), according to the manufacturer's instructions. The DNA concentration and quality were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, San Jose, CA, USA).

Molecular detection of *Sarcocystis* spp. based on the ITS-1, *cox1*, *SAG2*, *SAG3* and *SAG4* gene fragments

Conventional PCR assays were performed to amplify *Sarcocystis* spp. DNA of the *loci* encoding *cox1*, *SAG2*, and ITS-1. Nested and hemi-nested PCR assays were used to amplify *loci* encoding *SAG3* and *SAG4*, respectively. Primers that amplify most of the open reading frames from *SAG2* and *SAG3* were used to maximize the likelihood of detecting polymorphisms at these *loci*. The primer sequences and cycling conditions are shown in Supplementary Table S1.

The first round of PCR was conducted in a 25-µL total reaction volume containing ~200 ng of target DNA, 0.2 mM mixed deoxynucleotide triphosphates, 3.0 mM $MgCl_2$, 1.25 U Taq Platinum DNA Polymerase (Life Technologies, Carlsbad, CA, USA), 0.4 µM of each primer, 2.5 µL of 10X reaction buffer, and sterile ultra-pure water. For *SAG2, SAG3* and *SAG4,* 2 µL of the product derived from the first amplification was used as a template in an additional round of conventional PCR, nested PCR, and hemi-nested PCR, respectively. A conventional thermocycler device (T100 Thermal Cycler, Bio-Rad, Hercules, CA, USA) was used to conduct the PCR. Ultrapure water and DNA from *S. neurona* merozoites obtained from the intestines of an opossum (*Didelphis virginiana*) (Lindsay et al., 2004) were used as negative and positive controls, respectively. PCR products were separated by electrophoresis on 1% agarose gels containing 0.5 µg/mL ethidium bromide. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System, Bio-Rad, Hercules, CA, USA) using the Image Lab Software v4.1 (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing

The ITS-1, *cox1*, and *SAG3* amplicons were cloned using the pGEM-T vector (Promega, Madison, WI, USA). Ligation reaction products were used to transform One Shot Mach1 T1 *Escherichia coli* cells (Invitrogen Cat # C8620-03) (109–1010 CFU/ng DNA) by thermal shocking. Plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). All the procedures were performed following the manufacturer's recommendations.

Up to three clones from each sample were selected for sequencing in an automatic sequencer (ABI Prism 310 Genetic Analyzer, Applied Biosystem/Perkin Elmer) (Sanger et al., 1977), using primers M13 F and M13 R (Lau et al., 2010) that flank the cloning site of the pGEM-T vector. Additionally, primers ITS-720R19 (Valadas et al., 2016) and *cox1*-275F22 (Gondim et al., 2019) were used to sequence an internal region of ITS-1 and *cox1* fragments to increase the Phred quality of the consensus sequence. The amplicons obtained from the *SAG2*- and *SAG4*-based PCR assays were purified using EXOSAP-IT (Applied Biosystems) and sequenced with the same primer pairs used in the PCR assays.

Sequence editing

Consensus sequences were obtained through analysis of electropherograms with Phred-base calling and the Phrap-assembly tool available in the suite Codoncode aligner v.4.2.1. (Codoncode Corp. Dedham, MA, USA) with a Phred quality score of ≥ 20 (99% accuracy of the base call). Consensus sequences were submitted to BLASTn (http://www.ncbi.nlm.nih.gov) to determine sequence identity by comparison with the sequences available in the GenBank database. The significance of the alignments was determined based on the E-value analysis. A BLAST search, using the newly generated sequences as a query, allowed the retrieval of homologous fragments from *Sarcocystis* spp. with similar lengths.

Identification of genetic relationship of *Sarcocystis* spp.

Genetic diversity was assessed with sequences from all five molecular markers used in this study. The sequences were aligned and used to calculate nucleotide diversity (π), polymorphism level (allele diversity [ad]), number of alleles (a), average number of nucleotide differences (K), and number of variable sites (v), using DnaSP v5.10 (Librado & Rozas, 2009). The alleles were then aligned with sequences available in the GenBank database using ClustalW (Thompson et al., 1994), and adjusted using Bioedit v. 7.0.5.3. (Hall, 1999). Phylogenetic reconstructions using the maximum likelihood (ML) method were performed on MEGA-X software (Tamura et al., 2011). The model for evolutionary distances was also calculated using the MEGA-X software and was applied to identify the most appropriate model for nucleotide substitution. The robustness of the ML tree was statistically evaluated using bootstrap analysis with 1.000 replicates. Representative genomic DNA sequences for each *Sarcocystis* spp. allele observed in this study were deposited in GenBank under the following accession numbers: OL830303–OL830329 (ITS-1), OL780806–OL780816 and OL780818–OL780820 (*cox1*), OL809965–OL809968 (*SAG2*), OP321544–OP321555 and OP811212 (*SAG3*), and OL862280–OL862286 (*SAG4*).

Results

Sarcocystis spp. sporocysts were observed in the intestinal scrapings of 18 out of 37 opossums: 13 *D. albiventris* from Campo Grande, and five *D. aurita* from São Paulo. From the 18 sporocysts suspensions, 12 presented more than 1×10^4 sporocysts/mL and were subjected to *in vitro* culture. Replication of *Sarcocystis* spp. was observed in six cultures: four inoculated with *D. albiventris*-derived sporozoites and two inoculated with *D. aurita*-derived sporozoites. Therefore, from six samples, DNA was obtained from culture-derived merozoites, and from 12 samples, DNA was obtained from sporocysts.

Sequencing could not be performed for all samples due to unsuccessful amplification or low DNA concentration (faint bands) in some PCR amplicons. At least one molecular marker was sequenced from 16 samples, and sequences from all five molecular markers were obtained from 8 samples. From two sporocyst samples none of the molecular markers were successfully sequenced. After cloning, 29 sequences were obtained from ITS-1, 39 from *cox1,* and 13 from *SAG3*. Additionally, nine sequences were obtained from *SAG2* and 15 from *SAG4*. After genetic diversity assessment using DnaSP v5.10, 27 allele variants were obtained for ITS-1, 14 for *cox1*, four for *SAG2*, 13 for *SAG3*, and seven for *SAG4. SAG3* was the molecular marker with the highest allele diversity (1.00 ± 0.03), nucleotide diversity per site (0.055 ± 0.00), and number of nucleotide differences between all sequences (55.4) (Table 1). Samples were named with alphanumeric code (Dal00-CG or Dau00-SP), and allele variants from each locus were designated with Arabic numerals (Table 2).

After BLASTn search for ITS-1, all 27 alleles disclosed identity ranging from 98.98–100% with a sequence of *S. falcatula* from a rainbow lorikeet (*Trichoglossus moluccanus*) from the United States (MH626538), with a query cover value of 100% (Supplementary Table S2). The phylogenetic reconstruction of ITS-1 exhibited three clades (Figure 1). The first clade was formed by the 27 alleles from this study and sequences from *S. falcatula* detected in rainbow lorikeet (*Trichoglossus moluccanus*, MH626538), brown boobies (*Sula leucogaster*, MW822665, MW822670), and ducks (*Anas* sp., OL323100), and sequences of *Sarcocystis* sp. detected in Magellanic penguins (*Spheniscus magellanicus*, MG493471) and opossums (*D. virginiana*, AY082647, and *D. aurita/D. marsupialis*, MK803362). The second clade was formed by sequences from *S. neurona* (MN172273, AF252407, AF081944, and AF204230) and *S. speeri* (KT207458). A third clade was formed exclusively with sequences from *S. falcatula* from the United States (AF098244, AF098242, AY082639, and AY082638).

Table 1. Polymorphism and genetic diversity of *Sarcocystis* spp. detected in wild-caught opossums (*Didelphis albiventris* and *Didelphis aurita*) captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively.

Molecular marker	Size of the fragment analyzed*	Number of sequences analyzed	Number of variable sites (VS)	Number of Alleles (a)	GC content (%)	Allele Diversity (mean ± SD)	Nucleotide Diversity per site = π (mean ± SD)	Average Number of Nucleotide Differences between All Sequences (K)
ITS-1	1075	29	54	27	46.3	0.99 ± 0.01	0.006 ± 0.00	7.32
cox1	972	39	53	14	37.2	0.81 ± 0.04	0.013 ± 0.00	13.01
SAG2	676	9	5	4	54.9	0.80 ± 0.08	0.003 ± 0.00	2.27
SAG3	1106	13	159	13	51.8	1.00 ± 0.03	0.055 ± 0.00	55.4
SAG4	280	15	22	7	58.9	0.87 ± 0.05	0.034 ± 0.00	9.71

*The number of nucleotides in all the sequences was standardized based on the length of the shorter sequence.

Table 2. *Sarcocystis* spp. detected in wild-caught opossums *Didelphis albiventris* and *Didelphis aurita* captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Midwestern, and Southeastern Brazil, respectively. Numbers represent the alleles identified at ITS-1 (27), *cox1* (14), SAG2 (4), SAG3 (13), and SAG4 (7) at genetic diversity assessment. Superscribed letters refer to each of the clones sequenced.

Samples		Species of opossum	Molecular Markers				
ID	Origin of DNA		ITS-1	*cox1*	SAG2	SAG3	SAG4
Dal1-CG	Sporocyst	*Didelphis albiventris*	N	N	N	NS	NS
Dal3-CG	Sporocyst	*Didelphis albiventris*	NS	N	NS	1	1
Dal5-CG	Sporocyst	*Didelphis albiventris*	N	N	NS	NS	2
Dal6-CG	Sporocyst	*Didelphis albiventris*	1^a, 2^b	1^a, 2^b, 3^c	2	2	3
Dal9-CG	Sporocyst	*Didelphis albiventris*	3^a, 4^b, 5^c	$2^{a,b}$	1	NS	4
Dal11-CG	Sporocyst	*Didelphis albiventris*	6^a, 7^b, 8^c	$2^{a,c}$, 4^b	2	3	1
Dal12-CG	Merozoite	*Didelphis albiventris*	9^a, 10^b, 11^c	$5^{a,b,c}$	2	4	2
Dal13-CG	Sporocyst	*Didelphis albiventris*	N	NS	NS	N	N
Dal16-CG	Sporocyst	*Didelphis albiventris*	NS	2^a, 6^b, 7^c	N	N	N
Dal18-CG	Merozoite	*Didelphis albiventris*	12^a, 13^b	$5^{a,b,c}$	2	5	2
Dal23-CG	Merozoite	*Didelphis albiventris*	12^a	$2^{a,b,c}$	3	6	1
Dal26-CG	Sporocyst	*Didelphis albiventris*	N	8^a $9^{b,c}$	N	7	5
Dal27-CG	Merozoite	*Didelphis albiventris*	14^a, 15^b, 16^c	$2^{a,b,c}$	3	8	1
Dau28-SP	Sporocyst	*Didelphis aurita*	17^a, 18^b	10^b, 2^c, 5^a	N	9	4
Dau29-SP	Sporocyst	*Didelphis aurita*	19^a, 20^b	2^b, 11^a,	1	10	4
Dau30-SP	Sporocyst	*Didelphis aurita*	17^b, 21^a	12^a, 13^b	NS	11	6
Dau36-SP	Merozoite	*Didelphis aurita*	22^a, 23^b, 24^c	$9^{a,b,c}$	NS	12	7
Dau37-SP	Merozoite	*Didelphis aurita*	25^a, 26^b, 27^c	14^a, $5^{b,c}$	4	13	6

N = Negative at PCR. NS = Positive at PCR, but not sequenced due to low DNA concentration (faint band).

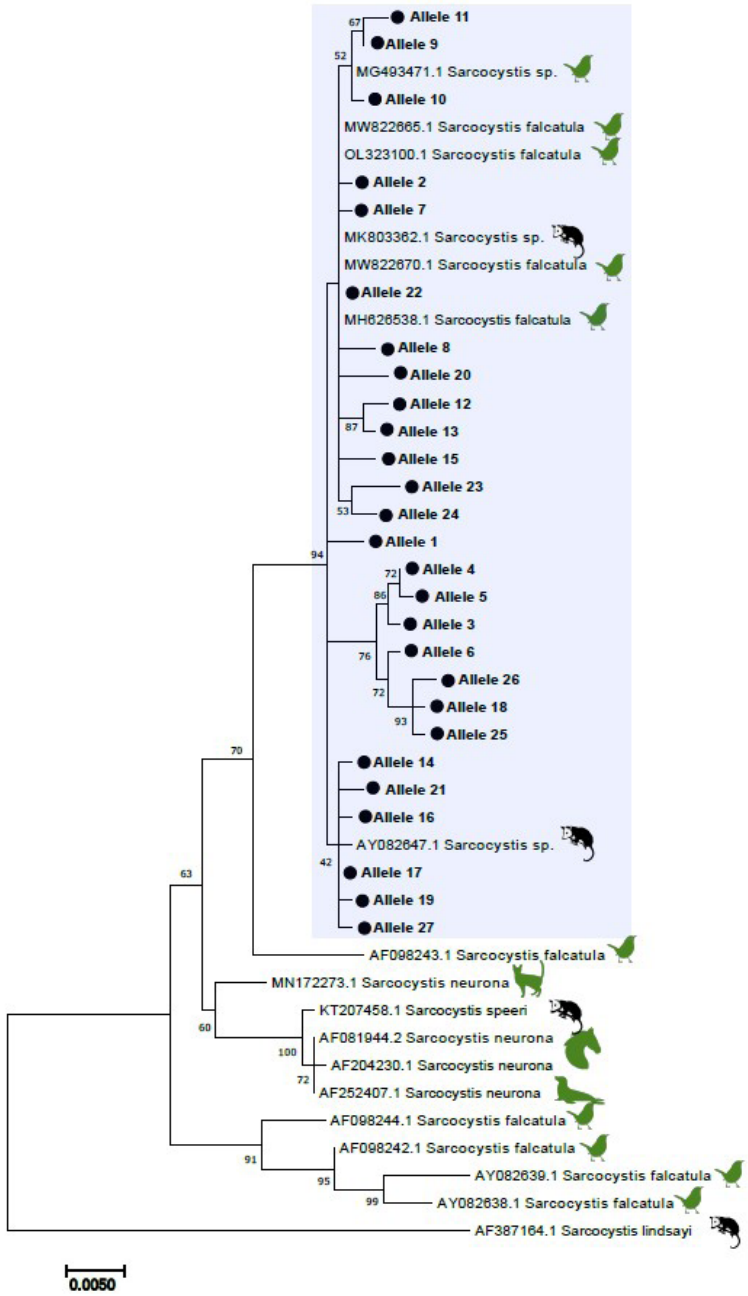

Figure 1. Phylogenetic relationships within the *Sarcocystis* genus based on 965 bp fragment of ITS-1. The tree was inferred by using the maximum likelihood (ML) method with the evolutionary model Kimura 2-parameter with gamma distributed rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. A sequence from *Sarcocystis lindsayi* was used as an outgroup. The sequences from the present study are marked in bold.

In the BLASTn analysis of *cox1*, alleles 8, 9, and 11 disclosed identities ranging from 96.40–96.81% with *S. rileyi* from a common eider (*Somateria mollissima*) from Norway (KJ396582), with a query cover value of 100% (Supplementary Table S3). The remaining alleles showed identities ranging from 99.69–100% with sequences from *S. falcatula* (MH665257) and *S. speeri* (KT207461), with a query cover value of 100%. The phylogeny for *cox1* positioned the alleles from this study into two well-separated clades (Figure 2). The first clade (clade A) was formed by alleles 8, 9, and 11 and a sequence from *Sarcocystis rileyi* Stiles, 1893 (*Somateria mollissima*) (KJ396582) found in avian muscle tissues. Another clade was formed with sequences of *Sarcocystis* spp. from birds, *S. arctica* from red fox (*Vulpes vulpes*) (MF596306), *S. caninum* from domestic dogs (MH469240), *S. canis* from polar bear (*Ursus maritimus*) (KX721495), and *S. lutrae* from otters (*Lutra lutra*) (KM657808). The second clade (clade B) was formed by the remaining alleles, and sequences from *S. falcatula* detected in the muscle tissues of psittacine birds (*Trichoglossus*

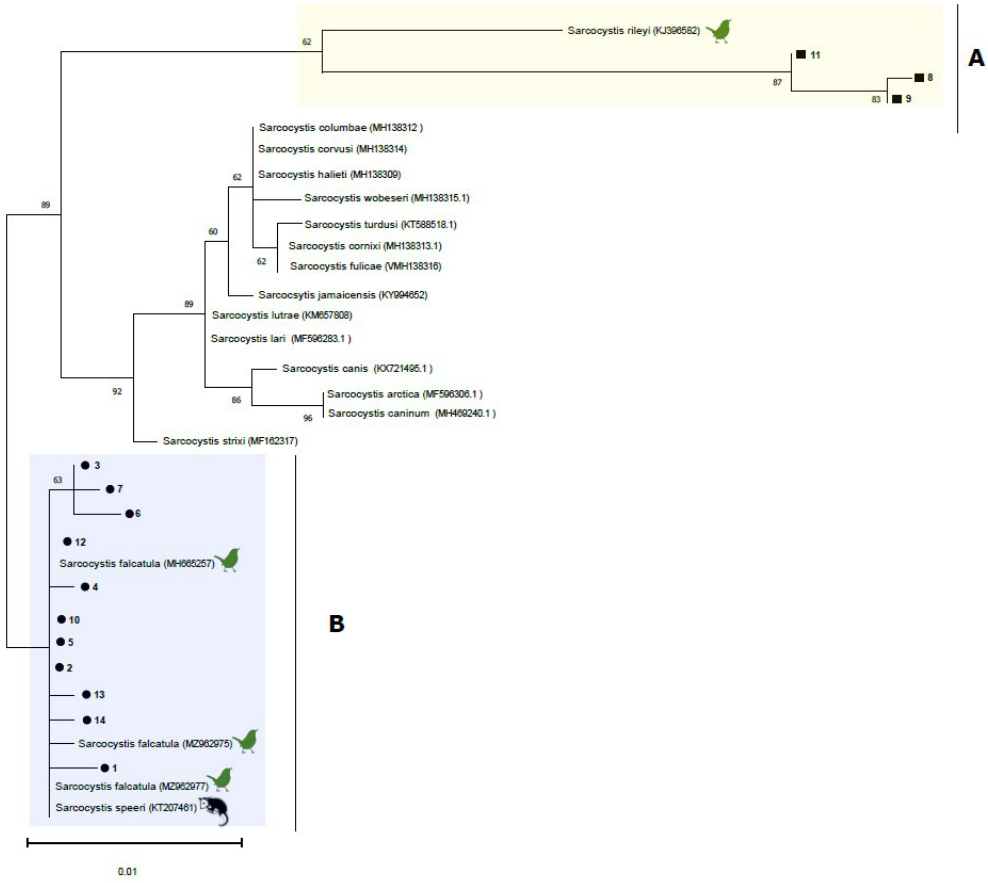

Figure 2. Phylogenetic relationships within the *Sarcocystis* genus based on 916 pb fragment of *cox1*. The tree was inferred by using the maximum likelihood (ML) method with the evolutionary model Tamura 3-parameter with gamma distributed rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. Sequences from *Toxoplasma gondii, Neospora caninum* and *Hammondia heydorni* were used as an outgroup (hidden). The sequences from the present study are marked in bold.

moluccanus, MH665257; *Polytelis alexandrae*, MZ962977; *Psittacula krameri*, MZ962975) and a sequence from *S. speeri* detected in opossums intestines (*D. albiventris*, KT207461).

Although the *SAG2* and *SAG3* nucleotide sequences from this study contained most of the open reading frame of the genes, the majority of the sequences available in GenBank were substantially shorter. Therefore, two phylogenetic reconstructions were performed for these *loci*: one with short fragments and other composed exclusively of longer sequences. The *SAG2* phylogeny based on 211 bp fragment showed four clades with high statistical support (Figure 3). All alleles obtained in this study were positioned in clades along with sequences of *Sarcocystis* sp. detected in sporocysts shed by opossums (*Didelphis* spp.) and *S. falcatula* detected in experimentally and naturally infected birds. The *SAG2* phylogenetic tree based on 645 bp (Supplementary Figure S1) fragment positioned the alleles from this study in the same clade as *S. falcatula* (GQ851953). The other clade was formed exclusively with sequences from *S. neurona* (BLASTn information on Supplementary Table S4).

For *SAG3,* five alleles (1, 2, 4, 5, and 10) were related to *S. falcatula* (GQ851956), with nucleotide identities ranging from 90.4–99.9% (Supplementary Table S5). The remaining eight alleles (3, 6, 7, 8, 9, 11, 12, and 13) were monophyletic and closely related to a sister clade exclusively formed by the *S. neurona* genetic sequences (Supplementary Figure S1). The alleles related to *S. neurona* were typically larger than the rest and showed the presence of "AT" repetitions (approximately 90 nucleotides long), which were absent in the *S. falcatula SAG3*-related alleles. In the phylogenetic reconstruction based on 317 bp fragment, the alleles split into six highly supported clades (Figure 3). All *SAG3* sequences in this study were phylogenetically related to *Sarcocystis* sp. and *S. falcatula*, either shed by opossums and/or detected in birds. One of the six *SAG3* clades was formed exclusively the *S. neurona* genetic sequences.

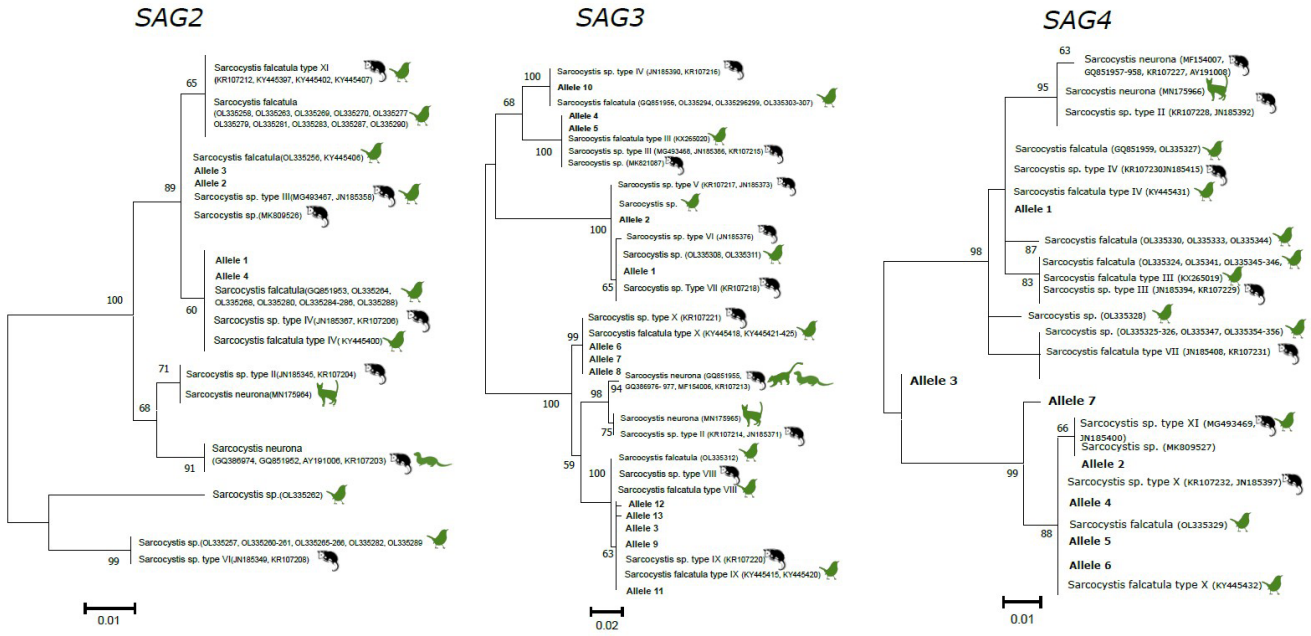

Figure 3. Phylogenetic relationships within the *Sarcocystis* genus based on *SAG2, SAG3* and *SAG4*. The trees were inferred by using the maximum likelihood (ML) method with the evolutionary models Jukes-Cantor (*SAG2* and *SAG4*) and Kimura 2-parameter (*SAG3*) with uniform rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. The sequences from the present study are marked in bold.

Phylogenetic reconstruction of *SAG4* failed to fully resolve branching in some samples. As with other SAG phylogenies, *S. neurona*-derived sequences were grouped into a highly statistically supported branch. The majority of *SAG4* alleles were closely related to sequences annotated as *Sarcocystis* sp. or *S. falcatula*, either detected in *Didelphis* spp. or birds. Allele 3 was the most divergent taxon and could not be associated with any known *Sarcocystis*-derived sequences (Figure 3) (BLASTn information on Supplementary Table S6).

None of the molecular markers used in this study demonstrated host specificity for different *Sarcocystis* lineages or alleles, as the distribution of the alleles among *D. aurita* and *D. albiventris* was random. Alleles derived from both species clustered together on several occasions.

Discussion

Previous studies have shown that *S. falcatula* constitutes a heterogeneous population (Marsh et al., 1999; Dubey et al., 2001d; Valadas et al., 2016; Gondim et al., 2017, 2019; Cesar et al., 2018; Llano et al., 2022). Thus far, two lineages of *S. falcatula* have been described in the Americas, based on ITS-1. One was described only in North America, while the other was described in both North and South America. In this study, the ITS-1-based phylogeny separated the two *S. falcatula* lineages into two well-supported clades. All 27 alleles obtained in this study belonged to the same lineage and positioned in a clade along with sequences from *S. falcatula*-like detected in naturally infected birds from Brazil (MG493471, MK803362, MW822665, MW822670, OL323100, MH626538,) and North America (AY082647). The second clade comprised *S. falcatula* sequences detected only in birds from North America. Most *Sarcocystis* found in Brazil were identified as *S. falcatula*-like, as they were phylogenetically related to *S. falcatula* and infective for birds, but had molecular differences compared with *S. falcatula* described in North America. Several *Sarcocystis,* primarily found in opossums and described as *Sarcocystis* spp., were after observed in natural and experimental infections in birds (Valadas et al., 2016; Gondim et al., 2017, 2019; Konradt et al., 2017; Acosta et al., 2018; Cesar et al., 2018; Gallo et al., 2018; Llano et al., 2022).

All the *cox1* alleles from this study showed BLASTn identity (> 96%) with a wide variety of *Sarcocystis* species. Phylogenetic reconstruction positioned the alleles in two well-separated clades with high statistical support (Figure 2). The fragment from *cox1* was too conserved to allow differentiation between the alleles from this study

and *S. falcatula* sequences present in clade B from *S. speeri*. Llano et al. (2022) also reported the failure of *cox1* to differentiate *Sarcocystis* species obtained from the muscle tissue of wild birds, even though the phylogeny based on ITS-1 demonstrated the presence of different species of *Sarcocystis*. Herein, while only *Sarcocystis falcatula*-like was detected in ITS-1, phylogeny based on *cox1* demonstrated the presence of two species of *Sarcocystis: Sarcocystis falcatula*-like sequences from clade B and *Sarcocystis* sp. phylogenetically related to *S. rileyi*, positioned in clade A. The evolutionary divergence revealed between clades B and C suggests that alleles present in the later branch belong to some species not yet described in opossums.

Mixed *Sarcocystis* infections may occur in the intestines of opossums; therefore, more than one *Sarcocystis* species may be detected in DNA obtained from sporocysts (Dubey et al., 2000). Allele 11 from *cox1* was identical to allele 2 from bases 1–193 and 768–972, and to alleles 8 and 9 from bases 194–767. These findings suggest that allele 11 might be a chimera, that is, an artifact sequence formed by the incorrect junction of two biological sequences. Chimeras may occur during PCR using mixed templates and do not represent sequences that exist in nature (Kalle et al., 2014). The presence of chimeras could lead to the misinterpretation of the results. Therefore, the scientific community should be aware of the need to avoid polluting public databases with artifact sequences.

Twenty-one new *SAG* alleles were found in comparison with those previously described: four at *SAG2*, 13 at *SAG3* and four at *SAG4*. This result reinforces that *Sarcocystis* spp. from opossums in Brazil exhibit a diverse portfolio of surface antigen-encoding genes (Monteiro et al., 2013, Valadas et al., 2016; Cesar et al., 2018). The amplification of the entire open reading frame of *SAG2* and *SAG3* demonstrated that the use of primers that amplify short fragments of *SAGs* may be underestimating the variation in *SAG* alleles from *Sarcocystis* shed by opossums in Brazil. Most of the *SAG3* alleles from this study presented insertions of "AT" repetitions of ~90 nucleotides in the region of the intron, resembling a micro-satellite pattern. Alleles presenting "AT" repetitions disclosed identity with *S. neurona* (96.3–96.7%), but phylogeny positioned them in a separate clade. "AT" repeats have been previously described in sequences from *S. neurona* obtained from sea others (*Enhydra lutris nereis* - GQ851954 and GQ851955) and white-nosed coati (*Nasua narica molaris*- MF154006), and in *Sarcocystis* sp. types VIII, IX, and X obtained from opossums in Brazil (Valadas et al., 2016). Sequences identical to those observed in *Sarcocystis* sp. types VIII, IX, and X have also been found in *S. falcatula* infecting budgerigars (*M. undulattus*) (Gondim et al., 2017; Cesar et al., 2018). Interestingly, *S. falcatula* that present "AT" repeats were more similar to *S. neurona* than to the other *S. falcatula*. Among the three *SAGs* evaluated in this study, *SAG3* had the highest number of alleles, similar to that previously observed by Valadas et al. (2016). The phylogeny based on *SAG4* failed to fully resolve branching in some samples. However, it was possible to notice that none of the *SAG4* alleles from this study was phylogenetically related to *S. neurona*. All the *SAG4* alleles in this study were closely related to *S. falcatula.*

The diversity of South American fauna acting as definitive hosts for *Sarcocystis* spp. is higher than that of North America. A single species of opossum *D. virginiana* is found in North America, while, in South America, five species, grossly divided into white-eared opossums (*D. albiventris, D. pernigra*, and *D. imperfecta*) and black-eared opossums (*D. aurita* and *D. marsupialis*), have been described (Cerqueira, 1985; Lemos & Cerqueira, 2002). In this study, host specificity for different *Sarcocystis* species was not observed, as the distribution of the alleles among *D. aurita* and *D. albiventris* was random. This suggests that both species of opossum might present the same susceptibility as definitive hosts for different *Sarcocystis* species.

Although *D. albiventris* is considered the definitive host for *S. neurona* in South America, only on one occasion *S. neurona* was isolated from *D. albiventris* in Brazil (Dubey et al., 2001e). Hammerschmitt et al. (2020) reported *S. neurona* causing meningoencephalitis in domestic cats. The authors observed significant molecular differences related to *S. neurona* detected elsewhere in the Americas. At three *SAG* loci (2, 3, and 4), the parasite found in the cat was identical to *Sarcocystis* sp. genotype II derived from opossums (*D. albiventris* and *D. aurita*) in the state of Rio Grande do Sul (Monteiro et al., 2013). None of the molecular markers used in this study demonstrated the presence of *S. neurona* in the sampled opossums. The results observed here, along with numerous other studies performed in Brazil, imply that *Sarcocystis* spp. that use opossums as definitive hosts in Brazil are different from those found in North America. Furthermore, the frequency of infection caused by *S. falcatula*-related organisms in opossums from Brazil is much higher than that of *S. neurona* infection (Monteiro et al., 2013; Valadas et al., 2016; Acosta et al., 2018; Cesar et al., 2018; Gondim et al., 2017, 2019).

Conclusions

This study reported the occurrence, genetic diversity, and phylogenetic relationships of *Sarcocystis* spp. derived from the wild-caught opossums *D. albiventris* and *D. aurita* sampled in midwestern and southeastern Brazil. *S. falcatula*-like was detected by ITS-1, whereas *S. falcatula*-like and *Sarcocystis* spp. were detected by *cox1.* The assessment of phylogenetic inferences based on *SAG2, SAG3* and *SAG4* revealed the genetic richness of *Sarcocystis* spp. occurring among opossums and highlighted the presence of several genotypes infecting these animals in the country.

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Ethics declaration

The present study was approved by the Ethics Committees in the Use of Animals of the (hidden) (protocol number 013444/2019) and (hidden) (1558030821), and by Institute Chico Mendes for Conservation of Biodiversity (ICMBio numbers 49662-8 and 76295-1).

Conflict of interest

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table S1: Oligonucleotide primers utilized for DNA amplification of ITS-1, cox1, SAG2, SAG3, and SAG4 from Sarcocystis spp. detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil.

Supplementary Table S2. BLASTn analysis of each Sarcocystis ITS-1 detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil.

Supplementary Table S3. BLASTn analysis of each Sarcocystis cox1 sequence detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil

Supplementary Table S4. BLASTn analysis of each Sarcocystis SAG2 sequence detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil.

Supplementary Table S5. BLASTn analysis of each Sarcocystis SAG3 sequence detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil.

Supplementary Table S6. BLASTn analysis of each Sarcocystis SAG4 sequence detected in wild-caught opossums Didelphis albiventris and Didelphis aurita captured in Campo Grande state of Mato Grosso do Sul, and São Paulo, state of São Paulo, Brazil.

Supplementary Figure S1. Phylogenetic relationships within the Sarcocystis genus based on SAG2 and SAG3. The trees were inferred by using the maximum likelihood (ML) method with the evolutionary model Kimura 2-parameter with uniform rates. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1000 replicates. The sequences from the present study are marked in bold.

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