

## ***Sarcocystis* sp. parasites in the Mexican Great-tailed Grackle (*Quiscalus mexicanus*), Bronzed Cowbird (*Molothrus aeneus*), and Stripe-headed Sparrow (*Aimophila ruficauda*)**

Félix Domingo Sánchez Godoy<sup>a\*</sup>  
Fernando Chávez Maya<sup>a</sup>  
Adriana Méndez Bernal<sup>c</sup>  
Gary García Espinosa<sup>a</sup>  
Cristina Guerrero Molina<sup>b</sup>  
Néstor Ledesma Martínez<sup>a</sup>  
Elizabeth Morales Salinas<sup>c</sup>

<sup>a</sup> Departamento de Medicina y Zootecnia de Aves

<sup>b</sup> Departamento de Parasitología

<sup>c</sup> Departamento de Patología

Facultad de Medicina Veterinaria y Zootecnia,  
Universidad Nacional Autónoma de México.  
Avenida Universidad 3000, 04510 México, D.F.,  
México.

**\*Corresponding author:**  
Tel: + 52 55 5622 5867 / 5616 6923  
Email address: [spuma91@hotmail.com](mailto:spuma91@hotmail.com)

### **Abstract**

The objective of this study was to describe the morphological and ultrastructural characteristics, the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) results, the sequences and the phylogenetic analysis of a specific fragment of internal transcribed spacer 1 (ITS-1), amplified using the 25/396 primers, of the *Sarcocystis* sp. parasites identified in the muscles of wild great-tailed grackles, bronzed cowbirds, and stripe-headed sparrows in Mexico. Fifteen birds with sarcocystosis in their skeletal muscles were studied: 7 great-tailed grackles (*Quiscalus mexicanus*), 6 bronzed cowbirds (*Molothrus aeneus*), and 2 stripe-headed sparrows (*Aimophila ruficauda*). Histopathological analysis revealed thin-walled mature parasite cysts. Ultrastructurally, the cyst wall consisted of a granular layer with villar protrusions and numerous microtubules. The bradyzoites measured  $4.1 \times 1.6 \mu\text{m}$ , and micronemes appeared in the anterior third of the conoid. For molecular identification, PCR-RFLP was performed using sequences of a specific fragment of internal transcribed spacer 1 (ITS-1) using the primers 25/396 and *Hinf* I. *Hind* III did not cut this fragment. The sequencing results indicated a 100% similarity among the *Sarcocystis* parasites from the three bird species, and a BLAST search revealed 96% sequence similarity with *S. neurona*. The phylogenetic analysis shows that the sequences studied are topologically distant to those sequences reported for *S. neurona* in the United States and in South America and are not related to any group previously reported. Although our morphological and molecular analysis data provide strong evidence that *S. neurona* uses these bird species as intermediate hosts, future molecular studies with additional DNA fragments, combined with biological studies, will ultimately allow us to convincingly identify these parasites. This is the first report of a *Sarcocystis* sp. parasite in wild birds in Mexico that may be *S. neurona*.

**Keywords:** *Sarcocystis*; *Sarcocystis neurona*; *Quiscalus mexicanus*; *Molothrus aeneus*; *Aimophila ruficauda*; Histopathology; Ultrastructure; PCR-RFLP, Phylogenetic analysis.

Received: 2014-06-08  
Accepted: 2014-11-21  
Published: 2014-12-05

Additional information and declarations  
can be found on page 10

© Copyright 2014 Félix Domingo Sánchez Godoy et al.

open access 



Distributed under Creative Commons CC-BY 4.0

## Introduction

Parasites from the genus *Sarcocystis* are protozoans of the phylum Apicomplexa. These parasites affect mammals, birds, reptiles, amphibians, and fish (Munday *et al.*, 1979; Bolon *et al.*, 1989; Hillyer *et al.*, 1991; Dubey *et al.*, 2001a; Dubey *et al.*, 2003). *Sarcocystis falcatula* is the most prevalent species in birds, which serve as intermediate hosts, with opossums (*Didelphis virginiana* and *Didelphis albiventris*) serving as the definitive hosts (Box and Duszynski, 1978; Box and Smith, 1982; Dubey *et al.*, 2000; Dubey *et al.*, 2001b). Until 1995, the opossum was thought to be the definitive host of only *S. falcatula*; however, the opossum is known also to be the definitive host of *S. neurona*, *S. speeri* (Fenger *et al.*, 1997; Dubey *et al.*, 1998; Dubey *et al.*, 1999) and *S. lindsayi* (Dubey *et al.*, 2001c). Mansfield *et al.* (2008) reported *S. neurona* in brown-headed cowbirds. Dame *et al.* (1995) found a strong similarity between *S. neurona* and *S. falcatula* based on an analysis of the 18S rRNA gene and, thus, suggested that these are actually the same species. Subsequent biological studies, however, confirmed that these are indeed two different species (Marsh *et al.*, 1997a; Dubey and Lindsay, 1998). Genetic typing by various methods has established that *S. neurona* and *S. falcatula* are distinct species. Polymerase chain reaction (PCR) primers targeting the SSU rRNA gene were developed that distinguished *S. neurona* from the *Sarcocystis* found in skunks, raccoons, hawks, coyotes and cats (Fenger *et al.*, 1995). In another study, sequencing of the internal transcribed spacer 1 (ITS-1) region of the rRNA gene showed that *S. falcatula* may be composed of a heterogeneous population and that the ITS-1 region can be used to distinguish *S. neurona* from *S. falcatula* (Marsh *et al.*, 1999). Tanhauser *et al.* (1999) used ITS-1 to design specific primers (25/396) and restriction enzymes (*Hinf* I and *Hind* III) to perform PCR-restriction fragment length polymorphism (RFLP) analysis to differentiate *S. neurona* and *S. falcatula*. Likewise, Elsheikha *et al.* (2005) have used DNA markers 25/396 of ITS-1 for phylogenetic studies of *S. neurona* in the United States. Infections caused by *S. neurona* have been reported in a variety of species, such as cats (Butcher *et al.*, 2002), raccoons (Dubey *et al.*, 2001d), armadillos (Cheadle *et al.*, 2001a), skunks (Cheadle *et al.*, 2001b), seals (Miller *et al.*, 2001), and sea otters (Dubey *et al.*, 2003). In addition, *S. neurona* is an important cause of neurological problems in horses in the United States (Dubey *et al.*, 1991; MacKay *et al.*, 2000), although horses appear to be aberrant hosts (Dubey 2001b). Interestingly, schizonts have been observed in the brain and spinal cord and mature cysts have been observed in the skeletal muscle in a 4-year-old mare, suggesting that horses are intermediate hosts (Mullaney *et al.*, 2005). In 2008, the presence of parasitic cysts of *S. neurona* in the skeletal muscles of brown-headed cowbirds (*Molothrus ater*) was documented, indicating that these birds may be intermediate hosts (Mansfield *et al.*, 2008). In Mexico there are no data available on the presence of *S. neurona* in birds, but Yeargan *et al.*, in 2013, found a seroprevalence of 48.5% of *S. neurona* in horses in northern Mexico.

The objective of this study was to describe the morphological and ultrastructural characteristics, the PCR-RFLP results, the sequences and the phylogenetic analysis of a specific fragment of internal transcribed spacer 1 (ITS-1), which was amplified using the 25/396 primers, of the *Sarcocystis* sp. parasites identified in the muscles of wild great-tailed grackles, bronzed cowbirds, and stripe-headed sparrows in Mexico that may be *S. neurona*.

## Materials and methods

Fifteen wild birds (7 great-tailed grackles [*Quiscalus mexicanus*], 6 bronzed cowbirds [*Molothrus aeneus*], and 2 stripe-headed sparrows [*Aimophila ruficauda*]) found dead with suspected poisoning in the State of Morelos, Mexico, were submitted to the Diagnostic Laboratory and Research on Diseases of Birds of the Department of Medicine of Birds of the Faculty of Veterinary Medicine of the National Autonomous University of México (UNAM). During necropsy, parasitic structures were found in of the muscle.

## Necropsy and histopathology

All birds were submitted for necropsy, and samples were taken from muscle, fixed in 10% buffered formalin, processed by routine histological techniques, embedded in paraffin, cut into 4- $\mu$ m sections, and stained with hematoxylin and eosin (H&E).

## Electron microscopy

Sections (3 mm<sup>2</sup>) of striated skeletal muscle with parasitic cysts were taken from great-tailed grackles, fixed in 2.5% glutaraldehyde and, subsequently, post-fixed with 1% osmium tetroxide for 2 hours. Following washing with 0.1 M cacodylate buffer solution (pH 7.2), tissues were dehydrated with increasing concentrations of acetone. Sections were then embedded in epoxy resin (Epón 812, Electron Microscopy Sciences, Industry Road Hatfield, PA) and, finally, were polymerized at 60°C for 24 hours. Afterwards, 200- $\mu$ m thick semifine cuts were made using an ultramicrotome, and samples were mounted on slides and contrasted with toluidine blue (Hayat, 2000). Fine cuts were made to achieve 60- $\mu$ m samples, and the samples were then mounted on copper mesh grids, contrasted with uranyl acetate and lead citrate, and observed with an electron microscope (Zeiss EM-900, Zeiss, Oberkochen, Germany) at 80 kV.

## DNA extraction

To isolate the parasite DNA, muscle samples with cysts of the parasites were macerated with a pestle and suspended in 20% phosphate buffered saline (PBS). Then, 250  $\mu$ l of the suspension was mixed with 250  $\mu$ l of lysis buffer solution (EDTA-SDS-Tris HCL; GibcoBRL, Grand Island, NY, USA) and 25  $\mu$ l of proteinase K (20 mg/ml; Fermentas Inc., Glen Burnie, MD, USA). The samples were then incubated in a 37°C water bath for 2 hours followed by DNA purification with phenol-chloroform-isoamyl alcohol. The DNA was then precipitated with ethanol and hydrated.

## PCR

The following primers were used for the detection of *S. falcatula* or *S. neurona*: 25 5'-CAC ACA AAA CAC CTG AAA GTC ACG TAC TT-3' and 396 5'-CCT GCC TCA CTT CGA CAC AT-3' (Sigma-Aldrich Corp., St. Louis, MO, USA). These primers amplify a 334-bp fragment of ITS-1 of the ribosomal DNA gene. The PCR conditions were as follows: 2  $\mu$ l of Taq buffer, 0.4  $\mu$ l of dNTPs (0.2 mM), 1  $\mu$ l of primer (0.5 mM), 1.2  $\mu$ l of MgCl<sub>2</sub> (1.5 mM), 1  $\mu$ l of Triton (0.10%), 1  $\mu$ l of bovine serum albumin

(BSA) (0.015 mg/ml), 0.5 µl of Taq polymerase (2.5 U/µl; Fermentas Inc., Glen Burnie, MD, USA), 7.9 µl of diethylpyrocarbonate (DEPC) water and 5 µl (200 ng/µl) of DNA. The samples were amplified with a PCR Sprint Thermal Cycler (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, Inc., Waltham, MA, USA) the following conditions: 1 cycle at 94°C for 5 minutes; 30 cycles at 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 40 seconds; and 1 cycle at 72°C for 5 minutes. Then, 5 µl of each PCR reaction was electrophoresed on a 2% agarose gel, which was then stained with ethidium bromide and observed under ultraviolet light.

### Sequencing

The approximately 334-bp PCR fragment that was observed on the agarose gel was cut out and purified with a QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The purified fragment was visualized in a 1% agarose gel that was stained with ethidium bromide, along with the molecular weight marker GeneRuler (Fermentas Inc., Glen Burnie, MD, USA). PCR sequencing was performed with a BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The sequencing reactions were purified in CENTRI-SEP™ Spin Columns (CS-901, Princeton Separations, Adelphia, NJ, USA) following the manufacturer's instructions. Subsequently, the sequencing reactions were read with a 3130 Genetic Analyzer (Applied Biosystems Foster City, CA, USA). Sequencing of double-stranded DNA for the marker 25/396 was carried out to confirm the nucleotide sequence, and the primers 25/396 were used to obtain sense and antisense sequences.

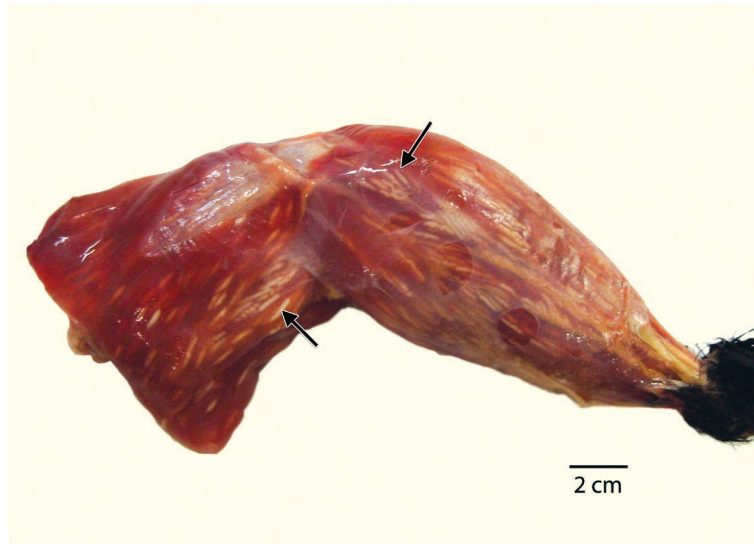
Raw sequences (sense and antisense) were edited using MEGA version 4 software (Tamura *et al.*, 2007). The consensus sequence yielded a file corresponding to each of the samples, and an alignment among the three sequences - the stripe-headed Sparrow (G1), the bronzed cowbird (T1), and the great-tailed grackle (Z5) - was carried out.

### Phylogenetic analysis

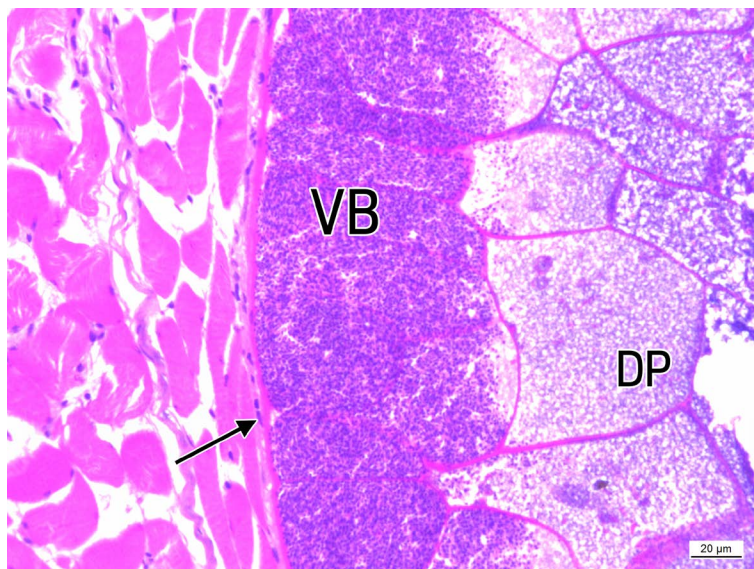
Phylogenetic analysis was based on sequences of the 25/396 DNA marker obtained from G1, T1 and Z5 in addition to the sequences obtained from GenBank. Sequences were aligned, and a phylogenetic tree was constructed with the neighbor-joining (NJ) method using the Kimura 2-parameter (K-2P) model. Bootstrap support for the results of the NJ analysis was based on 1,000 replicate datasets generated from the original multiple sequence alignment.

### RFLP

The approximately 334-bp PCR fragment that was observed on the agarose gel was digested with two restriction enzymes, *Hinf* I (Life Technologies, Carlsbad, CA, USA) and *Hind* III (Roche Applied Science, Indianapolis, IN, USA). The following RFLP conditions were used: 10 µl of PCR product, 3 µl of buffer, 1 µl of enzyme (*Hinf* I or *Hind* III), and 16 µl of DEPC water. The samples were incubated for 12 hours in a water bath at 37°C. Then, 15 µl of the reaction was electrophoresed on a 3% agarose gel that was stained with ethidium bromide and observed under ultraviolet light.



**Figure 1.** Skeletal muscle of the thigh and leg of a great-tailed grackle with abundant parasitic structures of *Sarcocystis* (arrows). Cysts are white in color, undulating, and oriented along the longitudinal axis of the muscle.



**Figure 2.** Histological section (H&E stain) of skeletal muscle of a great-tailed grackle containing a mature *Sarcocystis* cyst within the myofibers and no inflammatory reaction. Cysts have an eosinophilic wall (arrow) and contain numerous viable bradyzoites (VB). Degenerate protozoa are observed in the central part (DP).

## Results

The bodies of all birds were in good condition, and intramuscular parasitic cysts were found in the striated muscle of the breast, wings, and legs. These cysts were white in color, elongated, and undulating, and their dimensions ranged from 0.3 to 0.5 cm in length (Fig. 1). No pathological changes were evident in other organs.

Microscopic examination of the muscle tissue sections of the 15 birds showed multiple round (20 to 200 µm in diameter), elongated (100 to 4000 µm in length) cyst structures, each with a continuous thin wall (less than 2 µm) eccentrically compressing the myofibrils. Inside, the *Sarcocystis* parasites contained abundant bradyzoites that in some areas were separated by septa. In the central part of the larger cysts, an acellular, amorphous pale eosinophilic material that corresponded to degenerated protozoa was observed (Fig. 2). In 3 great-tailed grackles, discrete, multifocal, inflammatory cell aggregates composed of lymphocytes and plasma cells were observed around the cysts.

## Electron microscopy

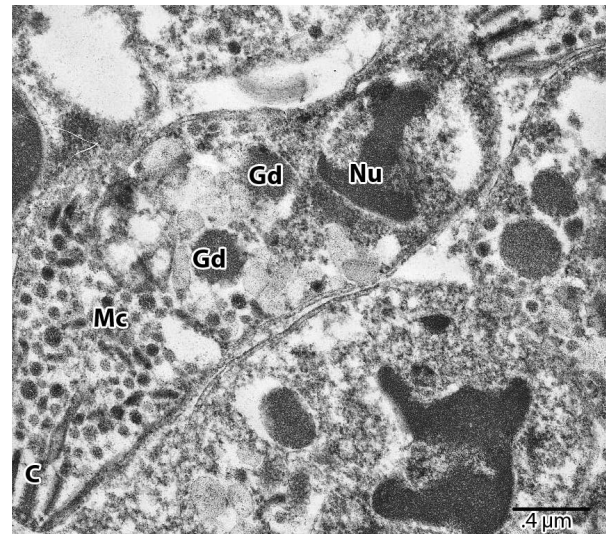
Striated skeletal muscle sections exhibited sarcoplasmic, thin-walled parasitic cysts consisting of a granular layer with villar protrusions containing numerous electron-dense microtubules that extended from the point to the base and beyond the underlying granular layer. The parasitic cysts showed some electrolucid merozoites beneath their walls, whereas toward the central portion, numerous mature bradyzoites were grouped together and separated by prolongations of the granular layer. Bradyzoites measured 4.1 µm in length and 1.6 µm in width and exhibited numerous micronemes in the anterior third of the conoid. The majority of nuclei are round with abundant electron-dense heterochromatin, which adhered to the internal nuclear sheath, and occasionally, a prominent nucleolus was noted (Fig. 3 y 4).

## PCR and sequencing

Following amplification with the 25/396 primer pair, 11 of the 15 skeletal muscle samples were positive (73%): 5 Mexican great-tailed grackles, 4 bronzed cowbirds, and 2 stripe-headed sparrows.



**Figure 3.** Transmission electron photograph of the skeletal striated muscle of a great-tailed grackle with sarcoplasm that exhibits a thin-walled, moderately electrolucid cyst (electrolucid basal substance, GI) with irregular projections (villar protrusions, V) and microtubules (\*) in the interior. Inside the cyst, numerous bradyzoites (Br) are shown. The parasitic membrane shows multiple protuberances (arrow). Adjacent to the cyst wall, distorted sarcomeres and numerous mitochondria (M) are observed. The nucleus is indicated (Nu). The sample was treated with a contrast technique that uses uranyl acetate and lead citrate. 7000× magnification.



**Figure 4.** Transmission electron micrograph of a bradyzoite in a longitudinal cut. Notice the conoid (C) and numerous micronemes (Mc). From the nucleus, round bodies of moderate electron density, which correspond to dense granules (Gd) intermingled with electrolucid granules are observed. The nucleus is located at the back of the conoid and exhibits abundant electron-dense heterochromatin (Nu). The sample was treated with a contrast technique that uses uranyl acetate and lead citrate. 12000× magnification.

Raw sequences (sense and antisense) of one specimen from each species were included in the study. The sequences were edited, and the fragment lengths were 338 bp for G1, 338 bp for T1, and 323 bp for Z5. Alignment among the 3 sequences was carried out, and 100% similarity was observed (fig. 5). In fact, all 3 sequences were identical. As the sequencing results contained only 323 bp for the Gt5 sequence, the final 15 nucleotides from the other 2 samples were not aligned with this sequence.

Using the Basic Local Alignment Search Tool (BLAST) from GenBank, a comparison of the sequences obtained in this study and the sequences contained in the database was performed. A 96% similarity was found between the sequences here and the sequences corresponding to the following GenBank accession numbers: AY627839, AY627841, AY627842, AY627845, AY627848, AY 627850, AY627851, AY627859, AF093159 and AY627852. All sequences corresponded to the fragment of the genome of *Sarcocystis neurona* with the exception of AY627852, which corresponded to a fragment of the genome of *Toxoplasma gondii* and AF093159, which corresponded to a fragment of the genome of *S. falcatula*.

### Phylogenetic and restriction fragment length polymorphism (RFLP) analysis

The sequences obtained in this study show a 100% homology between them. You may also notice that they are topologically more distant among the sequences

				111	111	111	122	222	222	222	223	333	333	333	444	444	444	455	555	555	556	666	666	666
	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	
G1	TCA	CAC	AAA	CAC	TGA	AAG	TCA	CGT	ACT	TAT	GAC	GGA	AAA	GCT	GCG	GTA	AGC	ACG	GGC	CAT	AAT	CAT	CAG	
T1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Z5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
												111	111	111	111	111	111	111	111	111	111	111	111	
	777	777	777	788	888	888	889	999	999	999	000	000	000	011	111	111	111	112	222	222	222	333	333	
	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	
G1	GAG	GAA	CTA	GTT	TGT	CAT	GTT	GTC	CCT	ACA	GAA	CCC	GAT	TCT	GCC	TAG	GCG	CCT	GAC	ACT	CTA	GCA	GAG	
T1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Z5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	122	222	222	
	344	444	444	445	555	555	555	666	666	666	677	777	777	778	888	888	888	888	999	999	999	900	000	
	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	
G1	AGT	GAC	GGA	TGG	AGC	AAC	TAA	AAG	GAC	TAA	GAG	TCG	TGC	AAG	TTT	CAT	TCG	GAG	CCA	GGA	GTC	TCA	ATG	
T1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Z5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	222	
	001	111	111	111	222	222	222	233	333	333	334	444	444	444	555	555	555	566	666	666	667	777	777	
	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	
G1	GAC	ACC	GCT	GCG	ACT	TAA	GAC	CTA	AGT	AGA	GAA	GCT	GGC	GGA	GGT	GAA	ACA	GTA	GAT	TTC	CTC	TTT	GTC	
T1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Z5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
	222	222	222	222	222	222	222	223	333	333	333	333	333	333	333	333	333	333	333	333	333	333	33	
	777	888	888	888	899	999	999	990	000	000	000	111	111	111	122	222	222	223	333	333	333	333	33	
	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	
G1	GAT	AAC	ACA	GGC	AGC	AAT	CAC	AAG	TGT	AAC	CAT	CGA	ATA	AAT	GTG	TCG	AAG	TGA	GGC	AGG	AG	AG	AG	
T1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
Z5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	

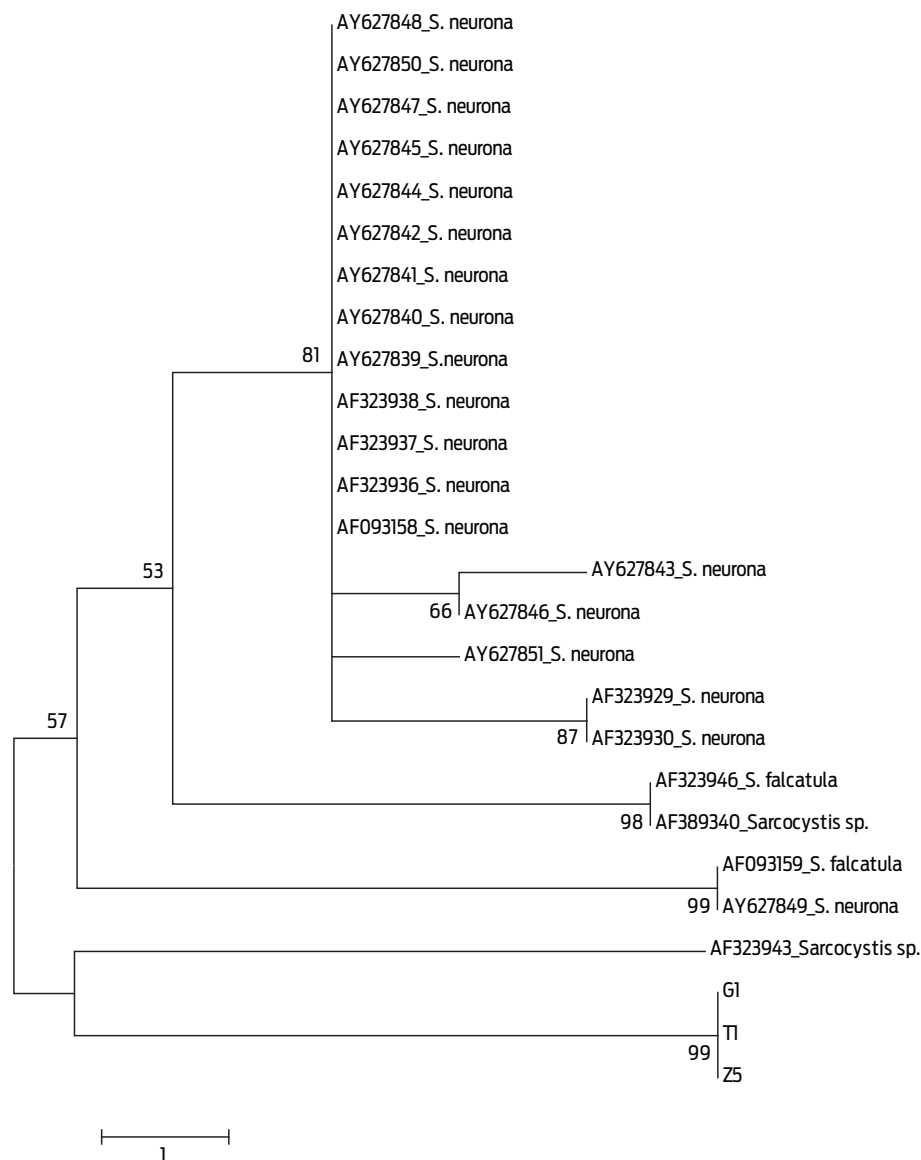
**Figure 5.** Sequences of a specimen from each species included in the study: stripe-headed sparrow (G1), bronzed cowbird (T1), and great-tailed grackle (Z5). Sequences were edited, and the lengths of the sequences were 338 bp for G1, 338 bp for T1, and 323 bp for Z5. Alignment between the three sequences was carried out, and 100% similarity was observed.

used to construct the phylogenetic tree, not being related to any group previously reported. The phylogenetic distance between the sequences obtained in this work, the sequences of previously reported *Sarcocystis neurona* and the sequence from a sample collected in Brazil (*Sarcocystis* spp.) have a similar magnitude (Fig. 6).

The banding pattern following digestion of the 334-bp fragment from all positive cases using the *Hinf* I restriction enzyme consisted of three fragments of 140, 108, and 62 bp. Digestion with the *Hind* III restriction enzyme did not affect the 334-bp product (Fig. 7).

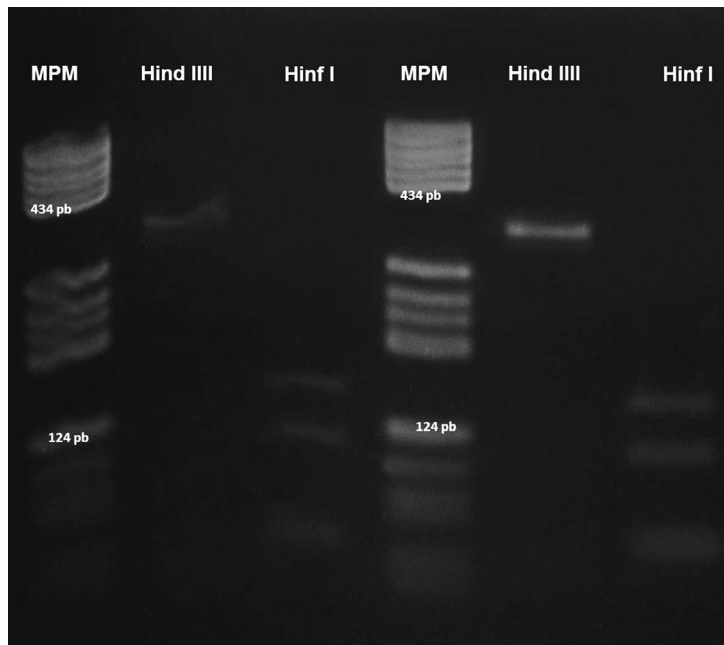
## Discussion

*Sarcocystis* were easily visualized in striated muscle that exhibited thin-walled parasitic cysts that measured between 0.3 and 0.5 cm in length. These observations differ from those reported by Mansfield *et al.* (2008). In that study, the investigators analyzed 381 brown-headed cowbirds (*Molothrus ater*) from the United States and noted macroscopically visible *Sarcocystis* only in the legs. In addition, the histopathological examination revealed 2 types of cysts: thin-walled and thick-walled. By electron microscopy, the thick-walled cysts were identified as *S. falcatula*, the thin-walled cysts were identified as *S. neurona*, and these findings were confirmed by PCR-RFLP. In the present study, macroscopic detection of *Sarcocystis* sp. parasites was common, possibly because the majority of the birds presented with chronic parasitic infection with mature *Sarcocystis* in the muscle fibers and degenerate bradyzoites in the interior, similar to the pattern in birds infected with



**Figure 6.** Neighbor-joining phylogenetic tree constructed from a matrix generated by the Kimura 2-parameter method using 1000 bootstrap replicates. The obtained sequences are identical and are phylogenetically distant from any previously reported sequence.

*S. falcatula*. Presentation can be acute or chronic, depending on the affected bird. The acute presentation is generally observed in Old World psittacines and in pigeons and causes high mortality associated with pneumonia and encephalitis without the development of parasitic cysts. The chronic presentation occurs in American Passeriformes, which are intermediate hosts. No mortality is observed, and the disease is characterized by the formation of parasitic cysts in skeletal muscle, without a consequent inflammatory reaction (Villar *et al.*, 2008). Histopathologic identification is subjective because *S. neurona* and *S. falcatula* have similar morphological characteristics, and literature data reports are inconclusive regarding the wall thickness of the parasitic cyst. Previously, however, Dubey *et al.* (2001e) reported the presence of a mature parasitic cyst with a 1 to 1.5- $\mu$ m wall in the cerebellum of an ibis (*Carphibis spinicollis*), corresponding to *Sarcocystis neurona*-like parasites and



**Figure 7.** RFLP analysis of a *Sarcocystis* sp. parasite. A 334-bp fragment was amplified with the 25/396 primers. Lanes 1 and 4: PBR322/DNA/BsuRI (HaeIII) molecular weight marker; lanes 2 and 5: bronzed cowbird and great-tailed grackle samples treated with HindIII; lanes 3 and 6: bronzed cowbird and great-tailed grackle samples digested with HinfI.

similar to the protozoa described in this study. In this work, electron microscopy was only used to visualize *Sarcocystis* in great-tailed grackles, and the ultrastructural characteristics of the wall and bradyzoites coincide with the descriptions of *S. neurona* in bronzed cowbirds (Mansfield *et al.*, 2008), horses (Mullaney *et al.*, 2005), mice, and cell culture (Speer and Dubey, 2001). In these reports, the main characteristic for the ultrastructural identification of *S. neurona* is the accumulation of micronemes in the anterior third of the conoid end and a granular wall with numerous microtubules in villar protrusions. These studies suggest that the *Sarcocystis* parasites observed in pectoral muscles, legs, and wings of the three species of birds (wild great-tailed grackles, bronzed cowbirds, and stripe-headed sparrows) are similar to *S. neurona*.

Regarding the molecular identification of *Sarcocystis* in great-tailed grackles, bronzed cowbirds, and stripe-headed sparrows using PCR-RFLP (Tanhauser *et al.*, 1999) with the 25/396 primers and *Hinf* I and *Hind* III restriction enzymes, a positive amplification product of 334 bp was observed. These products contained two *Hinf* I cutting sites in the same positions as the sequences of *S. neurona*. However, no recognition sites for *Hind* III

were present in the 334-bp band. This finding is consistent with the banding pattern of *S. neurona* that was previously reported (Tanhauser *et al.*, 1999; Mansfield *et al.*, 2008; Mullaney *et al.*, 2005; and Elsheikha *et al.*, 2005).

Alignment among the 3 sequences was carried out, and 100% similarity was observed. A 96% similarity was found between the sequences studied and *S. neurona*. An additional cutting site that was not detected using PCR-RFLP was detected by sequencing. The generated fragments were approximately 140, 108, 62, and 24 bp. The additional cut by the *Hinf* I enzyme may be due to a mutation or insertion of bases, due to independent genetic evolution during geographic isolation, as shown in the phylogenetic tree where the analyzed sequences are topologically separated between the US and South America. A similar result was observed by Elsheikha *et al.* (2005). These investigators examined the sequences of 25/396 fragments from 10 *Sarcocystis neurona* samples isolated and found mutations and base insertions, providing evidence for the presence of closely related genetic variants of *S. neurona* existing within the US and South America. Monteiro *et al.* (2013) suggest that it is possible that genetic groups of *S. neurona* and *S. falcatula* may exchange highly divergent alleles in sexual recombination.

However, because 25/396 marker sequence similarity is highly conserved among apicomplexan protozoans (*Sarcocystis neurona*, *S. falcatula*, *Toxoplasma gondii*, *Neospora caninum*), errors can occur in the interpretation of phylogenetic relationships. This finding emphasizes the importance of using more than just genetic or DNA markers for a robust phylogenetic analysis. In addition, these DNA studies should be supplemented with biological studies because, for example, mice

are more susceptible to *S. neurona* but are resistant to *S. falcatula* (Dubey and Lindsay, 1998), while Australian parakeets are susceptible to *S. falcatula* and are not affected by *S. neurona* (Marsh *et al.*, 1997b).

## Conclusions

The morphological, ultrastructural and PCR-RFLP characteristics suggest that the parasitic cysts observed in the studied birds are *S. neurona*. However, in the phylogenetic tree, sequences are topologically distant from the published sequences of *S. neurona* from the United States and South America, which suggests that this may be a new subspecies of *S. neurona*.

---

## Funding

The funding for this research was provided by Departamento de Medicina y Zootecnia de Aves, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México.

## Acknowledgements

The authors thank Mr. Alfredo A. Díaz Estrada for assistance with the histology and Emmanuel V. Ortega Martínez for the photographic support.

## Conflicts of interest

Gary García Espinosa is head of the Departamento de Medicina y Zootecnia de Aves, Facultad de Medicina Veterinaria y Zootecnia. The other authors declare that they have no conflicts of interest.

## Author contributions

Félix Domingo Sánchez Godoy: Designed the research and wrote the article.

Fernando Chávez Maya and Adriana Méndez Bernal: Contributed new reagents and analytical techniques.

Gary García Espinosa and Elizabeth Morales Salinas: Analyzed the data and wrote the article.

Cristina Guerrero Molina and Néstor Ledesma Martínez: Analyzed the data.

## References

- 1) Bolon B, Greiner EC, Mays MB. 1989. Microscopic features of *Sarcocystis falcatula* in skeletal muscle from a Patagonian conure. *Veterinary Pathology* 26:282-284.
- 2) Box ED, Duszynski DW. 1978. Experimental transmission of *Sarcocystis* from icterid birds to sparrows and canaries by sporocysts from the opossum. *Journal of Parasitology* 64:682-688.
- 3) Box ED, Smith JH. 1982. The intermediate host spectrum in a *Sarcocystis* species of birds. *Journal of Parasitology* 68:668-673.

- 4) Butcher M, Lakritz J, Halaney A, Branson K, Gupta GD, Kreeger J, Marsh AE. 2002. Experimental inoculation of domestic cats (*Felis domesticus*) with *Sarcocystis neurona* or *S. neurona*-like merozoites. *Veterinary Pathology* 107:1-14.
- 5) Cheadle MA, Tanhauser SM, Dame JB, Sellon DC, Hines M, Ginn PE, MacKay RJ, Greiner EC. 2001a. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. *International Journal for Parasitology* 31:330-335.
- 6) Cheadle MA, Yowell CA, Sellon DC, Hines M, Ginn PE, Marsh AE, MacKay RJ, Dame JB, Greiner EC. 2001b. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. *International Journal for Parasitology* 31:843-849.
- 7) Dame JB, MacKay RJ, Yowell CA, Cutler TJ, Marsh A, Greiner EC. 1995. *Sarcocystis falcatula* from passerine and psittacine birds: Synonymy with *Sarcocystis neurona*, agent of equine protozoal myeloencephalitis. *Journal of Parasitology* 81:930-935.
- 8) Dubey JP, Lindsay DS. 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. *International Journal for Parasitology* 28:1823-1828.
- 9) Dubey JP, Davis SW, Speer CA, Bowman DD, De Lahunta A, Granstrom DE, Topper MJ, Hamir AN, Cummings JF, Suter MM. 1991. *Sarcocystis neurona* n. sp. (Protozoa: Apicomplexan), the etiologic agent of equine protozoal myeloencephalitis. *Journal of Parasitology* 77:212-218.
- 10) Dubey JP, Johnson GC, Bermudez A, Suedmeyer KW, Fritz DL. 2001e. Neural Sarcocystosis in a Straw-necked Ibis (*Carphibis spinicollis*) associated with a *Sarcocystis neurona*-like organism and description of muscular *Sarcocystis* of an unidentified *Sarcocystis* species. *Journal of Parasitology* 87:1317-1322.
- 11) Dubey JP, Lindsay DS. 1999. *Sarcocystis speeri* n. sp. (Protozoa: Sarcocystidae) from the opossum (*Didelphis virginiana*). *Journal of Parasitology* 85:903-909.
- 12) Dubey JP, Lindsay DS, Rosenthal BM, Kerber CE, Kasai N, Pena HF, Kwok OC, Shen SK, Gennari SM. 2001b. Isolates of *Sarcocystis falcatula*-like organisms from South American opossums *Didelphis marsupialis* and *Didelphis albiventris* from Sao Paulo, Brazil. *Journal of Parasitology* 87:1449-1453.
- 13) Dubey JP, Lindsay DS, Saville WJ, Reed SM, Granstrom DE, Speer CA. 2001a. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). *Veterinary Parasitology* 95:89-131.
- 14) Dubey JP, Lindsay DS, Venturini L, Venturini C. 2000. Characterization of *Sarcocystis falcatula* isolates from the Argentinian opossum, *Didelphis albiventris*. *Journal Eukaryotic Microbiology* 47:260-263.
- 15) Dubey JP, Rosenthal BM, Speer CA. 2001c. *Sarcocystis lindsayi* n. sp. (Protozoa: Sarcocystidae) from the South American opossum, *Didelphis albiventris* from Brazil. *Journal Eukaryotic Microbiology* 48:595-603.
- 16) Dubey JP, Saville WJ, Stanek JF, Lindsay DS, Rosenthal BM, Oglesbee MJ, Rosypal AC, Njoku CJ, Stich RW, Kwok OC, Shen SK, Hamir AN, Reed SM. 2001d. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): evidence for natural infection with sarcocysts, transmission of infection to opossums (*Didelphis virginiana*), and experimental induction of neurologic disease in raccoons. *Veterinary Parasitology* 100:117-129.

- 17) Dubey JP, Speer CA, Lindsay DS. 1998. Isolation of a third species of *Sarcocystis* in immunodeficient mice fed feces from opossums (*Didelphis virginiana*) and its differentiation from *Sarcocystis falcatula* and *Sarcocystis neurona*. *Journal of Parasitology* 84:1158-1164.
- 18) Dubey JP, Zarnke R, Thomas NJ, Wong SK, Van Bonn W, Briggs M, Davis JW, Ewing R, Mense M, Kwok OC, Romand S, Thulliez P. 2003. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis-like* infections in marine mammals. *Veterinary Parasitology* 116:275-296.
- 19) Elsheikha MH, Murphy JA, Mansfield SL. 2005. Phylogenetic congruence of *Sarcocystis neurona* Dubey et al, 1991 (Apicomplexa: Sarcocystidae) in the United States based on sequence analysis and restriction fragment length polymorphism (RFLP). *Systematic Parasitology* 61:191-202.
- 20) Fenger CK, Granstrom DE, Langemeier JL, Stamper S, Donahue JM, Patterson JS, Gajadhar AA, Marteniuk JV, Xiaomin Z, Dubey JP. 1995. Identification of opossums (*Didelphis virginiana*) as the putative host of *Sarcocystis neurona*. *Journal of Parasitology* 81:916-919.
- 21) Fenger CK, Granstrom DE, Gajadhar AA, Williams NM, McCrillis SA, Stamper S, Langemeier JL, Dubey JP. 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* spp. from the opossum. *Veterinary Parasitology* 68:199-213.
- 22) Hayat M. 2000. *Principles and techniques of electron microscopy. biological applications*. 4<sup>th</sup> ed. Cambridge, USA: Cambridge University Press.
- 23) Hillyer EV, Anderson MP, Greiner EC, Atkinson CT, Frenkel JK. 1991. An outbreak of *Sarcocystis* in a collection of psittacines. *Journal of Zoo and Wildlife Medicine* 22:434-445.
- 24) MacKay RJ, Granstrom DE, Saville WJ, Reed SM. 2000. Equine protozoal myeloencephalitis. *Veterinary Clinics of North America: Equine Practice*. 16:405-425.
- 25) Mansfield LS, Mehler S, Nelson K, Elsheikha HM, Murphy AJ, Knust B, Tanhauser SM, Gearhart PM, Rossano MG, Bowman DD, Schott HC, Patterson JS. 2008. Brown-headed cowbirds (*Molothrus ater*) harbor *Sarcocystis neurona* and act as intermediate host. *Veterinary Parasitology* 153:24-43.
- 26) Marsh AE, Barr BC, Lakritz J, Nordhausen R, Madigan JE, Conrad PA. 1997a. Experimental infection of nude mice as a model for *Sarcocystis neurona*-associated encephalitis. *Parasitology Research* 83:706-711.
- 27) Marsh AE, Barr BC, Tell L, Bowman DD, Conrad PA, Ketcherside C, Green T. 1999. Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocystis neurona*. *Journal of Parasitology* 85:750-757.
- 28) Marsh AE, Barr BC, Tell L, Koski M, Greiner E, Dame J, Conrad PA. 1997b. *In vitro* cultivation and experimental inoculation of *Sarcocystis falcatula* and *Sarcocystis neurona* merozoites into budgerigars (*Melopsittacus undulatus*). *Journal of Parasitology* 83:1189-1192.
- 29) Miller MA, Sverlow K, Crosbie PR, Barr BC, Lowenstine LJ, Gulland FM, Packham A, Conrad PA. 2001. Isolation and characterization of two parasitic protozoa from a Pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalomyelitis. *Journal of Parasitology* 87:816-822.
- 30) Monteiro RM, Keid LB, Richtzenhain LJ, Valadas SY, Muller G, Soares RM. 2013. Extensively variable surface antigens of *Sarcocystis* spp. infecting Brazilian marsupi-

- als in the genus *Didelphis* occur in myriad allelic combinations, suggesting sexual recombination has aided their diversification. *Veterinary Parasitology* 196:64-70.
- 31) Mullaney T, Murphy AJ, Kiupel M, Bell JA, Rossano MG, Mansfield LS. 2005. Evidence to support horses as natural intermediate hosts for *Sarcocystis neurona*. *Veterinary Parasitology* 133:27-36.
- 32) Munday BL, Hartley WJ, Harrigan KE, Presidente PJ, Obendorf DL. 1979. *Sarcocystis* and related organisms in Australian wildlife: II. Survey findings in birds, reptiles, amphibians and fish. *Journal of Wildlife Diseases* 15:57-73.
- 33) Speer CA, Dubey JP. 2001. Ultrastructure of schizonts and merozoites of *Sarcocystis neurona*. *Veterinary Parasitology* 95:263-271.
- 34) Tamura K, Dudley J, Nei M, Kumar S. 2007. *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24:1596-1599.
- 35) Tanhauser SM, Yowell CA, Cutler TM, Greiner EC, Mackay RJ, Dame JB. 1999. Multiple DNA markers differentiate *Sarcocystis neurona* and *Sarcocystis falcatula*. *Journal of Parasitology* 85:221-228.
- 36) Villar D, Kramer M, Howard L, Hammond E, Cray C, Latimer K. 2008. Clinical presentation and pathology of *Sarcocystosis* in psittaciform birds: 11 cases. *Avian Diseases* 52:187-194.
- 37) Yeargan MR, Alvarado-Esquivel C, Dubey JP, Howe DK. 2013. Prevalence of antibodies to *Sarcocystis neurona* and *Neospora hughesi* in horses from Mexico. *Parasite* 20, 29. <http://dx.doi.org/10.1051/parasite/2013029> [published: September 10<sup>th</sup>].