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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *SARCOCYSTIS* INFECTIONS IN THE MUSCLES OF GRAY WOLVES (*CANIS LUPUS*) FROM MINNESOTA SUGGEST THEY MAY SERVE AS RESERVOIRS FOR INFECTION IN DOMESTICATED DOGS

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KEY WORDS

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Sarcocystis caninum
Sarcocystis svanai
Minnesota

ABSTRACT

Sarcocystis infections were found for the first time in the muscles of 3 of 3 gray wolves (*Canis lupus*) from Minnesota. Two kinds (thin-walled and thick-walled) of sarcocysts were detected, based on the appearance of the sarcocyst wall. In wolf 1, sarcocysts were thin-walled (<0.5 μm), and without any visible protrusions. Ultrastructurally, the sarcocyst wall was type 1a and identical to *Sarcocystis svanai* of the domestic dog (*Canis familiaris*). The second kind of sarcocyst, with a relatively thicker (>1 μm) sarcocyst wall, was detected in wolves 2 and 3. Ultrastructurally, the sarcocyst wall had undulating, pleomorphic villar protrusion of type 9c; these sarcocysts were identical to *Sarcocystis caninum* from the domestic dog. Molecularly, the 2 *Sarcocystis* species were characterized using *18S*, *28S*, *COI*, *ITS-1,* and *rpoB* genetic markers. All these markers showed 100% identity to either of the 2 species previously described from the domestic dog. The thick-walled sarcocyst corresponded to *Sarcocystis caninum*, whereas the thin-walled sarcocyst corresponded to *Sarcocystis svanai.*

Protozoa in the genus *Sarcocystis* parasitize virtually all endotherms, and a few species also occur in ectotherms (Dubey et al., 2016). Of more than 200 named species of *Sarcocystis*, a full life cycle is known for only a few. Some species of *Sarcocystis* are zoonotic, and some cause economic losses to livestock producers or undetermined wildlife conservation goals. *Sarcocystis* species have an obligatory 2-host life cycle, alternating between an intermediate host and a definitive host (Rommel et al., 1974). The sexual cycle is restricted to the intestines of the definitive host; the asexual cycle occurs in extraintestinal tissues of the intermediate host after it ingests water or vegetation contaminated with sporocysts. A definitive host consuming tissue contaminated with sarcocysts releases bradyzoites, which then transform into gamonts in its intestine. Fertilization produces oocysts in the lamina propria, where they sporulate in situ and often rupture, releasing sporocysts in the feces. Once an intermediate host ingests these sporocysts, parasites multiply in blood vessels and finally become encysted as sarcocysts, often in muscles. *Sarcocystis* species are generally host specific, especially for the intermediate host. In some definitive hosts, sarcocysts occur in extraintestinal tissues; life cycles for many species infecting wildlife remain incomplete.

Canids (dogs, foxes, coyotes) are known to act as definitive hosts for numerous *Sarcocystis* species (Dubey et al., 2016). They can excrete *Sarcocystis* sporocysts in their feces for months after ingesting *Sarcocystis*-infected tissues without suffering any ill effects. Until 2000, sarcocysts were only occasionally reported in the muscles of carnivores, including domestic dogs and cats; these were regarded as incidental findings. However, beginning in 2005, severe clinical, acute sarcocystosis was observed in 4 domestic dogs (Dubey et al., 2015). The affected dogs had fever, apathy, anorexia, muscle weakness, ataxia, and elevated liver and muscle enzymes. The dogs were from different geographical areas in North America. Initially, these dogs were suspected to have neosporosis or toxoplasmosis, but these diseases were ruled out by detailed diagnostic procedures (Dubey et al., 2015). Further histological examination revealed numerous sarcocysts associated with myositis in skeletal muscle. Based on transmission electron microscopy (TEM) examination and molecular characterization, 2 new species, *Sarcocystis caninum* and *Sarcocystis svanai* were proposed (Dubey et al., 2015). Naming species without the

Table I. Muscular *Sarcocystis* species in canids.

Host	Country	No. positive/ no. tested	*Sarcocystis* species	Morphology		Molecular	Remarks	Reference
				Light microscopy	TEM			
Gray wolf (*Canis lupus*)	Lithuania	4/15	*S. svanai* in 4	*S. svanai*-950–1,806 × 33–74, smooth cyst wall <1 μm thick	No data	*18S* rRNA, *28S* rRNA, *ITS1*, *COI*, *rpoB*	*S. arctica* and *S. svanai* could not be separated by *COI*. Species closely related to *Sarcocystis* from many noncanid hosts.	Juozaitytė-Ngugu et al. (2024)
			S. arctica-like remnants in 2, no intact cysts	No data	No data			
Alaskan wolf (*C. lupus*)	United States	1/1	*S. arctica*	Up to 900 μm long	Villi type 9c of Dubey et al. (2016).	*18S* rRNA, *28S* rRNA, *ITS1*, *COI*		Calero-Bernal et al. (2016)
Arctic fox (*Vulpes lagopus*)	Norway	2/2	*S. arctica*	1–12 mm × 100–250 μm Knob-like villar protrusions	No data	*18S* rRNA, *28S* rRNA, *ITS1*, *COI*	Species closely related to *Sarcocystis* from many noncanid hosts	Gjerde and Schulze (2014)
Arctic fox (*V. lagopus*)	Alaska, USA	9/56 (16.0%)	*S. arctica*–like	1,105–4,080 μm × 134–288 μm unstained tongue squash	Villi type 9c of Dubey et al. (2016)	*18S* rRNA, *28S* rRNA, *ITS1*, *CO1*	Closely related to species in dog, fox and wolf	Cerqueira-Cézar et al. (2017)
Pampas fox (*Lycalopex gymnocercus*)	Argentina	22/36 (61.1%) hunted foxes	*S. svanai*	220–310 × 28–42 μm	Yes, type 1 Dubey et al. (2016)	*18S* rRNA-PCR	High prevalence (61.1%)	Scioscia et al. (2017)
Red fox (*Vulpes vulpes*)	Czech Republic	3/6	*S. arctica*	1–7 mm long	No data	*18S* rRNA, *28S* rRNA, *ITS1*		Pavlásek and Máca (2017)
Red fox (*V. vulpes*)	Spain	6/22 (27.2%)	*S. arctica*		Yes	*18S* rRNA, *28S* rRNA, *ITS1*, *COI*, *rpoB*	Sarcocysts, not in tongue	Kirillova et al. (2018)
	Latvia	11/411 (1.4%)	*S. arctica*, *S. lutrae*	*S. arctica*-2,565–8,414 × 83–204 μm, sarcocyst wall 1.6-2.8 μm thick *S. lutrae* cysts 1,524–3,186 × 75–111 μm, wall 0.8–2.2 μm thick	No data			
	Lithuania	3/41 (7.3%)	*S. arctica*, *S. lutrae*		Yes			
Raccoon dog (*Nyctereutes procyonoides*)	Latvia	0/294						

Table II. Details of wolves from Minnesota tested for muscular *Sarcocystis*.

No.	Wolf ID	Sex	Age	Date died/shot	Date samples collected	Locality
1	W23-FDL015	Male	Older adult	12 December 2023	16 December 2023	Tamarak
2	20231213MS	Male	8–9 mo	12 December 2023	16 December 2023	Willow River
3	W23-709	Female	Adult (1–2 yr)	Shot 10 October 2023	12 December 2023	Trelipe

knowledge of the full life cycle allowed subsequent investigators to search for their occurrence elsewhere. Subsequently, *S. caninum* and *S. svanai* were reported associated with acute severe clinical sarcocystosis in a dog from Finland (Hagner et al., 2018). At the same time, Ye et al. (2018) found *S. caninum* sarcocysts in the muscles of 2 of 37 dogs in China; these dogs were adults, but their clinical histories were unknown, and they were purchased from a peddler's market. Sarcocysts were characterized, molecularly and ultrastructurally (Ye et al., 2018). Thus, *S. caninum* has been reported from North America, Europe, and Asia.

Sarcocysts resembling *S. caninum* and *S. svanai* have been reported from wolves (*Canis lupus*) in Alaska (Calero-Bernal et al., 2016) and Lithuania (Juozaitytė-Ngugu et al., 2024), red fox (*Vulpes vulpes*) from Czech Republic (Pavlásek and Máca, 2017), Baltic States and Spain (Kirillova et al., 2018), Arctic fox (*Vulpes lagopus*) in Norway (Gjerde and Schulze, 2014), and Pampas fox (*Lycalopex gymnocercus*) in Argentina (Scioscia et al., 2017). Available information on these reports is summarized in Table I. It is apparent that species of *Sarcocystis* infecting these carnids belonging to different genera are molecularly closely related and their identities cannot be established until they have been cultivated in vitro or assessed in vivo. Most species of *Sarcocystis* are believed to be host-specific, although *Sarcocystis neurona* and *Sarcocystis canis* have wide established host ranges, including dogs (Dubey et al., 2016).

The objective of the present study was the ultrastructural and genetic characterization of sarcocysts found in wolves from Minnesota.

MATERIALS AND METHODS

Naturally infected wolves

Wolves are protected in the United States, except in Montana and Wyoming. Muscle samples were collected from 3 wolves in Minnesota under permit 35816 for specimen possession and research issued by the State of Minnesota Department of Natural Resources, Division of Fish and Wildlife, St. Paul, Minnesota. Details of wolves are summarized in Table II. The cause of death was unknown for the 2 wolves that were found dead. The third wolf was illegally shot but the perpetrator was not found.

Samples of the tongue and limb muscle were collected, put in Ziplock bags, and transported to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture (USDA), Beltsville, Maryland for testing. Up to 9 days elapsed between collection and transport of samples to APDL (Table II).

Cytological and histological examination

Samples of muscles were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (HE), and examined microscopically for parasites. Parasites were photographed using an Olympus AX-70 microscope with a DP-73 digital camera and measurements were made digitally using Olympus Imaging Software cellSens Standard 1.18 (Olympus Optical Ltd., Tokyo, Japan).

Transmission electron microscopy

Sarcocysts were identified in muscle squash preparation between a cover slip and a glass slide. The cover slip was removed, and the infected muscle piece was washed with McDowells Trump fixative (10% formalin 37%, 1% glutaraldehyde in 1.16% sodium phosphate monobasic and 0.27% NaOH with deionized water), and transported by air to Dr. Rafael Calero-Bernal, Complutense, University of Madrid, Spain for TEM examination; samples were post-fixed as suggested by Kirillova et al. (2018). Ultrathin sections were examined at the Spanish National Centre for Electron Microscopy (Madrid, Spain) using a JEOL JEM 1400 Plus device at 80 kW.

DNA isolation and amplification

The individual sarcocysts isolated from the 3 wolves were fixed in 95% alcohol and stored at −20 C until further use. Genomic DNA was extracted using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the instructions specified by the manufacturer and stored at −20 C. The DNA purity and concentration were evaluated by spectrophotometric analysis using Nanodrop (ThermoFisher Scientific, Waltham, Massachusetts). PCR amplification of highly conserved regions of *18S* ribosomal RNA (*18S* rRNA), *28S* ribosomal RNA (*28S* rRNA), a mitochondrial cytochrome c oxidase subunit 1 (*COI*), highly variable internal transcribed spacer-1 (*ITS1*) along with the *rpoB* gene (encoding the RNA polymerase β subunit) were performed using custom primers designed to amplify *S. caninum* and *S. svanai* (Table III). The β′ subunit is the largest subunit of RNA polymerase and is encoded by *rpoC*. PCR reactions totaling 25 μl consisted of 2 μl DNA template, 12.5 μl of Platinum Hot Start PCR Master mix (Invitrogen, Waltham, Massachusetts), 1 μl of 10 pmol/μl of each primer (Integrated DNA Technologies, Coralville, Iowa) (Table III) and 8.5 μl of molecular-grade water. After initial denaturation at 94 C for 3 min; 35 cycles were performed consisting of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec, and elongation at 68 C for 20 min; terminal elongation incubated products at 68 C for 5 min. The PCR products were

Table III. Accession numbers and PCR primers used for the amplification and sequencing of different gene markers for *Sarcocystis caninum* and *Sarcocystis svanai* isolated from *Canis lupus* during the study.

Locus	Target bases	PCR amplification primers	Sequencing primers	Accession numbers	Reference
			Sarcocystis caninum		
18S	1554	562F-TTCCCTCGTGGAAGGGTAGT 946R-TCACCGGAACACTCAATCGG	562F-TTCCCTCGTGGAAGGGTAGT 320F-CTGGCATCCTCCTGATTGGT 807R-CGTGCAGCCCAGAACATCTA 946R-TCACCGGAACACTCAATCGG	PP814747	Designed during the study
	864	562F-TTCCCTCGTGGAAGGGTAGT 320R-TGATCGTCTTCGAGCCCCTA	562F-TTCCCTCGTGGAAGGGTAGT 946F-GGCAGCTGTCAGGACTGTTA 320R-TGATCGTCTTCGAGCCCCTA	PP814746	
28S	1458	137F-TCAAGCCCCTGATTCTTTCACT 869R-CCACGTCTTCCTACTCATTGC	137F-TCAAGCCCCTGATTCTTTCACT 869F-TGACGTGTACTTGTTGCATGG 869R-CCACGTCTTCCTACTCATTGC	PP814748	Designed during the study
COI	479	79F-TGATGCCCGCATTATTTGCAG 459R-TGGATGGCCAAAGAACCAGA	79F-TGATGCCCGCATTATTTGCAG 459R-TGGATGGCCAAAGAACCAGA	PP819576	Designed during the study
rpoB	375	135F-AACAGTAGGTGACAAATTATGTGGT 70R-AGAATACCTTGCAACTCCACAA	135F-AACAGTAGGTGACAAATTATGTGGT 135R-GAAGGAATACCTAAAGCTCCTACA 70R-AGAATACCTTGCAACTCCACAA	PP819577	Designed during the study
			Sarcocystis svanai		
18S	1259	SarCF-TTTAACTGTCAGAGGTGAAATTCTT SarBR-GGCAAATGCTTTCGCAGTAG	SarCF-TTTAACTGTCAGAGGTGAAATTCTT SarBF-GGGAGGTAGTGACAAGAAATAACAA SarCR-CCTGTTATTGCCTCAAACTTCC SarBR-GGCAAATGCTTTCGCAGTAG	PP814764	Kutkienė et al. (2010)
28S	772	KLP2F-AACCGACCCGTCTTGAAAC KLP2R-TGCTACTACCACCAAGATCTGC	KLP2F-AACCGACCCGTCTTGAAAC KLP1R-CCCAAGTTTGACGAACGATT KLP2R-TGCTACTACCACCAAGATCTGC	PP814765	Kutkienė et al. (2010)
CO1	939	SF1-ATGGCGTACAACAATCATAAAGAA SR5-TAGGTATCATGTAACGCAATATCCAT	SF1-ATGGCGTACAACAATCATAAAGAA SR4-CCACACCTGTAGTACCCCC SR5-TAGGTATCATGTAACGCAATATCCAT	PP819578	Gjerde (2013)
ITS1	210	P-ITSF-ATTGAGTGTTCCGGTGAATTA P-ITSR-GCCATTTGCGTTCAGAAATC	P-ITSF-ATTGAGTGTTCCGGTGAATTA P-ITSR-GCCATTTGCGTTCAGAAATC	PP816211	Prakas et al. (2014)
rpoB	854	RPObF-GCGGTCCAAAAAGGGTCAGTGGATATG RPObR-TCWGTATAAGGTCCTGTAGTTC	RPObF-GCGGTCCAAAAAGGGTCAGTGGATATG RPObFa-TAGTACATTAGAAATCCCTAAAC RPObR-TCWGTATAAGGTCCTGTAGTTC	PP819579	Wendte et al. (2010)

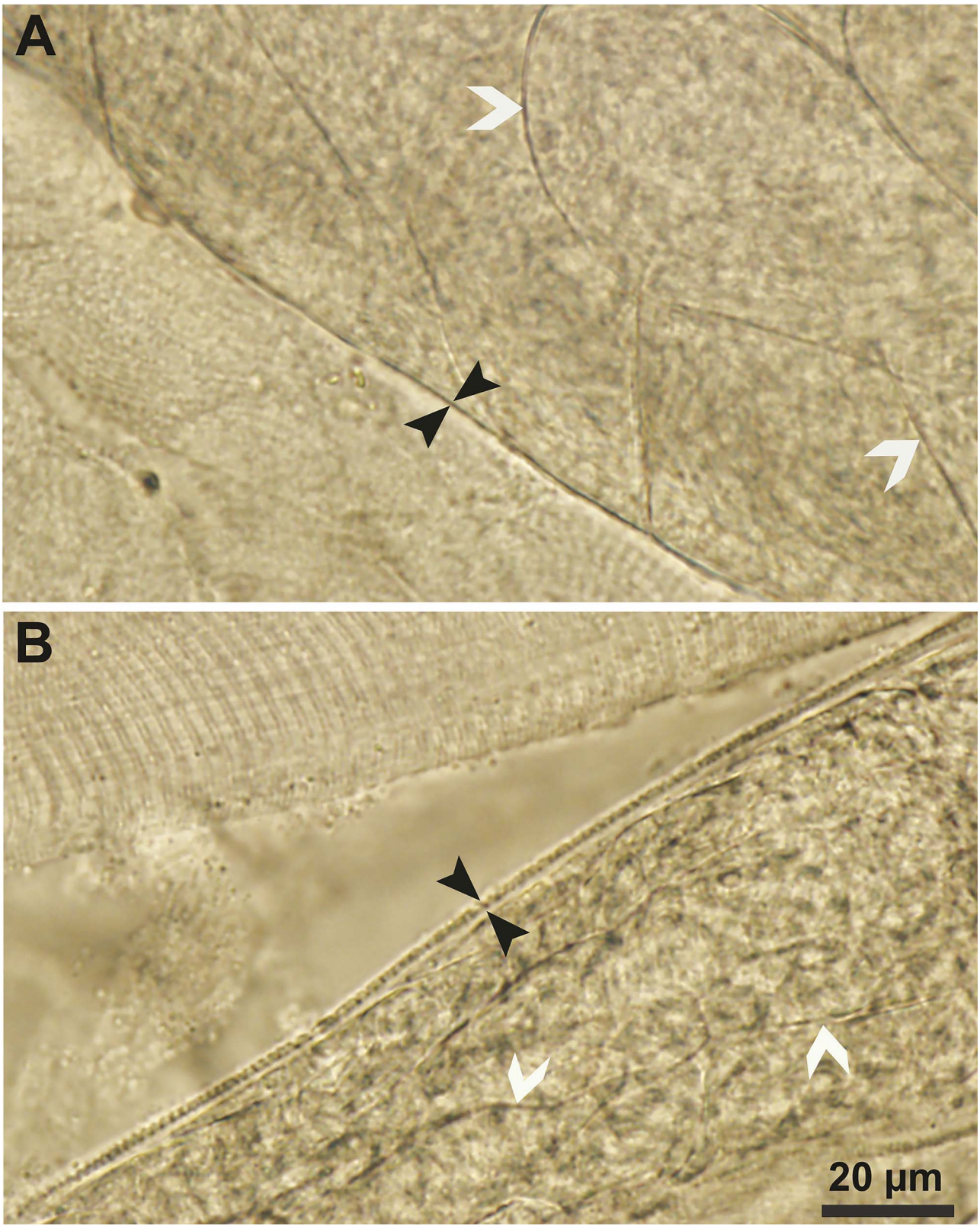

Figure 1. Sarcocyst walls of *Sarcocystis* sp. from the gray wolf in Minnesota. Unstained. Opposing black arrowheads point to sarcocyst walls and white arrowheads point to septa. The scale bar applies to both parts. (**A**) Wolf 1: Smooth, thin sarcocyst wall of *Sarcocystis svanai*. (**B**) Wolf 2: Saw-like serrations on the sarcocyst wall of *Sarcocystis caninum*. Color version available online.

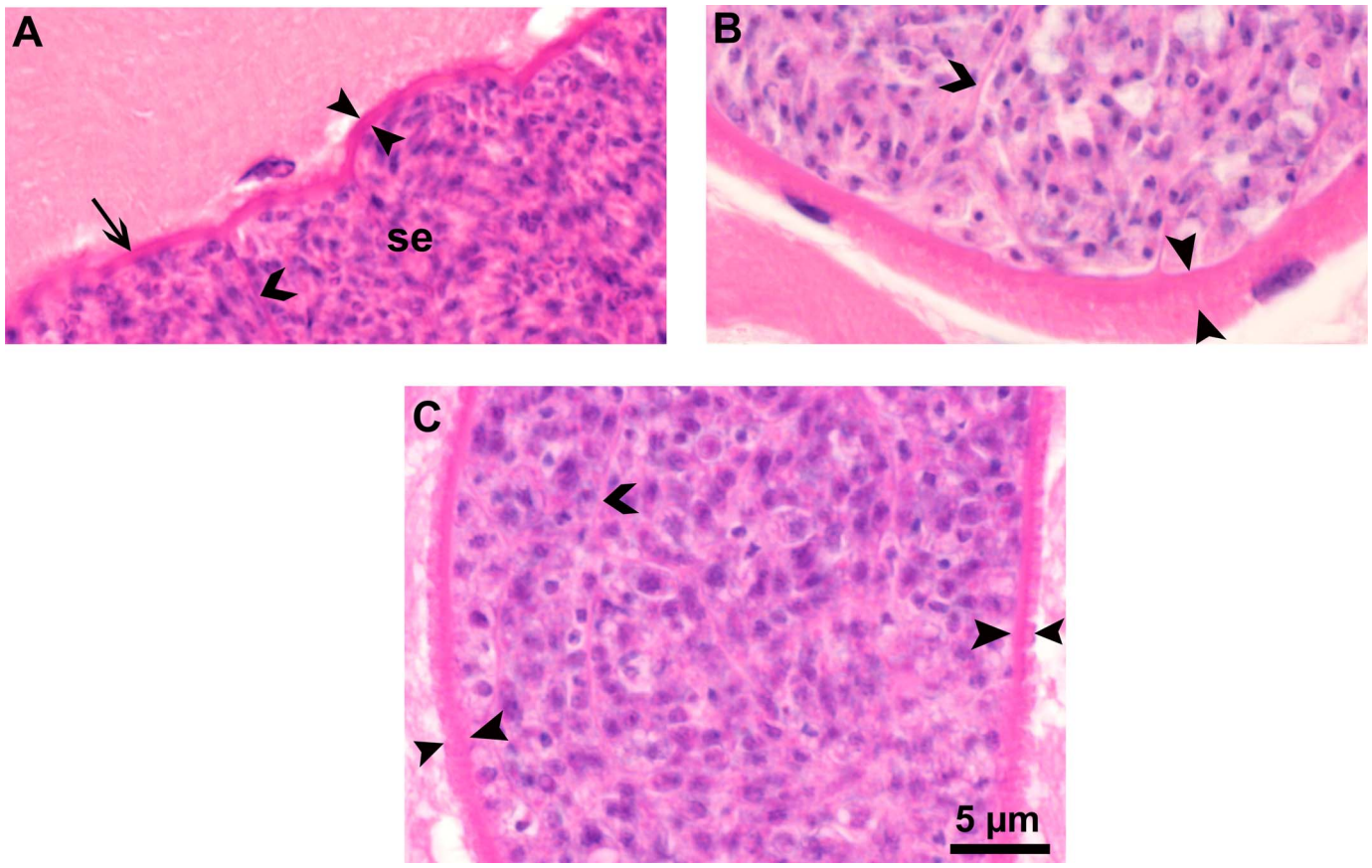

Figure 2. Sarcocyst walls of *Sarco-cystis* sp. in the gray wolf from Minnesota. Hematoxylin and eosin stain. The opposing black arrowheads point to the thickness of sarcocyst walls. Single arrowheads point to septa. Bar applies to all parts. (**A**) *Sarcocystis svanai*–like thin cyst wall. (**B**) *Sarcocystis caninum*–like thicker sarcocyst wall without visible serrations. (**C**) Longitudinal section of an unidentified sarcocyst with molar teeth-like protrusions. Color version available online.

analyzed on a 2% agarose gel and size was estimated by comparison with the 100–base-pair (bp) Plus DNA Ladder (Promega Corporation, Madison, Wisconsin). The PCR products were purified using the ExoSAP method (Bell, 2008). The final purified PCR products were subjected to bi-directional Sanger sequencing to Psomagen company (Rockville, Maryland) on an ABI 3500xl Genetic Analyzer (Applied Biosystems™, Waltham, Massachusetts) using the primer sets specially designed for this study.

Sequences were visualized, assembled, and edited using Geneious 11.1.5, and were submitted to GenBank. We compared sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Each sequence showed 100% identity to either *S. caninum* or *S. svanai* (Table III).

Phylogenetic reconstructions

Phylogenetic analyses were performed, independently, on nucleotide sequences of *18S* rRNA, *28S* rRNA, *COI*, *ITS1,* and *rpoB* gene. Each analysis included sequences obtained in this study as well as all sequences in the database with >95% homogeneity or above in the NCBI GenBank database using BLAST. The sequences identical to either *S. caninum* or *S. svanai* were also similar (but not identical) to each other: 99.42% (for *18S*); 98.36% (for *28S*); 99.16% (for *COI*); and 99.73% (for *rpoB*). We succeeded in amplifying the *ITS1* only for *S. svanai*, which proved 99.30% identical to a reference sequence for *S. caninum*. We constructed multiple sequence alignments using Clustal W 2.1 (Larkin et al., 2007) and MAFT with default parameters as implemented in Geneious Prime® 2024.0.5. All sequences were trimmed at each end before phylogenetic reconstruction. To determine the most suitable nucleotide substitution, a model test was performed using jModelTest 2.1.7 (Darriba et al., 2012). Maximum-likelihood phylogenetic trees were constructed using MrBayes One Model (Ronquist et al., 2012) in TOPALi v2 (Milne et al., 2009) software having 1,000 bootstrap values. A total of 100,000 generations were taken for the phylogenetic tree. Included codon positions were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated.

RESULTS

Sarcocysts were found in the muscles of all 3 wolves. Types 1 and 2 of sarcocysts were recognized based on the appearance of the sarcocyst wall (Fig. 1). Type 1 sarcocysts found in wolf 1 had a relatively thinner (<0.5 µm) cyst wall without any serrations (Figs. 1, 2). Sarcocysts were rare; 45 histological sections of 1 × 1–cm limb muscle yielded only 2 sarcocysts and none were found in the tongue. The 2 sarcocysts in HE-stained sections were 104 × 70 µm and 368 × 115 µm with a thin cyst wall (Fig. 2A). In

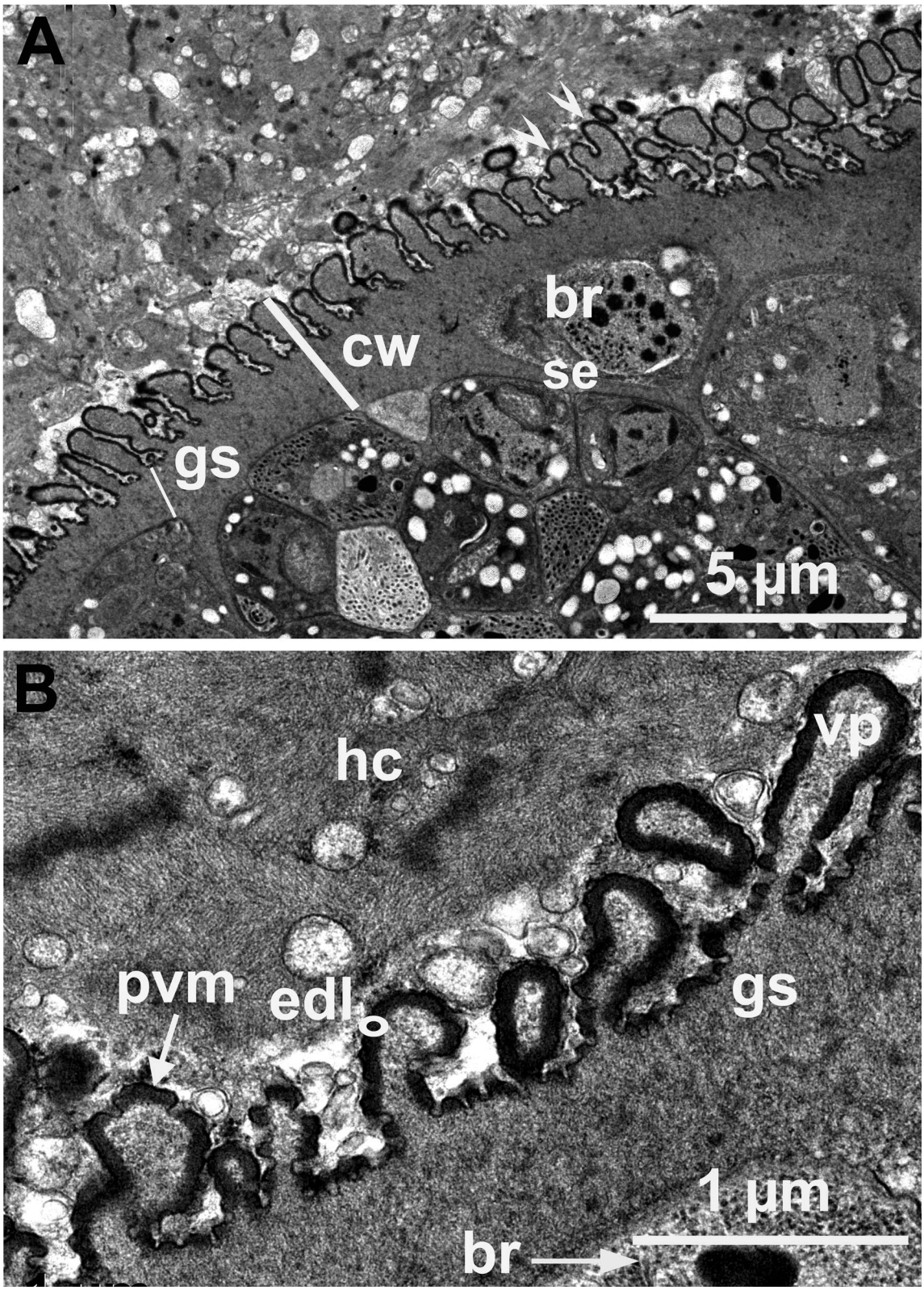

Figure 3. Transmission electron microscopic images of *Sarcocystis caninum* from Minnesota gray wolf. (**A**) Pleomorphic villar protrusions (vp) on the sarcocyst wall. Arrowheads point to bifid vp. The cyst wall (cw) thickness is variable. The ground substance (gs) is smooth and continues into the interior of the cyst as septa (se). Bradyzoites (br) are butted against the gs. (**B**) Higher magnification of sarcocyst wall showing a wavy parasitophorous vacuolar membrane (pvm) lined by an electron-dense layer (edl, white circle), villar protrusions (vp) without microtubules, a smooth ground substance (gs), and part of bradyzoite butted against the gs. Note host cell (hc).

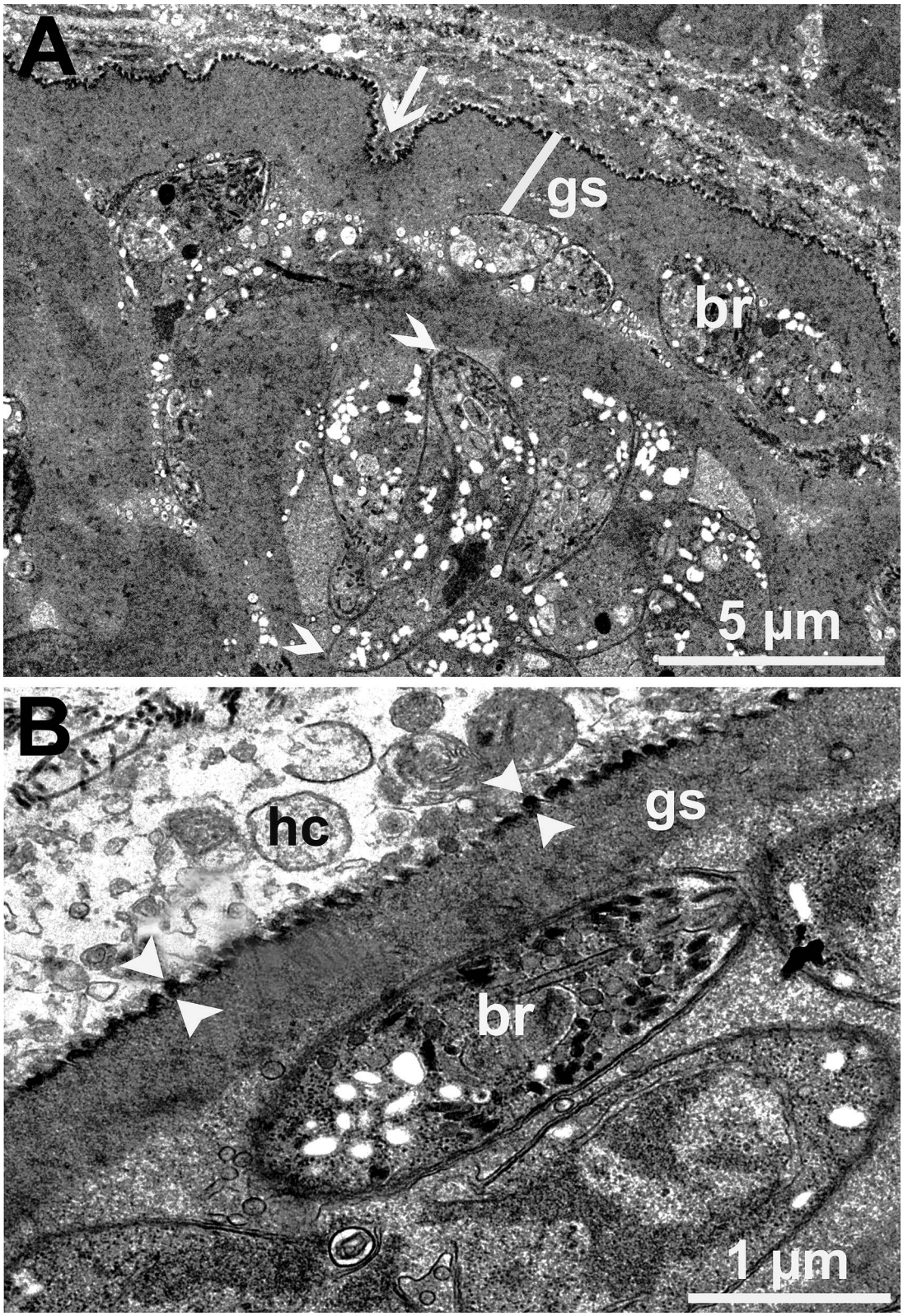

Figure 4. Transmission electron microscopic images of *Sarcocystis svanai* from Minnesota gray wolf 1. (**A**) Low-power image of the cyst wall showing invagination (arrow) of the cyst wall into ground substance (gs). The gs is thick and smooth, and bradyzoites (br) are butted against the gs. Note a longitudinally cut bradyzoite (arrowheads). (**B**) Higher magnification of the cyst wall shows small blebs (opposing arrowheads) with a stalk. The gs is thick and smooth, and a bradyzoite is butted against the gs. Note host cell (hc).

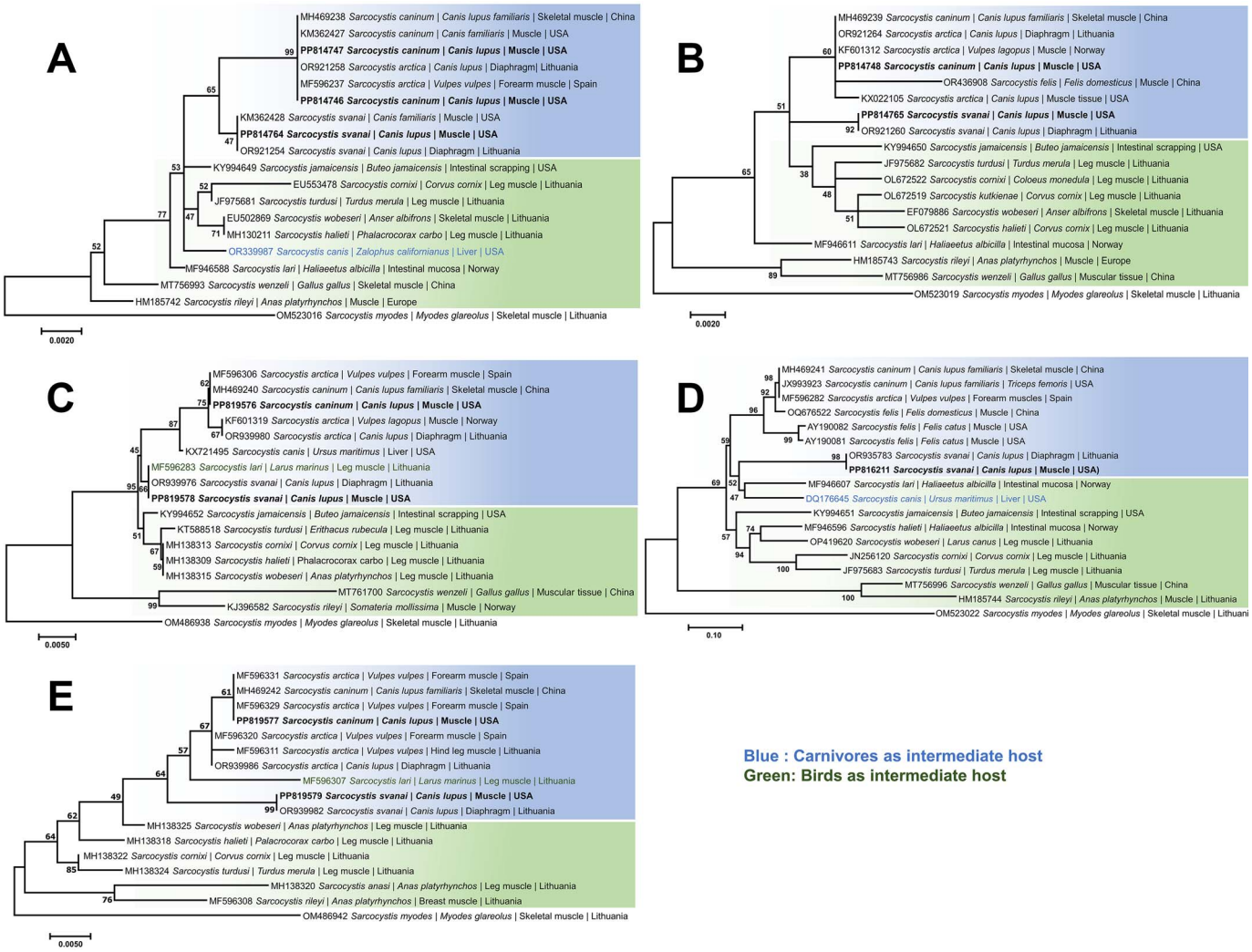

Figure 5. Phylogenetic relationships of *Sarcocystis caninum* and *Sarcocystis svanai* sequences obtained from the gray wolves (*Canis lupus*) inferred from various genetic markers (**A**) *18S* rRNA, (**B**) *28S* rRNA, (**C**) *COI*, (**D**) *ITS1*, and (**E**) *rpoB*. Branch supports are indicated near the corresponding nodes. Species in bold and highlighted are the ones obtained during the study. Color version available online.

unstained muscle squashes 27 cysts were found; the largest cyst was 3,450 × 138 μm.

Type 2 sarcocysts, from wolves 2 and 3, had a relatively thicker (>1 μm) cyst wall with serrations visible under optimal illumination of unstained squash preparations (Fig. 1B). The cyst wall in 1 unidentified sarcocyst had prominent molar tooth-like projections (Fig. 2C). In total, 52 sarcocysts were visible in unstained squashes (44 from wolf 2, 8 from wolf 3). The largest sarcocyst in unstained muscle squashes was 5,106 × 73 μm. One inflammatory focus associated with a degenerating sarcocyst-like structure was found in the limb muscle of wolf 2.

Four sarcocysts (2 from wolf 1 and 2 from wolf 2) were examined ultrastructurally. Both sarcocysts from wolf 2 were identical to *S. caninum* from the domestic dog (Dubey et al., 2015). The sarcocyst wall had undulating, pleomorphic villar protrusions (vp) on the sarcocyst wall like type 9c of Dubey et al. (2016). The vp lacked microtubules, and the ground substance was smooth with few scattered granules (Fig. 3).

By TEM, sarcocysts from wolf 1 appeared identical to sarcocysts of *S. svanai* from the domestic dog (Dubey et al., 2015). The wall was undulating (Fig. 4), like type 1a of Dubey et al. (2016); this type of sarcocyst wall (e.g., *Sarcocystis muris*) is thin and has blebs on the wall with a small stalk. However, in *S. svanai*, the wall invaginates into the ground substance (Fig. 4A); this feature was not noted in the original description of *S. svanai* of the dog (Dubey et al., 2015).

Phylogenetic analysis

The final data set included 19 taxa and 1,563 positions for *18S*; 18 taxa and 776 positions for *28S*; 17 taxa and 939 positions for *COI*; 18 taxa and 1,541 positions for *ITS1*, and 17 taxa and 1,279 positions for *rpoB*. Homologous sequences from isolates of *Sarcocystis myodes* infecting *Myodes glareolus* were taken as the outgroup. The root was placed between the most distant of these (selected as the outgroup) and the rest (Fig. 5). The phylogenetic trees reconstructed based on

Table IV. Homogeneity of various gene marker sequences of selected *Sarcocystis* spp. available in the GenBank with *Sarcocystis caninum* isolated during the study.*

Species	Locus	Host	Tissue	Accession no.	Country	Query cover	DNA sequence identity to *S. caninum*	Gaps
S. caninum	*18S*	*Canis familiaris*	M	KM362427	United States	99%	1,553/1,553 (100%)	0
		Canis lupus familiaris	SM	MH469238	China	100%	1,554/1,554 (100%)	0
	28S	*C. l. familiaris*	SM	MH469239	China	100%	1,458/1,458 (100%)	0
	COI	*C. l. familiaris*	SM	MH469240	China	100%	479/479 (100%)	0
	rpoB	*C. l. familiaris*	SM	MH469242	China	100%	375/375 (100%)	0
Sarcocystis arctica	*18S*	*C. lupus*	D	OR921258	Lithuania	100%	1,554/1,554 (100%)	0
		Vulpes vulpes	FM	MF596237	Spain	100%	1,554/1,554 (100%)	0
	28S	*C. lupus*	D	OR921264	Lithuania	99%	1,454/1,454 (100%)	0
		Vulpus lagopus	M	KF601312	Norway	100%	1,458/1,458 (100%)	0
		C. lupus	MT	KX022105	USA	99%	1,455/1,456 (99%)	0
	COI	*V. vulpes*	FM	MF596306	Spain	100%	479/479 (100%)	0
		V. lagopus	M	KF601319	Norway	100%	478/479 (99%)	0
		C. lupus	D	OR939980	Lithuania	100%	478/479 (99%)	0
	rpoB	*V. vulpes*	FM	MF596331	Spain	100%	375/375 (100%)	0
		V. vulpes	FM	MF596329	Spain	100%	375/375 (100%)	0
		V. vulpes	FM	MF596320	Spain	100%	374/375 (99%)	0
		V. vulpes	HM	MF596311	Lithuania	100%	373/375 (99%)	0
		C. lupus	D	OR939986	Lithuania	100%	374/375 (99%)	0
Sarcocystis felis	*28S*	*Felis domesticus*	M	OR436908	China	100%	1,452/1,460 (99%)	2
Sarcocystis svanai	*18S*	*C. familiaris*	M	KM362428	USA	99%	1,545/1,554 (99%)	2
		C. lupus	M	PP814764	USA	100%	1,254/1,259 (99%)	1
		C. lupus	D	OR921254	Lithuania	100%	1,546/1,555 (99%)	2
	28S	*C. lupus*	D	OR921260	Lithuania	100%	1,440/1,464 (98%)	7
		C. lupus	M	PP814765	USA	100%	767/772 (99%)	0
	COI	*C. lupus*	M	PP819578	USA	100%	934/939 (99%)	0
		C. lupus	D	OR939976	Lithuania	100%	474/479 (99%)	0
	rpoB	*C. lupus*	M	PP819579	USA	99%	757/765 (98%)	0
		C. lupus	D	OR939982	Lithuania	100%	367/375 (98%)	0
Sarcocystis jamaicensis	*18S*	*Buteo jamaicensis*	IS	KY994649	USA	100%	1,543/1,555 (99%)	1
	28S	*B. jamaicensis*	IS	KY994650	USA	100%	1,435/1,463 (98%)	5
	COI	*B. jamaicensis*	IS	KY994652	USA	100%	474/479 (99%)	0
Sarcocystis canis	*18S*	*Zalophus californianus*	L	OR339987	USA	95%	1,470/1,480 (99%)	1
	COI	*Ursus maritimus*	L	KX721495	USA	100%	473/479 (99%)	0
Sarcocystis lari	*18S*	*Haliaeetus albicilla*	IM	MF946588	Norway	100%	1,544/1,555 (99%)	1
	28S	*H. albicilla*	IM	MF946611	Norway	100%	1,444/1,465 (99%)	7
	COI	*Larus marinus*	LM	MF596283	Lithuania	100%	474/479 (99%)	0
	rpoB	*L. marinus*	LM	MF596307	Lithuania	100%	368/375 (98%)	0
Sarcocystis cornixi	*18S*	*Corvus cornix*	LM	EU553478	Lithuania	99%	1,541/1,555 (99%)	3
	28S	*Coloeus monedula*	LM	OL672522	Lithuania	98%	1,416/1,443 (98%)	8
	COI	*C. cornix*	LM	MH138313	Lithuania	100%	472/479 (99%)	0
	rpoB	*C. cornix*	LM	MH138322	Lithuania	100%	369/375 (98%)	0
Sarcocystis kutkienae	*28S*	*C. cornix*	LM	OL672519	Lithuania	100%	1,438/1,463 (98%)	6
Sarcocystis turdusi	*18S*	*Turdus merula*	LM	JF975681	Lithuania	100%	1,544/1,555 (99%)	1
	28S	*T. merula*	LM	JF975682	Lithuania	100%	1,441/1,465 (98%)	8
	COI	*Erithacus rubecula*	LM	KT588518	Lithuania	100%	471/479 (98%)	0
	rpoB	*T. merula*	LM	MH138324	Lithuania	100%	367/375 (98%)	0
Sarcocystis wobeseri	*18S*	*Anser albifrons*	SM	EU502869	Lithuania	100%	1,544/1,555 (99%)	2
	28S	*A. albifrons*	SM	EF079886	Lithuania	100%	1,438/1,463 (98%)	5
	COI	*Anas platyrhynchos*	LM	MH138315	Lithuania	100%	472/479 (99%)	0
	rpoB	*A. platyrhynchos*	LM	MH138325	Lithuania	100%	371/375 (99%)	0

Table IV. Continued.

Species	Locus	Host	Tissue	Accession no.	Country	Query cover	DNA sequence identity to *S. caninum*	Gaps
Sarcocystis halieti	*18S*	*Phalacrocorax carbo*	LM	MH130211	Lithuania	100%	1,544/1,555 (99%)	2
	28S	*C. cornix*	LM	OL672521	Lithuania	100%	1,434/1,463 (98%)	5
	COI	*P. carbo*	LM	MH138309	Lithuania	100%	472/479 (99%)	0
	rpoB	*P. carbo*	LM	MH138318	Lithuania	100%	368/375 (98%)	0
Sarcocystis anasi	*rpoB*	*A. platyrhynchos*	LM	MH138320	Lithuania	100%	360/375 (96%)	0
Sarcocystis rileyi	*18S*	*A. platyrhynchos*	M	HM185742	Europe	100%	1,536/1,555 (99%)	2
	28S	*A. platyrhynchos*	M	HM185743	Europe	100%	1,399/1,463 (96%)	15
	COI	*Somateria mollissima*	M	KJ396582	Norway	100%	460/479 (96%)	0
	rpoB	*A. platyrhynchos*	BM	MF596308	Lithuania	100%	361/375 (96%)	0
Sarcocystis wenzeli	*18S*	*Gallus gallus*	SM	MT756993	China	100%	1,537/1,555 (99%)	2
	28S	*G. gallus*	MT	MT756986	China	100%	1,408/1,461 (96%)	14
	COI	*G. gallus*	MT	MT761700	China	100%	452/479 (94%)	0
Sarcocystis myodes	*18S*	*Myodes glareolus*	SM	OM523016	Lithuania	100%	1,516/1,559 (97%)	5
(outgroup)	*28S*	*M. glareolus*	SM	OM523019	Lithuania	100%	1,373/1,477 (93%)	24
	COI	*M. glareolus*	SM	OM486938	Lithuania	100%	457/479 (95%)	0
	rpoB	*M. glareolus*	SM	OM486942	Lithuania	100%	358/375 (95%)	0

*M: muscle; SM: skeletal muscle; D: diaphragm; FM: forearm muscle; HM: hindleg muscle; IS: intestinal scraping; L: liver; IM: Intestinal mucosa; LM: leg muscle; BM: breast muscle; MT: muscular tissue.

maximum likelihood only differ in the position of a few low-supported branches (Fig. 5). These trees agree with previous studies on *Sarcocystis* spp. from carnivores (Gjerde and Schulze, 2014; Dubey et al., 2015; Calero-Bernal et al., 2016; Pavlásek and Máca, 2017; Hagner et al., 2018; Kirillova et al., 2018; Máca, 2018; Ye et al., 2018; Juozaitytė-Ngugu et al., 2024) (Table I).

The phylogenetic tree based on all the genetic markers utilized during the study precisely formed 2 clades in almost all the trees: 1 with all the carnivores as intermediate hosts, and the other with birds as intermediate hosts with strong bootstrap support and almost the same branching topology. Furthermore, the phylogenetic tree formed 2 sister clades subdividing species infecting carnivores from species infecting birds, and then basal to that, the clade including *Sarcocystis rileyi* and *Sarcocystis wenzeli*. The tree in Figure 5A and D represents a slight departure from this result by the different placement of certain branches, for example, *S. lari* (accession number MF596283) and *S. canis* (accession number DQ176645). Also, the placement of the 2 major clades showed some minor variations depending on the sequence variability and alignment settings, or variations in the representative sequences. These variations influenced the positioning of the clade containing *S. lari* and *S. canis* to be placed in between subsets of species with carnivores and birds as intermediate hosts. Interestingly, *S. caninum* (present study) formed sister clades with *Sarcocystis arctica*, *Sarcocystis canis*, and *Sarcocystis felis* with high bootstrap values (Tables IV, V), because these species are closely related.

The phylogenetic evaluation confirmed that *S. arctica* cannot be genetically differentiated from *S. caninum* in the 4/5 loci studied. The phylogenetic analysis further showed that *S. arctica* and *S. caninum* were placed together with *Sarcocystis* species using carnivores as their intermediate host (*S. canis*, *S. felis*, and *S. svanai*) and establish the monophyletic relationship between them. However, as stated earlier they have different intermediate hosts (Table I). The phylogenetic also tree reiterates the importance of host specificity in these highly enigmatic parasites.

DISCUSSION

For reasons stated in the introduction, we wish to restrict the present discussion to *Sarcocystis* infections in the wolf. *Sarcocystis* infection was first described in an Alaskan wolf from the United States (Calero-Bernal et al., 2016). The Alaskan wolf (*Canis lupus arctos*) is a subspecies of the gray wolf, and its habitat is more geographically distinct than the gray wolf in the continental United States. Calero-Bernal et al. (2016) called the parasite *S. arctica* and did not discuss *S. caninum*. In retrospect, the parasite resembles *S. caninum*.

Here we provide the first record/description of *Sarcocystis* infections in the muscles of the gray wolf in Minnesota. Sarcocysts were found in all 3 wolves sampled. Although tissues were autolyzed, the structure of sarcocysts was preserved to obtain an ultrastructural description of the sarcocyst walls. The *Sarcocystis* species found were morphologically identical to *S. caninum* and *S. svanai* of the domestic dog (Dubey et al., 2015). *Sarcocystis caninum* and *S. svanai* have been associated with severe neuromuscular disorders in 2 dogs from British Columbia, Canada, 1 dog from Montana, and 1 dog from Colorado in

Table V. Homogeneity of various gene marker sequences of selected *Sarcocystis* spp. available in the GenBank with *Sarcocystis svanai* isolated during the study.*

Species	Locus	Host	Tissue	Accession no.	Country	Query cover	DNA sequence identity to *S. svanai*	Gaps
Sarcocystis caninum	*18S*	*Canis familiaris*	M	KM362427	United States	100%	1,253/1,259 (99%)	1
		Canis lupus	M	PP814747	United States	100%	1,254/1,259 (99%)	1
		C. lupus	M	PP814746	United States	100%	1,253/1,259 (99%)	1
		C. lupus familiaris	SM	MH469238	China	100%	1,253/1,259 (99%)	1
	28S	*C. lupus*	M	PP814748	United States	100%	767/772 (99%)	0
		C. l. familiaris	SM	MH469239	China	100%	767/772 (99%)	0
	COI	*C. l. familiaris*	SM	MH469240	China	100%	933/939 (99%)	0
		C. lupus	M	PP819576	USA	100%	934/939 (99%)	0
	ITS1	*C. l. familiaris*	TM	JX993923	USA	70%	135/149 (91%)	3
		C. l. familiaris	SM	MH469241	China	93%	185/198 (93%)	2
	rpoB	*C. l. familiaris*	SM	MH469242	China	89%	748/765 (98%)	0
		C. lupus	M	PP819577	United States	99%	757/765 (98%)	0
Sarcocystis arctica	*18S*	*C. lupus*	D	OR921258	Lithuania	100%	1,253/1,259 (99%)	1
		Vulpes vulpes	FM	MF596237	Spain	100%	1,253/1,259 (99%)	1
	28S	*C. lupus*	D	OR921264	Lithuania	100%	749/754 (99%)	0
		Vulpes lagopus	M	KF601312	Norway	100%	767/772 (99%)	0
		C. lupus	MT	KX022105	United States	97%	752/756 (99%)	0
	COI	*V. vulpes*	FM	MF596306	Spain	100%	933/939 (99%)	0
		V. lagopus	M	KF601319	Norway	100%	933/939 (99%)	0
		C. lupus	D	OR939980	Lithuania	100%	933/939 (99%)	0
	ITS1	*V. vulpes*	FM	MF596282	Spain	56%	107/120 (89%)	2
	rpoB	*V. vulpes*	FM	MF596331	Spain	86%	726/742 (98%)	0
		V. vulpes	FM	MF596329	Spain	86%	726/742 (98%)	0
		V. vulpes	FM	MF596320	Spain	86%	727/742 (98%)	0
		V. vulpes	FM	MF596311	Spain	86%	726/742 (98%)	0
		C. lupus	D	OR939986	Lithuania	86%	727/742 (98%)	0
Sarcocystis svanai	*18S*	*C. familiaris*	M	KM362428	United States	100%	1,259/1,259 (100%)	0
		C. lupus	D	OR921254	Lithuania	100%	1,259/1,259 (100%)	0
	28S	*C. lupus*	D	OR921260	Lithuania	100%	772/772 (100%)	0
	COI	*C. lupus*	D	OR939976	Lithuania	100%	939/939 (100%)	0
	ITS1	*C. lupus*	D	OR935783	Lithuania	62%	132/132 (100%)	0
	rpoB	*C. lupus*	D	OR939982	Lithuania	100%	742/742 (100%)	0
Sarcocystis felis	*ITS1*	*Felis domesticus*	M	OQ676522	China	93%	183/201 (91%)	5
		Felis catus	M	AY190081	United States	70%	135/152 (89%)	4
		F. catus	M	AY190082	United States	70%	134/152 (88%)	4
Sarcocystis jamaicensis	*18S*	*Buteo jamaicensis*	IS	KY994649	United States	96%	1,214/1,218 (99%)	0
	28S	*B. jamaicensis*	IS	KY994650	United States	100%	765/772 (99%)	0
	COI	*B. jamaicensis*	IS	KY994652	United States	100%	936/939 (99%)	0
	ITS1	*B. jamaicensis*	IS	KY994651	United States	92%	181/196 (92%)	2
Sarcocystis canis	*18S*	*Zalophus californianus*	L	OR339987	United States	100%	1,257/1,259 (99%)	0
	COI	*Ursus maritimus*	L	KX721495	United States	100%	932/939 (99%)	0
	ITS1	*U. maritimus*	L	DQ176645	United States	99%	195/208 (94%)	0
Sarcocystis lari	*18S*	*Haliaeetus albicilla*	IM	MF946588	Norway	100%	1,255/1,259 (99%)	0
	28S	*H. albicilla*	IM	MF946611	Norway	100%	765/772 (99%)	0
	COI	*Larus marinus*	LM	MF596283	Lithuania	100%	939/939 (100%)	0
	ITS1	*H. albicilla*	IM	MF946607	Norway	100%	193/213 (91%)	3
	rpoB	*L. marinus*	LM	MF596307	Lithuania	86%	724/742 (98%)	0
Sarcocystis cornixi	*18S*	*Corvus cornix*	LM	EU553478	Lithuania	100%	1,252/1,261 (99%)	2
	28S	*Coloeus monedula*	LM	OL672522	Lithuania	99%	762/769 (99%)	3
	COI	*C. cornix*	LM	MH138313	Lithuania	100%	936/939 (99%)	0
	ITS1	*C. cornix*	LM	JN256120	Lithuania	54%	105/115 (91%)	2
	rpoB	*C. cornix*	LM	MH138322	Lithuania	86%	728/742 (98%)	0

Table V. Continued.

Species	Locus	Host	Tissue	Accession no.	Country	Query cover	DNA sequence identity to *S. svanai*	Gaps
Sarcocystis kutkienae	*28S*	*C. cornix*	LM	OL672519	Lithuania	99%	761/766 (99%)	0
Sarcocystis turdusi	*18S*	*Turdus merula*	LM	JF975681	Lithuania	100%	1,256/1,259 (99%)	0
	28S	*T. merula*	LM	JF975682	Lithuania	98%	754/759 (99%)	0
	COI	*Erithacus rubecula*	LM	KT588518	Lithuania	100%	935/939 (99%)	0
	ITS1	*T. merula*	LM	JF975683	Lithuania	62%	118/136 (87%)	5
	rpoB	*T. merula*	LM	MH138324	Lithuania	86%	726/742 (98%)	0
Sarcocystis anasi	*rpoB*	*Anas platyrhynchos*	LM	MH138320	Lithuania	86%	698/742 (94%)	0
Sarcocystis wobeseri	*18S*	*Anser albifrons*	SM	EU502869	Lithuania	100%	1,256/1,259 (99%)	0
	28S	*A. albifrons*	SM	EF079886	Lithuania	100%	764/772 (99%)	0
	COI	*A. platyrhynchos*	LM	MH138315	Lithuania	99%	935/938 (99%)	0
	ITS1	*Larus canus*	LM	OP419620	Lithuania	62%	114/132 (86%)	0
	rpoB	*A. platyrhynchos*	LM	MH138325	Lithuania	86%	727/742 (98%)	0
Sarcocystis halieti	*18S*	*Phalacrocorax carbo*	LM	MH130211	Lithuania	100%	1,256/1,259 (99%)	0
	28S	*C. cornix*	LM	OL672521	Lithuania	100%	764/772 (99%)	0
	COI	*P. carbo*	LM	MH138309	Lithuania	100%	937/939 (99%)	0
	ITS1	*H. albicilla*	IM	MF946596	Norway	92%	183/196 (93%)	2
	rpoB	*P. carbo*	LM	MH138318	Lithuania	86%	728/742 (98%)	0
Sarcocystis rileyi	*18S*	*A. platyrhynchos*	M	HM185742	Europe	100%	1,252/1,259 (99%)	0
	28S	*A. platyrhynchos*	M	HM185743	Europe	100%	758/772 (98%)	6
	COI	*Somateria mollissima*	M	KJ396582	Norway	100%	912/939 (97%)	0
	ITS1	*A. platyrhynchos*	M	HM185744	Lithuania	53%	81/115 (70%)	14
	rpoB	*A. platyrhynchos*	BM	MF596308	Lithuania	86%	704/742 (95%)	0
Sarcocystis wenzeli	*18S*	*Gallus gallus*	SM	MT756993	China	100%	1,252/1,259 (99%)	0
	28S	*G. gallus*	MT	MT756986	China	100%	757/772 (98%)	6
	COI	*G. gallus*	MT	MT761700	China	99%	896/938 (96%)	0
	ITS1	*G. gallus*	MT	MT756996	China	90%	153/190 (81%)	13
Sarcocystis myodes	*18S*	*Myodes glareolus*	SM	OM523016	Lithuania	100%	1,234/1,263 (98%)	5
(Outgroup)	*28S*	*M. glareolus*	SM	OM523019	Lithuania	100%	751/772 (97%)	3
	COI	*M. glareolus*	SM	OM486938	Lithuania	100%	895/939 (95%)	0
	ITS1	*M. glareolus*	SM	OM523022	Lithuania	5%	12/12 (100%)	0
	rpoB	*M. glareolus*	SM	OM486942	Lithuania	86%	707/742 (95%)	0

*M: muscle; SM: skeletal muscle; D: diaphragm; TM: triceps femoris; FM: forearm muscle; HM: hindleg muscle; IS: intestinal scraping; L: liver; IM: intestinal mucosa; LM: leg muscle; BM: breast muscle; MT: muscular tissue.

the United States (Dubey et al., 2015), and 1 dog from Helsinki, Finland (Hagner et al., 2018). Of interest is that in 2 of the 5 dogs, *S. caninum* and *S. svanai* occurred concurrently, suggesting common epidemiological risk factors. Nothing was known concerning the cause of death in 2 wolves in the present study (Table I).

In the present study, only 1 species of *Sarcocystis* (either *S. caninum* or *S. svanai*) was found in each wolf. Domestic dogs and gray wolves are phylogenetically related and share common parasites (Lesniak et al., 2017). The occurrence of *S. caninum* and *S. svanai* from distant locations suggests that dogs and wolves are not accidentally infected with these *Sarcocystis* species but likely have an unrecognized cycle. There are not many predators of wolves to have a regular *Sarcocystis* cycle, but a wide range of scavenger species consume dead wolves or prey on young wolves and might act as definitive hosts.

Originally, *S. svanai* was discovered accidentally while reviewing TEM images of 2 dogs infected with *S. caninum*; therefore, a light-microscope description of the sarcocyst was unavailable (Dubey et al., 2015). Here, a light microscopic description of *S. svanai* sarcocyst was added both in unstained muscle squashes as well as in HE-stained sections. Additionally, we added to the ultrastructural description of the sarcocyst wall; the parasitophorous vacuolar membrane invaginated in ground substance, and here we designated the wall type as type 1a-1 to the wall type classification of Dubey et al. (2016).

Sarcocystis caninum and *S. svanai* infections were recently reported in gray wolves from Lithuania (Juozaityé-Ngugu et al., 2024) (Table I). Sarcocysts were detected in 4 (26.7%) of 15 gray wolves and sarcocysts were numerous. In 2 wolves, thin-walled *S. svanai*–like sarcocysts were detected, and in the other 2 wolves, thin-walled sarcocysts were mixed with remnants of *S. caninum*–like bradyzoites (Juozaityé-Ngugu et al., 2024); TEM was not performed. This investigation listed the pros and cons of different genetic markers for the diagnosis of *S. caninum*– and *S. svanai*–related *Sarcocystis* species (Table I).

Sarcocystis caninum and *S. svanai* were previously characterized molecularly, using loci such as *18S*, *28S*, *COI*, *ITS1*, and *rpoB* (Dubey et al., 2015). The phylogenetic analyses based on these loci provided insights into the relationships among *Sarcocystis* spp. infecting carnivores (intermediate hosts), and how they are related to birds and rodents highlighting the close genetic relationships between them, as well as their distinctness from other related species, shedding light on the host–parasite interactions and potential transmission patterns. In another report, *S. caninum* and *S. svanai* were molecularly characterized and described from a clinically affected dog (Hagner et al., 2018) in Finland; Pampas fox (Scioscia et al., 2017); 2 domestic dogs from China (Ye et al., 2018) and gray wolves from Lithuania (Juozaitytè-Ngugu et al., 2024). These studies show the importance of canids as a potential source of *Sarcocystis* spp. infections and their affinities to phylogenetically related host species in close proximity. Our data further implicate natural cycles, between wild canids and some (still unknown, possibly avian) definitive hosts, as reservoirs for infection in domesticated dogs.

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