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PROTOZOOLOGY - ORIGINAL PAPER

Molecular and morphological description of *Sarcocystis kutkienae* sp. nov. from the common raven (*Corvus corax*)

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Abstract

Until now, two *Sarcocystis* species, *S. cornixi* and *S. corvusi,* were known to employ members of the family Corvidae as intermediate hosts. Between 2013 and 2019, having examined leg muscles of 23 common ravens in Lithuania, sarcocysts were detected in 18 birds (78.3%). Using light microscopy, transmission electron microscopy (TEM), and molecular analysis (three genetic loci, 18S rDNA, 28S rDNA, and ITS1), sarcocysts found in the common raven were described as a new species *S. kutkienae*. Under a light microscope, the observed sarcocysts were ribbon-shaped (1500–8147 × 53–79 μm) and had a wavy striated cyst wall that reached up to 1.5 μm. Lancet-shaped bradyzoites were 7.7 × 2.2 μm (6.1–9.0 × 1.2–3.0 μm) in size. Ultrastructurally, the sarcocyst wall was 1.5–1.8 μm in thickness and had conical-like protrusions with minute invaginations of a parasitophorous vacuolar membrane. The cyst wall was type 1e-like. Limited genetic variability was observed between the 18S rDNA and 28S rDNA sequences of *S. kutkienae* and other *Sarcocystis* spp. using birds as intermediate hosts. In contrast, *S. kutkienae* could be clearly identified by comparing sequences. At this locus, sequences of *S. kutkienae* shared the highest similarity (89.5–89.7%) with those of *S. cornixi*. Phylogenetic analysis showed that *S. kutkienae* was most closely related to *Sarcocystis* spp. that employs birds as intermediate and definitive hosts. The issue relating to which species might serve as definitive hosts of *S. kutkienae* in Lithuania is addressed.

Keywords *Sarcocystis* · Common raven · Electron microscopy · rDNA · ITS1 · Phylogeny

Introduction

Members of the genus *Sarcocystis* are intracellular protozoan parasites distinguished by a two-host prey-predator life cycle. Asexual multiplication with sarcocyst formation occurs in the intermediate host, while sexual stages develop in the small intestine of the definitive host. Some *Sarcocystis* species are harmful to humans and domestic and wild animals (Dubey et al. 2016).

In general, granivorous, insectivorous, and omnivorous birds are intermediate hosts for numerous *Sarcocystis* species, while birds of prey serve as definitive hosts for these parasites (Kutkienė et al. 2009, 2010, 2012a, b; Gjerde and Dahlgren 2010; Prakas et al. 2011, 2013, 2014, 2018a, b, 2020). However, it was shown that predatory birds might act as intermediate hosts for some *Sarcocystis* species (Lindsay and Blagburn 1999; Krone et al. 2000). Birds of the family Corvidae may serve as intermediate or definitive hosts for some *Sarcocystis* species. Sarcocysts were found in the muscles of the hooded crow (*Corvus cornix*), American crow (*Corvus brachyrhynchos*), rook (*Corvus frugilegus*), jay (*Garrulus glandarius*), raven (*Corvus corax*), Tasmanian raven (*Corvus tasmanicus*), jackdaw (*Corvus monedula*), Canada jay (*Perisoreus canadensis*), and magpie (*Pica pica*) (Drouin and Mahrt 1979; Munday et al. 1979; Černá 1984; Pak and Eshtokina 1984; Pinayeva et al. 1998; Kutkienė et al. 2009; Prakas et al. 2013). Oocysts/sporocysts of *S. ovalis* were detected in the intestinal lamina propria of the magpie and Japanese jungle crow (*Corvus macrorhynchos*) (Gjerde and Dahlgren 2010; Irie et al. 2017). The moose (*Alces alces*), red deer (*Cervus elaphus*), and sika deer (*Cervus nippon*) were confirmed as intermediate hosts of this species (Dahlgren and Gjerde 2008, 2010). It is assumed that corvids are

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involved in the transmission of many more *Sarcocystis* species (Gjerde and Dahlgren 2010).

Members of the family Corvidae are known to be intermediate hosts of two valid *Sarcocystis* species, *S. cornixi* and *S. corvusi* (Kutkienė et al. 2009; Prakas et al. 2013). The present paper provides a morphological and molecular description of a new *Sarcocystis* species from the common raven in Lithuania.

Material and methods

Sample collection and morphological analysis

Between 2013 and 2019, a total of 23 common ravens were studied for *Sarcocystis* spp. Muscle tissues of dead common ravens were received from taxidermists. The bird tissue samples were kept frozen (20 °C) until a microscopical examination was conducted.

Leg muscles of birds were examined for the presence of sarcocysts. The prevalence and intensity of *Sarcocystis* infections were evaluated in stained muscle samples. For this purpose, 28 pieces of muscle (about 1 g) were cut off, stained with 0.2% methylene blue solution, clarified with 1.5% acetic acid solution, pressed into a glass compressor, and studied under a light microscope. Sarcocysts were morphologically characterised in squashed preparations after the cysts had been isolated from the muscle fibres with the help of two preparation needles. Overall, 18 sarcocysts were extracted from leg muscle tissues from 18 individual common ravens (isolates CcLt1-CcLt4; CcLt7-CcLt12; CcLt15; CcLt17-CcLt23) and preserved in individual microcentrifuge tubes containing 96% ethanol.

For transmission electron microscopy (TEM) analysis, a single mature sarcocyst was isolated from the leg muscle of one common raven (CcLt22). Sarcocysts were fixed in 2% glutaraldehyde fixative, postfixed in 1% osmium tetroxide, dehydrated, and infiltrated in epoxy resin. Sections were cut with a Leica UC6 ultramicrotome and stained with 4% uranyl acetate and 3% lead citrate solution. Grids were examined under the Morgagni 268 TEM (FEI, Hillsboro, Oregon, USA). TEM analysis was carried out at the National Centre of Pathology (Vilnius, Lithuania).

Molecular analysis

Genomic DNA was extracted from individual sarcocysts using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer's recommendations. The 18S rDNA was amplified using SarAF/SarBR and SarCF/SarDR primer pairs; partial 28S rDNA was amplified with the help of the KL-P1F/KL-P2R primer pair (Kutkienė et al. 2010). Meanwhile, the complete ITS1 (internal transcribed spacer) region was amplified using the SU1F/5.8SR2 primer pair (Gjerde 2014). Each PCR mixture consisted of 25 μl containing 12.5 μl of Dream Taq PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 0.5 μM of each primer, 4-μl template DNA, and nuclease-free water. Amplification reactions were carried out using the same thermal protocol, starting with the initial hot start at 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, annealing at 54–60 °C, depending on the primer pair, for 60 s and 72 °C for 80 s, and a final extension at 72 °C for 10 min. PCR products were evaluated using a 1.5% agarose gel, visualized via UV light after staining with 0.05 μg/ml ethidium bromide, and purified with the help of exonuclease *Exo*I and alkaline phosphatase *FastAP* (Thermo Fisher Scientific, Vilnius, Lithuania). Visualisation, purification, and sequencing of PCR products were carried out using a previously described protocol (Prakas et al. 2016).

The 18S rDNA, 28S rDNA, and ITS1 sequences obtained in this study were compared with those of various *Sarcocystis* spp. using the nucleotide BLAST program megablast option (http://blast.ncbi.nlm.nih.gov/) for the purpose of detecting remarkably similar DNA sequences. The 18S rDNA, 28S rDNA, and ITS1 sequences were aligned using the MUSCLE algorithm loaded in MEGA7 software (Kumar et al. 2016). TOPALi v2.5 software (Milne et al. 2004) was used for phylogenetic analysis. The 1802-bp-long 18S rDNA, 1479-bp-long 28S rDNA, and 880-bp-long ITS1 region sequences of *Sarcocystis kutkienae* from the common raven generated in the present study were deposited in GenBank under accession numbers MT495321–MT495322 and MT495389–MT495406.

Results

Morphological characteristics of *S. kutkienae*

Sarcocysts were detected in 78.3% (18/23) of leg muscles from Lithuanian common ravens. The average parasite load was 29.0 cysts/g of muscle (median 44.9 cysts/g of muscle), observed in methylene blue-stained samples. The parasite load ranged from 2 to 160 cysts/g of muscles. The highest load was observed in the common raven collected in the Šilutė district (isolate CcLt9).

Sarcocysts detected in unstained squashed muscles of 18 infected individuals were morphologically similar and most likely represented one species. They were microscopic, ribbon-shaped, and measured 4331 × 63 μm (1500–8147 × 53–79 μm). Under a light microscope, the cyst wall was striated and reached up to 1.5 μm in thickness (Fig. 1a). Septa divided sarcocysts into compartments filled with lancet-shaped bradyzoites, 7.7 × 2.2 μm (6.1–9.0 × 1.2–3.0 μm) in size (Fig. 1b).

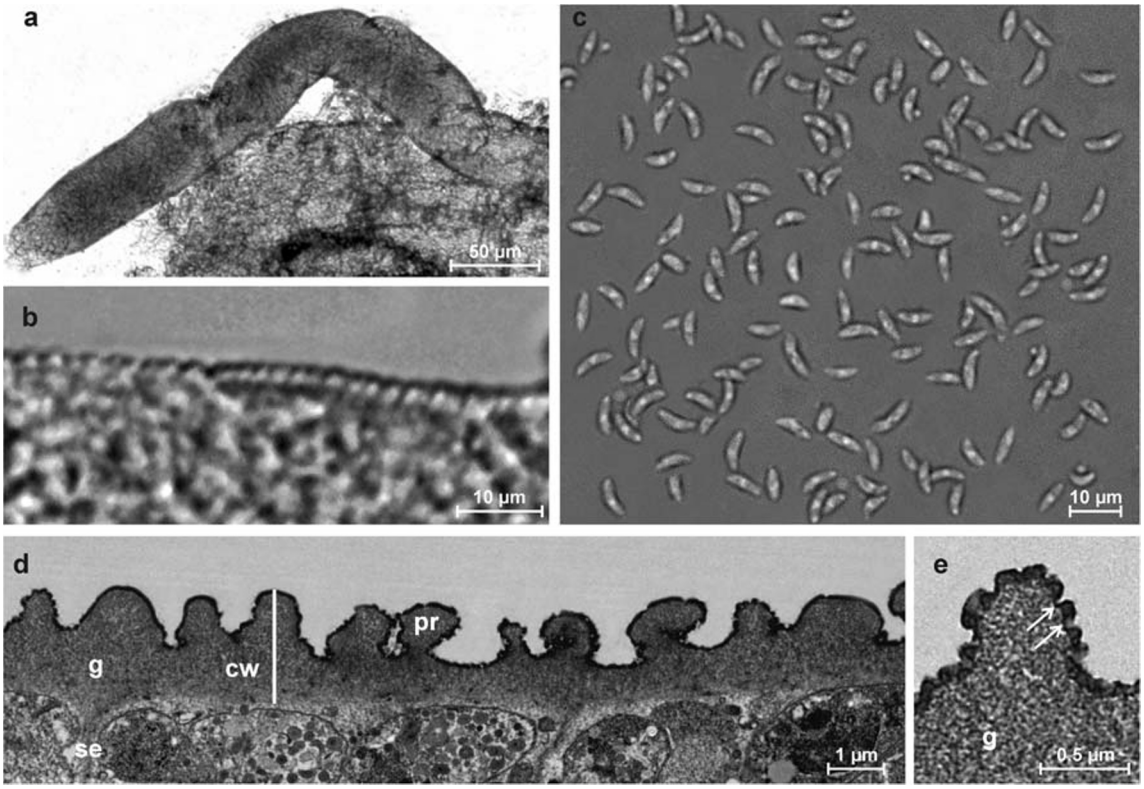

Fig. 1 Morphology of *Sarcocystis kutkienae* n. sp. from muscle tissue of the common raven (*Corvus corax*). **a-c** Light micrographs. Fresh preparations. **a** Fragment of the ribbon-shaped sarcocyst. **b** A portion of sarcocyst; note striated cyst wall. **c** Lancet-shaped bradyzoites. **d, e** TEM micrographs. **d** Fragment of cyst wall (*cw*) with conical protrusions (*pr*) of similar height, but different width; arranged at uneven distances. **e** High magnification of *pr*; note minute invaginations of parasitophorous vacuolar membrane (arrows). *g* Ground substance, *se* septa

Under TEM, the sarcocyst wall was 1.5–1.8-µm thick, had conical protrusions of similar height (0.7–0.9 µm) but different width (0.5–1.0 µm), and was arranged at uneven distances (0.1–0.8 µm) (Fig. 1c). The ground substance layer measured 0.6–0.9 µm and continued into the interior of the sarcocyst as septa. The parasitophorous vacuolar membrane had many minute invaginations (Fig. 1d). The cyst wall was type 1e-like (Dubey et al. 2016).

Cyst wall type 1 was detected in several *Sarcocystis* species employing wild birds as intermediate hosts (Prakas et al. 2018a, b). Therefore, molecular methods were used for complete identification of the *Sarcocystis* sp. under investigation. Based on DNA analysis, it was proposed to refer to sarcocsyts detected in the common raven in Lithuania as *Sarcocystis kutkienae* n. sp.

Molecular characteristics and phylogenetic placement of *S. kutkienae*

Eighteen *S. kutkienae* isolates were identical in almost complete 18S rDNA and partial 28S rDNA. Intraspecific genetic diversity of the examined *S. kutkienae* isolates was revealed only within the ITS1 region. The obtained ITS1 sequences of *S. kutkienae* differed by one-two transitions (A/G) at nucleotide positions 349 and 412. As expected, by comparing the obtained sequences of the 18S rDNA gene with the homologous ones of *Sarcocystis* species, limited genetic diversity was observed (99.5–99.7% similarity with *S. fulicae*, *S. turdusi*, *S. cornixi*, *S. columbae*, *S. jamaicensis*, *S. lari*, *S. halieti*, *S. calchasi*, and *S. corvusi*). On the basis of the 28S rDNA sequences, *S. kutkienae* demonstrated 99.2% similarity with *S. cornixi* (GenBank: EF079884) from the hooded crow, 99.1% similarity to several *Sarcocystis* spp., namely *S. turdusi* (JF975682) from the common blackbird (*Turdus merula*), *Sarcocystis* sp. ex *Accipiter nisus* (GU253888), *S. calchasi* (FJ232949) from the common pigeon (*Columba livia*), and *S. wobeseri* (EF079886, GQ922887-GQ922888 and HM159420) from several Anseriformes species and the herring gull (*Larus argentatus*). ITS1 was the most variable genetic region for the discrimination of *Sarcocystis* spp. that employs birds as intermediate hosts. At this genetic region, sequences of *S. kutkienae* shared the highest similarity (89.5–89.7%) to those of *S. cornixi* (JF520781 and JN256120).

The phylogenetic tree based on 28S rDNA sequences showed a close relationship between *S. kutkienae* and numerous *Sarcocystis* species using birds, predatory mammals, and rodents as intermediate hosts. In the 28S rDNA phylogenetic tree, *S. kutkienae* was placed in one cluster with *S. calchasi*, *S. wobeseri*, *S. corvusi*, *S. halieti*, *S. columbae*, *S. cornixi*, *S. turdusi*, and *S. fulicae* (Fig. 2a).

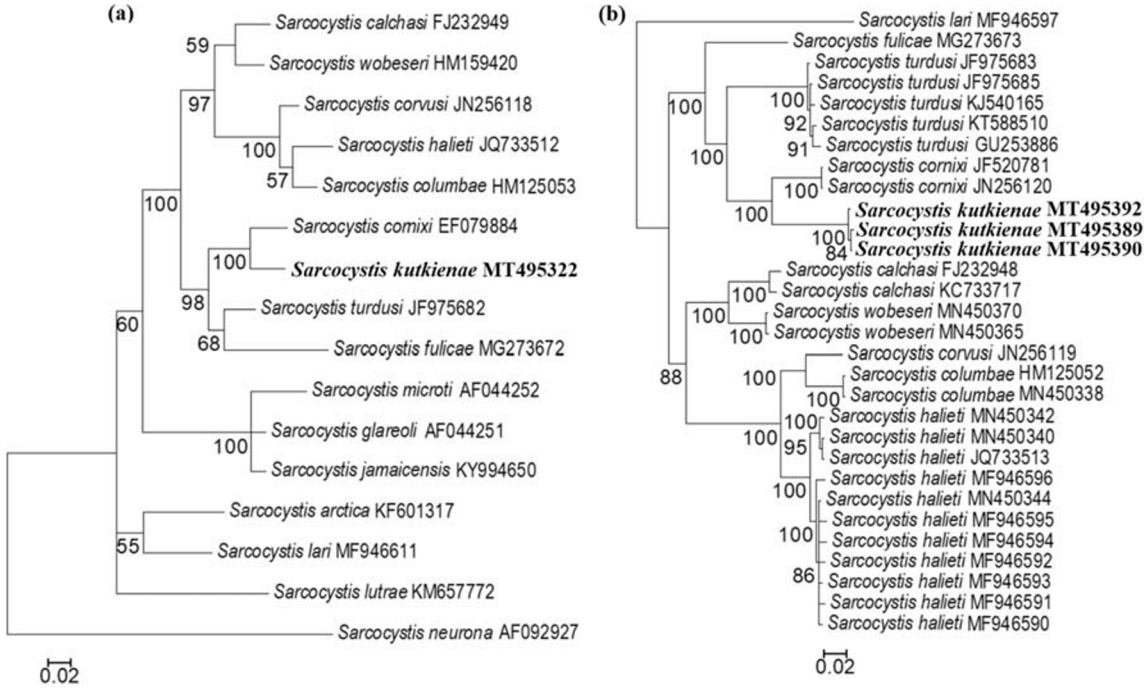

Fig. 2 The phylogenetic trees of selected *Sarcocystis* species based on 28S rDNA (**a**) and ITS1 (**b**) sequences. The tree was constructed using Bayesian methods, scaled according to the branch length, and rooted on *S. neurona* (**a**) and *S. lari* (**b**). The final alignment of 28S rDNA contained 16 taxa and 1,445 aligned nucleotide positions, whereas the alignment of ITS1 contained 30 taxa and 994 aligned nucleotide positions. The figures next to the branches show posterior probability support values. The GenBank accession numbers of sequences are given behind the species name. Three ITS1 sequences of *S. kutkienae* with different nucleotide composition were used in phylogenetic analysis

The ITS1 phylogenetic tree was supported by high probability values (84–100). Based on the sequences of ITS1, *S. kutkienae* was placed together with *S. cornixi*, *S. fulicae,* and *S. turdusi* (Fig. 2b). At ITS1, *S. kutkienae* was most closely related to *S. cornixi*, while *S. turdusi* was a sister species to the *S. kutkienae* and *S. cornixi* clade.

Taxonomic summary of *S. kutkienae* n. sp.

Type intermediate host: Common raven (*Corvus corax*).

Definitive host: Unknown.

Locality: Lithuania (Šilutė district).

Specimens deposited: TEM material was deposited at the National Centre of Pathology, Vilnius, Lithuania. Sequences were deposited in NCBI GenBank with accession numbers MT495321–MT495322 and MT495389–MT495406.

Etymology: This species has been named in honour of Lithuanian parasitologist Dr. Liuda Kutkienė who worked in the field of cyst forming coccidia.

Discussion

European populations of common ravens are estimated at 611,000–1,160,000 pairs (BirdLife International 2015); however, investigations into *Sarcocystis* infection in these birds are scarce. *Sarcocystis kutkienae* is the first described species of *Sarcocystis* in the common raven. Thus far, the structure of the sarcocyst wall has been the main morphological criteria for separating *Sarcocystis* species in intermediate hosts (Dubey et al. 2016). Under a light microscope, two main morphological types of sarcocysts detected in the muscles of corvids could be distinguished (Drouin and Mahrt 1979; Munday et al. 1979; Černá 1984; Pak and Eshtokina 1984; Pinayeva et al. 1998; Kutkienė et al. 2009; Prakas et al. 2013). Some sarcocysts detected in different corvid species had a relatively thin (0.3–1.0 μm) and smooth cyst wall, whereas other sarcocysts were characterized by a striated cyst wall, about 1.5–2.5 μm in thickness. Until now, two *Sarcocystis* species were known to employ corvids as intermediate hosts: *S. cornixi* characterised by a striated sarcocyst wall (Kutkienė et al. 2009) and *S. corvusi* with a thin and smooth sarcocyst wall (Prakas et al. 2013). According to morphological characteristics of sarcocysts defined by light microscopy and TEM analysis, *S. kutkienae* was similar to *S. cornixi*. Under a light microscope, sarcocysts of *S. cornixi* from the hooded crow were ribbon-shaped, up to 6-mm long and 300-μm thick, and had a striated sarcocyst wall up to 2.5-μm thick. Under TEM, the sarcocyst wall of *S. cornixi* was measured up to 2.1 μm, had stump-like protrusions differing in size and shape, and had a relatively thick (up to 1.5 μm) ground substance (Kutkienė et al. 2009). Sarcocysts of *S. cornixi* and *S. kutkienae* were of similar size and shape. However, sarcocysts of these two species had some differences in their

ultrastructure. The sarcocyst wall of *S. kutkienae* was slightly thinner (1.5–1.8 μm), was characterised by protrusions of different shapes (conical-like and stump-like protrusions of *S. kutkienae* and *S. cornixi*, respectively), and had a seemingly thinner (0.6–0.9 μm) ground substance layer. Furthermore, the parasitophorous vacuolar membrane of *S. cornixi* sarcocysts had clearly visible electron-dense microprojections, which were not observed in sarcocysts of S. *kutkienae*.

A comparison with other studies revealed an apparently high prevalence of *Sarcocystis* infection in common ravens from Lithuania (18/23, 78.3%). In Canada, the infection prevalence in the magpie (23/38, 60.5%) and Canada jay (4/10, 40%) were reported as relatively high, while a low infection prevalence (5/32, 15.6%) was determined in the American crow (Drouin and Mahrt 1979). Different infection prevalence values were reported in corvids from Lithuania, 35.9% (14/39) in the hooded crow, 25% (2/8) in the jackdaw, and 5% (1/20) in the rook (Kutkienė et al. 2009; Prakas et al. 2013). A relatively low prevalence of *Sarcocystis* spp. infection was detected in the Tasmanian raven (1/18, 5.6%) from Australia (Munday et al. 1979). It is difficult to draw final conclusions about the prevalence of infection in Corvidae birds due to the small number of samples examined. Furthermore, the parasite load observed in this study (median = 44.9 cysts/g of muscle) could be estimated as relatively high.

The observed *Sarcocystis* species from the common raven were genetically characterized at three genetic loci (18S rDNA, 28S rDNA, and ITS1). Based on 18S rDNA and 28S rDNA sequences, *S. kutkienae* was similar to some *Sarcocystis* species employing birds as intermediate hosts. However, using these genetic loci, differences between some *Sarcocystis* species were less than 1%. Many recent investigations showed that ITS1 was the most appropriate genetic region for the discrimination of *Sarcocystis* spp. using birds as intermediate hosts (Olias et al. 2010b; El-Morsey et al. 2015a, b; Prakas et al. 2013, 2014, 2018a, b, 2020). In the present study, the obtained ITS1 sequences of *S. kutkienae* demonstrated more than 10% differences as compared with other *Sarcocystis* spp.

On the basis of 28S rDNA and ITS1, *S. kutkienae* was placed together with eight *Sarcocystis* spp. (*S. calchasi*, *S. columbae*, *S. cornixi*, *S. corvusi*, *S. fulicae*, *S. halieti*, *S. turdusi*, and *S. wobeseri*) that employ birds of the orders Anseriformes, Charadriiformes, Columbiformes, Gruiformes, Passeriformes, Psittaciformes, and Suliformes as intermediate hosts. The northern goshawk (*Accipiter gentilis gentilis*) and/or the Eurasian sparrowhawk (*Accipiter nisus*) were confirmed as the final hosts of *S. calchasi*, *S. columbae*, *S. cornixi*, *S. halieti*, and *S. turdusi* (Olias et al. 2010a, 2011; Mayr et al. 2016). Furthermore, oocysts of *S. halieti* were identified in the intestine of the white-tailed eagle (Gjerde et al. 2018). By contrast, the definitive hosts of *S. corvusi*, *S. fulicae*, and *S. wobeseri* are still unknown. Thus, phylogenetic data suggests that birds of prey are the final hosts of *S. kutkienae*.

Diet analysis and direct behavioural observations showed that eagles (eastern imperial eagle (*Aquila heliaca*), golden eagle (*Aquila chrysaetos*), Steller's sea-eagle (*Haliaeetus pelagicus*), and white-tailed eagle (*Haliaeetus albicilla*)), falcons (gyrfalcon (*Falco rusticolus*)), owls (Eurasian eagle-owl (*Bubo bubo*)), and hawks (the northern goshawk (*Accipiter gentilis*)) are predators of common ravens (Jenkins 1978; Wille and Kampp 1983; Malafosse 1985; Utekhina et al. 2000; Chavko et al. 2007). In Lithuania, among birds of prey, northern goshawks, white-tailed eagles, Eurasian eagle-owls, golden eagles, and gyrfalcons could be predators of the common raven (Winkler et al. 2020). Thus, the said bird species are suspected to be definitive hosts of *S. kutkienae* in Lithuania. Scavenging birds are also likely to be involved in transmitting the examined *Sarcocystis* species.

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Data availability The 18S rDNA, 28S rDNA and ITS1 sequences generated in the present study were submitted to the GenBank database under accession numbers MT495321–MT495322 and MT495389–MT495406.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines were followed for collecting common raven muscle samples.

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