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MORPHOLOGIC AND GENETIC IDENTIFICATION OF *SARCOCYSTIS FULICAE* N. SP. (APICOMPLEXA: SARCOCYSTIDAE) FROM THE EURASIAN COOT (*FULICA ATRA*)

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ABSTRACT: One morphologic type of sarcocyst was found in 26% (7/27) of Eurasian Coots (*Fulica atra*) and was described as *Sarcocystis fulicae* n. sp. using morphologic, 18S ribosomal (r)DNA, 28S rDNA, and internal transcribed spacer 1 (ITS1) analysis. By light microscope, cysts were ribbon-shaped and measured 7.3 mm in length by 116 µm wide. Histologically, the cyst wall reached up to 1.2 µm in thickness and seemed smooth. The detected sarcocysts were packed with relatively small banana-shaped bradyzoites that were 6.7×2.0 µm in size. Ultrastructurally, the cyst wall amounted to 1 µm and had many conical protrusions but seemed almost smooth in some places. The parasitophorous vacuolar membrane appeared undulating, with knob-like blebs and invaginations. The cyst wall belonged to type 1d. Morphologically, cysts of *S. fulicae* differed considerably from cysts of *Sarcocystis atraii* previously described in the same host but were indistinguishable from those of *Sarcocystis corvusi*, *Sarcocystis lari*, and *Sarcocystis wobeseri*, which use birds as intermediate hosts. According to the phylogenetic and ecologic data, birds of prey, mostly the Western Marsh Harrier (*Circus aeruginosus*) and the White-tailed Eagle (*Haliaeetus albicilla*), are presumed to be definitive hosts of *S. fulicae*.

Key words: 18S rDNA, 28S rDNA, Eurasian Coot, ITS1, phylogeny, *Sarcocystis fulicae*, transmission electron microscopy.

INTRODUCTION

Sarcocystis spp. infections are prevalent in many species of birds, mammals, and reptiles. The genus *Sarcocystis* has an obligatory two-host life cycle. Sexual stages (oocysts-sporocysts) develop in the small intestine of the definitive host whereas an asexual development with a formation of sarcocysts occurs in the extraintestinal tissues of the intermediate host. Most species of *Sarcocystis* are host-specific for their intermediate rather than for their definitive hosts (Mehlhorn and Heydorn 1978; Dubey et al. 2016).

At present, about 25 *Sarcocystis* species that form sarcocysts in the muscle tissues of birds belonging to at least 13 orders are known (Dubey et al. 2016). Some avian *Sarcocystis* spp., for instance *Sarcocystis calchasi* and *Sarcocystis falcatula*, are pathogenic (Olias et al. 2014; Konradt et al. 2017). To date, members of the order Gruiformes are known to be intermediate hosts of two valid *Sarcocystis* spp. These two species, *Sarcocystis chloropusae* from the Common Moorhen (*Gallinula chloropus*) and *Sarcocystis atraii* from the Eurasian Coot (*Fulica atra*), have been recently described in Egypt (El-Morsey et al. 2014, 2015a, b).

The Eurasian Coot breeds in Eurasia, Australia, and Africa (del Hoyo et al. 1996). The global population of the species is estimated at about 9 million individuals (Wetlands International 2018). It is a common and abundant species in Lithuania, with an estimated breeding population of 20,000–30,000 pairs (Kurlavičius 2006). Eurasian Coots have a wide range of predators and they are important game birds in many countries (del Hoyo et al. 1996). Therefore, Eurasian Coot infections with various parasites may have an epidemiologic impact. We describe a new *Sarcocystis* species from the Eurasian Coot in Lithuania based on morphologic and DNA investigations.

MATERIALS AND METHODS

Sample collection and morphologic analysis

In the period 2015–17, a total of 27 Eurasian Coots (*Fulica atra*) were legally hunted in central Lithuania, necropsied, and examined for *Sarcocystis* cysts. The bird samples obtained were kept frozen (–20 C) until a morphologic investigation of sarcocysts was carried out.

To detect *Sarcocystis* cysts, samples of the leg muscles of each bird were examined. For this purpose, 28 pieces of muscle (about 1 g) were cut off, stained with 0.2% methylene blue solution, lightened with 1.5% acetic acid solution, pressed into a glass compressor, and studied under a light microscope (LM). To evaluate infection intensity, sarcocysts were counted in about 1 g of stained sections of leg muscles. A morphologic characterization of the cysts was carried out in fresh-squashed preparations after the cysts had been isolated from the muscle fibers with the help of two fine preparation needles. Seven cysts were extracted from the leg muscle tissues from seven individual Eurasian Coots (isolates FaLt2, FaLt6, FaLt8, FaLt10, FaLt11, FaLt12, and FaLt19), preserved in individual microcentrifuge tubes containing 96% ethanol, and kept frozen at –20 C for a molecular investigation.

For transmission electron microscopy (TEM) analysis, a single mature sarcocyst was isolated from the leg muscle of one Eurasian Coot (FaLt19) and fixed in 2% glutaraldehyde fixative, postfixed in 1% osmium tetroxide, dehydrated, and infiltrated in epoxy resin. Ultrathin sections were stained with uranyl acetate/lead citrate solution and examined under the Morgagni 268 TEM (FEI, Hillsboro, Oregon, USA).

Molecular analysis

Genomic DNA was extracted using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and eluted in 60 μL of nuclease-free water. Nearly complete 18S ribosomal (r)DNA was amplified using SarAF/SarBR and SarCF/SarDR primer pairs; partial 28S rDNA was amplified with the help of a KL-P1F/KL-P2R primer pair; and the internal transcribed spacer 1 (ITS1) region was amplified using a P-ITSF/P-ITSR primer pair (Kutkienė et al. 2010). Each PCR was performed in a final 25-μL volume consisting of 0.5 μM of each primer, 12.5 μL DreamTaq PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), 0.04 μg template DNA, and nuclease-free water. All amplification reactions were carried out using the same thermal protocol, starting with the initial hot start at 95 C for 15 min followed by 45 cycles of denaturation at 94 C for 45 s, annealing at 53–58 C depending on the primer pair for 45 s, elongation at 72 C for 90 s, and ending with the final extension at 72 C for 10 min. The amplified products were visualized using 1.7% agarose gel electrophoresis and purified with the help of ExoI and FastAP (Thermo Fisher Scientific Baltics). The PCR products were sequenced directly using the same forward and reverse primers as for the PCR. Sequencing reactions were performed using the Big-Dye® Terminator v3.1 Cycle Sequencing Kit and the 3500 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) according to the manufacturer's recommendations. The resulting sequences were compared with those of various *Sarcocystis* spp. using Nucleotide BLAST program megablast option (National Center for Biotechnology Information [NCBI] 2017). The multiple sequence alignments were obtained using MEGA7 (Kumar et al. 2016). Phylogenetic analysis using Bayesian methods was performed with the help of TOPALi v2.5 software (Milne et al. 2004).

RESULTS

Morphologic characteristics of *S. fulicae*

We found sarcocysts in 26% (7/27) of the Eurasian Coots that we examined. No macroscopic lesions were observed in the organs of the birds infected with *Sarcocystis* cysts. The number of sarcocysts in 1 g of leg muscle varied from 7 to 30 (mean=20; SD±8.4). The cysts that we detected were morphologically similar. They were microscopic, ribbon-shaped, and measured 5,400×70 μm (3,450–7,260×32–116 μm; *n*=19; Fig. 1A). By LM, the cyst wall seemed to be thin (up to 1.2 μm), without visible protrusions (Fig. 1B). Banana-shaped bradyzoites were 6.7×2.0 μm (5.4–8.3×1.4–3.4 μm; *n*=52) in size (Fig. 1C). Ultrastructurally, the cyst wall amounted up to 1 μm in thickness and had many conical protrusions of variable heights (Fig. 1D); however, in some places the cyst wall seemed almost smooth (Fig. 1E). The thickness of the ground substance layer varied between 0.4–0.8 μm. The parasitophorous vacuolar membrane had knob-like blebs with rounded ends and regularly arranged invaginations (Fig. 1F). The cyst wall belonged to type 1d according to the classification of Dubey et al. (2016). The cyst wall type 1 was detected in several *Sarcocystis* spp. that use wild birds as

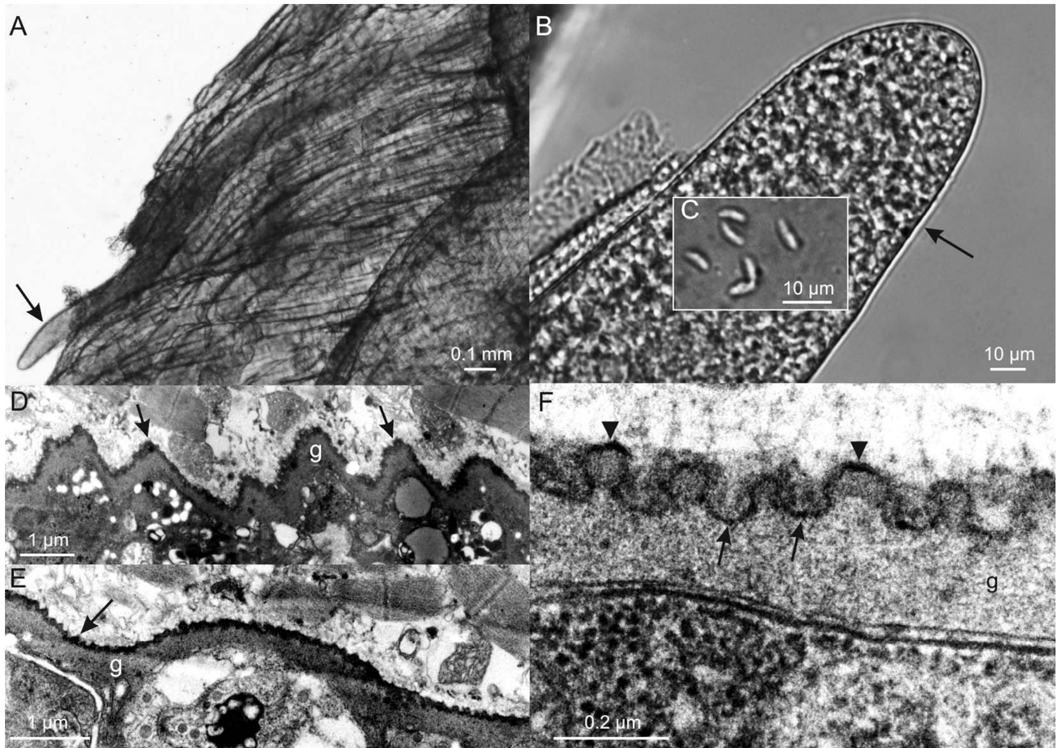

FIGURE 1. Morphology of *Sarcocystis fulicae* n. sp. from muscle tissue of Eurasian Coot (*Fulica atra*). (A–C) Light micrographs. Fresh preparations: (A) Cyst of *S. fulicae* (arrow) in the leg muscles of the Eurasian Coot (*Fulica atra*). (B) Apical portion of sarcocyst showing the thin cyst wall (arrow). (C) Banana-shaped bradyzoites. (D–F) Transmission electron microscopy micrographs: (D) Fragment of cyst wall with conical protrusions of different height (arrows); g=ground substance. (E) Fragment of cyst wall showing the parasitophorous vacuolar membrane with minute undulations (arrows); g=ground substance. (F) High magnification of the cyst wall; the parasitophorous vacuolar membrane has knob-like blebs with rounded ends (arrowheads) and invaginations of the parasitophorous vacuolar membrane (arrows); g=ground substance.

intermediate hosts. Therefore, the morphologic analysis performed was insufficient for the complete identification of the *Sarcocystis* strain under investigation. Based on DNA analysis, the sarcocysts detected from the Eurasian Coot were genetically different at the interspecific level from all *Sarcocystis* species available in NCBI GenBank and were proposed as *Sarcocystis fulicae* n. sp.

Molecular characteristics and phylogenetic placement of *S. fulicae*

Seven *S. fulicae* isolates obtained from seven different Eurasian Coots were identical at the almost-complete 18S rDNA (1,782 base pairs [bp] long), partial 28S rDNA (1,449 bp long), and the complete ITS1 region (850 bp long). Sequences of *S. fulicae* are available in NCBI GenBank under accession numbers MG273671–MG273673. The 18S rDNA was not variable enough to identify *S. fulicae* because 18S rDNA sequences of this species shared >99% similarity with sequences of more than 10 avian *Sarcocystis* spp. Based on the 28S rDNA, *S. fulicae* showed the strongest similarity (99.0%) with *Sarcocystis turdusi* (JF975682) from the Common Blackbird (*Turdus merula*). The ITS1 region was suitable for the discrimination of *S. fulicae* from other *Sarcocystis* spp. that use birds as intermediate hosts. Using this genetic region, *S. fulicae* demonstrated the highest sequence identity values (84.6–84.7%) with *Sarcocystis*

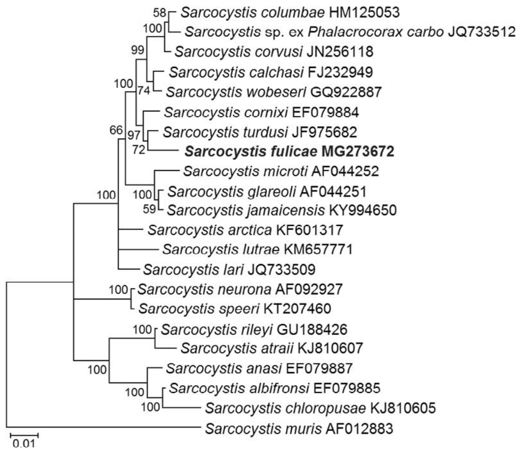

FIGURE 2. The phylogenetic tree of selected *Sarcocystis* species based on 28S rDNA sequences. The tree was constructed using Bayesian methods, scaled according to the branch length, and rooted on *Sarcocystis muris*. The final alignment contained 22 taxa and 1,425 aligned nucleotide positions. The GTR+G evolutionary model was set for the phylogenetic analysis. The figures next to the branches show the posterior probability support values. The GenBank accession numbers of sequences are given behind the *Sarcocystis* species name.

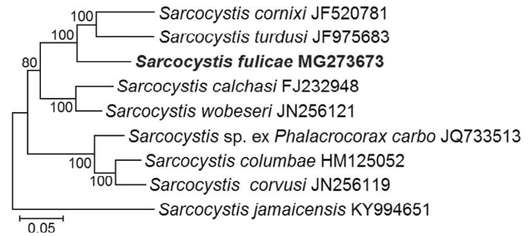

FIGURE 3. The phylogenetic tree of selected *Sarcocystis* species based on ITS1 sequences. The tree was constructed using Bayesian methods, scaled according to the branch length, and rooted on *Sarcocystis jamaicensis*. The final alignment contained nine taxa and 970 aligned nucleotide positions. The HKY+G evolutionary model was set for the phylogenetic analysis. The figures next to the branches show the posterior probability support values. The GenBank accession numbers of sequences are given behind the *Sarcocystis* species name.

cornixi (JF520781, JN256120) from the Hooded Crow (*Corvus cornix*).

The phylogenetic analysis showed a close relationship of *S. fulicae* with *Sarcocystis* spp. that use birds as intermediate hosts, predatory mammals as intermediate hosts, and use rodents as intermediate and predatory birds as definitive hosts. Based on 28S rDNA sequences, *S. fulicae* was placed together with seven *Sarcocystis* spp. (*Sarcocystis columbae*, *Sarcocystis halieti*, *Sarcocystis corvusi*, *Sarcocystis calchasi*, *Sarcocystis wobeseri*, *Sarcocystis cornixi*, and *Sarcocystis turdusi*) using birds of the orders Anseriformes, Columbiformes, Passeriformes, Psittaciformes, and Suliformes as intermediate hosts (Fig. 2). Two other *Sarcocystis* spp. (*S. atraii* and *S. chloropusae*) from birds of the order Gruiformes, together with three species from the order Anseriformes (*Sarcocystis rileyi*, *Sarcocystis anasi*, and *Sarcocystis albifronsi*), formed another cluster in the 28S rDNA phylogenetic tree (Fig. 2). Based on the sequences of ITS1, the most variable locus among the analyzed loci, *S. fulicae* was a sister species to *S. cornixi* and *S. turdusi* (Fig. 3).

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

Type intermediate host: The Eurasian Coot (*Fulica atra*).

Definitive host: Unknown.

Locality: Central Lithuania.

Specimens deposited: The TEM material was deposited at the National Centre of Pathology, Vilnius, Lithuania. Sequences deposited in NCBI GenBank with accession numbers MG273671–MG273673.

Etymology: The Latin name of genus *Fulica* is used for the species name.

DISCUSSION

Some research on *Sarcocystis* species diversity in the Eurasian Coot has been carried out before. In 2015, microcysts found in muscles of 5% (2/43) Eurasian Coots examined in Egypt were described as *S. atraii* on the basis of LM, TEM, and DNA investigations (El-Morsey et al. 2015a). In histologic sections, cysts were 165–850×50–85 µm in size; the cyst wall was wavy and had minute undulations. The elongated banana-shaped bradyzoites measured 7.5–14.0×1.5–2.5 µm. Under a TEM, the cyst wall amounted to 1–3 µm in thickness and had many mushroom-like

protrusions; type 24. Thus, two *Sarcocystis* species from the Eurasian Coot, *S. fulicae* described in the present study (Fig. 1) and *S. atraii*, differ in the sarcocyst wall structure as well as in the size of cysts and bradyzoites. In contrast, the cysts of *S. fulicae* were similar to those found in the muscles of the Eurasian Coot from Sorbulak Lake in Kazakhstan (Pak and Eshtokina 1984). A *Sarcocystis* species was found in 16% (8/51) of Eurasian Coots hunted in Kazakhstan. The sarcocysts were up to 100×1,190 µm, had a thin (0.5–0.7 µm) and smooth cyst wall, and banana-shaped bradyzoites and amounted to 2.7–5.4×1.0–1.4 µm. *Sarcocystis* species were also detected in the Eurasian Coot in Germany (Wenzel et al. 1982) and repeatedly in Kazakhstan (Pinayeva et al. 1998), but a detailed morphologic examination of sarcocysts found in these studies was not performed.

Two species identified in the Eurasian Coot, *S. atraii* and *S. fulicae*, were placed in separate clusters in the 28S rDNA phylogenetic tree (Fig. 2). Based on ITS1 sequences, *S. fulicae* characterized in the present study was most-closely related to *S. cornixi* and *S. turdusi* (Fig. 3). Morphologically, *S. fulicae* differed markedly from the latter species. Under TEM, the cyst wall of *S. fulicae* was up to 1 µm thick, had conical protrusions of different heights, and the parasitophorous vacuolar membrane had knob-like blebs and represented type 1d (Dubey et al. 2016; Fig. 1). Ultrastructurally, the cyst wall of *S. cornixi* measured 2.1 µm thick and had stump-like protrusions located at irregular distances and differed greatly in size and shape (Kutkienė et al. 2009), representing type 1g (Dubey et al. 2016). By TEM, the cyst wall of *S. turdusi* was thick (2.5–4.4 µm), had club- or irregularly-shaped and sometimes branched protrusions that differed in size and were situated at different distances from one another (Kutkienė et al. 2012), similar to type 18b (Dubey et al. 2016). The cyst wall type 1d established for *S. fulicae* was also defined for the *S. corvusi*, *S. lari*, and *S. wobeseri* that used birds as intermediate hosts (Kutkienė et al. 2010; Prakas et al. 2013, 2014). Ultrastructurally, the cyst wall of *S. fulicae*, *S. lari*, and *S. wobeseri* in some places appeared almost smooth (Kutkienė et al. 2010; Prakas et al. 2014). *Sarcocystis fulicae* was also similar to other avian *Sarcocystis* spp: *S. calchasi*, *S. columbae*, a *Sarcocystis* sp. from the Black-crowned Night-heron (*Nycticorax nycticorax*), a *Sarcocystis* sp. from the Common Goldeneye (*Bucephala clangula*), a *Sarcocystis* sp. from the Snow Goose (*Anser caerulescens caerulescens*), and a *Sarcocystis* sp. from the White-rumped Swift (*Apus caffer*) according to the morphologic characteristics of sarcocysts (Wobeser et al. 1981; Kaiser and Markus 1983; Kutkienė et al. 2008; Olias et al. 2010a, c; Prakas et al. 2011). Thus, *S. fulicae* could not be reliably separated on the basis of morphologic investigations from several *Sarcocystis* spp. using birds as intermediate hosts.

Based on the phylogenetic analysis, *S. fulicae* was placed in one cluster together with *S. calchasi*, *S. columbae*, *S. cornixi*, *S. corvusi*, *S. turdusi*, *S. wobeseri*, and *S. halieti* (Figs. 2, 3). Experimental studies indicated the Northern Goshawk (*Accipiter gentilis*) as a final host of *S. calchasi* (Olias et al. 2010b). Later, using species-specific PCR, German Northern Goshawk and Eurasian Sparrowhawk (*Accipiter nisus*) were positive for oocysts-sporocysts of *S. calchasi* and *S. columbae* (Olias et al. 2011). Based on sequencing of the ITS1 region or species-specific PCR, infection with *S. calchasi*, *S. columbae*, *S. cornixi*, *S. turdusi*, *S. cornixi*, and *S. halieti* in the intestinal tract or feces of Northern Goshawks and European Sparrowhawks from Germany has been confirmed (Mayr et al. 2016). Recently, *S. halieti* was molecularly identified in the intestine of the White-tailed Eagle (*H. albicilla*) in Norway (Gjerde et al. 2017). Meanwhile, the final hosts of *S. corvusi* and *S. wobeseri* are still unknown.

Hence, phylogenetic results suggest birds of prey to be definitive hosts of *S. fulicae*. Based on ecologic data, *S. fulicae* is most likely to be transmitted by the Western Marsh Harrier (*Circus aeruginosus*) and the White-tailed Eagle. In the Baltic States, the Western Marsh Harrier predates on adult coots, mostly on incubating individuals (Blūms 1973; Log-

minas 1990). In Latvia, the Eurasian Coot in some sites accounts for up to 40% of all food items of the Western Marsh Harrier diet (Blūms 1973). The Western Marsh Harrier is widespread in Lithuania and its breeding population is estimated at about 3,000 pairs (Kurlavičius 2006). In many regions of Europe (including Lithuania), Eurasian Coots dominate also in the diet of the White-tailed Eagle (Sandor et al. 2015). The growing breeding population of the White-tailed Eagle in Lithuania is estimated at more than 120 pairs (Treinys et al. 2016). Certain other avian predators catch coots only accidentally or locally (Blūms 1973; Kosheliov 1984). Among mammals, only the American mink (*Neovison vison*) is known to be an important predator of the Eurasian Coot in certain regions of Europe, particularly in the sites where these birds breed almost colonially (Ferreras and Macdonald 1999; Bartoszewicz and Zalewski 2003).

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