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The Richness of *Sarcocystis* Species in the Common Gull (*Larus canus*) and Black-Headed Gull (*Larus ridibundus*) from Lithuania

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Abstract: The common gull (*Larus canus*) and the black-headed gull (*Larus ridibundus*) are common waterbird species in Lithuania. Until now, the composition of *Sarcocystis* species in these birds was unknown. The current study aimed to identify *Sarcocystis* spp. by the morphological examination of sarcocysts found in the muscle tissues and by DNA sequence analysis. Between 2011 and 2019, the leg muscles of 42 common gulls and 63 black-headed gulls were tested for *Sarcocystis* spp. Based on the methylene blue staining of squashed muscle samples, sarcocysts were detected in six common gulls (14.3%) and in six black-headed gulls (9.5%). Under a light microscope, one type of microcyst was observed. Sarcocysts were thread-like (2860–8250 × 40–180 µm) and had a smooth and thin (0.8–1.4 µm) cyst wall, while bradyzoites were banana-shaped and 5.0–9.2 × 1.3–2.4 µm in size. The sequencing of complete *ITS1* showed the presence of *S. columbae*, *S. halieti* and *S. wobeseri* in the common gull and *S. columbae* and *S. halieti* in the black-headed gull. The highest intraspecific genetic variability was established for *S. halieti*, which is characterized by a wide host range. This species is considered to be pathogenic, therefore further histopathological examination of the various organs of gulls is needed.

Keywords: *Sarcocystis*; gulls; infection rates; *ITS1*; genetic variation

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1. Introduction

Protozoans belonging to the genus *Sarcocystis* are intracellular parasites infecting mammals, birds and reptiles [1]. These apicomplexan parasites have an obligatory two-host prey–predator life cycle. Sarcocysts are mainly formed in the muscles or CNS of an intermediate host, while oocysts/sporocysts develop in the intestinal mucosa of a definitive host. Intermediate hosts usually acquire a *Sarcocystis* infection through the accidental ingestion of sporocysts in contaminated feed or water, whereas definitive hosts become infected through the ingestion of sarcocysts via predation or scavenging [1,2]. Birds serve as intermediate or definitive hosts for many *Sarcocystis* species [1,3–5]. Some of them are pathogenic for their intermediate host [1,6]. *Sarcocystis falcatula* and *S. calchasi*, each with distinct morphologies and biology, are two well-investigated pathogenic *Sarcocystis* spp. that are hazardous for birds [1,7–9].

The members of the family Laridae are abundant and adaptable birds are distributed worldwide. This family consist of around 100 species arranged into 22 genera [10,11]. Gulls are found mainly on the coast in summer, but some species also nest inland, especially in winter [10–13]. Gulls are an example of opportunistic birds that have successfully adapted to urban environments, and some species have become more common in urban areas and have even established breeding populations in cities around the world [14,15]. Common gulls (*Larus canus*) and black-headed gulls (*Larus ridibundus*) are opportunistic predators of marine invertebrates, fishes, insects and birds, as well as opportunistic scavengers of dead animals, who are capable of roosting in urban landfills and other waste sites [12,13,16,17].

The common gull and black-headed gull are common bird species breeding in various bodies of water and marshes in Lithuania [12,13]. Common gulls are more prevalent near water bodies, while black-headed gulls are also found near human settlements [18]. To date, four *Sarcocystis* species, *S. columbae*, *S. halieti*, *S. lari* and *S. wobeseri*, have been identified in the muscles of larids. All four of these species were confirmed in the herring gull (*Larus argentatus*), while *S. lari* was described in the great black-backed gull (*Larus marinus*) [18–20]. The richness of *Sarcocystis* species in common gulls and black-headed gulls is unknown. Therefore, the aim of the present study was to identify *Sarcocystis* spp. from the leg muscles of common gulls and black-headed gulls by the morphological examination of sarcocysts and the *ITS1* (internal transcribed spacer 1) region sequence analysis of isolated parasites.

2. Results

2.1. Morphological Examination of Sarcocystis spp. Detected in Common Gull and Black-Headed Gull

Based on methylene blue staining, sarcocysts were found in 12 of the 105 (11.4%) birds (6/42, 14.3% common gulls and 6/63, 9.5% black-headed gulls) examined in Lithuania. The parasite load ranged from 1 to 107 cysts/g of the muscle samples. In most muscle samples the parasite load was low, since the median parasite load was 2.5 cysts/g of muscle.

In fresh muscle samples, sarcocysts were detected in nine birds (five common gulls and four black-headed gulls). Due to low parasite load sarcocysts were not detected in fresh muscle samples of one common gull (specimen LcLt31) and two black-headed gulls (LrLt24, LrLt35).

An examination of 36 sarcocysts under LM revealed that they were quite similar by light microscope (LM). Sarcocysts were microscopic, thread-like, 2860–8250 × 40–180 µm (5992 ± 1832 × 99 ± 30 µm; n = 36) in size (Figure 1a), with a thin (0.8–1.4 µm; n = 36) and apparently smooth cyst wall (Figure 1b). Septa divided sarcocysts into compartments filled with banana-shaped bradyzoites, which were 5.0–9.2 × 1.3–2.4 µm (7.2 ± 0.9 × 2.0 ± 0.4 µm; n = 150) in size (Figure 1c).

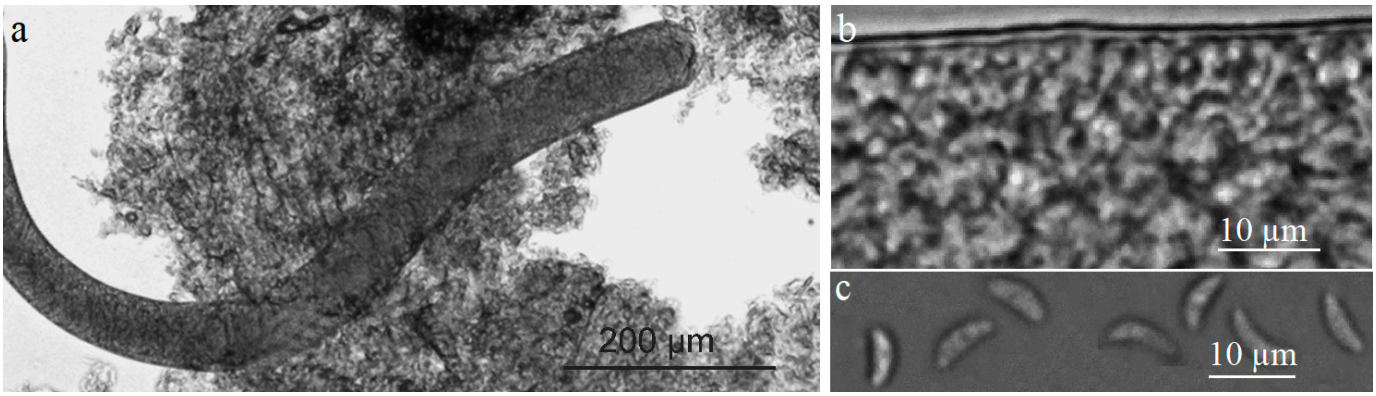

Figure 1. Morphology of sarcocyst and bradyzoites isolated from muscle tissue of common gull (*Larus canus*) in fresh preparations. (**a**) Fragment of the ribbon-shaped sarcocyst. (**b**) A portion of sarcocyst having a thin and apparently smooth cyst wall. (**c**) Banana-shaped bradyzoites.

2.2. Genetic Identification and Intraspecific Variation of Sarcocystis spp.

Based on the comparison of complete *ITS1* sequences obtained in this study, *S. columbae*, *S. halieti* and *S. wobeseri* were identified in the leg muscles of two of the examined larid species. All these *Sarcocystis* species were confirmed in the common gull, while two of them (*S. halieti* and *S. columbae*) were detected in the black-headed gull (Table 1). Furthermore, of the 16 sarcocysts isolates excreted from the black-headed gulls, 14 were attributed to *S. halieti* and only 2 isolates were identified as *S. columbae*. Whereas the distribution of the identified *Sarcocystis* species in common gulls was more uniform. In general, four, seven and nine sequences of *S. columbae*, *S. wobeseri* and *S. halieti* were obtained, respectively.

Co-infections with two *Sarcocystis* species were detected in a single common gull (isolate LcLt34) and in a single black-headed gull (isolate LrLt53).

Table 1. The genetic identification of *Sarcocystis* spp. from common gulls and black-headed gulls using the sequence comparison of complete *ITS1* region.

Bird Species	Isolate Numbers	*Sarcocystis* Species and GenBank Accession Numbers		
		S. columbae	***S. halieti***	***S. wobeseri***
Common gull	LcLt17.1–17.4		OP419605–OP419608	
Common gull	LcLt20.1–20.4	OP419609–OP419612		
Common gull	LcLt34.1–34.4		OP419613	OP419614–OP419616
Common gull	LcLt38.1–38.4			OP419617–OP419620
Common gull	LcLt41.1–41.4		OP419621–OP419624	
Black-headed gull	LrLt7.1–7.4		OP419625–OP419629	
Black-headed gull	LrLt13.1–13.4		OP419630–OP419633	
Black-headed gull	LrLt49.1–49.4		OP419634–OP419637	
Black-headed gull	LrLt53.1–53.4	OP419640–OP419641	OP419638–OP419639	

A total of 23 sequences of *S. halieti* comprised 3 haplotypes sharing 97.59–99.88% identity between each other, and 97.11–100% identity compared with other *S. halieti* sequences available in GenBank. *ITS1* sequences of *S. halieti* from two gull hosts showed great similarity to three unnamed *Sarcocystis* spp., *Sarcocystis* sp. isolate Skua-2016-CH (95.90–96.14%), *Sarcocystis* sp. ex *Corvus corax* (94.59–94.77%) and *Sarcocystis* sp. ex *Accipiter cooperii* (93.16–93.40%), and less than 93% similarity compared to other named *Sarcocystis* spp. In total, 6 sequences of the *S. columbae* determined in the present study were 100% identical, demonstrating 99.40–100% identity to *S. columbae* isolated from other hosts, and displaying the greatest similarity to *Sarcocystis* sp. ex *Accipiter cooperii* (96.04%) and *S. columbae* (93.80%). Finally, 4 haplotypes differing up to 0.47% were defined by 7 *ITS1* sequences of *S. wobeseri*. These 4 haplotypes shared 99.41–100% identity with those of *S. wobeseri* deposited in GenBank and showed the greatest similarity (92.54–93.30%) to *S. calchasi*.

The comparison of *ITS1* sequences of *Sarcocystis* spp. employing birds as intermediate hosts showed that *S. halieti*, which was also determined in the current study, was distinguished by the highest intraspecific variation reaching almost 3% (Figure 2). The intraspecific genetic differences of other avian *Sarcocystis* spp. did not exceed 1.2%. The lowest interspecific differences slightly less than 4% were determined comparing *S. columbae*, *S. halieti*, *Sarcocystis* sp. ex *Accipiter cooperii* and *Sarcocystis* sp. isolate Skua-2016-CH with other *Sarcocystis* spp. However, for all examined *Sarcocystis* spp., interspecific and intraspecific genetic differences did not overlap, showing that *ITS1* is a useful genetic marker for the discrimination of avian *Sarcocystis* spp.

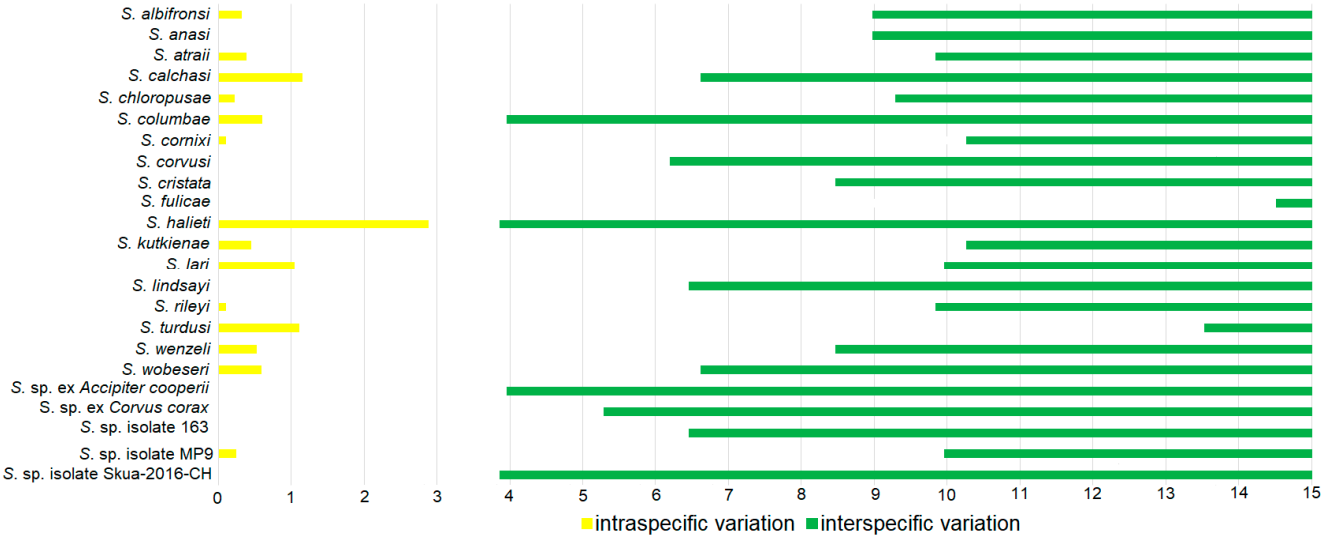

Figure 2. The intraspecific and interspecific genetic variation of *Sarcocystis* species using birds as intermediate hosts within *ITS1* region. The X axis shows genetic differences expressed in percentages, which was calculated 100% minus genetic similarity values obtained by BLAST analysis. Notably, *S. falcatula* was not included into the analysis, since it is assumed that this parasite is species-complex [21,22]. Based on sequence comparison, *Sarcocystis* sp. isolate MP9 and *Sarcocystis* sp. isolate 471 should belong to the same species.

3. Discussion

3.1. Similar Morphology of Saroccystis spp. in Gulls

Sarcocystis species are described in intermediate hosts on the basis of morphological examination, life cycle studies and/or DNA analysis. The morphology of the sarcocyst wall is the main diagnostic feature for morphological characterisation and distinguishment of *Sarcocystis* species in intermediate hosts. In the latest review of *Sarcocystis* species, 82 different types or subtypes of the sarcocyst wall were distinguished under transmission electron microscopy (TEM) [1]. Since studies on the ultrastructure of sarcocyst walls are expensive and time consuming, usually the morphology of the cyst wall is evaluated in fresh preparations or histological sections under a LM. The morphology of the sarcocyst wall becomes of little significance if morphologically indistinguishable sarcocysts occur in more than one taxonomically closely related intermediate hosts. Furthermore, the structure of the sarcocyst wall is a non-informative feature in case more than one *Sarcocystis* species having the same type of sarcocysts wall is detected in a single animal [18,23–26].

In the present study, three *Sarcocystis* species, *S. columbae*, *S. halieti* and *S. wobeseri*, were identified in common gulls and black-headed gulls from Lithuania. The abovementioned *Sarcocystis* species detected had thread-like sarcocysts with smooth cyst walls, banana-shaped bradyzoites and were indistinguishable from one another under LM (Figure 1). Furthermore, by LM, two inseparable *Sarcocystis* species were found in the two gulls examined (Table 1). Sarcocysts of *Sarcocystis* spp. detected in the common gull and in the black-headed gull in the present study were morphologically indistinguishable from those of parasite species identified in the herring gull from Lithuania [18]. Furthermore, sarcocysts observed in gulls from Lithuania were morphologically quite similar to those recorded in Canada in the muscle of the California gull (*Larus californicus*). Sarcocysts isolated from the California gull were characterised by a thin (0.8 µm) and smooth cyst wall [27]. Sarcocysts described as having smooth and thin cyst walls were also found in Kazakhstan in the muscles of the black-headed gull and the common gull [28]. Thus, it is likely that the *Sarcocystis* spp. identified in gulls from Lithuania may be present in Asia and America; however, molecular examinations are needed to confirm such an assumption. In conclusion, LM analysis of sarcocysts isolated from gulls has little diagnostic value.

On the contrary, four *Sarcocystis* species forming microcysts in the muscles of birds of the order Anseriformes (*S. anasi*, *S. albifronsi*, *S. platyrhynchosi* and *S. wobeseri*) can be discerned on the basis of the sarcocyst wall appearance and morphometric parameters of bradyzoites [29–31].

Under TEM, the sarcocyst walls of the three *Sarcocystis* identified in this study are attributed to the same type, but different subtypes (1a *S. columbae*) [32,33], (1d *S. wobeseri*) [29] and (1e *S. halieti*) [34]. However, morphological differences between these three species are minor, somewhat ambiguous and require extensive scientific research. Further studies on the morphological differences in the apical complex or other structures of bradyzoites could help to distinguish *Sarcocystis* species, which are very similar by LM.

3.2. The Richness and Possible Pathogenicity of Sarcocystis Species in Gulls

In the current work, *S. halieti* was the most commonly identified species in the leg muscles of common gulls and black-headed gulls (Table 1). This species was also distinguished by the highest intraspecific variation among *Sarcocystis* spp. employing birds as intermediate hosts (Figure 2). The high intraspecific variability of *S. halieti* could be explained by a wide range of intermediate hosts [18,22,25,34–40] and the broad geographical distribution of this *Sarcocystis* species [22]. As reported by Maier-Sam et al. [36], *S. halieti* might be pathogenic for birds, as it has been associated with encephalitis in a small owl (*Athene noctua*).

There is a gap in the knowledge regarding the pathogenicity of *Sarcocystis* spp. in birds in Europe. Previously, *Sarcocystis* species detected in the muscles of birds in Europe were thought to be non-pathogenic. The first pathogenic *Sarcocystis* species, *S. calchasi*, was described in Germany in 2010 [8]. Currently, it is known that *S. calchasi* may parasitise birds belonging to at least five different orders, Columbiformes, Galliformes, Piciformes, Psittaciformes and Suliformes [1,8,9,38,41–47]. Meanwhile, the pathogenicity of *S. halieti* in birds was only revealed in 2021 with the detection of granulomatous cerebral encephalitis [36]. Notably, mainly the leg muscles of birds were examined in the studies during which *S. halieti* have been identified [18,25,34,37]. Extending research on the pathogenicity of *Sarcocystis* spp. requires the analysis of internal organs such as the brain, liver, lungs and spleen.

Presently, 28 *Sarcocystis* spp. have been identified using birds as intermediate hosts [5,31,48]. Gulls (Charadriiformes: Laridae) and corvids (Passeriformes: Corvidae) serve as intermediate hosts for four *Sarcocystis* spp. [18,25]. The great *Sarcocystis* spp. diversity determined in gulls can be related to the different habitats and ecosystems of the common gull and black-headed gull [11–17]. The ability of gulls to adapt to the human impacts associated with cities leads to the establishment and growth of urban bird populations worldwide. Urban ecosystems can provide abundant food resources for gull species, as well as a stable urban microclimate and fewer natural predators than natural environments [14,15,49]. Naturally, both the common gull and black-headed gull breed colonially near water or in marshes, or on islands in lakes, nesting on the ground [12,13]. Conjointly, gulls are omnivorous scavengers of small prey and they are capable of roosting in urban landfills and other waste sites [12,16,17].

4. Materials and Methods

4.1. Collection of Samples

A total of 105 birds (42 common gulls and 63 black-headed gulls) from Lithuania (54–55° N, 21–24° W) were sampled in the period of 2011–2019. All birds were found dead (killed by motor vehicles on highways, as a result of collisions with power lines, etc.) and received from Kaunas Tadas Ivanauskas Zoology Museum (the national authority responsible for the monitoring of wild birds found dead). The leg muscle samples were kept frozen (−20 °C) until a microscopical examination was conducted.

4.2. Morphological Examination of Sarcocysts

The leg muscles of birds were examined for the presence of *Sarcocystis* spp. sarcocysts. The prevalence and intensity of *Sarcocystis* infections were evaluated in methylene-blue-stained preparations. For this purpose, 1 g (±0.1) of the leg muscle was cut into 28 oat-sized fragments, stained with 0.2% methylene blue solution, clarified with 1.5% acetic acid solution, pressed into a glass compressor and examined under a light microscope (LM). Eventually, sarcocysts were morphologically characterised in squashed preparations after the cysts had been isolated from the muscle fibres with the help of preparation needles. Overall, 36 sarcocysts were excreted from the leg muscle tissues from 4 individual common gulls and 5 black-headed gulls (isolates LcLt7.1–LcLt7.4; LcLt28.1–LcLt28.4; LcLt34.1–LcLt34.4; LcLt38.1–LcLt38.4; LcLt41.1–LcLt41.4; LrLt7.1–LrLt7.4; LrLt13.1–LrLt13.4; LrLt49.1–LrLt49.4; LrLt53.1–LrLt53.4) (Table 1). The isolated sarcocysts were stored in individual 1.5 mL microcentrifuge tubes containing 96% ethanol until DNA extraction.

4.3. Molecular Analysis of Sarcocystis spp.

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 instructions. The complete *ITS1* region was amplified using the SU1F/5.8SR2 primer pair [50]. 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. The cycling conditions began with one cycle at 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 60 °C for 60 s and 72 °C for 80 s, and a final extension step at 72 °C for 7 min. The visualisation, purification, and sequencing of PCR products were carried out using the previously described protocol [51]. In order to detect essentially similar DNA sequences and evaluate the intraspecific and interspecific genetic variability of avian *Sarcocystis* spp., the *ITS1* sequences generated in this study were compared with those of various *Sarcocystis* spp. using the nucleotide BLAST program with the megablast option (http://blast.ncbi.nlm.nih.gov/, accessed on 10 March 2023). The 830-844 bp-long *ITS1* sequences for *Sarcocystis* spp. from gulls generated in the present study are deposited in the GenBank database under the following accession numbers OP419605–OP419641.

5. Conclusions

Based on PCR targeting *ITS1* and subsequent sequencing, for the first time, three *Sarcocystis* spp., *S. columbae*, *S. halieti* and *S. wobeseri* were identified in the leg muscles of two larid species, the common gull and the black-headed gull. The relatively high richness of *Sarcocystis* species in gulls is related to the abundance of habitats and ecosystems occupied by these birds. Notably, the *Sarcocystis* species detected in the current work were indistinguishable under LM. Therefore, for the morphological discrimination of *Sarcocystis* spp. found in the muscles of the bird family Laridae, detailed TEM analysis, including examination of the sarcocyst wall, bradyzoites and apical complex, are required. Furthermore, for a comprehensive genetic characterisation of the identified *Sarcocystis* species in gulls, more genetic markers should be developed.

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Data Availability Statement: The *ITS1* sequences of the three *Sarcocystis* species (*S. columbae*, *S. halieti* and *S. wobeseri*) generated in the current work are available in the GenBank database under accession numbers OP419605–OP419641.

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Conflicts of Interest: The authors declare no conflict of interest.

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