The Role of Birds of the Family Corvidae in Transmitting Sarcocystis Protozoan Parasites

Evelina Juozaitiê-Ngugu, Saulius Švažas, Donatas Šneideris, Eglè Rudaiytêt-Lukošienêt, Dalius Butkauskas and Petras Prakas *

Nature Research Centre, Akademijos Str. 2, LT-08412 Vilnius, Lithuania; evelina.ngugu@gamtc.lt (E.J.-N.); saulius.svazas@gamtc.lt (S.Š.); donatas.sneideris@gamtc.lt (D.Š.); egle.rudaiyte@gamtc.lt (E.R.-L.); dalius.butkauskas@gamtc.lt (D.B.)
* Correspondence: petras.prakas@gamtc.lt

Simple Summary: Members of the genus Sarcocystis are protozoan parasites that infect mammals, birds, and reptiles. Sarcocystis spp. have an obligatory two-host prey-predator life cycle. Sarcocysts form in the muscles and central nervous system of the intermediate host, while oocysts and sporocysts develop in the small intestine of the definitive host. There is a lack of studies on omnivorous birds of family Corvidae as potential definitive hosts of Sarcocystis spp. Until now, only S. ovalis has been confirmed to be transmitted via corvids. In the current study, 91 small intestine samples from six corvid species from Lithuania were examined for the presence of Sarcocystis spp. that use birds, carnivorous mammals, and cervids as intermediate hosts. Oocysts of Sarcocystis spp. were observed in 43 samples (47.3%) using a light microscope. Based on molecular methods, 11 Sarcocystis spp., (S. columbae, S. cornixi, S. halieti, S. kutkienae, S. lari, S. turdusi, S. wobeseri, S. arctica, S. lutrae, S. ovalis, and S. oviformis) were identified. These results indicate that corvids may transmit some species of Sarcocystis that use birds and mammals as intermediate hosts.

Abstract: Members of the family Corvidae are ecologically flexible omnivorous birds, particularly adaptive to urban habitats, and living in proximity to humans; these birds may serve as definitive hosts (DH) for Sarcocystis spp., but research about this is lacking. In the present study, intestinal samples from 91 corvids collected in Lithuania were molecularly tested by species-specific PCR targeting the ITS1 and cox1 genes and subsequently sequenced for the presence of Sarcocystis spp. Under a light microscope, oocysts of Sarcocystis spp. were observed in 43 samples (47.3%), while molecular methods, detected Sarcocystis spp. in 77 birds (84.6%). Eleven Sarcocystis spp. (S. columbae, S. cornixi, potentially pathogenic S. halieti, S. kutkienae, S. lari, S. turdusi, S. wobeseri, S. arctica, S. lutrae, S. ovalis, and S. oviformis) were identified in the intestinal samples from six corvid species from Lithuania. Infections with multiple Sarcocystis spp. were detected in 79.2% of the infected corvid birds. Three of the identified Sarcocystis spp. use corvids as intermediate hosts (IH); therefore, corvids may serve as IH and DH of the same Sarcocystis species. Based on molecular results and on corvid diet, omnivorous corvids may play an important role in transmitting Sarcocystis spp.

Keywords: Sarcocystis; corvids; definitive host; intermediate host; ITS1; cox1; molecular identification

1. Introduction

Representatives of the genus Sarcocystis (Apicomplexa: Sarcocystidae) are parasitic protozoa widespread in reptiles, birds, and mammals. They are characterised by an obligatory prey-predator two-host life cycle [1]. Asexual multiplication with formation of sarcocysts occurs in extra-intestinal tissues, mainly the muscles, of the intermediate host (IH), while sexual stages of the parasite’s life cycle (oocysts and sporocysts) develop in the small intestine of the definitive host (DH) [2]. The sarcocyst structure is one of the most important criteria for describing Sarcocystis spp., and species cannot be distinguished according to the morphology of parasite sexual stages observed in the DH [1].
Birds have been shown to be DH of at least 17 Sarcocystis spp. worldwide [1–8]. Birds of prey have been reported as DH of Sarcocystis spp. that mainly use birds and small mammals (rodents, lagomorphs, etc.) as IH [1,8–10]. The role of omnivorous birds in transmitting Sarcocystis spp. is unclear; however, based on phylogenetic studies, omnivorous birds are likely to be involved in transmitting several Sarcocystis spp. that use carnivores and ungulates as IH [11–13].

Corvidae is a family of ecologically flexible omnivorous birds, particularly adaptive to urban habitats close to humans [14–17]. Carrion of mammals and birds constitutes a significant part of the corvid diet, particularly of common ravens (Corvus corax) and hooded crows (C. cornix) [14,18]. Based on their diet, corvids may act as DH of Sarcocystis spp. [19], but they have received little attention [4,20]. Currently, only one Sarcocystis species, S. ovalis, is confirmed to be transmitted by corvids [20,21]. This species employs cervids such as moose (Alces alces), red deer (Cervus elaphus) and sika deer (C. nippon) as IH [22–24] and common magpie (Pica pica) [21] and Japanese jungle crow (C. macrorhynchos) as DH [20].

The aim of the current study was to molecularly identify Sarcocystis spp. in intestinal samples from six corvid species from Lithuania.

2. Materials and Methods

2.1. Animal Collection and Oocysts/Sporocysts Isolation

A total of 91 birds from the Corvidae family were collected between 2015 and 2021. All birds were found dead (as a result of collisions with motor vehicles, power lines, buildings, etc.) and obtained from the Kaunas T. Ivanauskas Zoology Museum, the Lithuanian national authority responsible for monitoring dead or wounded wild birds. Bird samples were kept frozen at −20 °C until a microscopic examination had been conducted. Intestinal samples of 33 hooded crows, 25 common ravens, 21 western jackdaws (Coloeus monedula), 5 rooks (Corvus frugilegus), 4 common magpies and 3 Eurasian jays (Garrulus glandarius) were examined for Sarcocystis spp. Oocysts/sporocysts of Sarcocystis spp. were isolated from the intestinal mucosa of each bird using previously described methodology [25]. All samples underwent further molecular analysis, regardless of whether oocysts/sporocysts were visible under a light microscope.

2.2. Molecular Analysis

Sixteen Sarcocystis spp. with birds as confirmed or presumed DH were tested for whether they could be found in the intestinal samples of corvids from Lithuania (Table 1). Ten of these species, (S. calchasi, S. columbae, S. cornixi, S. corvusi, S. fulicae, S. halieti, S. lari, S. turdusi, S. wobeseri, and S. kutkienae) are found in the muscles of birds as IH; two species, (S. arctica, and S. lutrae) use carnivorous mammals as IH; and four remaining species (S. frondea, S. hardangeri, S. ovalis and S. oviformis) employ cervids as IH [1,11,12,26,27].

Approximately 200 µL of intestinal sediment was taken from each sample and prepared for DNA extraction using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). Nested PCR (nPCR) was used to amplify DNA fragments of all parasite species in the study. The external primers SU1F/5.8SR2 amplified the internal transcribed spacer 1 (ITS1) region in Sarcocystis spp. that use birds and carnivores as IH [28]. The SF1/SR5 primer pair was applied for the amplification of partial cytochrome c oxidase subunit I (cox1) gene of Sarcocystis spp. that use cervids as IH [29]. Internal nPCR primers, which were developed and used in this study, are listed in Table 1. PCR reactions were conducted using DreamTag PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer’s protocol. The PCR cycling conditions were as previously described [25], with modified annealing temperatures (57–65 °C) depending on the primer pairs used. PCR products were visualised, purified, and directly sequenced as previously described [30]. Nucleotide BLAST was used to compare the obtained sequences [31]. The sequences generated in the present study are available in GenBank with Acc. No. OK481182–OK481382.
Table 1. The internal primers used for the nPCR of selected *Sarcocystis* spp.

| Species            | Internal Primers | Product Size (bp) |
|--------------------|------------------|-------------------|
|                     | Name             | Sequence (5′-3′)  |
| *S. calchasi*       | GsScalF          | ATGAACTGCTTTTTTTCCTTCCATT | 508 |
|                     | GsScalR          | GCCGGTCAAAATGTCTTCTTCTCT | 579 |
| *S. columbae*       | GsScolF          | AATATGATTTATAATTATCATCTT | 483 |
|                     | GsScolR          | TTACCTTTTAAACACCTTCGCTGAG | 524 |
| *S. cornixi*        | GsScornF2        | AGTTGTTAGCTTGGATTGAGGATCT | 449 |
|                     | GsScornR2        | CCATCCCTTTTTCTAAGAGTATATACTGAAA | 644 |
| *S. fulicae*        | GsSfulF          | CAAAGATGAAGAAGTATACGTGAA | 561 |
|                     | GsSfulR          | CTTTACTTGAAGAAGCAGCGAGTGA | 545 |
| *S. halicti*        | GsHaliF          | CACACAGGCTGAGTTGATATGAC | 625 |
|                     | GsHaliR2         | CCATCCCTTTTTCTAAGAGGAGT | 528 |
| *S. lari*           | GsLarF           | TCTTTACTCTTTTACAGGTTGCTT | 532 |
| *S. turdus*         | GsTurF           | CTTTTTACATGCCTTTCTACTCCTT | 567 |
|                     | GsTurR           | ATCTAATATGCTCTTTCCTTCCTT | 565 |
| *S. wobeserii*      | GsWobF           | ATGAACTGCTTTTTTTCCTTCATTT | 532 |
| *S. arctica*        | GsArctF          | CAAAGCACAATATGGATATCTGTTA | 524 |
| *S. lutreia*        | GsLutF           | TCTTTTTACTTCTCAAATGATTCTC | 508 |
| *S. frondea*        | GsFrondeF        | GACACAGGTAGAAATGATGATGAT | 495 |
| *S. hardangeri*     | GsHardangeriF    | ATCTATGCTAAATGATGGATGAT | 528 |
| *S. oviformis*      | GsOviformisF     | ATGAACTGCTTTTTTTCCTTCATTT | 567 |

2.3. Statistical Analysis

The statistical analyses were performed with Quantitative Parasitology 3.0 software [32]. The Sterne’s exact method was used to calculate 95% confidence interval (CI) for prevalence [33]. To compare *Sarcocystis* spp. detection rates in the analysed host species, it was estimated the overall frequency of positive parasite cases, which was calculated as the ratio of positive cases from the host species sample size multiplied by the 16 *Sarcocystis* species tested. The unconditional exact test, which is more sensitive in detecting differences, especially in small sample sizes, evaluated differences in the prevalence of the detected *Sarcocystis* spp. and in the frequency of positive parasite cases [34].

3. Results

3.1. *Sarcocystis* spp. Identification in Intestine Samples of Birds of Family Corvidae

Microscopic examination detected, *Sarcocystis* spp. oocysts in 47.3% (43/91) of birds examined (Table 2). Oocysts measuring 20.4 × 19.3 µm (12.3–25.8 × 12.2–23.7 µm; n = 69) were seen under a light microscope in intestinal mucosa, while free sporocysts were not detected. The molecular method detected *Sarcocystis* spp. 1.8 times more often (77/91, 84.6%), which was significantly higher (p < 0.001) than the microscopic method. In four cases (two hooded crows and two western jackdaws), *Sarcocystis* spp. were observed microscopically, but species-specific nPCR did not detect amplified DNA fragments of parasites. Molecular examination revealed high *Sarcocystis* spp. rates in examined corvid
species, varying from 66.7% (in western jackdaw and Eurasian jay) to 100% (in common raven and common magpie). Comparing *Sarcocystis* spp. infection prevalence obtained in three corvid species with adequate sample size to draw reliable conclusions (N > 20), molecular analysis detected significantly lower prevalence (p = 0.0014) in western jackdaw (14/21, 66.7%) than in common raven (25/25, 100%).

| Table 2. Detection rates of *Sarcocystis* spp. established by microscopic and molecular analyses. |
|----------------|----------------|----------------|---------------|
| **Bird Species** | **N** | **Microscopy** | **Molecular Analysis** |
| | | **n** | **%** | **95% CI** | **n** | **%** | **95% CI** |
| Hooded crow | 33 | 18 | 54.5 | 37.8–71.5 | 28 | 84.8 ** | 68.4–93.8 |
| Common raven | 25 | 16 | 64.0 | 43.9–80.4 | 25 | 100 ** | 86.6–100 |
| Western jackdaw | 21 | 7 | 33.3 | 15.9–55.1 | 14 | 66.7 * | 44.9–84.1 |
| Rook | 5 | 0 | 0 | 0–50.0 | 4 | 80 * | 34.3–99.0 |
| Common magpie | 4 | 2 | 50 | 9.8–90.2 | 4 | 100 ** | 86.6–100 |
| Eurasian jay | 3 | 0 | 0 | 0–63.2 | 2 | 66.7 NS | 13.5–98.3 |
| Overall | 91 | 43 | 47.3 | 36.9–57.7 | 77 | 84.6 *** | 75.4–90.8 |

*N*—number of studied birds; *n*—number of infected birds; *p* < 0.05, **p** < 0.01, ***p** < 0.001, NS—not significant.

All amplified samples were sequenced and identified as belonging to the species for which the species-specific primers were designed. Comparing ITS1 or *cox1* sequences generated in the current study revealed the presence of 11 out of 16 examined *Sarcocystis* spp. (*S. columbae, S. cornixi, S. halieti, S. kutkienae, S. lari, S. turdusi, S. wobeseri, S. arctica, S. lutrae, S. ovalis, and S. oviformis*) (Table S1). Sequence similarity values were calculated for obtained isolates, within species (sequences obtained during the current study were compared to sequences of the same species available in NCBI GenBank) and, in comparison, to the most closely related *Sarcocystis* species (Table 3). Detected *Sarcocystis* spp. were reliably identified, since sequence similarity within specific species did not overlap in values compared to other valid *Sarcocystis* species. Analysing ITS1 sequences, the highest intraspecific genetic differences were estimated for *S. halieti* (0–3.5%) and *S. lutrae* (0–4.6%). The GenBank accession numbers of analysed sequences of *Sarcocystis* spp. listed in Table 2 are provided in the supplementary material (Table S2).

| Table 3. Identification and genetic variation of detected *Sarcocystis* spp. |
|----------------|----------------|----------------|---------------|
| **Species** | **Genetic Region** | **Comparing Sequences of the Same Species Obtained in the Present Study** | **Comparing Sequences of the Same Species Available in GenBank** | **Comparing Isolated with Most Closely Related Species * ** |
| | | **Sequence Similarity %** | | **Sequence Similarity %** |
| *S. columbae* | ITS1 | 98.9–99.8 | 98.9–99.8 | *S. coreus* 93.0–93.2 |
| *S. cornixi* | ITS1 | 98.6–100 | 98.6–100 | *S. kutkienae* 89.1–90.3 |
| *S. halieti* | ITS1 | 98.7–100 | 96.3–100 | *S. columbae* 91.2–92.4 |
| *S. kutkienae* | ITS1 | 99.0–100 | 99.0–100 | *S. cornixi* 88.4–89.1 |
| *S. lari* | ITS1 | 99.2–100 | 98.8–100 | *S. jaamaicensis* 77.9–78.3 |
| *S. turdusi* | ITS1 | 99.0–100 | 98.6–100 | *S. kutkienae* 83.6–86.3 |
| *S. wobeseri* | ITS1 | 99.2–100 | 99.2–100 | *S. calclusi* 91.9–92.7 |
| *S. arctica* | ITS1 | 100 | 98.6–100 | *S. felis* 88.6–90.0 |
| *S. lutrae* | ITS1 | 95.4–100 | 98.1–100 | *S. canis* 73.2–75.5 |
| *S. ovalis* | Cox1 | 100 | 99.8–100 | *S. hardangeri* 91.7–92.1 |
| *S. oviformis* | Cox1 | 100 | 99.8–100 | *S. ovalis* 89.6–90.6 |

*Comparison with valid *Sarcocystis* species; - only one sequence was obtained.*

3.2. Distribution of *Sarcocystis* spp. in Examined Hosts

All 11 *Sarcocystis* spp. identified in this study were detected in hooded crow samples (Table 4). Nine *Sarcocystis* spp. were found in common raven, six in common magpie, five in western jackdaw and three each in rook and Eurasian jay. *Sarcocystis wobeseri* (n = 51), *S. halieti* (n = 48) and *S. kutkienae* (n = 45), were detected more often than other examined species (Table 4). *Sarcocystis cornixi* (n = 16), *S. lari* (n = 13) and *S. turdusi* (n = 13)
were detected slightly less, and other *Sarcocystis* spp. were identified in no more than five birds. *Sarcocystis* spp. that use carnivores and cervids as IH were detected only in hooded crow and common raven; however, the number of investigated individuals of other host species was lower, except for western jackdaw (*n* = 21). When comparing the frequency of positive parasite cases (Table 4) between three corvid species (hooded crow, common raven, and western jackdaw) with adequate sample size to draw reliable conclusions, hooded crow (85/528, 16.1%, *p* = 0.0054) and common raven (60/400, 15.0%, *p* = 0.0254) had significantly higher infection rates than western jackdaw (32/336, 9.5%).

**Table 4.** Identification of *Sarcocystis* spp. in examined corvid samples from Lithuania.

| *Sarcocystis* Species | The Family of IH | Host Species |
|-----------------------|-----------------|-------------|
|                       | The Family of IH | Hooded Crow (*n* = 33) | Common Raven (*n* = 23) | Western Jackdaw (*n* = 21) | Rook (*n* = 5) | Common Magpie (*n* = 4) | Eurasian Jay (*n* = 3) | Overall (%) |
| *S. calcasi* * | Cacatuidae; Columbidae; Phalacrocoracidae; Picidae; Pauletidae | - | - | - | - | - | 0 (0) |
| *S. columba* | Columbidae; Laridae | 2 | 2 | - | - | - | 4 (4.4) |
| *S. cornixi* | Corvidae | 6 | 6 | 2 | 1 | 1 | 16 (17.6) |
| *S. corvus* | Corvidae | - | - | - | - | - | 0 (0) |
| *S. falci* | Accipitridae; Corvidae; Laridae | - | - | - | - | - | 0 (0) |
| *S. halieti* | Coronado; Phalacrocoracidae; Strigidae | 20 | 18 | 7 | 3 | - | 48 (52.7) |
| *S. lutetiae* | Corvidae | 18 | 11 | 10 | 1 | 3 | 2 | 45 (49.4) |
| *S. lari* | Laridae | 4 | 3 | 2 | 3 | 1 | - | 26 (13.6) |
| *S. tardusi* | Turdidae; Muscicapidae; Accipitridae; Anatidae; Laridae | 4 | 3 | 4 | 2 | - | 13 (14.3) |
| *S. topo* | Laridae | 25 | 12 | 9 | 4 | 1 | 51 (56) |
| *S. arctica* | Caridae | 3 | 2 | - | - | - | - | 5 (5.5) |
| *S. latres* | Mustelidae | 1 | - | - | - | - | 1 (1.1) |
| *S. hardangeri* * | Cervidae | - | - | - | - | - | 0 (0) |
| *S. frondos* | Cervidae | - | - | - | - | - | 0 (0) |
| *S. oviformis* | Cervidae | 1 | 3 | - | - | - | 4 (4.4) |
| Overall (%) ** | - | 85 (16.1) | 60 (15.0) | 32 (9.5) | 6 (7.5) | 14 (21.9) | 4 (8.3) | 201 (13.8) |

* Positive DNA controls were not available in this study. ** Frequency of positive parasite cases was estimated as the ratio of positive cases from the host species sample size multiplied by the 16 *Sarcocystis* species tested.

### 3.3. *Sarcocystis* spp. Mixed Infections

Overall, 84.6% (77/91) of examined corvid birds were positive for at least one *Sarcocystis* spp. Multiple species of *Sarcocystis* were common in all corvid host species (Figure 1). Infection with multiple species were found in 79.2% (61/77) of the infected corvid birds, with usually two to four *Sarcocystis* species in one sample. Six bird each had more than four *Sarcocystis* species, and a single common magpie and hooded crow contained the most diverse *Sarcocystis* species, with six and seven parasite species, respectively.
4. Discussion

4.1. Differences in Sarcocystis spp. Detection Using Microscopic and Molecular Methods

Microscopic analysis is essential to describe and characterize Sarcocystis spp. in IH, since parasite species cannot be differentiated by the stages found in DH (oocyst or sporocyst) [1]. Whereas molecular methods can help to identify Sarcocystis spp. from intestinal and faecal samples of predators or omnivorous animals [7,8,10,20,21,25,35,36]. However, detection of Sarcocystis spp. DNA from intestinal and/or faecal samples does not conclusively prove the role of tested animal as DH of these parasites. The detected DNA may also belong to Sarcocystis spp. that had been present in the carrion the bird has been feeding on without infecting analysed animal species. Thus, life cycle experiments are necessary to confirm the results obtained [1]. In the current study, 11 Sarcocystis spp. were identified in mucosal scrapings of corvids based on species-specific nPCR targeting ITS1 or cox1 and subsequent sequencing (Table 3). The overall Sarcocystis spp. detection rate was significantly higher ($p < 0.001$) by molecular methods (84.6%) than by microscopic examination (47.3%) (Table 2). The study’s findings are consistent with previous research [25,36], showing that molecular methods should be used to examine all samples, rather than just those that are microscopically positive.

4.2. Corvids as Possible DH of some Sarcocystis spp. That Use these Birds as IH

Representatives of the genus Sarcocystis have a diheteroxenous life cycle, meaning different animal species serve as IH and DH [1]. However, some Sarcocystis spp. that use mice, rats and lizards as IH (S. cymruensis, S. dugesii, S. gallotiae, S. muris, S. simonyi, and S. stehlinii) have been shown to have both diheteroxenous and dihomoxenous life cycles, allowing transmission via cannibalism [37–42]. Corvids are known to be IH of three Sarcocystis spp. identified in the present study, (S. cornixi [43], S. kutkienae [44] and S. halieti (MZ707148-49, unpublished data) [10]. Sarcocystis cornixi and S. kutkienae form sarcocysts in muscles of corvids, while S. halieti is multi-host adapted, employing birds of several different orders as IH [45,46]. Birds of prey are identified DH of S. cornixi and S. halieti [8,10], and based on phylogenetic placement, birds are the presumed DH of S. kutkienae [44]. As mentioned before, the detected DNA may belong to the sarcocyst from IH that was eaten and was present in intestines without infecting the DH. However, the other possibility that corvids may act both as IH and DH for the same Sarcocystis spp. is intriguing, and more research is needed to reveal this interesting phenomenon. Our data indicate that corvids can feed on carrion of other birds of the Corvidae family, including their conspecifics. For
example, groups of hooded crows were observed feeding on carcasses of hooded crows and rooks (presumably killed by poisoning) in a waste disposal area of Klaipeda, Lithuania in January 2016. In several cases, common ravens were recorded feeding on carrion of rooks and western jackdaws killed by cars in Lithuania.

4.3. Occurrence of Sarcocystis Species in the Intestinal Samples of Corvids

In the present study, ten Sarcocystis spp. (S. columbae, S. cornixi, S. halieti, S. kutkienae, S. lari, S. turdusi, S. wobeseri, S. arctica, S. lutrae and S. oviformis) were identified in the intestinal samples of corvids (Table 4) for the first time. It should be noted that DH of S. kutkienae, S. wobeseri, S. arctica, S. lutrae and S. oviformis were unknown [11,12,44,47,48]. Sarcocystis ovalis, which was detected during the current study, was previously determined in faecal and intestinal mucosa samples of common magpie [21] and intestinal mucosa samples of Japanese jungle crow [20]. Of the Sarcocystis spp. identified in this work, S. halieti is potentially pathogenic. Recently, S. halieti-associated encephalitis was reported in a juvenile free-ranging little owl (Athene noctua) from Germany [49]. In this work, S. halieti was one of the most commonly detected species; it was confirmed in hooded crows (n = 20), in common ravens (n = 18), in western jackdaws (n = 7) and in common magpies (n = 3) (Table 4). Thus, corvids are likely to be involved in the transmitting pathogenic S. halieti.

In the current study, Sarcocystis spp. were identified in all six studied bird species (hooded crow, common raven, western jackdaw, rook, common magpie, and Eurasian jay). Hooded crow and common raven had significantly higher frequency of positive Sarcocystis spp. cases than western jackdaw. This observation agrees with diet studies of corvids, since common raven and hooded crow are the main scavengers among corvids in Lithuania [50,51]. Carcasses of dead birds and mammals form an important part of the diet of common raven and hooded crow, particularly at waste disposal areas in the winter [51,52]. Common magpie and rook are also frequently observed feeding on carrion of birds [18,51]. Western jackdaws have been observed feeding on small parts of carrion remnants at a waste disposal area in Klaipeda, Lithuania in heavy winter, while Eurasian jays were frequently observed in forests eating the smallest parts of muscles and intestine left in carrion previously used by larger corvids or birds of prey. Based on available data on the corvid diet and the results of this study, corvids may play an important role in transmitting Sarcocystis spp.

Oocysts or sporocysts of S. calchasi, S. corvusi, S. fulicae, S. hardangeri, and S. frondea were not detected in corvids in this study. Pathogenic S. calchasi infects birds of several orders and has been detected in Germany [53,54], USA [55,56], and Japan [57]. This species is also transmitted by Accipiter hawks [53,54] prevalent in Lithuania [50,52]; however, S. calchasi has not been confirmed in Lithuania yet, may be explained by the possibility that corvids are not the DH of this parasite, or this species is absent or rare in Lithuania. In 2013, S. corvusi was described in the leg muscles of two migrating jackdaws from Lithuania [58], and afterward, there were no more records on this species. Therefore, we hypothesise that this species is absent or rare in the area under investigation. Sarcocysts of S. fulicae were identified solely in muscles of Eurasian coot (Fulica atra) [30], a water bird rarely occurring on land; therefore, corvids have less access to carrion of this species, except for dead individuals rarely found on frozen water bodies in winter [50–52]. Sarcocystis hardangeri was identified only in cervices from specific regions of Norway and Iceland [23,59], so, this species is unlikely to be distributed in regions south of Norway. Out of five cervid species examined in Lithuania, S. frondea was found only in introduced sika deer [26]. Therefore, we suppose that S. frondea was not observed in examined corvids due to this species’ low prevalence in Lithuania.

4.4. Molecular Identification of Sarcocystis spp. in Naturally Infected DH

One DH can harbour multiple Sarcocystis species [1,8,10,25]. The present study revealed mixed Sarcocystis spp. infections in 79.2% of the infected corvid birds. The mixed
infections in intestinal or faecal samples of naturally infected wild animals cause issues with identifying *Sarcocystis* spp. In this work, species-specific PCR was applied to detect *Sarcocystis* spp. Despite the high *Sarcocystis* spp. detection rate (86.0%), identification of species is sure to be limited. For instance, four samples were microscopically positive for *Sarcocystis* spp., but molecular analysis did not identify the examined species. These results indicate the presence of other *Sarcocystis* spp. not tested in the intestinal samples of corvids. Other authors have applied methods apart from species-specific PCR, such as cloning, and metabarcoding, to detect *Sarcocystis* spp. from samples containing oocysts or sporocysts [60–63]. However, previously conducted studies were also limited and revealed only a partial diversity of *Sarcocystis* spp. in certain hosts [8,10,36]. In summary, more sensitive molecular identification techniques of *Sarcocystis* spp. in intestines and faecal samples of omnivorous animals and predators should be further developed.

5. Conclusions

Based on species-specific nPCR targeting ITS1 or *cox1* and subsequent sequencing 11 *Sarcocystis* spp. that employ birds (*S. columbae*, *S. cornixi*, *S. haliei*, *S. kutkienae*, *S. lari*, *S. turdusi* and *S. wobeseri*), cervids (*S. ovalis* and *S. oviformis*) and carnivorous mammals (*S. arctica* and *S. lutrae*) as their IH were identified in the intestinal samples of six corvid species. Ten of these *Sarcocystis* species were confirmed in corvid intestines for the first time. Therefore, the present study’s results indicate that widespread omnivorous corvids, which live close to humans, could be involved in transmitting these *Sarcocystis* spp., including potentially pathogenic *S. haliei*. However, the question remains whether corvids are DH of these identified *Sarcocystis* spp. or the detected DNA was a residue of food particles present in the intestine of tested birds.

In four cases, light microscopy detected oocysts of *Sarcocystis* spp. that were not identified by molecular methods. Several other studies also reported that molecular methods were not efficient in revealing the full diversity of *Sarcocystis* spp. in the intestinal and faecal samples of predators or omnivorous animals. Thus, more sensitive *Sarcocystis* spp. identification techniques from naturally infected DH must be developed.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/ani11113258/s1. Table S1: Species of *Sarcocystis* validated in various corvid birds using ITS1 or *cox1*, Table S2: *Sarcocystis* species and GenBank accession numbers of sequences used in comparison analysis (Table 3). Sequences obtained in the present study are in boldface.

**Author Contributions:** Conceptualization, P.P., D.B. and S.Š.; methodology, P.P. and E.R.-L.; software, P.P.; validation, D.B. and P.P.; formal analysis, P.P.; investigation, E.J.-N., D.Š. and E.R.-L.; resources D.B.; data curation, P.P.; writing—original draft preparation, E.J.-N., S.Š., D.Š., E.R.-L., D.B. and P.P.; writing—review and editing, E.J.-N., S.Š., D.Š., E.R.-L., D.B. and P.P.; visualization E.J.-N. and D.Š.; supervision, P.P.; project administration, P.P. and D.B.; funding acquisition, D.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by the Research Council of Lithuania (grant number S-MIP-20-24).

**Institutional Review Board Statement:** Samples were collected with the permission of the Ministry of Environment of the Republic of Lithuania (2017-03-23 no.26-A4-3119; 2019-03-01 no. 26-A4-1535; 2021-03-31 nr. (26)-SR-89).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data supporting the conclusions of this article are included in the article. The sequences generated in the present study were submitted to the GenBank database under accession numbers OK481182–OK481382.

**Acknowledgments:** This study was supported by the Open Access research infrastructure of the Nature Research Centre under the Lithuanian open access network initiative. The authors are grateful to Kaunas T. Ivanauskas Zoology Museum for providing bird samples, also to V. Pabrinkis for his great support in bird sampling.

**Conflicts of Interest:** The authors declare that they have no conflict of interest.
29. Gjerde, B. Phylogenetic relationships among Sarcocystis species in cervices, cattle and sheep inferred from the mitochondrial cytochrome C oxidase subunit 1 gene. Int. J. Parasitol. 2013, 43, 579–591. [CrossRef] [PubMed]

30. Prakas, P.; Butkauskas, D.; Švažas, S.; Juozaitytė-NGugu, E.; Stanevičius, V. Morphologic and genetic identification of Sarcocystis fulicae n. sp. (Apicomplexa: Sarcocystidae) from the Eurasian coot (Fulica atra). J. Wildl. Dis. 2018, 54, 765–771. [CrossRef] [PubMed]

31. Allisch, S.E.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. J. Mol. Biol. 1990, 215, 403–410. [CrossRef]

32. Rözza, L.; Reiczigel, J.; Majoros, G. Quantifying parasites in samples of hosts. J. Parasitol. 2000, 86, 228–232. [CrossRef]

33. Reiczigel, J. Confidence intervals for the binomial parameter: Some new considerations. Stat. Med. 2003, 22, 611–621. [CrossRef]

34. Reiczigel, J.; Abonyi-Toth, Z.; Singer, J. An exact confidence set for two binomial proportions and exact unconditional confidence intervals for the difference and ratio of proportions. Comput. Stat. Data Anal. 2008, 52, 5046–5053. [CrossRef]

35. Prakas, P.; Laiugaudaitė, S.; Kutkienė, L.; Sruoga, A.; Švažas, S. Molecular identification of Sarcocystis rileyi sporocysts in red foxes (Vulpes vulpes) and raccoon dogs (Nyctereutes procyonoides) in Lithuania. Parasitol. Res. 2015, 114, 1671–1676. [CrossRef] [PubMed]

36. Prakas, P.; Rudaitytė-Lukošienė, E.; Šneideris, D.; Butkauskas, D. Invasive American mink (Neovison vison) as potential definitive host of Sarcocystis elongata, S. entzerothi, S. japonica, S. truncata and S. silva using different cedvid species as intermediate hosts. Parasitol. Res. 2021, 120, 2243–2250. [CrossRef]

37. Matuschka, F.R.; Bannert, B. Cannibalism and autotomy as predator-prey relationship for Monoxenous Sarcosporidia. Parasitol. Res. 1987, 85, 815–821. [CrossRef] [PubMed]

38. Matuschka, F.R. Studies on the life cycle of Sarcocystis dugesii in the madeiran wall lizard Podarcis (syn. Lacerta) dugesii. Parasitol. Res. 1988, 75, 73–75. [CrossRef]

39. Matuschka, F.R.; Bannert, B. Recognition of cyclic transmission of Sarcocystis stehlinii n. sp. in the Gran Canarian giant lizard. J. Parasitol. 1989, 75, 383–387. [CrossRef] [PubMed]

40. Bannert, B. Sarcocystis simonyi sp. nov. (Apicomplexa Sarcocystidae) from the endangered Hierro giant lizard Gallotia simonyi (Reptilia Lacertidae). Parasitol. Res. 1992, 78, 142–145. [CrossRef] [PubMed]

41. Koudela, B.; Modrý, D. Sarcocystis muris possesses both Diheteroxenous and Dihomoxenous characters of life cycle. J. Parasitol. 2000, 86, 877–879. [CrossRef]

42. Hu, J.J.; Liao, J.Y.; Meng, Y.; Guo, Y.M.; Chen, X.W.; Zuo, Y.X. Identification of Sarcocystis cyprhuensis in wild Rattus flavipectus and Rattus norvegicus from Peoples Republic of China and its transmission to rats and cats. J. Parasitol. 2011, 97, 421–424. [CrossRef]

43. Kutkienė, L.; Prakas, P.; Sruoga, A.; Butkauskas, D. Sarcocystis in the birds family Corvidae with description of Sarcocystis cornixi sp. nov. from the hooded crow (Corvus cornix). Parasitol. Res. 2009, 104, 329–336. [CrossRef]

44. Prakas, P.; Butkauskas, D.; Juozaitytė-NGugu, E. Molecular and morphological description of Sarcocystis kutkienae sp. nov. from the common raven (Corvus corax). Parasitol. Res. 2020, 119, 4205–4210. [CrossRef]

45. Prakas, P.; Butkauskas, D.; Juozaitytė-NGugu, E. Molecular identification of four Sarcocystis species in the herring gull, Larus argentatus, from Lithuania. Parasit. Vectors 2013, 6, 1–6. [CrossRef]

46. Prakas, P.; Bea, A.; Juozaitytė-NGugu, E.; Olano, I.; Villanúa, D.; Švažas, S.; Butkauskas, D. Molecular identification of Sarcocystis haleti in the muscles of two species of birds of prey from Spain. Parasit. Vectors 2021, 14, 414. [CrossRef]

47. Dahlgren, S.S.; Gjerde, B. Sarcocystis in Norwegian roe deer (Capreolus capreolus): Molecular and morphological identification of Sarcocystis oviformis n. sp. and Sarcocystis gracilis and their phylogenetic relationship with other Sarcocystis species. Parasitol. Res. 2009, 104, 993–1003. [CrossRef] [PubMed]

48. Shadbolt, T.; Pocknell, A.; Sainsbury, A.W.; Egerton-Read, S.; Blake, D.P. Molecular identification of Sarcocystis wobeseri-like parasites in a new intermediate host species, the white-tailed sea eagle (Haliaeetus albicilla). Parasitol. Res. 2021, 120, 1845–1850. [CrossRef] [PubMed]

49. Maier-Sam, K.; Kaiponen, T.; Schmitz, A.; Schulze, C.; Bock, S.; Hlinak, A.; Olias, P. Encephalitis associated with Sarcocystis halieti infection in a free-ranging little owl (Athene noctua). J. Wildl. Dis. 2021, 57, 712–714. [CrossRef] [PubMed]

50. Ivanusauskaitė, T. Lithuanian Birds; Mokslos Press: Vilnius, Lithuania, 2015.

51. Logminas, V.; Nedzinskas, V.; Drobels, E.; Petraitis, A.; Patapavičius, R.; Žalakevičius, M.; Valius, M.; Šablevičius, B.; Gražulevičius, G.; Raudonikis, L.; et al. Lithuanian Fauna. Birds; Mokslos Press: Vilnius, Lithuania, 1990.

52. Kurlavičius, P.; Preiška, Ž.; Skuja, S.; Krastukas, M.; Brazaitis, G.; Stanevičius, V.; Mačiulis, M.; Jusys, V.; Butleris, A.; Raudonikis, L.; et al. Lithuanian Breeding Bird Atlas; Lututė Press: Kaunas, Lithuania, 2006.

53. Olias, P.; Gruber, A.D.; Hafez, H.M.; Heydorn, A.O.; Mehllhorn, H.; Lierz, M. Sarcocystis calcilis sp. nov. of the Domestic pigeon (Columba livia f. domestica) and the Northern goshawk (Accipiter gentilis): Light and electron microscopical characteristics. Parasitol. Res. 2010, 106, 577–585. [CrossRef] [PubMed]

54. Parmentier, S.L.; Maier-Sam, K.; Failing, K.; Enderlein, D.; Gruber, D.A.; Lierz, M. Prevalence of Sarcocystis calcilis in free-ranging host species: Accipiter hawks and common woodpigeon in Germany. Sci. Rep. 2018, 8, 17610. [CrossRef] [PubMed]

55. Wünschmann, A.; Armien, A.G.; Reed, L.; Gruber, A.D.; Olias, P. Sarcocystis calcilis-associated neurologic disease in a domestic pigeon in North America. Transbound. Emerg. Dis. 2011, 58, 526–530. [CrossRef]

56. Rimoldi, G.; Speer, B.; Wellenham, J.F., Jr.; Bradway, D.S.; Wright, L.; Reavill, D.; Barr, B.C.; Childress, A.; Shivapradasad, H.L.; Chin, R.P. An outbreak of Sarcocystis calcilis encephalitis in multiple psittacine species within an enclosed zoological aviary. J. Vet. Diagn. Invest. 2013, 25, 775–781. [CrossRef]
57. Ushio, N.; Watanabe, K.; Chambers, J.K.; Shibato, T.; Nakayama, H.; Uchida, K. Sarcocystis calchasi encephalitis in a rock pigeon. J. Vet. Med. Sci. 2015, 77, 1523–1526. [CrossRef]
58. Prakas, P.; Kutkienė, L.; Butkauskas, D.; Sruoga, A.; Žalakevičius, M. Molecular and morphological investigations of Sarcocystis corvus sp. nov. from the Jackdaw (Corvus monedula). Parasitol. Res. 2013, 112, 1163–1167. [CrossRef]
59. Dahlgren, S.S.; Gjerde, B.; Skirnisson, K.; Gudmundsdottir, B. Morphological and molecular identification of three species of Sarcocystis in Reindeer (Rangifer tarandus tarandus) in Iceland. Vet. Parasitol. 2007, 149, 191–198. [CrossRef]
60. Lau, Y.L.; Chang, P.Y.; Subramaniam, V.; Ng, Y.H.; Mahmud, R.; Ahmad, A.F.; Fong, M.Y. Genetic assemblage of Sarcocystis spp. in Malaysian snakes. Parasit. Vectors 2013, 6, 257. [CrossRef] [PubMed]
61. Moré, G.; Maksimov, A.; Conraths, F.J.; Schares, G. Molecular identification of Sarcocystis spp. in foxes (Vulpes vulpes) and raccoon dogs (Nyctereutes procyonoides) from Germany. Vet. Parasitol. 2016, 220, 9–14. [CrossRef] [PubMed]
62. Lesniak, I.; Franz, M.; Heckmann, I.; Greenwood, A.D.; Hofer, H.; Krone, O. Surrogate hosts: Hunting dogs and recolonizing grey wolves share their endoparasites. Int. J. Parasitol. Parasites Wildl. 2017, 6, 278–286. [CrossRef] [PubMed]
63. Basso, W.; Alvarez Rojas, C.A.; Buob, D.; Ruetten, M.; Deplazes, P. Sarcocystis infection in red deer (Cervus elaphus) with eosinophilic myositis/fasciitis in Switzerland and involvement of red foxes (Vulpes vulpes) and hunting dogs in the transmission. Int. J. Parasitol. Parasites Wildl. 2020, 13, 130–141. [CrossRef]