Description of *Sarcocystis turdusi* sp. nov. from the common blackbird (*Turdus merula*)

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SUMMARY

Cysts of *Sarcocystis* species were found in 24 of 44 (54·5%) examined blackbirds (*Turdus merula*). Under the light microscope, only 1 morphological type of cyst was found in all birds investigated. Ribbon-shaped cysts were long (the largest fragment found amounted to 7 mm) and of different thickness (25–206 μm). A cyst wall reached up to 3·5 μm and had finger-like protrusions. Under the transmission electron microscope, a single cyst isolated from 1 blackbird was studied. The cyst wall was 2·5–4·4 μm thick, had club- or irregularly-shaped and sometimes branched protrusions that differed in size. The content of cysts was divided into large chambers by septa. Orange segment-shaped cystozoites were 6·2 × 1·4 (5·5–7·2 × 1·2–1·5) μm. This type of cyst wall has never been described in *Sarcocystis* species isolated from birds, thus far. The results of 18S rDNA, 28S rDNA and ITS–1 region sequences showed that *S. turdusi* was most closely related to *S. columbae, S. calchasi, S. wobeseri, S. cornixi* and *Sarcocystis* sp. ex *Accipiter nisus* parasitizing birds. Phylogenetic results suggest that predatory birds are the most probable definitive hosts of *S. turdusi*.

Key words: *Sarcocystis*, blackbird, cyst morphology, 18S rDNA, 28S rDNA, ITS–1.

INTRODUCTION

The genus *Sarcocystis* is a large heterogeneous group of cyst-forming coccidian parasites with over 210 named species found in mammals, birds and reptiles (Odening *et al*. 1998). These parasites are characterized by an obligatory prey-predator two-host life cycle, asexual multiplication with a formation of sarcocysts in the striated muscles of the intermediate host, sexual multiplication in the intestine of the definitive host and endogenous sporulation of oocysts (Dubey *et al*. 1989).

To our knowledge 30 named *Sarcocystis* species forming sarcocysts in the muscle tissues of birds belonging to at least 13 avian orders are known (Kutkienė *et al*. 2012). During the past 10–15 years, *Sarcocystis* species in birds were described combining the results of morphological and DNA investigations (Tanhauser *et al*. 1999; Dubey *et al*. 2001; Kutkienė *et al*. 2009, 2010, 2012; Olias 2010*a*, *b*).

Sarcocysts in the blackbird (*Turdus merula*) found in Europe were named *S. turdi* by Brumpt (1913). This species could be found in the taxonomic lists of *Sarcocystis* (Wenyon, 1926; Kalyakin and Zasukhin, 1975; Levine and Tadros, 1980; Levine, 1986) until it was crossed off by Odening (1998) as *nomen nudum*. To our knowledge, later *Sarcocystis* cysts in the blackbird were not discovered. They were found in the fieldfare (*Turdus pilaris*) in Russia, in the red-throated thrush (*Turdus ruficollis*) and in the black-throated thrush (*Turdus atrogularis*) in Kazakhstan, but more detailed investigations have not been carried out (Pak and Eshtokina, 1984; Grikienienė and Iezhova, 1998; Pinayeva *et al*. 1998).

The results of cyst morphology and DNA analysis of *Sarcocystis turdusi* sp. nov. from the blackbird are presented in this paper.

MATERIALS AND METHODS

Material

Between 2003 and 2011, 44 blackbirds from different districts of Lithuania were investigated for the presence of *Sarcocystis* cysts. Samples of dead birds were obtained from the bird ringing stations or taxidermists.

Light microscopy

In order to detect and morphologically characterize *Sarcocystis* cysts, samples of leg muscles of each bird were examined using previously described methods (Kutkienė *et al*. 2010).

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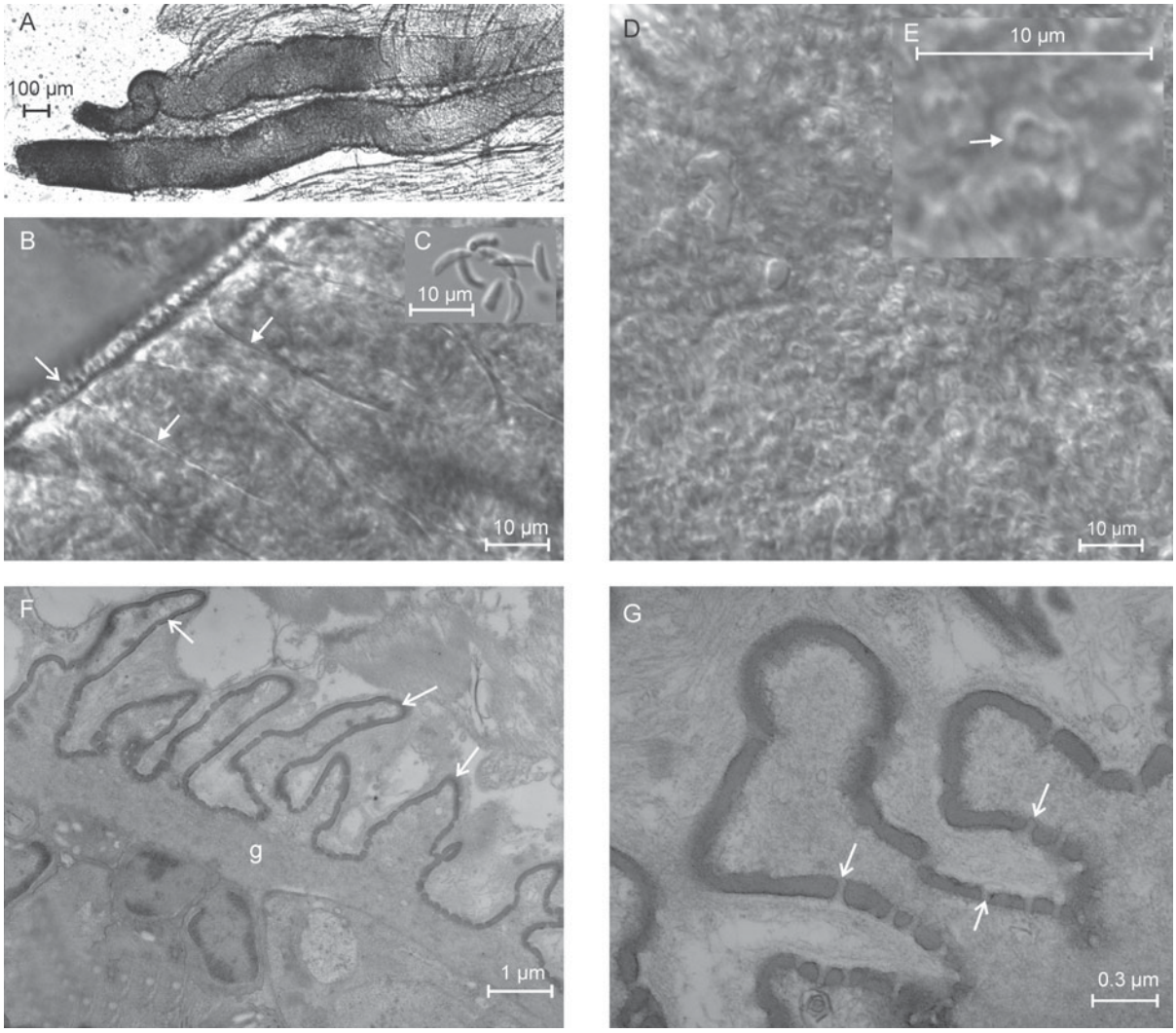

Fig. 1. Morphology of *Sarcocystis turdusi* sp. nov. (A) Cysts of *S. turdusi* in the leg muscles of the blackbird. Fresh preparation. (B–E) Light micrographs (computerized image analysis system). Fresh preparations. (B) Fragment of the cyst. Note cyst wall with protrusions (arrow) and clearly visible septa (arrows with white arrowheads). (C) Cystozoites. (D) Cyst wall surface with protrusions. (E) High magnification of the top of cyst wall protrusions. Note irregular shape and concavity of the top of the protrusion (arrow). (F, G) Electron micrographs of the cyst wall fragments. (F) Club-or irregularly shaped cyst wall protrusions (arrows). (G) High magnification of protrusions. Arrows pointed at invaginations of parasitophorous vacuolar membrane. g, ground substance.

Transmission electron microscopy (TEM)

A mature sarcocyst from 1 blackbird was fixed and examined by TEM in the previously described way (Kutkienė *et al*. 2010).

DNA analysis

Genomic DNA was extracted from a few mature sarcocysts, which were isolated from the leg muscle fibres of 3 infected blackbirds using the NucleoSpin Tissue Kit, (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's recommendations. Fragments of 28S rDNA, full length 18S rDNA and ITS–1 region were amplified using 7 primer pairs KL-P1F\KL-P1R, KL-P2F\KL-P2R, SarAF\SarAR, SarBF\SarBR, SarCF\SarCR, SarDF\SarDR, P-ITSF\P-ITSR (Kutkienė *et al*. 2010). PCRs were performed in the final 25 μl volume comprised of 1 PCR buffer (with 50 mM KCl), 0·2 mM dNTP, 0·2 μM of each primer, 2·5 mM $MgCl_2$, 1 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), and 0·04 μg template DNA. PCRs were carried out with an initial denaturing at 95 °C for 5 min, 5 cycles at 94 °C for 45 s, at 64 °C for 60 s, at 72 °C for 70 s, followed by 30 cycles at 94 °C for 45 s, at 58 °C for 60 s, at 72 °C for 70 s and ended with the final extension at 72 °C for 10 min. The amplification products were analysed using 1·7% agarose gel and purified with the help of exonucleases ExoI and FastAP. PCR products were sequenced directly with an ABI Prism 377 automatic DNA sequencer using the same primers as for PCR reactions. The identified sequences were compared with the sequences listed in the GenBank by searching with the BLAST program megablast (http://www.ncbi.nlm.nih.gov/BLAST/).

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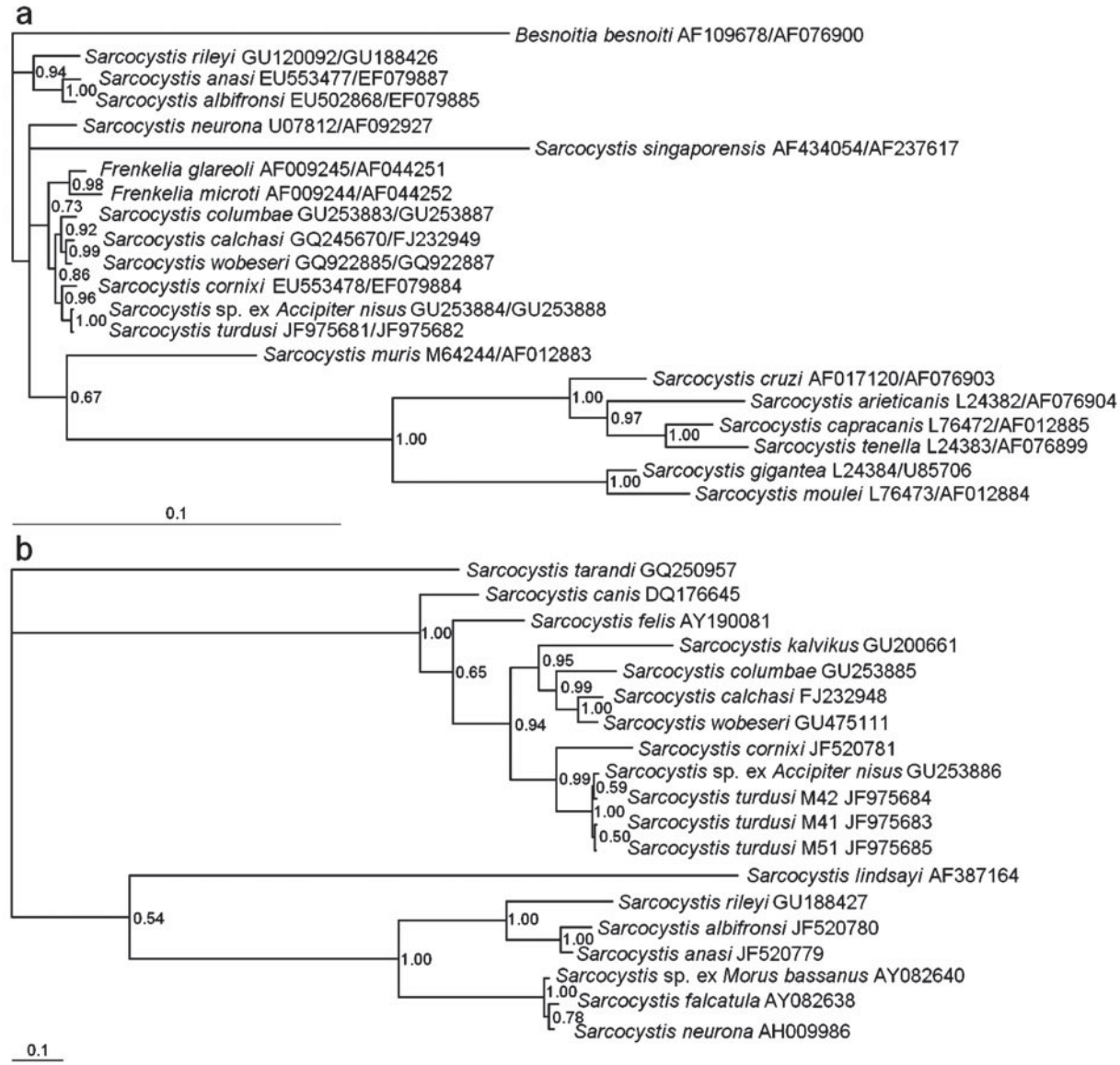

Fig. 2. The phylogram of the genus *Sarcocystis* species based on (a) fused partial 18S rDNA and 28S rDNA sequences and (b) ITS-1 region sequences. Multiple sequence alignments consisted of (a) 3425 nucleotide positions including gaps, from which 1817 belonged to 18S rDNA and 1608 belonged to 28S rDNA and of (b) 691 nucleotide positions including gaps. The phylogenetic tree was constructed using the Bayesian methods, scaled according to the branch length and rooted on (a) *Besnoitia besnoiti* and (b) *S. tarandi*. The numbers in the figure show the posterior probability support values. GenBank Accession numbers of all taxa are given after the species name.

Sequence identity values were determined on the European Molecular Biology Open Software Suite (http://www.ebi.ac.uk/emboss/align/). Sequences were aligned using MUSCLE algorithm (Edgar, 2004). Phylogenetic relationships were assessed using the Bayesian method by the MrBayes program, version 3. 1. 2 (Ronquist and Huelsenbeck, 2003). The most complex evolutionary model available, i.e. GTR + I + G model, was chosen for a phylogenetic analysis.

RESULTS

Having studied 44 blackbirds, cysts of *Sarcocystis* were found in 24 (54·5%) individuals. Infection intensity fluctuated from 1 to 200 (mean = 21, median = 5) cysts in 28 oat grain size (~ 1 g) sections of leg muscles. Under the light microscope, only 1 morphological type of cyst was found in all birds studied. By TEM, 1 cyst from 1 blackbird was examined and proposed as a new *Sarcocystis* species.

Sarcocystis turdusi sp. nov.

Description. Cysts were ribbon-shaped, long (the largest fragment found amounted to 7 mm) and of different thickness (25–206 μm) (Fig. 1A). Under the light microscope, the wall of the sarcocyst amounted up to 3·5 μm, had clearly visible finger-like protrusions and looked like a band sewn on the surface of the

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Table 1. Variable sites within the ITS-1 region of *Sarcocystis turdusi* and *Sarcocystis* sp. ex *Accipiter nisus*

Species	GenBank Accession number	Site number						
		292	296	314	463	730	758	790–795
S. turdusi M41	JF975683	T	C	A	A	C	A	TGAATG
S. turdusi M42	JF975684	C	A	A	T	C	A	TGAATG
S. turdusi M51	JF975685	T	N	A	T	C	A	TGAATG
Sarcocystis sp. ex *Accipiter nisus*	GU253886	C	C	G	T	T	T	TAA—G

cyst (Fig. 1B). The cyst content was divided into large chambers by septa (Fig. 1B). With the help of a computerized image analysis system it was possible to see irregularly shaped protrusions with concave tops on the cyst wall surface (Fig. 1D, E). Orange segment-shaped cystozoites were 6·2×1·4 (5·5–7·2×1·2–1·5) μm (n=13) (Fig. 1C). By TEM, a cyst wall was 2·5–4·4 μm thick (with the ground substance), had club or irregularly shaped, sometimes branched protrusions, which differed in size and were situated at different distances from one another (Fig. 1F). The parasitophorous vacuolar membrane had many indentations and was lined by the electron-dense layer that, in some places, was interrupted (Fig. 1G). The ground substance layer continued into the interior of the cyst as septa. The cyst wall of this sarcocyst resembled somewhat cyst wall type-4 (Dubey and Odening, 2001).

Molecular analysis. Three *S. turdusi* isolates from 3 different blackbird individuals were identical in the 1793 bp of the 18S rDNA and in the 1469 bp of the 28S rDNA. The complete 18S rDNA and 28S rDNA sequences were deposited in GenBank with Accession numbers JF975681 and JF975682, respectively. Furthermore, *S turdusi* and *Sarcocystis* sp. ex *Accipiter nisus*, isolated from sporocysts found in sparrowhawks (*Accipiter nisus*) had the same nucleotide composition within overlapping 1450 bp long fragments of 28S rDNA and 1630-bp long fragments of 18S rDNA. The investigated sequences were most similar to the sequences of *Sarcocystis* species parasitizing birds and also to 2 *Frenkelia* species. According to a phylogenetic tree of the fused sequences of 18S rDNA and 28S rDNA, *S. turdusi* was grouped with *S. columbae, S. calchasi, S. wobeseri, S. cornixi* and *Sarcocystis* sp. ex *Accipiter nisus*, with high bifurcation support (Fig. 2a). These species formed a sister group to 2 representatives of genus *Frenkelia*. Other *Sarcocystis* species from birds, i.e. *S. rileyi, S. anasi* and *S. albifronsi*, were grouped separately.

The complete ITS–1 region sequences of *S. turdusi* obtained from 3 blackbirds were deposited in GenBank with Accession numbers JF975683-JF975685. Sequences of 3 isolates differed from each other by 2–3 nucleotide substitutions (99·6–99·8% sequence identity) (Table 1). Sequence identities between *S. turdusi* and *Sarcocystis* sp. ex *Accipiter nisus* were a bit lower and ranged between 99·0% and 99·1% (sequences differed by 5–6 nucleotide substitutions). Comparing ITS–1 region sequences, the extent of genetic similarity of *S. turdusi* with other *Sarcocystis* species was significantly smaller and accounted for <78%. On the basis of the phylogram constructed from ITS–1 region sequences, high support was established to joining 3 *S. turdusi* isolates with *S. kalvikus, S. columbae, S. calchasi, S. wobeseri, S. cornixi* and *Sarcocystis* sp. ex *Accipiter nisus*, whereas *S. felis* and *S. canis* were sister species (Fig. 2b) Inside this phylogenetic group the lowest sequence identity value between two *Sarcocystis* species, i.e. *S. calchasi* and *S. wobeseri* was 93%. Genetic data of ITS–1 region shows that sequence differences between *S. turdusi* M41, M42, M51 isolates and *Sarcocystis* sp. ex *Accipiter nisus* must be regarded as genetic differences of the intraspecific level. On the basis of the results of a genetic investigation the sparrowhawk can be said to be a possible definitive host of *S. turdusi*.

Taxonomic summary

Type intermediate host: common blackbird (*Turdus merula*).

Locality: Šilutė district (near the Baltic Sea), western Lithuania.

Definitive host: unknown.

GenBank Accession numbers: JF975681 (18S rDNA), JF975682 (28S rDNA), JF975683-JF975685 (ITS-1 region).

Specimens deposited: histological preparations, TEM material and DNA samples are deposited at the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

Etymology: the Latin name of Turdidae family is used for a species name.

DISCUSSION

Despite the fact that cysts of *Sarcocystis* were found in the blackbird as far back as the end of the nineteenth century, no detailed investigations into these parasites have been conducted (Bütschli, 1880–82).

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According to the results of this study, prevalence of *S. turdusi* infection in this bird species is high and exceeds 54·5%. High prevalence of infection is evidently caused by nutrition peculiarities. Blackbirds pick insects, grubs, earthworms and molluscs on the loose soil and with this food they might ingest oocysts/sporocysts excreted by the definitive hosts. Potential definitive hosts of *S. turdusi* might be the main predators of the blackbird such as the sparrowhawk and the red fox (*Vulpes vulpes*) (Logminas, 1990).

When comparing the results of a morphological examination of sarcocysts obtained in this work with analogous data in the literature about other species of *Sarcocystis* in birds, it can be stated that a cyst wall of similar morphology has not been identified for any known *Sarcocystis* species parasitizing birds (Odening, 1998). The cyst wall ultrastructure of *S. turdusi* from the blackbird faintly resembles cyst wall type-4 of *S. sigmodontis* from the rodent hispid cotton rat (*Sigmodon hispidus*) (Dubey and Odening, 2001). However the majority of protrusions of *S. turdusi* were longer and had a more diverse shape. According to Odening (1998), cyst wall type-4 was established for a few more *Sarcocystis* species, i.e. *S. chalcidicolubris, S. felis, S. gongyli, S. murinotechis, S. podarcicolubris* infecting lizards, rodents, felids.

Phylogenetic analysis showing the evolutionary relatedness to the definitive host rather than the intermediate host inside the phylogenetic groups of Sarcocystinae has been reported several times (Doležel *et al*. 1999; Holmdahl *et al*. 1999; Šlapeta *et al*. 2003; Morrison *et al*. 2004; Dahlgren *et al*. 2008). In the present study, *Sarcocystis* species from birds were placed in 2 separate well-supported phylogenetic groups in the phylogenetic trees; the first group encompassed *S. turdusi, S. cornixi, S. wobeseri, S. calchasi* and *S. columbae* and the second group contained *S. rileyi, S. anasi, S. albifronsi* and *S. falcatula*. On the basis of transmission experiments the hypothesis can be suggested that birds serve as definitive hosts of the first phylogenetic group and mammals serve as definitive hosts of the second one. Furthermore, closely related *S. turdusi, S. columbae, S. calchasi, S. wobeseri* and *S. cornixi* also share similar morphometric characteristics of cystozoites which are relatively small (1·4–1·8 × 6·1–8·1 μm) and are banana- or lancet-shaped (Kutkienė *et al*. 2009, 2010; Olias 2010*a*, *b*). Cystozoites of *S. falcatula* had similar dimensions (2·2 × 6·9 μm), but were a little wider; whereas cystozoites of *S. rileyi, S. anasi* and *S. albifronsi* were significantly larger (Box *et al*. 1984; Kutkienė *et al*. 2006, 2008).

Birds of prey act as definitive hosts of numerous *Sarcocystis* species (Černá, 1984; Svobodova, 1996; Yabsley *et al*. 2009). Recently, it was shown that sparrowhawks and goshawks (*Accipiter gentilis*) that originated from Northern Germany were highly infected with *S. calchasi, S. columbae* and *Sarcocystis* sp. ex *Accipiter nisus* (Olias *et al*. 2011). On the basis of the results of the DNA analysis presented in this article, *S. turdusi* and *Sarcocystis* sp. ex *Accipiter nisus* may be the same species; however cross-transmission experiments are needed for the final approval of this hypothesis. Across most of Lithuania, sparrowhawks and goshawks are a fairly common breeding species that have stable populations and are regarded as short- or medium-distance migrants (Stratford, 1999; Žalakevičius, 2007). Small passerine birds, thrushes, finches, tits, warblers, starlings, buntings and sparrows are the most important preys of sparrowhawks. On the contrary, goshawks are less specialized as predators of birds, they feed on birds – jays, finches, thrushes, woodpeckers, doves, jackdaws, hooded crows and small mammals – voles, moles, squirrels (Logminas, 1990). According to the genetic results available, *S. turdusi* is most likely transmitted by European *Accipiter* hawks, and ecological data are in agreement with this hypothesis.

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