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Regional report

Molecular characterization of three *Sarcocystis* spp. from wild sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan

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ABSTRACT

Diaphragm samples from 65 hunted sika deer (*Cervus nippon yesoensis*) from Hokkaido, Japan were examined for the presence of sarcocysts based on histological sections. Morphologically, the detected sarcocysts grouped into three types: (Type 1) 108.0–305.0 μm in width, thick-walled (4.3–7.0 μm) with tombstone-like protrusions; (Type 2) 25.0–69.5 μm in width, thick-walled (3.8–8.0 μm) with finger-like protrusions; and (Type 3) 22.5–55.0 μm in width, thin-walled (under 1 μm) with no visible protrusions under light microscopy. All samples contained at least one sarcocyst type, and multiparasitism was apparent in 58 samples. Morphologically, Type 1 sarcocysts were found in 19 (29.2%) samples, Type 2 in 62 (95.4%) samples, and Type 3 in 60 (92.3%) samples. The sarcocysts were collected using laser microdissection, the DNA extracted from them was PCR-amplified, and their 18S ribosomal RNA and cytochrome *c* oxidase subunit 1 genes were sequenced. Phylogenetic analysis showed that, for both genes, each morphological sarcocyst type (Types 1, 2, and 3) aligned most closely with *S. silva/S. truncata*, *S. tarandi/S. elongata*, and *S. pilosa*, respectively. Based on the sequence identities between taxa and the molecular information for sarcocysts in *C. nippon centralis*, the sarcocyst types were presumed to be *S. truncata*-like (Type 1), *S. tarandi*-like (Type 2), and *S. pilosa* (Type 3). The phylogenetic analyses based on the present comprehensive molecular characterization of three *Sarcocystis* spp. from *C. nippon yesoensis* in Hokkaido suggest that canids (e.g., wild foxes) may be the definitive hosts for *S. pilosa*, and felids (or unknown species) the definitive hosts for the other two species.

1. Introduction

Coccidian protozoan parasites that belong to the genus *Sarcocystis*, which are characterized by an obligatory heteroxenous prey–predator two-host life cycle, are found in mammals, birds, and reptiles, and there are over 220 species (Prakas and Butkauskas, 2012). Distinguishing species based on only microscopy is difficult, because some species have similar morphological features, such as *S. tarandi* and *S. elongata* (Gjerde, 2014). Therefore, the use of morphological and molecular analyses is recommended to identify sarcocyst species; in particular, analysis of the cytochrome *c* oxidase subunit 1 (COI) gene is known to be helpful (Gjerde, 2013; Gjerde et al., 2017).

Cervids serve as intermediate hosts of numerous *Sarcocystis* species, and at least seven species were recorded in muscle from *Cervus nippon* in Lithuania (Prakas et al., 2016; Rudaitytė-Lukošienė et al., 2018). Two subspecies of wild sika deer (*C. nippon centralis* and *C. nippon yesoensis*) inhabit Japan, and both are known to have several morphologically distinct *Sarcocystis* species (Saito et al., 1998b). *Sarcocystis* spp. in *C. nippon centralis*, which exists on the main island of Japan, have been morphologically described as *S. sybillensis* and *S. wapiti* (Saito et al., 1998b; Arai et al., 2010); however, there has been disagreement regarding their original description (Dahlgren and Gjerde, 2010a). Therefore, the actual *Sarcocystis* species composition among those deer remains unclear, mainly because of a lack of molecular information. Recently, molecular studies on sarcocysts isolated from *C. nippon centralis* using the 18S ribosomal RNA (rRNA) gene (Matsuo et al., 2016)

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and the COI gene were conducted, and the latter revealed the presence of at least five *Sarcocystis* species, including one known species, *S. pilosa* (Abe et al., 2019; the sequences are registered as *Sarcocystis* sp. NA in the GenBank/EMBL/DDBJ databases).

However, there has not yet been characterization of sarcocysts in *C. nippon yesoensis,* which inhabit Hokkaido. Among several morphologically distinct sarcocysts, only one species was well studied and identified as being *S. ovalis* based on molecular study, with corvids recognized as the definitive hosts (Takano et al., 2006; Abe, 2014; Irie et al., 2017). Narisawa et al. (2008) described three morphologically different sarcocysts, and Dahlgren and Gjerde (2010a) suggested that the three sarcocyst types might be *S. rangiferi*-type (described as a Type 1 sarcocyst by Narisawa et al., 2008), *S. tarandi*-type (Type 2), and *S. hjorti*-type (Type 3); however, the species assignments lacked molecular confirmation. Molecular studies on *Sarcocystis* spp. in *C. nippon yesoensis* are scarce, but partial 18S rRNA gene sequences from one species are available in the aforementioned databases (unpublished study that generated sequences registered as *Sarcocystis* sp. HM050622). Phylogenetic analysis using those sequences suggested that a species related to *S. truncata* and *S. rangiferi* may be present in Hokkaido (Dahlgren and Gjerde, 2010a). However, with no morphological information available, species identification was not undertaken. Therefore, there is a need for more comprehensive morphological and molecular studies of *Sarcocystis* spp. from *C. nippon yesoensis* in Hokkaido.

The objective of the present study was to identify *Sarcocystis* spp. from *C. nippon yesoensis* in Hokkaido with molecular methods. In wild sika deer in Japan, co-infections with different species is often suspected, and the significance of the isolation methods used for different sarcocyst types was described (Matsuo et al., 2016). In this study, therefore, morphologically distinct sarcocysts were collected using laser microdissection (LMD) after histological observation of the diaphragm samples from *C. nippon yesoensis*, and were characterized based on molecular analyses of the 18S rDNA and COI genes. Identifying the prevalent and dominant *Sarcocystis* species in Hokkaido and elucidating those definitive hosts based on phylogenetic and epidemiological information could help clarify their life cycle.

2. Materials and methods

2.1. Diaphragm collection from deer

In collaboration with local hunters, diaphragm muscle samples were collected from 65 hunted wild sika deer, *C. nippon yesoensis*, in Hokkaido, Japan, from October 2015 to July 2018. Each sample was cut into pieces, and three blocks (each about 1-cm square) were fixed in phosphate-buffered saline–formalin solution, and the remaining samples were stored at −30 °C.

2.2. Histological examination of the diaphragm

In order to increase the number of muscular fibers observed, the formalin-fixed diaphragm samples were oriented vertically when embedded in paraffin blocks, so that the muscle fibers would be cut in cross section. The sections were then stained with hematoxylin and eosin for histological analysis. From each animal, two sequential sections from three different parts of the diaphragm (six sections altogether) were examined. Sarcocysts were morphologically classified as follows using light microscopy according to the descriptions by Narisawa et al. (2008): (Type 1) < 360 μm in width, thick-walled (4–7 μm) with tombstone-like protrusions; (Type 2) < 78 μm in width, thick-walled (4–8 μm) with finger-like protrusions; and (Type 3) < 76 μm in width, thin-walled (under 1 μm) with hair-like protrusions, which are recognizable with transmission electron microscopy (TEM) (no visible protrusions were noted with light microscopy).

2.3. DNA extraction, PCR, and sequencing

Because Type 1 sarcocysts were large enough to be observed macroscopically, six individual cysts from different diaphragm samples were excised by blunt dissection using ophthalmology scissors and tweezers, which also resulted in the removal of a small amount of the adjacent host tissue. Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sarcocysts with Type 2 or 3 morphologies were collected for molecular analysis using LMD. Briefly, paraffin-embedded tissue was cut into 6-μm-thick sections and placed onto membrane slides (Carl Zeiss, Oberkochen, Germany). After deparaffinization, dehydration, and staining with toluidine blue, each morphological sarcocyst type was individually excised using the MicroBeam Rel.4.2 (Carl Zeiss, Oberkochen, Germany) LMD system, and then placed into a 0.6-ml tube. Approximately 200 sections from several sarcocysts of the same morphological type in sequential tissue samples were pooled. Genomic DNA was extracted from them using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Genes were PCR-amplified using the 18S rRNA gene-specific (around 1600 bp) primers 1L (Dahlgren and Gjerde, 2007) and R6 (current study: 5′-CGGAACACTCAATCGGTAGG-3′). Sequencing was performed using same primers with two additional primers, F5 (forward: 5′-GGGTGTGYACWTGRTSAATT-3′) and R5 (reverse: 5′-GCAAA TGCTTTCGCAGTAG-3′). For the COI gene (around 1000 bp), PCR sequencing was conducted using the SF1 forward primer, and SR9 or SR8D reverse primers (Gjerde, 2013, 2014). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). For phylogenetic analysis, sequences other than *Sarcocystis* spp. whose lengths exceeded 1500 bp for the 18S rRNA gene and 900 bp for the COI gene were obtained from the GenBank/EMBL/DDBJ databases and with reference to Rudaitytė-Lukošienė et al. (2018) and Abe et al. (2019) (Supplementary Table S1). The multiple sequence alignment of the 18S rRNA gene was generated with the default values for gap openings and extension penalties in the MUSCLE program (Edgar, 2004). The deduced amino acid sequences of the COI gene were aligned using the same program, and then re-converted back into nucleotide sequences. After the best-fit model of nucleotide substitution was tested, a maximum likelihood tree was constructed using MEGA7 (Kumar et al., 2016).

3. Results

The 65 diaphragm samples all contained at least one sarcocyst type. The morphology of each sarcocyst type, as observed across tissue sections, was as follows: Type 1 cysts: 108.0–305.0 μm in width, a 4.3–7.0 μm thick wall with tombstone-like protrusions; Type 2 cysts: 25.0–69.5 μm in width, a 3.8–8.0 μm thick wall with finger-like protrusions; and Type 3 cysts: 22.5–55.0 μm in width, a thin wall (under 1 μm) with no protrusions visible under light microscopy (Fig. 1). The number of samples comprising Type 1 sarcocysts was 19 (29.2%), Type 2 was 62 (95.4%), and Type 3 was 60 (92.3%). Cysts with morphologies different from these three types were not observed. Sarcocysts of two and three morphological types were observed in samples from 39 and 19 of the deer, respectively (Supplementary Table S2).

Among these samples, six representative genomic DNA samples from each morphological sarcocyst type were PCR-amplified and sequenced at the 18S rRNA and COI genes. Sequences were deposited in the GenBank/EMBL/DDBJ nucleotide sequence database (accession numbers: LC466166–LC466201, Supplementary Table S3). Phylogenetic trees based on the 18S rRNA and COI genes are shown in Figs. 2 and 3, respectively. The 18S rRNA gene sequences from Type 1 sarcocysts clustered with the sequences from *S. truncata*, *S. silva*, *Sarcocystis* sp. NA-1, and *Sarcocystis* sp. HM050622. By contrast, the COI gene sequences from Type 1 sarcocysts clustered with those of

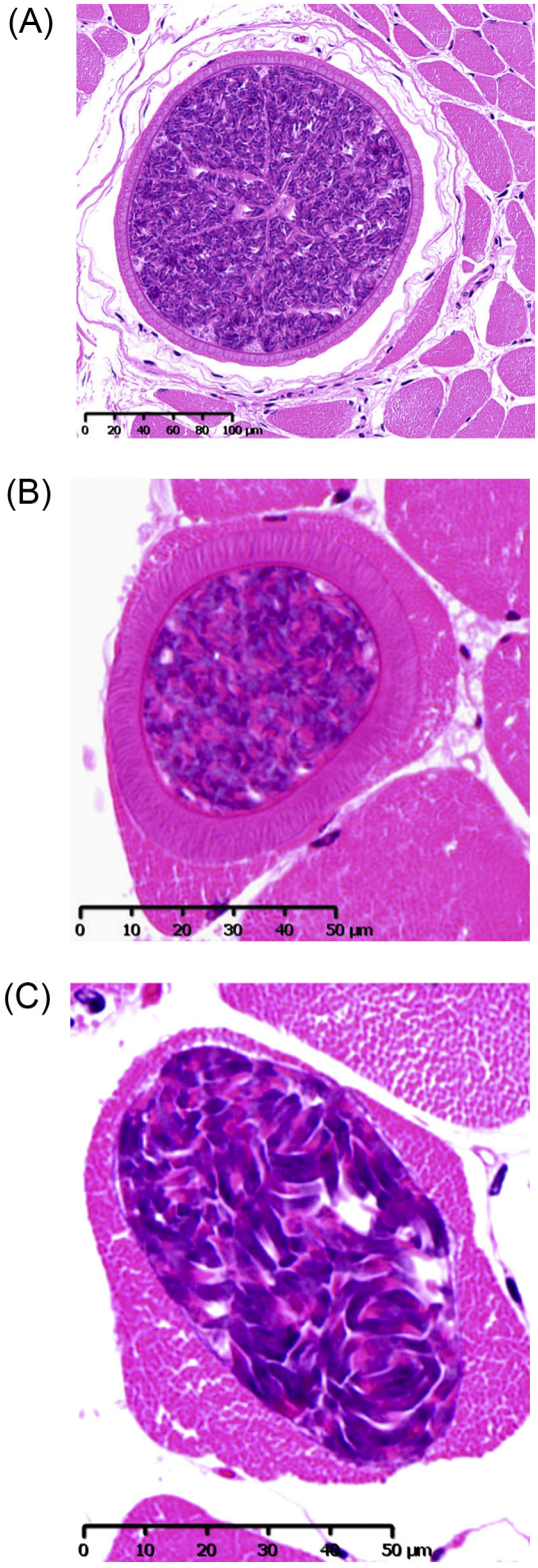

Fig. 1. Sarcocyst morphology under light microscopy. A: Type 1 cyst, thick-walled with tombstone-like protrusions, B: Type 2 cyst, thick-walled with finger-like protrusions, and C: Type 3 cyst, thin-walled (under 1 µm) with no visible protrusions.

Sarcocystis sp. NA-1 only, and these were distinct from those of *S. silva* and *S. truncata*. The 18S rRNA sequences of the Type 1 species were 99.5% and 99.3% identical to those of *S. truncata* and *S. silva*, respectively, and the COI sequences were 96.6% and 96.7% identical to these species. The sequence identity values for *S. truncata* and *S. silva* were similar for both genes (Supplementary Table S3). The 18S rRNA gene sequences from the Type 2 sarcocysts clustered together with *S. tarandi, S. elongata*, and *Sarcocystis* sp. NA-2, while those from the COI gene clustered with sequences from *Sarcocystis* sp. NA-2 only, and these were sister taxa to sequences of *S. tarandi*. Slightly higher sequence identities for both genes from the Type 2 sarcocysts were ascribed to *S. tarandi* than to *S. elongata* (Table S3). For the Type 3 sarcocysts, the sequences of both genes clustered with *S. pilosa* and *Sarcocystis* sp. NA-4, with sequence identities exceeding 99% for these taxa (Table S3).

4. Discussion

Sarcocysts were detected in all 65 diaphragm samples from *C. nippon yesoensis* in Hokkaido, Japan. The morphological features of the three sarcocyst types were determined as described by Narisawa et al. (2008). To better understand the parasite species in this study, the three sarcocyst types we isolated were molecularly analyzed according to their 18S rRNA and COI genes. Notably, we found that sarcocysts exhibiting Type 3 morphology were molecularly identical to *S. pilosa*, which was originally isolated from *C. nippon* in Lithuania (Prakas et al., 2016), and the species has previously also been detected in *C. nippon centralis* in Japan (Abe et al., 2019). The Type 3 sarcocysts described by Narisawa et al. (2008) might represent *S. pilosa* sarcocysts, because TEM-based morphology of the sarcocysts was consistent with the original description, in which *S. pilosa* sarcocysts are characterized by hair-like protrusions arising from its microtubule-lacking dome-shaped base. The definitive hosts for *S. pilosa* are suspected to be Canidae family members, because our phylogenetic analysis revealed that *S. pilosa* fell within the clade comprising *Sarcocystis* spp. using Canidae as the definitive host. This accords with the finding that sarcocysts from the related species *S. hjorti* have been experimentally shown to reproduce in foxes (Dahlgren and Gjerde, 2010b). Sarcocysts that are morphologically similar to *S. pilosa* have been described in *C. nippon centralis* and *C. nippon yesoensis* in Japan, and these types of sarcocysts are able to infest and reproduce in dogs (Saito et al., 1995, 1998b; Arai et al., 2010). Large populations of the wild fox (*Vulpes vulpes schrencki*) inhabit Hokkaido (Uraguchi, 2015), making them a potential definitive host animal for *S. pilosa* in Hokkaido. To clarify the life cycle and the reason (s) for the high prevalence of *S. pilosa* among deer, surveys on wild foxes are warranted.

The 18S rRNA and COI gene sequences from the Type 1 and 2 sarcocysts were nearly identical to those recently obtained from *C. nippon centralis* (Abe et al., 2019). Whereas the 18S rRNA gene tree placed Type 1 sarcocysts with *S. truncata/S. silva* and Type 2 with *S. tarandi/S. elongata*, these species formed different clades in the COI gene tree. As mentioned by Dahlgren and Gjerde (2010a), these Type 1 and 2 sarcocysts were morphologically categorized as *S. rangiferi*-type and *S. tarandi*-type, respectively. The former is now assigned as *S. truncata* based on morphological and molecular studies (Gjerde, 1985, 2014). *Sarcocystis tarandi* and *S. elongata* sarcocysts were described as morphologically indistinguishable, but molecularly distinguishable via COI gene analysis (Gjerde, 2014). Furthermore, the molecular identification of a Type 1 sarcocyst in a recent analysis by Abe et al. (2019), which is identical to the one from the present study, indicate that Type 1 sarcocysts represent a new species that is closely related to *S. truncata/S. silva*; whereas Type 2 sarcocysts might represent a new species or *S. tarandi*. Therefore, our Type 1 and 2 sarcocysts are assumed to be *S. truncata*-like and *S. tarandi*-like, respectively. Although these two species phylogenetically cluster in a clade represented by an unknown/felid definitive host group, the definitive hosts for *S. tarandi* remain unknown (Dahlgren and Gjerde, 2010a), as are the hosts for *S. truncata*, but Felidae are phylogenetically and epidemiologically the suspected host family (Gjerde, 2014). Only one felid species, *Felis catus*, is present in Hokkaido (Izawa and Nakanishi, 2015). Whether this felid has the opportunity to ingest deer meat (e.g., from a carcass) is unclear;

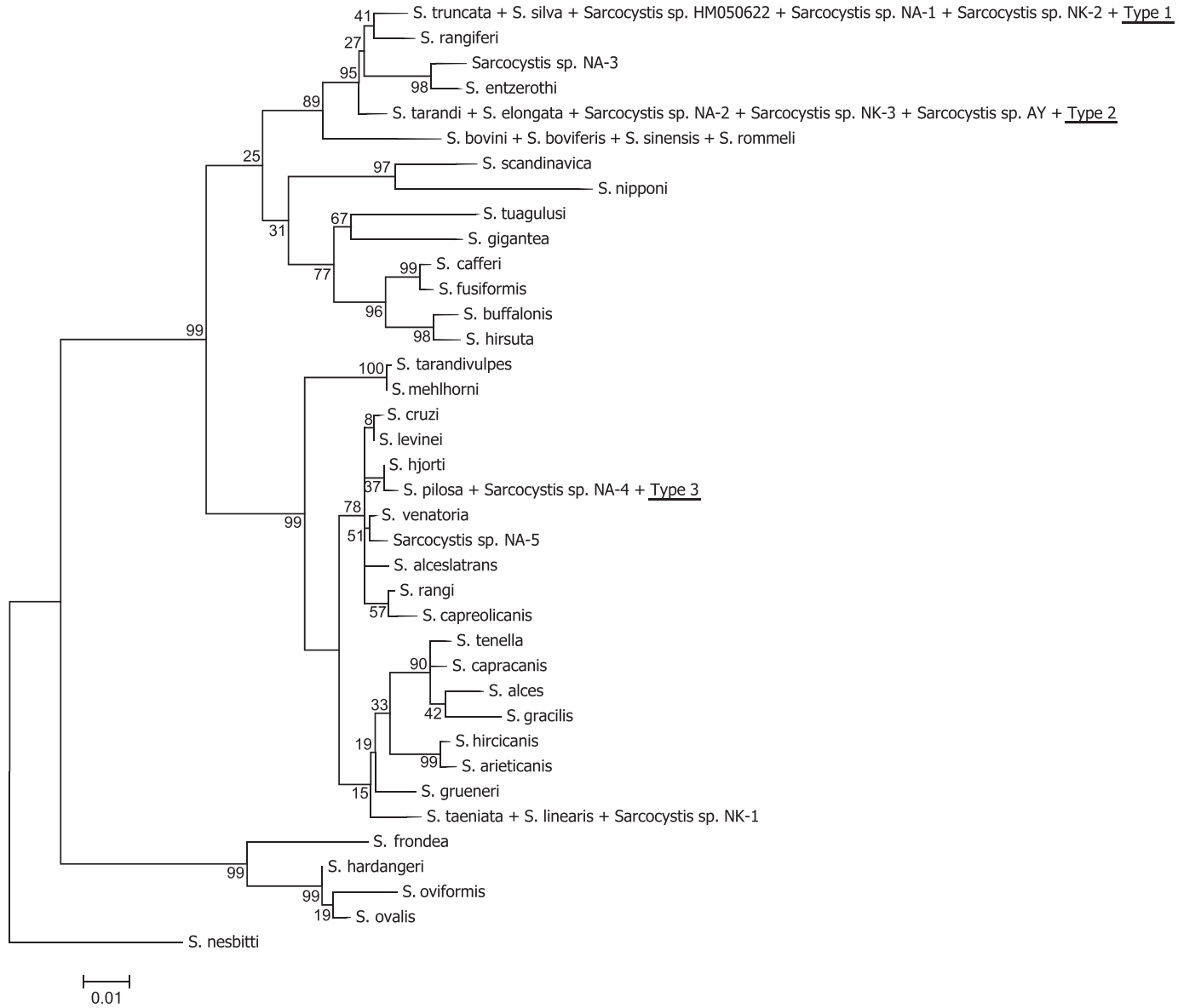

Fig. 2. Maximum likelihood tree for *Sarcocystis* based on 18S rRNA gene sequences that was constructed using the GTR + G + I model. Bootstrap scores are expressed as percentages of 1000 replications and are shown on each node. Sequences from the present study are underlined. GenBank accession numbers of all sequences included in the analysis are given in Supplementary Table S1.

therefore, examining the dietary habits and *Sarcocystis* prevalence in wild cats is of interest.

In the examined diaphragm samples, *S. tarandi*-like sarcocysts were most commonly detected (95.4%) followed by *S. pilosa* (92.3%), with *S. truncata*-like being the least common (29.2%). This prevalence difference might reflect different predilections of these species for different muscle groups; *S. truncata*-like is less common in the diaphragm than *S. tarandi*-like and *S. pilosa*. In fact, different *Sarcocystis* species parasitize different muscle types; for example, *S. cruzi* in cattle are mostly harbored in the heart, followed by the diaphragm (Saito et al., 1998a; Matsuo and Goto, 2013), whereas *S. sybillensis* and *S. wapiti* in deer are mostly found in the diaphragm but not in the heart (Saito et al., 1998b). This was similar to *S. ovalis,* of which sarcocysts have been isolated from deer in Hokkaido (Takano et al., 2006; Abe, 2014), and this parasite's life cycle is known to be maintained in the Corvidae definitive host (Irie et al., 2017); however, we did not detect *S. ovalis* in our specimens. One possible reason for this might be related to muscle type examined, because Takano et al. (2006) sampled skeletal muscles and we sampled diaphragm muscles. Furthermore, low intensities of parasitism of such corvid-transmitted species have been suggested for deer (Dahlgren and Gjerde, 2008, 2010a). Thus, examining different muscle types is now needed to fully evaluate the prevalence of the different *Sarcocystis* species in Hokkaido.

In conclusion, all of the deer were positive for sarcocysts in the present study. Although similar sarcocysts to those from our study have previously been detected morphologically, they were not molecularly evaluated. Using the LMD method, which can clearly connect morphological and molecular information, we molecularly characterized three types of sarcocysts from *C. nippon yesoensis* as *S. truncata*-like, *S. tarandi*-like, and *S. pilosa.* These three species were already suspected to be commonly prevalent in *C. nippon centralis* in Japan. Based on the life cycles reported for related species, candidates for the definitive hosts in our surveyed area are suspected to be canids (e.g., wild foxes) for *S. pilosa* and felids (or unknown) for the other two species. To clarify why *Sarcocystis* species are highly endemic among deer, determining the main definitive hosts for the different *Sarcocystis* species and evaluating their prevalences should be undertaken.

Supplementary data to this article can be found online at https://

Fig. 3. Maximum likelihood tree for *Sarcocystis* based on COI gene sequences that was constructed using the K2 + G model. Bootstrap scores are expressed as percentages of 1000 replications and are shown on each node. Sequences from the present study are underlined. GenBank accession numbers of all sequences included in the analysis are given in Supplementary Table S1.

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Ethical statement

We have read and understood the International Guiding Principles for Biomedical Research Involving Animals.

Our investigation in this study has not included animal experiment. For the research, we used specimens from wild deer, and we assuredly handled those specimens with dignity.

Declaration of Competing Interest

There are no relationships or support that might be perceived as constituting a conflict of interest.

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