Parasitology Research (2019) 118:1975–1979
https://doi.org/10.1007/s00436-019-06319-5

GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Molecular differentiation of five *Sarcocystis* species in sika deer (*Cervus nippon centralis*) in Japan based on mitochondrial cytochrome c oxidase subunit I gene (*cox1*) sequences

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Received: 28 December 2018 / Accepted: 9 April 2019 / Published online: 18 April 2019

Abstract

Several surveys of *Sarcocystis* infection in sika deer in Japan have shown a high prevalence, but the identification has been unclear because molecular data have been lacking or have been limited to 18S ribosomal RNA gene sequences. Thus, in our previous study based on such sequences, some *Sarcocystis* isolates from sika deer were not clearly separated from other species in the phylogenetic analysis. In the present study, we therefore characterized sarcocyst isolates from sika deer (*Cervus nippon centralis*) at the mitochondrial cytochrome c oxidase subunit I gene (*cox1*). Moreover, we developed a multiplex PCR based on *cox1* sequences of all species found, so that we could rapidly identify sarcocysts of these species. Twenty-one sarcocysts from nine sika deer were examined. Five distinct *cox1* sequence types, each with a high sequence identity (> 99%), were found, and the sarcocysts could thus be classified into five species. Based on the sequence comparisons and the phylogeny, *Sarcocystis* spp. of types 1, 3, and 5 are considered to represent three new species, which were most closely related to *Sarcocystis silva/Sarcocystis truncata*, *Sarcocystis entzerothi*, and *Sarcocystis iberica/Sarcocystis venatoria*, respectively. There was a slight uncertainly whether *Sarcocystis* sp. with type 2 sequences represented a new species or was identical to *Sarcocystis tarandi*. Type 4 sequences showed 99% identity with those of *Sarcocystis pilosa* from sika deer in Lithuania and have therefore been assigned to this species. In the multiplex PCR, type-specific fragments were successfully amplified for all five *Sarcocystis* spp., indicating that this assay may be useful for a rapid identification of sarcocysts of these species.

Keywords *Sarcocystis* · *Cervus nippon centralis* · Japan · Cytochrome c oxidase subunit I gene · Multiplex PCR

Introduction

The genus *Sarcocystis* comprises intracellular protozoan parasites that require two hosts to complete their life cycle. Carnivores act as definitive hosts, whereas herbivores, omnivores, and carnivores act as intermediate hosts, harboring the sarcocyst stage in their muscles. In the intermediate hosts, it is not possible to discriminate between *Sarcocystis* spp. with

Section Editor: Daniel K. Howe

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-019-06319-5) contains supplementary material, which is available to authorized users.

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similar sarcocyst morphology by light and electron microscopy. Therefore, molecular characterization using the appropriate markers has become an essential tool for accurate identification of *Sarcocystis* species and for research on the phylogenetic relationships of these species. As regards *Sarcocystis* species in cervids, nucleotide sequences of the nuclear 18S ribosomal RNA gene (18S rDNA) and/or the mitochondrial cytochrome c oxidase subunit I gene (*cox1*) of about 30 named species are currently available for comparative molecular studies (Gjerde et al. 2017b; Rudaitytė-Lukošienė et al. 2018). Of these markers, *cox1* has been found to be superior to 18S rDNA in resolving unclear species boundaries of closely related *Sarcocystis* species in various ruminant intermediate hosts (Gjerde 2013, Gjerde et al. 2017a, b; Rudaitytė-Lukošienė et al. 2018).

In Japan, several surveys of *Sarcocystis* infection in sika deer have shown a high prevalence (Narisawa et al. 2008; Matsuo et al. 2014; Saito and Hagiwara 2013; Saito et al. 1998). In these studies, the isolates have only been tentatively identified on the basis of their morphological similarity to other named species, and no comparative molecular studies with already known *Sarcocystis* spp. have been performed. However, some isolates from sika deer have been well characterized at the 18S rRNA, as determined from sequences deposited in GenBank, but no accompanying morphological data have been published. Recently, Irie et al. (2017) identified *Sarcocystis ovalis* in sika deer based on an 18S rDNA sequence. Likewise, in our previous study (Matsuo et al. 2016), we characterized isolates from sika deer at the 18S rDNA locus and subjected the sequences to a phylogenetic analysis. Our isolates then seemed to belong to four or five species, but it could not be determined if they represented new species or previously known species. Moreover, subsequent to that study, seven *Sarcocystis* spp. have been characterized molecularly from sika deer in Lithuania (Prakas et al. 2016; Rudaitytė-Lukošienė et al. 2018).

Multiplex PCR consisting of multiple primer sets within a single PCR mixture may produce amplicons of varying sizes depending on primer pair. If species-specific primers are used, a preliminary identification to species may be rapidly achieved through electrophoresis of the amplicons on agarose gels and thus without sequencing. This methodology may be useful for screening sarcocysts in order to rapidly determine which *Sarcocystis* species they belong to. As regards *Sarcocystis* infection in animals, multiplex PCR (targeting the 18S rDNA locus) has so far only been applied for identification of sarcocysts in cattle (Moré et al. 2013).

The major aim of the present study was to characterize sarcocyst isolates from sika deer in Japan at *cox1* and then use sequence comparisons and phylogenetic analysis to determine speciation and the relationships of these species to other *Sarcocystis* spp. characterized at this locus previously. A second aim was to develop a multiplex PCR using species-specific primers targeting *cox1* of the species found by standard PCR in order to be able to rapidly identify additional sarcocysts of these species in subsequent studies.

Materials and methods

Portions of the skeletal muscles were obtained from nine sika deer that had been legally hunted in the Gifu prefecture between May 2015 and October 2016 (Matsuo et al. 2016). The muscle samples were examined under a stereo microscope and a total of 21 sarcocysts were excised using a fine needle and tweezers (Table S1 in Supplementary material). It was noticed that the size and shape of the sarcocysts varied (thread-like, fusiform, nearly oval), but the cyst wall morphology of the collected sarcocysts was not recorded (Matsuo et al. 2016).

A 1085-bp-long portion of *cox1* was amplified using primer pair SF1/SR9 (Gjerde 2013, 2014). Details about the PCR amplification, the evaluation and purification of PCR products, their cloning into a vector, sequencing, and use of the new *cox1* sequences in the phylogenetic analysis are given in Supplementary material. The same applies to details about the primers (Table S2) and procedures used in the multiplex PCR.

Results and discussion

A total of 39 positive clones of *cox1* were obtained and sequenced from 21 sarcocysts from nine sika deer. All *cox1* sequences obtained were 1038 bp long (primers not included) and have been deposited in DDBJ under accession numbers LC349938–LC349976 (Table S1). The sequences could be classified into five types (types 1–5), each with a high sequence identity (> 99%) and were therefore considered to represent five *Sarcocystis* spp. The most common species among the 21 isolated sarcocysts was that with type 1 sequences, which was identified in 12 sarcocysts from five sika deer, whereas types 2, 3, 4, and 5 were identified from 1, 1, 2, and 5 sarcocysts, respectively. Since the PCR products were cloned, types 1–5 were represented by 22, 3, 2, 3, and 9 *cox1* sequences, respectively (Table S1).

Sequence comparisons revealed which of the previously known species each *cox1* sequence type shared the highest identity with (Table S3). As expected, a similar relationship between the species was evident from the phylogenetic analysis (Fig. 1). Thus, sequences of type 1 were sister to those of *S. silva*, whereas sequences of type 2 were sister to those of *S. tarandi*, and the latter two taxa were clearly separated from sequences of *S. elongata*. Sequences of type 3 were separated with near maximum support from those of *S. entzerothi*. Sequences of type 4 clustered close to but separately from previous sequences of *S. pilosa*, and thus seemed to represent a separate subpopulation of this species. Sequences of type 5

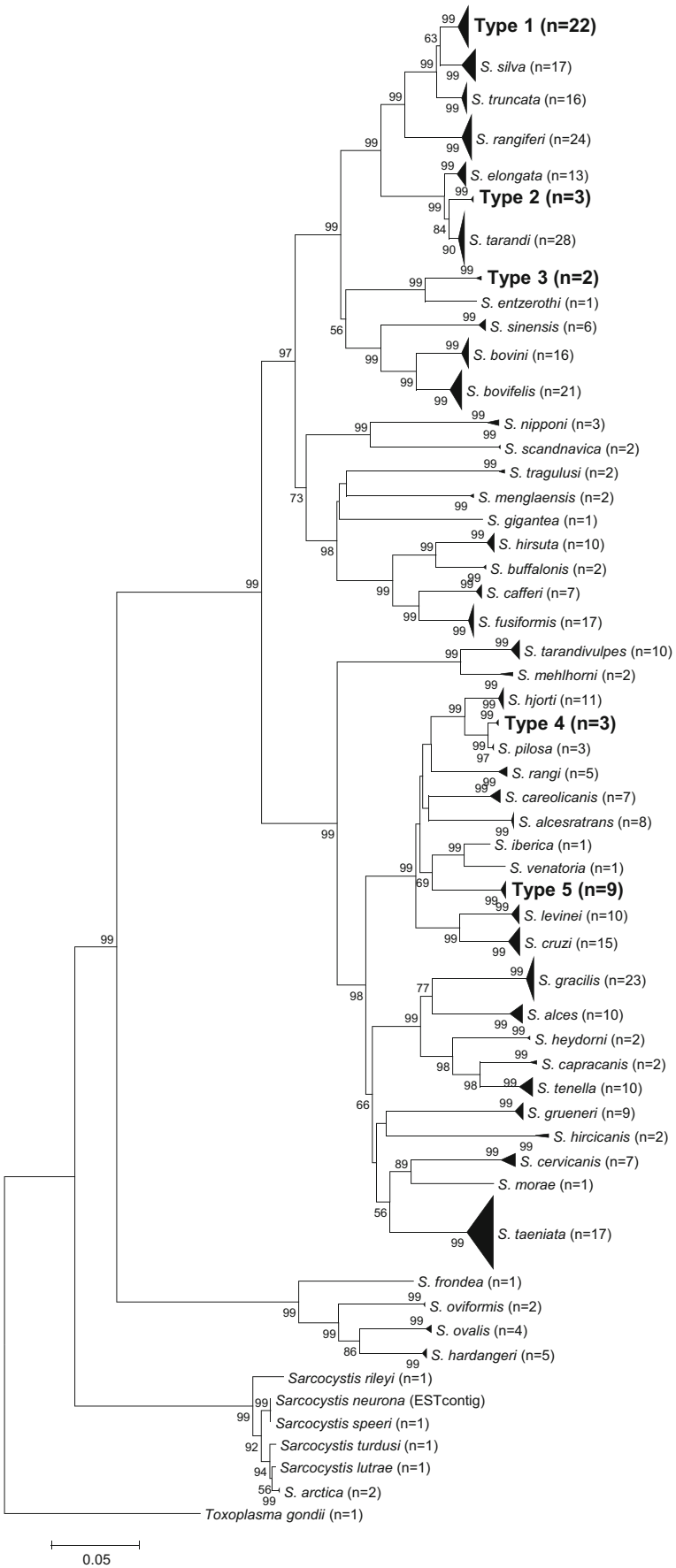

Fig. 1 Phylogenetic tree for members of the Sarcocystidae based on 425 partial sequences of *cox1* of 55 taxa including the five *Sarcocystis* species from this study (types 1 to 5; in bold) and inferred using the neighbor-joining method and with evolutionary distances computed using the p-distance method. Bootstrap support (> 50% for 1000 replicates) is shown at each node. Subtrees formed by two or more haplotypes of the same species have been collapsed, and the number of haplotypes included is given in parentheses. The GenBank accession numbers of all sequences except those from the present study (see Table S1) and from *S. nipponi* and *S. frondea* are the same as in Gjerde et al. (2017b)

were sister to sequences of *S. iberica* and *S. venatoria* within a clade comprising species with hair-like cyst wall protrusions, including *S. pilosa*.

The multiplex PCR assay using the type-specific primers proved to be able to discriminate between the five *Sarcocystis* species found by standard PCR. Thus, the type-specific fragments were only amplified from the *Sarcocystis* sp. targeted by the different primer pairs (Fig. S1). This was confirmed by sequencing of the amplicons generated with the type-specific primers used in the multiplex PCR. These short sequences corresponded to the homologous portions of the *cox1* sequences obtained from the same sarcocysts when amplified with primer pair SF1/SR9 by standard PCR.

Using the *cox1* marker, the present study revealed that the examined sarcocysts from sika deer represented five separate *cox1* sequence types and thus five distinct *Sarcocystis* spp. Sequences of type 4 could be assigned to *S. pilosa*, whereas sequences of type 2 could not be clearly separated from those of *S. tarandi*, a species predominantly found in reindeer (Gjerde 2014). Since only three *cox1* sequences from a single type 2 isolate were available for comparison, additional sequences from more isolates of this type have to be obtained before assigning them to a particular species.

Sequences of types 1, 3, and 5, on the other hand, were distinct from all *Sarcocystis* spp. previously characterized at *cox1*, and thus seem to represent three new species. However, these species may have been found in sika deer in Japan previously, but have then only been characterized morphologically or at the 18S rDNA locus. By contrast, in our previous study based on partial 18S rDNA sequences (Matsuo et al. 2016), sequence groups 1–4, corresponding to the present *cox1* types 1–4, respectively, could not be clearly distinguished from closely related *Sarcocystis* species. Moreover, 18S rDNA sequences of *S. entzerothi* and *S. pilosa* were not available for comparison at that time. The previous study showed, however, that sequences of group 1 were closely related to GenBank sequences of the unnamed *Sarcocystis* sp. HM050622 from Hokkaido sika deer, and it is therefore likely that the latter species is identical with the species with type 1 sequences from the present study. The sarcocysts of *Sarcocystis* sp. type 1 were fairly large and thick (see Fig. 2 in Matsuo et al. 2016), and from the close phylogenetic relationship of this species with *S. silva* and *S. truncata*, the sarcocysts might be expected to have fairly thick finger-like cyst wall protrusions (Gjerde 2014; Gjerde et al. 2017a; Rudaitytė-Lukošienė et al. 2018). Hence, this species might be the same as *Sarcocystis* sp. type 1 described morphologically by Narisawa et al. (2006).

The *cox1* sequences of *Sarcocystis* sp. type 3 were most similar to those of *S. entzerothi* but are considered to be distinct from this species. In previous studies (Prakas et al. 2017; Rudaitytė-Lukošienė et al. 2018), *cox1* sequences of *S. entzerothi* could not be amplified using reverse primer SR9, whereas sequences of type 3 could be successfully amplified with this primer, suggesting additional differences in the target area of primer SR9 between these species. In our previous study (Matsuo et al. 2016), 18S rDNA sequences of the present *Sarcocystis* sp. type 4 shared 99% identity with *Sarcocystis hjorti*, and they could not be clearly distinguished from that species. Subsequent to that study, *S. pilosa* was described from sika deer and found to indistinguishable from *S. hjorti* at the 18S rDNA locus (Prakas et al. 2016). When using *cox1*, however, type 4 sequences shared an identity of only 96% with those of *S. hjorti*, but an identity of > 99% with those of *S. pilosa*, and could therefore be assigned to the latter species.

Sarcocystis sp. type 5 was most similar to *S. iberica* and *S. venatoria* in red deer, but it was distinct from these and other named species and therefore represents a new species. The aforementioned two species in red deer have hair-like cyst wall protrusions (Gjerde et al. 2017b), and in the phylogenetic analysis (Fig. 1), our type 5 sequences were placed within a subclade comprising several *Sarcocystis* spp. with hair-like protrusions, including *S. pilosa* (Prakas et al. 2016). Hence, it may be inferred that sarcocysts of *Sarcocystis* sp. type 5 also possess hair-like protrusions and will probably be indistinguishable from those of *S. pilosa*. If so, the previously reported *Sarcocystis* sp. with hair-like protrusions in sika deer in Japan might be either *S. pilosa* or *Sarcocystis* sp. type 5, or a mixture of both species. In previous studies, the species with hair-like cyst wall protrusions has been designated *Sarcocystis* sp. type 3 (Narisawa et al. 2006), *Sarcocystis* sp. 2 (Saito et al. 1998), and *Sarcocystis wapiti* (Saito and Hagiwara 2013).

At least two more *Sarcocystis* spp. than those found in the present study have been identified in sika deer in Japan by molecular methods comprising sequencing of the 18S rDNA. Thus, *S. ovalis* has been identified both as sarcocysts in skeletal muscles of Hokkaido sika deer as an intermediate host and as oocysts in the intestinal mucosa of a Japanese jungle crow (Irie et al. 2017). In addition, an 18S rDNA sequence of the unnamed *Sarcocystis* sp. T18 from sika deer in Hyogo Prefecture is available in GenBank (AB698065). Based on this sequence, this species was found to be indistinguishable from *Sarcocystis cervicanis* in red deer (Gjerde et al. 2017b).

Molecular identification of morphologically similar sarcocysts from wild deer has traditionally been performed by standard PCR followed by sequencing of *cox1* and/or 18S rDNA. In the present study, all sarcocyst isolates were successfully typed by multiplex PCR using type-specific primers, indicating that this methodology may be useful for a rapid screening of DNA from individual sarcocysts for the presence of the five *Sarcocystis* species identified in this study.

Funding information This study was supported by a Grant-in-Aid for Scientific Research (C) (17K09185) from the Japan Society for the Promotion of Sciences (JSPS).

Compliance with ethical standards

The sika deer used in the present study were legally hunted (Matsuo et al. 2016).

Conflict of interest The authors declare that they have no competing interests.

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