

Morphological and molecular characteristics of six *Sarcocystis* spp. from red deer (*Cervus elaphus*) in Spain, including *Sarcocystis cervicanis* and three new species

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Abstract Samples of muscle tissue from the diaphragm, oesophagus and/or heart of eight adult red deer (*Cervus elaphus hispanicus*) from the Quintos de Mora Park in Toledo, Central Spain, were screened for sarcocysts by means of the compression method. From positive samples, individual sarcocysts were excised and examined in wet mounts under a light microscope (LM) in order to study their basic morphology before being preserved for molecular studies. In all red deer examined, only microscopic sarcocysts were found. Those in the diaphragm and oesophagus were spindle-shaped and about 1×0.1 mm in size, while those in cardiac muscle were sac-like and $500\text{--}800 \times 80\text{--}180$ μm . By LM, the sarcocysts either had densely packed, about 8- μm -long, hair-like protrusions (type 1), sparsely distributed indistinct projections (fuzzy outline; type 2) or no visible protrusions (smooth surface; type 3). In cardiac muscle, only sarcocysts without visible protrusions were found. One of the latter sarcocysts was examined by scanning electron microscopy (SEM) and found to possess thin ribbon-like protrusions. Forty-eight sarcocysts isolated from the diaphragm, oesophagus and heart of one red deer, as well as 55 sarcocysts from the heart of three

other red deer, 103 sarcocysts in total, were characterized molecularly through PCR amplification and sequencing of the partial cytochrome c oxidase subunit I gene (*cox1*) of the mitochondrial genome, revealing the presence of six major *cox1* sequence types. Each type comprised either a single sequence (three types) or a collection of several identical or nearly identical sequences. From selected isolates possessing each of these *cox1* sequence types, the complete 18S ribosomal RNA (rRNA) gene was amplified and sequenced directly and/or after cloning of the 5' end half. Supported by the sequence data from the latter gene, as well as the morphology of the sarcocysts from which the sequences originated, the six *cox1* sequence types were considered to represent six separate *Sarcocystis* spp. Two *cox1* sequence types were identified as belonging to the previously characterized species *Sarcocystis hjorti* (one sequence/sarcocyst) and *Sarcocystis linearis* (38 sequences/sarcocysts), respectively, whereas four sequence types were new. One of the latter types was assigned to the previously named species *Sarcocystis cervicanis* from red deer, since this sequence type was obtained from 52 sarcocysts from cardiac muscle, which matched the original morphological description (smooth surface) and habitat of this species. The remaining three sequence types were assigned to the three new species *Sarcocystis iberica* (one sequence/sarcocyst) *Sarcocystis venatoria* (10 sequences/sarcocysts) and *Sarcocystis morae* (one sequence/sarcocyst), respectively. The two species *S. iberica* and *S. venatoria* shared the same sarcocyst morphology (type 1) and habitat (diaphragm) and had virtually identical 18S rRNA gene sequences, but differed by 4% at *cox1*, which was considered sufficient to regard them as separate species. The single sarcocyst of *S. morae* (from the oesophagus) examined by LM had a smooth wall and this species was therefore believed to have the same type of ribbon-like protrusions (ultrastructurally) as sarcocysts of

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S. cervicanis and *S. linearis*, which were also the species most closely related to *S. morae* at *cox1*. Thus, there seems to be three species with similar ribbon-like cyst wall protrusions in red deer (*S. cervicanis*, *S. linearis*, *S. morae*), as well as three species with similar hair-like protrusions (*S. hjorti*, *S. iberica*, *S. venatoria*). Sarcocysts of *S. cervicanis* were only identified in cardiac muscle, whereas sarcocysts of *S. linearis* were found mainly in the diaphragm and oesophagus and rarely in the heart. The relative number of *cox1* haplotypes was greater among sequences/isolates of *S. linearis* (17/38) than among isolates of *S. cervicanis* (7/52). Four of the species examined (*S. cervicanis*, *S. linearis*, *S. iberica*, *S. venatoria*) possessed considerable intra-isolate (intra-genomic) sequence variation (insertions/deletions, substitutions) in the 18S rRNA gene.

Keywords Red deer · *Cervus elaphus* · Spain · Cytochrome c oxidase subunit I gene (*cox1*) · 18S rRNA gene · Phylogeny · Scanning electron microscopy · *Sarcocystis cervicanis* · *Sarcocystis hjorti* · *Sarcocystis iberica* · *Sarcocystis linearis* · *Sarcocystis morae* · *Sarcocystis venatoria*

Introduction

Protozoan parasites belonging to the genus *Sarcocystis* have an obligatory two-host life cycle comprising mainly carnivores as definitive hosts and herbivores and omnivores as intermediate hosts. Free-ranging cervids may serve as intermediate host for many *Sarcocystis* spp. (Gjerde, 2013; Gjerde et al., 2017). Previous molecular and/or morphological studies in several countries have indicated that the European red deer, *Cervus elaphus* (comprising five subspecies), may act as intermediate host for eight or nine different *Sarcocystis* spp. forming sarcocysts of five major morphological types (Table 1). Thus, some of these sarcocyst types represent more than one species. On the other hand, the species designated *Sarcocystis* cf. *hofmanni* by Wesemeier & Sedlaczek (1995a) seems to comprise two morphological types, corresponding to sarcocysts of *Sarcocystis elongata* and *Sarcocystis truncata*, respectively (Dahlgren & Gjerde, 2010a; Gjerde, 2014a). Likewise, it is not clear to which of the latter two morphological types one should assign sarcocysts initially designated *Sarcocystis hofmanni*-like2 (Prakas, 2011), but later believed to belong to *Sarcocystis entzerothi* (Prakas et al., 2017). In Table 1, this species has provisionally been grouped with sarcocysts of the *S. truncata* type.

Two *Sarcocystis* spp. have so far been reported from the North American elk or wapiti (*Cervus elaphus canadensis*) in the USA, of which *Sarcocystis wapiti* (Speer & Dubey, 1982) is morphologically indistinguishable from *Sarcocystis cervicanis* in European red deer (Table 1). The second named species from wapiti, *Sarcocystis sybillensis*, was reported by

Dubey et al. (1983) to have thick-walled (8 µm) sarcocysts when examined by light microscopy (LM) in histological sections, which suggested the presence of finger-like protrusions. By transmission electron microscopy (TEM), however, sarcocysts of allegedly the same species seemed to have hair-like protrusions like those of *Sarcocystis hjorti*, but sarcocysts with such protrusions are known to be thin-walled in histological sections. Dahlgren & Gjerde (2010a) therefore believed that *S. sybillensis* as described by Dubey et al. (1983) represented a mixture of at least two species with different sarcocyst morphology, which is why this taxon has not been included in Table 1.

In a recent study, 61 red deer hunted in the Quintos de Mora Park of the National Wildlife Reserves, Toledo, Central Spain, were examined for sarcocystosis, mainly for the purpose of comparing two diagnostic techniques, that is, compression of fresh muscle tissue and standard histology (Luzón et al., 2015). In addition, samples of the diaphragm and heart of three heavily infected red deer were examined under a stereo microscope, and sarcocysts were isolated and identified to morphological type in wet mounts under a light microscope. The prevalence study revealed that 59 of 61 (97%) red deer examined were infected with sarcocysts, whereas the limited morphological study of isolated sarcocysts, revealed the presence of two morph types. All sarcocysts isolated from cardiac muscle and about 70% of those from the diaphragm, had a thin wall with no visible protrusions, and were thus consistent with sarcocysts assigned to the species *S. cervicanis* by Hernández-Rodríguez et al. (1981a, b), whereas about 30% of the sarcocysts from the diaphragm had thin hair-like protrusions and were consistent with sarcocysts of *S. hjorti* as described from red deer and moose in Norway (Dahlgren & Gjerde, 2010a, b).

The abovementioned findings prompted the present study, in which the major aim was to identify which *Sarcocystis* spp. the different sarcocyst types in red deer from the Quintos de Mora reserve belonged to through the use of molecular methods, that is, sequencing of the complete nuclear 18S ribosomal (r) RNA gene and the partial mitochondrial cytochrome c oxidase subunit I gene (*cox1*). We were particularly interested in examining the *S. cervicanis*-like sarcocysts from red deer in this region by molecular methods in order to determine their relationship to the previously characterized and named species of this morphological type (*Sarcocystis grueneri*, *Sarcocystis taeniata*, *Sarcocystis linearis*) (Gjerde et al., 2017) through sequence comparisons and phylogenetic analyses. Thus, Hernández-Rodríguez et al. (1981a, b) originally described the species *S. cervicanis* on the basis of sarcocysts mainly from cardiac muscle of red deer from Sierra Morena, Córdoba, which lies about 200 km to the south of Quintos de Mora.

Table 1 Overview of *Sarcocystis* spp. previously reported from European red deer and North-American wapiti grouped according to their sarcocyst type

Sarcocyst type	Species designation ¹	Country	Studies performed ²	References
Microscopic sarcocysts with no visible protrusions by LM, but ribbon-like by TEM	<i>Sarcocystis cervicanis</i>	Spain	LMw, TEM; transmission to dogs	Hernández-Rodríguez et al., 1981a, b
	<i>Sarcocystis wapiti</i>	USA	LMh, TEM	Speer & Dubey, 1982
	<i>Sarcocystis</i> sp.	Hungary	TEM	Entzeroth et al., 1983
	<i>Sarcocystis</i> cf. <i>grueneri</i>	Germany	LMw, TEM	Wesemeier & Sedlaczek, 1995a
	<i>Sarcocystis</i> cf. <i>grueneri</i>	Lithuania	LMw	Kutkienė, 2003
	<i>Sarcocystis</i> cf. <i>grueneri</i>	Korea	LMh, TEM	Son et al., 2009
	(<i>Sarcocystis grueneri</i> -like), <i>Sarcocystis taeniata</i>	Lithuania	LMw; Mol: 18S rRNA gene	Prakas, 2011; Prakas et al., 2016
<i>Sarcocystis taeniata</i>	Argentina	LMh, LMw, TEM; Mol: 18S rRNA gene	Reissig et al., 2016	
Microscopic sarcocysts with hair-like protrusions	<i>Sarcocystis</i> cf. <i>capreolicanis</i>	Germany	LMw, TEM	Wesemeier & Sedlaczek, 1995a
	<i>Sarcocystis</i> cf. <i>capreolicanis</i>	Lithuania	LMw	Kutkienė, 2003
	<i>Sarcocystis hjorti</i>	Norway	LMw, SEM; transmission to foxes (from moose); Mol: 18S rRNA gene, <i>cox1</i>	Dahlgren & Gjerde, 2010a, b; Gjerde, 2013, 2014a
	<i>Sarcocystis hjorti</i>	Lithuania	LMw; Mol: 18S rRNA gene	Prakas, 2011
Macroscopic oval sarcocysts with slanting tongue-like protrusions	<i>Sarcocystis ovalis</i>	Norway	LMw, SEM; Mol: 18S rRNA gene, <i>cox1</i>	Dahlgren & Gjerde, 2010a; Gjerde, 2013, 2014a
	<i>Sarcocystis hardangeri</i>	Norway	LMw, SEM; Mol: 18S rRNA gene, <i>cox1</i>	Dahlgren & Gjerde, 2010a; Gjerde, 2013, 2014a
Microscopic, spindle-shaped sarcocysts with thin finger-like protrusions	(<i>Sarcocystis tarandi</i>), (<i>Sarcocystis</i> cf. <i>tarandi</i>), <i>Sarcocystis elongata</i>	Norway	LMw, SEM; Mol: 18S rRNA gene, ITS1, <i>cox1</i>	Dahlgren & Gjerde, 2010a; Gjerde, 2013, 2014a
	<i>Sarcocystis tarandi</i>	Norway	LMw; Mol: 18S rRNA gene, <i>cox1</i>	Gjerde, 2014a
	<i>Sarcocystis</i> cf. <i>hofmanni</i>	Germany	LMw; TEM	Wesemeier & Sedlaczek, 1995a
Macroscopic, cigar-shaped sarcocysts with thick finger-like protrusions	<i>Sarcocystis</i> cf. <i>hofmanni</i>	Lithuania	LMw	Kutkienė, 2003
	(<i>Sarcocystis rangiferi</i>), (<i>Sarcocystis</i> cf. <i>rangiferi</i>), <i>Sarcocystis truncata</i>	Norway	LMw, SEM; Mol: 18S rRNA gene, ITS1, <i>cox1</i>	Dahlgren & Gjerde, 2010a; Gjerde, 2013, 2014a
	(<i>Sarcocystis hofmanni</i> -like2), <i>Sarcocystis entzerothi</i>	Lithuania	LMw; Mol: 18S rRNA gene	Prakas, 2011; Prakas et al., 2017

¹ Preliminary species designations that were later changed by the same author(s) are in parentheses

² LMw light microscopic examination of isolated fresh sarcocysts in wet mounts; LMh light microscopic examination of sarcocysts in histological sections; TEM examination of sarcocysts by transmission electron microscopy; SEM examination of sarcocysts by scanning electron microscopy; Mol molecular identification of sarcocysts comprising sequencing of the given DNA regions; 18S rRNA gene the nuclear 18S ribosomal RNA gene; ITS1 the internal transcribed spacer 1 region; *cox1* the mitochondrial cytochrome c oxidase subunit 1 gene

Materials and methods

Collection of muscle samples and isolation of sarcocysts

Muscle samples were collected from eight adult red deer (*Cervus elaphus hispanicus*) that were killed during selective hunting in November 2014 in the Quintos de Mora park of the National Wildlife Reserves, Toledo, Central Spain (39° 25' N, 4° 04' W). For details about the park, see Santín-Durán et al. (2004). Portions of the heart (inter-ventricular septum), oesophagus and diaphragm (diaphragmatic pillars) of each

animal were placed in labelled plastic bags and transported in a cool box to the Animal Health Department at the Veterinary Faculty, Complutense University of Madrid, where the samples were kept at – 20 °C until further examined. Thawed samples were subsequently examined for sarcocysts by the compression technique used for detection of *Trichinella* larvae as previously described (Luzón et al., 2015). Briefly, sets of 28 oat-grain-sized muscle samples (about 2 mm × 10 mm), which had been cut from the full sample with scissors, were compressed between the two glass plates of a compressorium until they became translucent and

examined for sarcocysts using a conventional light microscope at $\times 100$ magnification. Individual samples comprising sarcocysts were released from the glass plates and transferred to separate microscope slides, covered with a drop of distilled water and kept inside a moist chamber at 4 °C for 1–2 h. The moistened muscle samples were then examined under a stereo microscope at $\times 20$ –30 magnification and the sarcocysts were excised from the muscle tissue using a fine insulin needle attached to a syringe. Isolated sarcocysts were further examined in a drop of distilled water under a Nomarski microscope at $\times 100$ –400 magnification and morphologically classified on the basis of the appearance of the cyst surface and the characteristics of the surface protrusions, if present, following the cyst type descriptions of Hernández-Rodríguez et al. (1981a, b) for *S. cervicanis* and of Dahlgren & Gjerde (2010a) for the other *Sarcocystis* species in red deer. Digital photographs were recorded of some sarcocysts at this stage. From all eight red deer, samples of the heart were examined, whereas samples of the diaphragm and oesophagus were examined from five red deer (Table S1 in the Supplementary Material). A total of 212 sarcocysts were excised and classified to morphological type. The cysts were then retrieved from the slides and placed into labelled 1.5-ml micro-centrifuge tubes. Sarcocysts isolated from the first red deer examined (C57/CeS1) were pooled according to morphological type and tissue of origin into nine 1.5-ml micro-centrifuge tubes containing 1 ml of distilled water, which were then stored in a freezer, whereas sarcocysts isolated from the other animals were placed separately into tubes containing 1 ml of 70% ethanol. A first batch of 67 sarcocysts from the oesophagus, diaphragm and heart of red deer CeS1 (C57) were shipped to the first author in Norway for molecular examination in March 2015, following replacement of the distilled water in the tubes with 70% ethanol. A second batch of 67 cysts isolated from the heart of three other red deer (CeS2/C28 = 26 cysts; CeS3/C82 = 18 cysts; CeS4/C85 = 23 cysts) were shipped in May 2016 (Table S1 in the Supplementary Material).

Scanning electron microscopy

A study of the sarcocysts by scanning electron microscopy (SEM) was initially not intended, and hence, no sarcocysts were specifically fixed for this purpose when sarcocysts were isolated from the muscle samples. Only during the course of the study the opportunity to examine some sarcocysts by SEM in Norway arose, and such a study was therefore performed on sarcocysts fixed in 70% ethanol for molecular studies. Two, three and two sarcocysts, isolated from the heart of red deer CeS2, CeS3 and CeS4, respectively, were pooled into one tube, and then further dehydrated in an ascending ethanol series comprising 80–100% ethanol. The ethanol was then gradually replaced by hexamethyldisilazane (HMDS) in

several steps until the sarcocysts became immersed in a small volume of 100% HMDS, which was allowed to evaporate at room temperature. The dried sarcocysts were then further processed and examined by SEM as previously described (Gjerde et al., 2017).

Molecular examination of sarcocysts

The molecular examination of sarcocysts was basically as described previously (Gjerde, 2013, 2014a, b, 2016; Gjerde et al., 2017), the major difference being that the sarcocysts had been kept in 70% ethanol rather than in distilled water following isolation from the muscle samples. Moreover, the 67 sarcocysts isolated from red deer CeS1 had been pooled into nine micro-centrifuge tubes. In order to extract DNA from individual sarcocysts, the contents of the latter tubes were pipetted in small portions into an inverted snap cap removed from a micro-centrifuge tube, the fluid was examined under a stereo microscope and individual sarcocysts were pipetted in small volumes of alcohol from the cap and transferred to separate tubes. Some small sarcocysts were apparently lost during this procedure and only 55 of 67 putative sarcocysts were retrieved. The alcohol in all tubes containing individual sarcocysts were allowed to evaporate at room temperature and then about 20 μ l of distilled water was added to each tube. Finally, genomic DNA was extracted from about 115 putative sarcocysts using the QIAmp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's tissue protocol as previously described (Gjerde, 2013). The resulting DNA samples were kept frozen at -20 °C in between their use as templates for PCR amplification of the complete 18S rRNA gene and/or the partial *cox1*. For all available DNA isolates, attempts were made to amplify a 1085-bp-long portion of *cox1* with primer pair SF1/SR9 in order to identify the sarcocysts to species and study the variability of this marker. For some isolates, this primer pair resulted in poor amplification and fairly short sequence reads and these isolates were therefore amplified as two overlapping fragments with primer pairs SF1/SR66 and SF4/SR9 as previously described (Gjerde et al., 2017). In addition, two such isolates were amplified with primer pair SF1/SR5. Following the identification of the sarcocyst isolates through sequencing of *cox1*, the complete 18S rRNA gene (1858–1920 bp) of one or more isolates of each of the six putative species found were amplified as two overlapping fragments using primer pairs ERIB1/S2r and S3f/Primer BSarc. Due to intra-isolate sequence variation detected through initial direct sequencing, amplicons of the 5' end half of the 18S rRNA gene of five species were subsequently cloned into a plasmid vector before sequencing in order to obtain unambiguous sequences, whereas amplicons of one species (*S. hjorti*) were only sequenced directly.

The sequences of the primers used are given in the supplementary material of Gjerde et al. (2017), except that of primer

SR5 (Gjerde, 2013). The PCR reactions were performed and the PCR products were evaluated and purified as described previously, as were the procedures and the reagents used for cloning and purification of plasmid DNA (Gjerde, 2014b, 2016). Purified amplicons and plasmid DNA (from cloning) were sent to Eurofins Genomics, Germany, for sequencing on both strands as previously described (Gjerde et al., 2017). Forward and reverse sequence reads, as well as overlapping sequences, were assembled into complete consensus sequences using the Alignment Explorer of the MEGA7 software (Kumar et al., 2016). Newly assembled sequences were compared with each other and with related sequences in GenBank using the Nucleotide BLAST program as previously described (Gjerde, 2013). The different sequences obtained by cloning of the 5' end half of the 18S rRNA gene of five species were also aligned in MEGA7 by ClustalW and carefully examined by eye for the purpose of describing the intraspecific variation. In addition, all new complete 18S rRNA gene sequences were similarly compared with previous sequences of the same or closely related species after multiple alignments had been generated. The software package DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado & Rozas, 2009) was used for the analysis of sequence variation and DNA divergence within and between different populations (types) of *cox1* sequences found in the present and previous studies, in order to characterize these populations and assign them to different species, if possible.

Phylogenetic analyses

Phylogenetic analyses were conducted separately on nucleotide sequences of *cox1* and the 18S rRNA gene, respectively, by means of the MEGA7 software (Kumar et al., 2016). In all analyses, the phylogeny was tested with the bootstrap method, using 1000 bootstrap replications. All sequences included in the analysis of each gene and their GenBank accession numbers are listed in Table S2 in the Supplementary Material.

For *cox1*, a total of 747 partial sequences from 53 taxa were initially included in the analysis, including 28 new sequences generated from the six *Sarcocystis* spp. found in the present study. However, in order to reduce computation time, identical superfluous sequences were removed, so that each haplotype (based on nucleotides 1–1020) was represented by a single sequence only. Hence, for the final analysis, a total of 386 sequences (haplotypes) were used. A codon-based multiple alignment of all sequences was obtained by using the ClustalW program integrated in MEGA7 as described previously (Gjerde, 2013). Sequences longer than 1020 bp were truncated at their 3' end, so that the final alignment comprised 1020 positions with no gaps. The phylogenetic tree was reconstructed using the Neighbour-Joining (NJ) method with the p-distance algorithm. All codon positions were used. The

intestinal coccidium *Eimeria tenella* (family: Eimeriidae) was used as outgroup species to root the tree.

As regards the 18S rRNA gene, a total of 235 near full-length sequences from 57 taxa were used in the main analysis, including 66 new sequences of the six *Sarcocystis* spp. found in the present study. A multiple sequence alignment was generated with the ClustalW program integrated in MEGA7, using a gap opening penalty of 10 and a gap extension penalty of 0.2 and 0.1 for the pairwise and multiple alignments, respectively. The resulting alignment was checked by eye and slightly corrected for inconsistencies in the treatment of closely related sequences. Most sequences were truncated slightly at both ends, so that most sequences started and ended at the same homologous nucleotide positions, corresponding to positions 75 and 1852, respectively, of GenBank sequence KY973355 of *S. linearis*. The final alignment comprised 2018 aligned positions, including gaps. The phylogenetic tree was reconstructed using the maximum parsimony (MP) method with the subtree-pruning-grafting (SPR) algorithm. All sites were used. The intestinal coccidium *E. tenella* was used as outgroup species to root the tree. An additional analysis was performed on a smaller dataset comprising 28 taxa and 175 nucleotide sequences, of which 171 sequences belonged to taxa with canines as known or presumed definitive hosts. One sequence each of *Sarcocystis hardangeri*, *Sarcocystis ovalis* and *Sarcocystis oviformis* were used as outgroup species to root the tree (Table S2 in the Supplementary Material). A multiple alignment was generated with the same settings as for the larger dataset, resulting in 1942 aligned positions. The phylogenetic tree was reconstructed using the MP method with the SPR algorithm.

Results

Light microscopic appearance of sarcocysts

All sarcocysts were microscopic in size and were only detected using the compression method. The light microscopic examination of isolated sarcocysts in wet mounts revealed three types of surface morphology. Type 1 sarcocysts had densely packed, distinct hair-like protrusions (Fig. 1a–c) and resembled sarcocysts of *S. hjorti* from Norwegian red deer (Dahlgren & Gjerde, 2010a); type 2 sarcocysts displayed a fuzzy outline, which seemed to be due to loosely packed structures resembling short hairs (Fig. 1d); whereas type 3 sarcocysts had a smooth surface with no visible protrusions (Fig. 1 e–h) as reported for *S. cervicanis* (Hernández-Rodríguez et al., 1981a, b). Type 1 and 2 sarcocysts were found in the diaphragm of one, and in the oesophagus of two of the five red deer from which these tissues were examined, but they were not found in cardiac muscle of any of the eight red deer. Type 3 sarcocysts were found in cardiac muscle of

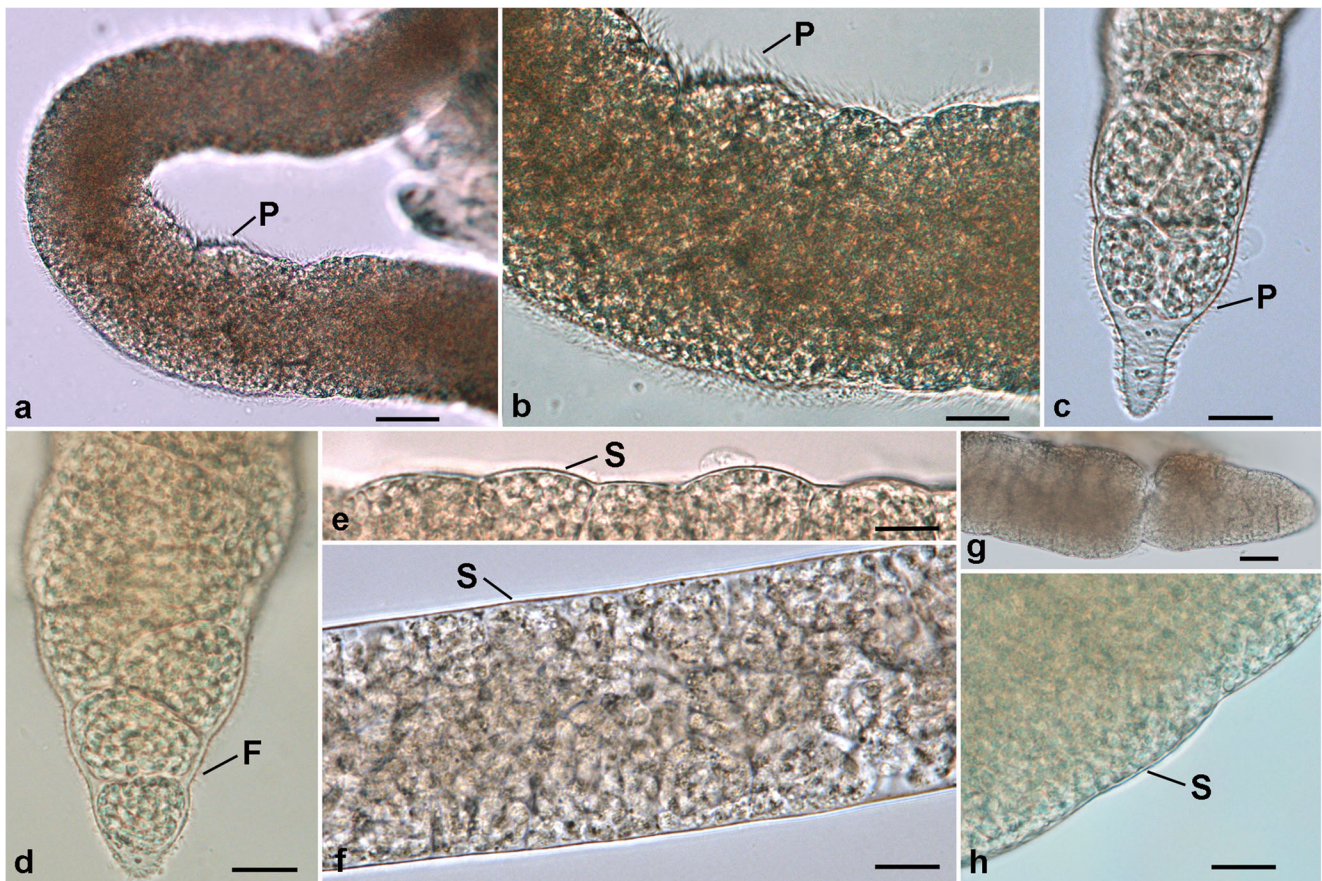


Fig. 1 Light microscopic appearance of unstained sarcocysts isolated from red deer from Quintos de Mora, Central Spain. **a–c** Sarcocysts of type 1 with hair-like protrusions (P). **a** Portion of a spindle-shaped small sarcocyst from the diaphragm of red deer CeS1, probably of either *S. iberica* or *S. venatoria*. **b** Higher magnification of sarcocyst in (a) showing surface with tightly packed delicate hair-like protrusions (P). **c** Tip of sarcocyst with hair-like protrusions (P) from the oesophagus of red deer CeS1, possibly of *S. hjorti*. **d** Sarcocyst of type

2 with a fuzzy outline (F) from the oesophagus of red deer CeS1, possibly of *S. linearis*. **e–h** Sarcocysts of type 3 with a smooth surface (S) and no visible protrusions. **e** Peripheral portion of sarcocyst from the oesophagus of red deer CeS1, possibly of *S. linearis*. **f** Middle portion of fusiform sarcocyst from the diaphragm of red deer C233. **g** Tip of sarcocyst from the heart of red deer CeS1, probably of *S. cervicanis*. **h** Peripheral portion of sarcocyst from the heart of red deer C100, probably of *S. cervicanis*. Scale bars **a, g** 40 μm and **b–f, h** 20 μm

seven of eight red deer, as well as in the diaphragm and oesophagus of three of five animals (Table S1 in the Supplementary Material). In the oesophagus and diaphragm, sarcocysts of all three types were spindle-shaped with pointed ends and about 90- μm wide in the middle. Their full length could not be clearly established, since only a few fairly complete cysts were isolated and measured. Based on the largest cyst fragments and a few intact cysts, the full length was estimated to be 650–1500 μm . In type 1 sarcocysts, the hair-like protrusions were about 8- μm long. Sarcocysts of type 3 in cardiac muscle were sac-like and about 500–800 \times 80–180 μm in size (Fig. 1g, h).

Scanning electron microscopy

In only one of the seven sarcocysts processed for SEM had portions of the cyst surface become exposed through removal of the surrounding host cell, whereas the other mounted

cysts were still completely covered by host cell material. In the partially denuded sarcocyst, regularly distributed thin ribbon-like protrusions were running along the exposed cyst surface (Fig. 2a, b). Many of the protrusions seemed to have become twisted and distorted and to have been torn off at different distances from their point of origin on the cyst surface. Still, their basic configuration was evident. They were band-like and seemed to have a nearly uniform width and thickness throughout their length. They were about 0.3–0.4- μm wide, about 0.02–0.03- μm thick and the longest intact portions were up to 2.5- μm long. The protrusions had a smooth surface, whereas the surface of the cyst itself was provided with regularly distributed small pits, about 0.05 μm in diameter, separated by low ridges. From the bottom of the larger pits, smaller pores seemed to arise and form minute invaginations into the cyst wall. Together, the ridges and pits gave the sarcocyst surface a porous or reticulated appearance (Fig. 2b). Based on the molecular characterization of other sarcocysts from

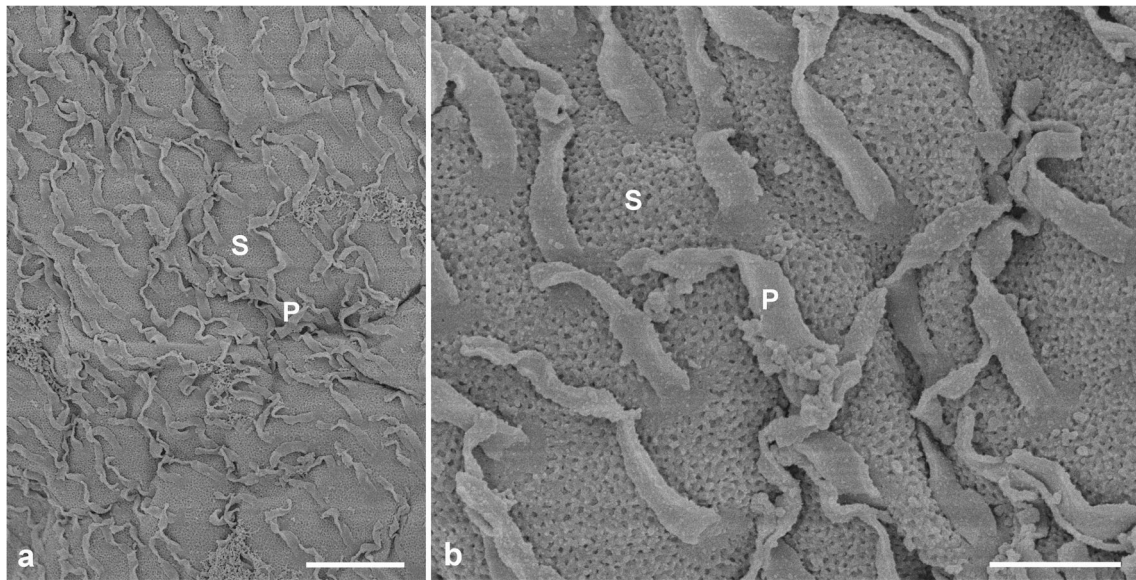


Fig. 2 Scanning electron micrographs of the surface of a sarcocyst from cardiac muscle of red deer, probably of *S. cervicanis*. **a** Numerous ribbon-like protrusions (P) are running along the sarcocyst surface (S) in the same direction, but some protrusions have become twisted and distorted and their distal end have been torn off during removal of the surrounding host

cell material. **b** Higher magnification of cyst in (a), showing that the ribbon-like protrusions (P) have a fairly uniform width throughout their length. The sarcocyst surface (S) in between the bases of the protrusions have a reticulated appearance due to low ridges and invaginations into the sarcocyst wall. Scale bars 3 μm (a) and 1 μm (b)

cardiac muscle of the same three red deer (Table S3 in the Supplementary Material), the sarcocyst examined by SEM most likely belonged to *S. cervicanis*.

Molecular characterization of sarcocysts

Partial *cox1* gene

A total of 103 sarcocysts were unambiguously characterized through PCR amplification and sequencing of the partial *cox1* gene (1038–1053 bp), revealing the presence of six major sequence types, with each type consisting either of a single sequence from a single sarcocyst isolate or a collection of several identical or nearly identical sequences derived from different sarcocysts. On the basis of sequence comparisons using BLAST and the program DnaSP, as well as their phylogenetic placement and the morphological features of the sarcocysts from which they originated, the sequence(s) of different types were considered to represent six different *Sarcocystis* spp. (Table 2). The molecularly identified sarcocysts comprised 48 sarcocysts from red deer CeS1 (32 from the diaphragm, 13 from the oesophagus, 3 from the heart); and 21, 14 and 20 sarcocysts from cardiac muscle of red deer CeS2, CeS3 and CeS4, respectively (Table S3 in the Supplementary Material).

Of the six major *cox1* sequence types found, two types had a high sequence identity to the previously characterized species *S. hjorti* and *S. linearis*, respectively, and thus belonged to these species. The four other sequence types

differed considerably from previous sequences of known species, as did the corresponding 18S rRNA gene sequences, and they were therefore considered to represent three new species and *S. cervicanis*, respectively. The species found and the number of sarcocysts/sequences belonging to each of them were: *Sarcocystis iberica* n. sp. ($n = 1$), *Sarcocystis venatoria* n. sp. ($n = 10$), *S. hjorti* ($n = 1$), *S. cervicanis* ($n = 52$), *S. linearis* ($n = 38$) and *Sarcocystis morae* n. sp. ($n = 1$) (Table 2; Tables S3 and S4 in the Supplementary Material). The 103 sequences comprised 28 haplotypes, and 28 sequences representing these haplotypes were deposited in GenBank (KY973286–KY973313).

The 11 *cox1* sequences obtained from type 1 sarcocysts (hair-like protrusions) in the diaphragm of red deer CeS1, were of two types, comprising one (isolate CeS1.1) and ten identical sequences (isolates CeS1.2–CeS1.11), respectively (Table S4 in the Supplementary material). The two sequence types differed at 42 of 1038 nucleotide (nt) positions (96% identity) and were considered to belong to two separate species, even though the corresponding 18S rRNA gene sequences could not be separated from each other (see later). Since neither the *cox1* nor the 18S rRNA gene sequences from these isolates matched those of any previously examined species, they were considered to represent two new species. The *cox1* sequence from isolate CeS1.1 (KY973286) was assigned to *S. iberica* n. sp., whereas those from isolates CeS1.2–CeS1.11 (KY973287) were assigned to *S. venatoria* n. sp. Both of these sequence types shared the highest identity (about 91% for *S. iberica* and about 90% for *S. venatoria*)

Table 2 Overview of the molecular identification and characterization at *cox1* and the 18S rRNA gene of 103 sarcocyst isolates from four red deer from Spain (CeS1–CeS4) and the GenBank accession numbers of the submitted sequences

Species	Isolates examined at <i>cox1</i> and the <u>18S rRNA gene</u> ¹	<i>cox1</i> sequences (S/H) ²	18S rRNA gene sequences
<i>S. iberica</i>	CeS1.1	KY973286 (1/1)	KY973314–KY973323 ³
<i>S. venatoria</i>	CeS1.2 , <u>CeS1.3–CeS1.6</u> –CeS1.11	KY973287 (10/1)	KY973324–KY973331 ³
<i>S. hjorti</i>	CeS1.48	KY973288 (1/1)	KY973332
<i>S. cervicanis</i>	<u>CeS1.54</u> , CeS1.55 , CeS1.56; CeS2.12; CeS4.1, CeS4.3, <u>CeS4.4</u> , CeS4.5, CeS4.7–CeS4.10, <u>CeS4.12</u> , CeS4.14, CeS4.16, CeS4.17; CeS4.19–CeS4.21	Type A: KY973289–KY973292 (19/4)	KY973333–KY973353 ³
	CeS2.1– CeS2.4 , CeS2.7, CeS2.8, CeS2.10, CeS2.11, CeS2.13– <u>CeS2.15</u> –CeS2.24; CeS3.1–CeS3.5, CeS3.7, <u>CeS3.8</u> –CeS3.10, CeS3.12–CeS3.14	Type B: KY973293–KY973294 (32/2)	KY973354
	<u>CeS4.11</u>	Type C: KY973295 (1/1)	
<i>S. linearis</i>	CeS1.13– CeS1.16 – CeS1.21 –CeS1.30, CeS1.32, CeS1.33, CeS1.35–CeS1.37, CeS1.40, CeS1.42– <u>CeS1.44</u> , CeS1.46, CeS1.49, CeS1.50–CeS1.52; CeS3.6, CeS3.11; <u>CeS4.2</u> , <u>CeS4.13</u> ; CeS4.15; CeS4.18	KY973296–KY973312 (38/17)	KY973355–KY973372 ³
<i>S. morae</i>	CeS1.47	KY973313 (1/1)	KY973373–KY973379 ³

¹ Isolates examined at the 18S rRNA gene are underlined; those from which sequences were submitted to GenBank are in boldface

² The total number of *cox1* sequences (S) obtained and the number of submitted sequences or haplotypes (H) of each species are given in parentheses behind the accession numbers

³ Sequences in which the 5' end half of the gene was obtained by cloning

with sequences of other species from cervids with hair-like cyst wall protrusions (*Sarcocystis capreolicanis*, *Sarcocystis rangi*, *S. hjorti*, *Sarcocystis pilosa*).

One sarcocyst (isolate CeS1.48) from the oesophagus of red deer CeS1, which by LM had seemed to have a smooth wall, was found to belong to *S. hjorti* from its *cox1* sequence (KY973288), which were 99.4–99.7% identical with 22 previous *cox1* sequences of this species (KC209635–KC209643; KF241342–KF241354) derived from sarcocysts in red deer and moose in Norway (Gjerde, 2013; Gjerde, 2014b). The new *cox1* sequence of *S. hjorti* was 90.7 and 90.1% identical with the abovementioned new sequences of *S. iberica* and *S. venatoria*, respectively.

A total of 38 sarcocysts were identified as belonging to *S. linearis*, including 21 and 11 from the diaphragm and oesophagus, respectively, of red deer CeS1, and two and four from the cardiac muscle of red deer CeS3 and CeS4, respectively (Table 2; Tables S3 and S4 in the Supplementary material). The majority of these cysts (31) had been recorded as being of type 3 when examined by LM, but three and four cysts from the oesophagus had been assigned to type 1 and 2, respectively. The 38 new *cox1* sequences of *S. linearis* comprised 17 haplotypes (KY973296–KY973312), which differed from each other at 1–13 of 1038 nt positions (98.7–99.9% identity), whereas they differed at 6–19 nt positions (98.2–99.4% identity) from the five previous *cox1* haplotypes of *S. linearis* obtained from 19 sarcocyst isolates (KY018969–KY018987) from roe deer in Italy (Gjerde et al., 2017). The 22 haplotypes of *S. linearis* from both hosts were thus 98.2–99.9% identical with each other, whereas they shared an

identity of 95.8–97.1% (on average 96.5%) with the 17 known haplotypes of *S. taeniata* derived from 25 sarcocyst isolates from moose and sika deer (Gjerde, 2014b; Prakas et al., 2016). There were 18 fixed nucleotide differences between the two populations, that is, positions were all haplotypes of *S. linearis* differed from all haplotypes of *S. taeniata*. The 22 haplotypes of *S. linearis* further shared an identity of 83.5–85.0% with sequences (KC209615–KC209624) of the morphologically similar species *S. grueneri* from reindeer (Table S5 in the Supplementary material).

From 52 of the 58 type 3 sarcocysts from cardiac muscle of the four red deer examined, a new major *cox1* sequence type was obtained, whereas six other sarcocysts of this morphological type belonged to *S. linearis* as mentioned above (Tables S3 and S4 in the Supplementary material). The 52 sequences of the new major type, comprised three subtypes, which were provisionally designated types A, B and C, respectively. In each red deer, sequences of either type A or B predominated (Table 2; Table S3 in the Supplementary material). The 19 type A sequences comprised four haplotypes (KY973289–KY973292), which differed from each other at 1–3 of 1038 nt positions (99.7–99.9% identity), whereas the 32 type B sequences comprised only two haplotypes (KY973293–KY973294), differing at two positions (99.8% identity). In addition, there was a single sequence/haplotype of type C (KY973295). Type A haplotypes differed from type B haplotypes at 17–21 nt positions (98.0–98.4% identity), including 17 fixed nt differences, and from the single type C haplotype at 17–19 positions (98.2–98.4% identity; 17 fixed nt differences), whereas type B and C haplotypes differed at

10 nt positions (99% identity; nine fixed nt differences). Thus, the single type C sequence seemed to represent an intermediate type between the two other types, but it was more similar to type B than to type A. The finding of this intermediate type suggested that there might have been additional intermediate types between type A and B if more isolated had been examined. Hence, all three subtypes were considered to belong to a single species. Moreover, since this species predominated in cardiac muscle, from which *S. cervicanis* was originally described (Hernández-Rodríguez et al., 1981a, b), they were attributed to this species. The seven *cox1* haplotypes assigned to *S. cervicanis* thus differed at 1–19 nt positions and were 98.0–99.9% identical. These sequences shared the highest identity (88.4–88.9%) with the new sequence of *S. morae* (see below) followed by 86.7–87.4% identity with sequences of *S. taeniata* (Table S5 in the Supplementary material).

From a type 3 sarcocyst (isolate CeS1.47) from the oesophagus of red deer CeS1, still another new *cox1* sequence type was obtained. Likewise, sequences of the 18S rRNA gene from this isolate did not match any previous sequences in GenBank. These sequences were therefore attributed to the new species *S. morae*. The *cox1* sequence (KY973313) showed the highest identity with the seven new sequences of *S. cervicanis* (see above), followed by identities of 86.3–86.9% with all sequences of *S. linearis* from red deer and roe deer and 86.0–86.8% identity with *S. taeniata* and *S. grueneri* (Table S5 in the Supplementary material). The identity was about the same with sequences of *S. hjorti* (86.0–86.5%) and *S. capreolicanis* (85.8–86.3%) and slightly less with sequences of several other species, including *S. iberica* (85.4%) and *S. venatoria* (85.4%).

Complete 18S rRNA gene

Following the identification of six major *cox1* sequence types among the 103 isolates examined, the complete 18S rRNA gene was amplified and sequenced from one or more isolates of each of the six species these types were considered to represent (Table 2). Attempts were initially made to sequence directly the PCR products of the two overlapping gene fragments of all selected isolates. It turned out that the 5' end half of the gene of *S. iberica*, *S. venatoria*, *S. linearis* and *S. cervicanis* comprised one or more indels (insertions/deletions), which made the sequence reads ambiguous or unintelligible downstream of these variations. Hence, this portion of the gene of one or two isolates of each of these species, was subsequently cloned before sequencing. The 5' end half of the gene of *S. hjorti* and *S. morae*, on the other hand, was sequenced directly with good results, but amplicons of the latter species were nevertheless cloned and sequenced for comparative purposes. As regards to the 3' end half of the gene, amplicons of all species, except some isolates of *S. cervicanis* (see later), were sequenced in both directions

with good results. A total of 66 sequences of the (near) complete gene were finally obtained and submitted to GenBank under the accession numbers KY973314–KY973379 (Table 2).

The 18S rRNA gene of the single available isolate of *S. iberica* and of three isolates of *S. venatoria* were initially amplified and sequenced directly (Table 2). Subsequently, ten and nine clones were generated and sequenced from the 5' end half of the gene of *S. iberica* (isolate CeS1.1) and *S. venatoria* (isolate CeS1.2), respectively. All clones of *S. iberica* differed slightly from each other, whereas two clones of *S. venatoria* were identical. Unique clones of each species were concatenated with the 3' end half of the gene, resulting in 10 and eight full-length sequences of *S. iberica* and *S. venatoria*, respectively (Table 2). The sequences of both species were 1896–1898-bp long; those of *S. iberica* shared an identity of 99.2–99.9% with each other, while those of *S. venatoria* were 99.5–99.9% identical. Moreover, sequences of the two species shared an identity of 99.2–100% with each other, that is, one sequence of *S. iberica* (KY973321) was identical with a sequence of *S. venatoria* (KY973327). The differences between individual sequences of both species were mainly due to three 2-bp-long indels between positions 745 and 765 in the multiple alignment of all sequences, which was 1902-bp long. In comparison to previously known species, *S. iberica* and *S. venatoria* were most similar (97–98% identity) to other species with hair-like cyst wall protrusions (*Sarcocystis alceslatrans*, *S. capreolicanis*, *Sarcocystis cruzi*, *S. hjorti*, *S. Sarcocystis levinei*, *S. pilosa*, *S. rangi*).

The near complete 18S rRNA gene of *S. hjorti* was PCR amplified and sequenced from the only available isolate of this species (Table 2). The new sequence (KY973332) was 99.8–99.9% identical with previous sequences of *S. hjorti* from moose (EU282017, JN256128, KF831294–KF831295) and red deer (GQ250990, JN256124) in Norway and Lithuania and it was 97.6–98.1% identical with the abovementioned new sequences of *S. iberica* and *S. venatoria*.

Six isolates of *S. linearis* were examined at the 18S rRNA gene through PCR amplification and direct sequencing of the amplicons. From two of the isolates, the 5' end half of the gene was cloned, and nine and ten clones were sequenced from isolates CeS1.16 and CeS1.21, respectively (Table 2). Two clones from the first isolate were identical, and hence 18 different clones were submitted to GenBank following concatenation with the 3' end half of the gene, which was identical in all six isolates examined. There was no indications of any sequence variation in the latter portion of the gene after direct sequencing. The 18 new complete (1878–1887-bp long) 18S rRNA gene sequences of *S. linearis* were 98.3–99.9% identical with each other and 97.8–99.7% identical with 24 previous sequences of *S. linearis* (KY019032–KY019055) from roe deer in Italy, which in turn were 98.1–99.9% identical with each other (Gjerde et al., 2017). Hence, the intraspecific

identity of all 42 available sequences of *S. linearis* was 97.8–99.9%. In comparison, these sequences were 97.9–99.7% identical with sequences of *S. taeniata* from moose and sika deer (KF831277–KF831293; KU753886–KU753890). The differences between individual sequences were in both species due to several indels and substitutions (single nucleotide polymorphisms, SNPs). When sequences of *S. linearis* and *S. taeniata* were compared in a multiple alignment, both species seemed to have a similar pattern of sequence variation with no consistent differences in the cloned 5' end half of the gene as described before (Gjerde et al., 2017). Near the 3' end of the gene, on the other hand, all the new sequences of *S. linearis* from red deer differed from previous sequences from roe deer in having only three versus five or six consecutive thymine bases (Ts) from nucleotide position 108 onwards from the 3' end of the gene (Fig. S1 in the Supplementary Material). Moreover, there seemed to be no intra-isolate variation with respect to the number of Ts in the six isolates from Spanish red deer, since both forward and reverse sequence reads were fine, whereas all roe deer isolates examined seemed to possess this indel, since the reverse sequence reads became illegible beyond this position (Gjerde et al., 2017). In addition, the new sequences of *S. linearis* from red deer differed from all available sequences of *S. taeniata* from moose and sika deer published in GenBank (KF831277–KF831293; KU753886–KU753890), which also possess six consecutive Ts. However, a re-examination of the sequencing chromatograms from the study of *S. taeniata* from Canadian moose (Gjerde, 2014b), revealed that there was actually an indel in this region, and thus five or six consecutive Ts as in the roe deer isolates of *S. linearis*. The illegible reverse sequence reads beyond this position had at that time been interpreted as being due to polymerase slippage during amplification or sequencing as a result of the presence of six consecutive Ts. The new sequences of *S. linearis* displayed two polymorphic sites in positions 95 and 96 from the 3' end of the gene. At both positions, the possible character states were T or C, as evidenced by double peaks in the chromatograms, but submitted sequences of *S. linearis* have either TT or CC, since either T or C predominated at both positions. In published sequences of *S. taeniata*, on the other hand, there are either TC or TT. However, the re-examination of the sequencing chromatograms of *S. taeniata* from moose, revealed that both positions were polymorphic with T and C as possible character states even in this species. Thus, there seems to be no consistent differences between the 18S rRNA gene sequences of *S. linearis* and *S. taeniata*, except perhaps between isolates of *S. linearis* from red deer and *S. taeniata*. Interestingly, the sequences from red deer in Lithuania and Argentina (JN256126–JN256127; KT626602) tentatively attributed to *S. taeniata* (Reissig et al., 2016; Prakas et al., 2016) also possessed only three Ts like the present isolates of *S. linearis* from red deer and may therefore belong to the latter species (Fig. S1 in the Supplementary material).

Six isolates of *S. cervicanis* were examined at the 18S rRNA gene through PCR amplification and direct sequencing of the amplicons (Table 2), but portions of the 5' end half of the gene could not be properly estimated from most sequence reads due to superimposed sequences caused by indels. A total of 23 clones were therefore generated and sequenced from isolate CeS1.55, from which *cox1* sequences of subtype A had been obtained. Two of these clones differed considerably (10–20%) from the others through a 59-bp-long deletion and several short stretches of atypical sequence composition. These clones were therefore considered to be aberrant and were not deposited in GenBank or included in the sequence comparisons. The 21 other clones were of two major types, with the major differences between them being found in the region spanning nucleotide positions 765 to 830 in the final sequences. In this region, there were a series of substitutions and six 1–3-bp-long indels, making the total length of this stretch of sequence differ by five nucleotides between the two types (e.g. KY973333 versus KY973335). In the clones, there were about 40 polymorphic sites in addition to the aforementioned differences, but at 35 of these sites, only one clone had an atypical nucleotide, suggesting that these deviations were not true SNPs, but a result of polymerase errors during PCR amplification prior to cloning. Near the 3' end of the gene, there were three additional polymorphic sites (true SNPs), as well as an indel, in three (CeS1.54, CeS1.55, CeS4.12) of four isolates having type A *cox1* sequences. In these isolates, there were both A and G (double peaks in chromatograms) in position 82 from the 3' end, both C and T at position 103, five or six consecutive Ts from position 107 onwards, and both C and T at position 114 or 115 (depending on number of Ts) from the 3' end (Fig. S2 in the Supplementary material). Thus, the reverse sequence reads became illegible beyond the indel as described for sequences of *S. linearis* from roe deer (Gjerde et al., 2017) and for sequences of *S. taeniata* from moose (see above), which had five or six consecutive Ts in the homologous region. In the fourth type A isolate (CeS4.4), as well as in the three type B isolates (CeS2.4, CeS2.15, CeS3.8) and the single type C isolate (CeS4.11) examined, there were no indications of any sequence variation at these sites (Fig. S2 in the Supplementary material). Due to the sequence variations described above, the complete 18S rRNA gene of *S. cervicanis* was 1913–1920 bp long in different clones and the alignment comprising all sequences was 1926 bp long. The 22 new sequences (KY973333–KY973354) shared an identity of 98.1–99.9% with each other. They were most similar (97.6–98.6% identity) to a sequence of the unnamed *Sarcocystis* sp. T18 from sika deer in Japan (AB698065), from which they mainly differed in the same variable region (nucleotide positions 765–830) that separated the clones from each other. The

new sequences of *S. cervicanis* were up to 95.8% identical with sequences of *S. linearis*, *S. taeniata* and *S. grueneri*.

From the only available isolate of *S. morae*, the complete 18S rRNA gene was initially amplified and sequenced directly with good results, but the 5' end half of the gene was nevertheless cloned in order to explore whether there might be some undisclosed sequence variation. Ten clones were processed and sequenced, resulting in four identical clones and six other clones that were slightly different from these. The seven different clones were concatenated with the 3' end half of the gene and the full-length sequences were submitted to GenBank (Table 2). The four identical clones, represented by sequence KY973374, were also identical with the sequence obtained by direct sequencing. The complete gene was 1858-bp long and the seven sequences differed from each other at 1–10 nucleotide positions (99.5–99.9% identity). These differences were due to various combinations of substitutions occurring at 17 polymorphic sites in the cloned region of the gene. However, at 16 of these sites, only a single sequence deviated from the majority, whereas at one site two sequences deviated from the five other sequences. Hence, most of these substitutions might be due to polymerase errors during the PCR amplification before cloning, and sequence KY973374, which was supported by four clones, might be regarded as the most accurate representation of the 18S rRNA gene of the examined isolate of *S. morae*. In comparison to sequences of previously characterized species available in GenBank, the most similar sequences were those of *S. cruzi* and *S. levinei* with up to 97.5 and 97.3% identity, respectively. With respect to the sequences of other species in the present study, the new sequences of *S. morae* shared identities of up to 96.7% with those of *S. linearis*, 96.4% with that of *S. hjorti*, 96.2% with those of *S. iberica* and *S. venatoria*, and up to 95.6% with those of *S. cervicanis*.

Phylogeny

Cox1

The phylogenetic analysis using NJ placed all *Sarcocystis* spp. with ruminant intermediate hosts into three major clades according to their known or presumed definitive hosts, that is, canines, felids and corvid birds (Fig. 3). Species with non-ruminant intermediate hosts formed a clade basal to these three clades. Sequences of the six species found in this study were placed within the canine clade. Those of *S. linearis* were placed with previous sequences of this species from roe deer into a monophyletic cluster, which with maximum bootstrap support was sister to all sequences of *S. taeniata*. Sequences of *S. cervicanis* were with high support sister to the single sequence of *S. morae*, and these taxa were sister to sequences of *S. grueneri* and *Sarcocystis hircanicus*. The latter four taxa in turn were a sister group to *S. linearis* and *S. taeniata*.

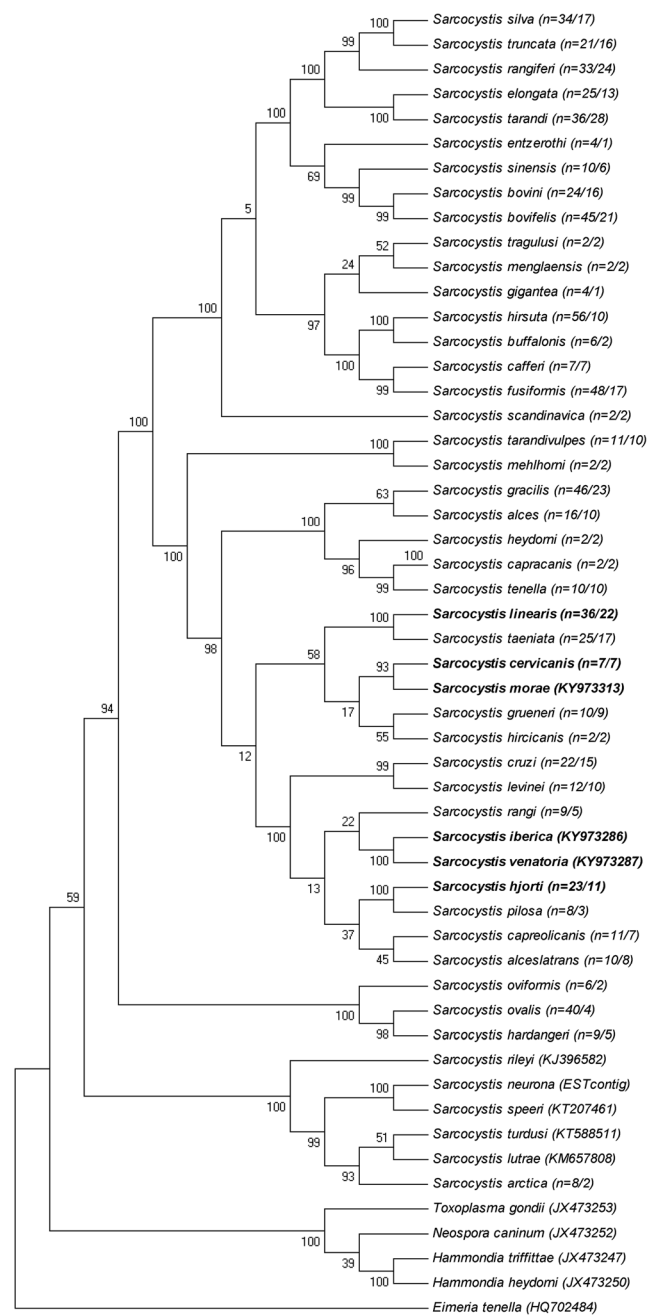


Fig. 3 Phylogenetic tree for members of the Sarcocystidae based on 386 partial sequences of *cox1* of 53 taxa and inferred using the neighbour-joining method and with evolutionary distances computed using the *p*-distance method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Subtrees formed by two or more sequences/haplotypes of the same species have been collapsed, but the number of sequences/haplotypes included is given in parentheses behind the taxon names, which are in boldface when comprising sequences from the present study. The GenBank accession numbers of all sequences are listed in Table S2 in the Supplementary material

However, the relationships between the different pairs of species were not well resolved. The sequences of *S. iberica* and *S. venatoria* were with maximum support sister to each other and both were sister to sequences of *S. rangi*, but the latter

relationship was not well supported. The single new sequence of *S. hjorti* clustered with previous sequences of this species. Sequences of *S. iberica*, *S. venatoria* and *S. hjorti* were placed within a subclade comprising species with hair-like cyst wall protrusions, whereas those of *S. cervicanis*, *S. morae* and *S. taeniata* were placed within a subclade comprising species with ribbon-like protrusions, except *S. hircicanis*, which has hair-like protrusions (Hu et al., 2016).

18S rRNA gene

The phylogenetic analysis using MP and the largest dataset placed all *Sarcocystis* spp. with ruminant intermediate hosts into three major clades according to their known or presumed definitive hosts (Fig. S3 in the Supplementary Material), as in the analysis using *cox1* sequences. Sequences of the six species found in the present study were placed within the canine clade. However, sequences of several sister species pairs (assignment based on *cox1* sequences), were not separated from each other, but rather formed paraphyletic clusters. Thus, within the canine clade, sequences of *S. iberica* were interleaved with those of *S. venatoria*, sequences of *S. cervicanis* were interleaved with the single available sequence of the unnamed *Sarcocystis* sp. T18 from sika deer in Japan (AB698065), and sequences of *S. linearis* were interleaved with those of *S. taeniata*. In this analysis, sequences of *S. morae* were placed within a subclade comprising species with hair-like protrusions. However, in the subsequent analysis based on the smaller dataset comprising taxa of the canine clade, sequences of *S. morae* were, albeit with low support, sister to the single sequence of *S. grueneri*, and both species were sister to the paraphyletic cluster comprising sequences of *S. linearis* and *S. taeniata* (Fig. 4). Moreover, these four taxa were sister to the paraphyletic cluster comprising sequences of *S. cervicanis* and the unnamed *Sarcocystis* sp. T18 from sika deer. The relationships between the species with hair-like cyst wall protrusions were largely the same in both analyses.

Taxonomic summary of the three new species and *Sarcocystis cervicanis*

Important taxonomic features of the three new species found in this study have been summarized in Table 3. Corresponding data for the previously named species *S. cervicanis* have also been included for comparative purposes since this study has re-defined this species through the provision of molecular data. Two of the new species have been named after the geographical location of the species, *S. iberica* from the Iberian peninsula, which includes Spain and the study area, and *S. morae* from the locality Mora and the wildlife reserve Quintos de Mora, from which the red deer examined originated. The name of the third species, *S. venatoria*, is related to hunting (ars venatoria). Venatoria is the Latin nominative of

the feminine form of the adjective venatorius. In addition, the word ‘venatus’ (animal hunted) is related to the Spanish word *venado*, which is a common name for all deer (Cervidae), but mainly used about red deer.

Discussion

The molecular characterization at *cox1* of 103 sarcocyst isolates from four red deer revealed that these sequences belonged to six major sequence types, which on the basis of their characteristics were considered to represent six separate *Sarcocystis* species. In addition, the corresponding 18S rRNA gene sequences and the morphology of the sarcocysts from which they originated were taken into consideration, but the latter features are not sufficient to discriminate closely related *Sarcocystis* spp. in ruminants from each other (Gjerde, 2014a, b, 2016; Gjerde et al., 2017). Hence, the identification and assignment of sarcocysts to species were based mainly on the new *cox1* sequences. In this manner, some sarcocysts were found to belong to the previously characterized species *S. hjorti* and *S. linearis*, whereas the remaining sarcocysts seemed to belong to species not previously known and/or characterized molecularly, since neither the *cox1* nor the 18S rRNA gene sequences derived from these sarcocysts showed a high identity to published sequences. Therefore, one of the four new *cox1* sequence types was attributed to the previously named species *S. cervicanis*, while the remaining new sequence types were assigned to the three new species *S. morae*, *S. iberica* and *S. venatoria*, respectively. In addition, the associated 18S rRNA gene sequences obtained from certain isolates of the latter four species, were also attributed to them. The rationale for these assignments will be discussed in the following.

Based on the sarcocyst morphology in wet mounts, there seemed to be only two or three species present in the red deer examined. The majority of the sarcocysts, including all cysts from cardiac muscle, seemed to have a smooth cyst wall with no visible protrusions (type 3), and were thus consistent with sarcocysts of *S. cervicanis* as described by Hernández-Rodríguez et al. (1981a, b) from red deer from Sierra Morena, Córdoba, which lies about 200 km to the south of Quintos de Mora. In addition, some sarcocysts from the diaphragm and oesophagus possessed hair-like protrusions (type 1) and were consistent with sarcocysts of *S. hjorti* as described from red deer and moose in Norway (Dahlgren & Gjerde, 2010a, b), and a few sarcocysts had an intermediate type of surface morphology (type 2; fuzzy surface). The latter appearance could have been due to aggregations of barely visible ribbon-like protrusions and host cell material, since a fuzzy surface was also noticed on sarcocysts of *S. linearis* from roe deer (Gjerde et al., 2017), or it could have been caused by partial removal of hair-like protrusions during

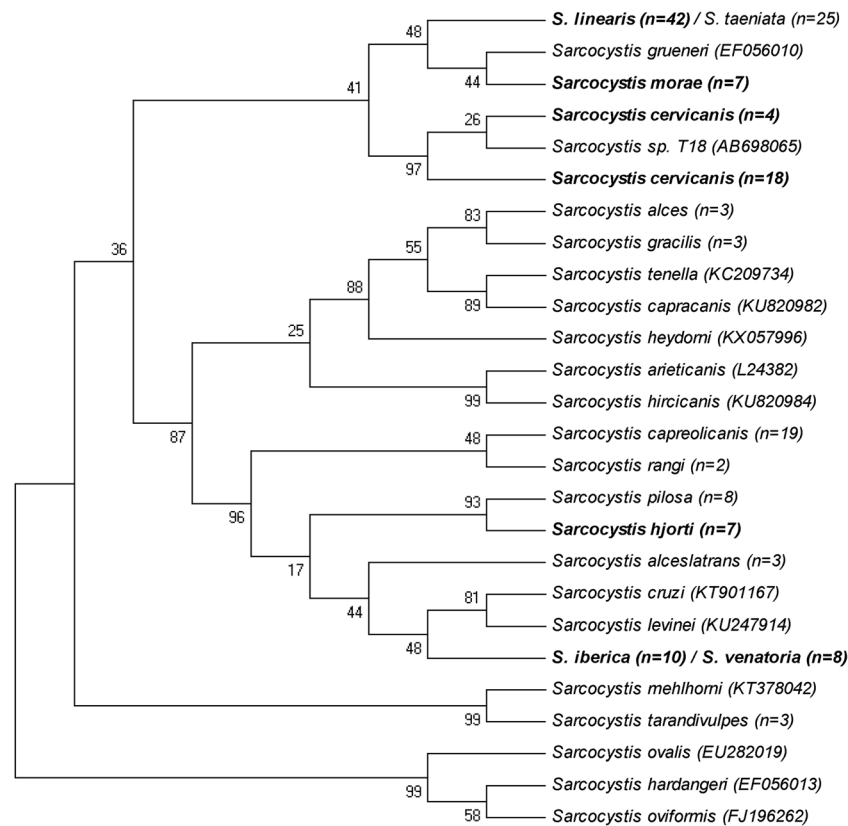


Fig. 4 Phylogenetic tree for selected members of the Sarcocystidae (mainly species known or believed to be transmitted by canids) based on near full-length sequences of the 18S rRNA gene and inferred using the maximum parsimony method with the Subtree–Pruning–Regrafting algorithm. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Subtrees formed by two or more sequences of the same or closely related species have been collapsed, but the number of sequences included are given behind the taxon names, which are in boldface when

comprising sequences from the present study. The GenBank accession numbers for all sequences are given in Table S2 in the Supplementary material. In the collapsed subtree formed by sequences of *S. linearis* and *S. taeniata*, sequences of the two species were interleaved. The same was true for sequences of *S. iberica* and *S. venatoria*. Note also that the single sequence of *Sarcocystis* sp. T18 from sika deer (AB698065) clusters in between sequences of *S. cervicanis*. A phylogenetic tree reconstructed from a larger dataset, comprising *Sarcocystis* spp. with different intermediate hosts, is shown in Fig. S3 in the Supplementary Material

isolation of the sarcocysts. In the preceding study of red deer from the Quintos de Mora reserve (Luzón et al., 2015), only *S. cervicanis*-like (type 3) and *S. hjorti*-like (type 1) sarcocysts were found.

However, the initial molecular characterisation of 48 sarcocysts from red deer CeS1, revealed that there were three major *cox1* sequence types associated with type 3 sarcocysts, and one of these types were nearly identical with sequences of a species found in Italian roe deer in a concurrent study, which were subsequently named *S. linearis* (Gjerde et al., 2017). The latter sequence type were found in 32 sarcocyst isolates from the diaphragm and oesophagus of red deer CeS1, but not in any of the three available sarcocyst isolates from cardiac muscle, which indicated that the two species had different tissue preferences. Moreover, Hernández-Rodríguez et al. (1981a, b) described and named *S. cervicanis* mainly on the basis of sarcocysts from cardiac muscle. Unfortunately, the type material deposited at the Faculty of Veterinary Medicine, University of Córdoba, in connection with the description of

that species (Hernández-Rodríguez et al., 1981a), could no longer be retrieved (Hernández-Rodríguez, personal communication 2016) and subjected to a molecular examination. We therefore focussed our efforts on identifying sarcocysts from cardiac muscle of three additional red deer (CeS2–CeS4). Of the 55 sarcocysts examined from these animals, 49 sarcocyst shared the same major sequence type as the three sarcocysts from cardiac muscle of red deer CeS1, while six sarcocysts shared the sequence type derived from sarcocysts in the diaphragm and oesophagus of that animal, as well as from sarcocysts in Italian roe deer (Table S3 in the Supplementary material). Hence, we attributed the sequence type predominating in sarcocysts from cardiac muscle to *S. cervicanis* in accordance with the original description of that species.

The sequence comparison and the phylogeny based on 18S rRNA gene sequences, however, suggested that an unnamed species from sika deer in Japan designated *Sarcocystis* sp. T18 (AB698065) might be identical with or a sister species to

Table 3 Taxonomic summary of the three new species *S. iberica*, *S. venatoria* and *S. morae* and the previously named species *S. cervicanis*, which was characterized molecularly for the first time in this study

Species	<i>Sarcocystis iberica</i> n. sp.	<i>Sarcocystis venatoria</i> n. sp.	<i>Sarcocystis morae</i> n. sp.	<i>Sarcocystis cervicanis</i>
Type intermediate host	Red deer, <i>Cervus elaphus</i>	Red deer, <i>Cervus elaphus</i>	Red deer, <i>Cervus elaphus</i>	Red deer, <i>Cervus elaphus</i>
Definitive host	Unknown, but probably canids	Unknown, but probably canids	Unknown, but probably canids	Dogs (Hernández-Rodríguez et al., 1981a, b)
Locality	Toledo, Spain	Toledo, Spain	Toledo, Spain	Córdoba and Toledo, Spain
Sarcocyst morphology	Microscopic (about 1 × 0.1 mm) spindle-shaped sarcocyst in diaphragm; hair-like protrusions, about 8 μm long (Fig. 1a, b)	Microscopic (about 1 × 0.1 mm) spindle-shaped sarcocysts in diaphragm; hair-like protrusions, about 8 μm long (Fig. 1 a, b)	Microscopic (about 1 × 0.1 mm) spindle-shaped sarcocyst in oesophagus; no visible protrusions by LM; phylogenetically related to species with ribbon-like protrusions	Microscopic sac-like sarcocysts (about 500–800 × 80–180 μm), mainly in cardiac muscle; no visible protrusions by LM (Fig. 1g, h); ribbon-like protrusions by TEM (Hernández-Rodríguez et al., 1981a, b) and SEM (Fig. 2)
Molecular characteristics (GenBank)	KY973286; KY973314–KY973323	KY973287; KY973324–KY973331	KY973313; KY973373–KY973379	KY973289–KY973295; KY973333–KY973354
Deposited material	Nucleotide sequences submitted to GenBank; genomic DNA stored at the Faculty of Veterinary Science, the Norwegian University of Life Sciences, Oslo, Norway	As for <i>S. iberica</i>	As for <i>S. iberica</i>	As for <i>S. iberica</i> . Type material deposited in original study (Hernández-Rodríguez et al., 1981a, b) no longer available
Etymology	Named after the Iberian peninsula, which includes Spain, the type locality of the species	Derived from the Latin adjective <i>venatorium</i> in the meaning hunting (<i>ars venatoria</i>). Also related to the Spanish word <i>venado</i> , a common name for cervids	Named after the locality Mora and the Quintos de Mora reserve, from which the infected red deer originated	Named after its intermediate and definitive hosts (Hernández-Rodríguez et al., 1981a, 1981b)

S. cervicanis, since this sequence clustered among sequences of the latter species. It will therefore be necessary to examine this species in sika deer at *cox1* to determine its precise relationship to *S. cervicanis*. Likewise, the relationship of *S. wapiti*-like sarcocysts in wapiti in North-America to the morphologically similar species in red deer in Europe and elsewhere (Table 1) might only be determined through extensive molecular studies of such sarcocysts.

Only one of the seven sarcocysts from cardiac muscle processed for SEM in this study had an exposed surface, which allowed an examination of its surface structure. The sarcocyst was found to possess ribbon-like protrusions (Fig. 2) similar to those revealed in previous SEM studies of sarcocysts of *S. grueneri* from reindeer (Gjerde, 1986), *S. taeniata* in moose (Gjerde, 2014b) and *S. linearis* in roe deer (Gjerde et al., 2017). Since sarcocysts of *S. cervicanis* predominated in the heart of the three red deer from which sarcocysts were processed for SEM, it is likely that this sarcocyst also belonged to *S. cervicanis*. In the recent study of *S. linearis* in roe deer, intact protrusions of this type could be examined, revealing that they were ribbon-like in their proximal portion before

terminating in a plate-like structure with an elliptic outline, together forming a seesaw-like structure (Gjerde et al., 2017). In the single sarcocyst examined in this study, only the ribbon-like proximal portion of each protrusion was present, probably because the delicate terminal plates had been torn off when the surrounding host cell was removed, as suggested by the distorted appearance of many protrusions. Thus, by TEM (Fig. 5 in Hernández-Rodríguez et al., 1981b), some of the protrusions of *S. cervicanis* are seen to have bifurcated (T-shaped) profiles, which represent sectioned terminal plates (Gjerde et al., 2017).

Although sarcocysts of *S. cervicanis* predominated in cardiac muscle of the red deer examined, a few sarcocysts of *S. linearis* were also identified. Likewise, three sarcocysts of *S. linearis* were identified in cardiac muscle of an Italian roe deer, whereas 16 sarcocysts were found in the diaphragm of another roe deer (Gjerde et al., 2017). Reissig et al. (2016) examined sarcocysts from both cardiac and skeletal muscle of red deer in Argentina and characterized a few of them at the 18S rRNA gene (KT626599–KT626602). The resulting sequences were assigned to *S. taeniata*, but as demonstrated in

the present study and in the previous comparison of *S. linearis* with *S. taeniata* (Gjerde et al., 2017), these species cannot be separated on the basis of their 18S rRNA gene sequences. Hence, the abovementioned four sequences from Argentinian red deer and the two similar sequences from Lithuanian red deer (JN356126, JN356127) might belong to *S. linearis* rather than to *S. taeniata* as assumed by Reissig et al. (2016) and Prakas et al. (2016), respectively. This question might be resolved by examining sarcocyst isolates from red deer in these countries at *cox1*, since *cox1* sequences seem to clearly separate the two species from each other. In addition, this study indicated that there might be consistent differences between sequences of *S. linearis* from red deer and sequences of *S. taeniata* from moose and sika deer near the 3' end of the 18S rRNA gene (three versus five or six consecutive T's). At *cox1*, the difference between the two lineages were slightly smaller after the addition of the new sequences from red deer, but there were still no overlap between the intra- and interspecific sequence variation, and sequences assigned to each species formed separate monophyletic clusters in the phylogenetic tree. Hence, we consider *S. linearis* in roe deer and red deer to be different from *S. taeniata* as reported from moose and sika deer (Gjerde, 2014b; Prakas et al., 2016).

From a type 3 sarcocysts from the oesophagus of red deer CeS1, unique sequences were obtained both at *cox1* and the 18S rRNA gene, and these sequences were assigned to the new species *S. morae*. Since only a single sarcocyst had been examined by LM, we could not be absolutely sure whether this sarcocyst actually lacked visible protrusions or had possessed hair-like protrusions that were hidden by host cell material. Simple sequence comparisons indicated that this species was most closely related to species with ribbon-like cyst wall protrusions (*S. cervicanis*, *S. taeniata*) at *cox1*, whereas at the 18S rRNA gene, *S. morae* showed a similar identity to species with either hair-like or ribbon-like protrusions. However, in the phylogenetic analysis based on sequences of the latter gene, *S. morae* clustered with species having ribbon-like protrusions when the dataset comprising mainly species transmitted by canids was used (Fig. 4), but not when a larger dataset was used (Fig. S3 in the Supplementary material). We believe that the phylogeny based on the smaller dataset better reflected the relationship among the species included, since the sequences probably were more correctly aligned than in the larger dataset. Thus, in the phylogeny based on *cox1* sequences (Fig. 3), *S. morae* were placed within a cluster comprising all species with ribbon-like protrusions, but also *S. hircicanis*. Based on these molecular findings, we believe that *S. morae* also possess ribbon-like cyst wall protrusions, and that its sarcocysts therefore might be indistinguishable by TEM and SEM from sarcocysts of *S. cervicanis* and *S. linearis* in red deer. Clearly, additional studies are necessary to characterize *S. morae* better morphologically, but the molecular data provided in this study,

particularly the *cox1* sequence, should be sufficient to delimit this species from other species with similar sarcocyst morphology in red deer or other hosts.

Three species with hair-like cyst wall protrusions were found in red deer CeS1. Of these, only *S. hjorti* was known from before, having been reported from both red deer and moose (Dahlgren & Gjerde, 2010a, b; Prakas, 2011; Stephan et al., 2012). However, when the single cyst of this species was examined by LM, no cyst wall protrusions were visible, which was also the case with several small sarcocysts of *S. hjorti* from cardiac muscle of red deer and moose in Norway (Dahlgren & Gjerde, 2010a). This was probably due to incomplete removal of the surrounding host cell material during cyst isolation. In the sarcocysts assigned to the two new species *S. iberica* and *S. venatoria*, on the other hand, hair-like protrusions were clearly seen by LM. Moreover, these species clustered with *S. hjorti* and other species with hair-like protrusions in the phylogenetic analysis. The *cox1* sequence obtained from one of the sarcocysts with hair-like protrusions differed by 4% from the 10 identical sequences obtained from the 10 other sarcocysts of this type. This difference was considered to be sufficient to attribute the two sequence types to separate species, that is, *S. iberica* and *S. venatoria*. In comparison, the difference between all *cox1* sequences assigned to *S. linearis* and those of *S. taeniata* was 2.9–4.2% and the difference between sequences of *S. elongata* in red deer and *Sarcocystis tarandi* in reindeer was 2.4–3.1% (Gjerde, 2014a). Moreover, the intraspecific divergence of *cox1* sequences of similar length of various *Sarcocystis* spp. is less than 2%, and frequently less than 1.5% (Gjerde, 2013, 2014a, b, 2016; Gjerde et al., 2017). Hence, it is unlikely that the *cox1* sequence attributed to *S. iberica* should represent an atypical sequence of *S. venatoria*, and even more so, since the 10 *cox1* sequences of the latter species were identical. Based on the 18S rRNA gene sequences, on the other hand, neither *S. iberica* and *S. venatoria*, nor *S. linearis* and *S. taeniata*, or *S. elongata* and *S. tarandi* could be separated from each other. The same is true with this marker for several other such sister species in ruminants as reviewed by Gjerde et al. (2017). It is also a characteristic of such closely related sister species that they have considerable intra-isolate (intra-genomic) sequence variation in the 18S rRNA gene, which makes attempts to separate them on the basis of this marker even more difficult.

The assignment of isolates with indistinguishable 18S rRNA gene sequences to separate species on the basis of their *cox1* sequences is based on the premise that differences at this marker above a certain level signify separate species. However, we still do not know the exact boundaries of each species at the *cox1* marker, and with respect to *S. iberica* and *S. venatoria*, additional isolates ought to be examined in order to ascertain that they represent distinct species. The low bootstrap support for many of the nodes in the phylogenetic tree based on *cox1* sequences (Fig. 3) seems to have been partially

due to the large number of sequences (386) included in the analysis. Thus, a reduction of the dataset to 80 sequences through inclusion of a single sequence of each species only, except those found in the present study, resulted in much higher support values, but also changed the relationship between some taxa (data not shown). Other studies have also shown that bootstrap support decreases when the number of sequences included in the analysis is increased, whereas the phylogenetic accuracy increases with increased taxon sample size (Soltis & Soltis, 2003).

Molecular identification of sarcocysts in recent years have revealed that many *Sarcocystis* spp. are not as intermediate host specific as once believed. Some *Sarcocystis* spp. may also recently have become adapted to new intermediate host due to confinement of their hosts to smaller areas and new opportunities for cross-transmission as a result of human activity. Hence, it is possible that some of the species found in red deer CeS1 in this study may use other cervids as their principal intermediate hosts. Thus, *S. morae*, *S. iberica* and *S. venatoria* could possibly use fallow deer (*Dama dama*) as their principal host, since this cervid is sympatric with red deer in Quintos de Mora (Santín-Durán et al. 2004), but less numerous. Given that the sarcocysts of *S. morae* have band-like protrusions, they would be similar to sarcocysts of an unnamed *Sarcocystis* sp. reported from fallow deer in Germany and Italy (Entzeroth et al., 1985; Poli et al., 1988; Wesemeier & Sedlacek, 1995b). No species with hair-like cyst wall protrusions have so far been reported from fallow deer, and none of the *Sarcocystis* spp. in fallow deer have so far been examined by molecular methods.

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