# *Sarcocystis* species in red deer revisited: with a re-description of two known species as *Sarcocystis elongata* n. sp. and *Sarcocystis truncata* n. sp. based on mitochondrial *cox1* sequences

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SUMMARY

In a previous investigation, five *Sarcocystis* species were described from Norwegian red deer and believed to be conspecific with species occurring in either reindeer or moose based on sarcocyst morphology and nucleotide sequences of the nuclear ribosomal DNA unit. The aim of the present study was to characterize numerous isolates of these sarcocyst types at the mitochondrial cytochrome c oxidase subunit I gene (*cox1*) in order to corroborate or refute previous species designations of *Sarcocystis* in red deer. The *Sarcocystis tarandi*- and *Sarcocystis rangiferi*-like taxa in red deer and reindeer, respectively, were thoroughly compared by sequencing 14–27 isolates of each type. Sequence comparisons revealed four distinct sequence types, which by phylogenetic analyses were placed in four monophyletic groups according to host origin, and they were therefore considered to represent four separate species. The two taxa of this type in red deer were named *Sarcocystis elongata* and *Sarcocystis truncata*, respectively. Sequencing of many isolates of *Sarcocystis hjorti* and *Sarcocystis ovalis* from red deer and moose confirmed that these species occur in both hosts. A revised description of the two new species is given and the current knowledge concerning all six *Sarcocystis* species in red deer is reviewed.

Key words: *Sarcocystis elongata*, *Sarcocystis hardangeri*, *Sarcocystis hjorti*, *Sarcocystis ovalis*, *Sarcocystis rangiferi*, *Sarcocystis tarandi*, *Sarcocystis truncata*, red deer, reindeer, *cox1*, species delimitation.

## INTRODUCTION

Protozoan parasites of the genus *Sarcocystis* (Apicomplexa, Sarcocystidae) have a two-host life cycle with mainly herbivores and omnivores as intermediate hosts and carnivores as definitive hosts. Cervid species like red deer, reindeer, roe deer and moose may each act as intermediate hosts for several *Sarcocystis* species, as revealed by differences in sarcocyst morphology and/or DNA sequences (Dahlgren, 2010). For many years, molecular delimitation of *Sarcocystis* species has been based almost solely on nucleotide sequences from the nuclear ribosomal DNA unit, particularly the 18S or small subunit (ssu) ribosomal RNA (rRNA) gene, and to a lesser extent the 28S rRNA gene and the internal transcribed spacer 1 (ITS1) region (see Gjerde, 2013*b*). Recently, however, the mitochondrial protein-coding cytochrome c oxidase subunit I gene (*cox1*) was established as a new marker for delimitation of *Sarcocystis* species in cervids, cattle and sheep (Gjerde, 2013*b*), but this gene may also be useful for differentiating between *Sarcocystis* species in other hosts.

In a previous study (Dahlgren and Gjerde, 2010*a*), free-ranging red deer (*Cervus elaphus*) from western Norway were found to host five different *Sarcocystis* species as determined from their sarcocyst morphology and near complete ssu rRNA gene sequences, i.e. *Sarcocystis hjorti, Sarcocystis hardangeri, Sarcocystis ovalis, Sarcocystis rangiferi* and *Sarcocystis tarandi*. There were some doubts, however, concerning the assignment of the latter two taxa to two species previously characterized from reindeer. Thus, isolates/clones of each of these sarcocyst types from both hosts showed a moderate sequence variation (0–1·1%) at the ssu rRNA gene, and the within-host and between-host sequence variation for each sarcocyst type overlapped. Moreover, both in the original (see Fig. 10 in Dahlgren and Gjerde, 2010*a*) and in subsequent (Gjerde, 2012, 2013*b*) phylogenetic analyses, ssu rRNA gene sequences of *S. tarandi* from reindeer were interspersed with sequences of the similar species in red deer, suggesting that a single species of this type infected both hosts. All sequences of *S. rangiferi* from reindeer, on the other hand, clustered in a monophyletic group, whereas those obtained from the similar species in red deer formed two other clusters together with sequences from an unnamed *Sarcocystis* sp. in sika

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deer, indicating the presence of at least two separate species infecting reindeer and red deer/sika deer, respectively (Dahlgren and Gjerde, 2010*a*; Gjerde, 2012, 2013*b*). In an attempt to resolve this question, the ITS1 region was also examined in the original study, but these sequences showed an even higher degree of within-host variation (0–12·8%), and clustered in a similar manner to the ssu rRNA gene sequences (see Fig. 11 in Dahlgren and Gjerde, 2010*a*). Moreover, partial sequences of the 28S rRNA gene were also generated from a few isolates of each species from both hosts, but these sequences also displayed some intraspecific variation, as well as a high similarity between the *S. tarandi*-type isolates and a slight difference between the *S. rangiferi*-type isolates from the two hosts (Dahlgren and Gjerde, 2010*a*; unpublished data).

However, when ∼1 kb long sequences of the mitochondrial *cox1* were obtained and used in phylogenetic analyses (Gjerde, 2013*b*), the seven characterized isolates of the *S. rangiferi*-like species in red deer and the six isolates of *S. rangiferi* from reindeer formed two separate monophyletic clusters, as did the seven isolates of the *S. tarandi*-like species in red deer and the eight isolates of *S. tarandi* from reindeer, respectively. Moreover, there was less sequence divergence between the isolates of each type from the same host, than between isolates from different hosts, particularly for the *S. rangiferi*-type. Each of these two species in red deer therefore seemed to be different from the two similar species in reindeer, and were provisionally referred to as *Sarcocystis* cf. *rangiferi* and *Sarcocystis* cf. *tarandi* in that paper (Gjerde, 2013*b*), pending an even more thorough comparison using more isolates of each taxon. The principal aim of the present study was therefore to obtain partial *cox1* sequences of additional isolates of each of these taxa from both red deer and reindeer, and use the new sequences together with the previously generated *cox1* sequences to establish the boundaries of these species through sequence comparisons and phylogenetic analyses. Provided that the results showed that the two species in red deer were distinct from those in reindeer, a second aim was to redefine these species by using previous and new data, and collate the complete species descriptions in a single paper for future reference. The *cox1* sequences previously obtained from a limited number of isolates of each *Sarcocystis* species (Gjerde, 2013*b*), seemed, on the other hand, to confirm that the three other species reported from red deer (Dahlgren and Gjerde, 2010*a*) were indeed conspecific with *S. hardangeri* in reindeer and with *S. hjorti* and *S. ovalis* in moose, respectively. Nevertheless, the present study also aimed at corroborating these findings by comparing more isolates of these taxa at *cox1* in order to make an updated review of all known *Sarcocystis* species in red deer.

## MATERIALS AND METHODS

### *Host origin and isolation of sarcocysts*

The host origin, identity and number of sarcocyst isolates of different types examined in the present study are given in Table 1. The main focus was on cysts of the *S. rangiferi*- and *S. tarandi*-types from red deer and reindeer and cysts of the *S. hjorti*- and *S. ovalis*-types from red deer and moose. However, for comparative purposes some cysts of *Sarcocystis alces* from moose and of *Sarcocystis capreolicanis*, *Sarcocystis gracilis* and *Sarcocystis silva* from roe deer were also included. All sarcocysts were isolated during 2005–2010 from the oesophagus, diaphragm and/or heart of semi-domesticated reindeer (*Rangifer tarandus*) in northern Norway (Dahlgren and Gjerde, 2007), free-ranging moose (*Alces alces*) in south-eastern Norway (Dahlgren and Gjerde, 2008, 2010*b*; Gjerde and Dahlgren, 2010), free-ranging roe deer (*Capreolus capreolus*) in south-eastern Norway (Dahlgren and Gjerde, 2009; Gjerde, 2012), and free-ranging red deer (*C. elaphus*) in western Norway (Dahlgren and Gjerde, 2010*a*). The red deer were adult animals from Rogaland and Hordaland counties in western Norway, which had been killed in September–October, 2006–2008 during the annual hunting season as described previously (Dahlgren and Gjerde, 2010*a*). The reindeer were semi-domesticated adult animals that had been slaughtered in 2005 and 2006 at abattoirs in Nord-Trøndelag, Nordland, Troms, and Finnmark counties in northern Norway as described for some of this material previously (Dahlgren and Gjerde, 2007, 2010*a*).

All muscle samples were frozen shortly after collection and/or arrival at the laboratory and stored at −20 °C for up to 2 years before examination. Samples from different cervid hosts were kept and processed separately. Upon thawing, the samples were examined under a stereo microscope and individual sarcocysts were excised and identified to cyst type/species under a light microscope as described previously (Dahlgren and Gjerde, 2007; Gjerde, 2013*b*). After cyst isolation and preliminary species identification, individual sarcocysts were placed in 20 μL of distilled water in labelled 1·5 mL micro-centrifuge tubes and kept frozen at −20 °C until DNA isolation weeks to years later.

### *DNA isolation, PCR and sequencing*

The majority of the sarcocyst isolates used in this study had been kept frozen for 5–7 years before genomic DNA was extracted in January–April 2013 from individual sarcocysts using QIAmp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's tissue protocol (Dahlgren and Gjerde, 2007). The remaining DNA samples had been extracted from sarcocysts in the same manner in 2005–2008 in connection with the characterization of the ssu rRNA

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Table 1. Overview of *Sarcocystis* isolates (sarcocysts) from red deer (Ce), reindeer (Rt), roe deer (Cc) and moose (Aa) from which partial *cox1* sequences were obtained in the present study and their associated GenBank accession numbers. The ratio between the number of isolates examined of each species and the number of haplotypes (Ha) found is also given, both for sequences obtained in the present (A) and a previous study (B)[a], as well as for both studies combined (A + B)

| Sarcocystis spp. | Host | Isolates examined at *cox1* | GenBank accession nos. | Isolates/Haplotypes | | |
|---|---|---|---|---|---|---|
| | | | | A | B[a] | A + B |
| *S. alces* | Moose | Aa14.4[b], Aa15.6[b] | KF241309–KF241310 | 2/1 | 10/7 | 12/7 |
| *S. capreolicanis* | Roe deer | Cc10.6[b] | KF241311 | 1/1 | 3/2 | 4/2 |
| *S. elongata* | Red deer | Ce3.7[b/c], Ce3.18[b], Ce6.10[b/c], Ce9.12[b/c], Ce9.22[b/c], Ce16.20[b/c], Ce16.23[b], Ce19.5[b], Ce19.12[b], Ce20.10[b], Ce20.12[b/c], Ce24.4[b], Ce25.5[b/c], Ce28.9[b], Ce31.5[b], Ce31.10[b], Ce31.21[b/c], Ce37.25[c] | KF241312–KF241329 | 18/9 | 7/6 | 25/13 |
| *S. gracilis* | Roe deer | Cc3.12[b], Cc3.31[b], Cc4.1[b], Cc4.2[b], Cc6.15[b], Cc7.7[b], Cc7.15[b], Cc9.1[c], Cc9.5[b], Cc11.12[b], Cc12.2[b], Cc12.10[b] | KF241330–KF241341 | 12/6 | 10/7 | 22/10 |
| *S. hjorti* | Red deer | Ce3.9[b], Ce6.1[b], Ce6.5[b], Ce9.19[b], Ce13.8[b], Ce15.8[b], Ce18.4[b], Ce23.6[b], Ce34.2[b], Ce35.12[b], Ce36.3[b] | KF241342–KF241352 | 11/4 | 5/4 | 16/6 |
| | Moose | Aa36.6[b], Aa37.8[b] | KF241353–KF241354 | 2/1 | 4/3 | 6/4 |
| *S. ovalis* | Red deer | Ce6.4[d], Ce8.2[d], Ce8.3[d], Ce9.17[d], Ce12.13[d], Ce16.10[d], Ce16.12[d], Ce19.30[e], Ce23.8[e], Ce29.10[e], Ce32.6[d], Ce34.22[e] | KF241355–KF241366 | 12/2 | 5/2 | 17/2 |
| | Moose | Aa20.16[e], Aa25.14[d/e], Aa28.10[e], Aa39.11[d/e], Aa42.10[e], Aa46.34[e], Aa47.6[e], Aa49.4[e], Aa49.26[e], Aa51.11[e], Aa52.13[e], Aa54.8[d/e], Aa55.7[e], Aa56.5[e], Aa57.4[e], Aa57.7[e] | KF241367–KF241382 | 16/1 | 6/1[f] | 22/1 |
| *S. rangiferi* | Reindeer | Rt21.1[c], Rt21.2[c], Rt23.2[c], Rt23.3[c], Rt32.6[b], Rt32.7[c], Rt52.17[b], Rt63.1[b], Rt65.12[b], Rt66.5[b], Rt111.4[c], Rt112.2[c], Rt112.22[b/e], Rt117.12[c], Rt119.8[c], Rt120.4[b/e], Rt124.4[c], Rt127.3[c], Rt127.4[c], Rt127.5[c], Rt142.19[b/c], Rt151.33[b], Rt152.24[b], Rt155.7[b], Rt171.21[b/e], Rt172.18[b/e], Rt211.6[c] | KF241383–KF241409 | 27/20 | 6/6 | 33/24 |
| *S. silva* | Roe deer | Cc11.19[b] | KF241410 | 1/1 | 2/2 | 3/3[g] |
| *S. tarandi* | Reindeer | Rt21.10[c], Rt21.12[c], Rt29.12[c], Rt33.6[c], Rt51.11[c], Rt52.13[c], Rt64.2[c], Rt65.9[c], Rt65.11[c], Rt68.2[c], Rt69.6[c], Rt69.7[c], Rt113.3[c], Rt117.13[c], Rt119.6[c], Rt125.10[c], Rt131.19[c], Rt141.18[c], Rt151.10[c], Rt152.18[c], Rt154.22[c], Rt155.16[c], Rt171.15[c], Rt173.12[c], Rt173.13[c], Rt200.13[c], Rt235.15[c] | KF241411–KF241437 | 27/22 | 8/8 | 36/24 |
| | Red deer | Ce34.14[c] | KF241438 | 1/1[h] | – | |
| *S. truncata* | Red deer | Ce3.22[b], Ce13.7[b], Ce16.4[b], Ce19.16[b], Ce19.25[b/c], Ce20.13[b], Ce21.8[b/c], Ce24.10[b], Ce28.12[b], Ce31.15[b], Ce34.5[b/c], Ce34.10[b], Ce34.19[b], Ce34.24[b] | KF241439–KF241452 | 14/11 | 7/7 | 21/16 |

[a] Data from Table 2 in Gjerde (2013*b*), in which *S. elongata* was designated *S.* cf. *tarandi* and *S. truncata* was designated *S.* cf. *rangiferi*.

[b–e]Reverse primers used together with forward primer SF1 to amplify the various isolates: [b]SR8D, [c]SR9, [d]SR10, [e]COIRm. Some isolates were amplified and sequenced twice with different reverse primers as indicated by two superscript letters; only the longest sequence in such pairs was submitted to GenBank.

[f] Haplotypes only from moose in Norway; a different haplotype from a Canadian moose is not included.

[g] Two additional haplotypes have been found for *S. silva* in moose (Gjerde, 2013*b*).

[h] The haplotype of *S. tarandi* in red deer was identical to a haplotype in reindeer.

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gene as reported previously (Dahlgren and Gjerde, 2007, 2008, 2009, 2010*a*).

A fragment of *cox1* was amplified using forward primer SF1 and one of the following reverse primers: SR8D (1029 bp long sequence, without primers), SR9 (1038 bp), SR10 (1092 bp) or COIRm (1095 bp), depending on species and isolate as detailed in Table 1. For some species and isolates reverse primers SR4, CoxS1R and SR5 were also attempted initially. Primer sequences have been published before (Gjerde, 2013*b*), except those of the two newly designed reverse primers SR9 (5′-atatccataccrccattgcccat-3′) and SR10 (5′-aacgacagcacaaaatggaagtg-3′). Each PCR reaction mixture contained 2·5 $\mu$L of the DNA solution, 18·75 $\mu$L HotStarTaq Master Mix (Qiagen GmbH, Germany), 10 pmol of each primer, 6 $\mu$g of bovine serum albumin, and RNase-free water to make a final volume of 37·5 $\mu$L. A negative control was included in each PCR run. PCR reactions were carried out in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories Inc., USA). Cycling conditions were: initial Hot Start at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 52–53 °C for 30 s (temperature depending on primer pair), extension at 72 °C for 90 s; and final extension at 72 °C for 10 min.

PCR products were evaluated, purified and sequenced and the resulting sequences were assembled as previously described (Gjerde, 2013*b*). All newly obtained *cox1* sequences of different species and isolates were compared pair-wise with each other and with those previously obtained from *cox1* of *Toxoplasma gondii, Neospora caninum, Hammondia heydorni* and *Hammondia triffittae* (Gjerde, 2013*a*) and various *Sarcocystis* spp. (Gjerde, 2013*b*) using the programme Nucleotide BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/). The software package DnaSP (DNA Sequence Polymorphism) version 5.10.01 (Librado and Rozas, 2009) was used for the analysis of nucleotide polymorphisms (sequence variation) among the *cox1* sequences, but these comparisons were confined to nucleotides 1–1020, which were present in all sequences. The analyses included enumeration of variable (polymorphic) sites and haplotypes among the sequences, and estimation of different measures of sequence divergence within and between species.

### *Phylogenetic analyses*

Phylogenetic analyses were conducted on *cox1* sequences by means of the MEGA5 (v5.10) software (Tamura *et al*. 2011). A total of 308 nucleotide sequences from 25 species were included in the analyses. They comprised the 144 new sequences from 10 *Sarcocystis* species generated in the present study as listed in Table 1 with their GenBank accession numbers. Furthermore, they comprised the 155 sequences of 22 *Sarcocystis* spp. (GenBank accession numbers KC209578–KC209732) from the previous study (see Table 2 in Gjerde, 2013*b* for details about these species/isolates), and sequences of the following six species: *H. triffittae* (JX473247–JX473249), *H. heydorni* (JX473250–JX473251), *N. caninum* (JX473252), *T. gondii* (JX473253) (see Gjerde, 2013*a*); *Eimeria necatrix* (HQ702482) and *Eimeria tenella* (HQ702484). The new partial *cox1* nucleotide sequences of various *Sarcocystis* spp. could easily be aligned against each other and against the codon-based multiple sequence alignment generated previously (Gjerde, 2013*b*), which resulted in a multiple alignment with no gaps, comprising individual *cox1* sequences of different *Sarcocystis* spp. varying in length between 1020–1095 nucleotides. Since all positions containing gaps and missing data were eliminated in the analyses, there were a total of 1020 positions in the final dataset. Phylogenetic trees were reconstructed by the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the p-distance model and 10 000 bootstrap replications (Felsenstein, 1985). All codon positions were used. The two *Eimeria* spp. from chickens (family: Eimeriidae) were used as outgroup species to root the trees. Other tree-building methods (maximum likelihood, maximum parsimony) were also tested for comparison.

## RESULTS

### *PCR amplification and sequence comparisons*

Initially, attempts were made to PCR-amplify isolates of all species, except those of the *S. ovalis*-type, using primer pair SF1/SR8D. This primer pair was found to work very well with isolates of *S. alces, S. capreolicanis, S. gracilis, S. hjorti, S. silva* and the *S. rangiferi*-like species from red deer, and also fairly well with some isolates of *S. rangiferi* from reindeer and some isolates of *S.* cf. *tarandi* from red deer. However, this primer pair resulted in very poor amplification of isolates of *S. tarandi*, and these products could not be used for sequencing. Three other reverse primers, SR4, CoxS1R and SR5 were then attempted with isolates of *S. tarandi*, but with similar poor results. This prompted the design of a new reverse primer, SR9, which in combination with forward primer SF1 resulted in excellent amplification and high-quality sequences not only of *S. tarandi*, but also of *S. rangiferi, S.* cf. *tarandi* and *S.* cf. *rangiferi*. However, many isolates of the latter three species had already been satisfactorily amplified with SR8D, and were therefore not amplified again with primer SR9. As regards the isolates of *S. ovalis*-type from red deer and moose, some of them were initially satisfactorily amplified

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with primer pair SF1/COIRm, but a more specific reverse primer, SR10, was subsequently designed and used for some isolates with excellent results. The latter primer targeted nearly the same sequence region as primer COIRm, but was designed from a sequence of *S. ovalis* covering the complete 3′ end of *cox1* (data not shown), and was therefore more specific than COIRm, which had primarily been designed for members of the Toxoplasmatinae (Gjerde, 2013*a*).

In total, new partial *cox1* sequences, 1029–1095 bp in length (primers not included) were obtained from a total of 144 sarcocyst isolates, which after sequence comparisons using BLAST, could be assigned to 10 different *Sarcocystis* species as listed in Table 1. These sequences were submitted to GenBank and have been issued accession numbers KF241309–KF241452. Sequence comparisons using BLAST and DnaSP revealed considerable differences between the species concerning intraspecific nucleotide diversity as partly reflected in the relative number of haplotypes, which has been summarized in Table 1, both for the new sequences, the previous sequences (Gjerde, 2013*b*), and for all available *cox1* sequences of these species. The sequences of *S. rangiferi* and *S. tarandi* displayed more polymorphic sites (42 and 40 of 1020 sites, respectively) than those of other species (*Sarcocystis tenella*, 35 sites; *S.* cf. *tarandi*, 27 sites; *S. silva*, 20 sites; *S.* cf. *rangiferi*, 19 sites; the remaining species, 0–18 polymorphic sites), but these numbers are also influenced by the total number and host origin of the isolates examined of each species.

Altogether 14 sarcocyst isolates from red deer could be assigned to *S.* cf. *rangiferi*, including three isolates from the oesophagus/diaphragm that had been tentatively identified microscopically as belonging to *S.* cf. *tarandi*, and one correctly identified isolate from cardiac muscle. Similarly, a total of 27 sarcocyst isolates from reindeer could be assigned to *S. rangiferi* based on *cox1* sequences, including three isolates from the oesophagus/diaphragm and one isolate from cardiac muscle that had been identified microscopically as sarcocysts of *S. tarandi*. A comparison of the sequences of *S.* cf. *rangiferi* from red deer with those of *S. rangiferi* from reindeer, revealed a sequence identity of ∼93%, both for the new sets of sequences (14 *vs* 27), and for all available sequences (21 *vs* 33) of these taxa, and there were 58 fixed nucleotide differences (nucleotide sites where all sequences of one population differed from all sequences of the other population) between the sets comprising all sequences. Furthermore, the 21 sequences of *S.* cf. *rangiferi* from red deer shared ∼96% identity (30 fixed nucleotide differences) with the five available sequences of *S. silva* (from moose and roe deer), whereas the 33 sequences of *S. rangiferi* from reindeer shared ∼92% identity (62 fixed nucleotide differences) with those of *S. silva*. In contrast, the sequence identities within each of these three taxa were >99%.

A total of 27 sarcocyst isolates from reindeer and one isolate from red deer could be assigned to *S. tarandi*. The single isolate of this species from red deer (Ce34.14; GenBank number KF241438) was completely identical to one isolate of *S. tarandi* from reindeer and shared >99% identity with the other reindeer isolates of this species. The sarcocyst of this particular isolate from red deer had been subjected to DNA extraction concurrently with nine other sarcocysts of the *S. tarandi*-type from this host, but this isolate showed a much weaker band on agarose gel than the nine other isolates following simultaneous PCR-amplification with primer pair SF1/SR8D. The isolate was subsequently amplified and sequenced twice on two separate occasions using reverse primer SR9, and two identical sequences were obtained. Hence, this isolate from red deer has been included with the sequences of *S. tarandi* from reindeer in the sequence comparisons, which showed 98·7–100% sequence identity among all 36 isolates of this type examined in the present (28 isolates) and the previous (eight isolates) study.

A total of 18 sarcocyst isolates from red deer could be assigned to *S.* cf. *tarandi* based on *cox1* sequences. When the seven previous isolates of this type were also included in the comparisons, the sequence identity among all 25 isolates was 98·5–100%. In contrast, the sequence identity between all available sequences of this type in red deer and all the above-mentioned sequences of *S. tarandi* was 96·9–97·6%, corresponding to differences between the two populations in 24–32 out of 1020 nucleotides (on average 27·6 nucleotides or 97·3% identity), including 14 fixed nucleotide differences. Most of the nucleotide differences were due to synonymous substitutions (silent mutations), and hence the two taxa differed at only two of 340 inferred amino acid residues.

The characterization of 13 new isolates of *S. hjorti*, 11 from red deer and two from moose, revealed some known and some new haplotypes compared with the nine isolates from the previous investigation (Gjerde, 2013*b*). None of the haplotypes in red deer were completely identical to any of the haplotypes in moose, but the differences between the sequences from either host were rather small, i.e. in 4–7 out of 1020 nucleotides (on average 5·3 nucleotides), and there was only one fixed nucleotide difference between the two populations.

The characterization of eight previously identified (by using the ssu rRNA gene) and four new isolates of the *S. ovalis*-type from red deer revealed that they all belonged to *S. ovalis*, whereas no isolates of the morphologically indistinguishable species *S. hardangeri* were found. Hence, only isolates of *S. ovalis* from moose were examined for comparison. The 12 newly examined isolates of *S. ovalis* from red deer

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belonged to the same two *cox1* haplotypes as the five previously examined isolates from this host (Gjerde, 2013*b*). A single nucleotide substitution separated the most common haplotype (14 isolates) from the rarer haplotype, which were found in three isolates from the same host animal (isolates Ce8.1, Ce8.2, Ce8.3; GenBank numbers KC209652, KF241356–KF241357), suggesting that these three sarcocysts had developed from the same infection event. The 16 new isolates of *S. ovalis* from moose were identical (confirmed by amplifying and sequencing some isolates a second time using new primer solutions, as well as doing it simultaneously with the slightly different isolates from red deer), and also identical to six previous isolates from this host (Gjerde, 2013*b*). All 22 isolates from Norwegian moose differed at the same two or three nucleotide positions from the abovementioned 17 isolates of *S. ovalis* from red deer.

The characterization of additional isolates of *S. alces* from moose and of *S. capreolicanis*, *S. gracilis* and *S. silva* from roe deer, recovered new haplotypes of the latter two species (Table 1), but the new types shared >99% sequence identity with the previously known haplotypes of each species. The sequencing results confirmed that all of these isolates had been correctly assigned to species based on sarcocyst morphology and host origin.

### *Phylogenetic analyses*

In the phylogenetic analysis using the NJ method, all taxa (sequence types) represented by two or more sequences formed monophyletic clusters, which have been collapsed in the NJ tree shown in Fig. 1. Such monophyletic clusters were also obtained when other tree-building methods (maximum likelihood, maximum parsimony) were used with the appropriate settings (data not shown). Of particular interest for the objective of this study was the fact that there was maximum support for the placement of all 25 *S. tarandi*-like isolates from red deer (=*Sarcocystis elongata*; see next section) as a sister taxon to all 35 isolates of *S. tarandi* from reindeer and the single isolate of this species from red deer. Likewise, there was maximum support for the placement of all 21 *S. rangiferi*-like isolates from red deer (=*Sarcocystis truncata*; see next section) as a sister taxon to the five isolates of *S. silva* from roe deer and moose, while both of these taxa formed a sister group to all 33 isolates of *S. rangiferi* from reindeer. Furthermore, all 16 isolates of *S. hjorti* from red deer formed a monophyletic group together with the six isolates of this species from moose, and all 17 isolates of *S. ovalis* from red deer formed a monophyletic group together with 22 isolates of this species derived from Norwegian moose and one isolate from a Canadian moose. Similarly, all nine isolates of *S. hardangeri* that were characterized in the recent study (Gjerde, 2013*b*), including one from red deer and eight from reindeer, formed a monophyletic group, which was sister taxon to the *S. ovalis* group.

As regards the overall phylogenetic relationships of the various taxa to each other, all *Sarcocystis* spp. formed a sister clade to the four members of the Toxoplasmatinae (*T. gondii*, *N. caninum*, *H. heydorni*, *H. triffittae*), and there was maximum bootstrap support for the placement of the various *Sarcocystis* spp. into three major clades, corresponding to their known or presumed definitive hosts, i.e. corvid birds (*S. hardangeri*, *S. ovalis*, *Sarcocystis oviformis*); canids (*S. alces*, *Sarcocystis alceslatrans*, *S. capreolicanis*, *Sarcocystis cruzi*, *S. gracilis*, *Sarcocystis grueneri*, *S. hjorti*, *Sarcocystis rangi*, *Sarcocystis tarandivulpes*, *S. tenella*); and felids/unknown (*Sarcocystis gigantea*, *Sarcocystis hirsuta*/*S. elongata*, *S. rangiferi*, *Sarcocystis scandinavica*, *S. silva*, *Sarcocystis sinensis*, *S. tarandi*, *S. truncata*) (Gjerde, 2013*b*). The relationships between some of the canine-transmitted species were, however, not clearly resolved in the phylogenetic analyses.

## TAXONOMIC SUMMARY

The sequence comparisons and phylogenetic analyses based on partial *cox1* sequences reported in the previous section clearly show that *S.* cf. *rangiferi* in red deer is different from *S. rangiferi* in reindeer, and is apparently more closely related to *S. silva* in roe deer and moose. The *cox1* data also strongly suggest that *S.* cf. *tarandi* in red deer is different from *S. tarandi*, which is mainly found in reindeer. Hence, *S.* cf. *rangiferi* and *S.* cf. *tarandi* in red deer will be redescribed in the following as *Sarcocystis truncata* n. sp. and *Sarcocystis elongata* n. sp., respectively, and their biological, morphological and molecular features will be summarized based on the results of the present study and those reported previously (Dahlgren and Gjerde, 2010*a*; Gjerde, 2013*b*).

*Name:* ***Sarcocystis elongata*** (syn. *S. tarandi*, *S.* cf. *tarandi*)

*Type intermediate host*: Red deer (*C. elaphus*).

*Type definitive host*: Unknown, but possibly felids based on phylogenetic placement and epidemiological data.

*Type locality*: Norway; Hordaland and Rogaland counties.

*Site of infection in intermediate host*: Mainly in skeletal muscles and oesophagus, occasionally in the heart.

*Description of sarcocysts*: Cysts in diaphragm and oesophagus slender and spindle-shaped with tapering pointed ends, measuring 1·0–2·3 × 0·07–0·15 mm (including protrusions). Due to their small diameter, cysts may be difficult to detect macroscopically. Cyst surface covered by densely packed, upright, thin,

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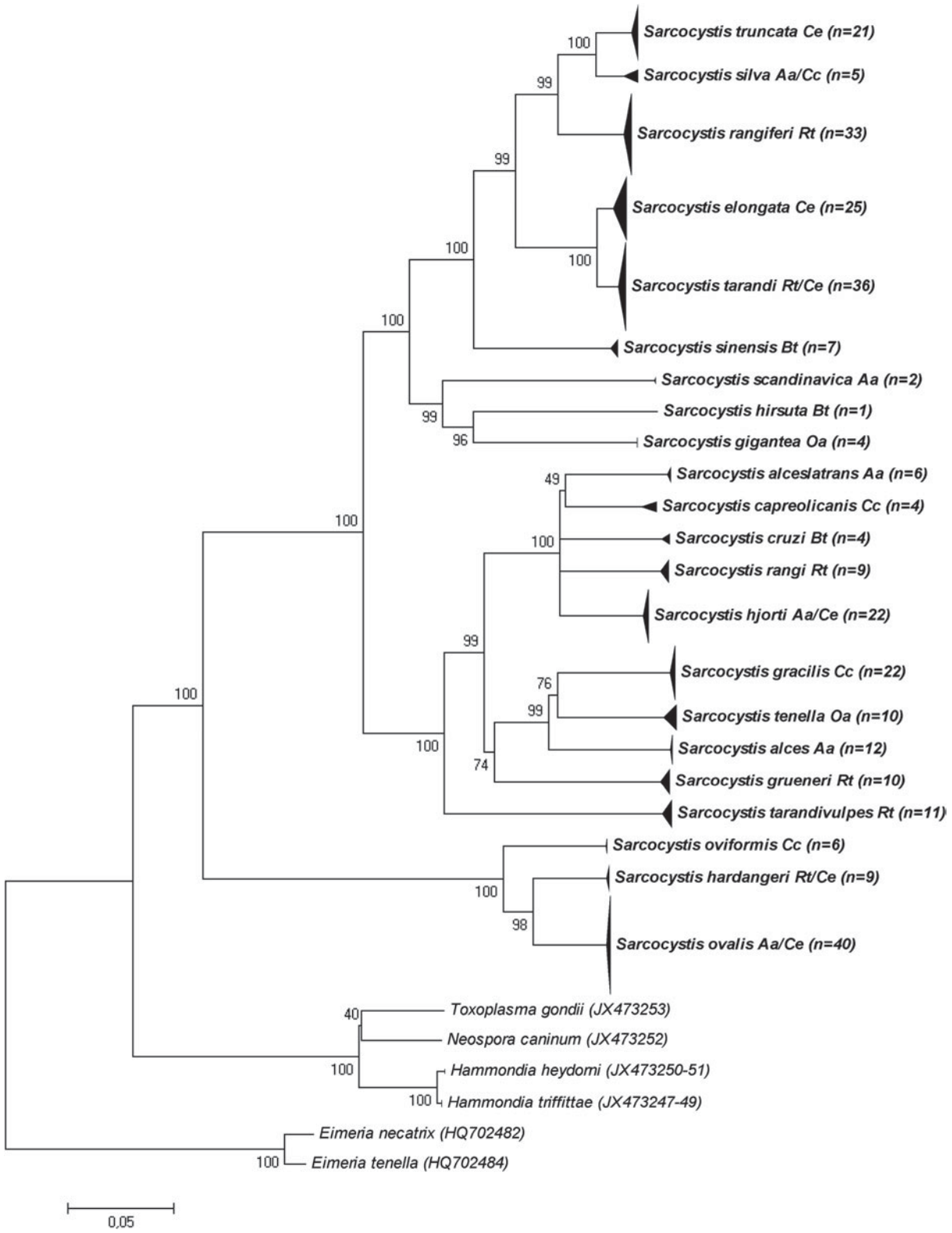


Fig. 1. Phylogenetic tree for members of the Sarcocystidae based on 308 partial sequences of *cox1* and inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Subtrees formed by two or more sequences of the same species have been collapsed, but the number of sequences included is given in parentheses behind the taxon names. The intermediate hosts from which sarcocysts were obtained for DNA isolation (or for infection of definitive hosts: two isolates of *S. gracilis* from foxes and two isolates of *S. ovalis* from a magpie) are also given, using the following abbreviations: Aa = *Alces alces* (moose); Bt = *Bos taurus* (cattle); Cc = *Capreolus capreolus* (roe deer); Ce = *Cervus elaphus* (red deer); Oa = *Ovis aries* (sheep); Rt = *Rangifer tarandus* (reindeer).

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finger-like protrusions, 7–8 $\mu$M long and 1·5–2 $\mu$M wide, with a round to polygonal outline, giving the cysts a thick, finely striated wall. Protrusions distributed in rows in a hexagonal pattern across cyst surface. Cysts in cardiac muscle, 0·2–0·3 × 0·06–0·13 mm in size, with about 5 $\mu$m long, finger-like protrusions. Such cysts are morphologically indistinguishable from those of *S. truncata*, making molecular identification of cardiac cysts necessary. The sarcocyst morphology as seen by light microscopy (LM) and scanning electron microscopy (SEM) is depicted in Figs 3a–d and 4 in Dahlgren and Gjerde (2010*a*).

*Type specimens*: Type material, consisting of a sarcocyst excised from muscular tissue of red deer, and photographs from LM and SEM examinations of the wall of other sarcocysts, have been deposited at the National History Museum, Oslo, Norway, with collection number NHMO-Prot00007 (originally deposited under the name *S. tarandi*).

*Molecular characteristics*: Ten nucleotide sequences (1727–1734 bp) of the near complete ssu rRNA gene differed from each other by 0·1–1·1%, and differed from 10 corresponding sequences of *S. tarandi* from reindeer (with 0·1–1·0% intraspecific variation) by 0·2–1·2%. Ten nucleotide sequences (~ 460–488 bp) of the ITS1 region differed from each other by 0–7·7%, and differed from 10 similar sequences of *S. tarandi* from reindeer (with 0·2–7·6% intraspecific variation) by 0·3–10·3%. Twenty-five partial *cox1* nucleotide sequences (1020–1038 bp) differed from each other by 0–1·5%, and differed from 36 sequences of *S. tarandi* by 2·4–3·1%.

*Nucleotide sequences deposited in GenBank*: GQ251011–GQ251020 (ssu rRNA gene; five clones from each of two cyst isolates from two red deer); GQ251001–GQ251010 (ITS1; five clones from each of two cyst isolates from two red deer); KC209705–KC209711 and KF241312–KF241329 (*cox1*; 25 sequences from 25 cyst isolates from 12 red deer).

*Etymology*: The species is named from its elongated, slender sarcocysts with pointed tips.

*Name:* ***Sarcocystis truncata*** (syn. *S. rangiferi, S.* cf. *rangiferi*)

*Type intermediate host*: Red deer (*C. elaphus*).

*Type definitive host*: Unknown, but possibly felids based on phylogenetic placement and epidemiological data.

*Type locality*: Norway; Hordaland and Rogaland counties.

*Site of infection in intermediate host*: Mainly in skeletal muscles and oesophagus, occasionally in the heart.

*Description of sarcocysts*: Cysts in diaphragm and oesophagus cigar-shaped to ellipsoidal with rounded tips, measuring 0·7–1·7 × 0·16–0·4 mm; mature cysts macroscopically visible. Cyst surface covered by densely packed, upright, fairly thick, finger-like protrusions, about 7–8 $\mu$m long and about 3 $\mu$m wide, with a round to polygonal cross-section, giving the cyst a thick, striated wall. Protrusions regularly distributed in rows in a hexagonal pattern across the surface. Cysts and their host cells do not seem to become enclosed by fibrous material. Cysts in cardiac muscle, 0·2–0·3 × 0·06–0·13 mm in size, with about 5 $\mu$M long, finger-like protrusions; morphologically indistinguishable from those of *S. elongata*, making molecular identification necessary. The sarcocyst morphology as seen by LM and SEM is depicted in Figs 3e, 5 and 6 in Dahlgren and Gjerde (2010*a*). The cyst from cardiac muscle shown in Fig. 3e (isolate Ce34.24) was found in the present study to belong to *S. truncata*.

*Type specimens*: Type material, consisting of a sarcocyst excised from muscular tissue of red deer, and photographs from LM and SEM examinations of the wall of other sarcocysts, have been deposited at the National History Museum, Oslo, Norway, with collection number NHMO-Prot00009 (originally deposited under the name *S. rangiferi*).

*Molecular characteristics*: Ten nucleotide sequences (1734–1739 bp) of the near complete ssu rRNA differed from each other by 0–1·0%, and differed from 10 corresponding sequences of *S. rangiferi* from reindeer (with 0–1·1% intraspecific variation) by 0·9–1·8%.Ten nucleotide sequences (~ 434–460 bp) of the ITS1 region differed from each other by 0·2–12·8%, and differed from 10 corresponding sequences of *S. rangiferi* from reindeer (with 0·2–10·0% intraspecific variation) by 5·3–14·1%. Twenty-one partial *cox1* nucleotide sequences (1029–1095 bp) differed from each other by <1%, and differed from 33 sequences of *S. rangiferi* from reindeer by ~7% and from five sequences of *S. silva* by ~4%.

*Nucleotide sequences deposited in GenBank*: GQ251021–GQ251030 (ssu rRNA gene; five clones from each of two cyst isolates from two red deer); GQ250991–251000 (ITS1; five clones from each of two cyst isolates from two red deer); KC209677–KC209683 and KF241439–KF241452 (*cox1*; 21 sequences from 21 cyst isolates from 14 red deer).

*Etymology*: The species is named from its thick, cigar-shaped sarcocysts with blunt, truncated tips.

## DISCUSSION

The present investigation has confirmed and expanded the findings in the previous study employing partial *cox1* sequences for delimitation of *Sarcocystis* spp. in red deer and other hosts (Gjerde, 2013*b*), and, in so doing, it has contradicted some of the original species designations of sarcocysts in Norwegian red deer, which were based on sarcocyst morphology and nucleotide sequences of the nuclear ribosomal DNA unit (Dahlgren and Gjerde, 2010*a*). In the following, the characteristics and relationships of the six

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*Sarcocystis* spp. in red deer to each other and to similar species in other cervid hosts will be discussed.

### *Sarcocystis truncata/S. rangiferi/S. silva*

Based on *cox1* sequences from many isolates, *S. truncata* of red deer seems to be clearly different from *S. rangiferi* in reindeer, and this species is apparently more closely related to *S. silva* in roe deer and moose than to *S. rangiferi*. Such a relationship was also suggested by phylogenetic reconstructions using ssu rRNA gene sequences, but only when more isolates of *S. silva* had been characterized at the latter gene (Gjerde, 2012, 2013*b*) than in the original study (Dahlgren and Gjerde, 2010*a*). In the previous phylogenetic analyses using ssu rRNA gene sequences (Dahlgren and Gjerde, 2010*a*; Gjerde, 2012, 2013*b*), isolates of *S. truncata* seemed to be even more closely related to an unnamed *Sarcocystis* sp. from sika deer (*Cervus nippon*) in Japan than to *S. silva* and *S. rangiferi*, which suggests that these *Cervus* spp. share the same *Sarcocystis* sp. Whether this is so, might be determined by sequencing *cox1* of this *Sarcocystis* sp. in Japanese sika deer.

The sarcocysts of *S. truncata* in red deer are quite similar to those of *S. rangiferi* in reindeer as regards the general size and shape of the cysts and their protrusions (Dahlgren and Gjerde, 2010*a*), but they do not seem to become encapsulated by a fairly thick layer of host-derived fibrous material, which is a consistent feature of *S. rangiferi* sarcocysts in reindeer (Gjerde, 1984*a*, 1985*b*, 1986). This difference supports the conclusion from this study that they are separate species, but it could also be due to differences in host reactions between red deer and reindeer. However, a similar encapsulation also occurs around cysts of *S. hardangeri* in both red deer and reindeer (Gjerde, 1984*b*, *c*, 1985*c*) and around cysts of *S. ovalis* in both red deer and moose (Dahlgren and Gjerde, 2008, 2010*a*), suggesting that this type of host reaction is induced by particular *Sarcocystis* spp. irrespective of their hosts. The sarcocysts of *S. truncata* have the same shape and the same type of surface protrusions as sarcocysts of *S. silva* in roe deer, but only a few cysts of the latter species have so far been examined morphologically, and only by LM (Gjerde, 2012). The unnamed *Sarcocystis* sp. from sika deer that was related to *S. truncata* based on its ssu rRNA gene sequences was not described morphologically in connection with the molecular characterization, but the Type 1 cysts described from other sika deer in Japan by Narisawa *et al*. (2008) are consistent with those of *S. truncata*.

### *Sarcocystis elongata/S. tarandi*

In previous phylogenetic analyses using ITS1 and/or ssu rRNA gene sequences, isolates of the *S. tarandi*-like species in red deer have clustered together with and been interspersed with isolates of *S. tarandi* from reindeer (Dahlgren and Gjerde, 2010*a*; Gjerde, 2012, 2013*b*). Hence, the isolates from red deer and reindeer seemed to be conspecific. However, based on *cox1* sequences from a considerable number of isolates from many different animals, it seems reasonable to conclude that two separate, but closely related species exist, and that the erection of a new species, *S. elongata*, is justified. The distinction between *S. elongata* and *S. tarandi* was not as clear-cut, however, as that between *S. truncata* and *S. rangiferi*, as also suggested by the differences in the clustering pattern of the ssu rRNA gene and ITS1 sequences between these species pairs (Dahlgren and Gjerde, 2010*a*). Nevertheless, the intraspecific sequence variation among *cox1* sequences assigned to either *S. elongata* or *S. tarandi* did not overlap with the small interspecific sequence variation between the two taxa, and the phylogenetic analyses consistently placed the isolates of each taxon in separate monophyletic clusters. The finding of only a single, but typical, isolate of *S. tarandi* in red deer, also suggests that there are two distinct populations, or species, with different affinity and infectivity for red deer and reindeer, respectively, i.e. *S. elongata* occurring only/mainly in red deer and *S. tarandi* occurring mainly in reindeer. Since the sarcocysts of *S. elongata* are morphologically indistinguishable from those of *S. tarandi* (Gjerde, 1984*a*, 1985*d*, 1986; Dahlgren and Gjerde, 2010*a*), and since *S. tarandi* may occur in red deer, a reliable species diagnosis of this sarcocyst type in red deer can only be achieved by molecular methods, and currently only by using *cox1* as the genetic marker.

### *Sarcocystis elongata/S. truncata*

By microscopy, four small cysts isolated from the heart of three of 37 red deer were identified as belonging to either *S. elongata* (*S.* cf. *tarandi*) or *S. truncata* (*S.* cf. *rangiferi*) due to the presence of upright finger-like protrusions (see Fig. 3d–e in Dahlgren and Gjerde, 2010*a*), but only one of these cysts were examined by molecular methods (partial ssu rRNA gene sequence) in the first study and identified as a cyst of *S. elongata*. In the recent study (Gjerde, 2013*b*), this identification was confirmed by using *cox1* sequences (GenBank number KC209707), whereas two additional cysts (GenBank numbers KC209680–KC209681) were identified as belonging to *S. truncata*. In the present study, the fourth sarcocyst of this type was examined, and found to belong to *S. truncata* (GenBank number KF241452). Thus, both *S. elongata* and *S. truncata* may occur in cardiac muscle, but with similarly sized sarcocysts and with indistinguishable protrusions since the growth of the cysts is limited by the small

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size of cardiac muscle cells. In skeletal muscle, on the other hand, mature cysts of *S. elongata* and *S. truncata* are clearly different in both size and shape, as reflected in the proposed new species names. Young and small cysts of *S. truncata* might, however, be confused with mature cysts of *S. elongata* when examined by LM. Thus, three sarcocysts from skeletal muscle that had been preliminary identified as *S. elongata* based on their morphology, turned out to belong to *S. truncata* from their *cox1* sequences. Such confusion may also occur between young sarcocysts of *S. rangiferi* and mature cysts of *S. tarandi* in reindeer.

It will often be necessary to distinguish between these two cyst types when examining muscle samples from red deer and reindeer, since individual red deer usually are concurrently infected with *S. elongata* and *S. truncata* (Dahlgren and Gjerde, 2010*a*), while individual reindeer are usually concurrently infected with *S. rangiferi* and *S. tarandi* (Gjerde, 1984*a*), rather than hosting only one species in each pair. This infection pattern suggests that these four *Sarcocystis* spp. use the same definitive host, and this notion has been further supported by their genetic similarity and phylogenetic placement. No definitive hosts have so far been determined for these species, but their phylogenetic placement and common occurrence suggest that felids like domestic cats and lynx (*Lynx lynx*) may act in this capacity (Dahlgren, 2010). The *cox1* primers and reference sequences generated in the previous (Gjerde, 2013*b*) and the present study may facilitate the molecular identification of the oocyst/sporocyst stage of these species, and thus the determination of their definitive hosts.

Species resembling *S. elongata* and *S. truncata* with respect to sarcocyst morphology have been reported from several cervid hosts in different countries as discussed in detail previously (Dahlgren and Gjerde, 2010*a*). Of particular interest is the *S. truncata*-like species in red deer referred to as *Sarcocystis* cf. *hofmanni* by Wesemeier and Sedlaczek (1995) and by Kutkienė (2003), and the *S. elongata*-like species from sika deer depicted in Fig. 1 of the paper by Saito *et al*. (1995). The relationships between *S. elongata* and *S. truncata* and the other similar species in different cervids can only be revealed through a molecular characterization of the other taxa.

### *Sarcocystis hjorti*

*Sarcocystis hjorti*, having small sarcocysts with delicate hair-like protrusions, was the most prevalent species in Norwegian red deer (Dahlgren and Gjerde, 2010*a*), and was found to be identical at the ssu rRNA gene with *Sarcocystis* sp. Type E previously detected in moose (Dahlgren and Gjerde, 2008). The latter sequence had been obtained from a small cyst in cardiac muscle with no visible protrusions, but when the smallest visible cysts in the diaphragm of several other moose were specifically targeted for isolation in a subsequent study, several small cysts of *S. hjorti* with typical hair-like protrusions were found (Dahlgren and Gjerde, 2010*b*). Thus, in moose, cysts of *S. hjorti* might be difficult to discern due to the concurrent presence of numerous cysts of *S. alces*, which look similar *in situ*, at least when not yet fully developed. The conspecificity of *S. hjorti*-like cysts in red deer and moose was confirmed by comparing *cox1* sequences in the recent study (Gjerde, 2013*b*), and that finding was further supported by examining 13 more isolates from red deer and moose in the present study (Table 1). Again, the phylogenetic analyses placed all the isolates from both cervids in a monophyletic group (Fig. 1), confirming that *S. hjorti* uses both red deer and moose as intermediate hosts. Experimental infections have shown that *S. hjorti* uses foxes as definitive hosts (Dahlgren and Gjerde, 2010*b*), which had been suggested by its common occurrence in red deer and moose and its phylogenetic placement (Dahlgren and Gjerde, 2010*a*, *b*). Sarcocysts of *S. hjorti* have the same type of delicate, hair-like protrusions as sarcocysts of *S. rangi* in reindeer (Gjerde, 1984*c*, 1986; Dahlgren *et al*. 2007), *S. alceslatrans* in moose (Dahlgren and Gjerde, 2008; Dahlgren, 2010) and *S. capreolicanis* in roe deer (Gjerde, 2012), and these species also cluster together in phylogenetic analyses using either ssu rRNA gene sequences or *cox1* sequences, suggesting a very close relationship among these four canine-transmitted *Sarcocystis* species of cervids. Recently, *S. hjorti* has been detected by molecular methods in red deer and moose in Lithuania (Prakas and Butkauskas, 2012) and in red deer in Switzerland (Stephan *et al*. 2012).

### *Sarcocystis ovalis*/*S. hardangeri*

In the first study of *Sarcocystis* spp. in red deer, partial or complete ssu rRNA gene sequences were obtained from 13 ovoid sarcocysts, and 12 of these sequences were near identical with that of *S. ovalis* in moose, whereas one sequence was near identical with *S. hardangeri* in reindeer (Dahlgren and Gjerde, 2010*a*). The species assignments of all of these 13 cyst isolates from reed deer have subsequently been confirmed by using *cox1* sequences, either in the previous (Gjerde, 2013*b*) or in the present study. Moreover, four additional cyst isolates of the *S. ovalis*-type were sequenced in this study and found to belong to *S. ovalis*. Thus, *S. ovalis* seems to be the more common of these two species in red deer, at least in western Norway. At *cox1*, there were consistent differences at two–three nucleotide positions between isolates of *S. ovalis* from the two hosts, but this might be due to sampling of geographically

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separated host populations, i.e. red deer in western Norway and moose in south-eastern Norway. The same might apply to the small nucleotide differences found between the isolates of *S. hjorti* in red deer and moose, respectively. However, the European magpie (*Pica pica*) has been found to act as a definitive host for *S. ovalis* from moose, and other corvid birds, particularly the hooded crow (*Corvus cornix*) and the common raven (*Corvus corax*) are assumed to act as additional definitive hosts for this species and the related species *S. hardangeri* and *S. oviformis* (Gjerde and Dahlgren, 2010). Thus, corvid birds might possibly disseminate these *Sarcocystis* spp. across considerable distances and between geographically separated deer populations.

### *Sarcocystis cervicanis*

Still another *Sarcocystis* sp. has been recorded in red deer and elk in other countries, having been referred to as an unnamed *Sarcocystis* sp. (Entzeroth *et al*. 1983), *Sarcocystis cervicanis* (Hernández-Rodríguez *et al*. 1981), *Sarcocystis wapiti* (Speer and Dubey, 1982) and *Sarcocystis* cf. *grueneri* (Wesemeier and Sedlaczek, 1995). The latter name stems from the fact that this species is morphologically indistinguishable from *S. grueneri* of reindeer (Gjerde, 1985*a*, 1986), which is very common in this host in Norway (Gjerde, 1985*a*; Dahlgren and Gjerde, 2007). The fact that such a species has not yet been found in Norwegian red deer, might indicate that it is different from *S. grueneri* in reindeer, but this can only be determined by a molecular characterization of this species in red deer.

## CONCLUSIONS

The findings of the present and the preceding study (Gjerde, 2013*b*) using *cox1* sequences for species delimitation, have in part confirmed and in part contradicted the findings of the original study on *Sarcocystis* spp. in Norwegian red deer (Dahlgren and Gjerde, 2010*a*). Altogether six *Sarcocystis* spp. have now been recorded in this host. Two of these species (*S. hjorti, S. ovalis*) seem to be fairly common in both red deer and moose, two species (*S. hardangeri, S. tarandi*) seem to mainly occur in reindeer, while two species (*S. elongata, S. truncata*) are common in red deer, but have so far not been recorded in other hosts. Thus, at least four of the six species recorded in Norwegian red deer are not strictly intermediate host specific, and the claim set forth in the title of the previous paper (Dahlgren and Gjerde, 2010*a*) is therefore still valid, and has even been further substantiated in the present study using *cox1* sequences. However, the two renamed species were not identical to their counterparts in reindeer as suggested from their sequence similarity at the ssu rRNA gene and ITS1 locus.

This study has also demonstrated that mitochondrial *cox1* sequences of appropriate length are better able to discriminate between some closely related and thus recently diverged *Sarcocystis* species in cervids than nucleotide sequences of the nuclear ribosomal DNA unit. Hence, it is recommended that *cox1* sequences are obtained in future molecular characterizations of different *Sarcocystis* spp. in various hosts, but not to the exclusion of the ssu rRNA gene, but as a supplement to this locus. It may, however, be better to use *cox1* as a genetic marker for molecular identification of oocysts/sporocysts in the intestines/feces of naturally or experimentally infected definitive hosts of various *Sarcocystis* spp., particularly when trying to identify species with a considerable sequence variation at the ssu rRNA gene.

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