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ORIGINAL PAPER

Muscular sarcocystosis in two arctic foxes (*Vulpes lagopus*) due to *Sarcocystis arctica* n. sp.: sarcocyst morphology, molecular characteristics and phylogeny

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Abstract The arctic fox (*Vulpes lagopus*) is a critically endangered species in Norway, and therefore, the small population is closely monitored, and most foxes found dead are subjected to necropsy. In two deceased foxes, thin-walled muscular sarcocysts were first detected in histological sections, and numerous sarcocysts were later found in frozen and thawed muscle samples from Fox 1. These sarcocysts measured 1–12×0.1–0.25 mm and had closely spaced, short, knob-like protrusions, giving the cysts a serrated outline. Genomic DNA was extracted from eight isolated sarcocysts (Fox 1) and two muscle samples (Fox 2) and subjected to polymerase chain reaction amplification at four loci: the nuclear 18S and 28S ribosomal RNA genes and internal transcribed spacer 1 region and the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*). Both foxes were infected by the same *Sarcocystis* sp., which displayed little or no genetic variation at the three nuclear loci (99.9–100 % identity) and slightly more variation at *cox1* (99.4–100 % identity). Sequence comparisons and phylogenetic analyses revealed that this species was distinct from other named *Sarcocystis* spp. but was closely related to various species using avian intermediate hosts and possibly identical to an unnamed species reported from two American dogs. The species described from the two arctic foxes was named *Sarcocystis arctica* n. sp.

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Introduction

Sarcocystis spp. have an obligatory two-host life cycle alternating between sexual development in the intestinal wall of the definitive host, terminating in oocyst/sporocyst excretion and asexual development in the extra-intestinal tissues of the intermediate host, terminating in the formation of sarcocysts, mostly within striated muscle cells. Intermediate hosts become infected via oocysts and sporocysts in fecally contaminated feed or water, whereas definitive hosts become infected by ingestion of fresh muscle tissue containing sarcocysts through predation or scavenging. Thus, carnivores typically act as definitive hosts, herbivores as intermediate hosts, whereas omnivores may act in both capacities, but usually not for the same *Sarcocystis* species. However, various carnivores, including canids, felids and mustelids, have also been found to act as intermediate hosts, harbouring muscular sarcocysts, as summarised by Kubo et al. (2009). Since that paper, there have been additional reports of muscular sarcocystosis in wolverines (Dubey et al. 2010), fishers (Larkin et al. 2011) and domestic dogs (Sykes et al. 2011). The pathogenic species *Sarcocystis neurona* may cause extra-intestinal sarcocystosis in certain carnivores (e.g. dogs, cats, raccoons, mink, fishers, striped skunks, armadillos, sea otters), as well as in horses, but its asexual multiplication is usually confined to the central nervous system of these intermediate or aberrant hosts, causing myeloencephalitis, without proceeding to sarcocyst formation (Gerhold et al. 2005; Dubey et al. 2006). Moreover, because of its dependence on opossums (*Didelphis* spp.) as definitive hosts, *S. neurona* is distributed only in the Americas.

In only a few cases of muscular sarcocystosis in carnivores have attempts been made to characterise the species involved by molecular methods and then mainly by sequencing fairly short fragments of the 18S ribosomal (r) RNA gene and/or the internal transcribed spacer 1 (ITS1) locus (e.g. Gillis et al.

2003; Dubey et al. 2010; Larkin et al. 2011; Sykes et al. 2011). Sequence comparisons reported in these papers have indicated that several *Sarcocystis* spp. may form sarcocysts in carnivores and that these species share a fairly high sequence identity at the 18S rRNA gene with various *Sarcocystis* spp. using birds as intermediate hosts and less so with *S. neurona*.

In Norway, the arctic fox, *Vulpes* (=*Alopex*) *lagopus*, is a critically endangered species, and the estimated total population for the past two decades has been approximately 50 adult animals but may have reached about 100 animals in 2011 (Flagstad et al. 2011). These foxes are separated into several smaller groups inhabiting various mountainous regions of the country. In order to save the wild arctic fox in Norway from extinction, both a national monitoring program and a captive breeding program have been in place for some years under the direction of the Norwegian Institute for Nature Research (NINA) in Trondheim (Flagstad et al. 2011; Landa et al. 2011). Moreover, as part of these programs, arctic foxes that are found dead are, whenever possible, examined post-mortem at the Norwegian Veterinary Institute, Trondheim. The examination of two such dead arctic foxes in 2011 and 2013, respectively, revealed the presence of many sarcocysts in histological sections of various muscle samples. The study reported herein was therefore conducted in order to determine the morphological and molecular characteristics and phylogenetic placement of this *Sarcocystis* sp. and hence its relationship to other *Sarcocystis* spp. and its possible definitive hosts.

Material and methods

Arctic foxes

Fox 1 was an adult male from Vadsø municipality in Finnmark County, north-eastern Norway, which was found on 25 March 2011 in a poor condition with a broken hind leg and therefore euthanised. Fox 2 was an adult male from Skaun municipality in Sør-Trøndelag County in central Norway (30 km southwest of Trondheim city), which was found dead in a frozen condition on 22 March 2013, presumably less than a week after it had died.

Pathological and histological examination

Fox 1 (autopsy case number: P174/11 / M405035) and Fox 2 (autopsy case number: P148/13 / M406233) were both submitted to the Norwegian Veterinary Institute, Trondheim, for a post-mortem examination in accordance with the national monitoring program and the captive breeding program for arctic foxes (Flagstad et al. 2011; Landa et al. 2011). At the necropsy, the animals were processed according to internal procedures (Schulze; unpublished) focussing on the assessment of parameters relevant for population management, such as age, body condition, reproductive status and any pathological findings. In addition, fresh muscle tissues were sampled and examined for the presence of *Trichinella* spp. larvae as part of a national surveillance of these parasites.

For the histopathological examination, samples of the skeletal muscles, diaphragm, right and left heart ventricle and heart septum (close to the heart valves), the skin, spleen, liver, kidneys, lungs and lymphonodus iliacus (only Fox 2) were fixed in 10 % neutral buffered formalin and embedded in paraffin. Sections were cut at 3 μm, stained with haematoxylin and eosin (H&E) and examined under a Leitz Dialux 22/22 EB microscope (Ernst Leitz Wetzlar, Germany). Digital images of sarcocysts in histological sections were recorded with a ProgRes C12plus microscope camera (Jenoptik, Germany) mounted on a Nikon Eclipse E600 microscope (Nikon, Japan) and operated through the ProgRes CapturePro v2.8.0 software (Jenoptik, Germany).

Light microscopic examination of fresh muscles samples for sarcocysts

Upon finding sarcocysts in histological sections, unprocessed portions of the diaphragm and the common digital extensor muscle of the forearm (musculus extensor digitalis communis) of both foxes, as well as a portion of the tongue from Fox 2, which had been sampled as part of the *Trichinella* spp. survey programme and thereafter kept frozen for several months, were shipped to the first author for further light microscopic and molecular examination. Individual sarcocysts were isolated as described previously (Dahlgren and Gjerde 2007; Gjerde 2013b) and examined in wet mounts under an Olympus BX51 light microscope (Olympus, Germany). Digital images were recorded of the majority of isolated sarcocysts, mainly of their outer wall, using a Colorview IIIu digital camera and the Cell^{B} image acquisition software (Olympus Soft Imaging Solutions, Germany). Following the microscopic examination, cysts were retrieved from the slides and placed individually in labelled 1.5 ml micro-centrifuge tubes containing 20 μl of distilled water and kept frozen at −20 °C until DNA isolation a few days later. Since no sarcocysts could be detected by microscopy in the samples from Fox 2, three small pieces, about 2 mm^3 in size, of the intrinsic tongue muscles were similarly placed in each of three 1.5 ml micro-centrifuge tubes and frozen.

Molecular characterisation

DNA extraction

Genomic DNA was extracted in January 2013 from eight randomly selected sarcocysts isolated from Fox 1 and in July 2013 from two tongue muscle samples from Fox 2 using QIAmp DNA Mini Kit (Qiagen, Germany) according to the

manufacturer's tissue protocol. The resulting DNA samples were subsequently kept frozen at −20 °C in between their use as templates for polymerase chain reaction (PCR) amplifications.

PCR primers and protocols

The ten genomic DNA samples were each subjected to PCR amplification of up to four genetic loci, the 18S rRNA gene, the ITS1 region and the 28S rRNA gene of the nuclear genome, as well as the cytochrome c oxidase subunit 1 gene (*cox1*) of the mitochondrial genome using the primers listed in Table 1. Thus, the complete 18S rRNA gene (~1,800 bp) was PCR-amplified in three overlapping fragments using primer pairs ERIB1/S2r, S5f/S4r and S3f/Primer B as described previously (Dahlgren and Gjerde 2007; Gjerde 2013b); the ITS1 region was amplified with primer pair 18S14F/ITS1-FR (Rosenthal et al. 2008; Dahlgren and Gjerde 2010), and ~1,500 bp of the 5′ end portion of the 28S rRNA gene was amplified using primer pair KL1/KL3 according to Mugridge et al. (1999). Moreover, a 1,103-bp portion from near the 5′ end of *cox1* was amplified with primer pair SF1/SR5 as described previously (Gjerde 2013b), following the initial amplification of two isolates with primer pair SF1/COIRm.

Each PCR reaction mixture contained 3 μl of the DNA solution, 18.75 μl HotStarTaq Master Mix (Qiagen, Germany), 10 pmol of each primer, 6 μg bovine serum albumin and RNase-free water to make a final volume of 37.5 μl. A negative control was included in each PCR run. PCR reactions were carried out in a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, USA). Cycling conditions were initial Hot Start at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 44–55.5 °C (temperature depending on primer pair) for 45 s, extension at 72 °C for 1–2 min (time depending on expected amplicon length) and final extension at 72 °C for 10 min.

Evaluation of PCR products and sequences

Aliquots of all PCR products were separated by electrophoresis on 1 % agarose gels and visualised under UV light after staining with ethidium bromide. PCR products of appropriate size and quantity were purified using the High Pure PCR Product Purification kit (Roche Diagnostics, Germany) and used for sequencing following evaluation of DNA concentration and purity on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Purified amplicons were sequenced on both strands by Eurofins MWG Operon, Germany, using the same primers as for PCR. The resulting forward and reverse sequences were imported into the Alignment Explorer application of the MEGA5 software (Tamura et al. 2011), checked against the chromatograms and merged into a single consensus sequence for each amplicon. The three overlapping partial sequences of the ssu rRNA gene of each isolate were assembled manually in MEGA5 into a contiguous sequence comprising the complete gene.

All newly obtained nucleotide sequences of different isolates from each locus were compared pair-wise with each other, as well as with homologous sequences available in the nucleotide sequence databases searched by the programme Nucleotide BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Since at the time of writing, no *cox1* sequences of *S. neurona* obtained through PCR amplification and sequencing were available in the nucleotide database of GenBank, a 1,095-bp long sequence (see Supplementary data) was

Table 1 Primers used to amplify various DNA regions in the present study

DNA region	Primer name	Primer sequence	References
18S rRNA gene	ERIB1	ACCTGGTTGATCCTGCCAG	Barta et al. 1997
	S2r	CTGATCGTCTTCGAGCCCCTA	Fischer and Odening 1998
	S5f	GTTCGATTCCGGAGAGGGAGC	Fischer and Odening 1998
	S4r	TATCCCCATCACGATGCATAC	Fischer and Odening 1998
	S3f	TTGTTAAAGACGAACTACTGCG	Fischer and Odening 1998
	Primer B	GATCCTTCTGCAGGTTCACCTAC	Fenger et al. 1995
ITS1	18S14F	AGTGTTCCGGTGAATTATTC	Rosenthal et al. 2008
	ITS1-FR	ACATCCATTGCTG	Rosenthal et al. 2008
28S rRNA gene	KL1	TACCCGCTGAACTTAAGC	Mugridge et al. 1999
	KL3	CCACCAAGATCTGCACTAG	Mugridge et al. 1999
cox1	SF1	ATGGCGTACAACAATCATAAAGAA	Gjerde 2013b
	SR5	TAGGTATCATGTAACGCAATATCCAT	Gjerde 2013b
	COIRm	CCCAGAGATAATACAAAATGGAA	Gjerde 2013b

artificially concatenated from two (CO747319.1, BU085375.1) of several similar expressed sequence tags (ESTs) retrieved from GenBank and used for sequence comparisons and phylogeny. These ESTs had originally been found through BLAST searches against the ESTs database of NCBI using *cox1* sequences of *Neospora caninum* (JX473252) and *Toxoplasma gondii* (JX473253) as query sequences in connection with a previous study (Gjerde 2013a), but these findings were not mentioned in that paper, nor in the subsequent paper on *cox1* of *Sarcocystis* spp. (Gjerde 2013b).

Phylogenetic analyses

Phylogenetic analyses were conducted separately on nucleotide sequences of the 18S rRNA gene and *cox1* by means of the MEGA5 software (Tamura et al. 2011).

18S rRNA gene sequences

A total of 62 sequences from 58 species were included in the analysis. They comprised five new sequences of the *Sarcocystis* sp. in the two arctic foxes in the present study (GenBank numbers KF601301–KF601305), as well as 57 other taxa and sequences, which are shown in Fig. 3 along with their GenBank accession numbers. A multiple sequence alignment was generated with the ClustalW programme within MEGA5, using a gap opening penalty of 10 and a gap extension penalty of 0.2. Most sequences were truncated slightly at both ends, so that nearly all sequences started and ended at the same (homologous) nucleotide positions, corresponding to positions 73 and 1,811, respectively, of sequence KC209734 of *Sarcocystis tenella*. The final alignment comprised 1,969 aligned positions.

Phylogenetic trees were reconstructed with the maximum likelihood (ML) and maximum parsimony (MP) methods, using all sites. The nucleotide substitution models with the best fit to the data set for the ML analysis were evaluated in MEGA5 and found to be the Tamura 3-parameter model (T92) with gamma distribution and invariable sites (T92+G+I) (Tamura 1992). The MP tree was obtained using the Subtree-pruning–regrafting (SPR) algorithm. The phylogeny was tested with the bootstrap method using 1,000 bootstrap replications. The intestinal coccidium *Eimeria tenella* (GenBank number U67121) from chickens was used as an outgroup species to root the trees.

Cox1 sequences

A total of 171 sequences from 29 species were included in the analysis. They comprised ten new sequences of the *Sarcocystis* sp. in the two arctic foxes in the present study (GenBank numbers KF601318–KF601327), as well as 160 GenBank sequences of 27 species as shown in Fig. 4 with their accession numbers. The artificially concatenated sequence of *S. neurona* (see Supplementary data) was also included in the analyses, since no *cox1* sequences of this species generated by PCR were available in GenBank.

A codon-based multiple alignment of all sequences was obtained by using ClustalW within MEGA5 as described previously (Gjerde 2013b). Sequences longer than 1,020 bp were truncated at their 3′ end, so that the final alignment comprised 1,020 positions with no gaps, corresponding to nt 1–1020 of the GenBank sequences of all *Sarcocystis* spp. included. Phylogenetic trees were reconstructed using the ML and MP methods. All codon positions were used. The nucleotide substitution model with the best fit to the data set for the ML analysis was found to be the general time reversible model with gamma distribution and invariable sites (GTR+G+I). The MP tree was obtained using the SPR algorithm. The phylogeny was tested with the bootstrap method using 1,000 bootstrap replications. The coccidium *E. tenella* (GenBank number HQ702484) was used as outgroup species to root the trees.

Results

Necropsy and histological findings

At necropsy, both foxes were found to be thin, corresponding to score 3 on Tufts body condition scale (http://www.neacha.org/resources/TACC.clear.pdf). While there was still a slight amount of fat tissue around the basis of the heart and kidneys of Fox 1, no body fat was visible in Fox 2. With the exception of two missing premolars in Fox 2, both animals featured a complete set of adult teeth in the correct position. There was no content in the stomach of either fox. Fox 1 had a recent open fracture of the tibia and fibula of a hind leg, a moderately hypertrophic prostate gland and above-average-sized testicles.

Examination of H&E-stained histological sections revealed the presence of basophilic bodies consistent with sarcocysts in both foxes. In Fox 1, such structures were found in sections of the myocardium, diaphragm and musculus extensor digitalis communis, and in Fox 2, in sections of the diaphragm, musculus extensor digitalis communis and musculus masseter. The sarcocysts were more numerous in Fox 1 than in Fox 2. The cysts appeared to be thin-walled, and there was no associated tissue reaction other than a slight compression of the neighbouring muscle fibres (Fig. 1). There were no pathological findings in the histological sections other than the sarcocysts, but the specimens were difficult to evaluate due to advanced autolysis.

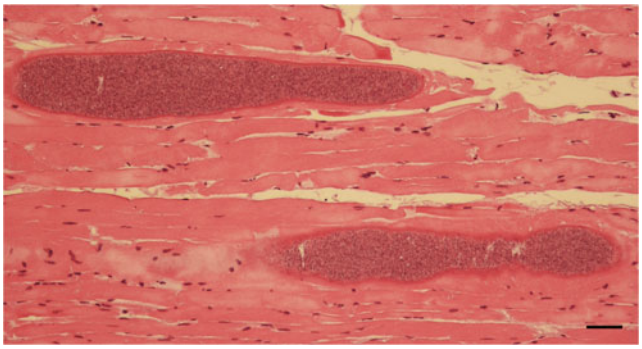

Fig. 1 Two thin-walled sarcocysts of *S. arctica* in H&E-stained histological section of skeletal muscle from Fox 1. *Scale bar* = 30 μm

Light microscopic examination of fresh muscle samples for sarcocysts

Fox 1

At low magnification under the stereo microscope, numerous sarcocysts could be seen, both in the sample of the extensor muscle and in the sample from the diaphragm. The cysts were spindle-shaped to thread-like, measuring 1–12 mm in length and 0.1– 0.25 mm in greatest diameter in the extensor muscle and 1–6×0.1–0.2 mm in the diaphragm. The largest cysts could also be seen grossly. Fifteen sarcocysts from the extensor muscle (isolates Vl1.1–Vl1.15) and 25 sarcocysts from the diaphragm (isolates Vl1.21–Vl1.45) were isolated and examined microscopically before being frozen and stored for subsequent molecular characterisation. All isolated sarcocysts had the same surface structure, displaying short knob-like or dome-shaped protrusions, giving the cysts a serrated outline (Fig. 2). The protrusions measured approximately 1–1.5 μm in width at their base and 0.5–1 μm in length. The interior of the sarcocysts was subdivided in the typical way by septa into many compartments, each containing numerous cystozoites.

Fox 2

No sarcocysts could be detected in the three muscle tissue samples (extensor muscle, diaphragm, tongue) when examined under the stereo microscope. Likewise, no cysts were detected when small pieces from these samples were compressed between two microscope slides and examined under a light microscope. Therefore, three pieces from the interior tongue muscles (isolates Vl2.1–Vl2.3) were excised and stored for DNA extraction and molecular examination.

Molecular characterisation

All ten isolates examined, including the two derived from the tongue muscle samples of Fox 2, were successfully amplified and sequenced at one or more loci as summarised in Table 2. All isolates could be assigned to the same *Sarcocystis* sp.

18S rRNA gene

Five isolates were amplified and sequenced at this gene, and five identical sequences were obtained. The complete 18S rRNA gene was 1,803 bp in length (including primers). When compared through BLAST searches with other (near) complete gene sequences available in GenBank, they shared the highest identity (99.0–99.4 %) with various *Sarcocystis* spp. using birds as intermediate hosts, i.e. *Sarcocystis columbae* (GU253883, HM125054), *Sarcocystis* sp. ex *Larus marinus* (JQ733508), *Sarcocystis* sp. ex *Phalacrocorax carbo* (JQ733511), *Sarcocystis turdusi* (JF975681), *Sarcocystis wobeseri* (GQ922886, HM159419), *Sarcocystis calchasi* (GQ245670) and *Sarcocystis cornixi* (EU553478). They were 98.8 % identical with *S. neurona* (U07812) and showed a higher sequence identity (~96 %) with several members of the subfamily Toxoplasmatinae (e.g. *T. gondii*, *N. caninum*, *Besnoitia besnoiti*, *Besnoitia jellisoni*, *Hammondia hammondi*, *Hammondia heydorni*, *Hammondia triffittae*) than with *Sarcocystis* spp. using ruminants as intermediate hosts (identity of ~93 % or less). When the new sequences were compared with two partial sequences (823–824 bp long; corresponding to nt 138–961 of the new sequences) obtained from two dogs with muscular sarcocystosis in the USA (Sykes et al. 2011), the sequence identities were found to be 99.9 % and 99.0 % to sequences JN256676 and JN256677, respectively.

In the multiple sequence alignment generated in MEGA5, it was noticed that the new sequences differed from all the other near-complete sequences used in the phylogenetic

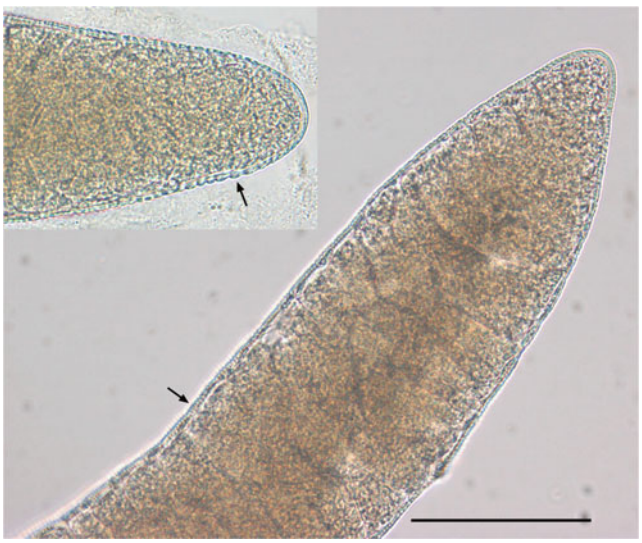

Fig. 2 Tip of two sarcocysts of *S. arctica* (*wet mounts*) isolated from the diaphragm of Fox 1. The cysts have short knob-like protrusions (*arrows*) giving the cysts a serrated outline. *Scale bar* = 100 μm

Table 2 Overview of DNA loci examined for each isolate and the resulting GenBank sequences

Host	Isolate	DNA region examined			
		18S rRNA gene	ITS1	28S rRNA gene	*cox1*
Fox 1	Vl1.1	nd	nd	nd	KF601318
	Vl1.5	nd	nd	nd	KF601319
	Vl1.10	KF601301	KF601306	KF601312	KF601320
	Vl1.15	nd	KF601307	KF601313	KF601321
	Vl1.22	nd	nd	nd	KF601322
	Vl1.26	KF601302	KF601308	KF601314	KF601323
	Vl1.28	nd	nd	nd	KF601324
	Vl1.40	KF601303	KF601309	KF601315	KF601325
Fox 2	Vl2.1	KF601304	KF601310	KF601316	KF601326
	Vl2.2	KF601305	KF601311	KF601317	KF601327

nd not done

analysis by three to four nucleotide substitutions and a deletion between nt 652–672. When this short stretch was used as a query sequence in BLAST, only eight matches were found, i.e. one of the aforementioned partial sequences from dogs (JN256676) and seven partial sequences (728–763 bp long; corresponding to nt 139–948) derived from fishers, *Martes pennanti*, with muscular sarcocystosis in the USA (Larkin et al. 2011). The sequences from fishers differed, however, from the new sequences at one to four other nucleotide positions, but only one of these differences was present in all of them.

ITS1

Six isolates were amplified and sequenced at the complete ITS1 region; five of the resulting sequences were identical, and these differed from the sixth isolate (Vl1.26) by a single nucleotide substitution (G/A) in position 683. The complete ITS1 region was 697 bp in length, encompassing nt 114–810 in the submitted sequences. Thus, there was a 113-bp-long overlap between these sequences and the abovementioned 18S rRNA gene sequences. In BLAST searches using the ITS1 region only as query sequence, 99.4–99.5 % sequence identity was found between the new sequences from foxes and two identical sequences obtained from muscle samples of two dogs in the USA (JX993923, JX993924). Actually, the five identical sequences from this study were identical to one possible variant of the two dog-derived sequences, since these differences were confined to three polymorphic sites, in which one of two possible character states was identical with the nucleotides found in the present sequences (C/Y, C/Y, A/R). The sequence identity to other available species in GenBank was fairly low, i.e. about 88 % to *Sarcocystis felis* (AY190081, AY190082, KC160213, KC160214) and less than 85 % to some species (e.g. *S. calchasi*, *S. turdusi*, *S. wobeseri*) with avian intermediate hosts.

28S rRNA gene

Six isolates were amplified and sequenced at this gene, and six identical sequences, 1,528-bp long (primers not included), were obtained. BLAST searches revealed that these sequences shared the highest identity, about 98 %, with various species using birds as intermediate hosts: *S.* sp. ex *Larus marinus* (JQ733509), *S. calchasi* (FJ232949), *S. wobeseri* (GQ922887, GQ922888), *S. turdusi* (JF975682), *S. cornixi* (EF079884), *S.* sp. ex *Phalacocorax carbo* (JQ733512), *S. columbae* (GU253887, HM125053) and *Frenkelia glareoli* (AF044251). The identity was about 97 % to *S. neurona* and 84–86 % to various *Sarcocystis* spp. with ruminants as intermediate hosts.

Cox1

All ten isolates examined were successfully amplified and sequenced at *cox1* when using SR5 as reverse primer, whereas only two of these isolates could be properly amplified and sequenced using reverse primer COIRm. The sequences obtained with reverse primers SR5 and COIRm were 1,053 and 1,095 bp long, respectively (primers not included). The ten sequences comprised three haplotypes, two from Fox 1 (isolates 1.5 and 1.10 vs. isolates 1.1, 1.15, 1.22, 1.26, 1.28 and 1.40) and one from Fox 2 (isolates 2.1 and 2.2). The two haplotypes from Fox 1 differed from each other at only 2 of 1,053 nucleotide positions (99.8 % identity), whereas they both differed from the single haplotype in Fox 2 at six positions (99.4 % identity). When compared with the artificially concatenated *cox1* sequence of *S. neurona*, the two haplotypes from Fox 1 differed at 16 nt positions (98.5 % identity), whereas the single haplotype from Fox 2 differed at only 10 of 1,053 nucleotide positions (99.1 % identity) and at only 2 of 351 inferred amino acid residues. In contrast, the new

sequences shared identities of about 70 % or less with *Sarcocystis* spp. using ruminants as intermediate hosts (GenBank numbers KC209578–KC209732) (Gjerde 2013b) and 73–75 % identity with members of the Toxoplasmatinae (GenBank numbers JX473247–JX473253) (Gjerde 2013a).

Phylogenetic relationships

18S rRNA gene

The topology of the phylogenetic trees obtained with the ML (Fig. 3) and MP (not shown) methods was identical concerning *Sarcocystis* spp. of ruminants and the Toxoplasmatinae but differed slightly in the branching order of the other taxa. By both methods, *Sarcocystis* spp. of ruminants were placed in three well-supported clades according to known or presumed definitive hosts, i.e. canids, felids/humans/unknown and corvid birds. Basal to these clades was a clade comprising three species using snakes as definitive hosts, including *Sarcocystis nesbitti* and *Sarcocystis singaporensis*, and further basal was a clade encompassing the new sequences from foxes and several species using either birds, rodents, marsupials or lizards as intermediate hosts, and also *S. neurona*. The relationships between most of the species of the latter clade were, however, not well resolved, neither by the ML (Fig. 3) nor by the MP analysis.

Cox1

The ML (Fig. 4) and MP (not shown) analysis resulted in almost identical trees, the only difference being the branching order of *Sarcocystis alces*, *Sarcocystis gracilis* and *S. tenella*. By both methods, the 22 *Sarcocystis* spp. with ruminant intermediate hosts were placed in three well-supported clades according to definitive hosts. There was also high bootstrap support for the placement of the new species and *S. neurona* together in a more basal clade, i.e. closer to the Toxoplasmatinae clade.

Taxonomic summary of *Sarcocystis arctica* n. sp.

Based on sequence comparisons using BLAST, the species found in the two arctic foxes in this study is different from all other named *Sarcocystis* spp. deposited in GenBank, but it might be identical to an unnamed *Sarcocystis* sp. from dogs in the USA. We therefore propose the new name *S. arctica* for this species in arctic foxes in Norway.

Type hosts The arctic fox, *Vulpes (=Alopex) lagopus*, is an intermediate host for *S. arctica*, whereas the definitive host is unknown.

Locality Sarcocysts of *S. arctica* were found in two free-ranging arctic foxes from Finnmark County in northern Norway and from Sør-Trøndelag County in central Norway, respectively.

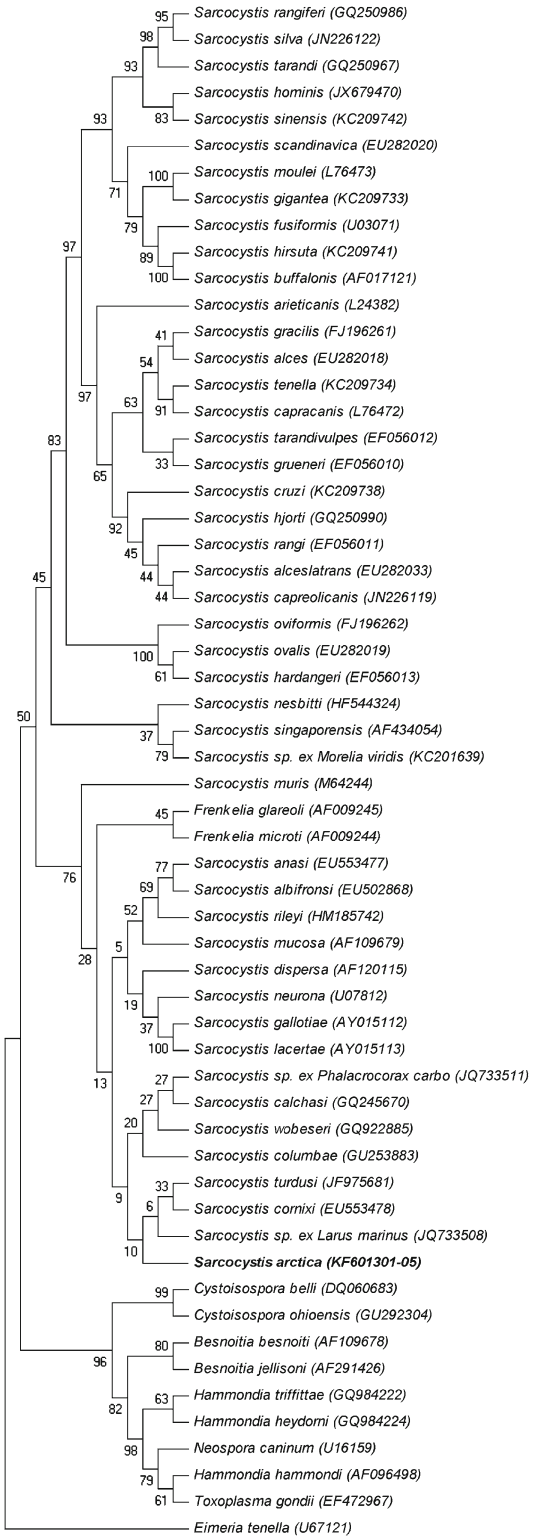

Fig. 3 Phylogenetic tree for members of the Sarcocystidae based on near-complete 18S rRNA sequences and inferred using the maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The subtree formed by the five new sequences of *S. arctica* has been collapsed. GenBank accession numbers for all sequences are given behind the taxon names

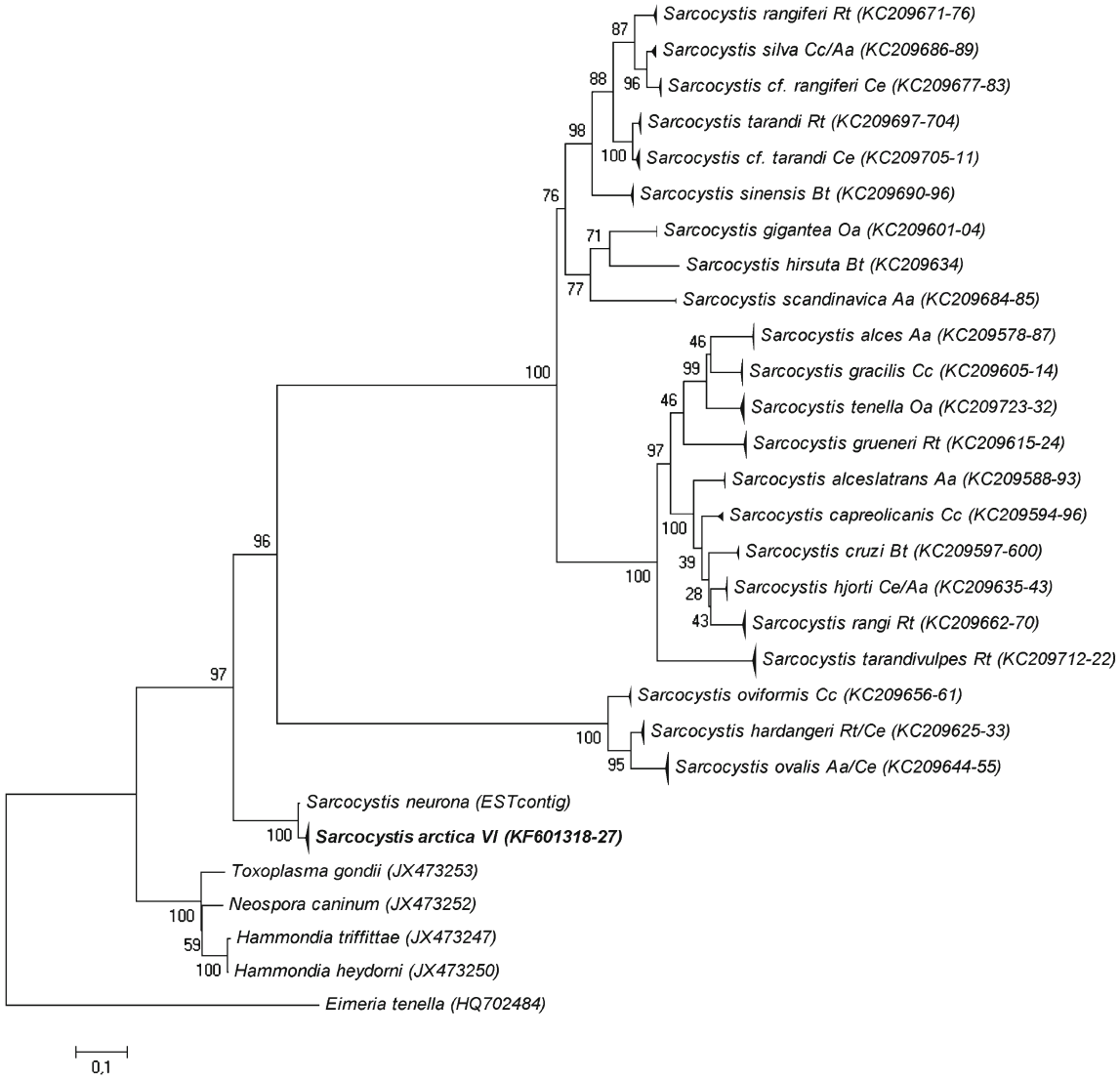

Fig. 4 Phylogenetic tree for members of the Sarcocystidae based on partial sequences of *cox1* and inferred using the maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. GenBank accession numbers for all sequences, except one, are given behind the taxon names. The sequence of *S. neurona* was artificially concatenated from two ESTs (see Supplementary data). Subtrees formed by two or more sequences of the same species have been collapsed, but the number of sequences included may be inferred from the GenBank accession numbers behind the taxon names. The intermediate hosts for different *Sarcocystis* spp. are also given behind the taxon names, 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); *Vl = V. lagopus* (arctic fox)

Sarcocyst morphology Sarcocysts spindle-shaped to thread-like, measuring 1–12×0.1–0.25 mm and having short knob-like or dome-shaped protrusions, approximately 1–1.5 μm wide and 0.5–1 μm long. In histological sections, the sarcocysts are thin-walled.

Molecular characterisation Nucleotide sequences of *S. arctica* were obtained from the complete 18S rRNA gene and ITS1 region and from the partial 28S rRNA gene and partial *cox1* of 5–10 isolates derived from two foxes and have been deposited in GenBank under accession numbers KF601301–KF601327.

Etymology The name is derived from the common name of the intermediate host, the arctic fox, as well as from the general area of distribution of this host.

Discussion

Molecular characterisation at four loci and subsequent sequence similarity searches using BLAST showed that the species causing muscular sarcocystosis in the two arctic foxes was different from all named *Sarcocystis* spp. from which

nucleotide sequences had been deposited in GenBank. According to Odening (1998), two *Sarcocystis* spp. using foxes in Kazakhstan as intermediate hosts have been named, i.e. *Sarcocystis corsaci* Pak, 1979, from the corsac fox (*Alopex corsac*), and *Sarcocystis vulpis* Pak, Sklyarova and Dymkova, 1991, from the red fox (*Vulpes vulpes*). Without molecular data, these species are not recognisable today, and they are therefore considered to be species inquirendae and their names are *nomina dubia*. Kubo et al. (2009), on the other hand, provided a fairly detailed morphological description of sarcocysts (see Fig. 2 in their paper) in the muscles of a Japanese red fox (*Vulpes vulpes japonica*), but these cysts seem to have a smooth cyst wall with no protrusions, and the species was not named. Thus, there were no recognisable matches either morphologically or molecularly with any named *Sarcocystis* sp., and the species described from the two arctic foxes in this study was therefore named *S. arctica* n. sp.

Moreover, with the exception of *S. neurona*, various *Sarcocystis* spp. causing extra-intestinal sarcocystosis in other carnivores than foxes have hitherto either not been molecularly characterised or have been subjected to a fairly limited molecular characterisation, encompassing only short fragments of the 18S rRNA gene and in a few cases the (near) complete ITS1 region. The short 18S rRNA gene sequences obtained from these species in carnivores have indicated a close relationship to *Sarcocystis* spp. using birds as definitive and/or intermediate hosts, many of which have been well characterised at the (near) complete 18S rRNA gene and the partial 28S rRNA gene, and recently also at the ITS1 locus (Olias et al. 2011). Thus, in order to ascertain whether the species in foxes had been reported previously in a recognisable manner, the isolates were examined at all of these loci of the nuclear ribosomal DNA unit, as well as at the mitochondrial *cox1*, which was recently established as an additional genetic marker for delimitation of *Sarcocystis* spp. in ruminants (Gjerde 2013b). Moreover, since BLAST similarity searches might not easily retrieve short sequences with a high identity to only a portion of a long query sequence, the fairly short 18S rRNA gene fragments of some *Sarcocystis* spp. reported from carnivores were specifically retrieved on the basis of accession numbers in relevant papers and compared pair-wise in BLAST with sequences of *S. arctica*. By this procedure, it was determined that the new species was 99.9 % identical with GenBank sequence JN256676 (*Sarcocystis* sp. MT1) obtained from a Rottweiler dog from Montana, USA, with muscular sarcocystosis, but only 99.0 % identical with sequence JN256677 (*Sarcocystis* sp. CO1) from a Golden Retriever dog from Colorado, USA (Sykes et al. 2011).

However, BLAST similarity searches using the complete ITS1 region of *S. arctica* as query sequence identified two sequences (*Sarcocystis* sp. 2 SN-2012) with 99.4–99.5 % identity, which had also been obtained from two dogs in the USA, apparently in an unpublished study. Based on the GenBank records, however, these dogs seem to be the same as those reported by Sykes et al. (2011), since sequence JX993923 had been obtained from a Rottweiler dog from Montana and sequence JX993924 from a Golden Retriever dog from Colorado. Thus, based on the 18S rRNA gene sequences, the two American dogs seem to host two separate *Sarcocystis* spp., of which only one is consistent with *S. arctica*, whereas based on ITS1 sequences, they seem to harbour the same *Sarcocystis* sp., which seems to be identical to *S. arctica* in the present study. Moreover, by transmission electron microscopy, the sarcocysts in these dogs were found to possess short villar protrusions (see Fig. 3 in Sykes et al. 2011), which are consistent with the protrusions seen by light microscopy on the sarcocysts from Fox 1 in this study.

A short stretch of the 18S rRNA gene sequences of *S. arctica* did not match any other available sequences of this gene, except one from the abovementioned Rottweiler dog in the USA and seven sequences originating from fishers, *M. pennanti*, with muscular sarcocystosis in the USA (Larkin et al. 2011). The species in fishers does not seem to have been characterised at the complete 18S rRNA gene or at other genetic loci, and its thin-walled sarcocysts have only been studied in histological sections. Hence, it is not yet possible to determine if this species is identical to *S. arctica* or not.

The phylogenetic placement of *S. arctica* as inferred from the 18S rRNA gene sequences was not well resolved, but it indicated a close relationship to several *Sarcocystis* spp. using birds as intermediate hosts, all of which shared a high sequence identity to *S. arctica*. Some of these species have been found to use raptorial birds as definitive hosts, e.g. *S. calchasi* and *S. columbae*, cycling between pigeons and *Accipiter* hawks (Olias et al. 2010, 2011), whereas one species, *Sarcocystis albifronsi*, has been found to use the arctic fox as definitive host (Kutkienė et al. 2012). This placement may indicate that *S. arctica* has a similar life cycle and that foxes are merely aberrant or incidental intermediate hosts of a species normally cycling among birds, or at least using birds as its preferred intermediate host. However, the fact that *S. arctica* was found in two unrelated and spatially separated foxes in this study and that possibly the same species was found in two unrelated dogs in the USA may also be taken to indicate that *S. arctica* primarily uses canids as intermediate hosts and mammalian or avian carnivores or scavengers as definitive hosts.

In Norway, the arctic fox may share its habitat with and thus be preyed or scavenged upon by the red fox (*V. vulpes*), the wolverine (*Gulo gulo*), the rough-legged buzzard (*Buteo lagopus*), the golden eagle (*Aquila chrysaëtos*), the white-tailed eagle (*Haliaeetus albicilla*) and to some extent also the brown bear (*Ursus arctos*) and the lynx (*Lynx lynx*) (Flagstad et al. 2011; superimposable species distribution maps provided by the Norwegian Biodiversity Information Centre at: http://artskart.artsdatabanken.no/FaneArtSok.aspx). Hence,

these species, as well as the arctic fox itself through cannibalism, are potential definitive hosts for *S. arctica*. If, on the other hand, the arctic fox is merely an aberrant host of a species mainly using birds or rodents as intermediate hosts, other raptorial birds present in Norway, e.g. the Northern goshawk (*Accipiter gentilis*) and the sparrow hawk (*Accipiter nisus*), might also be potential definitive hosts. The molecular data provided through this investigation might be used in future studies to search for potential definitive hosts for *S. arctica*.

The molecular comparisons showed that *S. arctica* shared a very high sequence identity at the 18S and 28S rRNA genes with several *Sarcocystis* spp. using avian intermediate hosts but were clearly different at the ITS1 locus. Moreover, there was little or no intraspecific variation among the isolates at either of these loci. Hence, the ITS1 locus might be better suited than the two rRNA genes for identification and delimitation of species within this assemblage, as already noted by Olias et al. (2011) for species with avian hosts. The mitochondrial *cox1* was recently found to be better able than the 18S rRNA gene to differentiate between closely related *Sarcocystis* spp. in ruminants (Gjerde 2013b), but since none of the *Sarcocystis* spp. with avian intermediate hosts have so far been characterised at this locus, it remains to be determined whether *cox1* can also reliably differentiate between species of this assemblage. It should also be noted that the addition of *cox1* sequences of *S. arctica* and *S. neurona* to the phylogenetic analyses only had a minor effect on the relationships among *Sarcocystis* spp. of ruminants compared with the previous analysis without these sequences included (Gjerde 2013b).

In the histological sections, there were no inflammatory reactions associated with the sarcocysts in the two arctic foxes. The same was true for the red fox in Japan with muscular sarcocystosis (Kubo et al. 2009). In contrast, the two American dogs that may have been infected with a species identical to *S. arctica*, suffered from severe myositis (Sykes et al. 2011), as did a Canadian dog infected with a morphologically similar species (Chapman et al. 2005). Vashisht et al. (2005) also reported myositis in an American dog with muscular sarcocysts, but the species involved could not be clearly separated from *S. neurona*. Most reported cases of muscular sarcocystosis in carnivores have, however, not been associated with inflammatory reactions (Kubo et al. 2009) or clinical disease. Moreover, clinical sarcocystosis and inflammatory reactions in intermediate hosts are usually associated with the initial asexual multiplication within vascular endothelial cells prior to cyst formation within muscle fibres. At present, it is impossible to decide whether the demise of the two arctic foxes in the present study in any way was related to the infection with *S. arctica*, but further studies should be undertaken in an attempt to determine the impact of this species on the arctic fox population in Norway and its occurrence in other carnivores, including domestic dogs.

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