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PROTOZOOLOGY - ORIGINAL PAPER

Prevalence and molecular characterisation of *Sarcocystis miescheriana* and *Sarcocystis suihominis* in wild boars (*Sus scrofa*) in Italy

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Abstract

A sample of the diaphragm was collected from each of 100 wild boars legally hunted in the Val Grande National Park in north-western Italy and examined for the presence of *Sarcocystis* infection by histological and molecular methods. In histological sections, thick-walled sarcocysts consistent with those of *Sarcocystis miescheriana* were detected in 32 wild boars. Genomic DNA extracted from diaphragm samples was initially subjected to PCR amplification of the internal transcribed spacer 1 (ITS1) region, and 97 wild boars were found to harbour a *Sarcocystis* infection at this screening. Selected DNA samples were then subjected to PCR amplification and sequencing of the ITS1 region and the 18S and 28S ribosomal RNA (rRNA) genes of the nuclear ribosomal DNA unit, while all positive samples were subjected to PCR amplification of the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene. *S. miescheriana* was identified in 97 wild boars (97%), while the zoonotic *Sarcocystis suihominis* was identified in one wild boar (1%), which also harboured *S. miescheriana*. Intra-specific sequence variation was found in all four DNA regions of *S. miescheriana* examined and in the 18S rRNA gene and ITS1 region of *S. suihominis*. The partial *cox1* gene was amplified and sequenced from 72 isolates of *S. miescheriana*, yielding 43 haplotypes with pairwise sequence identities of 97.6–99.9%. These haplotypes were 79.1–79.8% identical with the *cox1* sequence of *S. suihominis*. Phylogeny based on *cox1* sequences placed *S. miescheriana* and *S. suihominis* as sister species within a clade comprising mainly *Sarcocystis* spp. of ruminants with felids as known or presumed definitive hosts. The same was true for the phylogeny based on 18S rRNA gene sequences.

Keywords *Sarcocystis miescheriana* · *Sarcocystis suihominis* · Cox1 · Phylogeny

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Alessia Libera Gazzonis and Bjørn Gjerde contributed equally to this work.

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Introduction

Species within the coccidian genus *Sarcocystis* have an obligatory two-host life cycle comprising sexual development and formation of oocysts in the intestinal mucosa of their definitive hosts (carnivores and omnivores) and a biphasic asexual multiplication in vascular endothelial and striated muscle cells, respectively, of their intermediate hosts (herbivores, omnivores and carnivores). The multiplication within striated muscle cells leads to the formation of mature sarcocysts, which have a characteristic morphology (size, shape and surface configuration) for each species, and by which, definitive hosts become infected through ingestion of infected muscle tissue. The intermediate hosts, on the other hand, become infected via feed and water contaminated by oocysts and free sporocysts shed in the faeces of definitive hosts.

Domestic and wild pigs may act as intermediate hosts for two species of *Sarcocystis*, that is, *Sarcocystis miescheriana* (synonym: *Sarcocystis suicanis*) and *Sarcocystis suihominis*, having canids (dogs, foxes, wolves, jackals, raccoon dogs, raccoons) and humans (and non-human primates), respectively, as definite hosts (Dubey et al. 2016; Moré et al. 2016). In fact, these were among the very first *Sarcocystis* spp. to have their complete two-host life cycle elucidated (Rommel and Heydorn 1972; Rommel et al. 1974; Erber and Boch 1976; Erber 1977; Heydorn 1977a; Göbel et al. 1978). A third species was reported from the former Soviet Union to cycle between pigs and cats (Golubkov et al. 1974) and was therefore named *Sarcocystis porcifelis* by Dubey (1976), but its presence in pigs has not been unequivocally confirmed in other studies (Dubey et al. 2016).

Mature sarcocysts of *S. miescheriana* and *S. suihominis* differ in their surface morphology. Those of *S. miescheriana* possess tightly packed, erect, finger-like cyst wall protrusions, about 5 μm long and 1.3 μm wide, which together give the impression of a fairly thick and rigid palisade-like wall. Sarcocysts of *S. suihominis*, on the other hand, have longer and thinner (about 13 μm by 0.8 μm), almost hair-like protrusions, which usually run in an oblique or slanting direction along the cyst surface, and hence, their cyst wall may appear thinner than that in sarcocysts of *S. miescheriana*, particularly when viewed in histological sections (Saito et al. 1998). By light microscopy, the difference between the two species with respect to their cyst wall protrusions is therefore best seen in wet mounts of fresh cysts removed from their host cells (Erber 1977; Heydorn 1977a; Boch et al. 1978; Hinaidy and Supperer 1979; Chen et al. 2007). However, these differences are also evident by transmission electron microscopy (TEM) (Bergmann and Kinder 1974, 1976; Claveria et al. 2001; Chen et al. 2007; Yan et al. 2013) and, to some extent, also by scanning electron microscopy (SEM) (Saito et al. 1996, 1998). The sarcocysts described by TEM under the name *S. miescheriana* by Bergmann and Kinder (1974) were actually sarcocysts of *S. suihominis*, but when that study was published, only a single species, known as *S. miescheriana*, was believed to infect pigs.

Since the recognition of two *Sarcocystis* spp. in pigs in the 1970s, several studies have been undertaken to determine the presence and/or prevalence of *S. miescheriana* and *S. suihominis* in domestic and wild pigs in different countries. Before the molecular era, the identification of the two species was based mainly on the abovementioned differences in sarcocysts morphology (e.g. Boch et al. 1978; Hinaidy and Supperer 1979; Saito et al. 1998; Claveria et al. 2001), but during the past 20 years, molecular methods have been increasingly used for this purpose. The first molecular studies provided methods and reference sequences (Mugridge et al. 2000; Yang et al. 2001, 2002; GenBank sequences DQ839352 and EU327974 of *S. miescheriana* from unpublished studies), which were subsequently used by others as an aid for identification of newly characterised isolates from pigs. The latter studies have comprised investigations identifying a limited number of isolates (González et al. 2006; Caspari et al. 2011; Kia et al. 2011; Prakas 2011; Yan et al. 2013), as well as more comprehensive studies aimed at determining the prevalence of the two species in larger populations of pigs (Calero-Bernal et al. 2015, 2016; Coelho et al. 2015; Kaur et al. 2016; Imre et al. 2017). In addition, molecular methods have been used to identify oocysts of *S. miescheriana* in the intestine of naturally infected foxes and raccoon dogs (Moré et al. 2016).

The types of molecular methods used in the abovementioned studies have varied. Most studies have PCR-amplified a portion of the 18S ribosomal RNA (rRNA) gene using general primers targeting most *Sarcocystis* spp. (Yang et al. 2001), and then some or all samples yielding amplicons of expected size have been sequenced and assigned to either *S. miescheriana* or *S. suihominis* following sequence comparisons (González et al. 2006; Caspari et al. 2011; Kia et al. 2011; Prakas 2011; Yan et al. 2013; Kaur et al. 2016). However, some studies (Calero-Bernal et al. 2015, 2016; Imre et al. 2017) have initially tentatively identified the species present on the basis of the restriction fragment length polymorphism (RFLP) procedure established by Yang et al. (2002), in which PCR-amplified 18S rRNA gene sequences of *S. miescheriana*, but not those of *S. suihominis*, were digested (cut) by a restriction endonuclease. The RFLP-based identification has then been verified by sequencing of a few selected samples. Coelho et al. (2015) used a different approach, by which the muscle samples were first examined for the presence of *Sarcocystis* DNA through PCR with two general primers, and then the species present was identified by using PCR with primers specifically targeting the 18S rRNA gene of either *S. miescheriana* or *S. suihominis*, followed by sequencing of a subset of the samples to confirm the findings.

Thus, although molecular methods have been used in many recent studies on *Sarcocystis* infections in pigs, only a limited number of isolates have actually been sequenced and only some of the sequences generated seem to have been submitted to GenBank. Nevertheless, these studies have provided additional nucleotide sequences of both species in pigs, resulting in a total of 56 and 19 nucleotide sequences of *S. miescheriana* and *S. suihominis*, respectively, having been deposited in GenBank prior to the current investigation. However, all but one of these sequences have been of the nuclear 18S rRNA gene. The single exception has been the complete 28S rRNA gene of *S. miescheriana* (GenBank: AF076902), which was generated by Mugridge et al. (2000) in a phylogenetic study, but this marker does not seem to have been used for diagnostic purposes by anyone.

In a comprehensive study of 22 *Sarcocystis* spp. from cattle, sheep, red deer, reindeer, roe deer and moose, Gjerde (2013) established the mitochondrial cytochrome c oxidase subunit I (*cox1*) gene as a novel genetic marker for *Sarcocystis* spp. and found that partial *cox1* sequences performed better than 18S rRNA gene sequences for delimitation of closely related species in these hosts. Many subsequent studies by various groups have since confirmed that this is indeed the case with respect to *Sarcocystis* spp. using ruminants as intermediate hosts (e.g. Gjerde 2016; Gjerde et al. 2017a, b), whereas many *Sarcocystis* spp. using birds or carnivores as intermediate hosts, have differed very little, if at all, both at *cox1* and the 18S rRNA gene. Hence, the internal transcribed spacer 1 (ITS1) region of the nuclear ribosomal DNA unit is currently the best marker for identification and delimitation of the latter species (Gjerde et al. 2017c). So far, no *cox1* sequences of *S. miescheriana* or *S. suihominis* have been available in GenBank. Hence, it is not known if this marker clearly separates these species or if phylogenetic reconstructions based on *cox1* sequences might place the two species differently than in previous phylogenies based on 18S rRNA gene sequences.

In experimental infections of domestic pigs, both *S. miescheriana* and *S. suihominis* have caused a wide range of symptoms including reduced weight gain, cutaneous purpura, dyspnoea, muscle tremors, abortion and death during the early acute phase of the infection when fairly high numbers of sporocysts have been administered, whereas low doses of sporocysts mainly have resulted in subclinical infections (Heydorn 1977a; Boch et al. 1980; Barrows et al. 1982; Heydorn and Weniger 1988; Caspari et al. 2011; Dubey et al. 2016). The zoonotic *S. suihominis* may cause disease even in its definitive hosts. Thus, humans who have ingested fresh sarcocysts of *S. suihominis* in raw or undercooked pork (mainly volunteers in experimental settings) have experienced various gastrointestinal symptoms, including nausea, stomach ache, vomiting and diarrhoea, which may begin 6–12 h after consumption and last for 1–2 days (Heydorn 1977b; Fayer et al. 2015). Still, there are few reported cases of naturally acquired intestinal sarcocystosis in humans due to *S. suihominis*, either because clinical infections are underreported or misdiagnosed or because most infections are low-grade and asymptomatic (Poulsen and Stensvold 2014; Fayer et al. 2015). Likewise, only a single case of fatal acute sarcocystosis, which was caused by *S. miescheriana*, has been reported in naturally infected domestic pigs (Caspari et al. 2011). This may be due to low-grade infections at an early age followed by immunisation, making subsequent infections subclinical and/or unrecognised. The absence of clinical cases has obviously not been due to the absence of infections, since previous surveys have shown that many domestic pigs become infected and harbour sarcocysts. For instance, Boch et al. (1978) reported a prevalence of 35.5% in older pigs (mainly sows) in Germany, Hinaidy and Supperer (1979) found a prevalence of 31.6% in sows in Austria and Damriyasa et al. (2004) found a seroprevalence of 28.6% in sows in Germany. However, it is not known whether this is still the case, since there are no recent studies on the prevalence of *Sarcocystis* infections in domestic pigs kept under intensive indoor management systems.

Instead, the emphasis in recent years has been on prevalence studies in free-ranging pigs, including wild boars (Prakas 2011; Calero-Bernal et al. 2016; Coelho et al. 2015; Imre et al. 2017), feral pigs (Calero-Bernal et al. 2015) and domestic pigs (Kaur et al. 2016; Imre et al. 2017) kept outdoors. In the four surveys of wild boars, the prevalence of *Sarcocystis* infections ranged from 60.4 to 88.2%, but these values are not directly comparable since different diagnostic methods were used. In wild boars from Lithuania, Portugal and Romania, *S. miescheriana* was the only species detected (Prakas 2011; Coelho et al. 2015; Imre et al. 2017), and this species also predominated in wild boars in Spain, but *S. suihominis* was found in one of eight samples identified by molecular methods (Calero-Bernal et al. 2016). By contrast, *S. suihominis* was found to be quite common in domestic pigs in Germany (60.7% of 219 slaughter pigs; Boch et al. 1978) and Austria (7.5% of 348 sows; Hinaidy and Supperer 1979) in the 1970s. More recently, *S. suihominis* has been recorded in a few domestic pigs in Japan (Saito et al. 1998) and Spain (González et al. 2006) and seems to be quite common in domestic pigs in China (Yang et al. 2001; Chen et al. 2007) and India (Kaur et al. 2016).

So far, there have been no investigations into the occurrence and prevalence of *S. miescheriana* and *S. suihominis* in domestic or wild pigs in Italy. The number of wild boars hunted for human consumption has increased in Italy in recent years, and it is therefore of considerable public health interest to determine the presence of various food-borne zoonotic parasites in these game animals. Hence, in a previous study, the prevalence of *Toxoplasma gondii*, *Trichinella* spp. and *Alaria alata* was determined in muscle samples of 100 wild boars hunted in the Val Grande National Park in north-western Italy (Gazzonis et al. 2018). In the present study, samples from the same animals have been examined by molecular methods for the presence of *S. miescheriana* and *S. suihominis* in order to determine the prevalence of both species and thereby the risk for humans of acquiring the zoonotic *S. suihominis* through consumption of raw or undercooked wild boar meat. A further major aim of the study was to characterise isolates of *S. miescheriana* and/or *S. suihominis* molecularly, particularly with respect to the mitochondrial *cox1* gene, and determine for the first time the phylogenetic relationships of these species to each other and to *Sarcocystis* spp. of other hosts based on this molecular marker. Moreover, a characterisation of *cox1* would provide a novel diagnostic tool for the identification of sarcocysts of both species in pigs, as well as that of oocysts/sporocysts of *S. suihominis* in faecal samples from humans. In addition, a histological screening of muscle samples was included in the study in order to look for possible pathological changes associated with sarcocysts and to be able to compare the sensitivity of this method with the molecular methods used.

Materials and methods

Study area

The study was carried out on wild boars from the Val Grande National Park, which is located in the Piedmont region in the Central Italian Alps in north-western Italy (45° 56′ N, 8° 32′ E). The park comprises the largest “wilderness area” in Italy (Höchtl et al. 2005). Details about the topography, flora and fauna of the park area have been described previously (Gazzonis et al. 2018). The study area was chosen due to the high population density of wild boars and to the fact that a certain number of wild boars were allowed to be hunted each year as part of a recently implemented depopulation plan aimed at reducing the impact of wild boars on the natural environment of the park. The depopulation plan had been approved by the Institute for Environmental Protection and Research (ISPRA) and by the Italian Ministry of the Environment and Protection of Land and Sea (MATTM) and had also been authorised by the Piedmont region (decree no. 40-1967, 31 July 2015).

Sampling of wild boars

The sampling of wild boars was performed as described previously (Gazzonis et al. 2018). Briefly, during the hunting seasons (October–December) of 2015 and 2016, 100 muscle samples, each comprising about 150 g of the diaphragm, were collected from 100 legally hunted wild boars (33 females, 67 males) during the mandatory postmortem inspection of the carcasses at a game-handling establishment. Samples were placed individually in labelled plastic bags and were kept refrigerated while being shipped to the Laboratory of Parasitology, Department of Veterinary Medicine, Università degli Studi di Milano. An aliquot (25 g) of homogenised muscle samples, previously used for an epidemiological survey of three other food-borne parasites (Gazzonis et al. 2018) and stored at − 20 °C, and a small portion of tissues stored in buffered formalin were used in the present study for molecular and histological analyses, respectively.

In connection with the sampling, individual data (age, gender and weight) and the place where each wild boar had been shot were recorded. Age was estimated on the basis of a dental eruption table according to Boitani and Mattei (1991), categorising the animals into red (6–10 months), subadult (11–14 months), adult 1 (15–18 months), adult 2 (19–24 months), adult 3 (25–36 months) and adult 4 (> 36 months) as described previously (Gazzonis et al. 2018). The number of sampled animals belonging to each of the six age categories was 30, 7, 29, 12, 21 and 1, respectively. In this paper, the 37 animals belonging to age categories red and subadult have been collectively referred to as young animals (6–14 months), whereas the 63 animals belonging to the four adult categories (15 months and older) have been referred to as adults. Individual data on each wild boar are given in Table S1 of Supplementary file 1.

Histological examination of muscle samples

A small portion of each diaphragm sample was fixed in 10% buffered formalin and stored at room temperature until histopathological analyses. Fixed samples were embedded in paraffin wax and sectioned at 5 μm. Sections were stained with haematoxylin and eosin (HE) and microscopically examined at × 200 and × 400 magnifications. Only a single section from each animal was examined. *Sarcocystis*-like cysts were identified according to the morphological keys provided by Dubey et al. (2016) and counted per tissue section (i.e. the entire area under the 24 mm × 60 mm coverslip). From the histological data, descriptive statistics of the intensity of infection were obtained according to Bush et al. (1997).

Molecular examination of muscle samples

About 25 g of each diaphragm sample was examined by molecular methods for the presence of *Sarcocystis* infection. Upon thawing, each muscle sample was homogenised mechanically in a blender before total DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions.

All 100 DNA samples were initially screened for *Sarcocystis* DNA and the possible presence of more than one *Sarcocystis* species in each sample by using PCR with primer pair SU1F/5.8SR2, which amplifies the complete ITS1 region and small portions of the flanking 18S and 5.8S rRNA genes (Gjerde 2014b). The presence, amount and size of PCR products were determined through electrophoresis of the processed reaction mixture on 1% agarose gels stained with ethidium bromide, followed by examination of the gel under UV light. Based on the results from this screening, selected samples were subsequently amplified with various primers targeting four different DNA regions, followed by sequencing of the amplicons. Since the only DNA sample found to contain *S. suihominis* also contained *S. miescheriana*, it was necessary to design four new primers selectively targeting the former species in order to PCR amplify and sequence directly three of its DNA regions.

The complete 18S rRNA gene of *S. miescheriana* was initially amplified from three isolates as two overlapping fragments (about 1100 base pairs (bp) and 900 bp long, respectively) using primer pairs ERIB1/S2r and S3f/primer BSarc as in previous studies based on DNA extracted from individual sarcocysts (e.g. Gjerde 2016). However, some of the resulting sequences were of poor quality due to co-amplification of yeast DNA. Primers S2r and S3f were therefore substituted with primers S4r and D6f, respectively, since the latter primers have a very poor match against the 18S rRNA gene of all yeasts so far examined at this gene. The complete 18S rRNA gene of *S. miescheriana* was then re-amplified from two isolates (WB26, WB52) as two overlapping fragments, about 1670 bp and 1370 bp long, respectively, with primer pairs ERIB1/S4r and D6f/primer BSarc, whereas primers S2r and S3f were used as additional sequencing primers.

Two new primers were designed to selectively target the 18S rRNA gene of *S. suihominis* in the sample also containing *S. miescheriana*. Initially, the longest available nucleotide sequences of both species were downloaded from GenBank, aligned in the Alignment Explorer of MEGA7 (Kumar et al. 2016) and compared in order to identify regions in which the two species consistently differed. A stretch of sequence about 200 nucleotides from the 5′ end of the gene was then chosen as a target area for a new forward primer (SshF). About 1750 bp of the gene was subsequently amplified and sequenced with primer pair SshF/primer BSarc, while primers S2r and S3f were used as additional sequencing primers. Based on the new sequence and some previous sequences of *S. suihominis* in GenBank, a new reverse primer (SshR) was later designed and used in combination with forward primer ERIB1 for amplification and sequencing of about 850 bp of the gene beginning at the 5′ end and overlapping with the aforementioned new sequence.

The ITS1 region of *S. miescheriana* was amplified for sequencing and cloning from two isolates (WB26, WB52) using primer pair SU1F/5.8SR2 as in the initial screening of all samples mentioned above. In order to amplify the ITS1 region of *S. suihominis* from the mixed template, a semi-nested PCR was used. In the first round of amplification, the new forward primer SshF was used together with reverse primer 5.8SR2 (amplifying an ~ 2500-bp-long DNA fragment comprising the major portion of the 18S rRNA gene, the ITS1 region and a portion of the 5.8S rRNA gene), followed by amplification with primer pair SU1F/5.8SR2 in the second round.

The partial 28S rRNA gene (D2/D3 domain) of *S. miescheriana* was initially amplified as two overlapping fragments with primer pairs KL1/LS2R and LS1F/KL3, in which primers LS2R and LS1F mainly target members of the Sarcocystidae (Gjerde 2014b), whereas primers KL1 and KL3 are general. However, reverse sequences obtained with primer KL3 were difficult to interpret due to a mixture of two or more sequences, which at first was suspected to be due to co-amplification of yeast DNA. A new reverse primer, LS3R, was therefore designed to target a stretch of sequence about 170 bp downstream of reverse primer KL3 in which all GenBank sequences of the Sarcocystidae differed markedly from those of yeasts. Primer LS3R was then used together with primer LS1F instead of the general primer KL3 for reamplification and sequencing of this portion of the 28S rRNA gene of the four isolates under study (WB26, WB52, WB77, WB93). No attempts were made to amplify and sequence the 28S rRNA gene of *S. suihominis*.

Initial attempts to sequence directly the PCR products of the abovementioned three regions of the ribosomal DNA unit of *S. miescheriana* returned sequences that were fine for only a short distance from the PCR primers and then became mixed to various degrees, suggesting the presence of intra-specific sequence variation consisting of one or more indels. Purified PCR amplicons of primer pairs ERIB1/S4r and D6f/primer BSarc (18S rRNA gene of isolate WB26), SU1F/5.8SR2 (ITS1 region of isolates WB26 and WB52) as well as KL1/LS2R and LS1F/KL3 (partial 28S rRNA gene of isolate WB26) were therefore cloned into a vector. A total of 48 clones were cultured, followed by purification of the resulting plasmid DNA, whereas 33 clones were finally sequenced.

As regards PCR amplification of the partial *cox1* gene, DNA from two isolates of *S. miescheriana* (identified retrospectively) was initially tested with six previously used reverse primers in combination with forward primer SF1, as well as with primer pair SF6/SR10. A strong band of expected size was obtained both with SF1/SR66 and SF6/SR10, whereas a single weak band of expected size was obtained with SF1/SR10. A second round of amplification with SF1/SR10 yielded a strong band of expected size, but also an additional shorter band. Since primers SR66 and SF6 partially overlap (Gjerde 2016), they could not be used with primers SF1 and SR10, respectively, to obtain overlapping sequences of the entire region between SF1 and SR10. Four isolates were therefore amplified for sequencing with primer pairs SF1/SR66 and SF6/SR10, while eight other isolates were amplified with the latter primer pair only, in order to obtain partial sequences from which two new primers could be designed. The resulting sequences were fine, and a new forward primer, SF7, was designed to target nucleotides 108–126 downstream of primer SF1, whereas a new reverse primer, SR11, was designed to target nucleotides 1078–1099 from SF1. Primer SR11 thus included seven nucleotides of primer SR10 at its 5′ end. Primer pair SF1/SR11 was subsequently used to amplify the partial *cox1* of *S. miescheriana* from all positive isolates, while SF7 and SR11 were used as the main sequencing primers, but primers SR66 and SF6 were used as additional sequencing primers on a few isolates from which short sequence reads had been obtained with the PCR primers.

In order to amplify *cox1* of *S. suihominis* from the mixed template also containing *S. miescheriana*, two new primers were designed to selectively target the former species on the basis of a short sequence obtained with reverse primer SR66 on PCR products amplified with primer pair SF1/SR11. The new forward primer, SF8, targeting positions 286–305 downstream of SF1, was designed to be used together with reverse primer SR11, whereas a new reverse primer, SR13, targeting positions 487–505, was designed to be used together with forward primer SF1. The entire 1077-bp-long region between SF1 and SR11 was then obtained from the two overlapping sequences amplified with these primer pairs.

The sequences of all primers used are given in Table S2 in Supplementary file 1. The PCR reactions were performed, and the PCR products were evaluated and purified as described previously, as were the procedures and the reagents used for cloning and purification of plasmid DNA (Gjerde 2016). Purified amplicons and plasmid DNA (from cloning) were sent to Eurofins Genomics, Germany, for sequencing on both strands as previously described (Gjerde et al. 2017a). Forward and reverse sequence reads, as well as overlapping sequences, were manually assembled into complete consensus sequences using the Alignment Explorer of the MEGA7 software (Kumar et al. 2016). The similarity between the new sequences of each region and that between these and related sequences in GenBank were determined using the nucleotide BLAST program as previously described (Gjerde 2013). In addition, the software package DNA Sequence Polymorphism (DnaSP) (version 5.10.01) (Librado and Rozas 2009) was used for the analysis of sequence variation among the new *cox1* sequences of *S. miescheriana* and for comparing these sequences with the single new sequence of *S. suihominis*.

Phylogenetic analyses

Phylogenetic analyses were conducted separately on nucleotide sequences of the *cox1* and 18S rRNA genes by means of the MEGA7 software (Kumar et al. 2016). In both analyses, the intestinal coccidium *Eimeria tenella* (family Eimeriidae) was used as out-group species to root the trees and the phylogeny was tested with the bootstrap method, using 1000 bootstrap replications. The GenBank accession numbers of all sequences included in the analyses are given behind the taxon names in the phylogenetic trees (Figs. 1 and 2).

Concerning *cox1*, a total of 102 partial sequences from 60 taxa were used in the analysis, including 44 new sequences generated in the present study. A codon-based multiple alignment of all sequences was obtained by using the ClustalW program integrated in MEGA7 as described previously (Gjerde 2013). Sequences longer than 1020 bp (downstream of forward primer SF1) were truncated at their 3′ end, so that the final alignment comprised 1020 positions with no gaps except those caused by missing data in three sequences that were shorter than 1020 bp (Supplementary file 2). The phylogenetic tree was reconstructed using the neighbour joining method, in which evolutionary distances were computed according to the Kimura two-parameter model. All codon positions were used. Gaps due to missing data were treated with the pairwise deletion option.

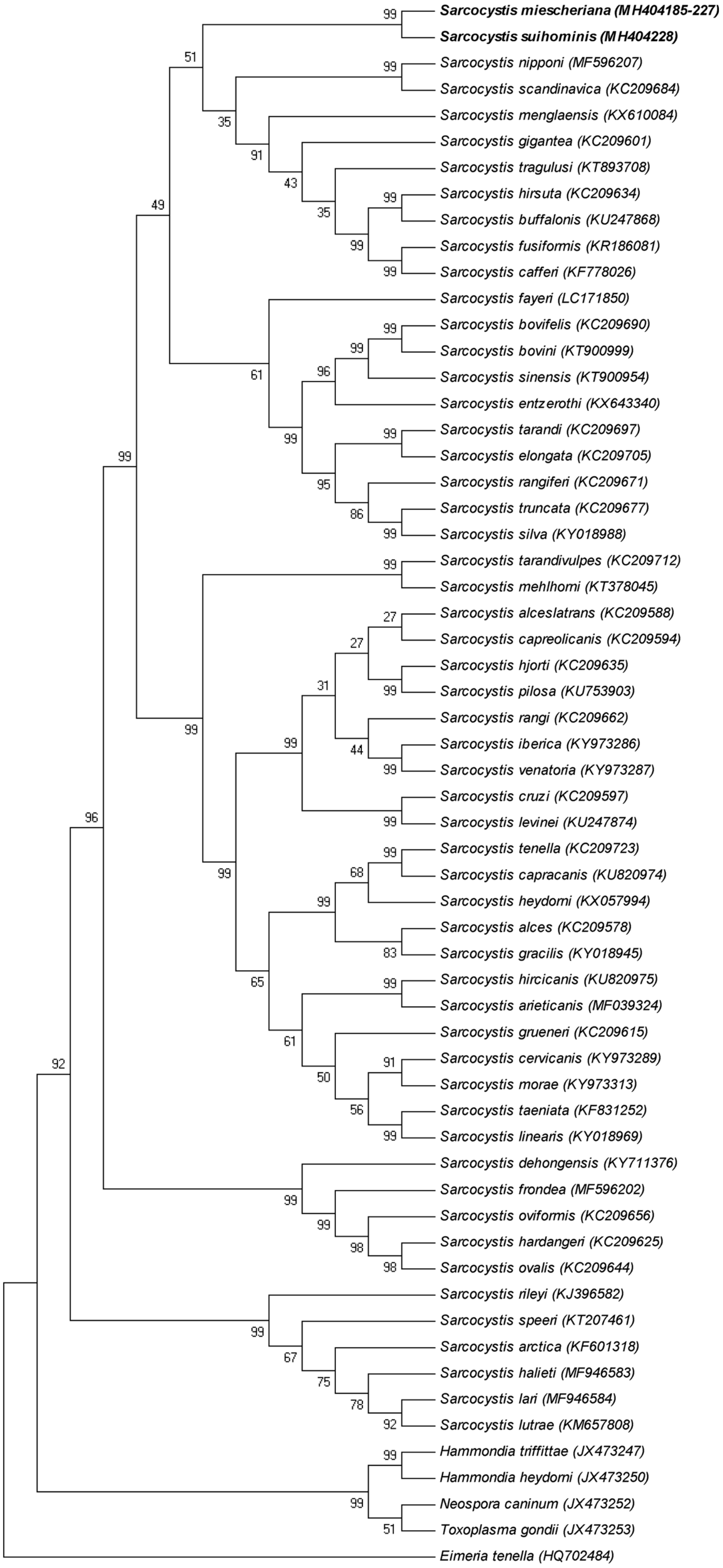
As regards the 18S rRNA gene, a total of 66 near-full-length sequences from 60 taxa were used in the analysis, including four new sequences from the present study. A multiple sequence alignment was generated with the ClustalW program within MEGA7, using a gap opening penalty of 10 and a gap extension penalty of 0.2 for both the pairwise and multiple alignments (Gjerde 2013). Most sequences were truncated slightly at both ends, so that most sequences started and ended at the same homologous nucleotide positions, corresponding to positions 74 and 1805, respectively, of the GenBank sequence KT901117 of *Sarcocystis bovifelis*. The final alignment comprised 2075 aligned positions, including gaps (Supplementary file 3). The phylogenetic tree was reconstructed using the maximum parsimony method with the Subtree–Pruning–Regrafting (SPR) algorithm. All sites were used.

Fig. 1 Phylogenetic tree for members of the Sarcocystidae based on 102 partial sequences (1020 bp) of the *cox1* gene from 60 taxa and inferred using the neighbour-joining method. Evolutionary distances were computed using the Kimura two-parameter method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers of all sequences included in the analysis are given behind the taxon names. The subtree formed by the 43 new sequences of *S. miescheriana* from this study has been collapsed. These sequences formed a sister group to the single new sequence of *S. suihominis* (both taxon names are in boldface) ▶

Results

Histology

Histology revealed the presence of typical *Sarcocystis* cysts in the diaphragm of 32 of the 100 wild boars examined. With respect to gender, sarcocysts were found in 12 of 33 females


99
Sarcocystis miescheriana (MH404185-227)
Sarcocystis suihominis (MH404228)
51
99
Sarcocystis nipponi (MF596207)
Sarcocystis scandinavica (KC209684)
Sarcocystis menglaensis (KX610084)
35
Sarcocystis gigantea (KC209601)
91
Sarcocystis tragulusi (KT893708)
43
99
Sarcocystis hirsuta (KC209634)
49
35
Sarcocystis buffalonis (KU247868)
99
Sarcocystis fusiformis (KR186081)
99
Sarcocystis cafferi (KF778026)
Sarcocystis fayeri (LC171850)
99
Sarcocystis bovifelis (KC209690)
99
Sarcocystis bovini (KT900999)
96
61
Sarcocystis sinensis (KT900954)
Sarcocystis entzerothi (KX643340)
99
Sarcocystis tarandi (KC209697)
99
Sarcocystis elongata (KC209705)
99
Sarcocystis rangiferi (KC209671)
95
Sarcocystis truncata (KC209677)
86
99
Sarcocystis silva (KY018988)
99
Sarcocystis tarandivulpes (KC209712)
Sarcocystis mehlhorni (KT378045)
27
Sarcocystis alceslatrans (KC209588)
27
Sarcocystis capreolicanis (KC209594)
Sarcocystis hjorti (KC209635)
31
99
Sarcocystis pilosa (KU753903)
Sarcocystis rangi (KC209662)
99
99
44
Sarcocystis iberica (KY973286)
99
Sarcocystis venatoria (KY973287)
Sarcocystis cruzi (KC209597)
96
99
Sarcocystis levinei (KU247874)
99
Sarcocystis tenella (KC209723)
68
99
Sarcocystis capracanis (KU820974)
99
Sarcocystis heydorni (KX057994)
Sarcocystis alces (KC209578)
83
Sarcocystis gracilis (KY018945)
65
99
Sarcocystis hircicanis (KU820975)
Sarcocystis arieticanis (MF039324)
Sarcocystis grueneri (KC209615)
61
92
91
Sarcocystis cervicanis (KY973289)
50
Sarcocystis morae (KY973313)
56
Sarcocystis taeniata (KF831252)
99
Sarcocystis linearis (KY018969)
Sarcocystis dehongensis (KY711376)
Sarcocystis frondea (MF596202)
99
99
Sarcocystis oviformis (KC209656)
98
Sarcocystis hardangeri (KC209625)
98
Sarcocystis ovalis (KC209644)
Sarcocystis rileyi (KJ396582)
Sarcocystis speeri (KT207461)
99
67
Sarcocystis arctica (KF601318)
Sarcocystis halieti (MF946583)
75
78
Sarcocystis lari (MF946584)
92
Sarcocystis lutrae (KM657808)
99
Hammondia triffittae (JX473247)
Hammondia heydorni (JX473250)
99
Neospora caninum (JX473252)
51
Toxoplasma gondii (JX473253)
Eimeria tenella (HQ702484)

(36.4%) and in 20 of 67 males (29.8%) (Table 1). With respect to age, cysts were found in 15 young (40.5%) and 17 adult (27.0%) animals. The sarcocysts were measured on average 222 μm × 86 μm in size, whereas the cyst wall was up to 6 μm in thickness and consisted of finger-like protrusions, which were up to 5 μm long (Fig. S1 in Supplementary file 1). All sarcocysts detected were morphologically similar and consistent with sarcocysts of *S. miescheriana*. The mean number of sarcocysts in each section was 2.8 (standard deviation = 5.35, variation = 1–31), with young females showing the highest burden of infection (Table 1). Samples showing the highest number of cysts (> 4 per section) belonged to young animals, while adults showed a lower burden of cysts per section.

Histopathological alterations of the muscle tissue consisting of myositis were found in five animals, three adult females and two young animals (one female and one male). In two of these animals (a young male and an adult female), no sarcocysts were found at the histological examination, but both animals were subsequently shown to be positive for *S. miescheriana* by molecular methods. Among the three other animals with myositis, two adult females had only one cyst per section, whereas a young female showed the highest burden of infection recorded (31 cysts per section). In the latter animal, only immature cysts containing metrocytes were detected. In addition, a wild boar that was negative for *Sarcocystis* at both the histological and molecular examinations showed moderate panniculitis.

Molecular identification and characterisation

The initial screening of all DNA samples for DNA of Sarcocystidae using PCR amplification of the ITS1 region with primer pair SU1F/5.8SR2, followed by evaluation of the results on agarose gels, revealed that 97 of 100 samples were positive. The three negative samples originated from animals that had also been negative by histology (Table S1 in Supplementary file 1). In all positive samples, the length of the amplicons seemed to be the same, since a single band occurred on the gels, whereas the strength of this band differed among samples. Four of the samples (WB26, WB52, WB77, WB93) were subsequently identified as comprising DNA of *S. miescheriana* on the basis of amplification and sequencing of the partial 28S rRNA gene followed by sequence similarity searches using BLAST, while two of these samples (WB26, WB52) were also identified through amplification and sequencing of the complete 18S rRNA gene. ITS1 sequences obtained from the latter two isolates were therefore also assigned to *S. miescheriana*, as were the *cox1* sequences obtained from all four isolates identified from previous sequences of the 18S and/or 28S rRNA genes. On the basis of these four *cox1* sequences, all other isolates yielding similar sequences were also considered to be positive for *S. miescheriana*. The finding of a *cox1* sequence markedly different from these in a sample containing *S. miescheriana* indicated that this sample also contained DNA of *S. suihominis*, the second major species in pigs, and this assumption was confirmed through sequencing of the 18S rRNA gene, followed by sequence similarity searches. In the following sections, the characteristics of the sequences from each DNA region examined will be described in more detail. The molecular characterisation resulted in a total of 70 nucleotide sequences being submitted to GenBank and issued accession numbers MH404185–MH404254.

Fig. 2 Phylogenetic tree for members of the Sarcocystidae based on 66 sequences of the near-full-length 18S rRNA gene of 60 taxa and inferred using the using the maximum parsimony method with the Subtree–Pruning–Regrafting algorithm. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers of all sequences included in the analysis are given behind the taxon names. The four new sequences from the present study are in boldface ▶

18S rRNA gene of *S. miescheriana* and *S. suihominis*

The complete 18S rRNA gene of *S. miescheriana* from isolates WB26 and WB52 was amplified twice as two overlapping fragments, initially with primer pairs ERIB1/S2r and S3f/primer BSarc and then with primer pairs ERIB1/S4r and D6f/primer BSarc, followed by direct sequencing, whereas amplicons from isolate WB26 generated with the latter two primer pairs were also cloned into a vector. A total of six clones were sequenced, including five clones comprising 1665–1669 bp from the 5′ end of the gene and one clone comprising 1363 bp from the 3′ end. The former six clones comprised three different sequence types, and each of these was concatenated with the non-overlapping portion (252 bp) of the latter clone to yield three full-length sequences of the gene, which were either 1917 bp or 1921 bp long (GenBank: MH404230–MH404232). The difference in length was due to a single 4-bp-long insertion (ATTT) in one clone (GenBank: MH404230; nucleotides 265–268) compared with the other two. In addition, the sequences differed from each other by one to three nucleotide substitutions at three polymorphic sites, and the overall identity between them was 99.7–99.8%. The four near-full-length sequences obtained by direct sequencing were identical and comprised the insertion (ATTT), which therefore presumably predominated among the gene copies, although it was present in only two of six sequenced clones. The four PCR-derived sequences, which were not submitted to GenBank, were identical with sequence MH404230, except that they showed a double peak (both T and C) at position 197, where the cloned sequence showed a T.

When compared with previous sequences in GenBank, the longest sequence (MH404230) differed at only one position (99.9% identity) from the near-complete sequence JN256123 of *S. miescheriana* from a wild boar in Lithuania (Prakas

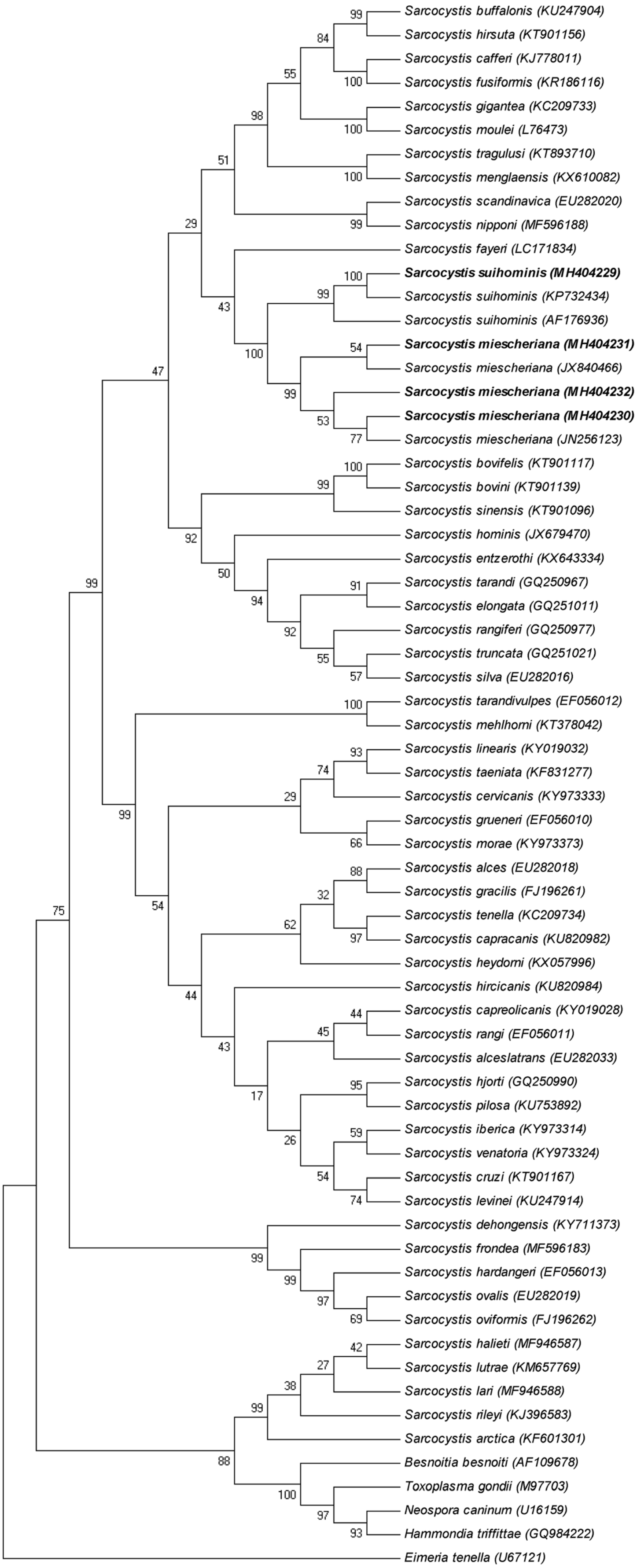

99
Sarcocystis buffalonis (KU247904)
84
Sarcocystis hirsuta (KT901156)
Sarcocystis cafferi (KJ778011)
55
100
Sarcocystis fusiformis (KR186116)
Sarcocystis gigantea (KC209733)
98
100
Sarcocystis moulei (L76473)
51
Sarcocystis tragulusi (KT893710)
100
Sarcocystis menglaensis (KX610082)
Sarcocystis scandinavica (EU282020)
29
99
Sarcocystis nipponi (MF596188)
Sarcocystis fayeri (LC171834)
100
Sarcocystis suihominis (MH404229)
99
Sarcocystis suihominis (KP732434)
43
Sarcocystis suihominis (AF176936)
54
Sarcocystis miescheriana (MH404231)
100
Sarcocystis miescheriana (JX840466)
47
99
Sarcocystis miescheriana (MH404232)
53
Sarcocystis miescheriana (MH404230)
77
Sarcocystis miescheriana (JN256123)
100
Sarcocystis bovifelis (KT901117)
99
Sarcocystis bovini (KT901139)
Sarcocystis sinensis (KT901096)
Sarcocystis hominis (JX679470)
92
Sarcocystis entzerothi (KX643334)
50
91
Sarcocystis tarandi (GQ250967)
99
94
Sarcocystis elongata (GQ251011)
92
Sarcocystis rangiferi (GQ250977)
55
Sarcocystis truncata (GQ251021)
57
Sarcocystis silva (EU282016)
100
Sarcocystis tarandivulpes (EF056012)
Sarcocystis mehlhorni (KT378042)
93
Sarcocystis linearis (KY019032)
74
Sarcocystis taeniata (KF831277)
29
Sarcocystis cervicanis (KY973333)
99
Sarcocystis grueneri (EF056010)
66
Sarcocystis morae (KY973373)
88
Sarcocystis alces (EU282018)
32
Sarcocystis gracilis (FJ196261)
54
75
Sarcocystis tenella (KC209734)
62
97
Sarcocystis capracanis (KU820982)
Sarcocystis heydorni (KX057996)
Sarcocystis hircicanis (KU820984)
44
44
Sarcocystis capreolicanis (KY019028)
45
Sarcocystis rangi (EF056011)
43
Sarcocystis alceslatrans (EU282033)
95
Sarcocystis hjorti (GQ250990)
17
Sarcocystis pilosa (KU753892)
59
Sarcocystis iberica (KY973314)
26
Sarcocystis venatoria (KY973324)
54
Sarcocystis cruzi (KT901167)
74
Sarcocystis levinei (KU247914)
Sarcocystis dehongensis (KY711373)
99
Sarcocystis frondea (MF596183)
99
Sarcocystis hardangeri (EF056013)
97
Sarcocystis ovalis (EU282019)
69
Sarcocystis oviformis (FJ196262)
42
Sarcocystis halieti (MF946587)
27
Sarcocystis lutrae (KM657769)
38
Sarcocystis lari (MF946588)
99
Sarcocystis rileyi (KJ396583)
Sarcocystis arctica (KF601301)
88
Besnoitia besnoiti (AF109678)
100
Toxoplasma gondii (M97703)
97
Neospora caninum (U16159)
93
Hammondia triffittae (GQ984222)
Eimeria tenella (U67121)

Table 1 Number and percentage of wild boars found positive for *Sarcocystis* infection according to age and gender in the histological and molecular screening of diaphragm samples

Gender	Age	*n*	Category		
			Histological screening[a]		Molecular screening[b]
			No. of positives (%)	Intensity of infection (mean ± SD)	No. of positives (%)
Female		33	12 (36.4)	4.67 ± 8.56	32 (96.9)
	Young	13	6 (46.1)	8.17 ± 11.48	12 (92.3)
	Adult	20	6 (30.0)	1.17 ± 0.41	20 (100)
Male		67	20 (29.8)	1.7 ± 0.92	65 (97.0)
	Young	24	9 (37.5)	1.78 ± 1.09	24 (100)
	Adult	43	11 (25.6)	1.64 ± 0.81	41 (95.3)

The mean number of sarcocysts per section (intensity of infection) in positive animals is also given

SD standard deviation

[a] One section of the diaphragm was examined from each animal

[b] Amplification of the ITS1 region with primers presumed to target all *Sarcocystis* spp. and verified in this study to be able to amplify the ITS1 region of both *Sarcocystis* spp. in pigs

2011), whereas the two shorter sequences without the insertion differed slightly more from this sequence (99.6% and 99.7% identity). Likewise, the new sequences were 98.9–99.6% identical with four 1667–1736-bp-long sequences of *S. miescheriana* (JX840464–JX840467) from domestic pigs in China (Yan et al. 2013). Moreover, the new sequences were identical with 19 short sequences of *S. miescheriana* derived from wild boars in Portugal (Coelho et al. 2015) and with 10 short sequences attributed to *S. miescheriana*, which had been obtained from oocysts/sporocysts in red foxes and raccoon dogs in Germany (Moré et al. 2016).

Isolate WB9, from which *cox1* sequences of both *S. miescheriana* and a second species had been obtained, was subjected to PCR amplification with the newly designed primers SshR and SshF targeting *S. suihominis* in combination with the terminal primers ERIB1 and primer BSarc, respectively. The sequencing of amplicons of primer pair SshF/primer BSarc yielded fine sequences in both directions with all four sequencing primers used. The forward and reverse sequence reads obtained from amplicons of primer pair ERIB1/SshR, on the other hand, were only fine to a certain point and then became mixed. A close examination of the chromatogram revealed that this was due to a 3-bp-long indel, and that the predominant sequence type lacked an insertion, whereas apparently, a minority of the gene copies possessed an insertion (ATT). Curiously, this insertion turned out to be located within the target region of forward primer SshF, and only gene copies without this insertion would therefore have been amplified with that primer. Hence, this insertion was not included in the final sequence submitted to GenBank (MH404229) but would have been located between positions 199 and 200 of that sequence. The new sequence of *S. suihominis* was 1930 bp long and represents the first full-length sequence of the 18S rRNA gene of this species. Sequence similarity searches revealed that this sequence was most similar (98.9–100% identical) to 13 sequences of 361–1521 bp long, assigned to *S. suihominis* from pigs in India (KP732433–KP732435; KC709514, KC709517, KC709519–KC709521; KT365500–KT365504), whereas the identity was 98.2% with two 1367-bp-long sequences (AF176936, AF176937) of *S. suihominis* from pigs in China (Yang et al. 2001) and 95.7% with three 504-bp-long sequences (AF261239–AF261241) of this species from pigs in Spain (González et al. 2006). The new sequence, as well as the 13 sequences from pigs in India, differed from the latter five sequences from pigs in China and Spain by four indels and several substitutions in an 80-bp-long portion of the gene, but was otherwise almost identical. Hence, the new sequence from isolate WB9 in this study was also assigned to *S. suihominis*. In comparison, the identity of the new sequence of *S. suihominis* with the three new complete sequences of *S. miescheriana* from this study was 97.6%.

ITS1 region of *S. miescheriana* and *S. suihominis*

A total of 17 clones of the ITS1 region of isolates WB26 and WB52 were sequenced. Fourteen of these were considered to be typical clones of *S. miescheriana* since the initial 120 nucleotides of the sequences, comprising the 3′ end of the 18S rRNA gene (exclusive of primer SU1F), completely matched the homologous portion of sequences obtained with primers targeting this gene (see previous section). Moreover, the 3′ end of the sequences, comprising 96 nucleotides of the 5.8S rRNA gene (exclusive of primer 5.8SR2), were typical of various *Sarcocystis* spp. from previous studies. Five of these clones were identical, and thus, there were 10 unique clones of this type. The 10 sequences (GenBank: MH404233–MH404242) were 869–878 bp long, of which the ITS1 region comprised

607–616 bp. The difference in length between the sequences was due to seven indels, occurring in various combinations. The overall identity of the 10 typical clones with respect to the ITS1 region only was 95.1–99.8%. By contrast, these clones were only about 85–87% identical with the three remaining clones (GenBank: MH404243–MH404245) and differed from the latter by 15 indels of 1–14 bp long and many substitutions, including some substitutions in the amplified portions of the 18S and 5.8S rRNA genes. Most of the indels were deletions in the latter clones, which were therefore only 844 bp long, of which the ITS1 region comprised 582 bp. However, there were also long stretches of sequence that were identical between both types of clones, and thus, sequences MH404243–MH404245 have tentatively been assigned to *S. miescheriana* but have been labelled as aberrant clones.

The ITS1 region of *S. suihominis* was amplified from the mixed isolate WB9 using a semi-nested PCR, which included forward primer SshF selectively targeting this species in the first round. However, when the PCR products were sequenced directly, the forward and reverse sequence reads were fine for only a short distance from primers SU1F and 5.8SR2, that is, until reaching about 20 and 107 nucleotides, respectively, into the 5′ and 3′ ends of the ITS1 region. From these points onwards, the sequences became mixed with two or more sequences being superimposed on each other, which seemed to be due to indels (intra-specific sequence variation). The total length of the amplified and sequenced region was about 900 bp, which was in line with the size of the band on agarose gel and was only slightly longer than the abovementioned typical clones of *S. miescheriana*. Thus, when isolate WB9 was amplified with primer pair SU1F/5.8SR2 in the initial screening, the single band on agarose gel from this mixed sample was of the same size as the bands from all the other isolates containing DNA of *S. miescheriana* only, and the presence of two species was not revealed. Due to limited time and resources, no attempt was made to clone the amplicons of the ITS1 region of *S. suihominis* for sequencing.

28S rRNA gene of *S. miescheriana*

The partial 28S rRNA gene (D2/D3 domain) of *S. miescheriana* was PCR-amplified from four isolates (WB26, WB52, WB77, WB93) as two overlapping fragments, using primer pair KL1/LS2R for the 5′ end portion, and forward primer LS1F together with either of the reverse primers KL3 and LS3R for the 3′ end portion. In addition, amplicons of both fragments from isolate WB26 were cloned, but only those generated with primer pair KL1/LS2R were successfully inserted into the vector. Nine such clones were sequenced; four of these were identical, and thus, a total of six unique clones were obtained and concatenated with the non-overlapping portion of the sequence obtained through direct sequencing of amplicons obtained with primer pair LS1F/LS3R and submitted to GenBank (MH404246–MH404251). Additionally, sequences obtained from isolates WB52, WB77 and WB93 through direct sequencing were submitted (GenBank: MH404252–MH404254).

In the cloned portion of the gene from isolate WB26, there were two indels comprising one and 14 positions, respectively, which, in sequence MH404251, were both present as insertions (G; ATTATTATTGTACT) at positions 515 and 870–883. The longer insertion was also present in sequence MH404247, whereas both indels appeared as deletions in the remaining cloning-derived sequences. By direct sequencing, the short indel was present as an insertion (G) in all four isolates and the forward sequences became mixed from this point onwards, whereas the longer indel did not appear as an insertion in any sequences, but there seemed to be some deterioration of the sequence quality downstream of its location, suggesting its presence. As regards the 3′ end portion of the targeted gene region, which was only obtained through direct sequencing, forward sequences with primer LS1F suffered from the abovementioned indels, particularly the G indel, and thus became mixed and unclear about 100 nucleotides downstream from the primer. Likewise, reverse sequence chromatograms obtained with primer KL3 became mixed almost immediately, whereas those obtained with the new reverse primer LS3R became mixed after about 210 nucleotides. A careful examination of the latter chromatograms revealed that this was caused by a 1-bp-long indel occurring within a homo-polymer consisting of either nine or 10 consecutive As. This homo-polymer was located from position 25 onwards downstream of primer KL3, and therefore, the sequences obtained with this primer seemed to be mixed from the very beginning, which was initially suspected to be due to co-amplification of the 28S rRNA gene of a yeast. This problem was then resolved by designing and using reverse primer LS3R targeting an area further away from the indel. Due to the indels, the length of the entire submitted sequences varied between 1780 and 1795 nucleotides. Other than the indels, the sequences differed by only two substitutions, and the overall identity between the nine new sequences was 98.9–99.9%. These sequences were 98.7–99.7% identical with the only previous sequence of the 28S rRNA gene of *S. miescheriana* (AF076902).

cox1 gene of *S. miescheriana* and *S. suihominis*

As mentioned before, the initial testing of previously used *cox1* primers on two isolates revealed that primer pairs SF1/SR66 and SF6/SR10 performed well, whereas primer pair SF1/SR10 resulted in less amplification. Hence, four and 12 isolates were initially amplified and sequenced with primer pairs SF1/SR66 and SF6/SR10, respectively, which facilitated the design of forward primer SF7 and reverse primer SR11 targeting *cox1* of *S. miescheriana*. Testing revealed that reverse primer SR11 performed well both with the new primer SF7 and with the more general forward primer SF1. In order to obtain as long sequences as possible, PCR amplification with primer pair SF1/SR11 was subsequently conducted on all 97 DNA samples that had been positive for Sarcocystidae DNA in the ITS1-based screening. From all 97 samples, amplicons of expected size (1123 bp) were produced, as determined on agarose gels. The amplicons from 72 isolates were sequenced, initially with primers SF7 and SR11, but those of some isolates were also sequenced with primers SR66 and/or SF6, in order to obtain the entire sequence between the amplification primers. In all sequenced isolates, except one, forward and reverse sequences were unambiguous and matched each other. From isolate WB9, the forward sequence obtained with primer SF7 was fine and closely similar to those of *S. miescheriana* in other isolates, whereas the reverse sequence chromatogram obtained with primer SR11 showed a mixture of two sequences, in which a sequence different from those of *S. miescheriana* predominated and could be fairly accurately estimated for about 300 nucleotides. This region was compared with the new sequences of *S. miescheriana* from other isolates, and forward primer SF8 and reverse primer SR13 were designed to target areas where the two sequence types clearly differed. The entire region between forward primer SF1 and SR11 was then amplified as to overlapping fragments with primer pairs SF1/SR13 and SF8 and SR11, resulting in a fine 1177-bp-long sequence of the second species in isolate WB9. This species was identified as being *S. suihominis* on the basis of its 18S rRNA gene sequence.

Since forward primer SF1 and reverse primer SR11 obviously had matched *cox1* of both *S. miescheriana* and *S. suihominis*, resulting in a mixed sequence when sequenced with SR11, the possibility existed that more isolates contained *S. suihominis* in addition to *S. miescheriana*. However, none of the other 71 isolates sequenced with reverse primer SR11 had yielded mixed sequences, and they were therefore considered to have contained *S. miescheriana* only. The 25 remaining isolates, which had not been sequenced but had yielded PCR products of expected size when using primer pair SF1/SR11, were subjected to PCR amplification with primer pair SF8/SR11, but no visible PCR products were seen on agarose gels from any of these samples, whereas a strong band was obtained from the positive control sample (WB9) containing *S. suihominis*. Hence, none of these 25 samples were considered to have contained DNA of *S. suihominis*.

A total of 72 *cox1* sequences of *S. miescheriana* were obtained, of which 60 were 1077 bp long (amplified with SF1/SR11 only) and 12 were 1092 bp long (isolates amplified with SF6/SR10 initially). The 72 sequences represented 43 haplotypes, which were submitted to GenBank (MH404185–MH404227). Thirty-five of these haplotypes were each represented by a single sequence/isolate, whereas seven haplotypes were represented by 2–12 sequences, the two most common being found in 12 and 10 isolates, respectively (represented by sequences MH404190 and MH404196). There were 55 polymorphic sites among the 1077 nucleotide positions covered by all 43 haplotypes, and many of these polymorphic sites were evident in individual isolates as double peaks in the sequencing chromatograms. The majority of the nucleotide differences were due to synonymous substitutions (silent mutations), and hence, there were only 13 variable amino acid residues among the 359 residues of the corresponding amino acid sequences. The 43 haplotypes differed from each other at 1–26 of these 1077 nucleotide positions, which corresponded to pairwise sequence identities of 97.6–99.9% (on average 98.9%). By contrast, the 43 sequences of *S. miescheriana* differed from the single new sequence of *S. suihominis* (MH404228) at 218–225 of 1077 nucleotide positions, corresponding to pairwise sequence identities of 79.1–79.8% (on average 79.4%). There were 199 fixed nucleotide differences between the two populations, that is, positions where all sequences of *S. miescheriana* differed from that of *S. suihominis*. When compared with previous *cox1* sequences of other *Sarcocystis* spp. in GenBank, sequences of *S. miescheriana* had the highest identity with those of *Sarcocystis rangiferi* (about 78%), whereas the sequence of *S. suihominis* shared the highest identity with those of *Sarcocystis truncata* and *Sarcocystis silva* (about 76%).

Phylogeny

In the phylogenetic tree based on sequences of *cox1* (Fig. 1), the 43 new sequences of *S. miescheriana* were sister to the single new sequence of *S. suihominis* within a clade comprising *Sarcocystis* spp. using ruminants (Bovidae, Cervidae) as intermediate hosts, as well as *Sarcocystis fayeri* (syn. *Sarcocystis bertrami*), cycling between horses and canids. As regards the species from ruminants, their definitive hosts are either felids or they are unknown, but suspected to be felids. This clade was separated with high support from a sister clade comprising *Sarcocystis* spp. from ruminants using canids as definitive host, except *Sarcocystis heydorni*, which allegedly use humans (Dubey et al. 2016). Basal to these clades was a clade comprising five *Sarcocystis* spp. with ruminant intermediate hosts that are known or suspected to use corvid birds as definitive hosts (Gjerde and Dahlgren 2010). The most basal clade of *Sarcocystis* spp. in the tree comprised taxa that use birds or carnivores as intermediate hosts (Gjerde et al. 2017c).

In the analysis based on near-complete 18S rRNA gene sequences (Fig. 2), the new sequences of *S. miescheriana* and *S. suihominis* clustered with previous sequences of each species; sequences of the two taxa were sister to each other, and both taxa were sister to *S. fayeri*. Again, these species were placed within a major clade comprising *Sarcocystis* spp. with ruminant intermediate hosts and felids as known or suspected definitive hosts.

Discussion

The examination of the muscle samples with molecular methods revealed that 97 of 100 wild boars were infected with *S. miescheriana*, and that one wild boar harboured both *S. miescheriana* and *S. suihominis*, making the prevalence rates of the two species 97% and 1%, respectively, and the overall prevalence of *Sarcocystis* infection 97%. In comparison, three recent studies on wild boars in Spain (910 animals), Portugal (103 animals) and Romania (101 animals) reported overall prevalence rates of 72.2%, 73.8% and 60.4%, respectively (Calero-Bernal et al. 2016; Coelho et al. 2015; Imre et al. 2017). The lower prevalence found in the other studies might simply be due to a lower occurrence of *Sarcocystis* spp. infection among wild boars in those countries but could, in part, also be due to the use of less sensitive detection methods than those employed in our study. Thus, only Coelho et al. (2015) used a similar PCR-based molecular detection of *Sarcocystis*-positive muscle samples and animals, whereas the studies by Calero-Bernal et al. (2016) and Imre et al. (2017), as well as the study by Calero-Bernal et al. (2015) on feral pigs in the USA, used microscopy to detect positive samples (presence of sarcocysts and/or cystozoites) following chopping/mincing or digestion (by pepsin or trypsin) of the muscle samples. In those surveys, molecular methods were only applied to identify the species present in some of the positive samples. Kaur et al. (2016) used a combination of microscopic and molecular detection on samples from farm and stray pigs in India and found that some muscle samples that had been negative in the microscopic screening after chopping and digestion of muscle samples were positive by PCR.

The type of muscle tissue examined for sarcocysts might also have affected the recorded prevalence rates. In this study, we examined samples of the diaphragm, as did Coelho et al. (2015), whereas the other studies examined cardiac muscle (Calero-Bernal et al. 2015; Kaur et al. 2016; Imre et al. 2017). However, Coelho et al. (2015) found that more histological sections of the diaphragm contained sarcocysts than sections from the oesophagus and heart. Likewise, Erber and Boch (1976), in comparing the frequency of *Sarcocystis* infection in different muscle tissues of wild boars from Germany and Poland, found that the tongue and sublingual muscles, the abdominal muscles and the diaphragm were more frequently infected (85%, 73% and 70%, respectively) than the oesophagus (37%) and the heart (24%). Hence, Boch et al. (1978) sampled both the sublingual muscles and the diaphragm in their comprehensive study of domestic pigs but found no differences in the intensity of infection between these tissues, and both *S. miescheriana* and *S. suihominis* were recorded. Likewise, Hinaidy and Supperer (1979) used samples of the diaphragm in their study of the prevalence of *S. miescheriana* and *S. suihominis* in domestic pigs in Austria and found both species. Thus, the diaphragm seems to be a good choice when examining the prevalence of the two *Sarcocystis* spp. in pigs.

The initial histological screening of sections of the diaphragm detected sarcocysts in only 32% of the 100 wild boars examined. The fairly low sensitivity of the histological detection method in comparison to the molecular assay was as expected, since a smaller volume of tissue is examined by the former method, at least when only a single muscle section from each animal is examined. Thus, Coelho et al. (2015) reported a prevalence of only 25.5% based on histology, but of 73.8% based on PCR. Likewise, Calero-Bernal et al. (2015) detected sarcocysts in only 25.0% of 1006 feral pigs examined histologically, whereas 49.0% of 148 heart samples were microscopically positive for cystozoites following pepsin digestion, which, as noted above, is a less sensitive method than molecular detection. Nevertheless, the initial histological screening provided data on the infection intensity (sarcocysts per section) of each animal, as well as that of the occurrence of histopathological changes associated with the sarcocysts. In this study, a mean of 2.8 cysts per histological section was found, and only one animal showed a high burden of infection (31 cysts per section). Moreover, the intensity of infection was higher in females than in males (4.7 versus 1.7 cysts per section). These results are similar to those reported from feral pigs in the USA (Calero-Bernal et al. 2015), in which a mean of 3.0 cysts (females: 4.0 cysts; males 1.8 cysts) and a maximum of 184 cysts per section were found. The histological screening also suggested a higher prevalence among females than among males, as well as among young animals compared with adult animals, but there was almost no difference in prevalence rates between any of these groups in the molecular examination, since nearly all animals were found to be infected. In other surveys of pigs, the prevalence rates have tended to increase with age (Boch et al. 1978; Hinaidy and Supperer 1979; Damriyasa et al. 2004; Calero-Bernal et al. 2015, 2016; Coelho et al. 2015; Imre et al. 2017), which is to be expected, since older animals have had more time to become infected, and since most sarcocysts, once established, are long-lived. As regards histopathological changes, myositis was found in only five infected wild boars, and these changes did not seem to be related to the intensity of infection (number of sarcocysts). Calero-Bernal et al. (2015), on the other hand, found a higher frequency of myositis in sections of the myocardium comprising a high number of sarcocysts than in sections with few sarcocysts. Coelho et al. (2015) did not report any histopathological changes at all in the 299 sections they screened for sarcocysts, but they may not have looked specifically for such lesions.

All the sarcocysts found in the histological screening were thick-walled (Fig. S2 in Supplementary file 1) and consistent with sarcocysts of *S. miescheriana*, and the predominance of this species was confirmed molecularly. The much higher prevalence of *S. miescheriana* (97%) compared to *S. suihominis* (1%) in this study is in agreement with the findings of other surveys of wild boars and feral pigs. Thus, only *S. miescheriana* was recorded from wild boars in Lithuania, Portugal and Romania (Prakas 2011; Coelho et al. 2015; Imre et al. 2017), as well as from feral pigs in the USA (Calero-Bernal et al. 2015). Moreover, *S. miescheriana* was identified in seven of eight isolates identified molecularly from wild boars in Spain, whereas a single isolate contained *S. suihominis* (Calero-Bernal et al. 2016). The high prevalence of *S. miescheriana* in free-ranging pigs is probably related to the abundance of suitable definitive hosts, that is, various canids, in their habitats and a frequent consumption of muscle tissues from pigs by these carnivores. Thus, *S. miescheriana* was among the most frequently identified *Sarcocystis* spp. (as oocysts) in the intestines of red foxes and raccoon dogs in Germany (Moré et al. 2016). As regards transmission of *S. miescheriana* to wild boars in the Val Grande National Park, red foxes are prevalent (Torretta et al. 2017) and presumably act as the major definitive host contaminating the environment with sporocysts. Dogs, on the other hand, probably play an insignificant role in the transmission, since there are no stray dogs in the park area, hunting dogs are only allowed during the hunting season and pet dogs have to be kept on a leash when visiting the park with their owners.

The low prevalence of *S. suihominis* both in this study and the aforementioned surveys of wild boars and feral pigs is likely due to a low prevalence of this species in humans in combination with a low degree of environmental contamination with human stools. Presumably, *S. miescheriana* may be transmitted continuously between wild pigs and wild canids in a sylvatic cycle independently of populations of the parasite cycling between domestic pigs and farm dogs. By contrast, wild boars probably mainly contract infections with *S. suihominis* from humans who have acquired their infection from eating infected pork from domestic pigs rather than from wild boars. Hence, the prevalence of *S. suihominis* in wild boars may, to some extent, reflect the prevalence of this parasite in domestic pigs in the same region or country. Thus, Bergmann and Kinder (1976), who studied sarcocysts from wild boars (and roe deer) in Germany by TEM, found that sarcocysts of type W1, which are consistent with sarcocysts of *S. suihominis*, were more frequent than sarcocysts of type W2, which seem to belong to *S. miescheriana*. Surveys conducted in Germany and Austria in the same time period found a rather high prevalence of *S. suihominis* in domestic pigs, particularly in older animals (Boch et al. 1978; Hinaidy and Supperer 1979), which was probably related to the common habit of humans in those countries of eating raw or undercooked pork in combination with inadequate sanitary measures to prevent human stools from being ingested by pigs. Indeed, poor sanitation may facilitate transmission of *S. suihominis* to pigs as reported from India (Kaur et al. 2016).

Recent data on the prevalence of *S. suihominis* in domestic pigs in Europe are lacking; the last reported finding of *S. suihominis* in domestic pigs was in Spain (González et al. 2006). Likewise, there are no recent reports from Europe of intestinal infections of humans with *S. suihominis* (Fayer et al. 2015). Nevertheless, wild boars may still become infected with *S. suihominis* as shown in this study and in the study of wild boars from Spain (Calero-Bernal et al. 2016). Hence, there is also a certain risk for humans of becoming infected with this parasite if they eat raw or undercooked pork from wild boars. As regards the wild boars from Val Grande National Park examined in this study, they had previously been found to harbour two other meat-borne zoonotic parasites, that is, *T. gondii* and *A. alata* (Gazzonis et al. 2018). Although 42 of 97 animals were positive for antibodies against *T. gondii* in meat juice samples, only three of them were positive in a molecular assay involving PCR amplification of a fragment of the ITS1 region (Gazzonis et al. 2018). The ITS1 assay used in the present study to screen the samples for the presence of *Sarcocystis* DNA also had the potential to amplify the ITS1 region of *T. gondii*, as demonstrated in a previous study of otters (Gjerde and Josefsen 2015). However, no amplification products consistent with those of *T. gondii* were seen on agarose gels from any sample, including the samples from the three wild boars previously found positive (Gazzonis et al. 2018).

The molecular characterisation of various isolates of *S. miescheriana* provided new sequences from three regions of the ribosomal DNA unit of the nuclear genome (18S and 28S rRNA genes, ITS1 region), as well as of the *cox1* gene of the mitochondrial genome. Previously, only sequences of the 18S and 28S rRNA genes of this species have been available in GenBank. In all three regions of the ribosomal DNA unit, some intra-specific sequence variation (substitutions, indels) was uncovered. Such variation has previously been found in several *Sarcocystis* spp. using ruminant intermediate hosts, and it has been considered to be intra-genomic, since DNA was extracted from individual sarcocysts (e.g. Gjerde 2016; Gjerde et al. 2017a, b). In this study, DNA was extracted from small pieces of muscle, which may have contained more than one sarcocyst of *S. miescheriana* and thus more than one haplotype per sample. Hence, the recorded sequence variation may only be referred to as intra-specific rather than intra-genomic. Likewise, there seemed to be intra-specific sequence variation in the 18S rRNA gene and ITS1 region of *S. suihominis*, since the sequence chromatograms revealed the presence of indels. As regards the 18S rRNA gene, such intra-specific variation was also evident when the new sequence of *S. suihominis* was compared with previous sequences of this species. Interestingly, the *S. suihominis*-specific primer (SsuihR) used by Coelho et al. (2015) for screening DNA samples from wild boars for this species had a very poor match against our new sequence due to indels in the targeted region and would probably not have detected *S. suihominis* in our sample.

The sequencing of the partial *cox1* gene of *S. miescheriana* from no less than 72 isolates revealed a rather high sequence variation even at this locus. Thus, there were 43 haplotypes with pairwise sequence identities of 97.6–99.9% and a total of 55 polymorphic sites among the 1077 sites covered. In comparison, other *Sarcocystis* spp., from which more than 10 *cox1* sequences of similar length (1020–1038 bp) have been obtained and compared, have shown intra-specific sequence identities of 98–100% and a maximum of 42 polymorphic sites (Gjerde 2013, 2014a, 2016; Gjerde et al. 2017a, b). Still, the *cox1* sequences of *S. miescheriana* were clearly different from the single sequence of *S. suihominis* (79.1–79.8% identity), and hence, the two species could be unambiguously identified on the basis of this marker. Moreover, three of the new *cox1* primers seemed to selectively amplify either *S. miescheriana* (SF7) or *S. suihominis* (SF8, SR13) even when used in combination with more general primers and may therefore be employed in assays to identify sarcocysts in pigs or oocysts from the definitive hosts. Thus, either of the three primer pairs SF8/SR13, SF8/SR11 or SF1/SR13 might be used to identify oocysts/sporocysts of *S. suihominis* in human stools or for screening of such samples for the presence of this parasite.

For the first time, the phylogenetic relationships of *S. miescheriana* and *S. suihominis* to each other and to other *Sarcocystis* spp. could be inferred from the protein-coding *cox1* gene of the mitochondrial genome rather than from the nuclear 18S rRNA gene, which, so far, has been the only available option. Moreover, a more complete 18S rRNA gene sequence of *S. suihominis* and a much broader selection of taxa were included in the latter analysis than in previous analyses focusing on these species in pigs (Yan et al. 2013; Calero-Bernal et al. 2015; Coelho et al. 2015; Kaur et al. 2016; Imre et al. 2017). In our analyses, the tree topologies obtained from the two genes were quite similar, but there were some minor differences in the branching order and placement of certain taxa (Figs. 1 and 2). In both analyses, *S. miescheriana* and *S. suihominis* were sister species and were placed within a clade comprising *Sarcocystis* spp. of ruminants that have felids as known or suspected definitive hosts, plus *S. fayeri*, cycling between horses and canids. Thus, while known *Sarcocystis* spp. of ruminants cluster according to definitive hosts into three major clades, those of pigs and horses seem to be placed independently of this grouping into the felid clade, while *Sarcocystis* spp. using birds or carnivores as intermediate hosts form a separate basal clade, within which they also seem to cluster largely according to definitive hosts (birds of prey/mammalian carnivores) (Gjerde et al. 2017c). The fact that *S. miescheriana* and *S. suihominis* are sister species in spite of using different definitive hosts is therefore noteworthy. A possible explanation for this relationship could be that they have descended from a common ancestor using only ancient canids as definitive host, and that two variants of this ancient species gradually separated and became adapted to extant canids and modern humans (probably via extinct *Homo* spp. or non-human primates), respectively. Another possibility is that *S. miescheriana* and/or *S. suihominis* are more closely related to one or more other *Sarcocystis* spp., which are still unknown or have not yet been characterised molecularly. Thus, *S. miescheriana* and *S. suihominis* differ markedly in sarcocyst morphology and at the *cox1* gene (difference of about 20%), whereas many closely related *Sarcocystis* spp. in ruminants share the same sarcocyst morphology and differ less at *cox1* (Gjerde 2014a, 2016; Gjerde et al. 2017a, b).

In conclusion, the present study has uncovered a very high prevalence rate of *S. miescheriana* in wild boars in Northern Italy, while *S. suihominis* occurs rarely but should still be considered as a possible cause of food-borne infections in humans. Both species have been thoroughly characterised molecularly, including the first characterisation of the mitochondrial *cox1* gene. Sequencing of *cox1* of *S. miescheriana* from 72 isolates revealed a fairly high sequence diversity at this locus. New primers designed for the amplification of *cox1* of *S. suihominis* might prove useful for identification of oocysts/sporocysts of this species in human stools. For the first time, the phylogenetic placement of *S. miescheriana* and *S. suihominis* was inferred from sequences of *cox1* but was found to be essentially the same as inferred from 18S rRNA gene sequences, that is, the two species were sister taxa within a clade comprising mainly *Sarcocystis* spp. of ruminants with felids as known or suspected definitive hosts.

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Compliance with ethical standards

The wild boars used in the present study were legally hunted as part of an approved depopulation plan for the Val Grande National Park (see the section "Materials and methods" and Gazzonis et al. 2018 for details).

Conflict of interest The authors declare that they have no conflicts of interest.

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