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ORIGINAL PAPER

Sarcocystis entzerothi n. sp. from the European roe deer (*Capreolus capreolus*)

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Abstract In the present study, we describe *Sarcocystis entzerothi* n. sp. from the European roe deer (*Capreolus capreolus*) based on the microscopical and DNA analysis. By light microscopy (LM), cysts of *S. entzerothi* were spindle-shaped with pointed tips, 950–1900 × 70–150 μm in size and had 5–6 μm long finger-like cyst wall protrusions. Cyst wall of *S. entzerothi* by transmission electron microscopy (TEM) was type 10a-like; villar protrusions were up to 1.2 μm wide, densely packed, lying about 0.1 μm between each other, had profuse microgranules and microfilaments, parasitophorous vacuolar membrane had many minute invaginations, and the ground substance layer measured up to 0.4 μm. This species is morphologically similar to *Sarcocystis silva*, previously found in the roe deer and the moose (*Alces alces*). By LM, cysts of *S. silva* were cigar-shaped with blunted tips, measured 1000–1500 × 130–184 μm, and had 7–8 μm long finger-like cyst wall protrusions. Under TEM, *S. silva* had no clear differences from *S. entzerothi* in their cyst wall ultrastructure. Having examined six roe deer hunted in Lithuania, cysts of *S. entzerothi* and *S. silva* were identified in four and two animals, respectively. These two *Sarcocystis* species could be morphologically differentiated according to the shape of the cysts and the length of protrusions. The species examined showed 95.6–96.1 % and 85.6–86.9 % sequence identity within 18S ribosomal DNA (rDNA) and *cox1*, respectively, and therefore they could be clearly distinguished by means of molecular methods. It should be noted that in the 18S rDNA phylogenetic tree, *S. entzerothi* from the roe deer was placed together with one sequence of *Sarcocystis* sp. from the Lithuanian red deer (*Cervus elaphus*) demonstrating the same species. Based on 18S rDNA and *cox1* sequences, *S. entzerothi* was more closely related to *Sarcocystis* species transmitted via felids than canids.

Keywords *Sarcocystis entzerothi* · *Sarcocystis silva* · Roe deer · Transmission electron microscopy · *cox1* · 18S rRNA · Phylogeny

Introduction

Protozoan parasites of the genus *Sarcocystis* (Apicomplexa: Sarcocystidae) have an obligatory two-host prey-predator life cycle. Oocysts/sporocysts develop in a small intestine of a definitive host, while sarcocysts are mainly formed in striated muscles of an intermediate host. Generally, herbivores and omnivores serve as intermediate hosts, while carnivores act as definitive hosts of these parasites (Dubey et al. 2015).

Up till now, the European roe deer (*Capreolus capreolus*) is known to be an intermediate host of four *Sarcocystis* species, *Sarcocystis gracilis*, *Sarcocystis capreolicanis*, *Sarcocystis oviformis*, and *Sarcocystis silva* (Rátz 1909; Erber et al. 1978; Dahlgren and Gjerde 2009; Gjerde 2012). The validity of these species was confirmed by molecular methods using 18S ribosomal DNA (rDNA) and *cox1* (mitochondrial gene encoding subunit I of cytochrome c oxidase) sequences analysis (Dahlgren and Gjerde 2009; Gjerde 2012, 2013; Kolenda et al. 2014). The first three species were observed exclusively in the roe deer, while *S. silva* was identified in the roe deer and the moose (*Alces alces*) (Dahlgren and Gjerde 2008; Gjerde 2012). The morphology of two *Sarcocystis* species from the roe deer, *S. gracilis* and *S. capreolicanis*, which are transmitted via canids (Blažek et al. 1978; Erber et al. 1978), were

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extensively examined by light microscopy (LM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM) (Bergmann and Kinder 1976; Erber et al. 1978; Entzeroth 1982, 1985; Sedlaczek and Wesemeier 1995; Kutkienė 2001; Spickschen and Pohlmeyer 2002; Gjerde 2012). Morphological characteristics of *S. oviformis* cysts have been studied by LM and SEM so far (Dahlgren and Gjerde 2009). The fourth species, *S. silva*, characterized by thick-walled cysts with finger-like protrusions, was described on the basis of the results of LM and DNA analysis (Gjerde 2012). Before the description of *S. silva*, some authors assigned cysts of this particular type found in the roe deer to *Sarcocystis hofmanni* (López et al. 2003), *S.* cf. *hofmanni* (Sedlaczek and Wesemeier 1995; Kutkienė 2001), or *S. hofmanni*-like (Prakas 2011) due to their resemblance to *S. hofmanni* cysts described in the European badger (*Meles meles*) (Odening et al. 1994).

In a previous study, Prakas (2011) molecularly examined 11 cysts with finger-like protrusions, 10 of them were isolated from the Lithuanian roe deer and one was isolated from the Lithuanian red deer (*Cervus elaphus*). Based on the 18S rDNA sequence comparison, cysts belonged to two species, *S. silva* (three identical sequences assigned to JN256132) and *Sarcocystis* sp. (JN256133–JN256137 from the roe deer and JN256125 from the red deer). Despite relatively high (3.8–4.0 %) 18S rDNA sequences variation between *S. silva* and *Sarcocystis* sp. extracted from the roe deer, clear taxonomically important morphological differences of these likely two species were not established by LM. Therefore, the aim of the present study was to determine whether cysts with finger-like protrusions found in the roe deer represented one or more *Sarcocystis* species based on their morphological (LM and TEM) and molecular (18S rDNA and *cox1*) characterization.

Material and methods

Samples

In 2015, diaphragm muscles of six roe deer hunted in Central Lithuania were examined for the *Sarcocystis* cysts. The muscle samples were kept frozen (−20 °C) until microscopic examination of the cysts.

Morphological analysis

The morphological analysis of sarcocysts and bradyzoites was performed in fresh-squashed preparations. By LM, the cysts were differentiated according to the size and shape of sarcocysts, the structure of the cyst wall and bradyzoites after the cysts had been isolated from muscle fibers with the help of two fine needles. Two morphologically different cysts, both with finger-like protrusions, were processed for TEM and examined in the previously described way (Prakas et al. 2016).

Molecular analysis

For molecular analysis, six cysts with finger-like protrusions on their surface were isolated from the muscle tissues, placed in individual 1.5 ml tubes containing 20 μl of 96 % ethanol and kept at −20 °C. Genomic DNA was extracted from individual sarcocysts using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The resulting DNA samples were used for PCR amplification of 18S rDNA and *cox1* sequences. SarAF/SarBR and SarCF/SarDR primer pairs were used for nearly complete 18S rDNA sequence amplification (Kutkienė et al. 2010), while partial *cox1* sequences were amplified with the help of SF1 forward primer in combination with one of the following reverse primers SR5 (Gjerde 2013) or in the present study newly designed SR12H (5′-AAATACCTTGGTGC CCGTAG-3′), depending on *Sarcocystis* species. Each PCR reaction mixture contained 12.5 μl DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), 0.05 μg template DNA, 1 μM of each primer, and nuclease-free water to make the final 25-μl volume. PCR cycling conditions, PCR products evaluation and sequencing were carried out as described previously (Prakas et al. 2016). The resulting sequences were imported into the MEGA7 (Kumar et al. 2016), edited manually if necessary and merged into a single fragment of the analyzed genes.

The 18S rDNA and *cox1* sequences obtained in this study were compared with those of various *Sarcocystis* spp. using the Nucleotide BLAST program megablast option (http://blast.ncbi.nlm.nih.gov/) in order to find remarkably similar DNA sequences and to compute the sequence identity values. Previously published sequences of selected *Sarcocystis* spp. using ruminants as intermediate hosts were retrieved from GenBank for the phylogenetic analyses. The multiple 18S rDNA and *cox1* sequence alignments were obtained using MUSCLE algorithm (Edgar 2004) implemented in the MEGA7 software. TOPALi v2.5 software (Milne et al. 2004) was used to select a nucleotide substitution model with the best fit to the aligned sequence dataset and to construct the phylogenetic trees under the Bayesian inference.

Results

Sarcocystis spp. found in Lithuanian roe deer

Under LM, five *Sarcocystis* species were found in the roe deer examined. Three of them that had previously been described, and morphologically well distinguished species (*S. gracilis*, *S. capreolicanis*, and *S. oviformis*) were not analyzed by

molecular methods. Detailed examinations were carried out with the particular *Sarcocystis* species having cysts with finger-like protrusions. According to the molecular results, these sarcocysts were identified as belonging to one of two distinct species, *Sarcocystis entzerothi* n. sp. or *S. silva*. Cysts of *S. entzerothi* were detected in four of six roe deer examined (isolates CcLttvz1, CcLttvz2, CcLttvz4, and CcLttvz6), whereas cysts of *S. silva* were found in two animals (isolates CcLttva2 and CcLttva3). One roe deer harbored both species (isolates CcLttvz2 and CcLttva2).

Description of *S. entzerothi* n. sp.

By LM, cysts of *S. entzerothi* were microscopic, spindle-shaped with pointed tips, and measured 1138.8 × 107.5 μm (950–1900 × 70–150; $n = 6$) (Fig. 1a). The cyst wall had 5–6 μm long finger-like protrusions (Fig. 1b). The contents of the cysts were divided by septa into large chambers filled with banana-shaped 10.7 × 3.3 μm (9.29–12.69 × 2.6–4.23; $n = 18$) bradyzoites (Fig. 1c). Under TEM, the cyst wall of *S. entzerothi* had thick, up to 4.5 μm long, finger-like villar protrusions. The width of the protrusions varied from 0.9 to 1.2 μm at the bases (Fig. 1d). The ground substance layer was thin, 0.4–0.5 μm, and seemed electron pale. The cyst wall protrusions of *S. entzerothi* had profuse microgranules and microfilaments; the protrusions were tightly packed, lying about 0.1 μm between each other (Fig. 1e). The surface of the protrusions appeared to be wavy, since the parasitophorous vacuolar membrane had many minute invaginations. The cyst wall was type 10a-like (Dubey et al. 2015).

Initially, the amplification of *cox1* fragment of *S. entzerothi* was unsuccessful using previously published reverse primers suitable for *Sarcocystis* spp. from cervids (Gjerde 2013, 2014b); therefore, a new reverse primer SR12H was designed. Thus, *cox1* sequences of *S. entzerothi* were amplified with SF1/SR12H primer pair. 18S rDNA (1821 bp long) and *cox1* (907 bp long) sequences of *S. entzerothi* were deposited in GenBank with accession numbers KX643334–KX643337 and KX643340–KX643343, respectively. Four *S. entzerothi* isolates examined were identical in the *cox1* and composed two 18S rDNA haplotypes differing in two substitutions. Comparing 18S rDNA sequences of *S. entzerothi* with *Sarcocystis* sp. from the Lithuanian red deer (JN256125) and the Lithuanian roe deer (JN256133–7), 99.7–100 % sequence identity indicating the same species was determined. It should be pointed out that *S. entzerothi* clearly genetically differed from *S. silva*, indicating 95.6–96.1 % and 85.6–86.9 % sequence identity in 18S rDNA and *cox1*, respectively. 18S rDNA sequences of *S. entzerothi* shared the highest identity values, similar to those calculated when *S. entzerothi* and *S. silva* were compared, with sequences of *Sarcocystis truncata* (95.7–96.2 %), *Sarcocystis elongata* (95.5–95.9 %), and *Sarcocystis tarandi* (95.5–95.9 %), whereas, *S. entzerothi* had the highest sequence identity within *cox1* to *Sarcocystis bovifelis* (86.6–86.9 %), *S. truncata* (86.1–86.6 %), *Sarcocystis bovini* (86.1–86.4 %), and *S. silva*.

Taxonomic summary of S. entzerothi n. sp.

Type intermediate host The roe deer (*Capreolus capreolus*).
Definitive host Unknown.
Locality Central Lithuania.

Sarcocyst morphology By LM, sarcocysts were spindle-shaped with pointed tips, 950–1900 × 70–150 μm in size, having 5–6 μm long finger-like cyst wall protrusions. Banana-shaped bradyzoites measured 10.7 × 3.3 μm. Under TEM, protrusions were tightly packed, with 0.1 μm distances between them, up to 4.5 μm long, 0.9–1.2 μm wide, filled with microfilaments and microgranules. The parasitophorous vacuolar membrane had many minute invaginations. The ground substance layer measured about 0.4–0.5 μm. The cyst wall was type 10a-like.

Specimens deposited TEM material deposited at the National Centre of Pathology, Vilnius, Lithuania. Sequences deposited in NCBI GenBank with accession numbers KX643334–KX643337 (18S rDNA) and KX643340–KX643343 (*cox1*).

Etymology Species was named after Prof. Rolf Entzeroth who contributed significantly to knowledge of *Sarcocystis* in cervids.

Morphological and molecular characteristics of *S. silva*

By LM, cysts of *S. silva* were cigar-shaped with blunted tips, 1200 × 154.6 μm (1000–1500 × 130–184; $n = 3$) in size (Fig. 2a). Finger-like cyst wall protrusions were 7–8-μm long (Fig. 2b). Bradyzoites were banana-shaped, 11.06 × 3.7 μm (10.08–12.25 × 3.2–4.91; $n = 9$) in size (Fig. 2c). Under TEM, protrusions of *S. silva* were up to 7 μm long, 0.9–1.5 μm wide, tightly packed, lying about 0.1 μm apart, and tapered at the distal ends (Fig. 2d). The cyst wall protrusions had numerous microgranules and microfilaments. The parasitophorous vacuolar membrane had many minute invaginations (Fig. 2e). The ground substance layer was thin, 0.4–0.7 μm. The ground substance and base of the protrusions seemed darker, probably due to a huge number of microgranules. The cyst wall was type 10a-like (Dubey et al. 2015).

Two newly obtained 1804-bp-long 18S rDNA sequences of *S. silva* (KX643338–KX643339) were identical with the previously published sequence of *S. silva* from the Lithuanian roe deer (JN256132), whereas sequence identity of two new 1053-bp-long *cox1* haplotypes of *S. silva* (KX643344–KX643345) accounted for 99.4 %. Comparing sequences of

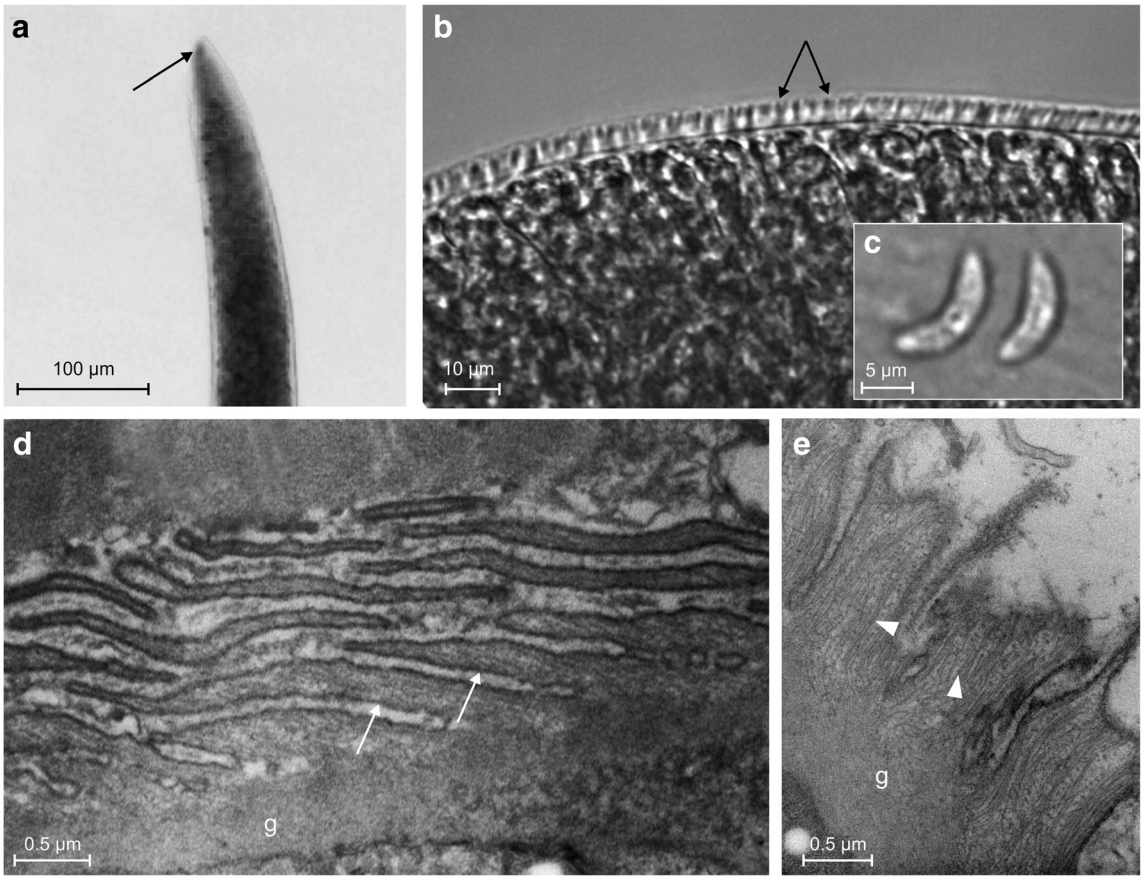

Fig. 1 Morphology of sarcocysts of *Sarcocystis entzerothi* n. sp. from the diaphragm muscles of the roe deer. **a**–**c** Light micrographs. Fresh preparations. **a** Fragment of the cyst. Note pointed tip of the cyst (*arrow*). **b** Image showing finger-like cyst wall protrusions (*arrows*). **c** Banana-shaped bradyzoites. **d**, **e** TEM micrographs. **d** Portion of the cyst wall showing finger-like protrusions (*arrows*). The image indicates atypical appearance of the cyst wall, whereas protrusions were squeezed by host myofibrils and consequently bent sideways. **e** Higher magnification of cyst wall protrusions at the proximal end; note the presence of numerous microfilaments and microgranules (*arrowheads*). Ground substance (*g*)

S. silva from the Lithuanian roe deer with other available *S. silva* sequences from the Norwegian and Polish roe deer and the Norwegian moose, 99.7–100 % and 98.4–99.5 % sequence identity for 18S rDNA and *cox1* was determined.

Phylogeny

Phylogenetic relationships of some *Sarcocystis* species, *S. truncata*, *Sarcocystis rangiferi*, *S. tarandi*, *S. elongata*, *S. bovifelis*, *S. bovini*, *Sarcocystis sinensis*, *Sarcocystis buffalonis*, and *Sarcocystis hirsuta* were not fully defined on the basis of 18S rDNA sequences (Fig. 3). In contrast, all *Sarcocystis* species analyzed formed monophyletic groups in the phylogram of *cox1* sequences (Fig. 4). In the 18S rDNA phylogenetic tree, *S. entzerothi* sequences interleaved with sequences, accession nos. JN256125, JN256133–7, and together formed one clade. Thus, the 18S rDNA phylogenetic analysis was in congruence with the molecular data demonstrating the isolates of *S. entzerothi* and *Sarcocystis* sp. from the roe deer and the red deer as the representatives of the same species. Based on 18S rDNA and *cox1* sequences, *S. entzerothi* was most closely related to *Sarcocystis* spp. using the representatives of the families Bovidae and Cervidae as an intermediate host. In the phylogenetic analyses, *S. entzerothi* did not group with *Sarcocystis* species employing canids as definitive hosts. Based on the 18S rDNA sequence analysis, *S. entzerothi* was placed with *S. tarandi*, *S. elongata*, *S. silva*, *S. truncata*, and *S. rangiferi*. However, *S. entzerothi* phylogenetic placement in the 18S rDNA sequences analysis is equivocal due to a poor posterior probability support value (58), whereas *S. entzerothi* was a sister taxon to *S. sinensis*, *S. bovini*, and *S. bovifelis* in the *cox1* phylogenetic tree. Definitive hosts of *S. truncata*, *S. rangiferi*, *S. silva*, *S. tarandi*, *S. elongata*, and *S. bovini* are unknown, while *S. sinensis* and *S. bovifelis* are transmittable via felids (Dahlgren et al. 2008; Gjerde 2012, 2014b, 2016a, 2016b; Gjerde and Hilali 2016). Therefore, *S. entzerothi* was more closely related to species transmitted by felids than canids.

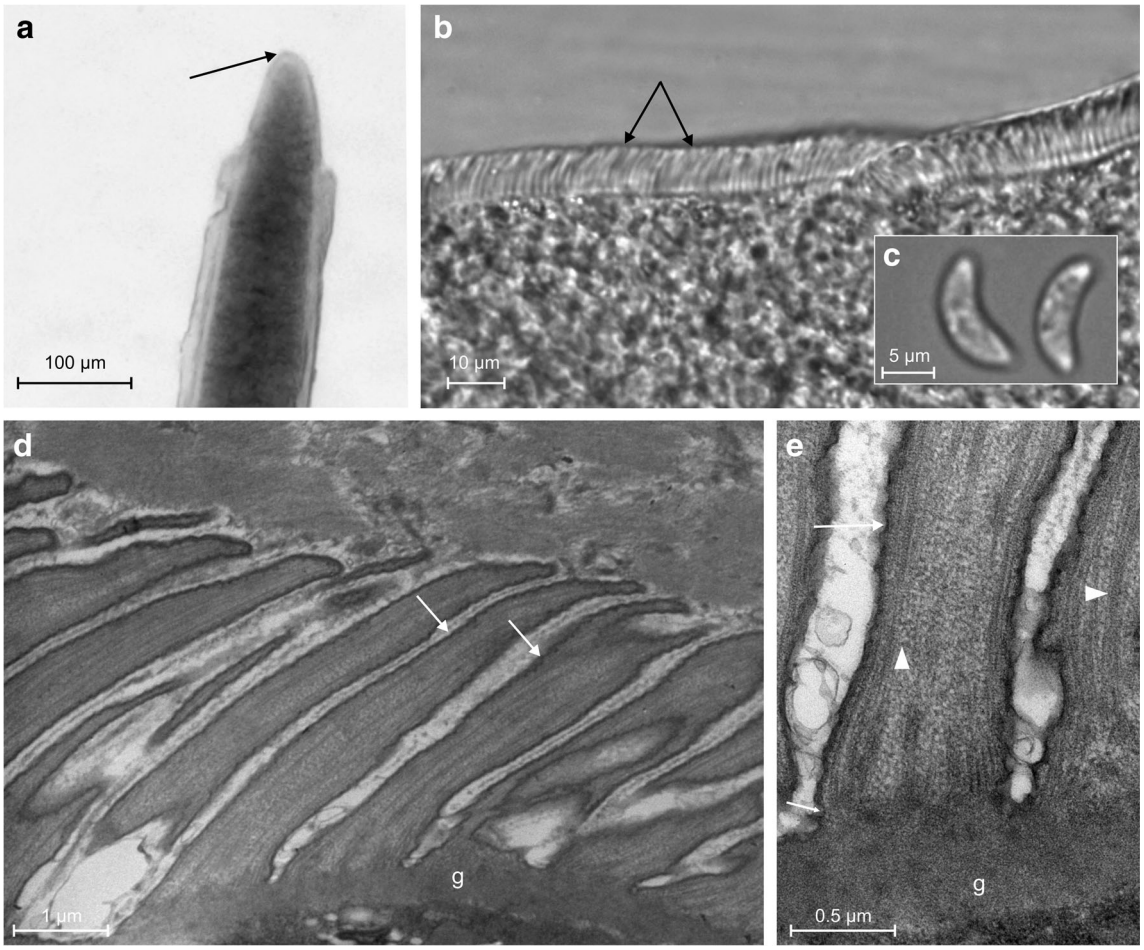

Fig. 2 Morphology of sarcocysts of *Sarcocystis silva* from the diaphragm muscles of the roe deer. **a**–**c** Light micrographs. Fresh preparations. **a** Fragment of the cyst. Note blunted tip of the cyst (*arrow*). **b** Image showing finger-like cyst wall protrusions (*arrows*). **c** Banana-shaped bradyzoites. **d**, **e** TEM micrographs. **d** Fragment of the cyst wall. *Arrows* pointed at the finger-like protrusions. **e** Higher magnification of the base of cyst wall protrusions; note invaginations of the parasitophorous vacuolar membrane (*arrows*), microfilaments, and microgranules (*arrowheads*). Ground substance (*g*)

Discussion

Numerous *Sarcocystis* species have been described in cervids (Gjerde 2014a, 2014b; Calero-Bernal et al. 2015; Prakas et al. 2016). Morphological criteria are mostly insufficient to differentiate *Sarcocystis* species found in cervids, since morphologically similar *Sarcocystis* spp. cysts are found in the same or closely related hosts (Dubey 1980; Colwell and Mahrt 1981; Speer and Dubey 1982; Dubey and Lozier 1983; Dubey et al. 1983; Dubey and Speer 1985, 1986; Gjerde 1986; Atkinson et al. 1993; Sedlaczek and Wesemeier 1995; Wesemeier and Sedlaczek 1995a, 1995b). DNA analysis has been shown to be a powerful tool for resolving taxonomic uncertainties of *Sarcocystis* spp. infecting the representatives of the family Cervidae (Dahlgren and Gjerde 2008, 2009, 2010; Gjerde 2012, 2014a, 2014b; Calero-Bernal et al. 2015; Prakas et al. 2016; Reissig et al. 2016). Therefore, it is necessary to combine morphological and molecular data in order to identify cryptic *Sarcocystis* species or avoid species oversplitting. Historically, 18S rDNA was the first molecular locus for the genetic characterization of *Sarcocystis* species with ruminants as intermediate hosts (Tenter et al. 1992; Jeffries et al. 1997; Holmdahl et al. 1999; Morrison et al. 2004; Dahlgren and Gjerde 2007). However, 18S rDNA is an inappropriate choice discriminating closely related *Sarcocystis* species employing cervids as intermediate hosts. Specifically, *S. tarandi* and *S. elongata* were not clearly separated by 18S rDNA as *S. rangiferi* from *S. truncata* and *Sarcocystis hjorti* from *Sarcocystis pilosa*, and these species were distinguished on the basis of the *cox1* sequence analysis (Gjerde 2014b; Prakas et al. 2016).

In the present study, we described *S. entzerothi* n. sp. based on LM, TEM, 18S rDNA, and *cox1* analysis. The newly characterized species is morphologically similar to *S. silva*, previously found in the roe deer (Gjerde 2012) and the moose (Dahlgren and Gjerde 2008). Gjerde (2012) described *S. silva* cysts in the roe deer as having tightly packed, upright, finger-like protrusions about 8 μm long and 1.5 μm wide. Having

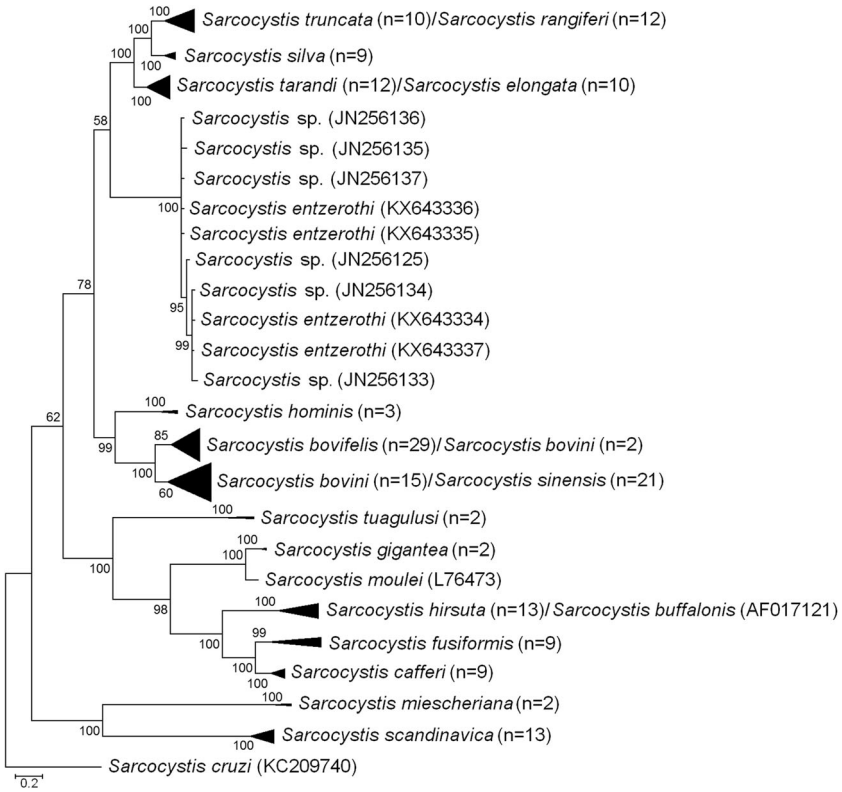

Fig. 3 The phylogenetic tree of selected *Sarcocystis* species based on 18S rDNA sequences. The tree was constructed using the Bayesian methods, scaled according to the branch length, and rooted on *S. cruzi*. The final alignment contained 186 sequences and 1777 aligned nucleotide positions. The HKY + G evolutionary model was set for the phylogenetic analysis. The figures next to branches show the posterior probability support values. GenBank accession numbers or the number of sequences for the corresponding taxon is given behind the *Sarcocystis* species name

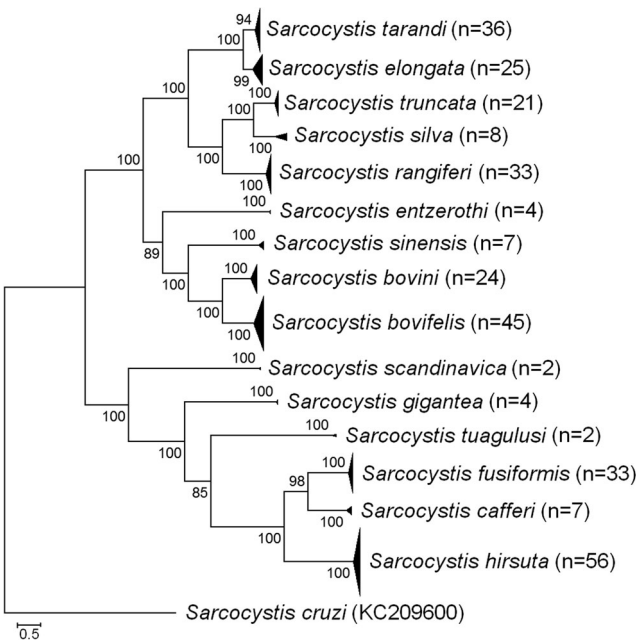

Fig. 4 The phylogenetic tree for selected *Sarcocystis* species based on *cox1* sequences. The tree was constructed using the Bayesian methods, scaled according to the branch length, and rooted on *S. cruzi*. The final alignment contained 308 sequences and 766 aligned nucleotide positions. The K80 + G evolutionary model was set for the phylogenetic analysis. GenBank accession numbers or number of sequences for the corresponding taxon is given behind the *Sarcocystis* species name

examined six roe deer hunted in Lithuania, we identified *S. entzerothi* cysts in four of them and *S. silva* in two animals. Cysts of *S. silva* were morphologically consistent with those of *S. silva* from the Norwegian roe deer (Gjerde 2012). In the present work, two species examined shared 95.6–96.1 % and 85.6–86.9 % sequence identity within 18S rDNA and *cox1*, respectively. Therefore, *S. entzerothi* could be clearly differentiated from *S. silva* using DNA analysis. Sarcocysts of both species discussed were similar in size, having densely packed finger-like protrusions on their surface (Figs. 1 and 2). However, *S. entzerothi* and *S. silva* were distinguishable by the shape of the cysts and by the length of villar protrusions. Cysts of *S. entzerothi* were spindle-shaped with pointed tips, whereas cysts of *S. silva* were cigar-shaped with blunt ends. It should be pointed out that the tips of the cysts of *S. entzerothi* sometimes seemed rounded in muscle fibers. Cyst wall protrusions of *S. entzerothi* were 5–6 μm long, while those of *S. silva* measured 7–8 μm. Nevertheless, the lengths of the protrusions may depend on the cyst state during fixation and on the age of the cyst. In conclusion, morphological separation between *S. entzerothi* and *S. silva* sometimes might be difficult; therefore DNA analysis is necessary for a definitive discrimination of these two species.

Cysts having finger-like cyst wall protrusions were reported in the roe deer in several publications before the descriptions of *S. silva* and *S. entzerothi*. The cysts of this type were morphologically characterized by LM in histological sections or fresh preparations and using TEM. Bergmann

and Kinder (1976) were the first to notice the thick-walled cysts with finger-like protrusions in the roe deer using LM and TEM analyses. Subsequently, Schramlová and Blažek (1978) studied the thick-walled cyst under TEM and characterized them as 4.49–7.49 μm in thickness, forming palisade-like protrusions. Erber et al. (1978) examined fresh muscle preparations of the roe deer under LM and distinguished three cyst types. Type 3 cysts had rigid 5–6 μm long and about 0.5-μm-thick finger-like protrusions. Afterwards, Entzeroth (1982) examined most exhaustively the wall ultrastructure of the cysts found in the roe deer and described six cyst wall types. The first three types were distinguished by finger-like protrusions varying in length and width. The author assumed that cyst walls of types 1–3 might belong to the same species at different stages of growth. Sedlaczek and Wesemeier (1995) found cysts with finger-like protrusions in the esophagus, heart, diaphragm, and skeletal musculature (thigh, loin, thorax, ribs) of 42 roe deer out of the 66 examined. Under LM, cysts had 7.8–8.6 μm long and 1.5–1.6 μm wide finger-like protrusions, whereas under TEM, the protrusions were 6.3–7.2 μm long and 1.2–1.7 μm wide. Kutkienė (2001) found cysts with tightly packed finger-like protrusions measuring up to 8.3 μm in length. The cysts showing a similar villar protrusions were also detected by Spickschen and Pohlmeyer (2002) and López et al. (2003). According to the contradictory morphological descriptions of the cysts with finger-like protrusions in the muscles of the roe deer, we could not state accurately, whether *S. entzerothi*, *S. silva*, or both of them were found in the above-mentioned. studies as no DNA analysis has been performed in those studies.

Earlier, one cyst with finger-like protrusions isolated from the Lithuanian red deer was characterized in 18S rDNA and the obtained sequence was deposited in GenBank with accession number JN256125 (Prakas 2011). Based on the results of the present study, this sequence belongs to *S. entzerothi*. Therefore, the case might be that *S. entzerothi* is not strictly host-specific and could employ both roe deer and red deer as intermediate hosts. However, molecularly based identification of *S. entzerothi* in one red deer is insufficient evidence suggesting that this *Sarcocystis* species uses red deer as another intermediate host. Furthermore, *S. entzerothi* cysts were not identified in numerous red deer examined in comprehensive studies in Norway (Dahlgren and Gjerde 2010; Gjerde 2014b). Therefore, it is necessary to carry out thorough investigations of red deer for the presence of *S. entzerothi*.

Based on 18S rDNA sequences, phylogenetic placement of *S. entzerothi* was unreliable (Fig. 3). By contrast, grouping of *S. entzerothi* together with *S. sinensis*, *S. bovini*, and *S. bovifelis* in the *cox1* phylogenetic tree was well-supported (Fig. 4). Two of these species, *S. bovifelis* and *S. sinensis*, use representatives of the family Bovidae as intermediate hosts and felids as definitive hosts (Gjerde 2016b; Gjerde and Hilali 2016). Thus, phylogenetic results suggest that definitive hosts of *S. entzerothi* might be felids. However, in previous experiments, domestic cats, wildcats (*Felis silvestris*), jungle cats (*Felis chaus*), bobcats (*Lynx rufus*), and the tiger (*Panthera tigris*) did not shed *Sarcocystis* sporocysts after ingesting roe deer meat infected with *Sarcocystis* spp. cysts (Entzeroth et al. 1978; Erber et al. 1978; Entzeroth 1981). Consequently, either felids do not act as intermediate hosts of *S. entzerothi* or the muscles of the roe deer used in these experiments did not contain *S. entzerothi* cysts.

In the present study, five *Sarcocystis* species, *S. entzerothi*, *S. silva*, *S. gracilis*, *S. capreolicanis*, and *S. oviformis*, in the diaphragm of the roe deer were found. According to the previous morphological investigations, the sixth *Sarcocystis* species might exist in this host. As observed under LM, the cysts of *Sarcocystis* sp. appeared to have a thin smooth wall (Kutkienė 2001), whereas the TEM analysis showed cysts having a highly folded primary cyst wall that formed hair-like protrusions running in parallel with the cyst surface (Schramlová and Blažek 1978; Entzeroth 1982; Santini et al. 1997). The cyst wall structure similar to that of *Sarcocystis* sp. from the roe deer was established for *Sarcocystis cervicanis* (Hernández-Rodríguez et al. 1981) and *Sarcocystis* sp. (Entzeroth et al. 1983) from the red deer, *Sarcocystis wapiti* from the wapiti (*Cervus canadensis*) (Speer and Dubey 1982), *Sarcocystis grueneri* from the reindeer (*Rangifer tarandus*) (Gjerde 1985), *Sarcocystis* sp. from the moose (Colwell and Mahrt 1981), and *Sarcocystis* sp. from the fallow deer (*Dama dama*) (Entzeroth et al. 1985; Poli et al. 1988). Comparative molecular studies are needed to determine the actual number of *Sarcocystis* species representing a cyst of this type found in different cervids.

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