Parasitology Research (2020) 119:1363–1370
https://doi.org/10.1007/s00436-020-06603-9

PROTOZOOLOGY - ORIGINAL PAPER

Sarcocystis spp. diversity in the roe deer (*Capreolus capreolus*) from Lithuania and Spain

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Received: 9 September 2019 / Accepted: 6 January 2020 / Published online: 11 January 2020

Abstract

The roe deer (*Capreolus capreolus*) has been identified as an intermediate host for six known *Sarcocystis* species, *S. capreolicanis*, *S. entzerothi*, *S. gracilis*, *S. linearis*, *S. oviformis*, and *S. silva*. In this study, we identified *Sarcocystis* species in the diaphragm and tongue muscles from the Lithuanian and Spanish roe deer, respectively, on the basis of a microscopic examination and DNA analysis. A total of 43 and 27 sarcocysts were isolated and characterized from the Lithuanian and Spanish roe deer, respectively. Overall six *Sarcocystis* species were identified in roe deer from Lithuania, and only three of them, *S. gracilis*, *S. linearis*, and *S. silva* were found to have infecting animals from Spain. The current paper represents first molecular results of *Sarcocystis* species in the Spanish roe deer. Furthermore, transmission electron microscopy examination revealed specific wall structure of sarcocysts studied, *S. linearis* was characterized by ribbon-like villar protrusions (vp) (type 8a), and *S. oviformis* was distinguished by elongated vp resembling spades or mushroom-like structures (type 39). Based on 18S rDNA and *cox1* sequences, *Sarcocystis* species from the roe deer showed considerable intraspecific genetic variability. However, similar values of intraspecific genetic variation were estimated at both genes analysed. The highest variability was observed for *S. capreolicanis* and *S. linearis* in both genes and for *S. silva* at *cox1*. Consequently, the level of genetic variability of *Sarcocystis* from the roe deer varied depending on species rather than on gene analysed or geographical area.

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

Introduction

Parasites of the genus *Sarcocystis* are characterized by an obligatory two-host life cycle (Dubey et al. 2016). Members of the family Cervidae may act as intermediate hosts for numerous *Sarcocystis* species. Overall, six *Sarcocystis* species infecting the European roe deer (*Capreolus capreolus*), *Sarcocystis capreolicanis*, *Sarcocystis entzerothi*, *Sarcocystis gracilis*, *Sarcocystis linearis*, *Sarcocystis oviformis*, and *Sarcocystis silva*, were adequately described by means of microscopical and molecular methods (Dahlgren and Gjerde 2009; Gjerde 2012; Gjerde et al. 2017; Prakas et al. 2017).

Pioneer European *Sarcocystis* infection surveys were carried out based on morphological methods (Erber et al. 1978; Schramlová and Blazek 1978; Entzeroth 1982; Santini et al. 1997; Kutkienė 2001; Spickschen and Pohlmeyer 2002; López et al. 2003); both scanning and transmission electron microscopy (SEM/TEM) analyses were conducted for sarcocysts of *S. capreolicanis*, *S. gracilis* and *S. silva*; however, only SEM data are available for *S. linearis* and *S. oviformis* and TEM for *S. entzerothi* (Dahlgren and Gjerde 2009; Gjerde et al. 2017; Prakas et al. 2017). Hence, further studies using electron microscopy would contribute to a better characterization of *Sarcocystis* species in the roe deer. Over the past decade, the necessity to use DNA markers to discriminate

Section Editor: Daniel K. Howe

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00436-020-06603-9) contains supplementary material, which is available to authorized users.

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Sarcocystis parasites infecting different cervids, including the roe deer, arose (Dahlgren and Gjerde 2009; Calero-Bernal et al. 2015; Prakas et al. 2016). The 18S rDNA and *cox1* markers have been shown to be suitable for differentiation between closely related *Sarcocystis* species using ruminants as intermediate hosts (Gjerde 2013, 2016). Based on these markers, the composition for presence of *Sarcocystis* species in roe deer has been revealed in several European countries, such as Italy, Lithuania, Poland, and Norway (Kolenda et al. 2014; Dubey et al. 2016; Prakas et al. 2017; Gjerde et al. 2017). Thus, more detailed researches are needed to elucidate species richness and genetic variability in distant areas and distinct ecosystems.

Previous studies on parasites of roe deer in Spain revealed a very high prevalence (99%) of *Sarcocystis* spp. infection (Pérez-Creo et al. 2013); however, no molecular analyses were performed. In the present study, we identified the *Sarcocystis* species in roe deer samples from Lithuania and Spain, ultrastructurally characterized sarcocysts of *S. linearis* and *S. oviformis,* and revealed differences in intraspecific genetic variability of *Sarcocystis* species found in distant countries.

Material and methods

Samples and light microscope (LM) examination

Diaphragm muscles of 40 roe deer from Lithuania and 41 tongues of roe deer from 10 provinces in Spain were collected between 2015 and 2018. About 50–100 g of diaphragms and 100–150 g of tongues were collected from animals examined. All studied animals had been legally hunted for trophy and meat consumption purposes, and samples, harvested the same day of hunt, were kept frozen until shipment to the corresponding laboratories. The morphological analysis of sarcocysts was performed in thawed-squashed preparations. Under LM, sarcocysts were isolated from muscle fibres and screened according to the size and shape of sarcocysts and the structure of the cyst wall (Kirillova et al. 2018). Isolated sarcocysts were separately preserved in ethanol for further DNA extraction and molecular analysis. *Sarcocystis* infection intensity from Lithuanian animals was evaluated in stained muscle samples by counting sarcocysts per gram of tissue (Prakas et al. 2019).

Histological examination

Histological examination was performed with Spanish specimens only. Muscle samples were cut into 1 cm^3 pieces, fixed in 10% buffered formalin, embedded in paraffin, and sectioned 5 μm thick; sections were stained with haematoxylin and eosin (H-E) and observed under the microscope. Intensity of infection was estimated as the number of tissue cysts per section (2.5 cm^2).

Transmission electron microscopy (TEM) analysis

Two excised sarcocysts, which by means of LM were similar to *S. linearis* and *S. oviformis*, were fixed in 2.5% glutaraldehyde and post-fixed in 1% buffered osmium tetroxide; thereafter sections were cut on Leica UC6 ultramicrotome and contrasted with 4% uranyl acetate and 3% lead citrate, as suggested by Trupkiewicz et al. (2016). Sections were stained with toluidine blue and examined under LM for the presence of cysts. Finally, ultra-thin sections were examined at the Spanish National Centre for Electron Microscopy (Madrid, Spain) using the JEOL JEM 1400 Plus device at 80 kW.

Molecular analysis

Genomic DNA was extracted from 70 individual sarcocysts (43 sarcocysts from 28 individual Lithuanian roe deer and 27 sarcocysts from 19 Spanish roe deer) using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Partial 18S rDNA sequences were amplified with SarAF/SarBR and SarCF/SarDR primer pairs (Kutkienė et al. 2010), while mitochondrial cytochrome c oxidase subunit I gene (*cox1*) sequences were amplified with SF1 forward primer in combination with one of the following reverse primers, SR8D/SR9/SR5/SR12H depending on *Sarcocystis* species (Gjerde 2013; Gjerde 2014; Prakas et al. 2017). PCR reactions were performed using DreamTaq PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The cycling conditions started for 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 60 s at 54–60 °C depending on the primer pair, and 80 s at 72 °C and ended with 10 min at 72 °C. PCR products were evaluated using 1.5% agarose gel electrophoresis and purified with exonuclease ExoI and alkaline phosphatase FastAP (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). Most of the samples ($n = 170$) were sequenced directly with the 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the same forward and reverse primers as for PCR, except for 18S rDNA fragments of *S. capreolicanis* and *S. linearis* that resulted in double peaks and poly signals within 18S rDNA. Thus, to obtain unambiguous sequences, the amplicons of *S. capreolicanis* and *S. linearis* ($n = 36$) were cloned into plasmids with the help of CloneJET PCR Cloning Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). Cloning reactions were chilled on ice for 1 min and transformed into 100 μl of chemocompetent *E. coli* DH10B cells. Clones were selected on LB agar plates supplemented with 50 μg/ml ampicillin. The recombinant clones were screened to obtain the insert of a desired size verified by colony PCR amplification. Individual colony was suspended in liquid LB media and used for colony PCR. Each PCR was performed in a final 50 μl volume consisting of 0.5 μM of vector primers pJET1.2 forward and reverse, dNTP mix (0.2 mM of each), 10 × DreamTaq buffer with

20 mM $MgCl_2$, 1.25 U DreamTaq polymerase (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), and 1 μl of the chosen clone colony suspended in liquid media and nuclease-free water. PCR fragments were analysed by agarose gel electrophoresis, and the remaining portion of the positive colony was used to inoculate liquid LB media with ampicillin for plasmid miniprep extraction. DNA plasmids were extracted from *E. coli* DH10B cells using GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) according to the manufacturer's instructions and afterwards sequenced using pJET1.2 forward and reverse vector primers. The number of segregating sites (S), the number of haplotypes (h) and haplotype diversity (Hd), and the average number of nucleotide differences (K) and nucleotide diversity (π) were calculated for the whole dataset and separate populations using DnaSP v6 (Rozas et al. 2017). The sequences used for the analysis of intraspecific genetic variation are given in Table S1 of the Supplementary material.

Results

Sarcocysts were detected in 95% (38/40) of diaphragm muscles and 100% (41/41) of tongues from Lithuanian and Spanish roe deer, respectively. Three types of microscopic sarcocysts having hair-like, finger-like, or no visible villar protrusions (vp) and one type of macroscopic sarcocyst were detected (Table 1). Based on molecular results, sarcocysts with no visible vp under LM belonged to *S. gracilis* or *S. linearis*. The sarcocysts of these species slightly differed in size as cysts of *S. linearis* were smaller to those of *S. gracilis*; however, the dimensions overlapped (Table 1). Molecular results confirmed that sarcocysts having hair-like vp belonged to *S. capreolicanis*, while sarcocysts with finger-like vp corresponded to *S. entzerothi* or *S. silva*, whose morphological differentiations were described in more detail by Prakas et al. (2017). Macroscopic, oval-shaped sarcocysts were identified as *S. oviformis*.

In Spanish animals, 100% of H-E-stained tissue sections were positive; there was an absence of myositis foci, and the average parasite load was estimated in 42.7 cysts/section (17.1 cysts/cm^2); parasite load records were established in four categories: 1–10 cysts (19.5% of animals), 11–50 cysts (48.8%), 51–100 cysts (22.0%), and more than 100 cysts (9.7%). The maximum load (194 cysts/section) was observed in the roe deer Cc25 collected in Burgos province. No secondary cyst wall was discovered in 1751 cysts examined, and two morphological types were observed: type-A, thin-walled (< 1 μm in thickness) with vp apparently absent, and type-B, thick-walled (> 5 μm) and finger-like 6–8 μm long vp; distribution of observed morphological types was 65.9% of type A and 2.4% of type B and 31.7% of mixed infections (Fig. 1). Mature bradyzoites but no metrocytes were observed in septated sarcocysts. In the infected Lithuanian animals, the average parasite load of 197.0 cysts/g (median 86.5 cysts/g) of muscle was observed in methylene blue-dyed samples. Parasite load records were divided into the same above-described categories to evaluate the intensity of infection: 1–10 cysts (7.9%), 11–50 cysts (26.3%), 51–100 cysts (21.1%), and more than 100 cysts (44.7%).

Two sarcocysts were processed for TEM analysis (Table 2); a thin-walled (0.5 μm wide) cyst was characterized by sectioned ribbon-like vp with tapered ends resembling tau crosses and corresponded to *S. linearis* (type 8a by Dubey et al. (2016)). Thick-walled (6.5 μm wide) cyst had elongated vp, which mostly appeared sectioned with condensation of electrondense material at the base and 1.2 μm long peduncle, resembling spades or mushroom-like structures and corresponded to *S. oviformis* (type 39 by Dubey et al. (2016)).

In the present study, the obtained sequences of *Sarcocystis* species from the roe deer are deposited in GenBank with accession numbers MN334245–MN334330 and MN339281–MN339350. Molecular analysis led to the identification of six *Sarcocystis* species in Lithuanian roe deer, *S. capreolicanis* (n = 8), *S. entzerothi* (n = 6), *S. gracilis* (n = 9), *S. linearis* (n = 5), *S. oviformis* (n = 5), and *S. silva* (n = 10) (Table 3). Three *Sarcocystis* species, *S. gracilis* (n = 18), *S. linearis* (n = 7), and *S. silva* (n = 2) were confirmed in roe deer from Spain. All isolates of the same *Sarcocystis* species were obtained from different animals. Intra-isolate sequence differences at 18S rDNA region were analysed for *S. capreolicanis* and *S. linearis* from Lithuania and for *S. linearis* from Spain. A total of 12 clones from three isolates of *S. capreolicanis* and 24 clones from six isolates of *S. linearis* were processed and sequenced.

Table 1 Morphological characteristics of *Sarcocystis* sarcocysts described by squeezing of thawed tissues and light microscopy examination in roe deer from Lithuania and Spain

Species	Number of cysts measured	Size (μm) and shape	Protrusions (vp) of the sarcocyst wall
S. capreolicanis	4	700–1645 × 30–60, ribbon-shaped	Hair-like, 6–9 μm long
S. entzerothi	5	950–1500 × 70–150, spindle-shaped	Finger-like, 5–6 μm long
S. gracilis	15	350–1800 × 60–170, spindle-shaped	No visible vp
S. linearis	7	300–850 × 45–90, spindle-shaped	No visible vp
S. oviformis	2	1100–2100 × 470–780, oval-shaped	Cyst surrounded by fibrous layer
S. silva	3	1000–1730 × 70–175, cigar-shaped	Finger-like, 7–8 μm long

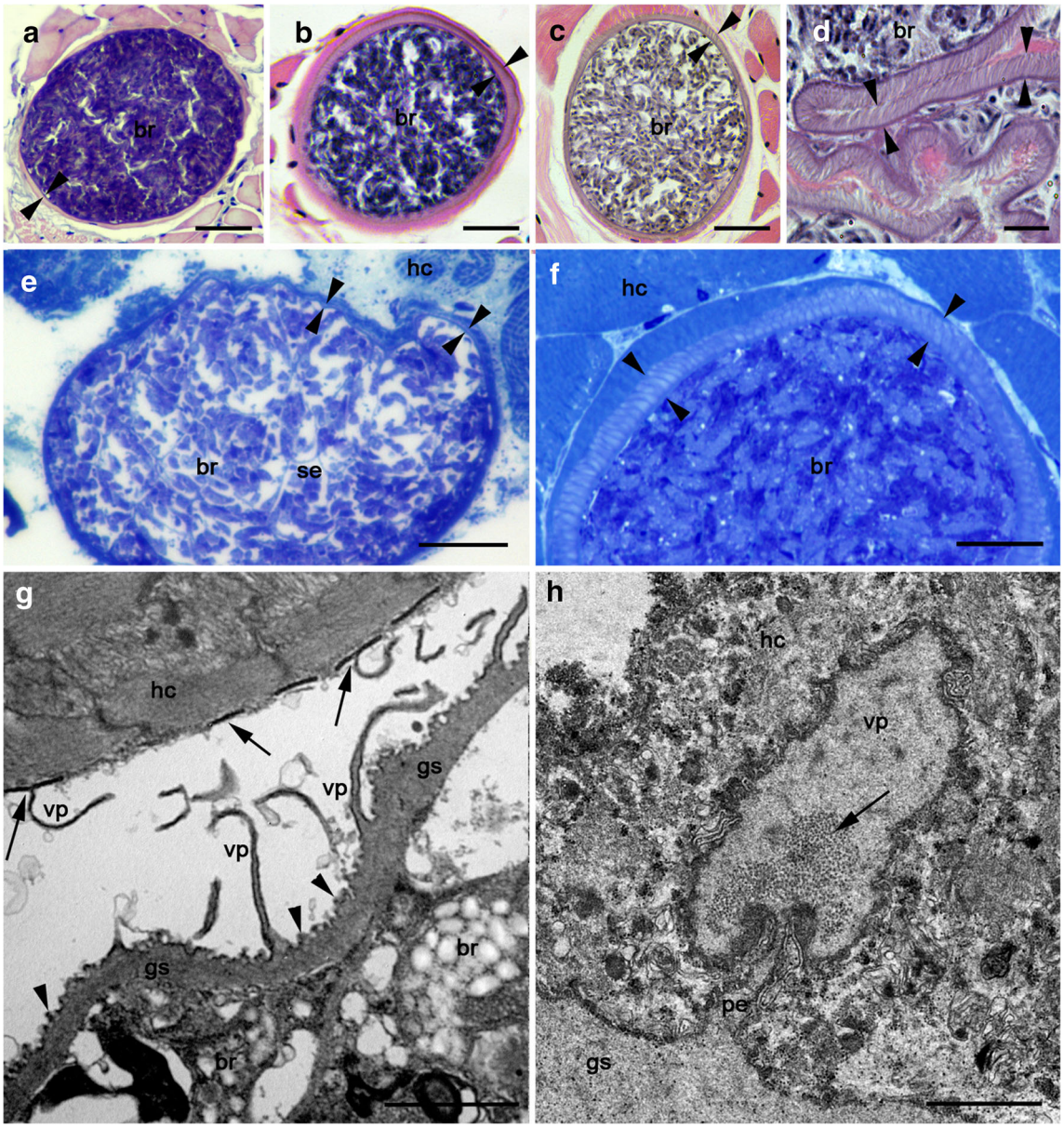

Fig. 1 Micrographs of *Sarcocystis* sarcocysts from the roe deer. (**a-d**) H-E-stained sections. Arrowheads point to cyst wall. Note, bradyzoites (br). (**e, f**) Toluidine blue-stained sections. Arrowheads point to cyst wall. Note, br, host cells (hc), and septae (se). (**g, h**) TEM micrographs. Note, br, hc, ground substance (gs), peduncle (pe), and villar protrusions (vp). Bars: **a-c** = 25 μm; **d** = 10 μm; **e**, **f** = 25 μm; **g**, **h** = 0.5 μm. (**a**) Thin-walled sarcocyst type A resembling *S. linearis* or *S. gracilis*. (**b-d**) Thick-walled sarcocysts, with finger-like vp resembling *S. silva*. (**e**) Evident thin-walled sarcocyst, identified later as *S. linearis*. (**f**) Evident thick-walled sarcocyst, identified later as *S. oviformis*. (**g**) Detailed view of the thin-walled *S. linearis*. Note, blebs (arrowheads) between vp and tapered ends of vp (arrows). (**h**) Details of the vp of *S. oviformis*. Note, pe and electrondense granules (arrow)

Sequence differences between the clones from the same isolate of *S. capreolicanis* and *S. linearis* from Lithuania and *S. linearis* from Spain were 0.43–1.45%, 0.49–1.52%, and 0.44–1.68%, respectively. Similarly, the range of genetic variation between clones from different isolates of the analysed species was broader, but with the overlapping values. Sequence differences of *S. capreolicanis* and *S. linearis* from Lithuania and Spain accounted for 0.22–2.20%, 0–1.63%, and 0.27–1.74%, respectively. In general, having evaluated intraspecific genetic variability in Lithuania and Spain, the highest DNA variability for *S. capreolicanis* was estimated at 18S rDNA, for *S. silva* at *cox1* and for *S. linearis* at both genes analysed (Table 4). A comparison of intraspecific genetic variations amongst *S. gracilis* and *S. linearis* species from both countries demonstrated a slightly higher variability in Lithuania.

Overall comparisons of intraspecific genetic variability at studied loci of *Sarcocystis* species from roe deer sampled in Lithuania and Spain, as well as Italy, Norway, and Poland, were

Table 2 Summarized information regarding TEM description of sarcocysts of *S. linearis* and *S. oviformis* infecting roe deer in Lithuania and Spain

Structure	Species	
	S. linearis	*S. oviformis*
Metrocytes	Not seen	Not seen
Bradyzoites	Not well-fixed, not described	Not well-fixed, not described
Primary cyst wall type[a]	8a	39
Total thickness	Thin, less than 500 nm	Thick, up to 6.5 μm
Ground substance	Up to 300 nm wide, clearly electrondense, especially in the areas close to the bradyzoites	Up to 2.5 μm wide, with visible electrondense mt
Septae (thickness)	Present, 100 nm	Present, 1.5 μm
Villar protrusions	Ribbon-like up to 1.5 μm long, with tapered ends, giving the aspect of tau crosses, 50 nm wide blebs interspersed the dome-shaped bases of vp at irregular distances	In sections vp resemble spades, with condensation of electrondense material at the base of vp and 1.2 μm long pe, giving mushroom-like appearance

[a] Dubey et al. (2016)

vp villar protrusions; *pe* peduncle; *mt* microtubules

made. The highest intraspecific genetic variability was detected in *S. capreolicanis* and *S. linearis* in both genes and in *S. silva* at *cox1*, while some less variability was revealed in *S. gracilis* at *cox1* and *S. silva* at 18S rDNA, whereas very low or none genetic variability was detected for *S. entzerothi* and *S. oviformis* at both loci examined and for *S. gracilis* at 18S rDNA. In general, similar genetic variability was observed within same *Sarcocystis* species from different countries. No relation between the level of intraspecific genetic variability and the sampling size was noticed.

Discussion

In the present study, very high infection prevalence was established in roe deer in Lithuania (95%) and Spain (100%). On the basis of morphological and molecular results, six *Sarcocystis* species, *S. capreolicanis*, *S. entzerothi*, *S. gracilis*, *S. linearis*, *S. oviformis,* and *S. silva*, were identified in Lithuanian roe deer, while only three species (*S. gracilis*, *S. linearis*, and *S. silva*) were found in the animals from Spain (Tables 1 and 3). *Sarcocystis gracilis* and *S. silva* have been molecularly identified in the roe deer from Italy ($n = 4$ animals), Poland ($n = 4$), Norway ($n = 9$), Spain ($n = 41$), and Lithuania ($n = 46$) (Dahlgren and Gjerde 2009; Gjerde 2012; Kolenda et al. 2014; Gjerde et al. 2017; Prakas et al. 2017; this study). These two are probably the most common *Sarcocystis* species employing the roe deer as an intermediate host. By means of molecular methods, sarcocysts of *S. capreolicanis* were confirmed in Italy, Lithuania, and Norway; however, they were not detected in Poland and Spain. Most likely this species was not detected in Poland due to a small sample size. Pérez-Creo et al. (2013) examined

Table 3 *Sarcocystis* spp. isolates from Lithuanian (LT) and Spanish (ES) roe deer and the GenBank accession numbers of the resulting sequences

Species	Country	Isolates	18S rDNA acc. no.	*cox1* acc. no.
S. capreolicanis	Lithuania	CcLT2.2, CcLT3.2, CcLT5.2, CcLT6.2, CcLT9.2, CcLT16.2, CcLT26.2, CcLT30.2	MN334245–MN334256	MN339281–MN339288
S. entzerothi	Lithuania	CcLT7.3, CcLT8.3, CcLT14.3a, CcLT16.3a, CcLT21.3, CcLT24.3a	MN334257–MN334262	MN339289–MN339294
S. gracilis	Lithuania	CcLT1.1, CcLT6.1, CcLT7.1, CcLT13.1, CcLT18.1, CcLT26.1, CcLT29.1, CcLT38.1, CcLT40.1	MN334263–MN334271	MN339295–MN339303
S. gracilis	Spain	CcES2.1, CcES2.3, CcES2.4, CcES2.5, CcES2.6, CcES2.7, CcES22, CcES24, CcES25, CcES26, CcES27, CcES28, CcES29, CcESB3, CcESB6, CcESB7, CcESB9, CcESB10	MN334272–MN334289	MN339304–MN339321
S. linearis	Lithuania	CcLT4.1, CcLT14.1, CcLT32.1, CcLT37.1, CcLT39.1	MN334290–MN334301	MN339322–MN339326
S. linearis	Spain	CcES2.9, CcES2.10, CcES11, CcES21, CcES23, CcESB5, CcESB8	MN334302–MN334313	MN339327–MN339333
S. oviformis	Lithuania	CcLT5.4, CcLT24.4, CcLT27.4, CcLT33.4, CcLT37.4	MN334314–MN334318	MN339334–MN339338
S. silva	Lithuania	CcLT3.3, CcLT4.3, CcLT9.3, CcLT11.3, CcLT14.3b, CcLT15.3, CcLT16.3b, CcLT24.3b, CcLT31.3, CcLT33.3	MN334319–MN334328	MN339339–MN339348
S. silva	Spain	CcES2.2, CcESB4	MN334329–MN334330	MN339349–MN339350

Table 4 Parameters of intraspecific genetic variability of six known *Sarcocystis* species from the roe deer

Species	Country	18S rDNA					*cox1*				
		h/n	Hd	K	S	π	h/n	Hd	K	S	π
S. capreolicanis	ITA	15/15	1.000	10.87619	44	0.00600	5/7	0.905	3.04762	7	0.00299
	LTU	13/13	1.000	12.75641	51	0.00709	5/8	0.786	2.14286	6	0.00210
	NOR	3/3	1.000	12.67777	19	0.00698	2/4	0.500	7.00000	14	0.00686
	Overall	31/31	1.000	11.61720	78	0.00645	12/19	0.936	5.52047	24	0.00541
S. entzerothi	LTU	4/15	0.657	1.26667	3	0.00070	1/10	0	0	0	0
S. gracilis	ESP	1/18	0	0	0	0	7/18	0.739	1.69935	7	0.00197
	ITA	1/2	0	0	0	0	13/24	0.880	2.50725	14	0.00290
	LTU	1/10	0	0	0	0	5/9	0.806	3.27778	10	0.00379
	NOR	1/5	0	0	0	0	10/20	0.868	2.00526	11	0.00232
	POL	1/1	–	–	–	–	4/6	0.800	2.00000	6	0.00231
	Overall	1/31	0	0	0	0	31/77	0.871	2.40738	36	0.00279
S. linearis	ITA	22/24	0.993	13.22464	56	0.00725	5/19	0.532	4.59649	17	0.00501
	ESP	12/12	1.000	12.54545	43	0.00689	5/7	0.857	2.80952	7	0.00306
	LTU	11/12	0.985	12.81818	45	0.00703	5/5	1.000	7.60000	17	0.00829
	Overall	45/48	0.997	13.26950	98	0.00730	15/31	0.822	6.44516	29	0.00703
S. oviformis	LTU	1/6	0	0	0	0	1/5	0	0	0	0
	NOR	1/4	0	0	0	0	2/6	0.600	0.60000	1	0.00061
	POL	1/1	–	–	–	–	1/3	0	0	0	0
	Overall	1/11	0	0	0	0	3/14	0.626	0.72527	2	0.00074
S. silva	ESP	2/2	1.000	2.00000	2	0.00117	2/2	1.000	13.00000	13	0.01408
	ITA	12/12	1000	9.84848	39	0.00580	10/27	0.883	9.54986	23	0.01035
	LTU	4/15	0.714	1.31429	4	0.00077	10/12	0.970	8.43939	24	0.00914
	NOR	4/4	1.000	4.00000	7	0.00235	3/3	1.000	1.33333	2	0.00144
	POL	1/1	–	–	–	–	4/4	1.000	7.66667	15	0.00831
	Overall	19/34	0.897	5.24955	44	0.00309	28/48	0.958	9.88918	39	0.01071

h the number of haplotypes, *n* the number of isolates, *Hd* the haplotype diversity, *K* the average number of nucleotide differences, *S* the number of segregating sites, π the nucleotide diversity, *ESP* Spain, *ITA* Italy, *LTU* Lithuania, *NOR* Norway, *POL* Poland

oesophagus, diaphragm, and heart of 101 roe deer hunted in north-western Spain and detected sarcocysts of *S. capreolicanis*, *S. gracilis*, *S. silva*, and *Sarcocystis* sp. by means of LM and/or TEM. During the present study, *S. capreolicanis* was not found in Spain, probably due to different muscles examined or because such species is rare in the investigated provinces. There is no data indicating that *S. linearis* is present in Norway or Poland, but since the species was only recently discovered (Gjerde et al. 2017), future researches are desirable, whereas *S. entzerothi* was confirmed only in Lithuanian roe deer, sika deer and fallow deer (Prakas et al. 2017; Rudaitytė-Lukošienė et al. 2018, 2020). The sika deer is native to East Asia and was introduced into different countries including Lithuania. It is possible that the roe deer is not a natural intermediate host of *S. entzerothi* as it was suggested by Gjerde et al. (2017), and the species could have spread in the area through the introduced sika deer. Macrocysts of *S. oviformis* were not found in Italy or Spain. We suppose that either *S. oviformis* was overlooked in Spanish roe deer and not detected in Italy due to a small number of animals examined or definitive hosts of *S. oviformis* are not prevalent in Southern Europe. Definitive hosts of *S. oviformis* are not revealed; however, based on phylogenetic placement of *S. oviformis*, corvid birds are assumed to be their final hosts (Gjerde et al. 2017).

In this study, LM morphological analysis was insufficient to discriminate sarcocysts of *S. gracilis* from those of *S. linearis*. The size ranges of these two *Sarcocystis* species corresponded to those reported in the previous study (Gjerde et al. 2017). Sarcocysts of *S. linearis* and *S. oviformis* were morphologically analysed using TEM. The ultrastructural description of sarcocysts provides valuable information when correlates with molecular data. In the present article, *S. linearis* and *S. oviformis* sarcocysts are described by TEM for the first time and supplement the previously published descriptions of these species based on SEM (Dahlgren and Gjerde 2009; Gjerde et al. 2017). The current *S. linearis* TEM pics represent ribbon-like vp with the tapered ends,

and these vp are separated by up to 1.3 μm wide segments of well-defined blebs; this was clearly visible in Fig. 4C provided by Gjerde et al. (2017). Several *Sarcocystis* sp. of similar ultrastructure have been reported in roe deer from Germany, Italy, and Spain, and sarcocysts of this unnamed species are likely to have belonged to *S. linearis* (Entzeroth 1982; Santini et al. 1997; López et al. 2003; Pérez-Creo et al. 2013). On the other hand, sarcocyst of *S. oviformis* displayed 6.5–10 μm long vp having the appearance of a sea anemone; this was initially shown by Dahlgren and Gjerde (2009) and Gjerde (2012) in Norwegian roe deer. TEM pics showed sectioned vp with thin peduncle and condensate material at the base of vp. In some micrographs, un-sectioned vp morphology resembled those tongue-shaped vp of *S. ovalis* from the moose (*Alces alces*). Bradyzoites of both species were unsuitable for description due to preservation of cysts.

In this study, *Sarcocystis* species examined revealed significant differences in intraspecific genetic variability. When compared with the molecular data of *Sarcocystis* species from the roe deer currently available from five European countries, the level of interspecific genetic variability depended more on the species rather than on locus or the geographical area examined. Our results are consistent with the findings of Kolenda et al. (2014) who observed high similarity in the level of genetic variability of a population of the same *Sarcocystis* species from roe deer living in different geographic regions.

The present survey provided new data on the complex epidemiology of *Sarcocystis* species infecting wild ungulates, especially on *S. oviformis* and *S. linearis*; in addition, the necessity to carry out further ultrastructural-molecular-combined investigations is emphasized in order to link previous research and current knowledge by adequately describing parasite species as an aid to unravel the role of possible definitive hosts.

Funding information This work was supported by the Research Council of Lithuania (grant number S-MIP-17-45) and Open Access to research infrastructure of the Nature Research Centre under Lithuanian open access network initiative.

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