**Sarcocystis** infection in red deer (*Cervus elaphus*) with eosinophilic myositis/fasciitis in Switzerland and involvement of red foxes (*Vulpes vulpes*) and hunting dogs in the transmission

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**ARTICLE INFO**

**Keywords:**
Sarcocystis
Red deer (*Cervus elaphus*)
Red fox (*Vulpes vulpes*)
Dog (*Canis familiaris*)
Myositis/fasciitis
Molecular identification

**ABSTRACT**

Red deer (*Cervus elaphus*) carcasses showing grey-greenish discolouration have been increasingly observed in the canton of Grisons, Switzerland. We investigated whether *Sarcocystis* infections were associated with this pathology, and whether wild and domestic canids were involved in their transmission. Meat from affected red deer (*n* = 26), faeces and intestines from red foxes (*Vulpes vulpes*) (*n* = 126), and faeces from hunting dogs (*n* = 12) from the region, were analysed. Eosinophilic myositis and/or fasciitis were diagnosed in 69% of the deer, and sarcocysts were observed in 89% of the animals. Molecular typing targeting a ~700bp variable region of the 18S rRNA gene revealed *Sarcocystis hjorti* in 73%, *S. venatoria/S. iberica* in 54%, *S. linearis/S. tenuata* in 12%, *S. pilosa* in 8% and *S. ovalis* in 4% of the deer samples. No inflammatory changes were observed in red deer carcasses with normal appearance (*n* = 8); however, sarcocysts were observed in one sample, and *S. hjorti, S. venatoria/S. iberica* or *S. silva* DNA was detected in five samples. Sarcocystis oocysts/sporocysts were observed in 11/106 faecal and 6/20 intestinal fox samples, and in 2/12 canine samples. *Sarcocystis tenella* (*n* = 8), *S. hjorti* (*n* = 2), *S. gracilis* (*n* = 2), and *S. miescheriana* (*n* = 1) were identified in foxes, and *S. gracilis* (*n* = 2), *S. capreoleicani* (*n* = 1) and *S. linearis/S. tenuata* (*n* = 1) in dogs. This study provides first molecular evidence of *S. pilosa* and *S. silva* infection in red deer and *S. linearis/S. tenuata* in dogs and represents the first record of *S. ovalis* transmitted by corvids in Central Europe. Although *Sarcocystis* species infecting red deer are not regarded as zoonotic, the affected carcasses can be declared as unfit for human consumption due to the extensive pathological changes.

1. Introduction

Members of the genus *Sarcocystis* (Apicomplexa, Sarcocystidae) are heteroxenous parasites with carnivores as definitive hosts (DH) and herbivores as intermediate hosts (IH) (Dubey et al., 2016; Deplazes et al., 2016). *Sarcocystis* spp. undergo a sexual reproduction in the intestine of the DH leading to production of sporulated oocysts, which are shed to the environment with the faeces, and serve as source of infection for IH. Definitive hosts may shed oocysts/sporocysts over several months, being responsible for prolonged environmental contamination (Dubey et al., 2016; Deplazes et al., 2016). Intermediate hosts acquire the infection by ingestion of sporocysts contaminating food or water. In the IH, the parasite undergoes an asexual merogonic reproduction infecting endothelial cells of several organs and later, muscle or nerve cells, in which tissue cysts (“sarcocysts”) containing numerous zoites (infectious for DH) are built (Dubey et al., 2016; Deplazes et al., 2016).

More than 200 species of *Sarcocystis* with variable pathogenicity have been described infecting mammals (including humans), reptiles and birds worldwide (Dubey et al., 2016). Intestinal infections in the DH are generally asymptomatic, except in humans (Fayer et al., 2015). In the IH the course of infection is frequently subclinical, but it may be severe and also fatal depending on the *Sarcocystis* species. Clinical signs such as fever, weakness, cyanosis, dyspnoea, neurological signs, abortion and death have been described in several animal species after experimental (Johnson et al., 1975; Koller et al., 1977) and natural (Caspari et al., 2011; Ravi et al., 2015) infections.

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https://doi.org/10.1016/j.ijppaw.2020.09.005
Received 16 July 2020; Received in revised form 24 September 2020; Accepted 28 September 2020
Available online 1 October 2020
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In cervids, clinical Sarcocystis infections are considered rare; however, some species were shown to be pathogenic in experimental infections. Mule deer (Odocoileus hemionus) fawns inoculated with 50,000 to 1 million sporocysts of S. hemionii latrantis became anorectic, showed incoordination, and died between 27 and 63 days post-inoculation (Koller et al., 1977). Rocky mountain elk (Cervus elaphus), inoculated with 250,000 sporocysts of Sarcocystis spp. (including S. sybillensis and S. wapiti) showed reduced weight gain associated with higher parasite burdens in different tissues, when compared with non-inoculated control animals (Foreyt et al., 1995). Furthermore, a natural case of clinical acute infection with S. alcesalutrans was recently described in a moose (Alces) calf presenting neurological signs and multisystemic inflammation with presence of intralesional schizonts in the brain, the uveal tract of both eyes, and also in lungs, heart and kidneys (Ravi et al., 2015).

In September 2010 and October 2011, two muscle samples of hunted red deer (Cervus elaphus) from Grisons, Switzerland, showing a grey-greenish discoulouration and a gelatinous change of the fasciae were sent to the Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Switzerland for analysis. Histopathologically, eosinophilic fasciitis was observed and S. hjorti was assumed as the causing agent of this pathology (Stephan et al., 2012). This finding was uncommon, but according to the Hunting and Fishing Department of Grisons and to regional meat inspectors, hunted red deer with greenish tissue discoulouration have been increasingly observed over the last few years, as it was also informed by the Journal for Swiss hunters (Deutz, 2013). Even though the Sarcocystis species found in red deer are not regarded as zoonotic, the affected carcasses can be declared unfit for human consumption due to the extensive pathological changes (Stephan et al., 2012). Association of Sarcocystis infection with greenish discoulouration of the carcasses due to eosinophilic myositis/fasciitis has been described in several animal species including cattle, sheep, horses and South American cameldids (Gajadhar et al., 1987; Wouda et al., 2006; Aráoz et al., 2019; Jensen et al., 1986; Vangeel et al., 2013; Herd et al., 2015; La Perle et al., 1999), but this was considered infrequent in cervids (Stephan et al., 2012).

Although the occurrence of Sarcocystis infections in cervids has been known for a long time, most Sarcocystis species have been only recently described, and many aspects about their epidemiology and significance remain still unknown (Dubey et al., 2016). At least eleven Sarcocystis species have been detected in European red deer: i.e. S. hjorti, S. hardangeri, S. ovalis, S. tarandii, S. cervicanis, S. truncata, S. elongata, S. linearis, S. iberica, S. venatoria and S. morae (Dahlgren and Gjerde, 2010a; Gjerde, 2014b; Hernández-Rodríguez et al., 1981; Gjerde et al., 2017b; Dubey et al., 2016), but the DH for only a few of these species have been identified so far (Dubey et al., 2016; Gjerde et al., 2017b; Irie et al., 2017; Dahlgren and Gjerde, 2010b).

This study aimed to identify the Sarcocystis species infecting red deer with eosinophilic myositis/fasciitis, and with normal carcass appearance in Switzerland, and to investigate the possible involvement of red foxes (Vulpes vulpes) and hunting dogs as definitive hosts of these species.

2. Materials and methods

2.1. Red deer samples

Meat samples from 26 red deer showing grey-greenish discoulouration and gelatinous changes in fasciae and muscles (Fig. 1)(age range = 1–13 years, mean 2.0 years; females n = 18, males n = 8) were collected for diagnosis of Sarcocystis infection by meat inspectors in four regions of the canton of Grisons, Switzerland (Davos (Deer 1–6); Rueun (Deer 7–9); Caniers (Deer 10–20) and Filsur (Deer 21–26)) during the hunting season 2015 (June to September). Additionally, samples from eight red deer with normal carcass appearance (Deer 27–34) (age range 2–3 years, mean = 2.5 years; females n = 2, males n = 6) hunted in the same region (i.e. Rueun) during the same hunting season were included as a control group. Estimated age and sex of the sampled animals, as well as the intensity of the observed macroscopical changes, are indicated in Table 1. Geographical coordinates of the hunting sites and sampling dates are registered in Supplementary Table 1. From each killed animal (n = 34), a sample (~10 × 15 × 5 cm) of the limb muscles was collected. In some cases (n = 11), samples from diaphragm showing grey-greenish discoulouration were additionally included (Table 1). All the samples were immediately refrigerated and sent to the Institute of Parasitology, University of Zurich for further examination.

2.2. Red fox samples

Faecal samples (n = 106; Fox 1–106) and intestines (n = 20; Fox 107–126) from red foxes were collected by hunters all over Grisons for two years (2013–2015). The geographic coordinates of the collection or hunting sites and age and sex of the hunted foxes were registered in Supplementary Table 2. All samples were frozen at −80 °C for at least two weeks to inactivate potentially present Echinococcus multilocularis eggs and were subsequently analysed for Sarcocystis infection. The foxes were hunted for reasons independent of this study and the hunters submitted the examined material on a voluntary basis.

2.3. Dog samples

In addition, in winter 2015, faecal samples from hunting dogs from Grisons (n = 12; Dog 1–12) were collected and analysed for Sarcocystis infection. The samples were directly submitted by the hunters to the Institute of Parasitology together with a questionnaire including data from the dogs (i.e. breed, age, sex, contact with deer, feeding habits and the possibility of eating raw meat or viscera from red deer and other hunted animals). The faecal samples were processed as indicated above for fox samples. Collected data from the dogs are displayed in Supplementary Table 3.

2.4. Histopathology

About 40 g of fresh meat from each red deer were fixed in 4% buffered formalin on the day of receiving the samples. Within 24 h, formalin-fixed tissues were embedded in paraffin, sectioned at 2.0–3.0 μm, and stained with haematoxylin and eosin (HE) for routine histopathological examination. All collected meat samples were evaluated for the presence of Sarcocystis and histological abnormalities.
2.5. Isolation of Sarcocystis oocysts/sporocysts from faeces and intestine

Faecal samples from foxes and dogs were processed for coproscopy by a flotation method using a concentrated sucrose solution (specific gravity of 1.3 g/l). After centrifugation at 500g for 5 min, three drops from the surface of the flotation fluid were examined at 100× and 400× magnification using a Leica DM 1000 LED microscope. In positive samples, the rest of the supernatant was collected and processed for isolation of Sarcocystis oocysts/sporocysts as previously described (Schares, 2007). Small intestines from red foxes were cut longitudinally and scrapings from the mucosa were taken using a disposable scalpel. The scrapings were homogenised and processed by flotation as indicated for faecal samples above.

2.6. DNA extraction

DNA was extracted from meat samples previously frozen at -20 °C (500 mg/animal including either limb muscles or limb muscles and diaphragm if this sample was additionally collected) using the QiAamp DNA mini kit (QIAGEN, Hilden, Germany) as described by (Glor et al., 2010). Besides, DNA was extracted from Sarcocystis oocysts/sporocysts isolated from fox or dog faeces concentrated in 200 μl aqueous solution using the ZR Fecal DNA MiniPrep kit (Zymo Research, USA), as indicated by the manufacturer.

2.7. Polymerase chain reaction (PCR) and sequencing

DNA samples obtained by the different extraction methods explained above were tested by a PCR targeting a variable ~700 bp region of the 18S rRNA gene of Sarcocystis using the primers SarcoF/SarcoR (Moré et al., 2011). The amplification reactions (initial denaturation step at 94 °C for 15 min followed by 40 cycles of 94 °C 40 s; 59 °C 30 s; 72 °C 1 min and a final extension step at 72 °C for 10 min) were performed in a thermocycler (SensoQuest Labcycler) in a final volume of 50 μl, using 25 μl QIAGEN Multiplex Mastermix; 19 μl QIAGEN RNase-free water; 0.5 μl of each primer (100 μM solution) and 5 μl DNA/sample. The amplification products were analysed by gel electrophoresis in 1.5% agarose stained with GelRed (Biotium). PCR products exhibiting the expected fragment length were purified using the MinElute PCR Purification Kit (Qiagen). The purified PCR products were cloned using the TOPO TA Cloning™ Kit (Invitrogen) and sequenced using the Sequencher (Gene Codes Corporation, Ann Arbor, USA) and the ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were deposited in GenBank® (accession numbers listed in Table 2) and compared with available sequences in GenBank® using the megablast function of BLASTn (http://blast.ncbi.nlm.nih.gov).

Table 1

| Hunting | Deer | Age (years) | Sex (F/M) | Macroscopical changes in the carcass | Histology in skeletal muscle samples | Sarcocystis PCR (Pos/Neg) | Sarcocystis species identification (Y/N/n) |
|---------|------|-------------|----------|-------------------------------------|-------------------------------------|--------------------------|------------------------------------------|
| Davos   | 1    | 2 M         | ++       | -/+                                 | +/                                 | Y/Y  Pos                 | N Y                                      |
| 2       | 2 M  | ++         | ++       | +/                                 | +/                                 | Y/Y  Pos                 | N Y                                      |
| 3       | 1 F  | ++         | ++       | +/                                 | +/                                 | Y/Y  Pos                 | N Y                                      |
| 4       | 1 F  | ++         | ++       | +/                                 | +/                                 | Y/Y  Pos                 | N Y                                      |
| 5       | 2 M  | +          | -/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 6       | 2 F  | +          | +/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| Riein   | 7    | 1 F        | ++       | +/++                              | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 8       | 4-6  | F          | ++       | ++/++                              | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 9       | 1 F  | ++         | ++       | +/                                 | +/                                 | N/N  Pos                 | N Y                                      |
| Cuners  | 10   | 1 F        | +        | -/n                                | -/n                                | Y/N  Pos                 | N Y                                      |
| 11      | 1 M  | ++         | +/n      | +/n                                | +/n                                | Y/N  Pos                 | N Y                                      |
| 12      | 2 M  | +          | -/n      | -/n                                | -/n                                | Y/N  Pos                 | N Y                                      |
| 13      | 2 F  | +          | -/n      | -/n                                | -/n                                | Y/N  Pos                 | N Y                                      |
| 14      | 1 F  | ++         | -/n      | -/n                                | -/n                                | Y/N  Pos                 | Y Y                                      |
| 15      | 1 F  | +          | +/n      | +/n                                | +/n                                | Y/N  Pos                 | N Y                                      |
| 16      | 1 F  | +          | -/n      | -/n                                | -/n                                | Y/N  Pos                 | Y n                                      |
| 17      | 3 M  | ++         | +/n      | +/n                                | +/n                                | Y/N  Pos                 | N Y                                      |
| 18      | 1 F  | +          | -/n      | -/n                                | -/n                                | Y/N  Pos                 | N Y                                      |
| 19      | 2 M  | ++         | -/n      | -/n                                | -/n                                | Y/N  Pos                 | N Y                                      |
| 20      | 1 F  | +          | -/n      | -/n                                | -/n                                | Y/N  Pos                 | Y Y                                      |
| Filisur | 21   | 1 F        | +        | +/                                 | +/                                 | Y/N  Pos                 | Y Y                                      |
| 22      | 1 F  | ++         | +/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 23      | 1 F  | ++         | +/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 24      | 1 F  | +          | +/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 25      | 1 F  | ++         | +/                   | +/                                 | +/                                 | Y/Y  Pos                 | Y Y                                      |
| 26      | 13 M | +          | -/        | -/                                 | -/                                 | Y/N  Pos                 | Y Y                                      |
| Riein   | 27   | 2 M        | +/n      | N/n                                | N/n                                | Neg                      | n n                                      |
| 28      | 3 F  | -          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |
| 29      | 2 M  | M          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |
| 30      | 2 F  | -          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |
| 31      | 3 M  | -          | -/n      | -/n                                | -/n                                | Neg                      | n n                                      |
| 32      | 3 M  | -          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |
| 33      | 3 M  | -          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |
| 34      | 2 M  | -          | -/n      | -/n                                | -/n                                | Pos                      | N Y                                      |

F: female; M: male; a) macroscopical changes: - normal carcass appearance, + slight, ++ moderate, +++: marked grey-greenish discolouration areas in the carcass.

b) microscopical changes: -: no pathological changes, + slight, ++ moderate, +++: severe fasciitis or myositis, Y: yes, N: no, n: not examined; L: limb muscle; D: diaphragm; Pos: positive PCR result for Sarcocystis DNA; Neg: negative PCR result for Sarcocystis DNA.
| Animal ID | Sequence ID | Sequence length (bp) | BLASTn identity (%) | GenBank® accession no. (reference sequences) | Reference | Sarcoctysis spp. (this study) | GenBank® accession no. (this study) |
|-----------|-------------|----------------------|---------------------|---------------------------------------------|-----------|-------------------------------|----------------------------------|
| Deer 1    | D1 clone1   | 652                  | S. hjorti           | 99.7                                        | Gjerde et al. (2017b) | S. hjorti | MT737809 |
|           | D1 clone2-D1 clone5 | 662-664 | S. venatoria / S. ibrica | 98.9-99.9 | Gjerde et al. (2017b) | Sarcoctysis sp. | MT737810 |
|           | D1 clone3   | 662-664 | S. hjorti           | 99.1-99.7                                   | Gjerde et al. (2017b) | Sarcoctysis sp. | MT737813 |
| Deer 2    | D2 clone1-D2 clone5 | 662-666 | S. venatoria / S. ibrica | 99.1-99.7 | Gjerde et al. (2017b) | Sarcoctysis sp. | MT737814 |
| Deer 3    | D3 clone5-D3 clone9 | 654 | S. hjorti           | 99.7-100                                    | GQ250990 | Dahlgren and Gjerde (2010a) | S. hjorti | MW019997 |
| Deer 4    | D4 clone2-D4 clone3 | 654 | S. hjorti           | 99.3-99.7                                   | GQ250990 | Dahlgren and Gjerde (2010a) | S. hjorti | MW020000 |
| Deer 5    | D5 clone1-D5 clone5 | 630 | S. hjorti           | 99.5-99.7                                   | GQ250990 | Dahlgren and Gjerde (2010a) | S. hjorti | MT737817 |
| Deer 6    | D6 clone1-D6 clone5 | 630 | S. ovalis           | 100                                         | GQ250998 | Dahlgren and Gjerde (2010a) | S. ovalis | MT737820 |
| Deer 7    | D7 clone1-D7 clone5 | 628 | S. hjorti           | 99.2-100                                    | GQ250990 | Dahlgren and Gjerde (2010a) | S. hjorti | MT737822 |
| Deer 8    | D8 clone6-D8 clone10 | 662-666 | S. venatoria / S. ibrica | 99.4-99.9 | GY973318, GY973321, GY973322, GY973327 | Sarcoctysis sp. | MT737822 |
| Deer 9    | D9 clone2- D9 clone 6 | 662-666 | S. venatoria / S. ibrica | 99.3-99.7 | GY973318, GY973321, GY973322, GY973327 | Sarcoctysis sp. | MT737823 |
| Deer 10   | D10 clone1-D10 clone3 | 662-664 | S. venatoria / S. ibrica | 99.4-99.9 | GY973318, GY973321, GY973322, GY973327 | Sarcoctysis sp. | MT737824 |
| Deer 11   | D11 clone1 | 652 | S. hjorti           | 100                                         | GY973332 | Gjerde et al. (2017b) | S. hjorti | MT737825 |
| Deer 12   | D12 clone1-D12 clone5 | 654 | S. venatoria / S. ibrica | 99.4-99.9 | KF831294 | Gjerde (2014a) | MT737826 |
| Deer 13   | D13 clone1-D13 clone5 | 658-660 | S. linearis/S. taimani | 97.9-99.9 | GY973371, GY973372, KU753890, KT626602, MN334301, KF831293 | Sarcoctysis sp. | MT737827 |
| Deer 14   | D14 clone4 | 654 | S. hjorti           | 99.2                                         | KF831294 | Gjerde (2014a) | S. hjorti | MT737828 |
| Deer 15   | D15 clone1-D15 clone5 | 654 | S. hjorti           | 98.7-99.9                                   | KF831294 | Gjerde (2014a) | S. hjorti | MT737829 |
| Deer 16   | D16 clone1-D16 clone5 | 654 | S. hjorti           | 100                                         | GQ250990 | Dahlgren and Gjerde (2010a) | S. hjorti | MT737830 |
| Deer 17   | D17 clone1-D17 clone5 | 654 | S. hjorti           | 99.7-100                                    | GY973332, KF831294 | Gjerde et al. (2017b) | Sarcoctysis sp. | MT737831 |
| Deer 18   | D18 clone1-D18 clone5 | 660 | S. linearis/S. taimani | 98.8-99.2 | GY973372, KU753890 | Sarcoctysis sp. | MT737832 |
| Deer 19   | D19 clone1-D19 clone5 | 664-666 | S. venatoria / S. ibrica | 99.3-99.9 | GY973318, GY973321, GY973322, GY973327 | Sarcoctysis sp. | MT737833 |

(continued on next page)
Table 2 (continued)

| Animal ID | Sequence ID | Sequence length (bp) | BLASTn identity (%) | GenBank® accession no. (reference sequences) | Reference | Sarcocystis sp. (this study) | GenBank® accession no. (this study) |
|-----------|-------------|----------------------|---------------------|---------------------------------------------|-----------|---------------------------|-----------------------------------|
| Deer 20   | D21 clone1  | 636                  | S. hjorti           | 100                                         | GQ250990  | S. hjorti                  | MT737896                          |
|           | D21 clone2  | 652–654              | S. hjorti           | 97.9–100                                    | KF831294, KY973322 | S. hjorti                  | MT737885                          |
|           | Deer 21    | D22 clone1–D22 clone5 | S. hjorti           | 95.9–100                                    | KF831294, KY973322 | S. hjorti                  | MT737890                          |
| Deer 22   | D23 clone2–D23 clone5 | S. hjorti | 97.7–99.9 | KF831294, KY973322 | S. hjorti | MT737906 |
| Deer 23   | D24 clone1–D24 clone4 | S. hjorti | 99.9–100 | KF831294, KY973322 | S. hjorti | MT737905 |
| Deer 24   | D25 clone1–D25 clone2 | S. hjorti | 99.3–99.7 | KY973318, KY973325 | S. hjorti | MT737907 |
| Deer 25   | D26 clone1–D26 clone5 | S. hjorti | 99.4–99.9 | KY973318, KY973322 | S. hjorti | MT737908 |
| Deer 26   | D27 clone1–D27 clone5 | S. hjorti | 99.4–99.7 | KY973318, KY973322 | S. hjorti | MT737909 |
| Deer 29   | D29 clone1–D29 clone5 | S. hjorti | 99.7 | KY973324, KY973327 | S. hjorti | MT737911 |
| Deer 30   | D30 clone1–D30 clone5 | S. hjorti | 99.1–99.9 | KY973318, KY973322 | S. hjorti | MT737912 |
| Deer 32   | D32 clone1–D32 clone5 | S. hjorti | 99.7–99.9 | KF831294 | S. hjorti | MT737923 |
| Deer 33   | D33 clone1–D33 clone5 | S. hjorti | 99.7–99.9 | KF831294, KY973322 | S. hjorti | MT737926 |
| Deer 34   | D34 clone1–D34 clone5 | S. hjorti | 99.1–99.9 | KF831294, KY973322 | S. hjorti | MT737927 |
|           |             |                      |                     |                                             |           | MT737928 |

The obtained sequences were assigned to a determined Sarcocystis species if: (i) they showed more than 99% unambiguous BLASTn identity with GenBank® entries for which the species was known; (ii) the reference GenBank® sequences were supported by morphological data; (iii) the obtained sequences clustered with the reference GenBank® sequences in a phylogenetical tree (see below). Sequences showing >99% similarity with GenBank® sequences of more than one named Sarcocystis species (as it is generally the case between S. linearis and S. taeniata or between S. venatoria and S. iberica and sometimes also between S. tenella and S. capracanis) were recorded as Sarcocystis sp. The highest homology for each species was indicated in Table 2 and Supplementary Table 4. Sequences with less than 99% identity with named GenBank® entries were recorded as Sarcocystis sp. To assess the relationship of the sequences among them and with reported reference GenBank® sequences, a phylogenetic tree was built using the neighbour-joining method with the software Geneious R10 (https://www.geneious.com) (Supplementary Fig. 1). For this analysis, the obtained sequences were trimmed from the primer binding regions. Nucleotide and haplotype diversity within species of Sarcocystis was calculated using the DnaSP v6 software (Rozas et al., 2017).

3. Results

3.1. Histopathological examination of muscle samples from red deer

Histologically, inflammatory changes characterized by eosinophilic myositis and eosinophilic lymphoplasmacellular fasciitis were diagnosed in 18 (69%) and 17 (65%) out of 26 examined deer showing a greenish discouloration of the carcass, respectively, and sarcocysts were observed in 23 (89%) of these animals (i.e. in 23/26 and 9/13 limb and diaphragm meat samples, respectively) (Fig. 2). Besides, the media of many veins were significantly thickened by hypertrophy and hyperplasia. The endothelial cells were flat or cuboidal and the lumen of some vessels was almost completely occluded (Fig. 2). Meat samples from animals with normal carcass appearance (control group from location Rueun, n = 8) did not show inflammatory changes and Sarcocystis was detected in only 1/8 (13%) of the limb samples. When the histopathological results of all 47 analysed muscle samples (i.e. 34 limb samples and 13 diaphragm samples) from red deer with and without macroscopic carcass changes were considered together, a positive association between the presence of sarcocysts and eosinophilic myositis was found (Table 1). Sarcocysts were more frequently detected in muscle samples in which eosinophilic myositis was observed (84.6% out of 26), than in those samples without inflammatory changes (52.4% out of 21) (Fisher’s exact test p = 0.0250). Detailed results of the histopathological examination are indicated in Table 1.

Microscopical examination of faecal and intestine samples from red foxes and faecal samples from hunting dogs.

By flotation in sucrose solution, Sarcocystis oocysts and/or sporocysts were microscopically detected in 10% (11/106) of the faecal samples and 30% (6/20) of the samples from the intestinal mucosa from red foxes (Supplementary Table 2), and in the faeces from two (Dog 5 and
Dog 6) (16.7%) out of 12 hunting dogs (Supplementary Table 3). Dog 5 was a female, 9-month-old Hanover Hound and Dog 6 was a male, 13-year-old, Magyar Vizsla. Both dogs were used for hunting purposes in the Canton of Grisons and were fed with fresh raw meat and heart from red deer and other hunted animals, besides receiving commercial feed.

Positive PCR results for Sarcocystis spp. were obtained in muscle samples from all 26 (100%) analysed red deer showing a grey-greenish discolouration of the carcass (Table 1). Direct sequencing and cloning of the obtained PCR products in a plasmid vector followed by sequencing allowed molecular species discrimination in eight and 25 animals, respectively (Tables 1 and 2). At least five different Sarcocystis species could be identified by molecular methods: S. hjorti, S. venatoria/S. iberica, S. linearis/S. taeniata, S. pilosa and S. ovalis (Table 2). Sarcocystis hjorti was the most frequently detected species in 19 (73%) of the deer, followed by S. venatoria/S. iberica in 15 (54%) animals, S. linearis/S. taeniata in three (12%), S. pilosa in two (8%) and S. ovalis in one (4%) animal. By cloning of the PCR products, one sole Sarcocystis species was detected in 14 of the animals (i.e. S. hjorti n = 9; S. venatoria/S. iberica n = 4 and S. ovalis n = 1). In three red deer (Deer 4, 14 and 20), in which direct sequencing indicated infection by only one species, the cloning technique revealed infection with two to three different Sarcocystis species (including the Sarcocystis species determined by direct sequencing) (Table 2), showing the higher diagnostic sensitivity of cloning over direct sequencing. Co-infections with two and three different Sarcocystis species were detected in nine and two animals, respectively (Tables 1 and 2 and Supplementary Table 4). In one of the samples (Deer 16), no further Sarcocystis species identification by cloning could be performed. While Sarcocystis DNA could be detected in muscle samples from 100% (26/26) of the animals with greenish macroscopic changes in the muscles, only 62.5% (5/8) of the samples from animals with normal carcass appearance yielded positive PCR results (Fisher’s exact test, p = 0.009). Direct sequencing did not allow species differentiation in any of the five animals. By cloning, sequences corresponding to S. hjorti and S. venatoria/S. iberica were detected in three and one animal, respectively. A further animal showed molecular evidence of co-infection with S. venatoria/S. iberica and S. silva (Deer 29) (Tables 1 and 2).

In red foxes, the presence of Sarcocystis DNA could be confirmed by PCR and sequencing in parasites isolated from 7/11 faecal samples and in 6/6 samples from intestine mucosa, in which Sarcocystis sporocysts/oocysts had been microscopically detected. By direct sequencing, amplicons obtained from 11 of the foxes showed 100% identity with GenBank® sequences of S. tenella (n = 7), S. gracilis (n = 2), S. hjorti (n = 1) or S. miescheriana (n = 1) (Table 3, Supplementary Table 5). Further cloning and sequencing of these PCR products allowed the confirmation of the results in all nine tested foxes (samples from Foxes 46 and 115 could not be cloned) and also revealed the presence of co-infection with further non-defined Sarcocystis species in six of these animals. These sequences were mainly related to S. capracanis/S. tenella (93.4–98.9% sequence identity) in Foxes 1, 10, 108, 112, 120 and 126, and to S. hircicantis/S. arcticans (94.5–95.2% sequence identity) in Fox 108. These sequences were deposited in GenBank® as Sarcocystis sp. (Table 3, Supplementary Table 5, Supplementary Fig. 1).

In two further foxes (Fox 6 and Fox 15), direct sequencing suggested co-infection with more than one Sarcocystis species (Table 1) and cloning was performed. In one of these foxes (Fox 6), sequencing of the obtained clones revealed co-infection by S. hjorti, and S. tenella/S. capracanis. In the other fox (Fox 15), sequences of S. capracanis/S. tenella and a non-defined Sarcocystis sp. (Fox15 clone5) with 96.5% and 96.1% sequence identity with GenBank® sequences of S. hircicantis and S. arcticans, respectively, were observed (Table 3, Supplementary Table 5).

Four out of 11 faecal samples in which Sarcocystis sporocysts had been observed after flotation were negative by PCR. These samples contained only very few sporocysts.

Both samples from hunting dogs, in which sporocysts of Sarcocystis spp. had been observed after flotation, were positive by PCR. By direct sequencing, a Sarcocystis mixed infection was assumed for both samples; therefore, these PCR products were cloned. In Dog 5, sequencing of the obtained clones allowed the detection of sequences with 99.1–99.4% and 99.4–99.6% identity with GenBank® sequences of S. gracilis and S. capreolica, respectively. In Dog 6, sequences with 99.5% identity with GenBank® sequences of S. gracilis and 99.1 and 98.9% % identity with S. linearis and S. taeniata, respectively, were found (Table 3).

Sequence analysis revealed high intraspecific variability in the 18S rRNA gene sequence obtained from Sarcocystis from all three different hosts. The number of isolates, number of identified variants for each Sarcocystis species, and further parameters of intraspecific genetic variability are shown in Table 4. A phylogenetic tree inferred using the neighbour-joining method shows the relationship of the obtained 18S rRNA partial sequences with reference Sarcocystis sequences annotated in GenBank® (Supplementary Fig. 1).

4. Discussion

Grey-greenish discolouration in meat has been attributed to several causes, such as eosinophilic inflammatory myopathies, and post-mortem microbial and non-microbial enzymatic processes leading to the production of hydrogen sulphide and alterations in the myoglobin pigments of striated muscles (Stephan et al. 1997, 2012). Besides, infections with Onchocerca spp. (Filarioidea; Onchocercidae) nematodes were associated with greenish discolouration and oedema of subcutaneous tissues in cervids (Laaksonen et al., 2017) and domestic species (Solimsan et al., 2008). Adult Onchocerca worms are localised free or in nodules in subcutaneous tissues and produce microfilariae in these sites (Bosch et al., 2016; Bojisen et al., 2017). Dead or dying adult worms or microfilariae may trigger inflammatory reactions characterized by infiltration of eosinophilic granulocytes and multifocal nodular lymphoplasmacytic aggregations around them, followed by calcification and fibrosis (Solimsan et al., 2008). Four Onchocerca species were described in red deer in Central Europe (i.e. O. skrjabini and O. garmisi free in subcutaneous tissues, and O. flexuosa and O. jakutenisi in subcutaneous nodules).
| Animal ID | Sequence ID | Sequence length (bp) | BLASTn identity (%) | GenBank® accession no. (reference sequence) | Reference | Sarcocystis sp. | GenBank® accession no. (this study) |
|-----------|-------------|----------------------|----------------------|---------------------------------------------|-----------|----------------|-----------------------------------|
| Fox 1     | Fox1 PCR    | 623                  | S. tenella 100        | KP263759, MK420019, KP263759                | Kolenda et al. (2015); Gjerde et al. (2020) | S. tenella | MTT379302, MTT20893, MTT20894, MTT20896, MTT20897 |
|           | Fox1 clone1 | 646-647              | S. tenella 99.7-99.9  | KU120987, MK420019                           | Hu et al. (2016); Gjerde et al. (2020) | Sarcocystis sp. | MTT20895 |
|           | Fox2 clone2 | 647                  | S. capracanis 93.8    | UF029329, L76472, JF029329                   | Jeffries et al., 1997; Hu et al., 2016; Hu et al., 2017; Gjerde et al., 2020 | Sarcocystis sp. | MTT37943, MTT37947 |
|           | Fox3 clone3 | 642                  | S. tenella 93.4       | KU120987                                    | Sarcocystis sp. | MTT37948, MTT37951 |
|           | Fox4 clone4 | 647                  | S. capracanis/S. tenella 98.5-99.9 | KU120987, MK420019 | Hu et al. (2016); Gjerde et al. (2020) | S. tenella | MTT37944, MTT37946 |
|           | Fox5 clone5 | 652-654              | S. hjorti 99.5-100    | KY973332, KF831294                          | (Gjerde, 2014a; Gjerde et al., 2017b) | S. hjorti | MTT37952 |
|           | Fox6 clone6 | 652                  | S. hircicola/S. areticamis 95.8-96.3 | KU120987, MK420017 | Hu et al., 2016; Gjerde et al. (2020) | Sarcocystis sp. | MTT208094, MTT208097 |
|           | Fox7 clone7 | 652                  | S. tenella 99.9       | KP263759                                    | Kolenda et al. (2015) | S. tenella | MTT379303 |
|           | Fox8 clone8 | 652                  | S. tenella 99.9-100   | KU820983, KP263759                          | Hu et al., 2016, 2017 | Sarcocystis sp. | MTT208988 |
|           | Fox9 clone9 | 652                  | S. capracanis/S. tenella 98.8-98.9 | MF039329, KP263759 | Hu et al., 2016; Gjerde et al. (2020) | Sarcocystis sp. | MTT37940, MTT37941, MTT20891, MTT208917, MTT208918 |
|           | Fox10 clone10| 647                 | S. tenella 99.2-99.7  | KP263759, KP263756                          | Kolenda et al. (2015); Gjerde et al., 2020 | S. tenella | MTT37940, MTT37941, MTT20891, MTT208917, MTT208918 |
|           | Fox11 clone10| 640                 | S. capracanis/S. tenella 98.6-98.9 | KU120987, MK420019 | Kolenda et al. (2015); Gjerde et al., 2020 | Sarcocystis sp. | MTT37940, MTT37941, MTT20891, MTT208917, MTT208918 |
|           | Fox12 clone10| 647                 | S. tenella 99.4-99.5  | KP263756, KP263756                          | Kolenda et al. (2015); Gjerde et al., 2020 | S. tenella | MTT37940, MTT37941, MTT20891, MTT208917, MTT208918 |
|           | Fox13 clone10| 647                 | S. capracanis/S. tenella 98.8-98.9 | KU120987, MK420019 | Kolenda et al. (2015); Gjerde et al., 2020 | Sarcocystis sp. | MTT37940, MTT37941, MTT20891, MTT208917, MTT208918 |

(continued on next page)
Table 3 (continued)

| Animal ID | Sequence ID | Sequence length (bp) | BLASTn identity (%) | GenBank® accession no. (reference sequence) | Reference |
|-----------|-------------|----------------------|---------------------|---------------------------------------------|-----------|
| Dog5 clone2, Dog5 clone3 | 675 | S. gracilis | 99.1–99.4 | MN334289 | (Gerde et al., 2017a; Rudaityte-Lukosiene et al., 2020) |
| Dog6 clone1, Dog6 clone3 | 656 | S. linearis/S. taeniata | 98.7–99 | MN334294, KU753890 | (Prakas et al., 2016; Rudaityte-Lukosiene et al., 2020) |
| Dog6 clone9 | 675 | S. gracilis | 99.8 | MN334289 | Rudaityte-Lukosiene et al. (2020) |

Table 4

Parameters of intraspecific genetic variability in the 18S rRNA gene in Sarcocystis species or groups of closely related species detected in red deer, red foxes and hunting dogs from Grisons, Switzerland.

| Sarcocystis species | h/n | Hd | Identity (%) | S | p |
|---------------------|-----|----|--------------|---|---|
| S. hjorti | 49/74 | 0.897 | 98.01–100 | 84 | 0.00416 |
| S. tenella | 21/31 | 0.882 | 99.9–100 | 66 | 0.00835 |
| S. venatoriensis/S. ibérica | 44/55 | 0.962 | 97.6–100 | 78 | 0.00480 |
| S. linearis/S. taeniata | 10/10 | 1 | 95.7–99.7 | 35 | 0.01497 |
| S. ovalis | 4/6 | 0.8 | 98.93–99.85 | 7 | 0.00372 |
| S. pilosa | 3/3 | 1 | 99.54 | 3 | 0.000378 |
| S. silva | 4/4 | 1 | 96.8–98.9 | 11 | 0.00868 |
| S. gracilis | 7/9 | 0.917 | 98.81–99.26 | 17 | 0.00619 |
| S. capricolum | 2/2 | 1 | 98.8% | 1 | 0.00151 |
| S. capracanis/S. tenella | 5/10 | 0.667 | 98.9–100 | 6 | 0.00294 |
| S. miescheriana | 3/5 | 0.7 | 99.9–100 | 3 | 0.00126 |

Total 152/209

(Bosch et al., 2016; Boijsen et al., 2017), and O. jakutensis infection was reported in 26% of red deer of the Grisons region (Bosch et al., 2016). Although the occurrence of Onchocerca spp. infections, as well as their putative involvement in the development of greenish discoloration in some of the carcasses in the present study cannot be completely ruled out, we have neither observed macroscopic nodules, nor the presence of adult worms or microfilariae in any of the histological slides analysed.

In our study, histopathological examination revealed eosinophilic myositis and/or eosinophilic lymphoplasmacellular fasciitis as the underlying cause for the observed macroscopic changes in 73% of the analysed animals, and Sarcocystis infection was detected in 89 and 100% of these samples by histopathology or molecular analysis, respectively (Table 1). Eosinophilic myositis is an inflammatory condition of striated skeletal and cardiac muscle, mainly characterized by infiltration with eosinophils, followed by myocytes degeneration and building of granulomas at later stages (Dubre et al., 2016). The observed thickening of the vessel walls could be related to the parasite multiplication in the endotelial cells, which may trigger a hypertrophic reaction; however, no parasites could be observed associated with these changes and further investigation is needed to support this hypothesis. Affected animals are in most cases asymptomatic and the pathology is first detected at the abattoir level, leading to carcass condemnation (Dubre et al., 2016).

Several studies provided evidence for a causal association between Sarcocystis spp. infection and eosinophilic myositis in different animal species such as cattle (Gajadhar et al., 1987; Wouda et al., 2006; Arãoz et al., 2019; Jensen et al., 1986; Vangeel et al., 2013), sheep (Jensen et al., 1986), horses (Her et al., 2015) and alpacas (La Perle et al., 1999), including the experimental reproduction of the lesions in cattle (Vangeel et al., 2012). However, the details of the pathological mechanisms of sarcosporidiosis causing eosinophilic myositis/fasciitis and grey-greenish tissue discoloration are mostly unknown. Possible triggers for the immune response may be the release of antigens after rupture of the sarcocyst wall (Jensen et al., 1986; Gajadhar and Marquardt, 1992; Vangeel et al., 2012), as well as hypersensitivity mechanisms (Granstrom et al., 1989). Sarcocystis antigens (lysed sarcocysts) inoculated intramuscularly in cattle were shown to induce local lesions at the injection site, characterized by massive infiltration of eosinophilic granulocytes, reactive macrophages, T-cells and B-cells, resembling natural eosinophilic myositis (Vangeel et al., 2012). Besides, a genetic predisposition of some individual animals was also suggested to play a role in the pathogenesis of eosinophilic myositis (Herd et al., 2015; Granstrom et al., 1989). This hypothesis would be in agreement with the low prevalence of eosinophilic myositis despite the high prevalence of Sarcocystis infection in certain animal species like cattle (Arãoz et al., 2019; Vangeel et al., 2013). Observation of damaged intralesional sarcocysts in histologic sections has been an argument in favour of the role of these parasites in the pathogenesis of eosinophilic myopathy (Vangeel et al., 2013; Gajadhar and Marquardt, 1992; Wouda et al., 2006). In this study, sarcocysts were only seldom detected in the middle of the lesions but were frequently observed in the surrounding areas. However, it must be considered that the sensitivity of histological examination for the detection of Sarcocystis is limited (Jensen et al., 1986), and that the specific immune response against this parasite and its subsequent destruction could also account for a decreased histological detection of sarcocysts in the lesions (Vangeel et al., 2013; Gajadhar and Marquardt, 1992). Sarcocysts were detected in only one out of eight meat samples with normal appearance by microscopy; however, when DNA extraction was performed on 0.5 g meat samples (a larger sample than that analysed by histology), Sarcocystis DNA could be detected in five of those samples. Therefore, the presence of a Sarcocystis infection could not be ruled out in the control animals, but we can presume that the density of tissue cysts was lower than in animals with greenish carcass discoloration. Considering only samples from limb muscle (as these were tested histopathologically in all 34 animals in the study), at least one sarcocyst was observed in H&E stained sections from 23 out of 26 (89%) animals with macroscopic carcass changes and in only in 1 out of 8 (13%) of the samples from animals with normal carcass appearance (Fisher’s exact test, p = 0.000056), suggesting a higher parasite burden in animals with pathological changes.

This study revealed that red deer in the canton Grisons may serve as an IH for at least six Sarcocystis species. Sarcocystis hjorti was the most frequently detected species in red deer showing a greenish discoloration of the carcass, and it had been reported in animals showing this pathology in this region before (Stephan et al., 2012). However, also other Sarcocystis species (i.e. S. venatoriensis/S. ibérica, S. linearis/S. taeniata, S. pilosa and S. ovalis) have been now detected, suggesting that different Sarcocystis species might be involved in the pathogenesis of eosinophilic fasciitis/myositis in red deer. Accordingly, a study in Belgium showed that four different Sarcocystis species were associated with eosinophilic myositis in slaughtered cattle (Vangeel et al., 2013).

To date, 11 different Sarcocystis species forming sarcocysts of five major morphological types have been described infecting European red deer (Gjerde et al., 2017b). Mixed natural infections with several Sarcocystis species seem to be very common in free-ranging cervids.
region, confirming that the red fox is a natural DH of parasite was also detected in two red foxes (Fox 6 and 46) from the same
2014b; Prakas et al., 2019), and it was shown experimentally, that red
Europe. This species uses red deer and moose (Gjerde, 2014b; Prakas et al., 2019) and sika deer (Gjerde and Dahlgren, 2010; Gjerde, 2014b; Gjerde and Dahlgren, 2010). The methodology based on the 18S rRNA gene amplification with subsequent cloning and sequencing has been successfully used to identify co-infections with several Sarcocystis spp. and to gather information on potential DH of these parasites (More et al., 2016). Cloning is necessary because in the case of mixed infections, direct sequencing alone would fail to identify the species involved as we could also observe in the present study (Tables 2 and 3, Supplementary Tables 4 and 5). For discrimination between some closely related species affecting red deer such as S. tarrandi/S. elongata, S. venatoria/S. iberaica and S. linearis/S. taeniata, differences at the 18S rRNA region may not be enough, and the use of a further genetic marker such ascox1 may be needed (Gjerde, 2014b; Gjerde et al., 2017b). The same applies to certain Sarcocystis species affecting small ruminants such as S. tenella and S. capracanina (More et al., 2016). The procedure used in this study, based on extraction of DNA from large (0.5 g) muscle samples, had the advantage (vs. isolation of DNA from individual sarcocysts), that it allowed molecular diagnosis of coinfections with several Sarcocystis species or genotypes, and that both large and small-sized sarcocysts (which could have been probably missed during microscopic isolation of individual sarcocysts from fresh muscle) had similar chances to be present in the samples.

Sarcocystis hjorti was the most frequently detected species by molecular methods in Swiss red deer with grey-greenish discoloration of the carcass. It was observed in 19 out of 26 (73%) animals from all four sampled regions in Grisons. This is also the most frequent Sarcocystis species in Norwegian red deer, where a prevalence of 95% (35/37) was recorded (Dahlgren and Gjerde, 2010a). Sarcocystis hjorti was also reported from red deer in Lithuania (Prakas and Butkauskas, 2012) and Spain (Gjerde et al., 2017b), suggesting a widespread distribution in Europe. This species uses red deer and moose (Alces) as IH (Gjerde, 2014b; Prakas et al., 2019), and it was shown experimentally, that red foxes and arctic foxes (Vulpes lagopus) could act as DH of S. hjorti isolated from moose (Dahlgren and Gjerde, 2010b). In the present study, this parasite was also detected in two red foxes (Fox 6 and 46) from the same region, confirming that the red fox is a natural DH of S. hjorti.

The second most frequently observed species was the cluster Sarcocystis venatoria/S. iberaica in 14 out of 26 (54%) animals from all four sampled regions (i.e. Davos, Ruessn, Cunters and Filisur). These species were detected between 2014 and 2016 in red deer in Spain (Gjerde et al., 2017b), and this is the first record outside de Iberian Peninsula. The DH are unknown but canids were suggested (Gjerde et al., 2017b). No infection with Sarcocystis venatoria/S. iberaica was recorded in foxes or dogs in this study.

In one of the analysed red deer (Deer 6), infection by S. ovalis was detected by both direct sequencing and cloning. This Sarcocystis species uses red deer, moose (Gjerde, 2014b; Prakas et al., 2019) and sika deer (Irie et al., 2017; Rudaitiene-Lukosiene et al., 2018) as IH and corvid birds as DH. So far, S. ovalis has been only detected in red deer from Norway (Dahlgren and Gjerde, 2010a; Gjerde, 2013), moose from Norway, Canada (Dahlgren and Gjerde, 2008) and Lithuania (Prakas et al., 2019) and sika deer from Japan (Irie et al., 2017) and Lithuania (Rudaityte-Lukosiene et al., 2018), and this represents the first record of S. ovalis in Central Europe. The European magpie (Pica) and the Japanese jungle crow (Corvus macrorhynchos) have been confirmed as definitive hosts for S. ovalis (Gjerde and Dahlgren, 2010; Irie et al., 2017). However, in Europe, other corvid birds, such as the carrion crow (Corvus corax) and the common raven (Corvus corax) are supposed to act as additional and possibly more important definitive hosts for S. ovalis, because they are the main corvid species feeding on carcasses of large animals (Gjerde and Dahlgren, 2010; Gjerde, 2014b; Gjerde and Dahlgren, 2010). Corvid birds are common in forest areas all over the canton Grisons and have a diverse diet, including carrion. We have found S. ovalis in only one out of 45 analysed muscle samples from Red deer, suggesting that sarcocysts of S. ovalis were not present in high numbers in the samples, or that this species does not frequently occur in the region. A low frequency of infections was also observed in studies from Lithuania, in which S. ovalis was only detected in 2 out of 33 (6.4%) examined sika deer (Rudaitie-Lukosiene et al., 2018). Accordingly, it was reported that S. ovalis and other Sarcocystis species using corvids as DH seem to produce only low-to-moderate-intensity infections in the IH, in contrast to species being transmitted by canids (Gjerde and Dahlgren, 2010). This could be due to a restricted ability of the parasite to multiply in the IH, or to reduced environmental contamination through the DH, leading to infections with few sporocysts (Gjerde and Dahlgren, 2010).

In two red deer (Deer 14 and 19) from the same sampling region (i.e. Cunters), sequences with 100 and 99.9% identity with GenBank® sequences of S. pilosa from sika deer (Cervus nippon)LC466183 (Irie et al., 2019) were detected. Sarcocystis pilosa has been so far described infecting sika deer in Lithuania (Prakas et al., 2016) and Japan (Abe et al. 2019a, 2019b; Irie et al., 2019), but there are no previous records of infection in red deer. Our findings are highly suggestive that red deer could also act as IH of this parasite. Recently, red foxes have been found to serve as DH for S. pilosa in Japan (Irie et al., 2020).

In three further red deer (Deer 11, 13 and 18) from the same sampling region in Grisons mentioned above (i.e. Cunters), sequences with 99.2–100% identity with GenBank® entries of S. linearis derived from red deer (KY973371, KY973372) (Gjerde et al., 2017b) and roe deer (MN334301) (Rudaitie-Lukosiene et al., 2020) were revealed by cloning. This recently described Sarcocystis species uses red deer (Gjerde et al., 2017b), roe deer (Capreolus) (Gjerde et al., 2017a; Rudaitie-Lukosiene et al., 2020) and moose (Prakas et al., 2019) as IH. Its DH was still not described, but based on its phylogenetic position, canids were suspected to play this role (Gjerde et al., 2017a). It is to note, that S. linearis shares a high degree of identity (97.9–99.7%) at the 18S rRNA sequence with S. taeniata (Gjerde et al., 2017b), a Sarcocystis species infecting moose (Dubey et al., 2016), and it is not possible to unequivocally separate both species on the sole basis of this gene (Gjerde et al., 2017b). Moreover, it was postulated that reported 18S rRNA sequences of Sarcocystis from red deer in Lithuania (JN256126–JN256127) (Prakas et al., 2016) and Argentina (KT626602) (Reissig et al., 2016), originally attributed to S. taeniata may actually correspond to the new described species S. linearis (Gjerde et al., 2017b). Accordingly, also the sequences obtained in our study showed a high similarity (98.8–99.9% identity) with GenBank sequences annotated as S. taeniata (Table 2, Supplementary Table 5). The high degree of identity as morphological information of individual sarcocysts was lacking, we have named these sequences as S. linearis/S. taeniata and annotated them as Sarcocystis sp. in GenBank®. Besides, we have provided a phylogenetical tree to show their relationship with other reported sequences (Supplementary Fig. 1). This shows that although the 18S rRNA gene marker has been widely used to differentiate Sarcocystis species it has some limitations. It has been also reported that S. venatoria and S. iberaica may share an identity of 99.2–100% at the 18S rRNA sequence...
would be needed to unequivocally discriminate between these closely related species affecting red deer (Gjerde et al., 2017b).

*Sarcocystis* was detected by PCR in five out of eight red deer with normal carcass appearance. By cloning of the PCR products, three species could be identified: *S. hjorti* in three animals and *S. venatoria/S. iberca* in two other animals. Interestingly, sequences with 99.7% identity with GenBank sequences of *S. silva* (KY190065) were identified in one of the animals co-infected with *S. venatoria/S. iberca* (Deer 29). This is noteworthy, because *S. silva* had been so far only reported from moose and roe deer (Dubey et al., 2016), and this would represent the first record of this species in red deer. The DH of *S. silva* is still unknown, but based on the phylogenetic position of this species, they do not appear to be canids (Gjerde, 2012). Our molecular findings of *S. silva* and also *S. pilosa* in red deer would need further morphological investigation, to confirm if these species infect red deer, or if these findings represent other still not described *Sarcocystis* species with similar sequence homology.

As it was already reported for various *Sarcocystis* species affecting cervids (Gjerde, 2012; Gjerde et al. 2017a, 2017b; Rudaihtyte-Lukišienė et al., 2020), a great intraspecific genetic variability at the 18S rRNA gene sequence level was also observed for *S. hjorti*, *S. venatoria/S. iberca*, *S. ovalis*, *S. pilosa*, *S. silva*, *S. capreolicanis* and *S. gracilis* in this study (Table 4).

Interestingly, no *Sarcocystis* species with felids as suspected DH based both on phylogenetical and epidemiological observations, such as *S. elongata*, *S. truncata* and *S. tarandi* (Gjerde, 2014b) were found in red deer in this study, suggesting that felids do not play a major role in the epidemiology of sarcocystosis in red deer in this region. This observation is supported by the fact that and only low numbers of wild felids such as lynxes are known to be present in the canton of Grisons (Bundesamt für Umwelt BAFU, 2016) and wild cats (*Felis silvestris*) are not supposed to occur in the region (https://www.wildtier.ch/projekte/wildkatzenmonitoring).

A further aim of the study was to investigate the involvement of red foxes and hunting dogs as definitive hosts of *Sarcocystis* species affecting red deer. Over the last few years, numerous studies have been performed to enlighten the life cycle of *Sarcocystis* affecting cervids (Dahlgren and Gjerde, 2010b; Irie et al., 2017, 2020; Gjerde and Dahlgren, 2010); however, for several species, the definitive hosts and many aspects about their epidemiology are still unknown. Due to the broad dietary habits, foxes may serve as DH for several *Sarcocystis* species (Table 4). In one of the dogs (Dog 5), molecular findings suggested a coinfection with *S. gracilis* and *S. capreolicanis*. These *Sarcocystis* species use roe deer as IH and dogs and foxes as DH (Dubey et al., 2016). In the further dog (Dog 6), cloned sequences with either 99.9% identity to GenBank sequences of *S. gracilis* (MN334289) or 98.8–99.1% identity with *S. linearis* (KY973371, MN334294) (Gjerde et al., 2017b; Rudaihtyte-Lukišienė et al., 2020) and *S. taeniata* (KU753890) (Prakas et al., 2016) were obtained. These findings suggest that domestic dogs may be DH of *S. linearis* and/or *S. taeniata*.

5. Conclusion

This study revealed a high frequency of *Sarcocystis* infection in red deer in Grisons and the occurrence of at least five *Sarcocystis* species (i.e. *S. hjorti*, *S. venatoria/S. iberca*, *S. pilosa*, *S. linearis/S. taeniata*, and *S. ovalis*) in animals with grey-greenish tissue discoulouration of the carcasses, and three species (i.e. *S. hjorti*, *S. venatoria/S. iberca* and *S. silva*) in animals with normal carcass appearance. First evidence of infection with *S. pilosa* and *S. silva* in red deer is provided; however, further morphological studies are needed to support these molecular findings.

Red foxes and hunting dogs from the region were shown to transmit *Sarcocystis* species affecting wild cervids, domestic ruminants and swine. Red foxes were confirmed as natural DH for *S. hjorti*, and hunting dogs are probably DH for *S. linearis/S. taeniata*. Moreover, also a *Sarcocystis* species transmitted by corvid birds (i.e. *S. ovalis*) was detected in red deer with eosinophilic myositis/fasciitis, representing the first record of this parasite in Central Europe.

Authors contribution

WB and PD designed and supervised the study. WB supervised and performed laboratory work, analysed the results, and wrote the manuscript. CAAR carried out molecular analysis, was responsible for cloning work and molecular result analysis. DB organised and performed the sampling, carried out preliminary laboratory work and provided a draft of the study within the frame of his Master thesis at the Vetsuisse Faculty, University of Zurich. MR performed the histopathological analysis.
All authors revised, contributed, and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to acknowledge Dr Georg J. Brosi, leader of the Department of hunting and fishing of Grisons, to all the gamekeepers and hunters and Olivia Beerli for their help with the sampling. Special thanks go to Eggenberger Erwin for his help with the organisation of the sampling and the documentation of the red deer carcasses and to Francesca Gori for her excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.09.005.

Direct sequencing of PCR products from Deer 3, 8, 9 and 26 revealed sequence ambiguities, suggesting mixed infection with different Sarcocystis species, but cloning of these products was not attempted. Samples from Deer 27, 28 and 31 (control group) yielded negative PCR results for Sarcocystis.

ID: identification; bp: base pairs; in Sequence ID: “PCR” refers to a sequence obtained by direct sequencing and “clone” to a sequence obtained by cloning of a PCR product in a plasmid vector. Detailed data to each sequence is presented in Supplementary Table 4.

ID: identification; bp: base pairs; in Sequence ID: “PCR” refers to a sequence obtained by direct sequencing and “clone” to a sequence obtained by cloning of a PCR product in a plasmid vector. Detailed data to each sequence is presented in Supplementary Table 5.

h/n = haplotypes/number of sequences (obtained by cloning and direct sequencing). Hdp = haplotype diversity, Identity = percentage of similarity between the sequences of the same species, S = number of segregating sites, \( \pi = \) nucleotide diversity. h: number of haplotypes. All compared sequences were trimmed from the primer regions and had the same length.

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