Sarcocystis spp. infection in South American deer huemul (Hippocamelus bisulcus) and pudu (Pudu pudu) from Patagonian National Parks, Argentina

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Abstract
Sarcocystis spp. are intracellular protozoan parasites with heteroxenous life cycles. This study described Sarcocystis spp. infection in adult South American native deer huemul (Hippocamelus bisulcus) and pudu (Pudu pudu). Heart, diaphragm, tongue, and skeletal muscle samples were collected from 5 huemuls and 2 pudus, found dead in National Parks. Direct microscopic examination, transmission electron microscopy, PCR, and sequencing were performed. Sarcocystis spp. microscopic thin-walled cysts were identified in 3 huemuls and 1 pudu. Several cysts from 1 huemul and 1 pudu were observed by TEM; ultrastructure was similar to previously reported as cyst wall type 17 and types 2 and 8, respectively. Fragments of the 18S rRNA and cytochrome c oxidase subunit I (cox1) genes were amplified and sequenced from 3 individual cysts from 2 huemuls and 2 cysts from the pudu. The sequences from huemuls showed a high identity among them (> 99%) at both amplified targets. The highest identities were > 99.7% at 18S rRNA and 93% at cox1 with S. tarandivulpes sequences. The 18S rRNA gene sequences from pudus showed an identity > 99.7% with Sarcocystis sp., S. taeniata, and S. linearis sequences, while the cox1 sequences were different, one showing 99.42% identity with S. venatoria and the other 98.22% with S. linearis. A single species, similar to S. tarandivulpes, was identified in all huemul samples while 2 molecularly different Sarcocystis spp. were found in 1 pudu with high similarities to either S. venatoria or to S. linearis, S. taeniata-like, and S. morae. Based on the cox1 sequence identities, at least the Sarcocystis sp. in huemuls might represent a new species, primarily occurring in this host. Additional sarcocyst isolates from both hosts need to be examined molecularly in order to firmly establish whether these species are indeed native to huemuls and/or pudus or are derived from introduced deer species.

Keywords Sarcocystis · Ultrastructure · PCR-sequencing · Native cervids/wild ungulates

Introduction
Sarcocystis spp. are intracellular protozoan parasites with heteroxenous life cycles, most of them using herbivores as intermediate host and carnivores/omnivores as definitive host (Dubey et al. 2016). Sarcocystosis on intermediate hosts is generally asymptomatic and chronic, producing muscle cysts or sarcocysts. During the last years, morphological characterization based on ultrastructure of cyst walls and molecular identification of several Sarcocystis species were performed in wild ungulates (Dubey et al. 2016). North American and European cervids, including elk (Cervus canadensis), white-tailed deer (Odocoileus virginianus), mule deer (Odocoileus hemionus), roe deer (Capreolus capreolus), moose (Alces alces), and red deer (Cervus elaphus) are intermediate hosts of several Sarcocystis species (Dahlgren et al. 2008; Dahlgren...
and Gjerde 2010; Gjerde 2013; Dubey et al. 2016; Chang Reissig et al. 2016; Gjerde et al. 2017,b).

Huemuls (Hippocamelus bisulcus) and pudus (Pudu puda) are South American native deer species. Both deer species have their natural habitat in the Andean Mountains (Duarte and González 2010) and are included in the International Union for Conservation Nature Red List of threatened species (IUCN 2020). A great variety of parasites were reported in huemuls and some have significant effects on their natural populations. Parasites commonly identified in domestic ruminants such as Ostertagia spp., Strongylus spp., Moniezia spp., Taenia spp., Fasciola hepatica, and Bovicola caprae were quoted in huemuls from Chile and Argentina. Eimeria spp. infection was reported in huemuls, which showed extreme susceptibility to clinical coccidiosis when sharing pastures grazed by sheep (Duarte and González 2010). However, little is known about intracellular protozoan infections in these Andean cervids, which generally require postmortem tissue samples for proper diagnosis. The presence of Sarcocystis spp. cysts in pudu muscles were previously identified in Chile (Lobão-Tello et al. 2017). However, morphological and molecular descriptions of Sarcocystis spp., using huemuls and pudus as intermediate hosts, have not been published.

The aim of this study was to describe the muscular parasitism by Sarcocystis spp. in huemuls and pudus, both native deer species from South America. To the best of our knowledge, this is the first study which describes and identifies the morphological and molecular features of Sarcocystis spp. cysts in pudus and huemuls.

Material and methods

Samples

Five adult huemuls (5 female) and 2 adult pudus (1 female and 1 male) were found dead by park rangers in 5 national parks: Los Alerces (LANP), Nahuel Huapi (NHPN), Lanín (LNP), Lago Puelo (LGP), and Los Glaciares (LGNP), all located in Patagonia, Argentina. Full necropsies were performed in all animals, and samples of heart (apical area), diaphragm, tongue, and skeletal muscle (hindquarter) were collected and refrigerated at 4–8 °C until microscopic examination was conducted.

Direct microscopic examination

The samples for each animal were processed as pooled muscle samples following the methodology of direct microscopic examination previously described (Moré et al. 2011). Briefly, 5 to 10 g of muscles were grounded in a tissue homogenizer with the addition of 50-ml phosphate buffered saline (PBS), pH 7.2, filtered and centrifuged. Around 3-ml homogenate aliquots were placed in a Petri dish, diluted with PBS, and observed in an inverted microscope (Nikon, TMZ). Samples containing at least one Sarcocystis sp. cyst or cyst portion were considered as positive. Observed sarcocysts were collected and glutaraldehyde fixed for transmission electron microscopy (TEM). Individual cysts or cyst portions were preserved frozen at −20 °C in 1.5-ml DNase-free microtubes for DNA extraction and molecular identification by polymerase chain reaction (PCR) and sequencing.

Transmission electron microscopy (TEM)

Several sarcocysts (around 10 from 1 huemul and 1 pudu positive sample) were collected using micropipettes during pooled muscle samples microscopic examination, fixed in 2% glutaraldehyde, and processed and analyzed by TEM as previously described (Moré et al. 2011).

PCR and sequencing

DNA extraction was performed from individual sarcocysts collected during the muscles’ direct microscopic examination, using a commercial kit according to manufacturer’s instructions (Wizard genomics, Promega, USA). A fragment of Sarcocystis spp. 18S rRNA gene was amplified by PCR using the primers SarcoFext and SarcoRext as previously described (Moré et al. 2013). Additionally, fragments of mitochondrial cytochrome oxidase subunit 1 gene (cox1) were amplified using the primers SF1 with 3 different reverse primers (SR5, COIRm, and SRD8) according protocols described by Gjerde (2013).

Amplification products (with an estimated concentration of at least 40 ng/μl) were purified using a commercial kit according to manufacturer instructions (Wizard SV clean up system, Promega), and submitted for sequencing to Macrogen Inc., South Korea (http://www.macrogen.com), with both primers used for each amplification. Sequences obtained were aligned and analyzed using the Geneious software (R9 version). Consensus sequences obtained were compared with others reported in GenBank by BLAST analysis.

The cox1 sequences obtained were aligned with other sequences from Sarcocystis spp. using cervids and ruminants as intermediate host and a phylogenetic tree was constructed using the neighbor-joining method based on Tamura-Nei genetic distance model, 1000 bootstraps, and no outgroup (Geneious, R9).

Results

Direct microscopic examination and TEM

Sarcocystis spp. infection was identified by direct microscopic examination of muscle samples of 3 huemuls from LANP (n =
2) and LGNP (n = 1) and 1 pudu from LNP. No sarcocysts were found in 2 huemul samples (from LGNP and LPNP) and from 1 pudu. All positive pool of muscles evidenced microscopical fusiform sarcocysts, clearly divided by septa. No macroscopic sarcocysts were observed.

Sarcocyst morphology is described in relation with cervid species:

**Huemul samples** A total of 12 sarcocysts or cysts portions were observed at optical microscopy (4 from each positive huemul pooled muscle samples). All cysts measured up to 650-μm long and 80-μm wide. The sarcocyst wall was relatively thin (≤ 2 μm) and no clearly visible protrusions were observed (Fig. 1a). A total of 3 cysts from one huemul sample were observed by TEM and evidenced an undulated cyst wall with regular knob or bulb-like protrusions interconnected by microfolds (Fig. 1b), similar to type 17 described by Dubey et al. (2016). The ground substance layer was 0.5-μm thick, without granules and microtubules. The bradyzoites were vacuolated and no proper descriptions were achieved (Fig. 1b).

**Pudu samples** A total of 8 sarcocysts or cyst portions were observed at microscopy from pudu muscles. All cysts measured up to 800-μm long and 70-μm wide. The sarcocyst wall was thin (≤ 1 μm) and short protrusions were observed (Fig. 2a). By TEM, 2 cysts were observed and showed a thin cyst wall formed by an undulated membrane from which arise bent thin ribbon-like protrusions folded over the cyst surface, giving a T-shape appearance (Fig. 2b). The ground substance layer measured 0.4–0.6 μm and lacked microtubules. The TEM cyst wall type is similar to the undulated primary cyst wall with regular knob like protrusions interconnected by microfolds (arrow). The bradyzoites appear vacuolated (double arrow).

**PCR and sequencing**

DNA from 7 individual cysts from the pudu sample and 11 cysts from 2 huemul samples were amplified by PCR. Out of all samples, only 3 huemul cysts (from 2 huemuls, both from LANP) and 2 cysts from the pudu produced amplicons with proper concentration for sequencing. The only primer combination which can produce acceptable cox1 amplicons was SF1/SR8D. Results from molecular analysis and BLAST comparisons are presented in Table 1. All consensus sequences were uploaded in GenBank. The 3 individual cysts from 2 huemul samples share 99.82–100% identity at 18S rRNA gene fragment sequences and 99.11–99.80% identity among them at the cox1 sequences. The 2 individual cysts from the pudu sample have a 100% identity at 18S rRNA gene fragment sequences and 93.99% identity at the cox1 fragment sequences. One of the samples (P13-1C) showed a poor-quality cox1 sequence; it was trimmed resulting in a shorter consensus (Table 1).

A multi-alignment comparing all sequences obtained from huemuls against the ones obtained from pudus showed a relative homology of 96.28–96.53% and 79.37–80.68% at 18S rRNA and the cox1 gene fragment sequences, respectively.

In the performed phylogenetic tree, the 3 cox1 sequences from huemuls were placed together and in a branch along with *S. tarandivulpes*, *S. mehlhorni*, and *Sarcocystis* spp. from

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**Fig. 1** Microscopical sarcocysts detected in huemul (*Hippocamelus bisulcus*) muscles. a Optical microscopy image from a cyst portion. b Transmission electron microscopy (TEM) image of the cyst wall. Note
mooose. The sequences from pudus were placed separated between them and from the obtained from huemuls. The sequence from sample P13-1A appears truncal in the tree together with *S. venatoria* and *S. iberica*, while the sequence P13-1C was placed in a branch (with low consensus support) along with *S. linearis*, *S. morae*, *S. taeniata-like*, *S. cervicanis*, and *S. grueneri* (Figure S1).

### Discussion

The fauna diversity of the South America Andean region is unique, harboring species such as the native cervids huemul and pudu (Duarte and González 2010). Huemuls and pudus are included as endangered and near threatened by the IUCN Red List (IUCN 2020), respectively. Their population trend is

![Fig. 2](image)

**Fig. 2** Microscopical sarcocysts detected in pudu (*Pudu puda*) muscles. **a** Optical microscopy image from a cyst portion. **b** Transmission electron microscopy (TEM) image of the cyst wall from a microscopical sarcocyst. Note the undulated primary cyst wall from which arise bent thin ribbon-like protrusions folded over the cyst surface with a T-shape (arrows). Electron lucid amyllopectin granules (am) are uniformly distributed within bradyzoites.

### Table 1  Sequencing and BLAST comparison results from huemul and pudu individual sarcocysts samples

| Sample ID | Sex | National Park | Cyst ID | Target gene | Bp   | BLASTn identity (#) | GenBank Accession number |
|-----------|-----|---------------|---------|-------------|------|---------------------|-------------------------|
| Huemul/13-1 | F   | Los Alerces   | H13-1A  | 18S rRNA    | 845  | 99.76% *S. tarandivulpes* (EF467657), 99.65% *S. mehlhorni* (KT378042) and *Sarcocystis* sp. (MK224430) | MT137767 |
|           |     |               | COI     |             | 1016 | 93.22% *S. tarandivulpes* (KC209718), 92.48% *S. mehlhorni* (KT378045), 91.16% *Sarcocystis* sp. (MK234169) | MT180293 |
| Huemul/14-2 | F   | Los Alerces   | H14-2   | 18S rRNA    | 829  | 99.88% *S. tarandivulpes* (EF467657), 99.76% *S. mehlhorni* (KT378042) and *Sarcocystis* sp. (MK224430) | MT137769 |
|           |     |               | COI     |             | 1017 | 93.50% *S. tarandivulpes* (KC209718), 92.87% *S. mehlhorni* (KT378045), 91.44% *Sarcocystis* sp. (MK234169) | MT180295 |
| Pudu/13-1  | M   | Lanin         | P13-1A  | 18S rRNA    | 834  | 100% *Sarcocystis* sp. (LC405946), 99.88% *S. taeniata* (KF831283) and *S. linearis* (KY019055) | MT137770 |
|           |     |               | COI     |             | 1030 | 99.42% *S. venatoria* (MT070691), 95.73% *S. iberica* (KY973286) | MT180296 |
|           |     |               | P13-1C  | 18S rRNA    | 839  | 99.88% *S. taeniata* (KF831283) and *S. linearis* (MN334301), 99.76% *Sarcocystis* sp. (LC405946) | MT137771 |
|           |     |               | COI     |             | 233  | 98.22% *S. linearis* (MN339331), 97.78% *S. taeniata* (LC481093) | MT180297 |

References: F= female, M= male, Bp= sequence base pairs. * Accession numbers of sequences retrieved by BLAST alignment.
Sarcocystis, was identified in 2 huemuls from LANP. However, morphological descriptions by optical and transmission electron sections of muscle samples (Lobão-Tello et al. 2017) previously recorded in pudu, based on the observation of histological sections of muscle samples (Lobão-Tello et al. 2017). In the Patagonia region, parasite infections, including Sarcocystis taeniata, Taenia ovis krabbei, and Fasciola hepatica, were described in exotic free-range red deer (Flueck and Jones 2006; Chang Reissig et al. 2016; Chang Reissig et al. 2018). Usually, red deer population and livestock cohabit with native Patagonian ungulates, even in protected areas, such as NHNP, LNP, and LANP. In the jurisdiction of these National Parks, the inhabitants carry out tourism activities and also agricultural livestock subsistence production. The infection with Sarcocystis spp. has been previously recorded in pudu, based on the observation of histological sections of muscle samples (Lobão-Tello et al. 2017). However, morphological descriptions by optical and transmission electron microscopy and molecular identification of Sarcocystis spp. from pudu and huemul muscles have not been performed.

A single species, morphologically and molecularly similar to S. tarandivulpes, was identified in 2 huemuls from LANP while 2 molecularly different Sarcocystis spp. were found in 1 pudu from LNP, with high similarities to either S. venatoria or to S. linearis, S. taeniata-like, and S. morae. Samples were obtained from animals with different decomposition degrees, which could hinder the diagnosis. Moreover, 2 huemuls and 1 pudu analyzed from LPNP, LGNP, and NHNP showed no Sarcocystis spp. cysts, but the infection may not be completely ruled out. The real prevalence could be underestimated when samples are autolytic, since cyst disruptions during homogenization avoid their direct microscopic observation (Moré et al. 2011, 2013). In addition, the samples processed from huemuls were partially autolytic; all cysts observed at TEM showed bradyzoites degeneration and the cyst wall TEM type description is not optimal (Fig. 1b). The same is true for the molecular identification, where most cyst DNA samples failed to amplify properly for further sequencing, probably due to a DNA degradation process. We used the 18S rRNA gene as the first step for species molecular identification using primers validated for cyst forming coccidia (Dahlgren et al. 2008; Moré et al. 2013). Once a positive amplicon was obtained from the mentioned gene, we have performed different PCRs (using different reverse primers) to obtain cox1 gene fragments as reported by Gjerde (2013). The combination of SF1 and SRD8 was the only producing amplicons both from huemul and pudu sarcocyst samples; the reverse SR5 and COIRm failed to render products. The same primer combination was successfully used to amplify cox1 fragments of S. morae in fallow deer (de Las Cuevas et al. 2019).

The sarcocysts obtained from huemuls showed a similar TEM type to S. tarandivulpes, as well as the highest identity at 18S rRNA gene with a sequence from this species isolated from reindeer (Dahlgren et al. 2007; Gjerde 1985). All the sequences were obtained from individual cysts from 2 huemuls and showed a high identity among them. The comparison of cox1 sequences evidenced a low identity (around 93%) with a S. tarandivulpes sequence (KC209718). In the phylogenetic tree, all huemul cox1 sequences were positioned, with high consensus support, along with sequences from S. tarandivulpes, S. mehlhorni, and Sarcocystis spp. from moose, suggesting a potential common ancestor for these species. Several Sarcocystis species have a conserved 18S rRNA gene sequence, but differ at cox1, and SAG genes, from which several haplotypes have been described (Gjerde 2013, 2014a, b; Gondim et al. 2017). At 18S rRNA sequences, a high identity with S. mehlhorni from black-tailed deer (KT378042) (Calero-Bernal et al. 2015) and a Sarcocystis sp. from moose diaphragm (MK224430) was also evidenced (Prakas et al. 2019). Analyzing altogether, we considered the species observed in huemuls as closely related with S. tarandivulpes and S. mehlhorni, but the sequence divergence at the cox1 gene was higher than expected for intraspecific sequence variation. Thus, other species using cervids as intermediate hosts have shown an intraspecific cox1 sequence identity higher than 98.5% (Gjerde 2013). Therefore, the Sarcocystis sp. detected in huemuls could be a new species using huemuls as intermediate hosts and probably canids as definitive hosts. The last assumption is based on the sequence homology with S. tarandivulpes (as well as the phylogenetic tree placement), and that these native cervids are not only consumed in the wild (as prey or carrion) by different native fox species but also frequently threatened by domestic canids (Canis lupus familiaris) (IUCN 2020). Additionally, this potentially new species should be named after proper morphological description became available as well as with more information and evidence about natural definitive hosts.

The Sarcocystis spp. obtained from 1 pudu differed both morphologically and molecularly from the one identified in huemul samples. The sarcocyst TEM wall type was similar to the described for S. wapiti (type 2) described in elk from the USA and the type 8a recorded for S. morae in fallow deer from Spain (Delgado de las Cuevas et al. 2019; Dubey et al. 2016). The T-shape of ribbon-like protrusions resembled the
structure reported by SEM for *S. linearis* from roe deer in Italy (Gjerde et al. 2017). However, sequence identities were higher with other species than with *S. morae*. The sequences of 2 individual cysts’ DNA samples have 100% homology at 18S rRNA gene (however were only 89% overlapping sequences) and showed the highest identity values with *Sarcocystis* sp. from sika deer meat (LC405946), *S. taeniata* from Canadian moose (KF831283), and *S. linearis* from roe deer in Italy (KY019055) and Lithuania (MN334301) (Gjerde 2014b; Gjerde et al. 2017; Sugita-Konishi et al. 2019; Rudaiytė-Lukošienė et al. 2020). On the other hand, the *cox1* sequences differ in around 10% between them and revealed different identities by BLAST. The cyst sample identified as P13-1A showed the highest identity (99.42%) with *S. venatoria* from red deer in Spain (KY973287). The consensus sequence from cyst identified as P13-1C showed the highest identity (98.22%) with *S. linearis* sequence from Capreolus capreolus in Spain (MN339331) followed by 97.78% identity with a *S. taeniata*-like sequence from sika deer (*Cervus nippon centralis*) in Japan (Abe et al. 2019). This difference was confirmed and reinforced by the neighbor-joining tree, where one sequence was related with *S. venatoria* and *S. iberca* while the other was in a low consensus branch along with *S. morae*, *S. taeniata*-like, *S. linearis*, *S. grueneri*, and *S. cervicanis*. The *cox1* sequences obtained from P13-1C cyst have low quality (special the one retrieved with reverse primer) and resulted in a shorter consensus which could partially explain the differences. However, we considered that the *cox1* sequences’ difference between the 2 cysts processed from one pudu is large enough and probably corresponds to different species. In addition, the different results obtained from 18S rRNA gene and *cox1* in sample P13-1A could indicate a mixture from two species DNA (potentially carried over during homogenization procedure) and that one or the other was amplified with each primer set, possibly because reverse primer SR8D matched the *S. venatoria*-like species better than the *S. linearis*/ *S. taeniata*-like species. Unfortunately, we have no morphological evidence to support such ideas since all the TEM-observed cysts from the pudu revealed the same cyst wall pattern, suggesting an “overrepresentation” of such morphotype in the sample. Using species-specific *cox1* primers could help to improve species identification in pudu samples in future studies. Processing more samples from pudus could help identify and differentiate the 2 species detected in the present study (Gjerde 2013). In summary, the high *cox1* sequence identity with *S. venatoria* sequences from red deer (sample P13-1A) suggests that pudus could be infected with *S. venatoria*-like species (probably originated from introduced red deer), while the *cox1* sequence of the *S. linearis*/ *S. taeniata*-like species is too short to make a clear identification. Hence, longer sequences are needed to determine if this is a new species different from *S. linearis* and/or *S. taeniata*.

In Argentina, investigations in parasitological diseases have determined high occurrences of sarcocystosis in livestock (Moré et al. 2011, 2013), and *S. taeniata* was previously described as having a significant prevalence in red deer (Chang Reissig et al. 2016). In northern Argentinean Patagonian National Parks, like LANP, NHNP, and LNP, domestic animals are present and, in some of them, red deer invasion is reported. In contrast, the other 2 National Parks, LGNP and LPNP, have limited and reduced domestic animals in their territory, and also no red deer invasion has yet been established. All the rural families from the National Park have dogs (*Canis familiaris*) for livestock work or companion, but also, the protected areas have the presence of native carnivores like the puma (*Puma concolor*), Andean fox (*Lycalopex culpaeus*), gray fox (*Lycalopex griseus*), Geoffroy’s cat (*Leopardus geoffroyi*), and güïña cat (*Leopardus guigna*). Our morphological and molecular findings suggest that a canid species may be a definitive host for the identified *Sarcocystis* spp. in huemuls and pudus. However, pumas are included as their main predator and could be also considered as a potential definitive host for *Sarcocystis* spp. producing sarcocysts in huemuls and pudus (Duarte and González 2010). Nevertheless, Felidae animals are considered as a definitive host from *Sarcocystis* spp. producing thick-walled and mainly macroscopic cysts in ruminants, which are not the case described here (Dubey et al. 2016). All the *cox1* sequences obtained in the present study were positioned in separated branches than species using felids as definitive hosts (Figure S1). On the other hand, the animals from which *Sarcocystis* spp. infected animals came from LANP (huemuls) and LNP (pudu) were interspecific interactions with domestic dogs are more frequent than other Patagonian National Parks. Therefore, the introduced red deer along with the wild canids and domestic dogs’ presence in National Parks could be considered as risk factors for huemul and pudu *Sarcocystis* spp. infection.

It has been suggested that there is an increase in the transmission of diseases in areas with habitat fragmentation where contact between wild, domestic animals and humans is most frequent (Gortázar et al. 2014). However, it is difficult to identify the interactions between wild and domestic animals in order to understand the ecology of parasitism and the role of the different hosts. South American cervids, such as the huemul and pudu, act as intermediate hosts of at least 2 different *Sarcocystis* spp. and we consider it highly probable that canids (like foxes and domestic dogs) playing a role as definitive hosts for these species, since they are frequently observed in the study area and predate/scavenge over these cervids.

Further studies should focus on identifying potential definitive hosts for the *Sarcocystis* spp. infecting huemuls and pudus as intermediate hosts and will allow us to get proper species description and naming.
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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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