Sarcocystis mehlhorni, n. sp. (Apicomplexa: Sarcocystidae) from the black-tailed deer (*Odocoileus hemionus columbianus*).

Rafael Calero-Bernal[1], Shiv K. Verma[1], Camila Cerqueira-Cézar[1], Laurence M. Schafer[2], Erna Van Wilpe[3], Jitender P. Dubey[1]

[1]United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Animal Parasitic Diseases Laboratory, Beltsville, Maryland, 20705-2350, USA.

[2]United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, 720 O'Leary Street NW, Olympia, WA 98502, USA.

[3]Department of Anatomy and Physiology, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, 0110, South Africa.

Corresponding author: J.P. Dubey, *jitender.dubey@ars.usda.gov*

Abstract

Infection with *Sarcocystis* is common in many species of wild cervids but none is reported from the black-tailed deer (*Odocoileus hemionus columbianus*). Here, we report *Sarcocystis* infection in two black-tailed deer from northwest USA for the first time. Sarcocysts were microscopic, up to 556 µm long and mature. The sarcocyst wall was up to 1.39 µm thick, and had rectangular 1.17 µm long villar protrusions, type 17, with thin (230 nm) electron dense ground substance layer. Molecular characterization and phylogenetic analysis indicated that *Sarcocystis* in the black-tailed deer is related to structurally distinct *Sarcocystis* species in cervids. A new name, *Sarcocystis mehlhorni*, is proposed for the *Sarcocystis* species in black-tailed deer.

Keywords

Black-tailed deer. *Odocoileus hemionus columbianus*. *Sarcocystis.* Ultrastructure. Phylogeny.

Introduction

Infection with *Sarcocystis* is common in many species of wild cervids but none is reported from the black-tailed deer (*Odocoileus hemionus columbianus*) (Dubey et al. 2015a). Here we report *Sarcocystis* infection in two black-tailed deer from northwest USA for the first time.

Material and methods

Tongue and myocardium were collected from two (#14183, #15030) adult black-tailed deer (*Odocoileus hemionus columbianus*) from Pierce County, Washington State, USA on 11 December 2014 and 19 April 2015. Animals were lethally removed by United States Department of Agriculture, Animal and Plant Health Inspection Service personnel during an authorized direct control project. Samples were immediately collected. Refrigerated tongues and hearts were shipped (overnight delivery) to the Animal Parasitic Diseases Laboratory, USDA, Beltsville, Maryland the following day, for testing for protozoal infections.

Microscopical examination

A portion of tissues was fixed in buffered formalin and processed for histology using hematoxylin and eosin (HE) stain of 5 µm-thick sections. The number of cysts per section (2 x 0.7 cm) was recorded. Fresh muscles were examined for sarcocysts presence by squeezing; individual cysts were excised and kept frozen until molecular assays. Five cysts detected in the HE sections of deer #14183 were excised and processed for transmission electron microscopy (TEM) as reported by Dubey et al. (2015b).

Molecular analyses

Thirteen morphologically similar individual cysts were excised from tissues in deer #15030 and subjected to DNA isolation using DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions. DNA quantification and quality were determined by Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The DNA was characterized by PCR amplification and sequencing of two regions of the nuclear ribosomal DNA unit, *18S rRNA*, and *28S rRNA*, and the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) locus. The complete regions of *18S rRNA* and *28S rRNA* were amplified using overlapping fragments and primer pairs; ERIB1/S2r, S5f/S4r, S3f/Primer Bsarc, and KL1/LS2R, LS1F/KL3, respectively as described previously (Gjerde and Josefsen 2015). The partial sequence of *cox1* locus was also amplified using primer pairs SF1/SR5 (Gjerde 2013, 2014, Gjerde and Josefsen 2015). The amplified PCR products were run on 2.5% (w/v) agarose gel.

The single PCR amplicons of *18S rRNA*, *28S rRNA*, and *cox1* were excised from the gel and purified using QIAquick Gel Extraction (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's recommendations. The purified PCR products were sent to Macrogen Corporation (Rockville, MD, USA) for direct sequencing using the same primer pair used in PCR amplification to obtain the both strands reads. The resulting sequences were edited manually if necessary, and analyzed using the software Geneious version 8.0.4 (Biomatters Ltd. Auckland, NZ). The sequences obtained were aligned against each other and the published sequences of various *Sarcocystis* spp. to detect intra-species and interspecies variation on these DNA regions respectively.

Phylogenetic trees were estimated to show relationship among reference *Sarcocystis* species by the Neighbor-Joining algorithm applied to Tamura-Nei genetic distances, as implemented by Geneious version 8.0.4. Trees were tested by selecting bootstrap method with value of 1,000 bootstrap replicates. Phylogenetic trees based on *18S rRNA* and *cox1* sequences were constructed using sequences obtained from the black-tailed deer sarcocysts and previously published sequences of various species of *Sarcocystis* in NCBI GenBank.

Results

In wet mounts, sarcocysts were fusiform and up to 600 μm (n=13); released bradyzoites from broken cysts were banana-shaped and 12 μm long (n=10). Microscopic examination of HE-stained tissue sections revealed 9 cysts in heart and 74 in tongue of deer #14183, and 19 sarcocysts (3 in heart and 16 in tongue) in deer #15030. Only 2 sarcocysts were cut longitudinally and they measured 330 and 556 μm long. In cross sections sarcocysts were 36.0-163 μm wide. Under lower magnification the sarcocyst wall appeared smooth but at 1000x illumination, polygonal or elongated villar protrusions were evident (Fig. 1). Bradyzoites were separated in groups by septa.

One mild focus of myocarditis was observed in deer #15030.

By TEM, the parasitophorous vacuolar membrane was folded into villar protrusions (vp) and lined by a 15-20 nm thick electron dense layer (Fig. 2a, b). Total thickness of primary cyst wall ranged from 29 to 44 nm. The vp were rectangular, type 17 (Dubey et al. 2015a), and measured 1.17 (1.50-0.92) μm long and 1.42 (1.98-1.20) μm wide (n=66). The ground substance layer was remarkably thin (184-278 nm; studied in 4 cysts) and electron dense, that continues as septa (88.0-312 nm wide). Up to 6 disc-shaped plaques were present in the vp (Fig. 2c). Structurally plaques were 200 nm wide, with at least 3 different layers, numerous microtubules connected them to the core of the vp, and several microfilaments can be seen arising from the parasitoforous vacuolar membrane (Fig.

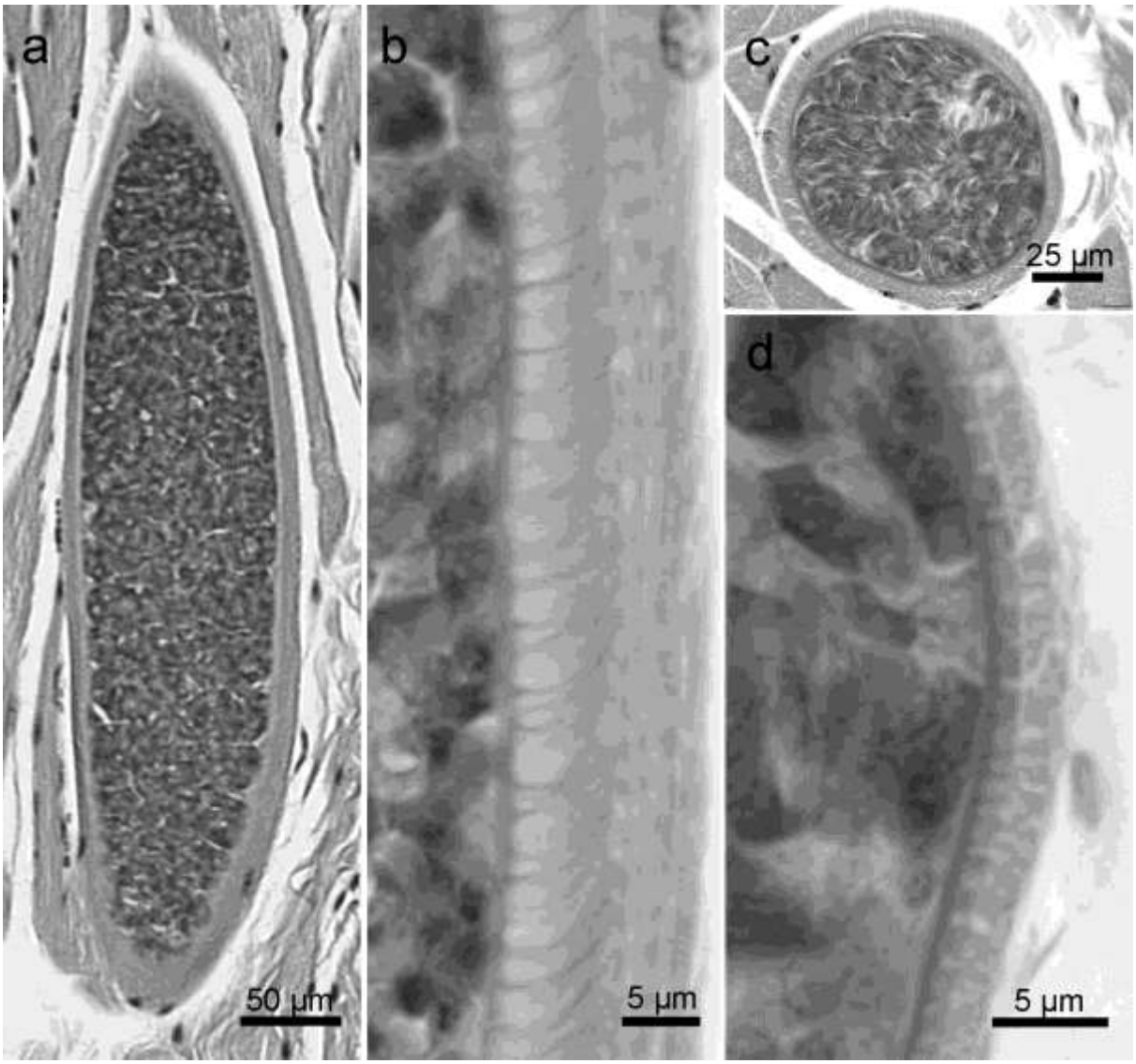

Fig. 1 Light micrographs of *Sarcocystis* sp. from black-tailed deer. **a, b** Longitudinally cut cysts. HE stain. **c, d** Transversally cut cysts. HE stain. **a** Note the septa and the thin wall. **b** Appearance of vp resembling keys of a piano. **c** Thin-wall sarcocyst. **d** Vp with rectangular appearance and remarkable dark ground substance

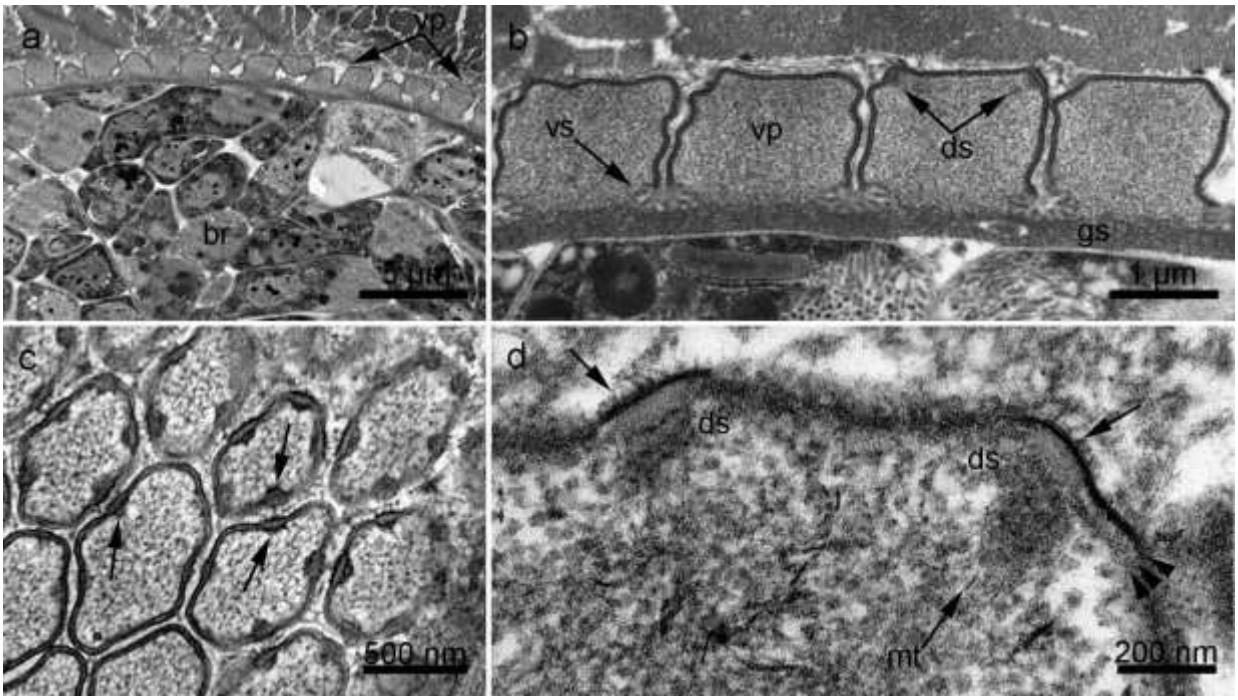

Fig. 2 TEM micrographs of *Sarcocystis* sp. from black-tailed deer. **a** Sarcocyst with polygonal-shaped villar protrusions (vp) on the wall, and enclosed bradyzoites (br). **b** Details of the cyst wall; note rectangular vp with disc-shaped plaques (ds), vesicles (vs), and thin electron dense ground substance (gs). **c** Cross/oblique section of vp showing several ds (arrows) from cyst #4. **d** Higher magnification of two ds; note the presence of microtubules (mt), three visible layers (arrowheads), and microfilaments (arrows) that arise from the parasitophorous vacuolar membrane

2d). The total thickness of the sarcocyst wall including the vp and gs was 1.39 (1.70-1.16) µm thick. Bradyzoites were 10.4 x 2.1 (8.3-11.5 x 1.6-2.7; n=12) µm in size and with a prominent mitochondrion , nucleus (nu), numerous micronemes (mn), dense granules (dg), amylopectin granules (am) (Fig. 3a), two rhoptries (rh1, rh2; Fig. 3b), and a conoid (co) with 22 subpellicular tubules and 7 internal tubules (Fig. 3c). Metrocytes, seen in the periphery, were 5.6 (5.0-6.0; n=4) µm in size but are not described in detail because they were not well preserved.

The *18S rRNA* (in three fragments), *28S rRNA* (in two fragments), and *cox1* loci were amplified by PCR using DNA obtained from the individual sarcocysts of black-tailed deer. DNA sequencing of PCR amplicons resulted the unambiguous sequences of three nuclear DNA regions; *18S rRNA* (1819 bp), *28S rRNA* (1598 bp), and the mitochondrial DNA loci; *cox1* (1022bp). These sequences were submitted to GenBank with accession numbers -

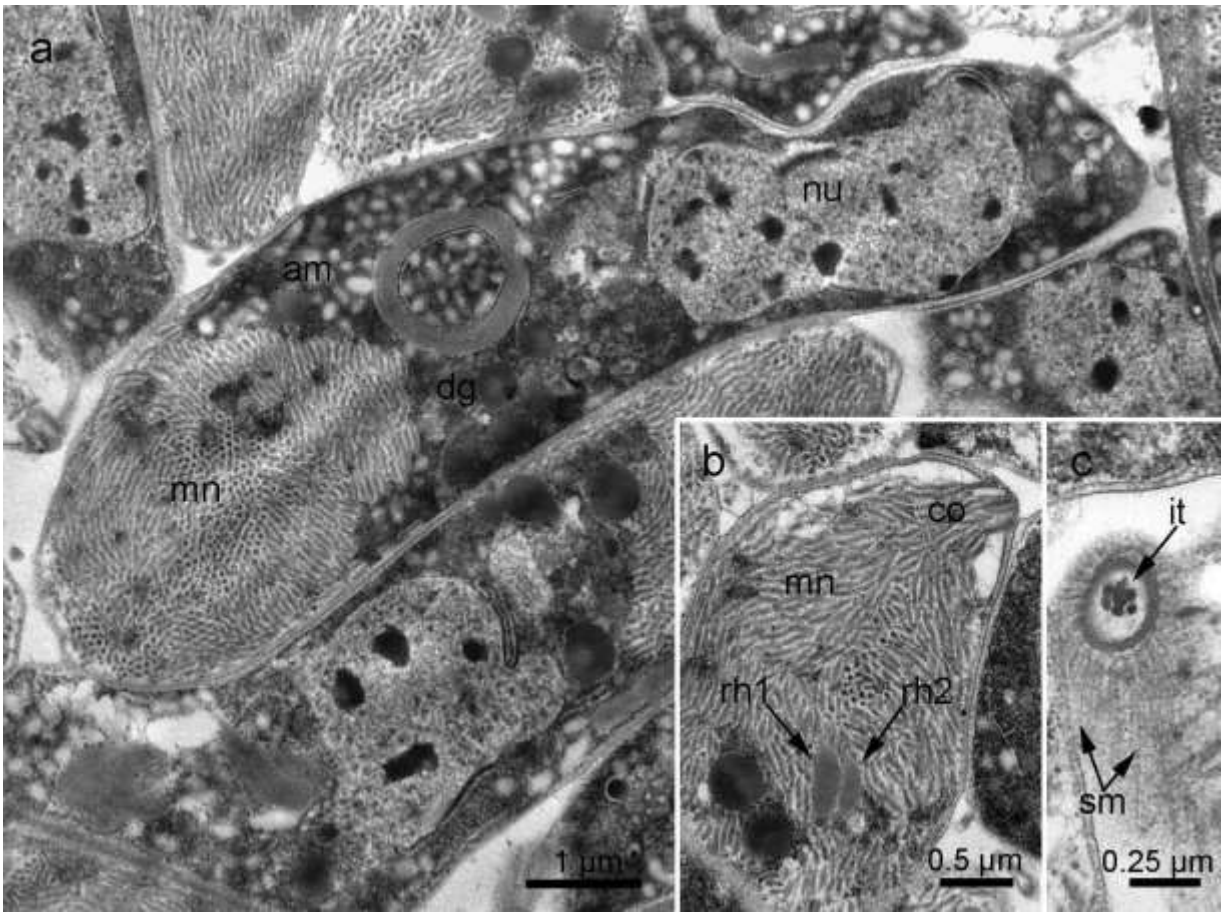

Fig. 3 TEM micrographs of *Sarcocystis* sp. from black-tailed deer. **a** Longitudinally cut bradyzoite, micronemes (mn), nucleus (nu), amylopectin (am) and dense granules (dg). **b** Conoidal part of a bradyzoite showing two rhoptries (rh1, rh2), conoid (co), and mn. **c** Detail of the co of a bradyzoite; note seven internal microtubules (it), and 22 subpellicular microtubules (sm)

-- (*18S rRNA*), ---- (*28S rRNA*), and --- (*cox1*). Phylogenetic analysis based on both the *18S rRNA* and the *cox1* sequences obtained from the DNA of individual sarcocysts of *S. mehlhorni* indicated an especially close relationship to another parasite in this genus that employs Canidae as their definitive host, *S. tarandivulpes* (Figs. 4, 5). The *18S rRNA*, *28S rRNA*, and *cox1*, sequences shared the highest identity with sequences of *S. tarandivulpes* (99.0%, EF056012), *S. tenella* (94.0%, AF076899), and *S. tarandivulpes* (92.0%, KC209718), respectively.

In addition, for *18S rRNA*, similarities were found for other *Sarcocystis* spp. that parasitize Cervidae, to note, *S. rangi* (EF056011) from reindeer (*Rangifer tarandus*) or *S. taeniata* (KF831278) and *S. alceslatrans*

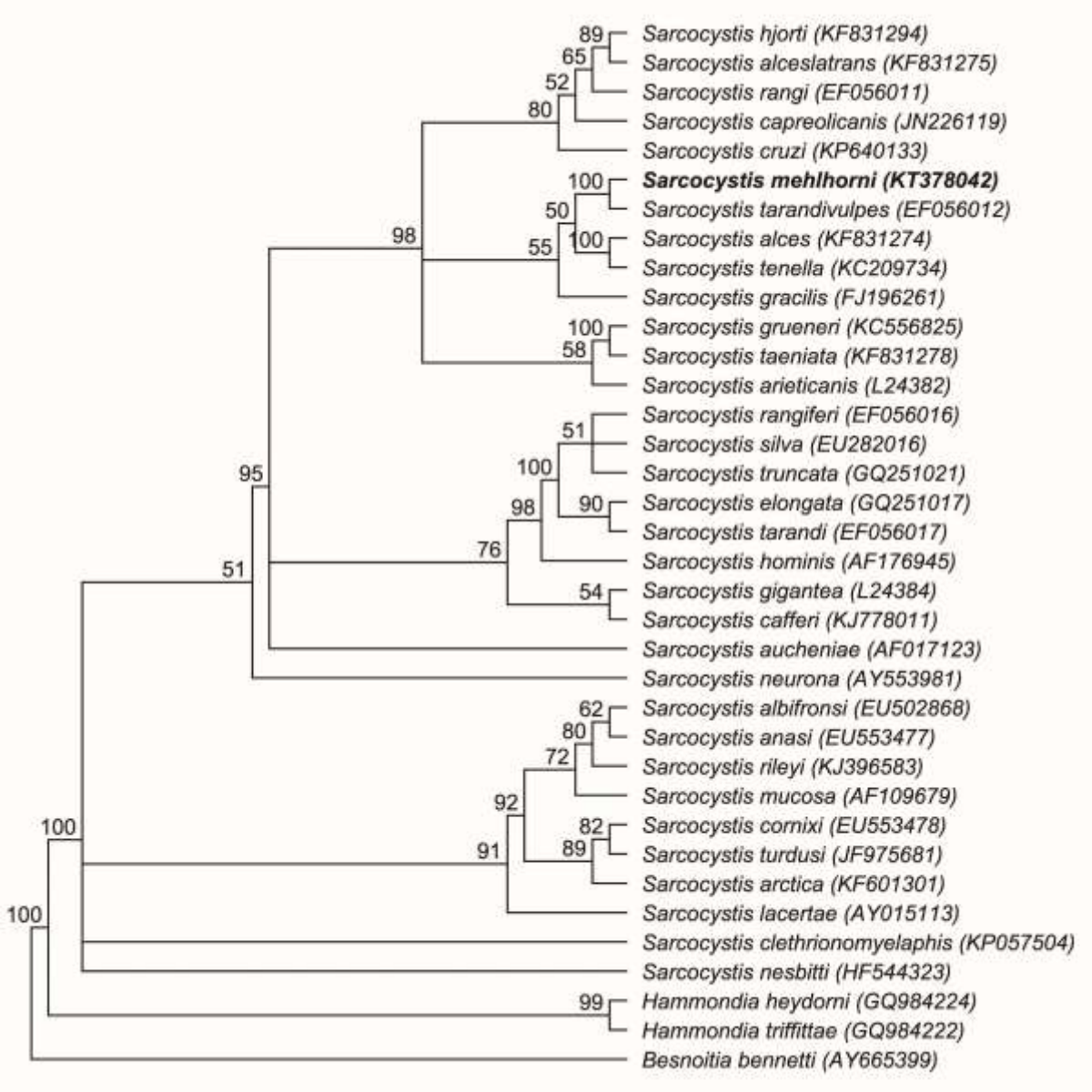

Fig.4 Phylogenetic tree of *18S rRNA* gene for *Sarcocystis mehlhorni* isolated from black-tailed deer in USA

(KF831275) from moose (*Alces alces*). (Fig. 4). When studying *cox1*, genetic relationship was observed with *S. taeniata* (KF831253), *S. grueneri* (KC209616) and *S. alceslatrans* (KF831248) (Fig. 5).

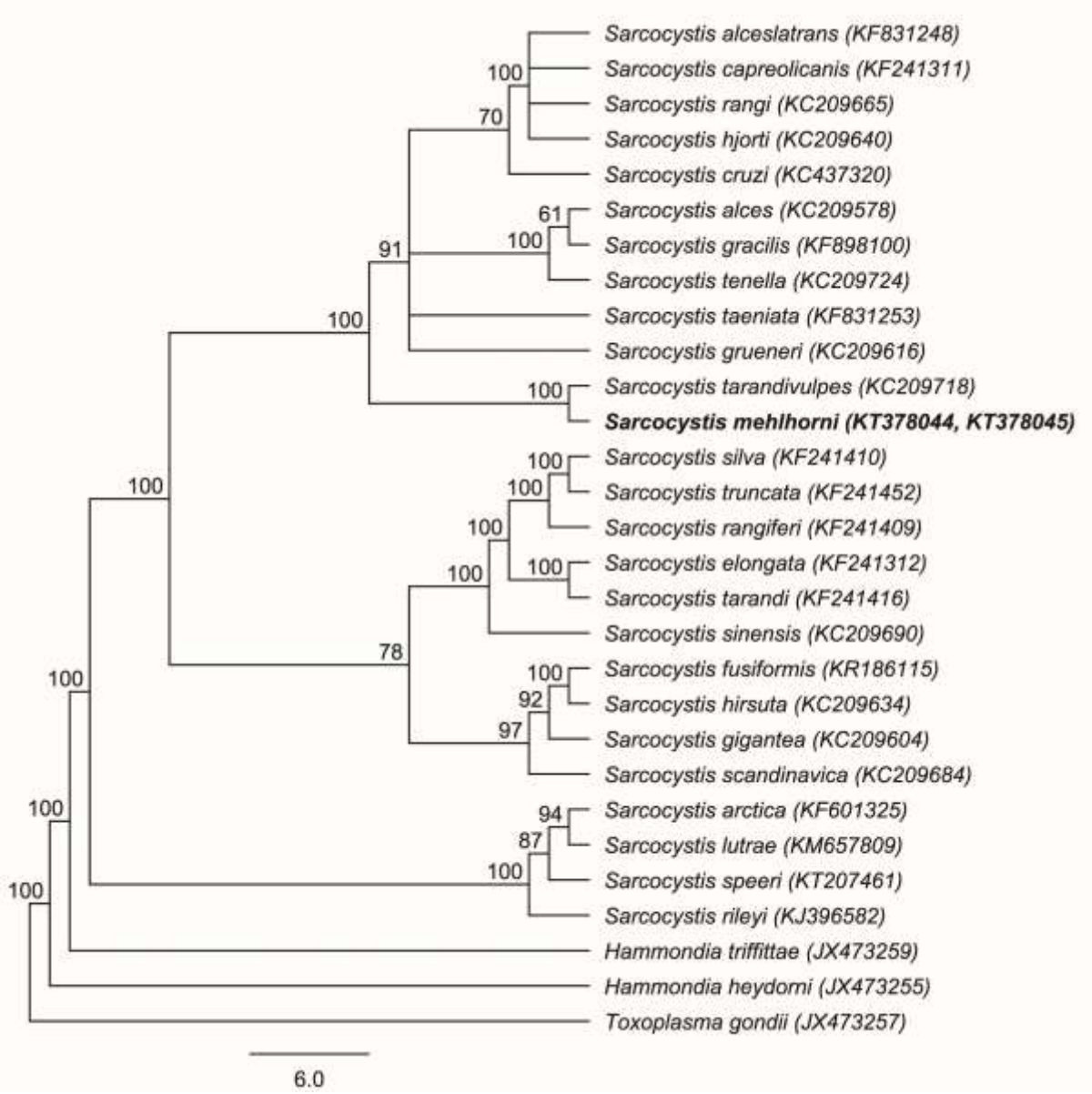

Fig. 5 Phylogenetic tree of *cox1* gene for *Sarcocystis mehlhorni* isolated from black-tailed deer in USA

Taxonomic summary of *Sarcocystis mehlhorni* n. sp. (Figs. 1-5).

Diagnosis Sarcocysts were microscopic, up to 556 µm long and mature. The sarcocyst wall was up to 1.39 µm thick, and had rectangular 1.17 µm long villar protrusions with 6 disc-shaped plaques, type 17, with thin (230 nm) electron dense ground substance layer.

Etymology Species named after the genus of its intermediate host.

Host Black-tailed deer (*Odocoileus hemionus columbianus*).

Distribution Washington State (USA), probably other areas in North America.

Definitive host Unknown, possibly Canidae.

Etymology Species named after Prof. Heinz Mehlhorn who, with Otto Heydorn and Michel Rommel contributed immensely to our knowledge on biology and structure of of *Sarcocystis.*

Specimens deposited Specimens deposited in the United States National Parasite Collection in the Division of Invertebrate Zoology and National Museum of Natural History, Smithsonian Institution, Washington, D.C. under (USNM-) include histological sections stained with H and E and Toluidine blue (--). Sequences were deposited in NCBI GenBank accession number -- (*18S rRNA*), -- (*28S rRNA*), and -- (*cox1*).

Discussion

Only one morphological type of sarcocyst was found in the black-tailed deer (BTD) examined, and is named *Sarcocystis mehlhorni*. Its sarcocysts resembled *S. odocoileocanis* from the white-tailed deer (WTD) from the USA (Table 1). Crum et al. (1981) named *S. odocoileocanis* for the parasite in WTD from Georgia with canids as the definitive hosts but did not describe its ultrastructure. Entzeroth et al. (1982) first reported the ultrastructure of sarcocysts in WTD from Michigan, USA. The sarcocyst wall was stated to be 15 to 20 μm thick (Entzeroth et al. 1982); however, the authors meant to say 1.5 to 2.0 μm thick (personal communication from B. Chobotar to J. P. Dubey, May 2015). The gs layer was 0.4-1.0 μm thick (Entzeroth et al. 1982); these measurements for the gs in BTD are thinner than in WTD. Otherwise the descriptions of villar protrusions in WTD and BTD appear similar, including description of *S. odocoileocanis* from WTD from Florida (Atkinson et al. 1993) and Montana (Dubey and Lozier 1983). One of the prominent feature of the sarcocyst in BTD was the presence of disc-shaped plaques on villar protrusions; these structures were not commented upon but are visible in illustrations provided by Entzeroth et al. (1982), Atkinson et al. (1993), and Dubey and Lozier (1983).

The disc-shaped plaques were first described by Speer and Dubey (1986) in vp of *S. hemionilatrantis* in mule deer from the USA. The ultrastructure of *S. odocoileocanis* from the BTD broadly resembles the structure of sarcocysts of *S. hemionilatrantis* in mule deer from the USA and *S. tarandivulpes* from the reindeer in Scandinavia (Gjerde 1984a, Gjerde 1985, Gjerde 1986) (Table 1).

For the morphological-related/similar species in wild ruminants, molecular information is only available for *S. tarandivulpes* (Dahlgren and Gjerde 2007, Dahlgren et al. 2007, Gjerde 2013). When examining the three

markers, molecular similarities were present. Further molecular characterization of *S. odocoileocanis* and *S. hemionilatrantis* will be of interest. Phylogenetic relationship of *Sarcocystis* sp. from the black-tailed deer with Canidae-transmitted *Sarcocystis* species in wild ruminants suggests that the present species is transmitted by these carnivores.

Transmission experiments are necessary to confirm the final identity of *Sarcocystis* spp. from the black-tailed deer. For example, *S. odocoileocanis* was infective to cattle and sheep (Crum et al. 1981) but not to goat (Lindsay et al. 1988).

Whether the differences on morphological description of parasites in white-tailed deer and mule deer from the USA and *S. tarandivulpes* in reindeer from Norway are related to techniques, description can not be resolved from the literature.

In conclusion, there is uncertainty concerning the identity of *S. odocoileocanis*-like sarcocysts in cervids. Available morphological and biological data are summarized in Table 1. It is evident that without extensive investigation and transmission experiments (that will not be easily done) the question of species can not be resolved. To facilitate further studies we propose new name, *S. mehlhorni*.

Acknowledgments

The authors thank Mr. Efrain Pérez and Joseph Madary, Joint Pathology Center, Veterinary Services, U.S. Army, Silver Spring, Maryland for excellent technical help with electron microscopy.

References

Atkinson CT, Wright SD, Telford SR, Mclaughlin GS, Forrester DJ, Roelke ME, McCown JW (1993) Morphology, prevalence, and distribution of *Sarcocystis* spp. in white-tailed deer (*Odocoileus virginianus*) from Florida. J Wildl Dis 29:73–84. doi:http://dx.doi.org/10.7589/0090-3558-29.1.73

Crum JM, Fayer R, Prestwood AK (1981) *Sarcocystis* spp. in white-tailed deer I. Definitive and intermediate host spectrum with a description of *Sarcocystis odocoileocanis* n. sp. J Wildl Dis 17:567–579. doi:http://dx.doi.org/10.7589/0090-3558-17.4.567

Dahlgren SS, Gjerde B (2007) Genetic characterisation of six *Sarcocystis* species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene. Vet Parasitol 146:204–213. doi:10.1016/j.vetpar.2007.02.023

Dahlgren SS, Gjerde B, Skirnisson K, Gudmundsdottir B (2007) Morphological and molecular identification of three species of *Sarcocystis* in reindeer (*Rangifer tarandus tarandus*) in Iceland. Vet Parasitol 149:191–198. doi:10.1016/j.vetpar.2007.08.015

Dubey JP, Kistner TP, Callis G (1983) Development of *Sarcocystis* in mule deer transmitted through dogs and coyotes. Can J Zool 61: 2904–2912. doi:10.1139/z83-378

Dubey JP, Lozier SC (1983) *Sarcocystis* infection in the white-tailed deer (*Odocoileus virginianus*) in Montana: Intensity and description of *Sarcocystis odoi* n. sp. Am J Vet Res 44:1738–1743

Dubey JP, Speer CA (1985) Prevalence and ultrastructure of three types of *Sarcocystis* in mule deer, *Odocoileus hemionus* (Rafinesque), in Montana. J Wildl Dis 21:219–228. doi:http://dx.doi.org/10.7589/0090-3558-21.3.219

Dubey JP, Speer CA (1986) *Sarcocystis* infections in mule deer (*Odocoileus hemionus*) in Montana and the description of three new species. Am J Vet Res 47:1052–1055

Dubey JP, Calero-Bernal R, Rosenthal BM, Speer CA, Fayer R (2015a) Sarcocystosis of animals and humans, 2nd Edn. CRC Press, Boca Raton

Dubey JP, Hilali M, van Wilpe E, Verma SK, Calero-Bernal R, Abdel-Wahab A (2015b) Redescription of *Sarcocystis fusiformis* sarcocysts from the water buffalo (*Bulbalus bubalis*). Parasitology 142:385–394. doi:10.1017/S003118201400122X

Entzeroth R, Chobotar B, Scholtyseck E (1982) Ultrastructure of *Sarcocystis* sp. from the muscle of a white-tailed deer (*Odocoileus virginianus*). Z Parasitenkd 68:33–38

Gjerde B (1984a) A light microscopic comparison of the cystss of four species of *Sarcocystis* infecting the domestic reindeer (*Rangifer tarandus*) in Northern Norway. Acta Vet Scand 25:195–204

Gjerde B (1984b) The fox as definitive host for *Sarcocystis* sp. Gjerde, 1984 from skeletal muscle of reindeer (*Rangifer tarandus*) with a proposal for *Sarcocystis tarandivulpes* n. sp. as replacement name. Acta Vet Scand 25:403–410

Gjerde B (1985) Ultrastructure of the cysts of *Sarcocystis tarandivulpes* from skeletal muscle of reindeer (*Rangifer tarandus tarandus*). Acta Vet Scand 26:91–104

Gjerde B (1986) Scanning electron microscopy of the sarcocysts of six species of *Sarcocystis* from reindeer (*Rangifer tarandus tarandus*). APMIS 94:309–317

Gjerde B (2013) Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. Int J Parasitol 43:579–591. doi:10.1016/j.ijpara.2013.02.004

Gjerde B (2014) Morphological and molecular characteristics of four *Sarcocystis* spp. in Canadian moose (*Alces alces*), including *Sarcocystis taeniata* n. sp. Parasitol Res 113: 1591-1604. doi:10.1007/s00436-014-3806-z.

Gjerde B, Josefsen TD (2015) Molecular characterisation of *Sarcocystis lutrae* n. sp. and *Toxoplasma gondii* from the musculature of two Eurasian otters (*Lutra lutra*) in Norway. Parasitol Res 114:873-886. doi:10.1007/s00436-014-4251-8.

Hudkins G, Kistner TP (1977) *Sarcocystis hemionilatrantis* (sp. n.) life cycle in mule deer and coyotes. J Wildl Dis 13:80–84. doi:http://dx.doi.org/10.7589/0090-3558-13.1.80

Lindsay DS, Blagburn BL, Mason WH, Frandsen JC (1988) Prevalence of *Sarcocystis odocoileocanis* from white-tailed deer in Alabama and its attempted transmission to goats. J Wildl Dis 24:154–156. doi:10.7589/0090-3558-24.1.154

Speer CA, Pond DB, Ernst JV (1980) Development of *Sarcocystis hemionilatrantis* Hudkins and Kistner, 1977 in the small intestine of coyotes. Proc Helminthol Soc Wash 47: 106–113

Speer CA, Dubey JP (1986) An unusual structure in the primary cyst wall of *Sarcocystis hemionilatrantis*. J Protozool 33:130–132. doi:10.1111/j.1550-7408.1986.tb05573.x