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The mallard duck (*Anas platyrhynchos*) as intermediate host for *Sarcocystis wobeseri* sp. nov. from the barnacle goose (*Branta leucopsis*)

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Abstract Morphometric and DNA investigation results of *Sarcocystis wobeseri* sp. nov. from the barnacle goose (*Branta leucopsis*) and *Sarcocystis* sp. (cyst type IV) from the mallard duck (*Anas platyrhynchos*) are presented. No significant morphometric differences between the investigated *Sarcocystis* species were found. ITS-1, 18S rRNA, and 28S rRNA gene sequences of these species showed 100% identity. The conclusion is drawn that it is one and the same *Sarcocystis* species in different intermediate hosts.

Introduction

Protists of the genus *Sarcocystis* are parasites with an obligatory prey-predator two-host life cycle. Herbivorous and omnivorous animals (prey) usually serve as intermediate hosts in the muscles of which asexual stages (sarcocysts) form. A sexual cycle with formation of oocysts/sporocysts occurs in the small intestine of carnivorous (predator) hosts. Birds can serve both as intermediate or definitive hosts for many species of *Sarcocystis*. Representatives of the order Anseriformes are intermediate hosts of these parasites.

Riley (1869) first found cysts of *Sarcocystis* in ducks. Stiles (1893) recorded macrocysts of these parasites in the shoveller (*Anas clypeata*) and named them *S. rileyi*. The striped skunk (*Mephitis mephitis*) was shown to be the definitive host for this species in North America (Cawthorn et al. 1981), although it is still unknown in Europe. Thus far, *S. rileyi* has been the only well investigated and named *Sarcocystis* species in ducks. Later, cysts of *Sarcocystis* were found in muscles of many species of ducks. Ducks also are intermediate hosts of other *Sarcocystis* species. Drouin and Mahrt (1980) found as many as five types of cysts in ducks, two of which had a smooth cyst wall of different thickness, and three types of microcysts had radial spines of different length and proximity on the outer surface of the cyst wall. It was established that the same hosts could be infected with more than one type of sarcocysts. Kutkienė and Sruoga (2004) present similar data. They discovered four types of microcysts in ducks: two of them had a smooth or wavy cyst wall that is difficult to distinguish by light microscopy and other two had protrusions of different length and shape on the wall surface (Kutkienė et al. 2008).

Geese have been investigated less extensively than ducks; however, sarcocysts have also been found in them. Wobeser et al. (1981) were the first to discover two types of sarcocysts in wild geese. The first type had finger-like protrusions on a wall surface within which fibrils were evident. This type was found in the lesser snow geese (*Anser caerulescens caerulescens*) and in the Ross geese (*A. rossii*). The other type was found in lesser snow geese and Canada geese (*Branta canadensis*) and had an undulating cyst wall with many small invaginations. Two types of sarcocysts (Kutkienė and Sruoga 2004) have also been found in the geese of Europe (Lithuania). One of them had teat- or finger-like protrusions on the wall surface and was found in the white-fronted geese (*A. albifrons*), in the lesser white fronted geese (*A. erythropus*), in the bean geese (*A. fabalis*) and in the gray-lag geese (*A. anser*). It has been established experimentally that one of the definitive hosts of this type of cysts was the Arctic fox (*Alopex lagopus*; Kutkienė et al. 2006). By light microscopy, cysts of another

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type had a relatively smooth cyst wall and were found in the white-fronted geese and in the gray-lag geese. Sarcocysts in gray-lag goose also were found in Kazakhstan (Pinayeva et al. 1998). Sarcocystis spp. found in all species of the geese examined thus far has been unnamed.

The identification and classification of Sarcocystis species have traditionally been based mainly on ultrastructure of the cysts and knowledge of the life cycle. Traditional morphological criteria could not always solve diagnostic problems of Sarcocystis, especially when similar morphological features of sarcocysts are found in taxonomically related species of hosts (Yang et al. 2001a). In recent years, DNA markers, mainly rRNA genes, have appeared to be able to overcome such complexity. Besides, the DNA analysis combined with the morphological one was successfully used to describe the new Sarcocystis species (Dubey et al. 2001; Dahlgren and Gjerde 2008a; Dahlgren and Gjerde 2008b; Kutkienė et al. 2009).

In the present paper, cysts morphology and DNA analysis results of Sarcocystis wobeseri sp. nov. from the barnacle goose (Branta leucopsis) and Sarcocystis sp. (cyst type IV) from the mallard duck (Anas platyrhynchos) are presented.

Material and methods

In the period of 2008 and 2009, 76 mallards (A. platyrhynchos) hunted in various districts of Lithuania or received from the National Food and Veterinary Risk Assessment Institute and three barnacle geese that were found dead after a fire on the island of Rusnė were investigated.

Light microscopy To detect Sarcocystis cysts, samples of neck muscles of each bird were examined. For this purpose 28 oath-size pieces of muscles were cut-off, stained with water (1:500) methylene blue solution, lightened with 1.5%

acetic acid solution, and pressed in a glass compressor. Sarcocystis infection intensity was evaluated having counted the cysts in 28 cross-sections of the muscles. The morphometric analysis of cyst walls and cystozoites was carried out in fresh preparations after the cysts had been isolated from muscle fibers by two preparation needles. The investigations were carried out using a computerized image analysis system. Cysts were differentiated on the basis of the wall thickness, the length and shape of protrusions and the length and shape of cystozoites.

Transmission electron microscopy For transmission electron microscopy (TEM), samples of neck muscles containing cysts from one barnacle goose and one mallard were fixed in Karnovsky's fixative, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and examined under the JEOL JEM-100B TEM. For TEM, only mature cysts were used.

DNA analysis Few cysts of Sarcocystis sp. (cyst type I) from one barnacle goose (B. leucopsis) and Sarcocystis sp. (cysts type IV) from one mallard (A. platyrhynchos) isolated from the neck muscles were placed separately in 1.5 ml Eppendorf tubes containing 75% ethanol. Genomic DNA was extracted from sarcocysts using Qiagen DNeasy tissue kit. Taxonomic status of the investigated Sarcocystis spp. was evaluated using highly variable first internal transcribed spacer (ITS-1). Phylogenetic analysis was performed using entire 18S rRNA gene and partial 28S rRNA gene sequences. Primers for ITS-1, 18S rDNA and 28S rDNA were designed from the conserved regions detected by the alignment of published sequences of the Sarcocystidae species using Primer3 program (Rozen and Skaletsky 2000; Table 1). Primers for the 28S rRNA gene containing variable D2 and D3 domains were picked. Phylogenetic trees of the family Sarcocystidae obtained

Table 1 Newly designed primers for amplification of ssu rRNA and lsu rRNA genes of Sarcocystis species

No	Primer pair	Region	Forward primer sequence	Reverse primer sequence	Position
1	SarAF\SarAR	18S rRNA	ctggttgatcctgccagtag	ttcccatcattccaatcact	4-23\506-525[a]
2	SarBF\SarBR	18S rRNA	gggaggtagtgacaagaaataacaa	ggcaaatgctttcgcagtag	462-486\942-961[a]
3	SarCF\SarCR	18S rRNA	tttaactgtcagaggtgaaattctt	cctgttattgcctcaaacttcc	899-923\1417-1438[a]
4	SarDF\SarDR	18S rRNA	ttccgttaacgaacgagacc	gcaggttcacctacggaaa	1322-1341\1775-1793[a]
5	KL-P1F\KL-P1R	28S rRNA	tacccgctgaacttaagcat	cccaagtttgacgaacgatt	1-20\989-1008[b]
6	KL-P2F\KL-P2R	28S rRNA	aaccgacccgtcttgaaac	tgctactaccaccaagatctgc	722-740\1551-1572[b]
7	P-ITSF\P-ITSR	ITS-1	attgagtgttccggtgaatta	gccatttgcgttcagaaatc	1581-1601\2070-2089[c]

[a] According to S. neurona (GenBank accession no. U07812)

[b] According to S. neurona (GenBank accession no. AF092927)

[c] According to B. besnoiti (GenBank accession no. FJ797432)

from D2 and D3 domains support the major grouping found for the full-length 28S rRNA gene analyses (Mugridge et al. 2000). Polymerase chain reactions (PCR) were carried out in the final 25-μl volume consisting of 5 μl 10× PCR buffer, 2.5 μl dNTP (2 mM), 0.2 μM each primer, 0.75 μl Taq polymerase, 2.5 μl MgCl$_2$ and 0.2 μg template DNA. Amplification was performed using "hot start" of 95°C for 10 min, followed by 35 cycles of 94°C for 45 s, 56°C (primer pairs 1, 2 and 3), 50°C (fourth primer pair) 53°C (fifth primer pair), 59°C (sixth primer pair) 58°C (seventh primer pair) for 45 s, 72°C for 2 min, and the final extension at 72°C for 10 min. Annealing temperatures and extension time varied depending on the amount of the amplified product and trying to avoid amplification of non-specific fractions. Amplification products were analyzed using 1.7% agarose gel and were purified using the Cyclo-Pure gel extraction kit (Amresco, USA). PCR products were sequenced directly with an ABI Prism 377 automatic DNA sequencer using the same primers as for the PCR reactions. Sequences identity values were determined on the European Molecular Biology Open Software Suite (http://www.ebi.ac.uk/emboss/align/) using the default options. Sequences were aligned using the ClustalW algorithm. The beginning and the end of some sequences were truncated to have all sequences beginning with and ending in the same nucleotide positions. The alignment was then checked manually in order to correct the ambiguously placed nucleotides. Sequenced isolates of the same species were primarily used separately in the phylogenetic analysis. All isolates of the same species were grouped together in the phylogenetic tree; then, they were merged into a consensus sequence using the IUPAC codes for those nucleotide positions with more than one possible character state in the consensus sequence. The phylogenetic trees were constructed using the Bayesian method by MrBayes program, version 3. 1. 2 (Ronquist and Huelsenbeck 2003). The phylogenetic relationships were assessed with the most complex available model, GTR+I+G evolutionary model, which allows all six possible substitutions to vary with a proportion of invariable sites and a gamma-shaped distribution of rates across the sites. The final trees were drawn using TreeView version 1.6.6 (Page 1996). Four species of different genera belonging to the subfamily Toxoplasmatinae and having the shortest evolutionary distance to the subfamily Sarcocystinae were chosen as outgroup of phylogenetic trees (*Besnoitia besnoiti*, *Hammondia hammondi*, *Neospora caninum*, and *Toxoplasma gondii*). The initial phylogenetic analysis revealed that all *Sarcocystis* species from birds were placed in one group of the phylogenetic tree. Those species that were grouped together with *Sarcocystis* parasitizing in birds served as the ingroup. Sequences of 18 representatives of the family Sarcocystidae were included in further phylogenetic analyses (Table 2).

S. dispersa, *S. mucosa*, and *Sarcocystis* sp. from the domestic pigeon were excluded from further analyses because of a lack of sequences of 18S rRNA or 28S rRNA genes.

Results

Having examined three barnacle geese, *Sarcocystis* cysts were found in one individual. Infection intensity was average and amounted to 35 cysts in 28 cross-sections of the neck muscles. One type of sarcocysts (temporarily called cyst type I) was found in this bird and proposed as a new species of *Sarcocystis*.

Sarcocystis wobeseri sp. nov

Description

Sarcocysts were ribbon-shaped, very long (the largest fragment found in fresh preparations measured about 5.2 mm) but relatively thin (up to 100 μm). They were divided into chambers by septa filled with cystozoites. By light microscopy, the cyst wall seemed smooth and amounted to 1.0 μm (Fig. 1a). Banana-shaped cystozoites were small and measured 6.4-7.9 μm in length (n=10; Fig. 1b). Ultrastructurally, the cyst wall amounted to 1.1 μm and was wavy (Fig. 1c). In some places, waves were high and resembled protrusions; however, in other places, the wall was almost smooth (Fig. 1d). The parasitophorous vacuolar membrane had many small invaginations (Fig. 1e). The ground substance layer continued into the interior of the cyst as septa. According to the classification of Dubey et al. (1989), these sarcocysts had type-1 cyst wall.

Sarcocystis sp. (cyst type IV) from the mallard

Cysts of *Sarcocystis* were detected in 11 (14.5%) mallards out of the 76 examined ones. Two types of cysts, which we temporarily called cysts type II and type IV, were found. Cyst type II was found during our earlier investigations into ducks and morphological, as well as DNA investigation results were presented in the publication Kutkienė et al 2008.

Sarcocysts type IV was ribbon-shaped, very long, and thick; the largest fragment found measured 8.0×0.2 mm. They were divided into large chambers by septa. By light microscopy, the cyst wall seemed wavy and reached up to 1.0 μm (Fig. 2a). Using a computerized image analysis system, it was defined that the cyst surface resembled a honeycomb (Fig. 2c). Banana-shaped cystozoites were small and measured 6.7-8.1 μm in length (n=20; Fig. 2b). Ultrastructurally, the cyst wall amounted to 1.6 μm and was

Table 2 Coccidia species used in phylogenetic analysis

Taxon name	18S rDNA accession no.	18S rDNA accession no.	Intermediate host	Definitive host
Besnoitia besnoiti	AF109678	AF076900	Cattle	Felidae
Frenkelia glareoli	AF009245	AF044251	Rodents	Aves
F. microti	AF009244	AF044252	Rodents	Aves
Hammondia hammondi	AF096498	AF101077	Rodents	Felidae
Neospora caninum	U17345	AF001946	Mammals	Canidae
Sarcocystis sp. 1 (cyst type I)	GQ922885	GQ922887	Barnacle goose	?
Sarcocystis sp. 2 (cyst type I)	EU502869	EF079886	White-fronted goose	?
Sarcocystis sp. 3 (cyst type IV)	GQ922886	GQ922888	Mallard	?
Sarcocystis sp. 4 (cyst type II)	EU553477	EF079887	Mallard	?
Sarcocystis sp. 5 (cyst type III)	EU502868	EF079885	White-fronted goose	Canidae
Sarcocystis sp. 6	AF513487-AF513488	AF513497, AF513498	Shrew	?
S. cornixi	EU553478	EF079884	Hooded crow	?
S. gallotiae	AY015112	AF513494	Lizard	Lacertidae
S. lacertae	AY015113	AF513495	Lizard	Serpentes
S. muris	M64244	AF012883	Rodent	Felidae, mustelidae
S. neurona	U33149, AY518209-AY518211, AY553981	AF092927	Horse, cat, raccoon, armadillo, skunk, seal, sea otter, cowbird	Marsupialia
S. rodentifelis	AY015111	AF513496	Rodent	Felidae, muridae
Toxoplasma gondii	U00458	AF076901	Mammals	Felidae

wavy (Fig. 2d). In some places, the waves were quite high and resembled protrusions; however, in other places, the wall was almost smooth (Fig. 2e). The parasitophorous vacuolar membrane had many small invaginations (Fig. 2f). The ground substance layer continued into the interior of the cyst as septa. These sarcocysts had type-1 cyst wall (Dubey et al. 1989).

DNA results ITS-1, 18S rRNA, and 28S rRNA gene sequences of *Sarcocystis* sp. (cyst type I) from the barnacle goose (we describe as *S. wobeseri* sp. nov.) and *Sarcocystis* sp. (cysts type IV) from the mallard were deposited in GenBank (GQ922885–GQ922888, GU475111, GU475112). ITS-1 sequences (844 bp long) gained from *S. wobeseri* (GU475111) and *Sarcocystis* sp. (cyst type IV) (GU475112) from the mallard were identical to each other. These sequences had low similarity within ITS-1 comparing with other *Sarcocystis* species. The highest identity within ITS-1 was found in *Sarcocystis* sp. from the domestic pigeon (*Columbia livia* dom.), *S. canis*, *Sarcocystis* sp. from wolverine (*Gulo gulo*) and *S. felis* species with following values: 93%, 75%, 70%, and 66%, respectively. These two identical sequences were especially different from other *Sarcocystis* species including *S. cruzi*, *S. dasypi*, *S. falcatula*,

S. lindsayi, *S. neurona*, *S. rangiferi*, and *S. tarandi*. A comparison of sequences of 18S rRNA and 28S rRNA genes revealed a 100% sequences identity between *S. wobeseri*, *Sarcocystis* sp. (cyst type IV) from the mallard and also *Sarcocystis* sp. (cyst type I) from the white-fronted goose. According to 18S rRNA and 28S rRNA gene sequences, these three *Sarcocystis* isolates have the closest sequence identity values to *S. cornixi* (99.6%) and to *Sarcocystis* sp. from the domestic pigeon, respectively (99.2%).

In the phylogenetic analysis, three datasets were generated; the first of the 18S rDNA sequences, the second of the 28S rDNA sequences, and the third of the 18S rDNA plus 2S rDNA sequences. The concatenated alignment contained 2,060 aligned nucleotide positions, with 1,598 and 462 aligned nucleotide positions belonging to the 18S rRNA and 28S rRNA genes, respectively. Unfortunately, only ~500 bp long sequences of the 28S rRNA gene for *S. gallotiae*, *S. lacertae*, *S. rodentifelis*, and *Sarcocystis* sp. from the shrew have been deposited in GenBank. Obtained phylogenetic trees present similar topologies (Fig. 3). According to phylogenetic analysis, *S. wobeseri* from barnacle goose is genetically identical to *Sarcocystis* sp. (cyst type IV) from the mallard and *Sarcocystis* sp. (cyst type I) from the white-fronted
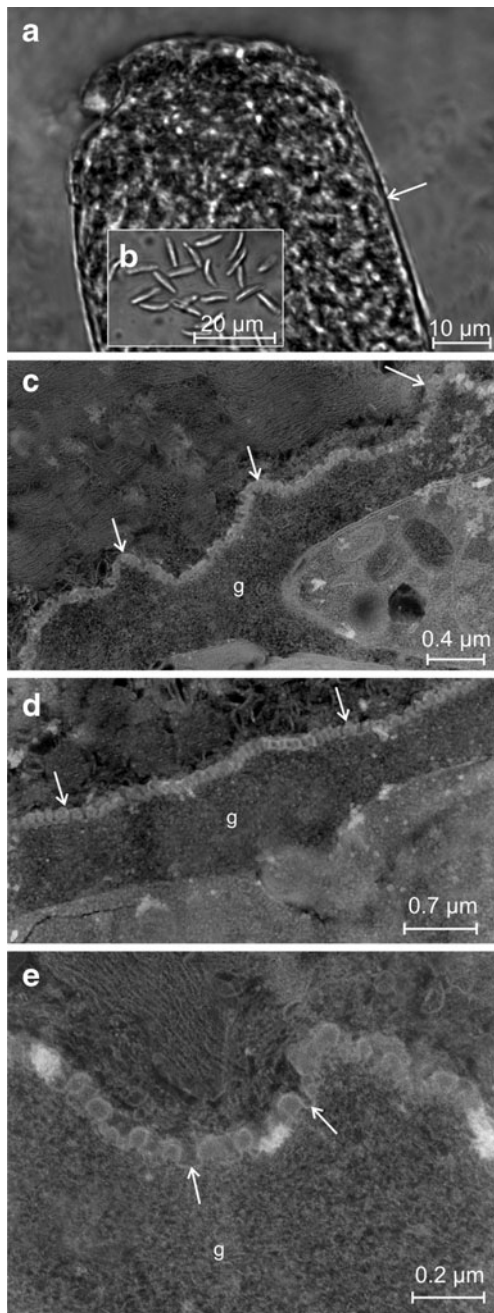
Fig. 1 a-e Structure of the *Sarcocystis wobeseri* sp. nov. from the neck muscle of the barnacle goose (*Branta leucopsis*). **a, b** Light micrographs (computerized image analysis system). Fresh preparations. **a** Fragment of the cyst; note smooth and thin cyst wall (*arrow*). **b** Cystozoites. **c-e** Electron micrographs of the cyst wall. **c** Fragment of the cyst wall with waves (*arrows*). **d** Fragment of the cyst wall without waves (*arrows*). **e** High magnification of the cyst wall; note minute invaginations of the parasitophorous vacuolar membrane (*arrows*). **g** Ground substance

goose and the most genetically related to *S. cornixi*, *Frenkelia glareoli*, *F. microti*, and *Sarcocystis* sp. from the shrew. In the general, phylogenetic tree *S. lacerate* and *S. gallotiae*; *S. muris*, and *S. rodentifelis*; *Sarcocystis* sp.

(cyst type III) from the white-fronted goose and *Sarcocystis* sp. (cyst type II) from the mallard are grouped together with 1.00 support value. The lowest resolution power was observed in 18S rDNA phylogram, where phylogenetic relationships inside *S. wobeseri* "group" were not robust.

Taxonomic summary

Type intermediate host: Barnacle goose (*B. leucopsis*)
    Definitive host: unknown
    Locality: Šilutė district (near the Baltic Sea), western Lithuania
    GenBank accession numbers: GenBank number as *Sarcocystis* sp. cyst type I ex *B. leucopsis* GQ922885, GQ922887, GU475111 for 18S rRNA, partial sequences of 28S rRNA genes and ITS-1, respectively
    Specimens deposited: TEM material is deposited at the Institute of Ecology, Vilnius, Lithuania.
    Etymology: the species has been named in honor of the famous Canadian scientist of veterinary pathology Gary Wobeser who (together with his co-workers) was the first to have determined this type of cyst wall in *Sarcocystis* of geese.

Discussion

The present work is a continuation of our earlier investigations into the birds order Anseriformes. In the period of 1997 and 2009, a total of 684 birds of this order were investigated and *Sarcocystis* cysts were found in 222 (32.5%) individuals (Kutkienė and Sruoga 2004 and unpublished data). By light microscopy, four types of sarcocysts have been determined, which were temporarily called type I, II, III, and IV, and *S. rileyi* was found. It was stated that cyst type II and III could be easily distinguished by light microscopy not only by the morphology of the cyst wall but also by a very specific shape of cystozoites. Meantime differentiation of cyst type I and IV by light microscopy was really complicated (Kutkienė and Sruoga 2004). Since the same types of cysts were found in different species of birds order Anseriformes, the hypothesis was put forward that the same species of *Sarcocystis* could parasitize different species of birds order Anseriformes. Later investigations into the ultrastructure of the cyst walls of some bird species and DNA analysis were undertaken (Kutkienė et al 2006; Kutkienė et al. 2008).

There are two opinions as to the host specificity of the species in the problem of sarcosporidiosis. According to the supporters of the first opinion (which is a prevailing one), the majority of species are specific to the intermediate host.
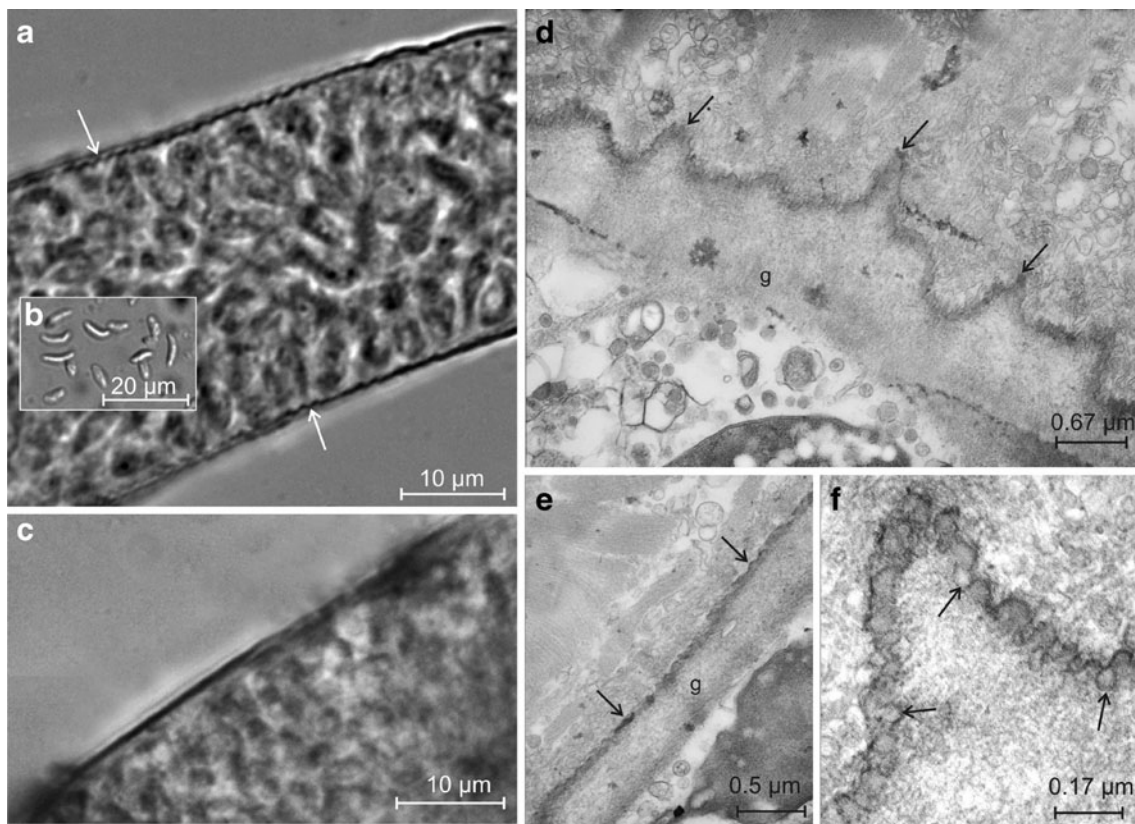
Fig. 2 **a–f** Structure of the *Sarcocystis* sp. (cyst type IV) from the neck muscle of the mallard (*Anas platyrhynchos*). **a–c** Light micrographs (computerized image analysis system). Fresh preparations. **a** Fragment of the cyst with wavy cyst wall (*arrows*). **b** Cystozoites. **c** Fragment of the cyst wall surface, which resembles a honeycomb. **d–f** Electron micrographs of the cyst wall. **d** Fragment of the cyst wall with waves, which resemble protrusions (*arrows*). **e** Fragment of the cyst wall without waves (*arrows*). **f** High magnification of the cyst wall; note minute invaginations of the parasitophorous vacuolar membrane (arrows). **g** Ground substance

In the opinion of other authors, the same *Sarcocystis* species can parasitize in taxonomically related species of hosts. The latter attitude is based on the morphological similarities of sarcocysts found in different species of hosts, as well as on the same possible definitive hosts (Wesemeier and Sedlaczek 1995; Odening 1998).

Box et al. (1984) experimentally showed that *S. falcatula* is a species of the passeriform, psitaciform, and columbiform birds. On the basis of these investigations, the idea was put forward that the *Sarcocystis* species parasitizing birds can be less host-specific than those parasitizing mammals.

Applying the DNA investigation methods in *Sarcocystis* diagnostics made the solution of the problem of the species specificity in intermediate hosts easier; however, thus far, the structure of the sarcosysts wall and morphology of cystozoites have remained one of the main criteria for diagnosing the *Sarcocystis* species. This is of especially great value in characterizing species in the same intermediate host (Mehlhorn and Heydorn 1978)

Cysts types I and IV under investigation in the present work are found by light microscopy in the majority of different species of birds order Anserifomes (Kutkienė and Sruoga

(2004) and unpublished data). However, as the earlier data and those presented in this article showed, the results of TEM can be at variance with the results of light microscopy. For example, *Sarcocystis* cyst walls of the common goldeneye (*Bucephala clangula*; Kutkienė et al. 2008) and the barnacle goose, which seemed to be smooth by light microscopy (according to our classification cyst type I), were clearly wavy by TEM. The case might be that only one cyst type, which has a thin wall with a characteristic wavy surface and many invaginations of the primary cyst wall (according to the classification of Dubey and Odening (2001)—cyst wall type-1) is found in the birds order Anseriformes. Besides, the shape and size of cystozoites of the cysts of types I and IV are the same.

Our investigations into *Sarcocystis* cysts of the barnacle goose and the mallard carried out by light microscopy showed some morphometric differences. The *Sarcocystis* wall of the mallard seemed wavy, whereas that of barnacle goose was smooth. The supposition that waviness is dependent on the quality of preparations cannot be rejected. These differences could not be of importance in the species diagnostics.
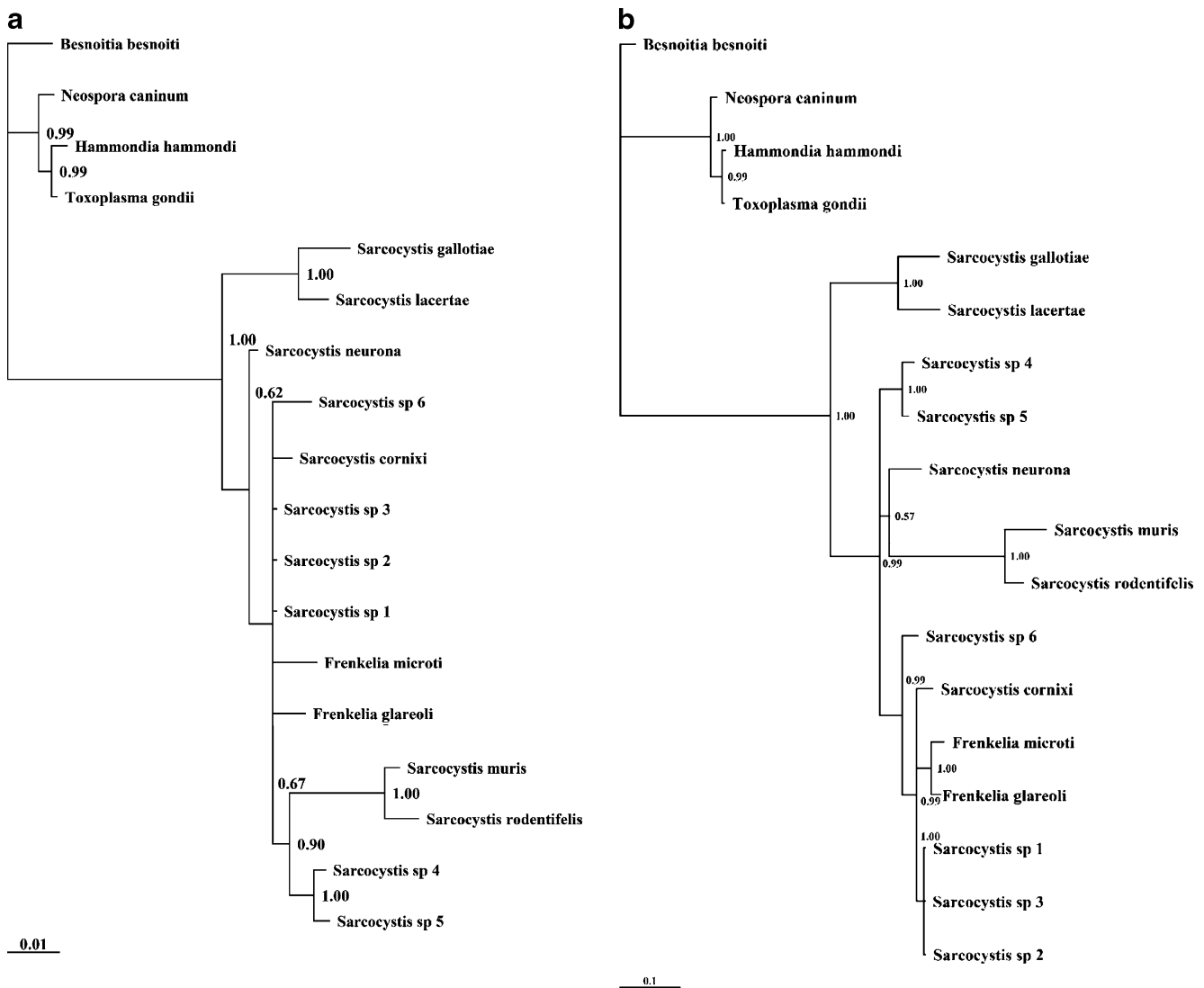
Fig. 3 **a** Phylogenetic tree for the subfamily Sarcocystinae based on 18S rRNA gene sequences. The tree was reconstructed using the Bayesian inference, rooted on *B. besnoiti*, *H. hammondi*, *N. caninum*, and *T. gondii* and scaled according to the branch length. The numbers in the figure show posterior probability support values. **b** Phylogenetic tree for the subfamily Sarcocystinae based on 28S rRNA gene sequences. The tree was reconstructed using the Bayesian inference, rooted on *B. besnoiti*, *H. hammondi*, *N. caninum*, and *T. gondii* and scaled according to the branch length. The numbers in the figure show posterior probability support values. **c** Phylogenetic tree for the subfamily Sarcocystinae based on the concatenated dataset of 18S rRNA and 28S rRNA gene sequences. The tree was reconstructed using the Bayesian inference, rooted on *B. besnoiti*, *H. hammondi*, *N. caninum*, and *T. gondii* and scaled according to the branch length. The numbers in the figure show posterior probability support values

When comparing the results of the ultrastructure of the barnacle goose and the mallard, it can be stated that no differences of significance to diagnostics of the species were found. Differences of wall thickness found in *Sarcocystis* species examined can be in dependence on cyst age. Sarcocyst walls from both species of birds correspond to cyst wall type-1 (Dubey and Odening 2001). Cyst wall ultrastructure of other anseriforms, i.e., of *S. rileyi* (Dubey et al. 2003), *Sarcocystis* sp. (cyst type II) from the mallard (Kutkienė et al. 2008), *Sarcocystis* sp. (cyst type III) from the white-fronted goose (Kutkienė et al. 2006) and sarcocysts (with finger-like protrusions on the cyst wall surface) from lesser snow and Ross geese (Wobeser et al. 1981) differed greatly from morphology of *Sarcocystis* isolates presented in this paper.

As has already been noted earlier, the wall type-1 (Dubey and Odening 2001) has been determined for sarcocysts from the lesser snow and Canada geese (Wobeser et al. 1981) and from the goldeneye (Kutkienė et al. 2008). An undulating cyst wall was seen in the white-rumped swift and the night heron from South Africa (Kaiser and Markus 1983). Thus, our findings and data from literature confirm once again the idea that the cyst wall of the same morphology is characteristic of sarcocysts parasitizing related and taxonom-
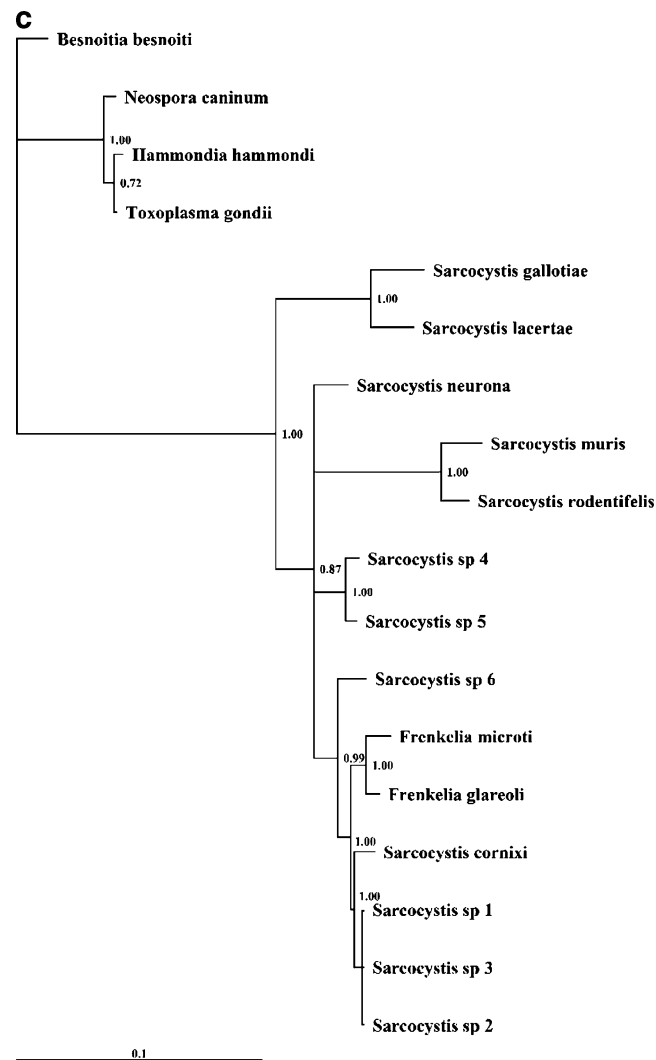
c

- Besnoitia besnoiti
- Neospora caninum
- Hammondia hammondi
- Toxoplasma gondii
- Sarcocystis gallotiae
- Sarcocystis lacertae
- Sarcocystis neurona
- Sarcocystis muris
- Sarcocystis rodentifelis
- Sarcocystis sp 4
- Sarcocystis sp 5
- Sarcocystis sp 6
- Frenkelia microti
- Frenkelia glareoli
- Sarcocystis cornixi
- Sarcocystis sp 1
- Sarcocystis sp 3
- Sarcocystis sp 2

0.1

Fig. 3 (continued)

ically distant species of animals including those of birds. Speaking about the possibilities of the same *Sarcocystis* species to parasitize taxonomically related species of animals (e.g., birds order Anseriformes) the morphological similarity of sarcocysts is only a primary feature enabling this supposition to be made. The results of the DNA investigation play an especially significant role in the solution of this problem. The 18S rRNA gene investigation results showed that several morphologically similar *Sarcocystis* species from the cattle and the water buffalo were the same species in both intermediate hosts (Yang et al. 2001a; Yang et al 2001b). Molecular studies confirm that *S. neurona* also use several species as intermediate hosts (Mullaney et al. 2005; Mansfield et al. 2008; Miller et al. 2009). Likewise, in our case, *S. wobeseri* from the barnacle goose and *Sarcocystis* sp. (cyst type IV) from the mallard had the same nucleotide composition in 4,143 bp long sequenced fragment of ITS-1, 18S rRNA,

and 28S rRNA genes. Thus, genetic investigations show that *S. wobeseri* from the barnacle goose and *Sarcocystis* sp. (cyst type IV) from the mallard are the same species. According to DNA results *S. wobeseri* is related but distinct to *S. cornixi*, *F. glareoli*, *F. microti*, and *Sarcocystis* sp. from the shrew and *Sarcocystis* sp. from the domestic pigeon. 18S rRNA and 28S rRNA gene sequences of *S. wobeseri* and *Sarcocystis* sp. (cyst type I) from the white-fronted goose showed 100% identity. We predict that *Sarcocystis* sp. (cyst type I) from the white-fronted goose also could be intermediate host for *S. wobeseri*. To confirm these hypotheses, ITS-1 sequences and cyst wall ultrastructure results of *Sarcocystis* sp. (cyst type I) from the white-fronted goose should be obtained and compared.

The 18S rRNA gene sequences were successfully used for the phylogenetic analysis within the family Sarcocysti-dae (Jeffries et al. 1997; Tenter and Johnson 1997; Doležel

et al. 1999; Jenkins et al. 1999; Holmdahl et al. 1999; Šlapeta et al. 2001; Šlapeta et al. 2002; Šlapeta et al. 2003; Morrison et al. 2004; Elsheikha et al. 2005; Dahlgren et al. 2008; Dahlgren and Gjerde 2008a; Dahlgren and Gjerde 2008b). In this taxon, several phylogenetic well-supported groups could be distinguished: the first group is made of representatives of the Toxoplasmatinae subfamily, the second group unites species studied in this work as an ingroup, the third group contains *Sarcocystis* species with a snake-rodent life cycle, and the fourth group comprises *Sarcocystis* species that uses an even-toed ungulate as their intermediate host and a canine, feline, or unknown predator as their definitive host. Species in the second group have very short branch length as compared with other Sarcocystidae groups, and phylogenetic relationships inside this group are not robust. Evolutionary relationships of some species inside the fourth group are also arguable. In the future perspective, when Sarcocystidae 18S rRNA gene database increases, new difficulties will arise in making a reliable phylogenetic inference. We support the previously stated opinion, that the 18S rRNA marker alone is insufficient to resolve all evolutionary relationships inside this family (Morrison et al 2004). In this study, reliable evolutionary relationships were revealed using entire 18S rRNA gene and partial 28S rRNA gene sequences. The concatenated 18S rRNA and 28S rRNA genes analysis have been successfully used in several studies within Sarcocystidae (Šlapeta et al. 2003; Kutkienė et al. 2009). The ITS-1 region is useful for closely related *Sarcocystis* species (Marsh et al. 1999; Dubey et al. 2006). However, due to huge sequences variability ITS-1 is a worthless determining evolutionary relationship of whole *Sarcocystis* genus. In order to reconstruct evolutionary relationships between closely related species of genus *Sarcocystis*, it could be valuable to use ITS-1 combining with 18S rRNA and/or 28S rRNA genes.

The comparative evaluation of the morphology of the cyst walls and the DNA investigation results enable us to state that *S. wobeseri* sp. nov. from the barnacle goose and *Sarcocystis* sp. (cyst type IV) from the mallard are one and the same species.

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