Beni-Suef University Journal of Basic and Applied Sciences

RESEARCH

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Morphological and molecular identification of *Sarcocystis* sp. from the little grebe, *Tachybaptus ruficollis* (Aves: Podicipediformes), for the first time in Egypt

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Abstract

Background: In Egypt, studies of sarcocystosis in migratory and wading birds specially Podicipediformes are scarce. Therefore, the occurrence of *Sarcocystis* spp. in the little grebe, *Tachybaptus ruficollis* (Podicipediformes: Podicipdidae) was investigated in Qena province, Upper Egypt. During the period from September 2017 to March 2018, muscle specimens of esophagus from 25 little grebes were examined for the occurrence of *Sarcocystis* spp. using light microscopy, transmission electron microscopy (TEM) and molecular tools including conventional PCR and phylogenetic analysis with both 18S rRNA and 28S rRNA genetic loci.

Results: *Sarcocystis* spp. were identified in 84.0% (21/25) of the *T. ruficollis* esophageal muscle samples by morphological analysis; each was long, ribbonlike and extended along the muscle fibers. TEM revealed a characteristic thin and wavy cyst wall and an undulating vacuolar membrane with villar protrusions of slightly variant sulci and gyri, as well as several septated compartments filled with bradyzoites. The applied 18S rRNA and 28S rRNA were not enough variable for the identification of *Sarcocystis* species.

Conclusion: This study is the first to report the occurrence of *Sarcocystis* sp. in little grebes in Egypt. Further studies are required to identify *Sarcocystis* spp. in various wild birds by ITS1 region in Egypt.

Keywords: *Sarcocystis* sp., *Tachybaptus ruficollis*, 18S rRNA, 28S rRNA, Egypt

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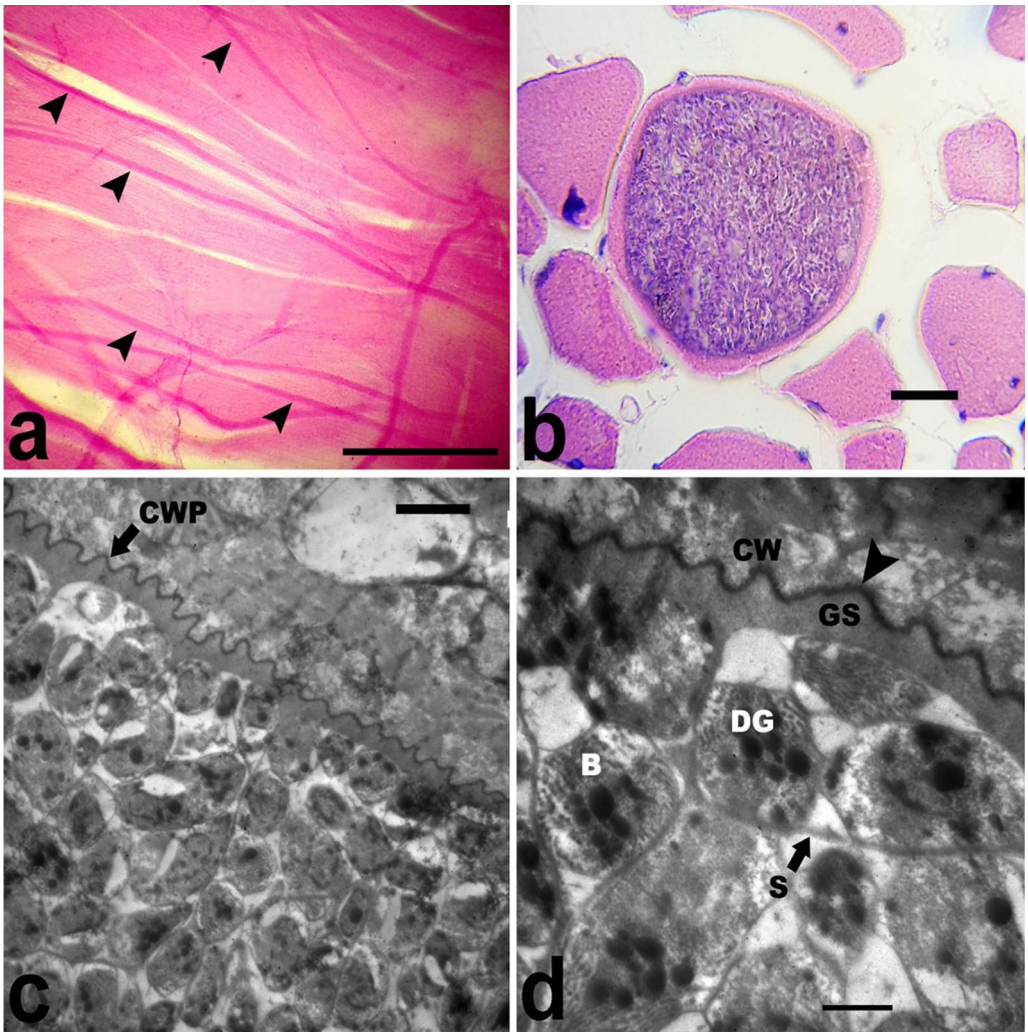
Graphical abstract



1 Background

Sarcocystis (Eucoccidiorida: Sarcocystidae) species are protozoan parasites of mammals, birds and reptiles that are distributed worldwide [1]. They are characterized by an obligatory two-host life cycle, consisting of prey as an intermediate host and a carnivore/omnivore as a definitive host [2]. The intermediate host becomes infected through the ingestion of oocysts/sporocysts in water or food contaminated with feces and the definitive host is infected via ingestion of muscles containing sarcocysts [3].

Birds may act as intermediate or definitive hosts for various *Sarcocystis* species [3–7]. More than 25 *Sarcocystis* species have been identified in birds acting as intermediate hosts; however, details about the life cycle of *Sarcocystis* have been reported in only a few species [3, 8]. Historically, *Sarcocystis* species have been described based on morphological analysis of sarcocysts by light microscopy (LM) and transmission electron microscopy (TEM), and through the detection of their definitive hosts. However, in recent decades, the majority of *Sarcocystis* species have been characterized with a combination of morphological and molecular analysis [9–21].

Few studies have reported *Sarcocystis* spp. infections in birds in Egypt. To date, only two *Sarcocystis* species, *Sarcocystis chloropusae* from the common moorhen, *Gallinula chloropus* (Gruiformes: Rallida), and *S. atraii* from the common coot, *Fulica atra* (Gruiformes: Rallidae) have been recorded [16, 17]. The aim of the present study was to detect and characterize *Sarcocystis* spp. in the little grebe, *Tachybaptus ruficollis*

(Podicipediformes: Podicipedidae), in Egypt using LM, TEM and molecular analysis.

2 Methods

2.1 Sampling

Twenty five little grebes, 11 males (one aged 6 months and ten adults aged 1 year) and 14 females (two birds aged 6 months and twelve adults aged 1 year), were sporadically found dead, probably due to unintentional catching, or died shortly on approaching, probably due to certain infections, and collected from different regions alongside the Nile River, Qena (26° 10′ 12″ N 32° 43′ 38″ E), Upper Egypt, during the period from September 2017 to March 2018.

2.2 Light microscopy

Birds were necropsied; then, small specimens with dimensions approximately 0.5 cm^3 of the esophageal muscles were obtained, compressed between two glass slides, fixed in 10% neutral buffered formalin, stained with potassium alum carmine and then photographed [22]. In addition, approximately 1 cm^3 of the same specimens was fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 5-µm-thick sections, stained with hematoxylin and eosin (HE), and then photographed using a digital microscope (Leica Microsystems, CH-9435 Heerbrugg, Ec3, Singapore) [23].

2.3 Transmission electron microscopy (TEM)

One sample (approximately 3 mm^3) of the esophageal muscle from each infected bird was fixed in 4% cold glutaraldehyde, washed in cacodylate buffer (pH 7.2), postfixed in 2% osmium tetroxide, dehydrated with ethanol and then embedded in resin. Semithin sections were obtained using an ultramicrotome. Ultrathin sections (thickness of 500–700 A) were produced, contrasted in uranyl acetate and lead citrate, examined under a JEM 100 CXII electron microscope at 80 kV and photographed using a CCD digital camera (model XR-41) [24].

2.4 Molecular analysis

2.4.1 DNA extraction and amplification

Genomic DNA was extracted from 5 specimens of *T. ruficollis* muscle tissues specimens containing sarcocysts. In addition, DNA of three identified macroscopic *S. fusiformis* isolated from an infected buffalo was extracted individually from each cyst to be used as positive control in the amplification. The extraction was carried out using a DNA extraction kit [Pure Link™ Genomic DNA Mini Kit (cat. no. K1820-01), Invitrogen, Thermo Scientific, USA] according to the manufacturer's instructions. The extracted DNA was stored at − 20 °C until use.

The genetic characteristics of *Sarcocystis* spp. were evaluated using partial 18S rRNA and 28S rRNA gene sequences. Both 18S rRNA and 28S rRNA gene fragments were amplified using two primer pairs (Table 1). A new primer targeting a long amplicon (812 bp) was designed using the oligoanalyzer tool (https://eu.idtdna.com/calc/analyzer) from the AF044252 GenBank deposition. For amplification, each 25 µL reaction contained 12.5 µL of 2X master mix (Geneaid Biotech Ltd., New Taipei City, Taiwan), 1 µL of pF (10 pmol/µL), 1 µL of pR (10 pmol/µL), µL of DNA sample and 7.5 µL of nuclease-free water. The PCR cycling conditions consisted of an initial activation at 95 °C for 5 min, followed by 37 cycles of 95 °C denaturation for 30 s, and 52 °C annealing and 72 °C extension for 1 min each. A final extension was completed at 72 °C for 7 min. PCR amplicons were electrophoresed through a 1% agarose gel in trisacetate EDTA buffer.

2.4.2 Nucleotide sequencing

PCR products were purified with a Thermo Scientific GeneJET PCR Purification Kit (Thermo Fisher Scientific Baltics, Vilnius (#K0701), Lithuania) according to the

Table 1 Primers used for amplification of DNA extracted from the identified sarcocysts using 18S and 28S rRNA regions, with expected amplicon size and melting temperature (MT)

Gene	Nucleotide sequences	bp	Melting temperature (°C)	Reference
Sarcocystis 18S rRNA fragment	1L (F) (5′-CCATGCATGTCTAAGTATAAGC-3′)	1391	51.2	Yang et al. [25]
	1H (R) (5′-TATCCCCATCACGATGCATAC-3′)		54.3	
	18S1F (5′-GGATAACCGTGGTAATTCTATG-3′)	1085	50.6	Rosenthal et al. [26]
	18S11R (5′-TCCCATGTCTGGACCTGGTGAG-3′)		54.5	
Sarcocystis 28S rRNA fragment	KL5a (5′-GAC CCT GTT GAG CTT GAC-3′)	645	53.2	Mugridge et al. [27]
	KL2 (5′-ACT TAG AGG CGT TCA GTC-3′)		51.3	
	Wf (5′-CTCTCTTAAGGTAGCCAAATGC-3′)	812	53.4	This study
	Wr (5′-CTTTTCAACTAGCCGCATTTAC-3′)		52.4	

manufacturer's instructions. The purified PCR products were sequenced on an Applied Biosystems 310 automated DNA sequencer using cycle sequencing ABI Prism Big Dye terminator chemistry (PerkinElmer/Applied Biosystems, Foster City, CA, USA). Forward and reverse PCR amplicon reads were used for sequence assembly, editing and correcting the ambiguously placed nucleotides using GeneStudio ™ Professional Edition V.2.2.0.0, and then comparison with related sequences in GenBank using the Nucleotide BLAST program [28]. The 18S rRNA and 28S rRNA region sequences of the recovered isolates were aligned using MUSCLE implemented in MEGA7 [29]; the alignments were then examined to detect intraspecific variation [30]. The start and the terminal ends of some sequences were manually truncated so that all sequences began with and terminated at the same nucleotide positions. The alignment was also manually checked to correct ambiguously placed nucleotides. The 18S rRNA and 28S rRNA region sequences were deposited in GenBank under accession numbers MH898961 and MH898981, respectively.

2.4.3 Phylogenetic analyses

Two sets of data were obtained and were phylogenetically analyzed with Bayesian inference (BI) using MrBayes V.-3.2.6 123 [31], and a best nucleotide substitution fit model was determined using MrModeltest 2.3, where the Akaike information criterion (AIC) indicated that SYM + G was the best estimator for BI settings in all datasets. Four simultaneous Markov chain Monte Carlo chains were sampled every 1000 generations for 1,000,000 generations; the first 3000 samples were discarded as burn. The average standard deviation of split frequencies was less than/equal to 0.01.

3 Results

3.1 Light and transmission electron microscopy

Of 25 examined little grebes, 21 (84.0%) birds (ten adult males and eleven adult females) were infected with the same *Sarcocystis* sp. by morphological analysis. There were no macroscopic lesions in the examined birds. Through LM analysis, sarcocysts in 21 specimens were observed to be long (5–12 × 0.05–0.07 mm), ribbonlike and extended along the muscle fibers (Fig. 1a). In HE-stained sections, the sarcocysts measured 40–62 µm in diameter (Fig. 1b). TEM analysis revealed a characteristic thin (0.6–0.8 µm) and wavy cyst wall. The outer cyst wall was smooth and thin with several bleb-like protrusions. The cyst contained numerous rounded to oval fragments of bradyzoites, which were appeared rounded toward the periphery (Fig. 1c, d). Beneath the cyst wall, a dense ground substance (0.6–0.7 µm thick) was found. The ground substance continued into the interior of the sarcocyst as septa which divided the cyst into several compartments.

3.2 Molecular identification

Amplification of extracted DNA from five esophagus muscle samples of five different birds using *Sarcocystis*-specific primers targeting the 18S rRNA gene revealed the presence of specific diagnostic bands at 1023 bp for specimens from *T. ruficollis* and the positive control *S. fusiformis*. Furthermore, PCR analysis using the same specimens with another *Sarcocystis*-specific primer and a designed primer targeting the 28S rRNA gene revealed specific band at 840 bp.

3.3 Phylogenetic analyses

Partial regions of the 18S rRNA and 28S rRNA genes from the present identified *Sarcocystis* sp. were sequenced, compared to those of other *Sarcocystis* taxa and aligned with two sets of data representing all appropriate species for which sequence data were available. In the first dataset, the present 18S rRNA isolate was compared to those from other *Sarcocystis* taxa infecting birds only: 30 sequences were obtained with 24 sequences representing 18 different known *Sarcocystis* taxa and 6 sequences that were isolated from avian hosts and identified as *Sarcocystis* spp. (Fig. 2). The second dataset included all 28S rRNA avian *Sarcocystis*. Eleven sequences were obtained containing six known and five unidentified avian *Sarcocystis* taxa (Fig. 3). For an out-group comparison, two sequences from a mammalian *Sarcocystis* species, *S. lutrae* and *S. speeri* (accession numbers KM657769 and KT205759, respectively) in the first data (18S rRNA) while the out-group in the second datasets (28S rRNA) included *S. capracanis* and *S. tenella* (accession numbers, KU820979 and MH413037, respectively).

In the present study which identified *Sarcocystis* sp.18S rRNA isolates at the highest query coverage (99%), 99.2% identity was shared with known *S. halieti* isolates (MH130211 and MF946587) and *Sarcocystis wobeseri* isolate (GQ922885) as well as some unidentified avian *Sarcocystis* spp. under accession numbers: JQ733511, GQ245670, KY348753 and EU502869. Furthermore, at the same query coverage, shared identity ranged from 99.1% to 97.9% with several avian GenBank isolates. The highest identity (99.4%) was observed in the least query coverage (92%) with *S. ramphastosi* (EU263366.1). In contrast, at 99% query coverage, shared identity with *S. chloropusae* (KJ810604) was lowest at only 97.9%.

In the identified 28S rRNA isolate, the highest query coverage was 96%. Shared identity percentage varied from 98.3% in *S. rileyi* (GU188426) to 99.4% in *Sarcocystis calchasi* (FJ232949) (Fig. 3). The highest shared identity was 99.4% (EU514792 and EU553480) at 79%

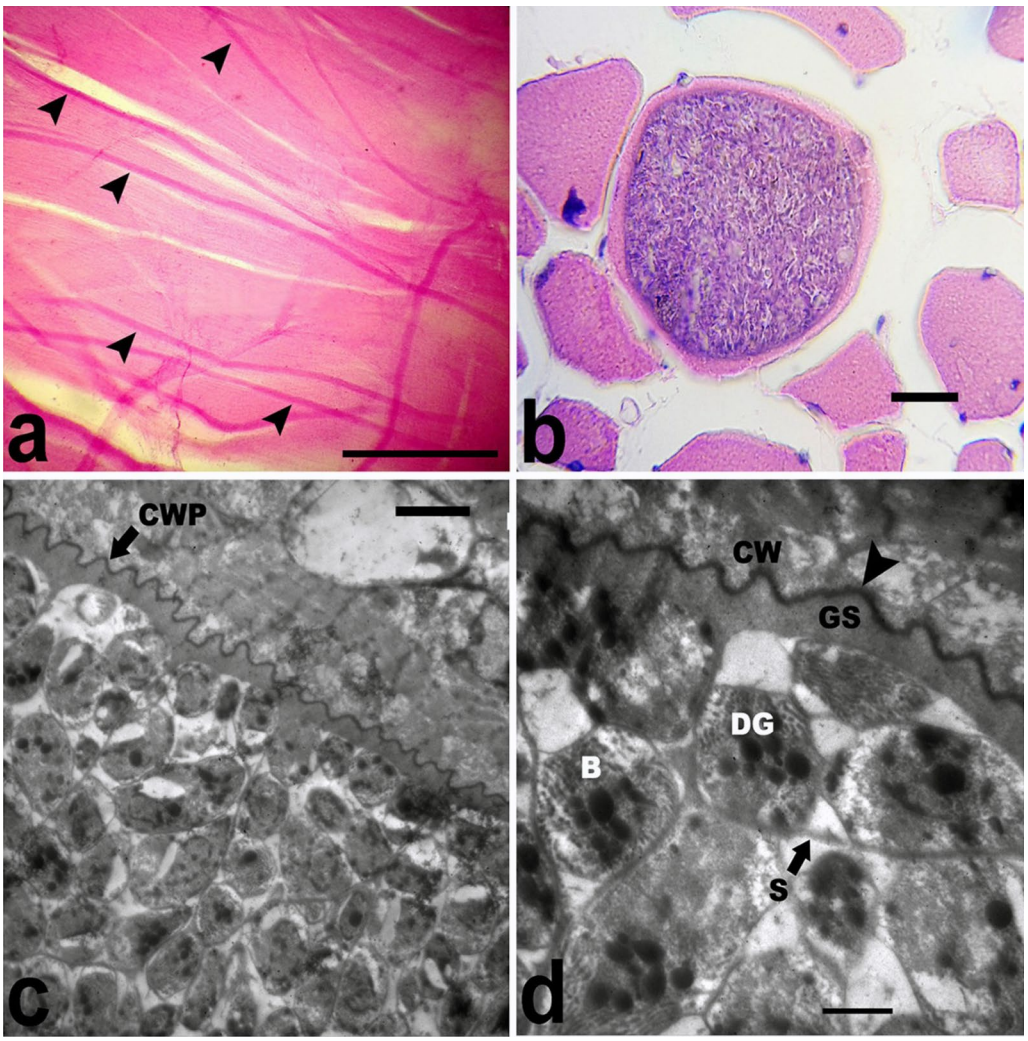


Fig. 1 *Sarcocystis* sp. obtained from the little grebe, *Tachybaptus ruficollis*. **a** A wet mount from esophageal muscles infected with sarcocysts stained with potassium alum carmine. Note the heavy infection with numerous ribbonlike sarcocysts (arrowheads). Scale bar = 1 mm. **b** Cross section of a thin-walled *Sarcocystis* sp. sarcocyst in a hematoxylin and eosin (HE)-stained histological section. Scale bar = 20 µm. **c** Transmission electron microscopy (TEM) image of *Sarcocystis* sp. showing zigzag-like or undulating vacuolar membrane with villar protrusions (VP) of slightly variant sulci and gyri. Scale bar = 1 µm. **d** A higher magnification of the TEM image reveals a cyst wall (CW) with several bleb-like protrusions (arrowhead), ground substances (GS), fragments of bradyzoites (B) with dense granules (DG) and septa (S). Scale bar = 0.5 µm

query coverage, while the lowest was 98% (EU553479) at 79% query coverage (Fig. 3). The highest cover (96%) and their corresponding identity values (97.9–99.4%) were refluxed in the 18S rRNA phylogenetic tree (Fig. 2), the identified isolate was clustered with those isolates as a part of polytomy and most closely related to *S. halieti* specimens while lesser query covers were separated from our isolate. The avian 28S rRNA phylogenetic tree (Fig. 3) revealed the identified isolate as a distinct clade, more closely related to avian *Sarcocystis* spp. than to mammalian *Sarcocystis* spp.

Based on the morphological characteristics of the *Sarcocystis* spp. obtained by direct microscopy and TEM, it was found that the revealed *Sarcocystis* sp. was closely related to *Sarcocystis halieti,* but unfortunately the used markers showed limited ability to confirm the precise *Sarcocystis* spp.

4 Discussion

Sarcocystis spp. have previously been found at considerably high levels in adult wading birds compared to other avian intermediate hosts [32]. The little grebe has not

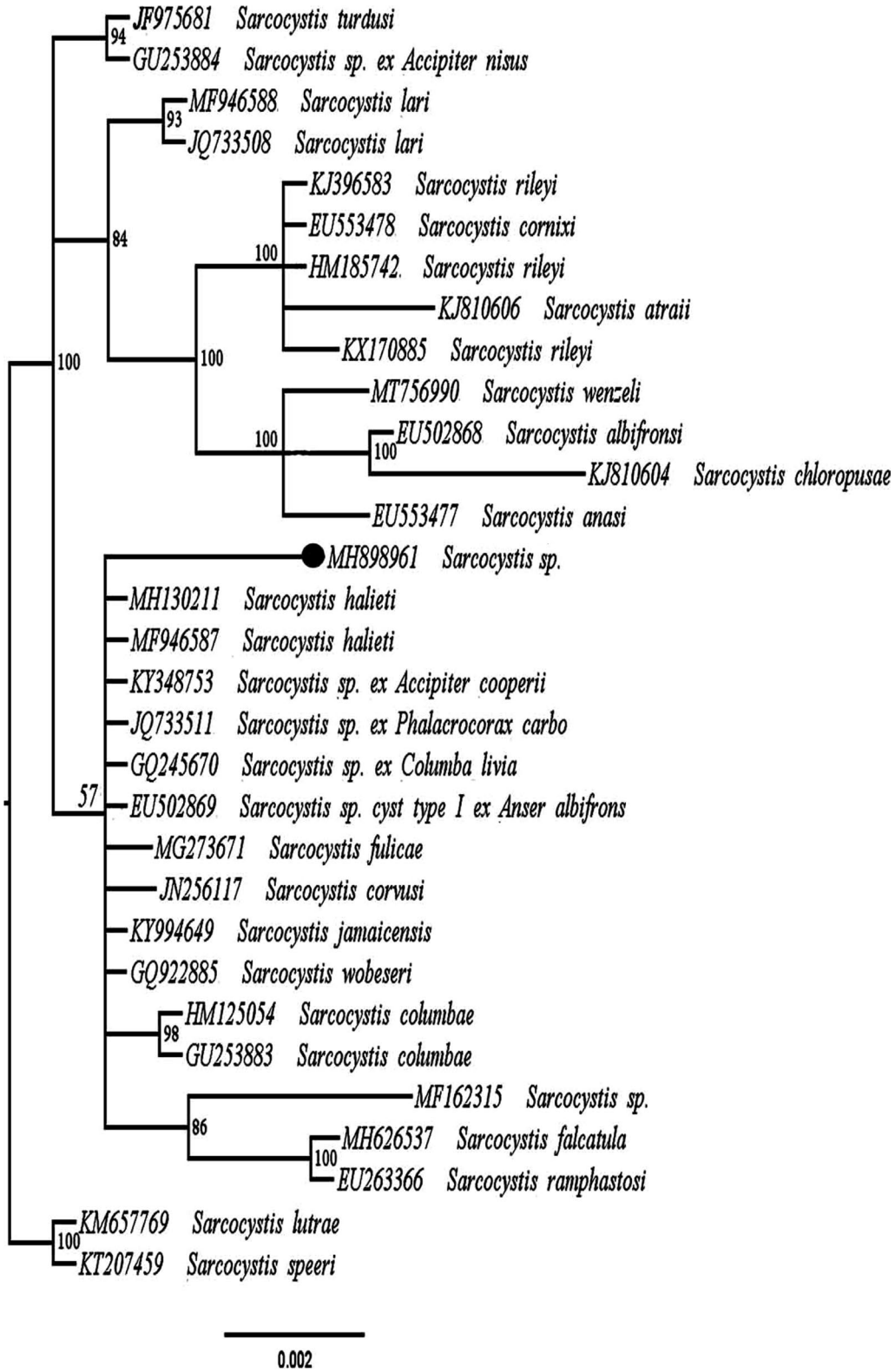


Fig. 2 Phylogenetic tree of the identified *Sarcocystis* sp., compared to other *Sarcocystis* taxa, using 18S rRNA fragment

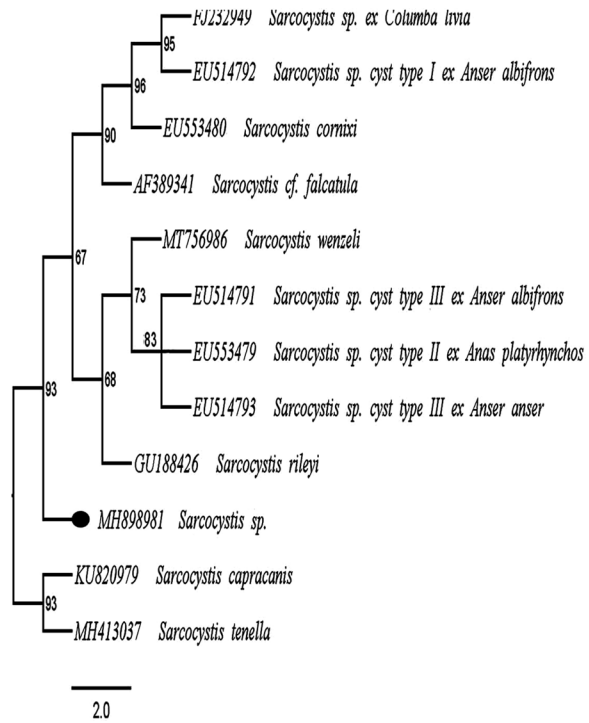


Fig. 3 Phylogenetic tree of the identified *Sarcocystis* sp., compared to other *Sarcocystis* taxa, using 28S rRNA fragment

previously been examined for the presence of sarcocysts. Herein, LM and TEM coupled with molecular analysis showed that the respective *Sarcocystis* sp. was found in the little grebes in Egypt.

The recovered sarcocysts were microscopic, ribbonlike and parallel to the longitudinal axis of muscle fibers. They measured 5–12 mm × 0.05–0.07 mm with a smooth and thin cyst wall measuring 0.6–0.8 µm in thickness. These morphometric features coincided with those reported by Gjerde et al. [7] (who identified *S. halieti* as a new *Sarcocystis* species, based on morphological characteristics) in *S. halieti* sampled from the cardiac muscles of the white-tailed sea eagle, *Haliaeetus albicilla*, in Norway. They were also in agreement with findings of Prakas et al. [21], who identified *S. halieti* in the leg, neck and breast muscles of the great cormorant, *Phalacrocorax carbo*, in Lithuania. The previous literature has reported the existence of several villar protrusions in the cyst wall that appear to have knob-like blebs. [7, 21]. The TEM results for *Sarcocystis* sp. from the current study support these previous findings. On the other hand, Lindsay et al. [33] found that *S. falculata* has a thick wall with fingerlike projections containing microtubules. In addition, *S. lindsayi* from budgerigars were found to be microscopic, long, up to 50 µm in width, and it had a cyst wall more than/equal to 2 µm thick with stylet-like protrusions [34]. In other studies, Kutkiene et al. [35] detected very long ribbon-shaped sarcocysts with a thin cyst wall and irregular villar protrusions that differed in size and shape; Prakas et al. [18] identified wavy wall sarcocysts in the jackdaw, *Corvus monedula*, and named the species *S. corvusi*; and El-Morsey et al. [24] reported that the wall of sarcocysts in *G. chloropus* contained fingerlike projections with a broader base and narrower tips. It is worth noting that in LM and TEM analysis, *S. halieti* appeared to be similar to several *Sarcocystis* species with birds as intermediate host, like *S. calchasi, S. columbae, S. corvusi, S. lari* and *S. wobeseri* [10, 14, 15, 18, 19, 21, 36]. Therefore, *S. halieti* is uniquely characterized by low intermediate host specificity [21, 37]. It must also be noted, however, that hosts are not definitively known for all *Sarcocystis* species included in the present phylogenetic analyses [19].

Accordingly, the development of phylogenetic analyses is critical to the delimitation of *Sarcocystis* species. Previous studies have shown that the use of nuclear ribosomal DNA unit loci (18S rRNA and 28S rRNA genes) can help accurately identify *Sarcocystis* spp., particularly those with avian intermediate hosts [19, 38]. These studies also showed that *S. halieti* is undistinguishable from some avian *Sarcocystis* spp. and almost identical when using the 28S rRNA gene (D2/D3 domain), with a higher degree of variable identity among *Sarcocystis* species than when using the 18S rRNA. In phylogenetic analysis conducted here, the GenBank-deposited isolates of *Sarcocystis* sp. (MH898961) based on the 18S rRNA locus showed 99.2% shared identity with *S. halieti* isolate Dkorm16 in the great cormorant in Lithuania (MH130211) [21] and *S. halieti* isolate Hal. 1.6 in the white-tailed sea eagle in Norway (MF946587) [7]. Interestingly, *Sarcocystis* sp. identified here were typically similar to other *Sarcocystis* spp. in various avian intermediate hosts, e.g., *Sarcocystis* sp. cyst type I ex *Anseralbifrons* isolate from the white-fronted goose, *Anser albifrons*, in Lithuania (EU502869) [39]; *S. wobeseri* isolate from the mallard duck, *A. platyrhinchos* (GQ922886) [10]; *S. calchasi* from domestic pigeons, *Columba livia* f. *domestica*, in Germany (GQ245670) [14], *S. columbae* isolate from the wood pigeon, *Columba palumbus*, in Lithuania (HM125054) [36]; and *Sarcocystis* sp. ex *A. cooperii* from a naturally infected *Cooper's hawk, A. cooperii*, in the USA (KY348753) [38]. In addition, our *Sarcocystis* sp. isolates showed 99.1% shared identity with *S. corvusi* isolate kuos1 from jackdaws in Lithuania (JN256117) [18] as well as 98.8% shared identity with *S. lutrae* isolate Ll1.11 found in the musculature of two Eurasian otters, *Lutralutra*, in Norway (KM657769) [40].

For the 28S rRNA locus, *Sarcocystis* sp. isolated in the current study (MH898981) was nearly identical (98.7%) to the recorded *Sarcocystis calchasi* from racing pigeons

(FJ232949) [41]. However, based on the 28S rRNA locus, no *S. halieti* was previously recorded in GenBank sequences. Authors plan to identify *Sarcocystis* spp. in various wild birds by ITS1 target gene in Egypt.

Little grebes live in colonies in vegetated areas near the edge of freshwater resources; they often move into more open water, occasionally even appearing on the coast in small water ponds. Grebes are excellent swimmers that predate fish and other aquatic vertebrates; however, as they do not walk well [42], other predatory birds often consume little grebes. Currently, the regions that near from the Nile River in Qena, Upper Egypt is an excellent environment for the breeding and colonization of little grebes, which are predicted to have a high prevalence of bird–bird transmission of *Sarcocystis* spp. In Egypt, sarcocystosis in wild birds are scarce, so authors intended to extremely examine wild birds for *Sarcocystis* species in order to explain the life cycle between those birds and mammals, therefore, accurately determine the possible routs of infection in terms of control.

5 Conclusion

This study denoted the occurrence of *Sarcocystis* sp. in the little grebe, *Tachybaptus ruficollis*, for the first time in Egypt. Findings based on morphological characteristics of the identified *Sarcocystis* spp., by light microscopy and TEM, as well as the molecular and phylogenetic analyses. Further study based on ITS1 marker will be considered to in future studies to overcome limitations of the used markers.

Acknowledgements
The authors acknowledge the veterinarians and colleagues who participated in the collection of examined birds.

Authors' contributions
KME was involved in data collection, sample preparing, microscopy, molecular analysis, photograph editing, manuscript writing and formatting and final editing. WMA was involved in molecular analyses, manuscript editing and microscopic examination. NMH was involved in sampling, data collection, microscopy, molecular analysis and drafting. All authors read and approved the final manuscript.

Funding
Not applicable.

Availability of data and materials
All data analyzed during this study are included in this article.

Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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Received: 8 July 2021 Accepted: 1 February 2022
Published online: 10 February 2022

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