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PROTOZOOLOGY - ORIGINAL PAPER

Morphological and molecular description of *Sarcocystis ratti* n. sp. from the black rat (*Rattus rattus*) in Latvia

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Abstract

Rodents have been widely studied as intermediate hosts of *Sarcocystis*; however, only a few reports on these parasites in the black rat (*Rattus rattus*) are known. Having examined 13 black rats captured in Latvia, sarcocysts were found in skeletal muscles of two mammals and were described as *Sarcocystis ratti* n. sp. Under a light microscope, sarcocysts were ribbon-shaped, 0.9–1.3 × 0.09–0.14 mm in size and had a thin (0.8–1.3 μm) and smooth cyst wall. The lancet-shaped bradyzoites were 8.3 × 4.3 (7.5–9.3 × 3.9–4.8) μm. Under a transmission electron microscope, the cyst wall was up to 1.3 μm thick, wavy, the ground substance appeared smooth, type 1a-like. Morphologically, sarcocysts of *S. ratti* were somewhat similar to those of *S. cymruensis*, *S. rodentifelis*, and *S. dispersa*-like previously identified in the brown rat (*Rattus norvegicus*). On the basis of 18S rDNA, 28S rDNA, and *cox1*, significant genetic differences (at least 2.3, 4.5, and 5.8%, respectively) were observed when comparing *S. ratti* with other *Sarcocystis* species using rodents as intermediate hosts. While ITS1 sequences of *S. ratti* were highly distinct from other *Sarcocystis* species available in GenBank. Phylogenetic and ecological data suggest that predatory mammals living near households are definitive hosts of *S. ratti*.

Keywords Black rat · *Sarcocystis ratti* · Transmission electron microscopy · rRNA · *cox1* · Phylogeny

Introduction

Members of the genus *Sarcocystis* are worldwide distributed apicomplexan parasites of reptiles, birds, and mammals. They are characterised by an obligatory two-host prey-predator life cycle. Sexual multiplication takes place in the small intestine of the definitive host, whereas asexual stages including sarcocysts develop in the extra-intestinal tissues of the intermediate host. Some *Sarcocystis* species are intermediate host specific, whereas others have a wide range of hosts (Dubey et al. 2016).

The brown rat was the most comprehensively examined for *Sarcocystis* infection among rat species and this rodent was shown to be an intermediate host for *S. cymruensis* Ashford, 1978, *S. murinotechis* Munday and Mason, 1980, *S. rodentifelis* Grikienienė et al., 1993, *S. singaporensis* Zaman and Colley (1975) 1976, *S. villivillosi* Beaver and Maleckar, 1981, *S. zamani* Beaver and Maleckar, 1981, *S. zuoi* Hu et al., 2005, and *S. dispersa*-like (Munday and Mason 1980; Beaver and Maleckar 1981; Munday 1983; Hu et al. 2005, 2011, 2012; Zaman and Colley 1975, 1976). The synonymy of *S. cymruensis* and *S. rodentifelis* has been discussed in the recent studies (Dubey et al. 2016; Antunes Murata et al. 2018). Snakes, birds of prey, and cats serve as definitive hosts of *Sarcocystis* species found in the muscles of rats (Munday 1977, 1983; Matuschka 1987; Jäkel et al. 1997; Koudela and Modrý 2000). Interestingly, rats can act as both the intermediate and definitive hosts of *S. cymruensis* and *S. rodentifelis* (Hu et al. 2011). Hence, *Sarcocystis* species from rats have a large variety of final hosts.

Two rat species, the black rat (*Rattus rattus* Linnaeus, 1758) and the brown rat, are known in Latvia. Both species dwell near human housing where their enemies are domestic animals, the European polecat (*Mustela putorius* Linnaeus,

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1758), the beech marten (*Martes foina* Erxleben, 1777), and birds of prey such as owls. Despite an omnivore lifestyle, black rats prefer food of plant origin, while brown rats choose animal food (Burnie and Wilson 2006; Kampe-Pērsone 2017). Limited data are available on *Sarcocystis* in black rats. Sarcocysts similar to *S. singaporensis* were detected in the Malaysian black rat (Kan and Dissanaike 1977), meanwhile Thailand black rat harboured *S. singaporensis* and *S. zamani* (Jäkel et al. 1997). Also, sarcocysts of *Sarcocystis* sp. were detected in skeletal muscles of black rats from Lithuania; however, no morphology of cysts was described (Grikienienė et al. 2001). In this paper, a new *Sarcocystis* species found in skeletal muscles of the black rat in Latvia is described based on morphological and DNA investigations.

Materials and methods

Sample collection and morphological examination

Thirteen black rats (*Rattus rattus*) captured in a trap near the farm from Latgale Region in November 2015 were necropsied. Skeletal muscles and such internal organs as the kidneys, the heart, the liver, and the lungs were examined for *Sarcocystis* infection.

To detect sarcocysts, fragments of muscle tissue were stained with 0.2% methylene blue solution, lightened with 1.5% acetic acid solution, placed in a glass compressor, and studied under a stereomicroscope at × 20 magnification. *Sarcocystis* infection intensity was evaluated by counting sarcocysts found in methylene blue–stained 28 oat-size pieces of muscle (~ 1 g).

Morphological analysis of sarcocysts observed was performed in fresh-squashed samples of the muscle. Sarcocysts with a small amount of host tissue were excised with the help of two preparation needles, and afterwards were characterised morphologically. Sarcocysts were described according to the size and shape of the cyst, the structure of the cyst wall, and morphometric parameters of bradyzoites. Cysts were measured under a stereomicroscope at × 20 magnification, and a detailed morphological characterisation was carried out under a light microscope (LM) at × 40–1000 magnification.

A single isolated sarcocyst was fixed in 2% glutaraldehyde and subjected to transmission electron microscopy (TEM) analysis. Sarcocyst was postfixed in 1% osmium tetroxide, dehydrated, and infiltrated in epoxy resin. Sections were cut on a Leica UC6 ultramicrotome and stained with 4% uranyl acetate and 3% lead citrate. Grids were imaged at 100 kV with the Morgagni 268 TEM (FEI, Hillsboro, OR, USA).

Molecular analysis

Two sarcocysts from two individuals of black rats were excised from fresh muscle preparations, preserved in individual microcentrifuge tubes containing 96% ethanol and kept at − 20 °C until molecular examination. The isolated sarcocysts were molecularly characterised at four genetic loci (18S rDNA, 28S rDNA, ITS1, and *cox1*).

Genomic DNA was extracted from sarcocysts using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nearly complete 18S rDNA was amplified using SarAF/SarBR and SarCF/SarDR primer pairs; partial 28S rDNA sequences were amplified using KL-P1F/KL-P2R primer pair (Kutkienė et al. 2010) and partial *cox1* sequences were amplified with the help of SF1/SR5 primers (Gjerde 2013) and the complete ITS1 region was amplified using SU1F/5.8SR2 primer pair (Gjerde 2014). PCRs for 18S rDNA, 28S rDNA, and *cox1* were performed in a final 25-μL volume consisting of 0.5 μM of each primer, 12.5-μL DreamTaq PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania), 0.04 μg template DNA, and nuclease-free water. Amplification reactions were carried out, starting with the initial hot start at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 54–60 °C depending on the primer pair for 45 s, elongation at 72 °C for 80 s, and ended with the final extension at 72 °C for 10 min. PCR amplification of ITS1 region was unsuccessful with DreamTaq polymerase; therefore, Platinum™ II Hot-Start Green PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) was used. PCR for ITS1 was performed in a final 20-μL volume consisting of 0.5 μM of each primer, 10-μL Platinum II Hot-Start Green PCR Master Mix, 0.04 μg template DNA, and nuclease-free water. The cycling conditions began with 1 cycle at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 s, at 60 °C for 15 s, and at 68 °C for 15 s. The amplified products were visualised using 1.5% agarose gel electrophoresis and purified with the help of ExoI and FastAP (Thermo Fisher Scientific Baltics, Vilnius, Lithuania). The PCR product visualisation, purification, and sequencing were carried out in the previously described way (Prakas et al. 2016).

The resulting sequences were compared with those of various *Sarcocystis* spp. using Nucleotide BLAST program megablast and blastn options (http://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments were obtained with the help of MUSCLE algorithm implemented in the MEGA7 (Kumar et al. 2016). Selection of nucleotide substitution model and phylogenetic analyses under Bayesian inference were conducted using TOPALi v2.5 (Milne et al. 2004).

Results

Infection rates and morphological characteristics of *S. ratti*

Sarcocystis infection was detected in 15.4% (2/13) of rats examined. Sarcocysts were found in skeletal muscles, while

no cysts were discovered in internal organs. The intensity of *Sarcocystis* infection varied in two animals and numbered from 2 to 67 sarcocysts in 1 g of muscle samples.

Under a light microscope, one morphological type of sarcocysts was observed. Sarcocysts were microscopic, ribbon-shaped with round tips, and measured 0.9–1.3 × 0.09–0.14 ($n = 5$) mm. The cyst wall was thin (0.8–1.3 μm; $n = 3$) and seemed smooth without visible protrusions (Fig. 1a). Lancet-shaped bradyzoites were 8.3 × 4.3 (7.5–9.3 × 3.9–4.8; $n = 35$) μm in size (Fig. 1b). Under the TEM, the cyst wall reached up to 1.3 μm in thickness, lacked protrusions, and appeared slightly wavy (Fig. 1c). The parasitophorous vacuolar membrane was about 100 nm thick and had small knob-like blebs (Fig. 1d). The ground substance appeared smooth and continued into the interior of the cyst as septae that subdivided the cyst in chambers filled with bradyzoites. The cyst wall was type 1a-like (Dubey et al. 2016). Based on DNA sequence comparison, sarcocysts found in black rats in Latvia were proposed as *S. ratti* n. sp.

Molecular characteristics of *S. ratti* and phylogeny

Two *S. ratti* isolates obtained from two black rats were identical at 1757-bp-long 18S rDNA (MK425189-MK425190), 1475-bp-long 28S rDNA (MK425192-MK425193), 1053-bp-long *cox1* (MK430072-MK430073), and 902-bp-long ITS1 (MK910965-MK910966). Considerable genetic differences, from 2.3, 4.5, and 5.8% within 18S rDNA, *cox1*, and 28S rDNA, respectively, were observed when comparing *S. ratti* with other *Sarcocystis* species using the rodent as intermediate hosts. While significant match was not obtained comparing ITS1 sequences of *S. ratti* with those of other *Sarcocystis* spp. available in GenBank. Thus, *S. ratti* is clearly molecularly separable at all four loci examined from other *Sarcocystis* species using rodents as intermediate hosts. The lowest interspecific variability was detected comparing 18S rDNA sequences. Based on this locus, *S. ratti* showed the greatest similarity (98.3%) to *S. rileyi* Stiles, 1893 (Dubey et al. 2003) (GU120092) and displayed > 97% similarity to more than 20 *Sarcocystis* species. At *cox1*, sequences of *S. ratti* demonstrated 95.5% identity with those of *S. strixi* Verma et al., 2017 (MF162317) and *S. cymruensis* (MG571085), 95.2% identity with *S. lutrae* Gjerde and Josefsen, 2015 (MF596285), *S. lari* Prakas et al., 2014 (MF596283), and *S. speeri* Dubey and Lindsay, 1999 (KT207461). Based on the 28S rDNA sequences, *S. ratti* had the strongest identity (94.2%) with *S. cymruensis* (MH564724) and disclosed 93.1–93.4% identity with *S. glareoli* (Erhardrová, 1955) Odening, 1997 (AF044251), *S. jamaicensis* Verma et al., 2017 (KY994650), *S. lari* (MF946611), *S. muris* (Railliet, 1886) Labbe, 1899 (AF012883), *S. fulicae* Prakas et al., 2018 (MG273672), and *S. turdusi* Kutkienė et al., 2012 (JF975682). In summary, *S. ratti* showed the greatest genetic similarity to *Sarcocystis* species distinguished by the rodent-cat (*S. cymruensis*, *S. muris*, and *S. rodentifelis*), the rodent-bird (*S. glareoli*, *S. jamaicensis*, and *S. strixi*), the rodent-opossum (*S. speeri*), the bird-bird (for instance, *S. halieti* Gjerde et al., 2018), the bird-carnivorans (for instance, *S. rileyi*), and the carnivorans-unknown (for instance, *S. lutrae* Gjerde and Josefsen, 2015) life cycle.

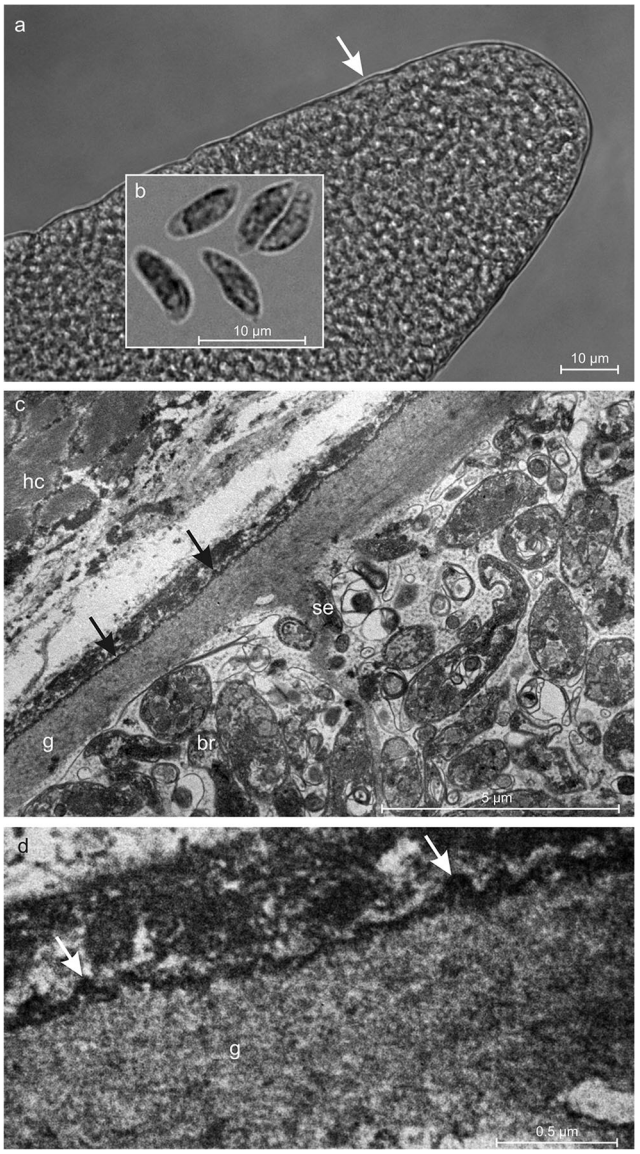

Fig. 1 Morphology of *Sarcocystis ratti* n. sp. from muscles of the black rat (*Rattus rattus*). **a**, **b** LM micrographs. Fresh preparations. **a** Portion of sarcocyst showing a thin and apparently smooth cyst wall (arrow). **b** Lancet-shaped bradyzoites. **c**, **d** TEM micrographs. **c** Fragment of a slightly wavy cyst wall (arrows); note muscular host cell (hc), bradyzoite (br), septae (se), and ground substance (g). **d** High magnification of the cyst wall; a parasitophorous vacuolar membrane has knob-like blebs (arrowheads), the ground substance seems smooth

The fragment of 18S rDNA analysed was not variable enough to robustly resolve phylogenetic relationships of selected *Sarcocystis* species closely related to *S. ratti* (Fig. 2).

The newly described species from the black rat formed a separate branch in the phylogenetic tree obtained using *cox1* sequences. It should be noted that 18S rDNA and 28S rDNA phylogenetic trees did not demonstrate any close relationship between *S. ratti* and *Sarcocystis* species employing rodents as intermediate and snakes as definitive hosts (*S. atheridis* Slapeta et al., 1999, *S. singaporensis*, *S. zamani*, *S. zuoi*, and *Sarcocystis* sp. AF513490). Based on 28S rDNA, *S. ratti* was a sister species to *S. muris* and *S. cymruensis*.

Taxonomic summary of *S. ratti* n. sp.

Type intermediate host: The black rat (*Rattus rattus*).

Definitive host: Unknown.

Locality: Eastern Latvia, Latgale region.

Specimens deposited: TEM material deposited at the National Centre of Pathology, Vilnius, Lithuania. Sequences deposited in NCBI GenBank with accession numbers MK425189-MK425190 (18S rDNA), MK425192-MK425193 (28S rDNA), MK430072-MK430073 (*cox1*), and MK910965-MK910966 (ITS1).

Etymology: the Latin name of genus *Rattus* is used for the species name.

Recorded in URN as urn:lsid:zoobank.org:act:28CFF2F6-D557-46FC-8F01-BEF9A2AAE88A.

Discussion

Here we describe *S. ratti* from the black rat in Latvia characterised by sarcocyst having a thin (up to 1.3 μm) cyst wall without clearly visible protrusions (Fig. 1). Ribbon-shaped sarcocysts of *S. ratti* were up to 1.3 × 0.14 mm in size, and lancet-shaped bradyzoites measured 8.3 × 4.3 (7.5–9.3 × 3.9–4.8) μm. Despite numerous reports on *Sarcocystis* in rodents, limited data have been acquired of *Sarcocystis* in the black rat. The reticulated python (*Python reticulatus* Schneider, 1801), definitive hosts of two species identified in the black rat, *S. singaporensis* and *S. zamani* (Jäkel et al. 1997), does not dwell in the territory of Latvia. Furthermore, the cyst wall structure of both species apparently differs from the detected one in the present investigation. Whereas, three *Sarcocystis* species (*S. cymruensis*, *S. rodentifelis*, and *S. dispersa*-like), which also have a thin and smooth sarcocyst wall, were found in the brown rat. Under TEM, *S. ratti* and *S. cymruensis* had a similar sarcocyst wall structure, and their parasitophorous vacuolar membranes contained numerous small blebs (Hu et al. 2011; Antunes Murata et al. 2018). However, there is a notable difference in the sizes of bradyzoites, as those of *S. cymruensis* were more elongated, measured 11.0–13.5 × 3.0–5.0 μm, in size

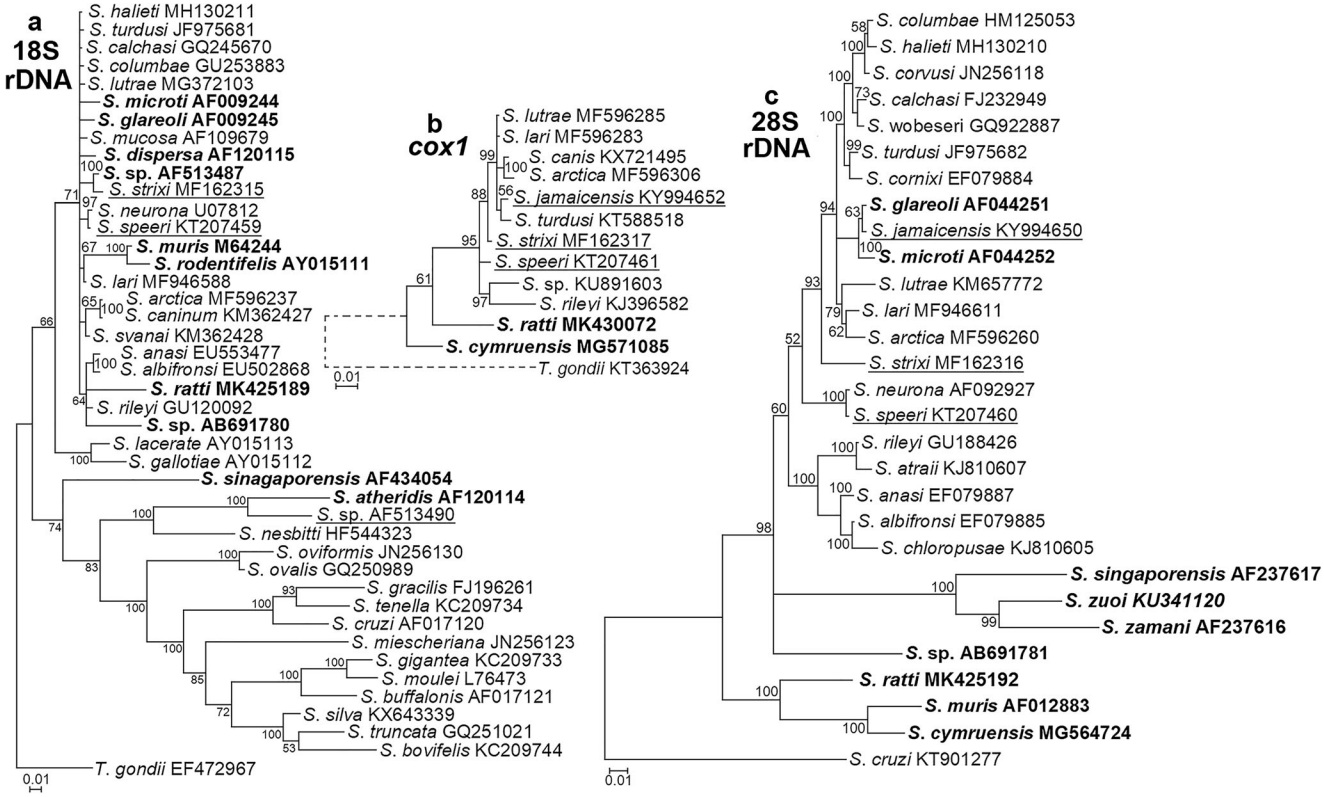

Fig. 2 Phylogenetic placement of *S. ratti* based on 18S rDNA (**a**), *cox1* (**b**), and 28S rDNA (**c**) sequences. The trees have been constructed using the Bayesian methods, scaled according to the branch length, and rooted on *Toxoplasma gondii* (**a** and **b**) or *S. cruzi* (**c**). *Sarcocystis* species using rodents as natural and experimental intermediate hosts are in boldface and underlined, respectively

(Hu et al. 2011). Under LM, a smooth cyst wall of *S. rodentifelis* was up to 1.0 μm in thickness, whereas, banana-like bradyzoites of this species were slightly longer and thinner (11.6–14.7 × 2.2–4.2 μm) than those belonging to *S. ratti* (Grikienienė et al. 1993). By TEM, sarcocysts of *S. dispersa*-like had simple wall structure, without protrusions on the surface, but with invaginations (Munday 1983). The DNA sequence comparison performed in this study indicated significant genetic differences between *S. cymruensis*, *S. rodentifelis*, *S. dispersa*-like, and *S. ratti* at 18S rDNA, 28S rDNA, and *cox1*. Based on the 18S rDNA sequences, *S. ratti* showed 97.5% identity to *S. dispersa*. Having compared sequences of *S. ratti* and *S. rodentifelis*, 97.1% (18S rDNA) and 89.9% (28S rDNA) identity was observed. Meanwhile sequences of *S. ratti* and *S. cymruensis* differed by 3.5%, 5.8%, and 4.5% within 18S rDNA, 28S rDNA, and *cox1*, respectively.

Despite a wide variety of *Sarcocystis* species using rodents as an intermediate host (Prakas and Butkauskas 2012), only some of them (*S. atheridis*, *S. cymruensis*, *S. dispersa*, *S. glareoli*, *S. jamaicensis*, *S. microti* (Findlay and Middleton, 1934) Modrý et al. 2004, *S. muris*, *S. rodentifelis*, *S. singaporensis*, *S. speeri*, *S. strixi*, *S. zamani*, *S. zuoi*, and several *Sarcocystis* sp.) have been characterised genetically. Predominantly, rDNA sequences were used for a genetic description and identification of these *Sarcocystis* spp. (Votýpka et al. 1998; Dolezel et al. 1999; Mugridge et al. 1999, 2000; Slapeta et al. 2001, 2003; Hu et al. 2012). Whereas other genes, like *cox1* and ITS1, were only occasionally applied to *Sarcocystis* using rodents as intermediate hosts (Dubey et al. 2015; Verma et al. 2017; Watthanakaiwan et al. 2017; Antunes Murata et al. 2018). Interestingly, in the present study, obtained ITS1 sequences of *S. ratti* had no considerable similarity to other *Sarcocystis* species; therefore, further molecular studies on ITS1 of *Sarcocystis* species from rodents are highly preferable. The current study indicated higher variability of *Sarcocystis* from rodents within *cox1* and 28S rDNA as compared with 18S rDNA. Furthermore, 18S rDNA sequences, in contrast to 28S rDNA and *cox1*, did not establish robust phylogenetic relationships among *Sarcocystis* spp. closely related to *S. ratti* (Fig. 2).

A phylogenetic analysis demonstrates no close relationship between *S. ratti* and *Sarcocystis* species characterised by a rodent-bird or rodent-snake life cycle (Fig. 2). Based on 28S rDNA, *S. ratti* was most closely related to *S. muris* and *S. cymruensis* using the cat as the natural definitive host. Whereas, *S. ratti* was placed separately from *S. cymruensis* in the *cox1* phylogenetic tree. Thus, on the basis of phylogenetic results, predatory mammals are presumed to be definitive hosts of *S. ratti*. Since the black rat is synanthropic species, possible definitive hosts of *S. ratti* could be predators living near households, such as the domestic cat, the domestic dog, the European polecat, or the beech marten.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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