The resurrection of a species, Sarcocystis asinus Gadaev 1978, in donkeys that is distinct from Sarcocystis bertrami/fayeri currently found in horses based on mitochondrial cox1 sequences

Junjie Hu (jjhu@ynu.edu.cn)
Yunnan University
Mingzhu Zhang
Yunnan University
Kaiwen Wei
Yunnan University
Zhipeng Wu
Yunnan University
Jun Sun
Yunnan University
Shuangsheng Deng
Yunnan University
Jianping Tao
Yangzhou University

Research Article

Keywords: Sarcocystis asinus , Donkey , Morphological and molecular characterization , Phylogenetic analysis

Posted Date: April 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1499716/v1

Abstract

There is considerable confusion concerning the relationships among species of Sarcocystis found in donkeys and horses. Here, sarcocysts obtained from donkeys (Eqqus asinus) were morphologically and molecularly characterized. Sarcocysts were diagnosed in 12 of 32 (37.5%) Chinese donkeys and could be divided into two forms, thin-walled and thick-walled, with the aid of light microscopy (LM). The thin-walled sarcocysts were macroscopic (up to 4856 × 320 μm in size) and had short club-like protrusions of up to 2.7 μm; the thick-walled sarcocysts were microscopic (up to 3750 × 135 μm in size) and had hair-like protrusions of up to 5.4 μm. The ultrastructures of the two forms exhibited highly similar morphological characteristics, including bundled microtubules in the core of the villus protrusions penetrating diagonally into the ground substance, similar to wall type 11. Three genetic markers, 18S rDNA, 28S rDNA, and mitochondrial cox1, were sequenced and analyzed in the two forms. The three genetic markers presented high intraspecific similarity between the two forms, ranging from 97.2–99.5% (average 97.8%), 97.8-99.6% (average 98.4%) and 99.0–99.9% (average 99.4%), respectively. The comparison of these sequences with those of Sarcocystis spp. previously deposited in GenBank showed that the newly obtained sequences of 18S rDNA, 28S rDNA, and mitochondrial cox1 presented identities of 90.0–97.5% (average 94.7%), 94.7–95.1% (average 94.9%) and 82.6–84.5% (average 83.4%), respectively, with those of S. bertrami/fayeri obtained from horses. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on the mitochondrial cox1 sequences of donkey and horse sarcocysts revealed three fragments (196, 243 and 641 bp) and two fragments (416 and 644 bp), respectively, upon digestion with the restriction enzymes HinifI and EcorRI. Phylogenetic analysis based on 18S rDNA or mitochondrial cox1 sequences revealed that donkey sarcocysts formed an individual clade that clustered with Sarcocystis spp. obtained from horses. Based on the divergence of the three sarcocyst genetic markers, especially mitochondrial cox1, between donkeys and horses, Sarcocystis asinus should be resurrected and regarded as a valid species in donkeys. The cross-infection of Sarcocystis between donkeys and horses needs to be attempted for further confirmation in the future.

Introduction

The sarcocysts infecting donkeys were first described and named *Sarcocystis asinus* by Gadaev (1978), although this was based on limited observations. Subsequently, on the basis of cross-infection between horses and donkeys and the morphological similarity of the sarcocysts found in the two hosts, *S. asinus* was questioned, and the species of *Sarcocystis* infecting the two hosts came to be regarded as the same species (Levine and Tadros 1980; Odening et al. 1995; Odening 1998). Thus, the name *S. bertrami* is currently used for the sarcocysts diagnosed in the muscles of donkeys by most authors (Dubey et al. 2016; Passantino et al. 2019).

The name *S. bertrami* was first proposed for the sarcocysts found in horses by Doflein (1901). After the identification of dogs as a definitive host of the parasite, *S. bertrami* was renamed *S. equicanis* by Rommel and Geisel (1975) following the nomenclature proposed by Heydorn et al., (1975). Two years later, Dubey et al., (1977) described *S. fayeri* as an additional *Sarcocystis* species transmitted by dogs

based mainly on differences in the size and prepatent period of the sporocysts from those of *S. equicanis*. Currently, the sarcocysts found in horses are divided into two forms by Dubey et al. (2016): a macroscopic form (up to 15 mm long) with a thin cyst wall, named *S. bertrami* (synonym *S. equicanis*), and a microscopic form (up to 990 μm long) with a thick cyst wall, named *S. fayeri*. However, the morphological characteristics of horse sarcocysts have been observed to undergo changes in various stages of development, showing long protrusions in the early phase and short protrusions in the later phase (Fayer et al. 1983). Therefore, there is considerable confusion concerning the relationship between *S. bertrami/ equicanis* and *S. fayeri* (Odening et al. 1995; Ma et al. 2020).

In recent decades, molecular analysis based on nucleotide sequences has been recommended as a useful and efficient tool for delineating or identifying species of *Sarcocystis* from the same or different hosts. There are currently only limited donkey sarcocyst 18S rDNA and mitochondrial *cox*1 sequences deposited in GenBank, which were provided by Zeng et al. (2018). Based on the high similarity of mitochondrial *cox*1 sequences, these authors proposed *S. bertrami* (syn. *S. fayeri*) as the descriptor of the parasites of both horses and donkeys. However, the reliability of the meat samples used in their study was based mainly on the applied version of meat Cutter (according to communication with the corresponding author of this paper, Dr. Yang). However, in local meat markets, meat sellers or butchers sometimes mix horse and donkey meat or replace donkey meat with horse meat to seek better returns because the price of donkey meat is higher than that of horse meat.

The morphological and molecular characteristics of *S. bertrami* sarcocysts isolated from horses in China have been investigated by our group previously (Ma et al. 2020). To clarify the relationship of the *Sarcocystis* species of donkeys and horses based on the molecular identification of meat samples, the aims of the present study were to investigate the morphological characteristics of donkey sarcocysts and to explore the relationship of the *Sarcocystis* species of donkeys and horses via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and phylogenetic analysis.

Materials And Methods

Meat sample collection and morphological examination

Donkey meat serves as a food source for humans and is commonly marketed in China. Muscle tissues from a total of 32 donkeys were purchased in a meat market in Kunming City located in Yunnan Province, China, in October 2019. Fresh muscle tissue (200 g each) from each donkey was examined for sarcocysts. In the laboratory, 10 specimens of approximately 10 × 3 mm in size from each collected sample were pressed and squeezed between two glass slides and then inspected using a stereomicroscope. Thereafter, individual sarcocysts were extracted and isolated from skeletal muscular fibers using needles and processed for light microscopy (LM), transmission electron microscopy (TEM) and DNA analysis.

To further confirm the identity of the meat samples, donkey meat and horse meat (collected previously and stored at -40 ℃) infected with sarcocysts were processed for molecular identification in the laboratory. DNA from each animal was extracted using a TIANamp Genomic DNA Kit (TIANGEN BIOTECH CO., LTD, Beijing, China) according to the manufacturer's instructions. Mitochondrial *cox*1 was amplified with the primer pair ML1F/MF1R (5'-ACCACAAAGACATCGGCACT-3'/5'-CGTTTGGATGCGAATGCT-3'), designed using Oligo 5.0 software (National Biosciences, Inc., Plymouth, MN, USA) based on the highly conserved areas of mitochondrial *cox*1 sequences of *Equus* spp. deposited in GenBank. PCR assays were carried out in 15 μl reaction with 50–100 ng of total DNA, 1 unit of Taq polymerase (TaKaRa, Dalian, China), and 20 pmol of each primer. The amplification program consisted of 35 cycles of 95 °C for 5 min, 54 °C for 30 sec, and 72 °C for 5 min. The PCR products were sequenced on an ABI 3730XL automatic DNA sequencer (Applied Biosystems, Inc., Foster City, California).

For TEM, four sarcocysts of each form were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C, postfixed in 1.0% osmium tetroxide in the same buffer, dehydrated in a graded alcohol series, and embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEM100-CX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

Molecular characterization

For DNA analysis, three individual sarcocysts of each form obtained from donkey meat and one sarcocyst isolated from identified molecularly horse meat were subjected to genomic DNA extraction using a TIANamp Genomic DNA Kit. Three genetic markers, 18S rDNA, 28S rDNA and mitochondrial *cox*1, were amplified from donkey sarcocysts with the primer sets S1/S4 (Fischer and Odening 1998), KL1/KL3, KL4/KL5b and KL6a/KL2 (Mugridge et al. 1999), and SF1/SR9 (Gjerde 2013, 2014), respectively. PCR products were gel purified, cloned, and sequenced using the methods detailed in a previous paper (Hu et al. 2016). Only mitochondrial *cox*1 was amplified from the sarcocyst obtained from horse meat using the SF1/SR9 primers for RFLP.

To establish the PCR-RFLP strategy for discriminating the sarcocysts of donkeys and horses, the internal endonuclease cleavage sites in the mitochondrial *cox*1 nucleotide sequences of the sarcocysts of the two hosts were screened using Premier 5.0 software (Premier, Canada). Three mitochondrial *cox*1 nucleotide sequences (MH025631–MH025633) of *S. bertrami* from horses morphologically identified in our laboratory (Ma et al. 2020) were used in the analysis of endonuclease cleavage sites. Based on the screening results, two restriction enzymes, EcoRI and HinfI, were selected to digest the amplified PCR products of mitochondrial *cox*1 from the sarcocysts of the two hosts because of their ability to produce different numbers of fragments from the sarcocysts of donkey (three, of 196, 243 and 641 bp) and horse (two, of 416 and 644 bp). Aliquots of 10 μl of the resulting PCR products were double digested in 20 μl reactions with 1 μl EcoRI and 1 μl HinfI and 10 × Cutsmart buffer following the recommendations of the

manufacturer (New England BioLabs). The digested products were analyzed via gel electrophoresis on 2% agarose gels stained with Goldenview at 100V in 0.5× TBE buffer.

Phylogenetic analyses were conducted separately on the nucleotide sequences of the 18S rDNA and mitochondrial *cox*1 sequences by using MEGAX software (Kumar et al. 2018). The maximum likelihood (ML) trees of 18S rDNA and mitochondrial *cox*1 were generated with the Tamura 3-parameter and Hasegawa-Kishino-Yano models, respectively, according to the Find Best DNA/Protein Models program integrated into MEGAX. The reliability of the maximum likelihood phylograms was tested via the bootstrap method using 1000 replications.

The 18S rDNA and mitochondrial *cox*1 sequences of *Sarcocystis* spp. from different hosts were downloaded from GenBank and aligned using the ClustalW program implemented in MEGAX. The final alignment of the 18S rDNA sequences consisted of 26 nucleotide sequences and 1045 aligned positions from 18 taxa. *Cystoisospora ohioensis* (GU292304), *Hammondia hammondi* (KF854253), and *Toxoplasma gondii* (U12138) were chosen as outgroups. The final alignment of mitochondrial *cox*1 sequences consisted of a total of 29 nucleotide sequences and 933 aligned positions from 20 taxa. *T. gondii* (JX473253), *Hammondia triffittae* (JX473247) and *Hammondia heydorni* (JX473251) were used as outgroup species to root the tree.

Results

Molecular identification of meat samples

The 14 meat samples (12 for donkeys and 2 for horses) infected with sarcocysts were molecularly identified based on mitochondrial *cox*1 sequences. The 14 newly obtained *cox*1 sequences were approximately 1350 bp long and shared 91.3–92.5% identity between donkeys and horses. At this locus, the 12 newly obtained nucleotide sequences from donkey meat and the 2 newly obtained nucleotide sequences from horse meat shared 98.7-99.7% and 98.4-99.9% identity, respectively, with those of *E. asinus* and *E. cabullus* previously deposited in GenBank.

Morphological observation of sarcocysts in donkeys

Sarcocysts were found in 12 of 32 (37.5%) donkeys. Using LM, the sarcocysts could be divided into two forms: thin-walled and thick-walled (Fig. 1a, c). The thin-walled sarcocysts were macroscopic, measuring 2350–4856 × 110–320 μm [average = 2787 (± 442) × 210 (±64) μm, (±SD); *n* = 20 isolated from five donkeys] in size, and exhibited numerous short club-like protrusions with lengths of 2.0 to 2.7 μm [mean = 2.4 ± 0.18 μm; *n* = 20 measurements from 10 sarcocysts); they were septate and contained bradyzoites measuring 14.5–17.4 × 3.5–5.0 μm [average =15.8 (±1.8) × 4.2 (±0.4) μm, *n* = 20 measurements from three sarcocysts) in size. The thick-walled sarcocysts were microscopic, measuring 1200–3750 × 45–135 μm [average = 2213 (± 126) × 98 (±21) μm, *n* = 20 isolated from four donkeys] in size and showed hair-like protrusions with lengths of 3.0 to 5.4 μm [mean = 4.2 (±0.25) μm, *n* = 20 measurements from 10

sarcocysts]; they were septate and contained bradyzoites measuring 12.1–16.2 × 2.5–4.7 μm [average = 14.5 (± 1.2) × 4.1 (±0.3) μm, *n* = 20 measurements from five sarcocysts) in size.

The ultrastructures of the two forms of sarcocysts exhibited similar morphological characteristics (Fig. 1 b, d): the primary cyst wall had numerous villus protrusions with bundled microtubules in the core, which penetrated diagonally into the ground substances and sometimes reached the interior border of the ground substance. Minute undulations were present over the entire sarcocyst surface. A layer of ground substance was present beneath the protrusions. Overall, the cyst wall type was similar to type 11 classified by Dubey et al. (2016).

Molecular characterization of sarcocysts in donkeys

The three selected genes (18S rDNA, 28S rDNA and mitochondrial *cox*1) were successfully amplified from six individual sarcocysts (three thin-walled cysts and three thick-wall cysts) isolated from four donkeys. The three 18S rDNA sequences (accession numbers OM971696–OM971698) of the thin-walled sarcocysts were 1591–1614 bp long and shared 97.7–99.8% identity (average 98.4%). The three 18S rDNA sequences (OM971699–OM971701) of the thick-walled sarcocysts were 1589–1607 bp long and shared 97.7–98.5% identity (average 98.2%). The similarity between the two forms was 97.2–99.5% (average 97.8%). The most similar sequences in GenBank to the newly obtained 18S rDNA sequences were those of *Sarcocystis* spp. obtained from donkeys and horses in different regions, including *S.* cf. *bertrami* (KX545381–KX545396) from Chinese donkeys (93.5–97.5% identity, average 96.6%), *S. bertrami* (MH025625–MH025628 and KX545397–KX545404) from Chinese horses (94.0–97.5% identity, average 96.3%), *S. fayeri* (LC171838) from an Italian horse (95.2–97.1%, average 96.3), *S. fayeri* (AB661437–AB661447) from Japanese horses (90.8–97.4% identity, average 94.7%), *S. fayeri* (AB972440–AB972443 and LC171831–LC171837) from Canadian horses (90.0–97.1% identity, average 94.1%), and *S. fayeri* (MF614956) from an Egyptian horse (93.7–93.8%, average 93.8%).

The three 28S rDNA sequences (OM971683–OM971685) obtained from thin-walled sarcocysts were 3441–3450 bp length and shared 97.7–98.5 identity (average 98.1%). Only two 28S rDNA sequences (OM971686 and OM971687) of thick-walled sarcocysts were successfully assembled. They were 3445 and 3446 bp in length and shared 98.8% identity. The similarity between the two forms was 97.8-99.6% (average 98.4%). The most similar sequences were those of S. *bertrami* (MH025629–MH025630) from Chinese horses (94.7–95.1% identity, average 94.9%), followed by those of *S. suihominis* (MK867471–MK867473) obtained from domestic pigs (90.0–91.2% identity, average 90.7%).

The three mitochondrial *cox*1 sequences (OM970235–OM970237) of thin-walled sarcocysts were 1085 bp in length and shared 99.2–99.7 identity (average 99.4%). The three mitochondrial *cox*1 sequences (OM970238–OM970240) of thick-walled sarcocysts were 1085 bp in length and shared 99.2–99.3% identity (average 99.3%). The identity between the two forms was 99.0–99.9% (average 99.4%). The most similar sequence in GenBank was that of *Sarcocystis* sp. (KY399759) obtained from a Chinese donkey (98.6–99.4% identity, average 98.8%), followed by those of *Sarcocystis* spp. obtained from horses and

donkeys in different regions, including *S. fayeri* (LC171840–LC171854) from Canadian horses (82.8–84.5% identity, average 83.9%), *S. bertrami* (MH025631–MH025633, KY399751–KY399755, KY399758, KY399760–KY399762, and MF152616–MF152619) from Chinese horses (82.6–84.5% identity, average 83.6%), *S. fayeri* (LC171857) from an Italian horse (83.1-83.8% identity, average 83.4%), *S.* cf. *bertrami* (KY399747–KY399750, KY399756, KY399757, MF152613–MF152615) from Chinese donkeys (83.1–83.8% identity with an average of 83.4%), and *S. fayeri* (LC171855 and LC171856) from Japanese horses (82.7–83.4% identity, average 83.1%).

PCR-RFLP based on mitochondrial *cox*1 obtained from donkey and horse sarcocysts

The PCR-amplified products of mitochondrial *cox*1 from donkey and horse sarcocysts were successfully digested by EcorRI and HinifI. This produced three fragments (196, 243 and 641 bp) and two fragments (416 and 644 bp) for donkey and horse, respectively (Fig. 2).

Based on the divergence of the 18S rDNA, 28S rDNA and mitochondrial *cox*1 sequences of donkey and horse sarcocysts, the *Sarcocystis* parasites of donkeys are different from those of horses. Hence, the name *S. asinus* needs to be resurrected for the sarcocysts of donkeys.

Phylogenetic analysis

Phylogenetic analysis based on the 18S rDNA and mitochondrial *cox*1 sequences of *S. asinus* sarocysts confirmed their association with *Sarcocystis* species. In the tree inferred from 18S rDNA sequences (Fig. 3), *S. asinus* formed an individual clade within a group comprising *Sarcocystis* spp. obtained from horses and donkeys originating from different areas, including *S. bertrami/ fayeri* (LC171835, KX545399, MH025625, LC171838) from horses and *S.* ef. *bertrami* (KX545386) from a donkey. In the tree inferred form mitochondrial *cox*1 sequences (Fig. 4), the newly obtained mitochondrial *cox*1 sequences of *S. asinus* formed an individual clade with *Sarcocystis* sp. (KY399759) obtained from a donkey within a group comprising *Sarcocystis* spp. from both horses and donkeys, including *S. bertrami/ fayeri* (MH02563, LC171856, LC171857, and LC171850) from horses and *S.* cf. *bertrami* from a donkey (KY399749).

Discussion

Sarcocystis is a common parasitic protozoan with a worldwide distribution found in a variety of mammals and birds, especially in domesticated food animals. Sarcocysts have been diagnosed in donkeys from the former USSR (Gadaev 1978), Austria (Hinaidy and Loupal 1982), Germany (Matuschika 1983), Morocco (Kirmse 1986), Egypt (Hilaili and Nasser 1987; Dubey et al. 2016), China (Hu et al. 2001; Zeng et al. 2018), and Italy (Passantino et al. 2019). In the present study, the prevalence of sarcocysts in the investigated Chinese donkeys was 37.5% (12/32), and it has been reported to be higher than 22.0%

(9/41) in Moroccan donkeys (Kirmse 1986) and 28.6% (40/140) in Italian donkeys (Passantino et al. 2019), but lower than 40% (8/20) in the former USSR (Gadaev 1978), 90.0% (18/20) in Egyptian donkeys (Hilaili and Nasser 1987) and 92.3 (24/26) in Chinese donkeys surveyed by our group 20 years ago (Hu et al. 2001).

In the present study, two forms of sarcocysts (thin-walled cysts and thick-walled cysts) were observed in the muscle tissues of donkeys under LM. The thin-walled sarcocysts were macroscopic (up to 4856 long and 320 μm wide) and had short club-like protrusions (up to 2.7 μm long); the thick-walled sarcocysts were microscopic (up to 3750 μm long and 135 μm wide) and had hair-like protrusions (up to 5.4 μm long). The two forms of sarcocysts were probably associated with the length of development. Fayer et al. (1983) experimentally infected ponies with sporocysts collected from dogs that had been fed horsemeat containing visible sarcocysts. On Day 127 post-infection (PI), some sarcocysts showed long protrusions (4.5 μm), and others showed short protrusions (1 to 2 μm). However, on Days 157 and 184 PI, all sarcocysts showed only short protrusions. Matuschka et al. (1986) performed a similar experimental infection and observed two forms of sarcocysts in ponies on Day 378 PI. However, on Day 1040 PI, only macroscopic sarcocysts were found, with sizes of up to 9 × 0.5 mm.

The ultrastructure of the sarcocyst wall is useful in evaluating the taxonomy of *Sarcocystis* species in a given host. Dubey et al. (2016) grouped sarcocysts into more than 40 types based on morphological characteristic. In our materials, the ultrastructures of the thin-walled and thick-walled sarcocysts presented similar morphological characteristics: the cyst wall presented villar protrusions, and bundled microtubules in the core of the protrusions penetrated into the ground substance, similar to type 11 according to the classification of Dubey et al. (2016). All descriptions of sarcocysts obtained from donkeys provided by different authors to date conform to these typical characteristics (Hilaili and Nasser 1987; Hu et al. 2001; Dubey et al. 2015; Zeng et al. 2015; Passantino et al. 2019). Therefore, it is presumed that only one species of *Sarcocystis* has been recorded in donkeys.

Gadaev (1978) first proposed the name *S. asinus* for the sarcocysts found in donkeys, although this was based on an inadequate description. For the reasons mentioned above, *S. asinus* has been regarded as invalid and synonymous with *S. bertrami*, which was the name originally proposed for *Sarcocystis* from horses. Morphologically similar sarcocysts frequently occur in different hosts, especially in closely related hosts, as observed in the sibling species *S. tenella* and *S. capracanis* found in sheep and goats, respectively, which sometimes creates controversy regarding species identification (Formisano et al. 2012; Dubey and Rosenthal 2013). Currently, PCR assays and sequencing procedures are considered much more practical, accurate, and reliable for the delineation and identification of *Sarcocystis* species than traditional methods based on morphological characteristics (Gjerde 2013). Therefore, a critical comparison of the molecular characteristics of *Sarcocystis* species in donkeys and horses should be performed to help reach a final conclusion.

In the present study, three genetic markers, 18S rDNA, 28S rDNA, and mitochondrial *cox*1, were sequenced and analyzed in the two forms of sarcocysts found in donkeys. The sequences of the three loci in the two

forms presented high intraspecific similarities of 97.2–99.5% (on average 97.8%), 97.8–99.6% (on average 98.4%) and 99.0–99.9% (on 99.4%), respectively. Therefore, combined with the similar morphological features observed under TEM, the two forms observed in donkeys are inferred to represent the same *Sarcocystis* species. The comparison of the newly obtained 18S rDNA, 28S rDNA and mitochondrial *cox*1 sequences with those deposited in GenBank showed identities of 90.0–97.5% (average 94.7%), 94.7–95.1% (average 94.9%) and 82.6–84.5% (average 83.4%), respectively, with those of *S. bertrami/ fayeri* sarcocysts obtained from horses. Only a few 18S rDNA sequences and mitochondrial *cox*1 sequences of sarcocysts obtained from donkeys have been deposited in GenBank as references, and all of these sequences were provided by authors from China (Zeng et al. 2018). Based on the high similarity (up to 99.3%) of mitochondrial *cox*1 sequences of sarcocysts obtained from horses and donkeys, the authors proposed *S. bertrami* (syn. *Sarcocystis fayeri*) as a common parasite of both horses and donkeys. However, our newly obtained 18S rDNA and mitochondrial *cox*1 sequences obtained from donkey sarcocysts shared identities of only 93.5–97.5% (average 96.6%) and 82.6–83.8% (average 83.2%), respectively, with those of *S.* cf *bertami* from donkeys provided by the authors. Notably, the newly obtained mitochondrial *cox*1 sequences shared a similarity (98.6–99.4%, average 98.8%) with that of *Sarcocystis* sp. (KY399759) from a donkey, which was also provided by the authors. However, the nucleotide sequence was not analyzed by these researchers. The meat samples used in the study were collected from the same area as those in the study of Zeng et al. (2018). While we found that the newly obtained mitochondrial *cox*1 sequences of sarcocysts in donkeys differed from those of *S.* cf. *bertrami* in donkeys provided by Zeng et al. (2018), all donkey meat samples infected with sarcocysts used in the present study were reappraised based on mitochondrial *cox*1 sequences to ensure reliability because meat from other animals is sometimes sold to consumers in local meat markets. Additionally, the difference between the donkey and horse sarcocysts was proven by PCR-RFLP based on the mitochondrial *cox*1 sequences of the two parasites. Therefore, the sarcocysts of donkeys do not belong to the species *S. bertrami/fayeri* found in horses; hence, the name *S. asinus* is valid and should be resurrected for sarcocysts of donkeys.

Cross-infection is a criterion for revealing whether different intermediate hosts harbor the same parasite. To date, there has been only one reported attempt to perform the cross-infection of *Sarcocystis* between donkey and horse (Matuschka 1983). Tissues from 20 horses naturally infected with sarcocysts were fed to a dog, and those of 10 donkeys were fed to another dog. Both dogs excreted sporocysts. Experimental infections were carried out in 4 ponies (#1-4). One pony (#1) fed donkey-derived sporocysts became febrile on Days 10 and 11 and 19 –21, but no sarcocysts were detected on Days 44 and 59 PI. The same pony was then fed horse-derived sporocysts on Day 117 and killed on Day 138 PI. Only immature sarcocysts (metrocytes) were identified in the carcass. The other three ponies (#2 –4) were fed horse-derived sporocysts and killed on Days 197, 212, and 21 PI, respectively. Mature sarcocysts (bradyzoites) were detected in ponies #2 and 3, but no sarcocysts were found in pony #4. These results suggest the possible transmission of the parasite in horses and donkeys. However, Fayer and Dubey (1982) found immature sarcocysts on Day 55 PI and mature sarcocysts on Day 77 PI in horses infected with horse-derived sporocysts. Therefore, the experiments performed by Matuschka (1983) are insufficient to

support the cross-infection of *Sarcocystis* between donkey and horse for reasons including the following: (1) transmission was based only on one pony (#1) fed donkey-derived sporocysts; (2) no mature sarcocysts (on Day 138 PI) were detected in the experimental donkey; and (3) immature sarcocysts (on Day 138 PI) were detected in the experimental donkey, however this animal was infected twice, first with donkey-derived sporocysts and second with horse-derived sporocysts (on Day 117 PI). Therefore, the cross-infection of *Sarcocystis* between donkey and horse should be attempted, and the available molecular evidence also needs to be supplemented in the future.

Declarations

Funding

This study was supported by the National Key R&D Program of China (grant 2017YFD0500400) and the Natural Sciences Foundation of China (Grant 31460557).

Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and material

No other data and material are provided

Authors' contributions

JH suggested the overall concept and design of the study, and drafted the manuscript. MZ, KW, and ZW, conducted specimen colllection and molecular work. JS and SD performed observation of sarcocysts and data analysis. JT provided suggestions for this manuscript. All authors reviewed the manuscript.

Ethics approval

The present study was approved by the Animal Ethics Committee of Yunnan University (permission number AECYU2018004)

Consent to participate

The authors declare that they have participated this work.

Consent for publication

The authors declare that they know the content of this manuscript and agree to submit it to Parasitology Research.

References

1. Doflein F (1901) *Sarcocystis bertrami* n. sp. In: Die Protozoen als Parasiten und Krankheitserreger, nach biologischen Gesichtspunkten dargestellt. Gustav Fischer, Jena, pp 219–220
2. Dubey JP, Streitel RH, Stromberg PC, Toussant MJ (1977) *Sarcocystis fayeri* sp. n. from the horse. J Parasitol 63:443–447. https://doi.org/10.2307/3279997
3. Dubey JP, Rosenthal BM (2013) *Sarcocystis capracanis*-associated encephalitis in sheep. Vet Parasitol 197:407–408. https://doi.org/10.1016/j.vetpar.2013.04.026
4. Dubey JP, Calero-Bernal R., Rosenthal BM, Speer CA, Fayer R (2016) Sarcocystosis of animals and humans, 2nd edn. CRC Press, Boca Raton, pp 249–256
5. Dubey JP, VAN Wilpe E, Verma SK, Hilali M (2016) Ultrastructure of *Sarcocystis bertrami* sarcocysts from a naturally infected donkey (*Equus asinus*) from Egypt. Parasitology 143:18–23. https://doi.org/ 10.1017/S0031182015001432
6. Fayer R, Dubey JP (1982) Development of *Sarcocystis fayeri* in the equine. J Parasitol 68:856–860. https://doi.org/10.2307/3280993
7. Fayer R, Hounsel C, Giles RC (1983) Chronic illness in a *Sarcocystis* infected pony. Vet Rec 113:216–217. https://doi.org/10.1136/vr.113.10.216
8. Fischer S, Odening K (1998) Characterization of bovine *Sarcocystis* species by analysis of their 18S ribosomal DNA sequences. J Parasitol 84: 50–54. https://doi.org/10.2307/3284529
9. Formisano P, Aldridge B, Alony Y, Beekhuis L, Davies E, Del Pozo J, Dunn, English K, Morrison L, Sargison N, Seguino A, Summers B A, Wilson D, Milne E, Beard PM (2013) Identification of *Sarcocystis capracanis* in cerebrospinal fluid from sheep with neurological disease. Vet Parasitol 193: 252–255. https://doi.org/10.1016/j.vetpar.2012.12.016
10. Gadaev A (1978) On sarcocysts of ass (*Equus asinus*). Akad. Nauk. Uzbecks. SSR. 1: 47–48
11. Gjerde B (2013) Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. Inter J Parasitol 43: 579–591. https://doi.org/10.1016/j.ijpara.2013.02.004
12. Gjerde B (2014) *Sarcocystis* species in red deer revisited: with a redescription of two known species as *Sarcocystis elongata* n. sp. and *Sarcocystis truncata* n. sp. based on mitochondrial cox1 sequences. Parasitology 141: 441–452. https://doi.org/10.1017/S0031182013001819
13. Heydorn AO, Gestrich R, Mehlhorn H, Rommel M (1975) Proposal for a new nomenclature of the Sarcosporidia. Z Parasitenkd 48: 73–82. https://doi.org/10.1007/BF00389639
14. Hilali M, Nasser AM (1987) Ultrastructure of *Sarcocystis* spp. from donkeys (*Equus asinus*) in Egypt. Vet Parasitol 23: 179–183. https://doi.org/10.1016/0304-4017(87)90003-3
15. Hinaidy HK, Loupal G (1982). *Sarcocystis bertrami* Doflein, 1901, a sarcosporidia of the horse. *Equus caballus* Zentralbl Veterinarmed B 29: 681–701. https://doi.org/10.1111/J.1439-0450.1982.TB01269.X
16. Hu JJ, Chen X. Zuo Y (2001) Ultrastructure of the cyst wall and experimental infectivity of *Sarcocystis* from donkeys. Chin J Vet Sci 21: 145–148.

17. Hu JJ, Liu TT, Liu Q, Esch GW, Chen JQ, Huang S, Wen T (2016) Prevalence, morphology, and molecular characteristics of *Sarcocystis* spp. in domestic goats (*Capra hircus*) from Kunming, China. Parasitol Res 115: 3973–3981. https://doi.org/10.1007/s00436-016-5163-6
18. Kirmse P (1986) Sarcosporidioses in equines of Morocco. Br Vet J 142: 70–72. https://doi.org/ 10.1016/0007-1935(86)90011-4
19. Kumar S, Stecher G, Li M, Knyaz C, Tamura, K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35: 1547–1549. https://doi.org/ 10.1093/molbev/msy096
20. Levine ND, Tadros W (1980) Named species and hosts of *Sarcocystis* (Protozoa: Apicomplexa: Sarcocystidae). Syst Parasitol 2: 41–59. https://doi.org/10.1007/BF00015094
21. Ma CL, Ye YL, Wen T, Huang ZM, Pan J, Hu JJ, Tao JP, Song JL (2020) Prevalence and morphological and molecular characteristics of *Sarcocystis bertrami* in horses in China. Parasite 27:1. https://doi.org/10.1051/parasite/2019078
22. Matuschka FR (1983) Infectivity of *Sarcocystis* from donkey for horse via sporocysts from dogs. Z Parasitenkd 69: 299–304. https://doi.org/10.1007/BF00927871
23. Matuschka FR, Schnieder T, Daugschies A, Rommel M (1986) Cyclic transmission of *Sarcocystis bertrami* by the dog to the horse. Protistologica 22: 231–234.
24. Mugridge NB, Morrison DA, Heckeroth AR, Johnson AM, Tenter AM (1999) Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. Int J Parasitol 29: 1545–1556. https://doi.org/10.1016/S0020-7519(99)00150-2
25. Odening K, Wesemeier HH, Walter G, Bockhardt I (1995) Ultrastructure of sarcocysts from equids. Acta Parasitologica 40: 12–20
26. Odening K (1998) The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). Syst Parasitol 41: 209–233. https://doi.org/10.1023/A:1006090232343
27. Passantino G, Lia RP, Latrofa S, Annoscia G, Šlapeta J, Otranto D, Rossi R, Zizzo N (2019) *Sarcocystis bertrami* in skeletal muscles of donkeys (*Equus africanus asinus*) from Southern Italy. Vet Parasitol Reg Stud Reports 16: 100283. https://doi.org/10.1016/j.vprsr.2019.100283
28. Rommel M, Geisel O (1975) Untersuchungen über die Verbreitung und de Lebenszyklus einer Sarkosporidienart des Pferdes (*Sarcocystis equicanis* n. spec.). Berl. Münch Tierärztl Wschr 88: 468–471.
29. Zeng W, Sun L, Xiang Z, Li N, Zhang J, He Y, Li Q, Yang F, Song J, Morris J, Rosenthal B M, Sun L, Liu H, Yang Z (2018) Morphological and molecular characteristics of *Sarcocystis bertrami* from horses and donkeys in China. Vet Parasitol 252: 89–94. https://doi.org/10.1016/j.vetpar.2018.01.024

Figures

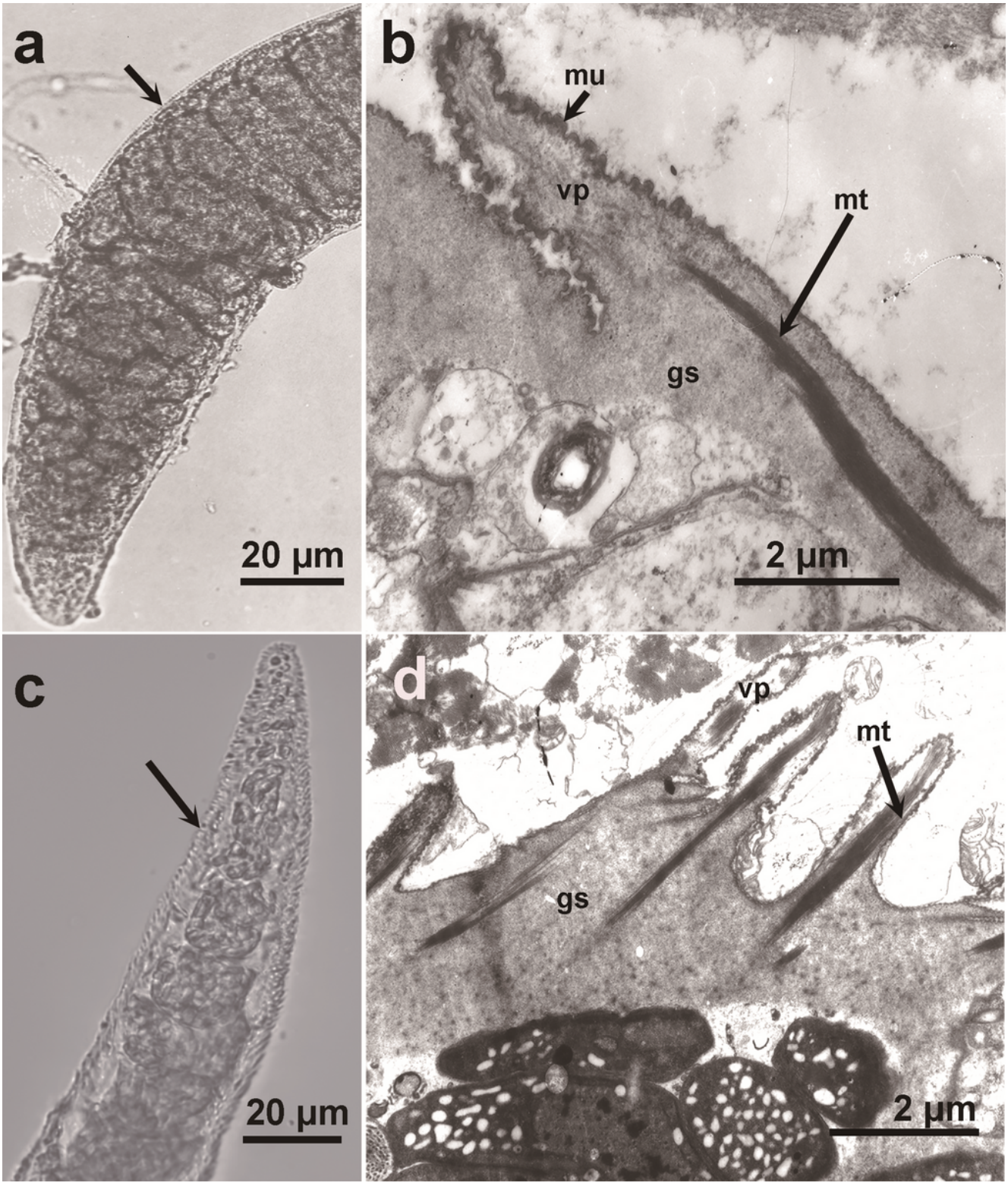

Figure 1

Morphological characteristics of *Sarcocystis asinus* sarcocysts isolated from skeletal muscles of donkeys. **a** Thin-walled sarcocyst (unstained, light microscopy, LM) bound by short club-like protrusions (arrow). **b** Diagonal section of a thin-walled sarcocyst (under transmission electron microscopy, TEM). The sarcocyst wall exhibits numerous villar protrusions (vps), which are often bent along the cyst surface. The vps contain bundled microtubules (mt) in their core, which penetrate diagonally into the

ground substance (gs). Minute undulations (mu) present over the entire sarcocyst surface. **c** Thick-walled sarcocyst (unstained, under LM) bound by hair-like protrusions (arrow). **d** Longitudinal section of a thick-walled sarcocyst (under TEM). The sarcocyst wall exhibits numerous vps, which are often bent along the cyst surface. The vps contain bundled microtubules (mt) in the core, which penetrate diagonally into the ground substance (gs).

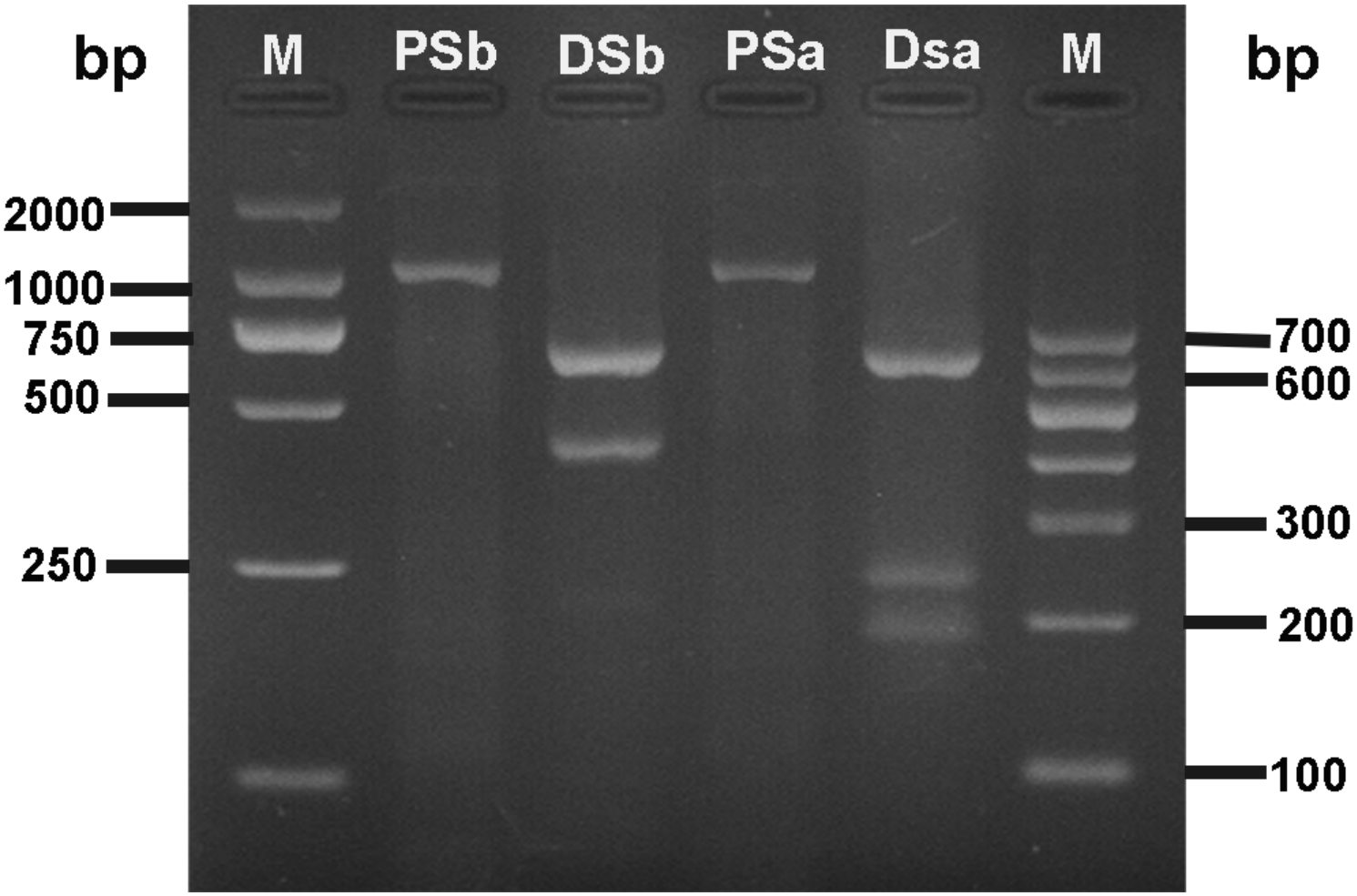

Figure 2

Results of PCR with primers SF1/SR9 and restriction enzyme digestion with HinifI and EcorRI for sarcocyst DNA from *Sarcocystis asinus* isolated from naturally infected donkey and *S. bertrami* from naturally infected horses. M, molecular mass marker; PSb, PCR product of *S. bertrami*; DSb, digestion of PCR product of *S. bertrami* with EcorRI and HinifI; PSa, PCR product of *S. asinus*; DSa, digestion of PCR product of *S. asinus* with HinifI and EcorRI.

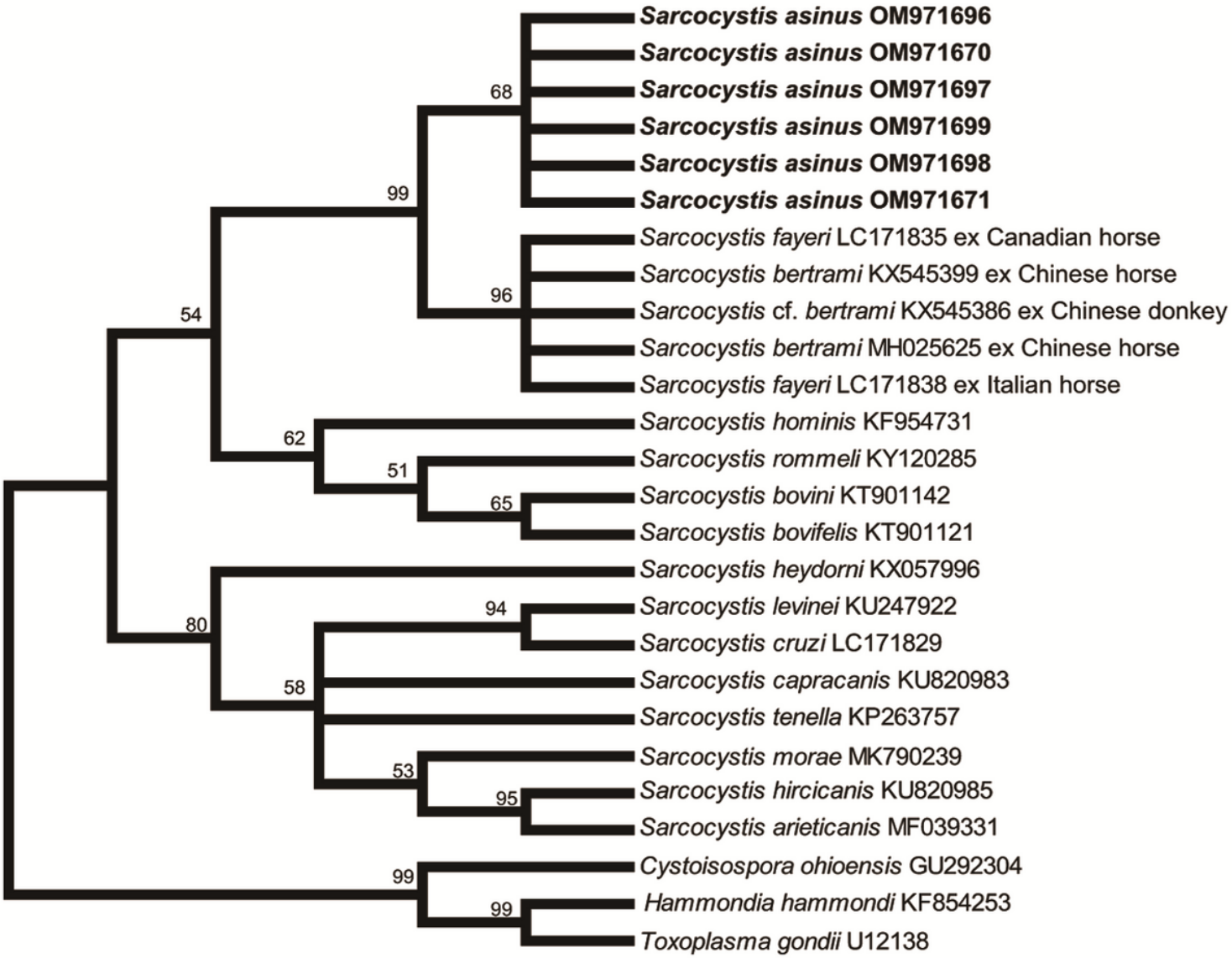

Figure 3

Phylogenetic tree based on 18S rDNA sequences. The tree was built using the maximum likelihood (ML) with the Tamura 3-parameter model. The analysis involved 26 nucleotide sequences (GenBank accession numbers behind the taxon names) and a total of 1045 aligned positions in the final dataset. The values between the branches represent bootstrap values per 1000 replicates, and values below 50% are not shown. The six new sequences of *Sarcocystis asinus* (OM971696–OM971671, shown in boldface) formed an individual clade within a group containing *S. bertrami/ fayeri* in horses and *S.* cf. *bertrami* in a donkey.

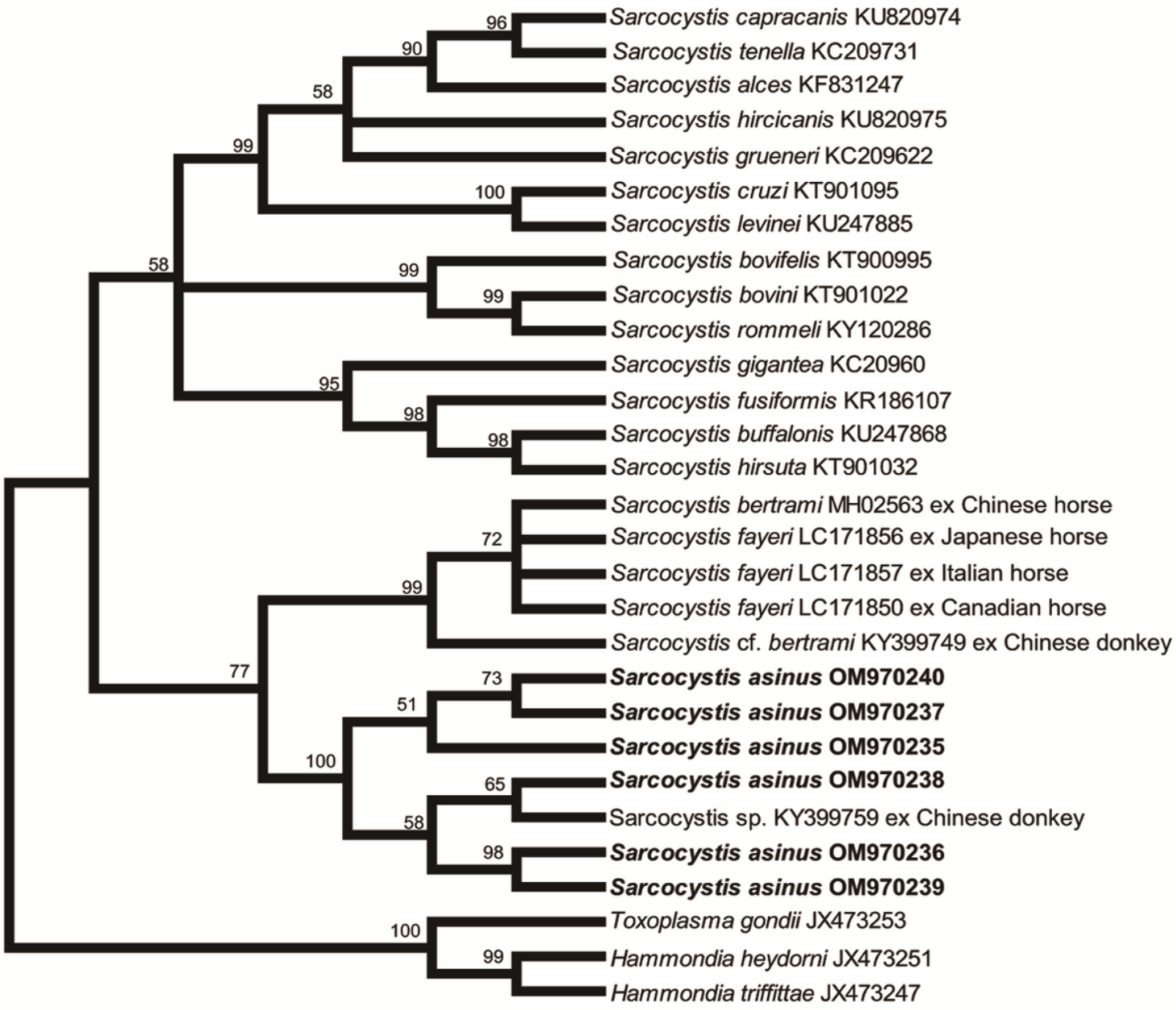

Figure 4

Phylogenetic tree based on mitochondrial *cox*1 sequences. The tree was built using the maximum likelihood (ML) with the Hasegawa-Kishino-Yano model. The analysis involved 29 nucleotide sequences (GenBank accession numbers behind the taxon names) and a total of 933 aligned positions in the final dataset. The values between the branches represent bootstrap values per 1000 replicates, and values below 50% are not shown. The six new sequences of *Sarcocystis asinus* (OM970235–OM970240, shown in boldface) formed an individual clade with *Sarcocystis* sp. ex *Equus asinus* within a group containing *S. bertrami/ fayeri* in horses and *S.* cf. *bertrami* in a donkey.