Morphological and Molecular Characterization of Sarcocystis Wenzeli in Chickens (Gallus gallus) in China

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Abstract

Background: There has been considerable confusion concerning the number and classification of *Sarcocystis* spp. found in chickens. To date, no nucleotide sequences of *Sarcocystis* spp. from chickens have been provided in GenBank. The present study aimed to investigate the morphological and molecular characteristics of *Sarcocystis* spp. in chickens in China.

Methods: Tissues from 33 chickens were collected in 2019. Sarcocysts were observed using light (LM) and transmission electron microscopy (TEM). Individual sarcocysts from different chickens were selected for DNA extraction, and five loci, 18S rDNA, 28S rDNA, the ITS-1 region (*ITS-1*), the mitochondrial cox1 gene (*cox1*), and the apicoplastic rpoB gene (*rpoB*), were amplified from each sarcocyst, cloned, sequenced and analyzed.

Results: Only *S. wenzeli* was found in 14 of 33 (42.4%) chickens. Under LM, the sarcocysts were microscopic and exhibited palisade-like villar protrusions measuring 1.5–2.8 μm. The bradyzoites were lancet-shaped, measuring 9.2–12.6 × 1.5–3.5 μm. Ultrastructurally, the sarcocyst wall contained numerous stubby hill-like villar protrusions that were up to 1.2 μm long and 1.0 μm wide. The protrusions included scattered microtubules, which extended from the tips of the protrusions into the ground substance. The intraspecific similarity of the five loci (18S rDNA, 28S rDNA, *ITS-1*, *cox1*, and *rpoB*) among the newly obtained sequences ranged from 99.8–100%, 99.7–100%, 99.0–99.9%, 100% and 98.9–100%, respectively. The five loci showed different levels of interspecific sequence similarity with closely related species of *Sarcocystis* (e.g., 99.8%, 99.0–99.2%, 89.3-89.7%, 98.5%, and 97.5%, respectively, with *S. anasi*). Phylogenetic analysis based on three of the loci (18S rDNA, *cox1*, and *rpoB*) revealed that *S. wenzeli* formed an independent clade with *Sarcocystis* spp. that employ geese or ducks as intermediate hosts and canines as the known or presumed definitive host.

Conclusions: The sequences of 18S rDNA, 28S rDNA, *ITS-1*, *cox1* and *rpoB* from *S. wenzeli* reported here constitute the first records of genetic markers of *Sarcocystis* from chickens made available in GenBank. Based on sequence analysis, *ITS-1* and *rpoB* are more suitable for discriminating *S. wenzeli* from closely related species of *Sarcocystis*. Phylogenetic analysis revealed that *S. wenzeli* presents a close relationship with *Sarcocystis* spp. from geese or ducks.

Background

Species of the genus *Sarcocystis* exhibit an obligate two-host life cycle, with sexual development in the small intestine of the definitive host and asexual development in the intermediate host, which are usually herbivores. To date, three species of *Sarcocystis*, *S. horvathis* Rátz 1908 [1], *S. gallinarum* Krause and Goranoff 1933 [2] and *S. wenzeli* (Wenzel, Erber, Boch, Schellner 1982) Odening 1997 [3, 4], with chickens (*Gallus gallus*) as the intermediate host have been named. However, there has been considerable confusion concerning the number and classification of species of *Sarcocystis* in chickens owing to the imperfection of the original description [5].

The correct identification of *Sarcocystis* species that might infect chickens is crucial for sarcocystosis control and prevention. The ultrastructure of sarcocysts is traditionally a reliable characteristic for identifying different *Sarcocystis* species in a given host. Currently, PCR assays and sequencing procedures are considered much more practical, accurate, and reliable methods for the delineation and identification of *Sarcocystis* species than traditional methods based on morphological characteristics [6, 7]. However, there are currently no records of the nucleotide sequences of *Sarcocystis* spp. obtained from chickens in GenBank to serve as references.

Therefore, the aims of the present study were to investigate the occurrence and morphological characteristics of *Sarcocystis* in chickens. Additionally, the sequences of five loci, 18S rDNA, 28S rDNA, the ITS-1 region (*ITS-1*), the mitochondrial cox1 gene (*cox1*), and the apicoplastic rpoB gene (*rpoB*), of the species of *Sarcocystis* found in chickens were sequenced and characterized for the first time to augment the descriptions of these parasites.

Methods

Morphological examination of sarcocysts from chickens

In total, tissues from 33 chickens were collected from Jiaojiaqing village, Shizong County, Yunnan Province, located in southwestern China, in July and December 2019. These chickens were free ranging and were raised by the local peasants. From each chicken, fresh tissue samples of the skeletal muscles and heart were examined for sarcocysts. In the laboratory, 20 pieces of 3-mm muscle from each collected sample were pressed and squeezed between two glass slides and inspected using a stereomicroscope. Individual sarcocysts were extracted and isolated from muscle fibers using dissection needles and processed for light (LM) and transmission electron microscopy (TEM) and DNA analysis. For TEM, four sarcocysts (two from chicken no. 4 and two from chicken no. 10) were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C and postfixed in 1% osmium tetroxide in the same buffer, then dehydrated in a graded alcohol series and embedded in an epon-alaldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEM100-CX transmission electron microscope at 80 kV. For DNA isolation, individual cysts were stored in sterile water at −20 °C prior to processing.

DNA isolation, PCR amplification, cloning, and sequence analysis

For DNA analysis, five individual sarcocysts obtained from different chickens were subjected to genomic DNA extraction using the TIANamp Genomic DNA Kit (Tiangen Biotech Ltd., Beijing, China) according to the manufacturer's instructions. The *Sarcocystis* species were characterized at five loci within the 18S rDNA, 28S rDNA, *ITS-1*, *cox1*, and *rpoB* sequences. The near-complete 18S rDNA sequence was amplified with the primer pair S1/SarDR [8, 9]; the near-full-length 28S rDNA sequence was amplified with the

primer sets KL1/KL3, KL4/KL5b, and KL6/KL2 [10]; *ITS-1* was amplified with the primer pair P-ITSF/P-ITSR [9]; *cox1* was amplified with the primer pair SF1/COIRm [6]; and *rpoB* was amplified with the primer pair rpoBF2/rpoBR2 (5'- ATTTTTGTGGATATGATTTTGAAGATGC-3'/5'- AGTTTAGATCCAGTTCTACCG-3'), designed using OLIGO 7.60 (Molecular Biology Insights, Inc, West Cascade, USA) based on highly conserved regions of the *rpoB* sequences of *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis* spp. deposited in GenBank. The PCR products were purified, cloned, sequenced, and assembled using the methods described in a previous report [11].

Phylogenetic analyses were conducted separately for the 18S rDNA, *cox1*, and *rpoB* sequences by using MEGA X software [12]. The maximum parsimony (MP) trees for the three genes were generated with a tree-bisection-regrafting (TBR) algorithm. The reliability of the MP phylograms was tested with the bootstrap method using 1000 replications. In the case of 18S rDNA, the 18S rDNA sequences of *Sarcocystis* spp. from various hosts were downloaded from GenBank and aligned with the ClustalW program integrated in MEGA X, applying a gap opening penalty of 10/10 and a gap extension penalty of 0.1/0.2 as pairwise and multiple alignment parameters, respectively. The alignment was subsequently checked visually; some sequences were slightly truncated at both ends, so all sequences started and ended at the same nucleotide positions, 1 and 1808, of the sequence for *S. tenella* (MF039329). The final alignment comprised a total of 29 nucleotide sequences and 2100 aligned positions from 26 taxa. *Besnoiti besnoiti* (DQ227418), *Neospora caninum* (U16159) and *Toxoplasma gondii* (U03070) were chosen as outgroups.

In total, 28 *cox1* nucleotide sequences from 28 species used in the analysis were aligned by the ClustalW program integrated in MEGA X. Some sequences were slightly truncated at both ends, so all sequences started and stopped at the same nucleotide positions, 1 and 1020, of the *S. tenella* (KC209731) sequence. The final alignment comprised 1020 aligned positions with no gaps. *Toxoplasma gondii* (JX473253), *H. triffittae* (JX473247), and *B. besnoiti* (XM029362743) were used as outgroup species to root the tree.

In the case of *rpoB*, 20 *rpoB* nucleotide sequences from 19 species were aligned with the ClustalW program integrated in MEGA X. Some sequences were slightly truncated at both ends, so that all sequences started and stopped at the same nucleotide positions, 24 and 711, of the *S. caninum* (MH469242) sequence. The final alignment comprised 694 aligned positions with no gaps. *Toxoplasma gondii* (AF095904) and *N. aninum* (AF138960) were used as outgroup species to root the tree.

Results

LM and TEM examination of *S. wenzeli* sarcocysts

Only sarcocysts resembling those of *S. wenzeli* were found in 14 of 33 (42.4%) chickens. Sarcocysts were found in skeletal muscles but not in the heart. Using LM, the sarcocysts of the parasite were observed to be microscopic, measuring 381–3585 × 48–154 μm (n = 30). The sarcocyst wall exhibited numerous short palisade-like villar protrusions measuring 1.5–2.8 μm (n = 40) in length (Fig. 1a). The cysts were

septate, and their interior compartments were filled with lancet-shaped bradyzoites measuring 9.2–12.6 × 1.5–3.5 μm (n = 40) (Fig. 1b).

Four sarcocysts from both chickens were examined using TEM, all of which appeared to have walls that were ultrastructurally similar and closely resembled the “type 9k” cyst wall. The sarcocyst wall contained numerous stubby hill-like villar protrusions that were up to 1.2 μm long and 1.0 μm wide and were lined with an electron-dense layer that appeared thicker at the tips of the protrusions (Fig. 1c). Within the protrusions, there were numerous scattered fine, electron-dense granules and scattered microtubules. The microtubules extended from the tips of the protrusions into the ground substance, where they crossed microtubules originating from neighboring protrusions (Fig. 1d). The protrusions were spaced at intervals of 0.3–1.1 μm from each other. Small invaginations of the primary wall were present on the lateral aspect of the protrusions and in the spaces between protrusions. The layer of ground substance beneath the protrusions was 0.3–0.4 μm in thickness; septa were evident within the cysts (Fig. 1c).

Molecular analysis

Genomic DNA was extracted from the five individual sarcocysts of *S. wenzeli* isolated from different chickens, and 18S rDNA, 28S rDNA, *ITS-1*, *cox1*, and *rpoB* were successfully amplified using their DNA as templates. The five 18S rDNA sequences of *S. wenzeli* were 1747 bp in length and shared an identity of 99.8–100% (average 99.8% identity). Therefore, only four sequences (MT756990–MT756993) were submitted to GenBank. The most similar sequence in GenBank was that of *S. anasi* (EU553477) from Mallard duck (*Anas platyrhynchos*) (99.8% identity), followed by *S. albifronsi* (EU502868) from white-fronted goose (*Anser albifons*) (99.7% identity) and *S. rileyi* (KJ396583) from common eider (*Somateria mollissima*) (99.5% identity).

The five 28S rDNA sequences of *S. wenzeli* were 3279 bp in length and shared 99.7-100% identity (average 99.9% identity). Therefore, only four sequences (MT756986–MT756989) were deposited in GenBank. The most similar sequence in GenBank was that of *S. albifronsi* (EF079885) (99.3-99.1% identity, average 99.2% identity), followed by *Sarcocystis* sp. (MH898978) from Temminck's stint (*Calidris temminckii*) (98.9–99.2% identity, average 99.1% identity), *S. anasi* (EF079887) (99.0–99.2% identity, average 99.1% identity), *S. cornixi* (EU553480) from hooded crow (*Corvus cornix*) (98.6-98.9% identity, average 98.8% identity) and *S. rileyi* (GU188426) (98.3-98.5% identity, average 98.4% identity).

The five *ITS-1* sequences (MT756994–MT756998) of *S. wenzeli* were 1186-1187 bp in length and shared 99.0-99.9% identity, with an average identity of 99.4%. The most similar sequence was that of *S. anasi* (JF520779) (89.3–89.7% identity, average 89.5% identity), followed by that of *S. rileyi* (KJ396584) (78.8-79.0% identity, average 78.9% identity).

The five *cox1* sequences of *S. wenzeli* were 1142 bp in length and were completely identical; therefore, only one sequence (MT761700) was submitted to GenBank. The most similar sequence in GenBank was

that of *S. albifronsi* (MH138310) (98.6% identity), followed by those of *S. anasi* (MH138311) (98.5% identity) and *S. rileyi* (KJ396582) (96.4% identity).

The five *rpoB* sequences of *S. wenzeli* were 844 bp in length and shared 98.9–100% identity, with an average identity of 99.3%, so only two sequences (MT761694 and MT761695) were submitted to GenBank. The most similar sequence in GenBank was that of *S. anasi* (MH138320) (97.5% identity), followed by those of *S. albifronsi* (MH138319) (97.4% identity) and *S. rileyi* (MF596308) (95.9% identity).

Phylogenetic analysis based on the 18S rDNA (Fig. 2), *cox1* (Fig. 3), or *rpoB* (Fig. 4) sequences revealed that *S. wenzeli* formed an individual clade with *S. anasi*, *S. albifronsi* and *S. rileyi* basal to a group comprising *Sarcocysits* spp. that mainly employ birds and canines as known or presumed definitive hosts.

Discussion

Sarcocystis spp. in chickens cause severe myositis [13] and occasionally neurological disease [14]. *Sarcocystis* infection in chickens has been reported in Hungary [1], Bulgaria [2], Russia [15], Papua New Guinea [13], Australia [13], Germany [3], the Czech Republic [16], Azerbaijan [17], China [18], Iran [19] and Brazil [20]. Three species of *Sarcocystis*, *S. horvathis*, *S. wenzeli*, and *S. gallinarum,* have been proposed to be responsible for the sarcocysts observed in muscle tissues of chickens. The sarcocysts found in chickens have been divided into two types based on the shape of the bradyzoites. Banana-shaped sarcocysts are considered to be produced by *S. horvathi*, described in 1909, which is synonymous with *S. gallinarum*; and lancet-shaped sarcocysts are attributed to *S. wenzeli*, described in 1982 [5]. The ultrastructure of the sarcocysts of *S. wenzeli* has been described in detail previously [18, 21] and is similar to the type 9k sarcocyst wall classified by Dubey et al. (2016) [5]. It is worth noting that morphologically similar sarcocysts have been observed in lesser snow geese (*Anser caerulescens*) in Saskatchewan, although the species has not been named [22]. The fine structure of the sarcocysts of *S. horvathis* and *S. gallinarum* is still unclear. In our materials, only the sarcocysts *S. wenzeli* were found and identified, based on the observation of lancet-shaped bradyzoites and the TEM analysis of sarcocysts. The 42.4% (14/33) prevalence rate of *Sarcocystis* identified in chickens was lower than the 94.78% (37/39) prevalence recently identified in Iran using the digestive method [19] but was higher than the 8.9% (17/191) prevalence based on microscopic detection reported in China in 2012 [18].

Nucleotide sequence analysis has proven to be a useful tool for delineating or identifying species of *Sarcocystis* from the same or different hosts, and different genetic markers have revealed different levels of intra- or interspecific sequence diversity [7, 11]. There are currently no nucleotide data for *Sarcocystis* spp. available in GenBank, which has led to an inability to identify the species of *Sarcocystis* infecting chickens using molecular methods [20]. Therefore, five loci (18S rDNA, 28S rDNA, *ITS-1*, and *cox1* and *rpoB*) from *S. wenzii* were sequenced and analyzed for the first time in the present study. In our analysis, the sequences of the five loci (18S rDNA, 28S rDNA, *ITS-1*, and *cox1* and *rpoB*) of this parasite presented high intraspecific similarities of 99.8-100%, 99.7-100%, 99.0-99.9%, 100% and 98.9-100%, respectively.

Additionally, they exhibited different levels of similarity compared with closely related *Sarcocystis* species, sharing 99.8%, 99.0–99.2%, 89.3-89.7%, 98.5%, 97.5% identity with the corresponding sequences of *S. anasi* at the five loci and 99.5%, 98.3-98.5%, 78.8-79.0%, 96.4% and 95.9% identity with those of of *S. rileyi*. Therefore, *ITS-1* and *rpoB* appeared to be more suitable to for distinguishing *S. wenzii* from other *Sarcocystis* spp., especially closely related species of *Sarcocystis*, than the 18S rDNA, 28S rDNA and *cox1* loci.

This study is the first to establish the phylogenetic relationships between *S. wenzii* and other *Sarcocystis* spp. based on 18S rDNA sequences, *cox1* sequences, or *rpoB* sequences. The topologies of the trees inferred from these sequences were highly similar and revealed that *S. wenzii* presents a close relationship with *S. rileyi*, *S. albifronsi* and *S. anasi*. These three species employ geese or ducks as their intermediate hosts, and the definitive hosts of *S. rileyi* and *S. albifronsi* are canines, but that of *S. anasi* is still unknown [23, 24]. Based on experimental infection, the definitive hosts of *S. wenzii* were confirmed to be both cats and dogs [3, 25], which is peculiar and differs from the situation for all known *Sarcocystis* spp. found in domestic animals, which use only either cats or dogs as their definitive host. However, sarcocyst sporocysts were not found in the feces of cats fed breast muscle sample from over 2000 chickens from grocery stores in the United States, although the muscle was not examined microscopically for sarcocysts or brasdyzoites [26].

Conclusions

In summary, we found a high prevalence rate of *Sarcocystis* in free-range chickens in China, and only *S. wenzii* was identified based on the cyst ultrastructure. Five loci (18S rDNA, 28S rDNA, ITS-1, *cox1* and *rpoB*) of the parasite were sequenced, analyzed and deposited in GenBank. Among these genetic markers, *ITS-1* and *rpoB* are more suitable for discrimination among closely related *Sarcocystis* species. Phylogenetic analysis revealed that *S. wenzii* shows a close relationship with *Sarcocystis* spp. that use geese and/or ducks as intermediate hosts and canids as definitive hosts.

Abbreviations

PCR: polymerase chain reaction; 18S rDNA: 18S ribosomal DNA; 28S rDNA: 28S ribosomal DNA; ITS-1: intergenic transcribed spacer region 1; cox1: cytochrome oxidase subunit 1; rpoB: RNA polymerase beta subunit; LM: light microscopy; TEM: transmission electron microscopy

Declarations

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Yunnan University (permission number AEC2015021), and all authors declare that they have participated in this work.

Acknowledgements

Not applicable

Authors’ contributions

Hu conceived the study and prepared the manuscript. Pan, Ma and Huang collected the samples and performed the molecular experiments. Ye and Zeng preformed the investigation of prevalence and morphological observation. Deng and Tao analyzed and interpreted the results. All authors read and approved the final manuscript.

Consent for publication

Not applicable

Availability of data and materials

The data collected for this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Figures

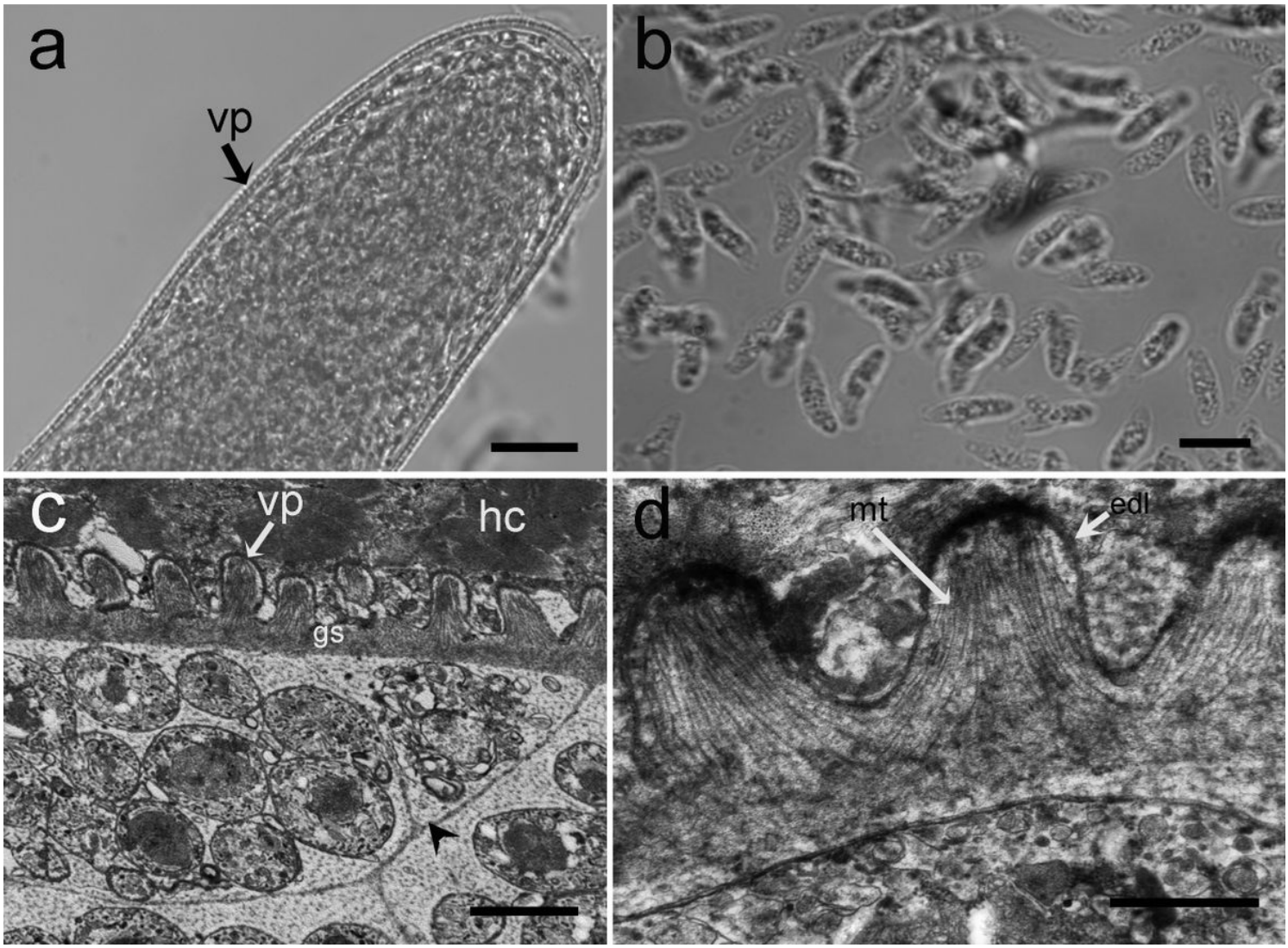

Figure 1

Morphological characteristics of Sarcocystis wenzeli isolated from skeletal muscle in chickens. a LM micrograph of a sarcocyst (unstained). Note the short palisade-like villar protrusions (vp). b LM micrograph of bradyzoites (unstained). Note the lancet-shaped bradyzoites. c TEM micrograph of a sarcocyst. Note the stubby hill-like vp, ground substance (gs), septa (arrowhead), and host cell (hc).d TEM micrograph of a sarcocyst. Note the electron-dense layer (edl) and the microtubules (mt). Scale bars: a 20 μm; b 10 μm; c 2 μm; d 1 μm

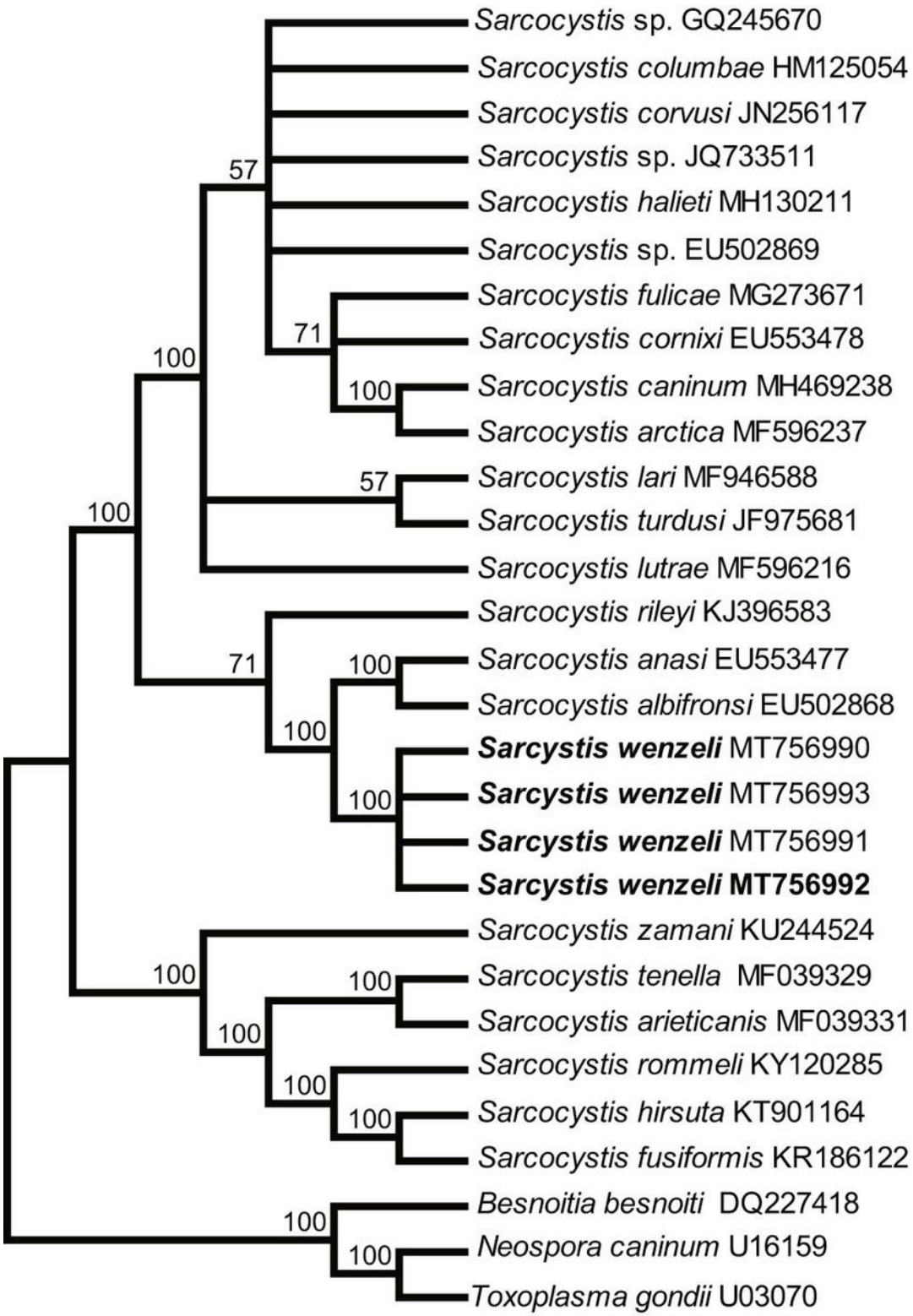

Figure 2

Phylogenetic tree of selected members of the Sarcocystidae family based on 18S rDNA sequences, inferred using the maximum parsimony (MP) method with the tree bisection-regrafting algorithm (TBR). The values between the branches represent the percent bootstrap value per 1000 replicates, and values below 50% are not shown. The GenBank accession numbers of all the sequences included in the analysis

are given after the taxon names. The four new sequences of Sarcocystis wenzeli (MT756990–MT756993) are shown in boldface.

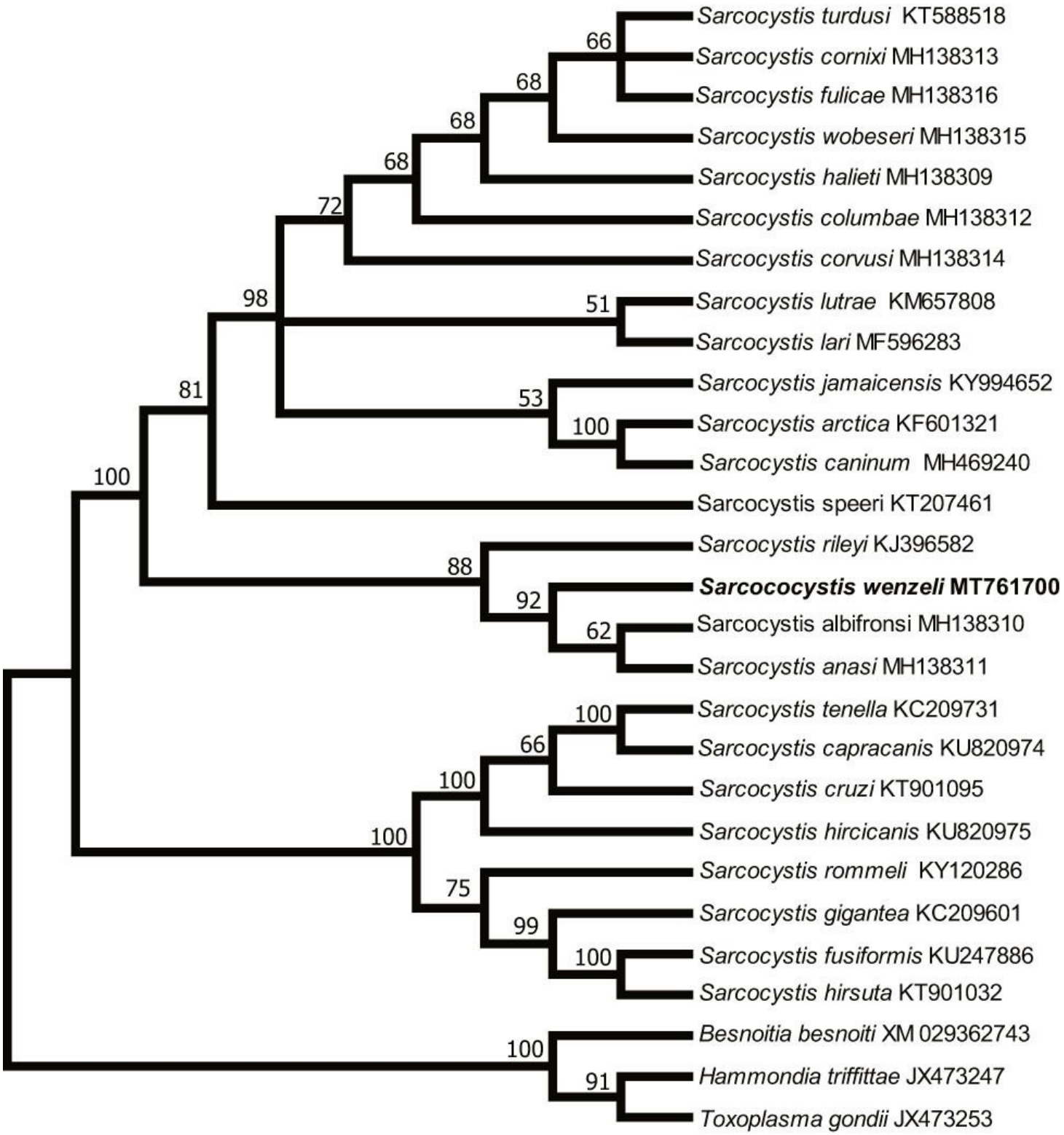

Figure 3

Phylogenetic tree of selected members of the Sarcocystidae family based on mitochondrial cox1 sequences, inferred using the maximum parsimony (MP) method with the tree bisection-regrafting algorithm (TBR). The values between the branches represent the percent bootstrap value per 1000

replicates, and values below 50% are not shown. The GenBank accession numbers of all the sequences included in the analysis are given after the taxon names. The new sequence of Sarcocystis wenzeli (MT761700) is shown in boldface.

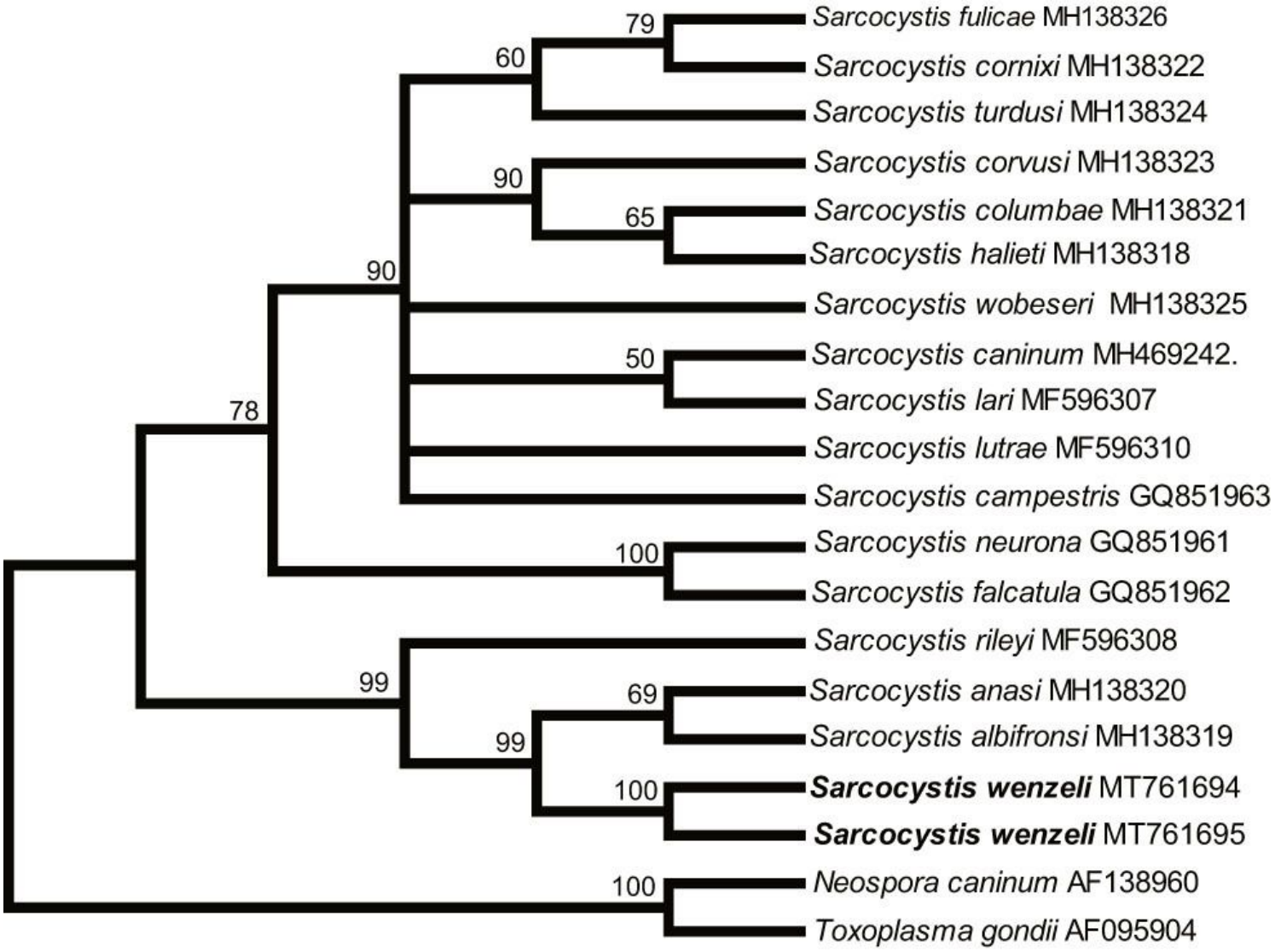

Figure 4

Phylogenetic tree of selected members of the Sarcocystidae family based on apicoplastic rpoB sequences, inferred using the maximum parsimony (MP) method with the tree bisection-regrafting algorithm (TBR). The values between the branches represent the percent bootstrap value per 1000 replicates, and values below 50% are not shown. The GenBank accession numbers of all the sequences included in the analysis are given after the taxon names. The two new sequences of Sarcocystis wenzeli (MT761694 and MT761695) are shown in boldface.