



# Morphological and molecular characterizations of *Sarcocystis miescheriana* and *Sarcocystis sui hominis* in domestic pigs (*Sus scrofa*) in China

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## Abstract

In this study, 36.8% (28/76) of tissue samples collected from domestic pigs (*Sus scrofa*) contained sarcocysts, as determined by light microscopy. The organisms were identified as *Sarcocystis miescheriana* and *Sarcocystis sui hominis* based on their morphological and molecular characteristics. Four genetic markers, i.e., 18S rDNA, 28S rDNA, ITS-1 region (*ITS-1*), and the mitochondrial COX1 gene (*COX1*), of the two parasites were sequenced and analyzed, and the 28S rDNA and *ITS-1* of *S. sui hominis* obtained from pigs constituted the first records of these markers in GenBank. The sequences of the four loci (18S rDNA, 28S rDNA, *ITS-1*, and *COX1*) of *S. miescheriana* shared high identities with those of *S. miescheriana* obtained from domestic and/or wild pigs in GenBank, with similarities of 99.6%, 99.6%, 95.9%, and 95.4%, respectively. The 18S rDNA sequences of *S. sui hominis* exhibited 99.4% identity with those of *S. sui hominis* from domestic and wild pigs. The comparison of the newly obtained sequences of the four genetic markers between the two parasites revealed that the interspecific similarities of 18S rDNA, 28S rDNA, *ITS-1*, and *COX1* were 97.7%, 96.6%, 80.3%, and 81.2%, respectively. Therefore, the two species could be better discriminated with *ITS-1* and mitochondrial *COX1* compared with 18S rDNA or 28S rDNA. The phylogenetic analysis using 28S rDNA indicated that the two *Sarcocystis* species in domestic pigs had a close relationship.

**Keywords** Domestic pig · *Sarcocystis* · Morphological and molecular characteristics · China

## Introduction

*Sarcocystis* spp. are cyst-forming intracellular protozoan parasites with an obligate two-host life cycle, with predators as definitive hosts and their prey animals as intermediate hosts. Domestic and wild pigs (*Sus scrofa*), as intermediate hosts, can acquire the parasite by ingesting environmentally resistant oocysts or sporocysts shed by the definitive hosts, and can

harbor sarcocysts of three *Sarcocystis* species, namely, *S. miescheriana*, *S. sui hominis*, and *S. porcifelis*, whose definitive hosts are canids, humans and nonhuman primates, and cats, respectively (Dubey et al. 2016). *Sarcocystis* sp. infection in domestic pigs can cause weight loss, abortion, and even death depending on the number of sporocysts ingested (Heydom 1977; Barrows et al. 1982). Human infections acquired by eating undercooked pork are most frequently characterized by diarrhea and stomachache (Li and Lian 1986).

The correct identification of *Sarcocystis* species that might infect domestic pigs 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. At present, 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 the morphological characteristics (Gjerde 2013). 18S rDNA sequences have been widely used for the identification of *Sarcocystis* species from various livestock and can reconstruct the phylogenetic relationships among *Sarcocystis* species; however, the

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discriminatory power of this gene has been shown to be unsuitable for the differentiation of closely related lineages of *Sarcocystis* in ruminants due to its highly conserved nature (Hu et al. 2016). To date, four genetic markers of *S. miescheriana*, including 18S rDNA, 28S rDNA, ITS-1 region (*ITS-1*), and the mitochondrial *COX1* gene (*COX1*), have been sequenced and analyzed, but most of these markers were recently obtained from wild pigs in Italy (Gazzonis et al. 2019). The sequences of 28S rDNA and *ITS-1* of *S. suihominis* have not yet been recorded, and only one sequence of the mitochondrial *COX1* of this zoonotic parasite from a wild pig is provided in GenBank.

Therefore, the aims of the present study were to investigate the occurrence of *Sarcocystis* spp. in domestic pigs based on morphological data and to characterize the sequences of the three nuclear DNA regions (18S rDNA, 28S rDNA, and *ITS-1*) and the mitochondrial *COX1* of *Sarcocystis* spp. found in domestic pigs to augment the descriptions of these species.

## Materials and methods

In total, tissue samples were collected from 76 domestic pigs from two abattoirs, one in Kunming city and one in Baoshan city, both of which are located in Yunnan Province, China, from February to May 2018. Fresh tissue samples (50 g each) from the esophagus, diaphragm, skeletal muscles, and heart of each animal were examined for sarcocysts. In the laboratory, 40 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 then isolated from muscular fibers using dissection needles and examined by light microscopy (LM). Each sarcocyst was identified according to the description provided by Dubey et al. (2016) prior to DNA analysis.

For DNA analysis, three individual sarcocysts of each species separated from muscle samples obtained from different domestic pigs 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 four loci located in 18S rDNA, 28S rDNA, *ITS-1*, and the mitochondrial *COX1*. The near-complete 18S rDNA was amplified with the primer pair S1/B, which was designed by Fischer and Odening (1998) and Medlin et al. (1988), respectively; the near full-length 28S rDNA was amplified with the primer sets KL1/KL3, KL4/KL5b, and KL6/KL2 designed by Mugridge et al. (1999); the *ITS-1* was amplified with the primer pair SU1F/5.8SR2 designed by Gjerde (2014); and the mitochondrial *COX1* was amplified with the primer pair SF1/SR66 designed by Gjerde (2013, 2016). The PCR products were purified, cloned, sequenced, and assembled using the methods described in a previous report (Hu et al. 2016).

Gazzonis et al. (2019) recently constructed phylogenetic trees based on the 18S rDNA sequences and the mitochondrial *COX1* sequences of *Sarcocystis* spp. in pigs. Therefore, in the present study, only the 28S rDNA sequences of *Sarcocystis* spp. in pigs were used to infer the relationships with other *Sarcocystis* spp. using MEGA7 software (Kumar et al. 2016). A maximum parsimony (MP) tree was created using a Tree-Bisection-Regrafting (TBR) algorithm. The reliability of the MP phylograms was tested using the bootstrap method with 1000 replications. The 28S rDNA sequences of *Sarcocystis* spp. were downloaded from GenBank and aligned using the ClustalW program integrated in MEGA7 with 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; for this analysis, some sequences were slightly truncated at both ends such that all sequences started and ended at the same nucleotide positions, i.e., 1 and 3497, respectively, of the *Sarcocystis cruzi* sequence (AF076903). The final alignment comprised a total of 34 nucleotide sequences and 3677 aligned positions of 19 taxa. *Hammondia heydorni* (AF159240), *Hammondia hammondi* (AF101077), and *Toxoplasma gondii* (AF076901) were selected as outgroups.

## Results

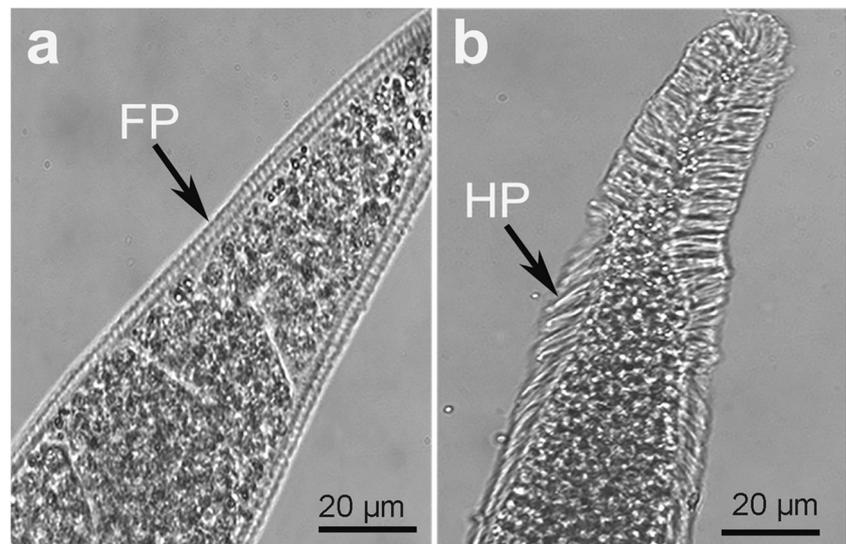
### Observations of sarcocysts by LM

Sarcocysts were detected in 28 of 76 domestic pigs (36.8%). Through LM, two morphologically distinct sarcocysts were observed in the muscle samples of domestic pigs. The *Sarcocystis miescheriana* sarcocysts were 745–5321 × 57–180 μm ( $n = 30$ ) in size and contained numerous, 2.4–4.5-μm ( $n = 40$ )-long, finger-like protrusions (Fig. 1a) that aligned almost vertical to the cyst surfaces. The *Sarcocystis suihominis* sarcocysts were 542–3025 × 49–192 μm ( $n = 30$ ) in size and presented numerous, 5.6–9.5 μm hair-like protrusions ( $n = 40$ ) that were inclined on the surface of the sarcocysts (Fig. 1b). *Sarcocystis miescheriana* was found in 25 domestic pigs (32.9%) and was more common than *S. suihominis*, which was found in 13 pigs (17.1%); their distribution in different muscle tissues is shown in Table 1.

### Molecular characterization of 18S rDNA

Genomic DNA was extracted from three individual sarcocysts of the two *Sarcocystis* species, and the 18S rDNA, 28S rDNA, *ITS-1*, and mitochondrial *COX1* genes were amplified and sequenced successfully using their DNA as templates. The three 18S rDNA sequences obtained from different isolates of *S. miescheriana* were 1867 bp in length and completely identical; as a result, only one sequence was submitted to GenBank

**Fig. 1** Morphological characteristics of *Sarcocystis* spp. isolated from skeletal muscles of domestic pigs under a light microscope (unstained). **a** Sarcocyst of *S. miescheriana* bounded by finger-like protrusions (FP). **b** Sarcocyst of *S. suihominis* surrounded by hair-like protrusions (HP)



(accession number: MK867454). The sequences in GenBank that exhibited the highest similarity with the 18S rDNA sequences obtained in this study were those of *S. miescheriana* (JN256123, JX840464–JX840467, KX929089–KX929094, and MH404230–MH404232) from domestic and wild pigs (99.1–99.8 identity, with an average of 99.6%).

The three 18S rDNA sequences of *S. suihominis* were 1878 bp in length, and only one sequence (MK867455) was deposited in GenBank due to their completely identical sequences. The three sequences shared 97.7% identity with the newly obtained *S. miescheriana* 18S rDNA sequences. In GenBank, the most similar sequences were those of *S. suihominis* (AF176936, AF176937, and MH404229) from domestic and wild pigs (98.8–99.8% identity, with an average of 99.4%).

### Molecular characterization of 28S rDNA

The three 28S rDNA sequences (MK867456–MK867458) of *S. miescheriana* were 3450–3454 bp in length and shared 99.5–99.8% identity (with an average of 99.7%). The sequences in GenBank that exhibited the highest similarity with

those obtained in this study were those of *S. miescheriana* (AF076902 and MH404246–MH404254) from domestic and wild pigs (98.8–99.9 identity, with an average of 99.6%).

The three 28S rDNA sequences (MK867471–MK867473) of *S. suihominis* were 3458–3466 bp in length and shared 99.4–99.7% identity (with an average of 99.5%). The identity between the newly obtained 28S rDNA sequences of the two parasites was 96.4–96.9% (with an average of 96.6%). The 28S rDNA sequences of *S. suihominis* shared an identity of 96.6–97.3% (with an average of 97.0%) with those of *S. miescheriana* (AF076902, MH404246–MH404254) from domestic and wild pigs in GenBank.

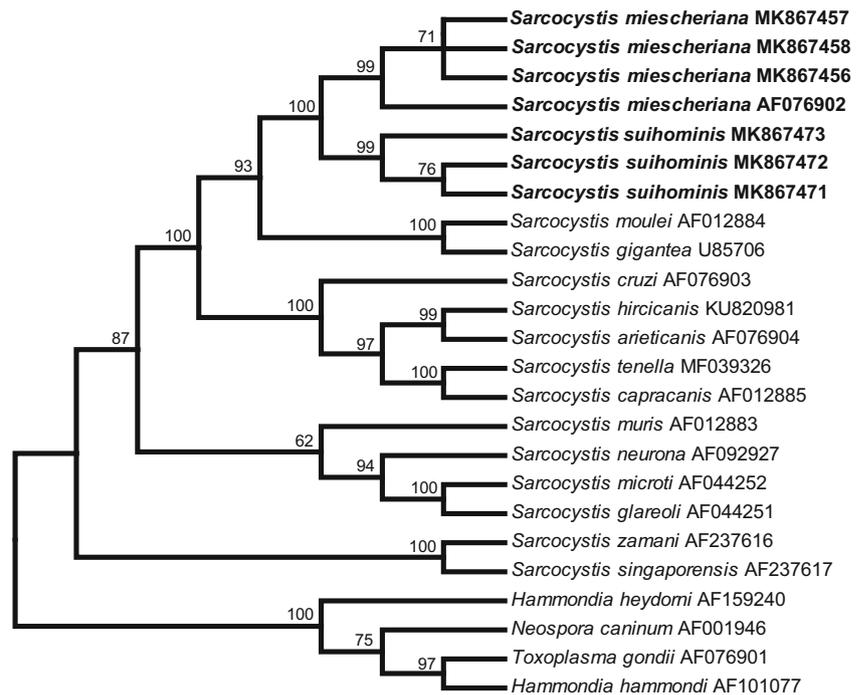
### Molecular characterization of ITS-1

The three *ITS-1* sequences (MK867468–MK867470) of *S. miescheriana* were 865–873 bp in length and shared 97.7–99.3% identity (with an average of 98.4%). The sequences in GenBank with the highest similarity to the three *ITS-1* sequences detected in this study were those of *S. miescheriana* (MH404234–MH404242) from wild pigs (94.2–97.0% identity, with an average of 95.9%).

**Table 1** Prevalence of *Sarcocystis* spp. in different muscular tissues of domestic pigs ( $n = 76$ ) collected from Yunnan Province, China

| Muscle infected        | <i>Sarcocystis</i> species                         |            |                        |            |                      |            |
|------------------------|--|------------|------------------------|------------|----------------------|------------|
|                        | <i>S. miescheriana</i> and/or <i>S. suihominis</i> |            | <i>S. miescheriana</i> |            | <i>S. suihominis</i> |            |
|                        | No. infected                                       | % infected | No. infected           | % infected | No. infected         | % infected |
| Esophagus              | 23   | 30.2       | 22                     | 28.9       | 9                    | 11.8       |
| Diaphragm              | 27   | 35.5       | 25                     | 32.9       | 12                   | 15.8       |
| Heart                  | 13   | 17.1       | 13                     | 17.1       | 0                    | 0          |
| Skeletal muscles       | 24   | 31.6       | 19                     | 25.0       | 13                   | 17.1       |
| Total infected animals | 28   | 36.8       | 25                     | 32.9       | 13                   | 17.1       |

**Fig. 2** Phylogenetic tree of select members of the Sarcocystidae family based on near-complete 28S rDNA sequences and inferred using the maximum parsimony method with the Tree-Bisection-Regrafting algorithm. The values between the branches represent the percent bootstrap value per 1000 replicates, and the 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 six new sequences of *Sarcocystis miescheriana* (MK867456–MK867458) and *S. sui hominis* (MK867471–MK867473) are shown in boldface



The three *ITS-1* sequences (MK867465–MK867467) of *S. sui hominis* were 859–864 bp in length and shared 97.4–99.5% identity (with an average of 98.4%). The identity between the newly obtained *ITS-1* sequences of the two parasites was 79.5–81.3% (with an average of 80.3%). At this locus, the sequences in GenBank with the greatest similarity to those obtained in this study were those of *S. miescheriana* (MH404234–MH404242) from wild pigs (82.8–88.5% identity, with an average of 84.5%).

### Molecular characterization of mitochondrial *COX1*

The three mitochondrial *COX1* sequences (MK867462–MK867464) of *S. miescheriana* were 713 bp in length and shared 99.0–99.8% identity (with an average of 99.3%). The sequences in GenBank with the highest similarity to the mitochondrial *COX1* sequences obtained in this study were those of *S. miescheriana* (MH404185–MH404227) from wild pigs (94.3–96.1% identity, with an average of 95.4%).

The three mitochondrial *COX1* sequences (MK867459–MK867461) of *S. sui hominis* were 713 bp and shared 99.0–99.2% identity (with an average of 99.1%). The identity between the newly obtained mitochondrial *COX1* sequences of the two parasites was 80.7–81.6% (with an average of 81.2%). These *COX1* sequences of *S. sui hominis* shared the most similarity with those of *S. sui hominis* (MH404228), a single *COX1* sequence of *S. sui hominis* obtained from a wild pig in GenBank. However, the identity was not high (only 88.1–88.2%, with an average of 88.2%).

### Phylogeny

A phylogenetic tree inferred from the 28S rDNA sequences revealed that *S. miescheriana* and *S. sui hominis* clustered together and formed a clade with *Sarcocystis gigantea* (U85706) and *Sarcocystis moulei* (AF012884) (Fig. 2).

### Discussion

*Sarcocystis miescheriana* is distributed worldwide (Dubey et al. 2016), whereas *S. sui hominis* has only been reported in Europe, India, Japan, and China (Hu et al. 2010; Dubey et al. 2016). An examination of sarcocysts in muscle samples from domestic pigs revealed that the prevalence rates of *S. miescheriana* and *S. sui hominis* were 32.9% and 17.1%, respectively, and the overall prevalence of *Sarcocystis* infection was 36.8%. In comparison, two recent studies of domestic pigs in India (250 animals) and Romania (64 animals) based on microscopic detection found overall prevalence rates of 58.4% and 23.4%, respectively (Kaur et al. 2016; Imre et al. 2017). In Yunnan Province, China, domestic pigs are usually left free ranging and are caught and slaughtered by their owners at maturity or when needed. Additionally, the dietary habits of inhabitants of this area are strongly related to the consumption of traditionally raw pork products, namely, “Shengpi,” for enjoyment. Thus, the conditions are favorable for the successful completion of the life cycles of *S. miescheriana* and *S. sui hominis* between their intermediate and definitive hosts.

Sequence analysis has been shown to be more sensitive and effective for detection of sarcocysts in muscle samples from domestic and wild pigs (Kia et al. 2011; Yan et al. 2013; Kaur et al. 2016; Gazzonis et al. 2019). However, the use of a single marker alone might be insufficient because some species exhibit low intraspecific sequence variation in a given region, as demonstrated recently (Hu et al. 2016). Therefore, four loci (18S rDNA, 28S rDNA, *ITS-1*, and mitochondrial *COXI*) of the two species of *Sarcocystis* in domestic pigs have been sequenced and analyzed. Among these loci, the sequences of 28S rDNA and *ITS-1* of *S. suihominis* were the first records of these genes deposited in GenBank. In our analysis, the newly obtained sequences of the four loci (18S rDNA, 28S rDNA, *ITS-1*, and mitochondrial *COXI*) obtained from the two species of *Sarcocystis* presented high intraspecific similarities, i.e., 100%, 99.7%, 98.4%, and 99.3%, respectively, among the *S. miescheriana* genes and 100%, 99.5%, 98.4%, and 99.1%, respectively, among the *S. suihominis* genes. The comparison of the newly obtained sequences of the four loci (18S rDNA, 28S rDNA, *ITS-1*, and mitochondrial *COXI*) of the two parasites revealed interspecific similarities of 97.7%, 96.6%, 80.3%, and 81.2%, respectively. Therefore, *ITS-1* and mitochondrial *COXI* appear to better distinguish *S. miescheriana* from *S. suihominis* compared with the results obtained with 18S rDNA or 28S rDNA.

The study constitutes the first establishment of the phylogenetic relationship among *S. miescheriana* and *S. suihominis* and with other *Sarcocystis* spp. based on their 28S rDNA sequences. The topology of the tree obtained with 28S rDNA was similar to those of the trees previously inferred with 18S rDNA and mitochondrial *COXI* (Gazzonis et al. 2019). The present analysis showed that *S. miescheriana* and *S. suihominis* are sister taxa and form a clade with *S. moulei* in domestic goats and *S. gigantea* in domestic sheep, whose definitive hosts are felids. This clade clusters together with a clade containing *Sarcocysts* spp. in ruminants, which have canids as their definitive hosts. The following possible explanation for this relationship has been proposed: *S. miescheriana* and *S. suihominis* descended from a common ancestor using only ancient species as definitive hosts, and two variants of this ancient species gradually separated and became adapted to extant canids and modern humans, respectively (Gazzonis et al. 2019).

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## Compliance with ethical standards

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

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