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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

First report of molecular characterization and phylogenetic analysis of *Sarcocystis tenella* from India

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Abstract

Sarcocystis tenella is a common tissue coccidian parasite of sheep. It is reported worldwide with high prevalence rate ranging from 9 to 100%. However, there are very limited reports of this parasite from the Indian context and those reports are totally based on the morphology alone. When it comes to molecular characterization, such studies are absent from India. The present communication reports the first characterization study of *S. tenella* from India. 18S rRNA ribosomal gene and mitochondrial cytochrome c oxidase subunit I (cox1) genes were used for molecular characterization and phylogenetic analysis alongside standard histopathology of sarcocysts. Five Indian isolates were characterized for each gene, and respective sequences were submitted in the NCBI. Two haplotypes were noticed, both for the 18S rRNA and cox1 gene showing 99.8–100.0% and 99.7–100.0% nucleotide homologies within themselves, respectively. When compared with other sequences of *S. tenella* across the globe, the present isolates showed 93.3–99.9% nucleotide homology based on 18S rRNA gene and 95.2–99.8% nucleotide homology based on cox1 gene, respectively. In both the 18S and cox1 phylogenetic trees, respective sequences of *S. tenella* were placed with monophyletic cluster which was sister to a cluster comprising of sequences of *S. gracilis* and *S. alces*.

Keywords *Sarcocystis tenella* · 18S rRNA · cox1 · Molecular characterization · Phylogenetic analysis

Introduction

Sheep (*Ovis aries*) are known to be intermediate hosts of at least four valid *Sarcocystis* species, viz., *S. tenella*, *S. arieticanis*, *S. gigantea*, and *S. medusiformis* (Dubey et al. 2016). *S. tenella* and *S. arieticanis* involve canids as definitive hosts and form microscopic sarcocysts while *S. gigantea* and *S. medusiformis* involve felids and form macroscopic sarcocysts (Dubey et al. 2016). Besides these four valid species, *S. mihoenis*, a macroscopic sarcocyst-forming species, is reported from Japan (Saito et al. 1997) while *S. microps*, a microscopic sarcocyst-forming species, is reported from China (Wang et al. 1988). Ovine sarcocystosis is reported from many countries across the globe with a very high prevalence rates ranging from 9 to 100% (Dubey et al. 2016). Although there are handful of reports of ovine sarcocystosis from India based on morphology alone (Chhabra and Samantaray 2013; Gopal et al. 2016), there is not even a single report about molecular characterization of sarcocysts of ovine origin from this part of the world. In the present study, molecular characterization of *S. tenella* was done on 18S rRNA ribosomal and mitochondrial cytochrome c oxidase subunit I (cox1) genes. Subsequently, sequence phylogenetic analysis of the present isolates was done with the sequences across the globe for both genes.

Materials and methods

Collection of samples

The heart tissues from sheep that were sent to the Department of Veterinary Pathology, DUVASU, for postmortem were collected over a period of 6 months. These tissues were routinely

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screened for sarcocysts through standard muscle squash method (Dubey et al. 1989). Numerous sarcocysts were found in different individual sheep. The sarcocysts were identified based on morphological features described elsewhere (Haziroglu et al. 2002; Dubey et al. 2016; Hu et al. 2017). Thereafter, tissue pieces of the heart samples were finely chopped and processed for DNA isolation.

DNA isolation and primer selection

DNA was isolated from pieces of heart tissue of five animals in duplicate using the commercial DNA isolation kit (Promega, USA) following the manufacturer's protocol. The primer sequences as well as the PCR conditions for both the genes were same as described by Kolenda et al. (2014). The PCR reaction mixture consisted of 25.0 μl of 2× Green Go Taq (Promega, USA) having 400 μM each of dATP, dGTP, dCTP, and dTTP and 3 mM $MgCl_2$ besides 10 pmol of each primer and 5 μl of DNA template. The final volume of PCR reaction mixture was made 50 μl using nuclease free water. The amplified products were visualized in 1.25% agarose gel incorporated with ethidium bromide. The PCR products were purified using gel purification and DNA clean up kit (Promega, USA) following the manufacturer's protocol. Thereafter, the purified products were sent for outsourced custom DNA sequencing in both directions using respective primer.

The sequences, hence obtained, were submitted into the NCBI, and corresponding accession numbers were obtained for both the genes. Thereafter, the sequences were analyzed using Mega 6 and Gene tool softwares. A multiple sequence alignment was generated with the Clustal W program within MEGA 6 software (Tamura et al. 2011) both the pairwise and multiple alignments. The sequences of respective genes were truncated at both ends, so that nearly all sequences started and ended at the same (homologous) nucleotide positions. The phylogenetic relationship of *S. tenella* with other isolates of *S. tenella* across the world was commutated based on nucleotide sequences of the 18S rRNA and cox1 gene using MEGA6 software (Fig. 1a, b). Phylogenetic tree for both the genes were reconstructed using the maximum parsimony (MP) method with tree-bisection-regrafting (TBR) algorithm and using all the sites. *Sarcocystis fusiformis* was used as out-group species to root the tree for both the genes. Likewise, the phylogenetic tree of both genes of present isolates with other species of *Sarcocystis* across the world was also constructed using the maximum parsimony (MP) method with the subtree-pruning-regrafting (SPR) algorithm (Fig. 2a, b). In both the analysis, phylogeny was tested with bootstrap method using 1000 bootstrap replications.

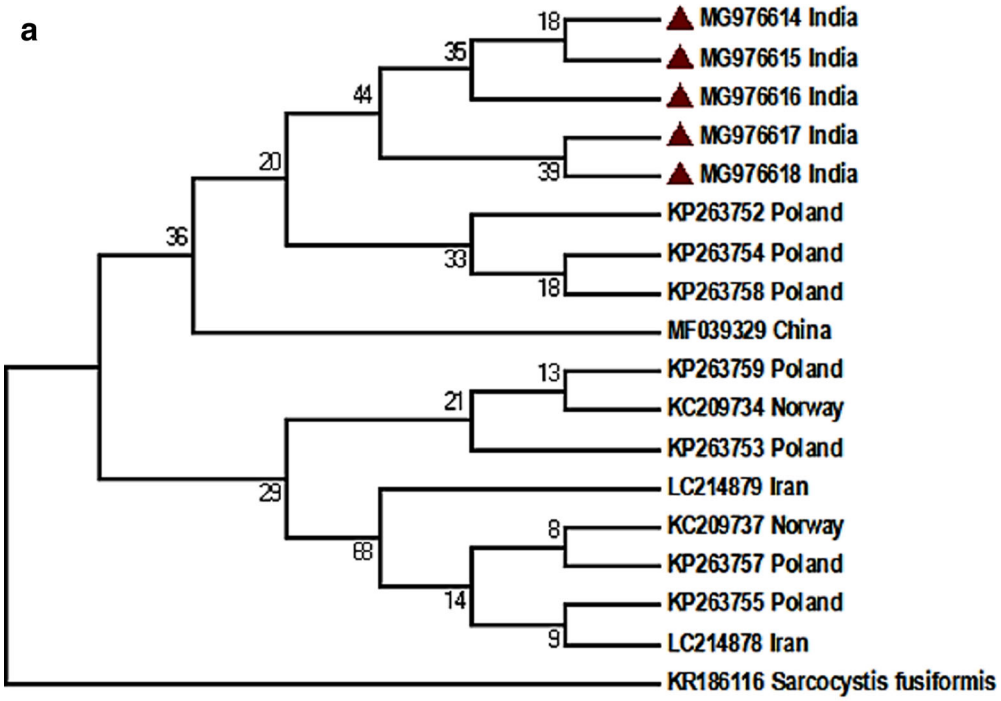
Fig. 1 Phylogenetic relationship of *S. tenella* Indian isolates with other isolates across the world based on 18S (**a**) and cox1 gene (**b**). All accession numbers corresponds to different *S. tenella* isolates followed by their country of origin. The sequences generated in the present study are marked as red triangle

Phylogenetic analysis of 18S and cox1 gene sequences of various *Sarcocystis* spp.

A total of 105 sequences from 52 taxa were used in the analysis of 18S gene, including 5 new sequences of *S. tenella* generated in the present study. A multiple sequence alignment was obtained with the Clustal W program within Mega6 (Tamura et al. 2011), using a gap opening penalty of 10 and gap extension penalty of 0.1 and 0.2 for the pairwise and multiple alignments, respectively. Sequences were trimmed at both ends, so that most sequences started and ended at the same (homologous) nucleotide positions, corresponding to positions 161 and 985, respectively, of the gene sequence KT901245 of *S. bovifelis*. The final alignment comprised 1036 aligned positions, with gaps. GeneBank sequence U67121 of *E. tenella* of chickens was used as out-group species to root the tree for 18S gene (Fig. 2a). Likewise, 571 sequences from 37 taxa were used in the analysis of cox1 gene, including 5 new sequences of the *S. tenella* generated in the present study. However, in order to reduce computation time, identical superfluous sequences were removed, so that each haplotype was only represented by a single sequence. Hence, for the final analysis, a total of 302 sequences (haplotypes) were used. A codon-based multiple sequence alignment of all sequences was generated with the Clustal W (codon) program within Mega6 (Tamura et al. 2011), using a gap opening penalty of 10 and gap extension penalty of 0.1 and 0.2 for the pairwise and multiple alignments, respectively. Sequences were trimmed at both ends, so that most sequences started and ended at the same (homologous) nucleotide positions, corresponding to positions 13 and 1016, respectively, of the gene sequence KT901245 of *S. bovifelis*. The final alignment comprised 1347 aligned positions, with gaps. The phylogenetic tree was reconstructed as described above using all codon positions. The GeneBank sequence HQ702484 of *E. tenella* was used as out-group species to root the tree for cox1 gene (Fig. 2b).

Results

The sarcocysts in the heart tissue were identified as that of *S. tenella* based to their morphological features. The cysts were found to be having thick walls. They were differentiated from *S. arieticanis*, another microscopic *Sarcocystis* spp. of sheep, owing to the absence of hair-like projections on their surface. The average size of cysts varied from 0.41 to 0.63 mm in length and 0.02 to 0.06 mm in width. Histopathologically, the sections of the heart myocardium revealed elliptical and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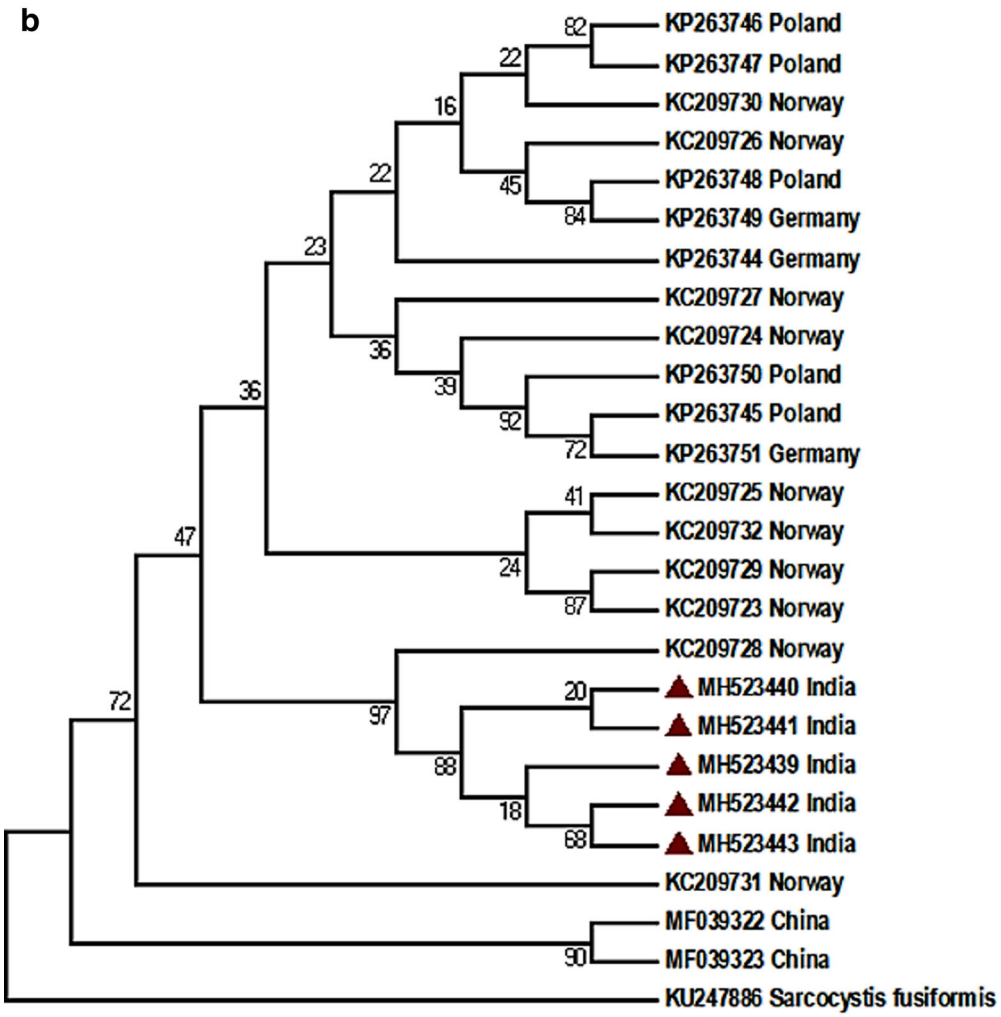
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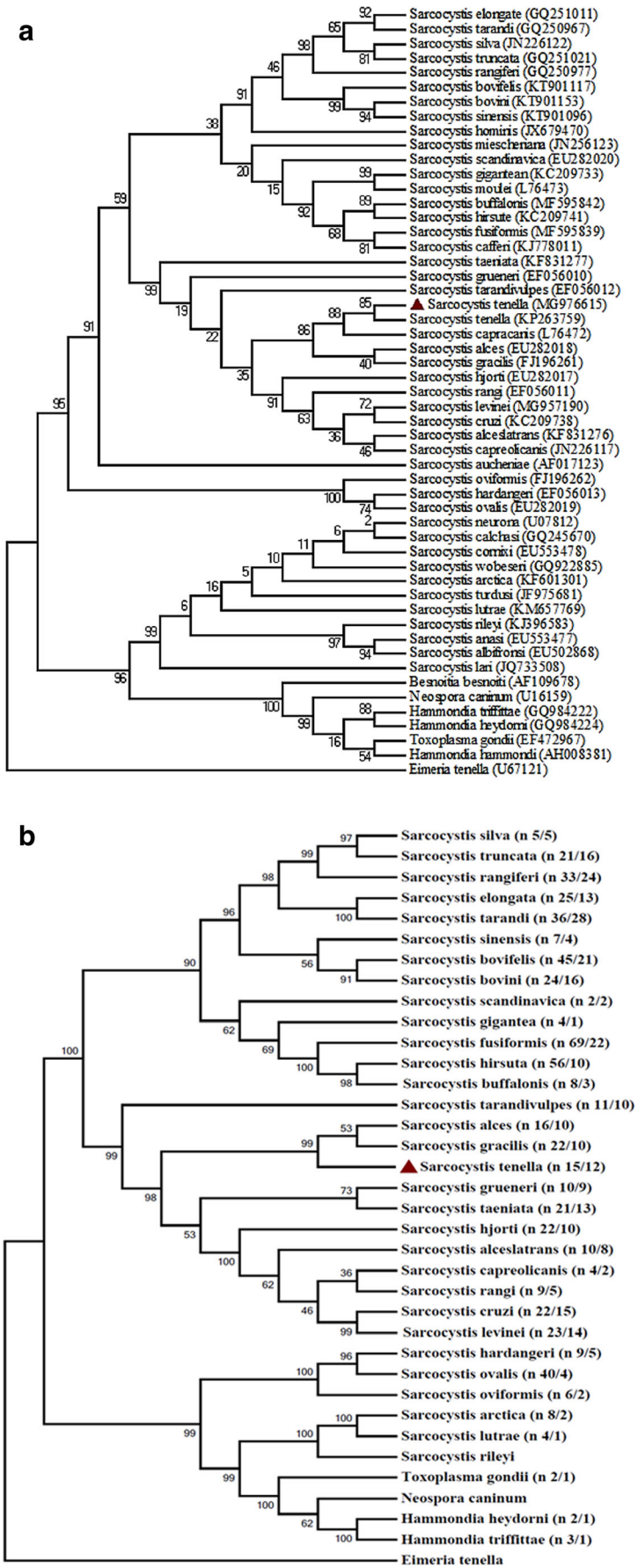

Fig. 2 Phylogenetic tree for selected members of the Sarcocystidae inferred using the maximum parsimony method (**a**) based on partial sequences of 18S (**b**) based on partial sequences of cox 1. Sub-trees formed by two or more sequences/haplotypes of the same species have been collapsed, but the numbers of sequences/haplotypes included are given behind the taxon names. The sequences generated in the present study are marked as red triangle

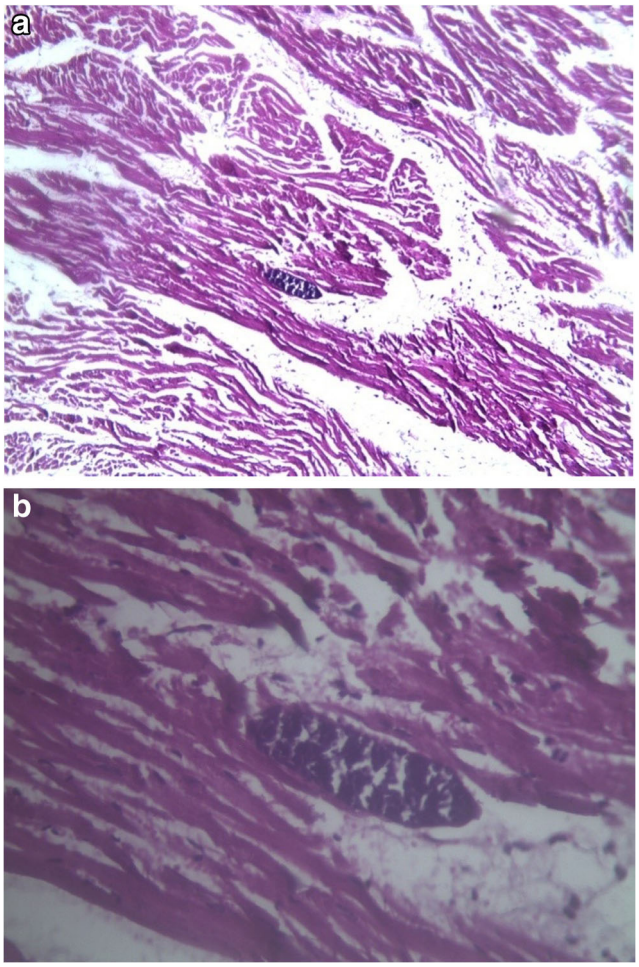

Fig. 3 Sarcocyst in myocardium of sheep. (**a**) The myocardial muscle bundles are intact with flat palisading nuclei in the muscle fibers (H&E, × 10). (**b**) Section of heart showing the cross section of sarcocyst (H&E, × 40)

circular thick-walled sarcocysts embedded in the cardiac muscle bundles (Fig. 3). The cardiac tissue surrounding the sarcocyst did not show any infiltration of inflammatory cells in the areas where the muscle bundles and muscle fibers were intact. The microscopic fields having sarcocysts lodging disintegrated muscle fibers showed infiltration of mononuclear cells suggestive of aged inflammatory lesions.

The 18S rRNA ribosomal and cox1 genes were resolved into a single amplicons of size 900 and 1100 bp, respectively. Upon submission of the various sequences, accession numbers were obtained for both 18S rRNA (MG976614–MG976618) and cox1 (MH523439–MH52343) genes, respectively. Two haplotypes were noticed among the 18S rRNA sequences wherein, MG976614-16 represented one haplotype and MG976617-18 represented the other and they showed 99.8–100.0% nucleotide homology within themselves. Likewise, again, two haplotypes were noticed wherein MH523439-41 showed one haplotype and MH52342-43 showed another with 99.7–100.0% nucleotide homology levels within the two haplotypes. When compared with other sequences of *S. tenella* across the globe, 18S rRNA and cox1 gene-based sequences showed variable nucleotide homologies of the range 93.3–99.9% and 95.2–99.8%, respectively. The two haplotypes of *S. tenella* based on 18S rRNA gene revealed variations at nucleotide position 39 (A-G) and position 639 (T-C). On similar lines, the two haplotypes based on cox1 gene revealed a variation at nucleotide position 350 (T-A) (Fig. 4).

In the phylogenentic tree inferred from the 18S rRNA gene sequence (Fig. 2a), the sequences of *S. tenella* were placed with strong support as a monophyletic sister group to the sequences *S. capracanis*. Both the taxas were sister to sequences of *S. alces* and *S. gracilis*. The phylogenetic analysis based on partial cox1 sequences (Fig. 2b), placed with near-maximum support, all sequences of *Sarcocystis* spp. into three, well supported the major clades. In the second major clade, all the sequences of *S. tenella* were placed with higher support in monophyletic cluster, which was sister to a cluster comprising sequences of *S. gracilis* and *S. alces*. Together, these three species were separated from the cluster comprising of nine other species that have been reported to have canids as definitive host.

Discussion

S. tenella is a well-known and widely prevalent *Sarcocystis* spp. infecting sheep characterized by enzootic tissue parasitoses and is often associated with neurological disorders especially in lambs (Dubey et al. 2016). The high prevalence of *S. tenella* throughout the world can be very well explained on the basis of its definitive host. The *Sarcocystis* spp. that are being transmitted by canids are more prevalent than those which are being spread by felids, partially because dogs are

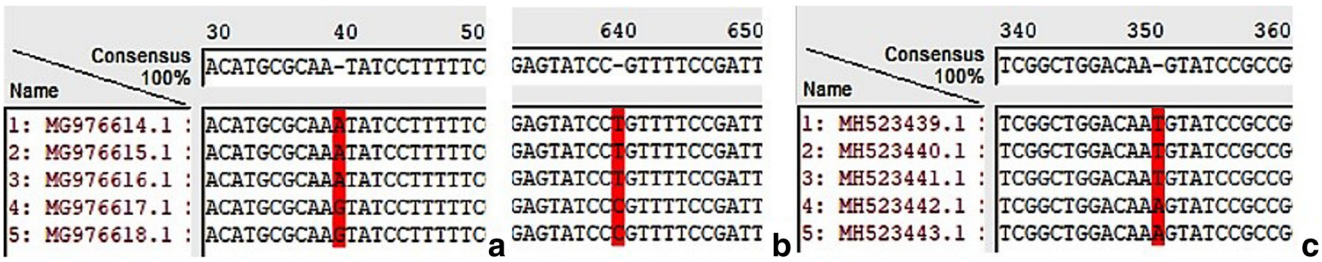

Fig. 4 Nucleotide variation in two haplotypes of *S. tenella* based on 18S rRNA gene at (**a**) nucleotide position 39 (A-G) and (**b**) = nucleotide position 639 (T-C) alongside cox1 gene (**c**) at nucleotide position 350 (T-A)

more efficient producer of sporocysts than cats, and partially because canid-transmitted sarcocysts do not require several months in the host to become infective unlike felid-transmitted sarcocysts (Dubey et al. 2016).

The cysts of *S. tenella* are reported from the heart, diaphragm, and esophagus of sheep (Dubey et al. 2016). Another *Sarcocystis* spp. of sheep—*S. arieticanis* is known to have a higher affinity for cardiac tissue (Haziroglu et al. 2002). The sole point of differentiation between *S. tenella* and *S. arieticanis* is the structure of the cyst wall. The cyst wall of *S. tenella* is thick with the absence of hair-like projections on its surface unlike *S. arieticanis* where hair-like projections are present (Haziroglu et al. 2002). However, during histopathological examination, the identification of these cysts is difficult owing to different angles of sarcocysts sectioned alongside alterations in structure due to fixation and tissue processing and similarity with cardiac muscle morphology (Haziroglu et al. 2002). Hence, cyst wall morphology should not be treated as confirmatory criterion alone but should be supplemented with other criteria like genetic characterization and sequencing.

The sequencing of 5′ end of the 18S rRNA gene of *Sarcocystis* spp. often results in sequences of poor quality due to sequence variation (indels) in certain regions of gene in many *Sarcocystis* spp. (Gjerde 2013; Sudan and Shanker 2018). Hence, 18S is nowadays not considered as sole molecular target for authentic characterization of *Sarcocystis* spp. (Gjerde 2013; Sudan and Shanker 2018). Two haplotypes were noticed, in the present study, based on 18S rRNA gene wherein MG976614-16 formed one haplotype and MG976617-18 formed the other. Among them, the difference was confined to a single substitution at two places, viz., position 39 (A-G) and position 639 (T-C). Interestingly, all the five Indian isolates formed a separate clade than the other isolates across the world. So far, as nucleotide homology is concerned, the two Indian haplotypes of *S. tenella* showed 99.8–100.0% homology within themselves. A high degree of nucleotide homology (93.3–99.9%) was noticed within all the isolates of *S. tenella* across the globe.

So far as cox1 gene is concerned, again, two haplotypes were noticed wherein sequences MH523439-41 represented one haplotype and MH52342-43 represented another. These isolates showed 99.7–100.0% homology levels within themselves. Interestingly, the same three isolates from 18S gene showed one haplotype and the two showed another haplotype with a single nucleotide change at position 350 (T-A). When compared with other sequences of *S. tenella* across the globe, the present isolates showed 95.2–99.8% nucleotide homologies with them. In both 18S and cox1 phylogenetic trees, respective sequences of *S. tenella* were placed with a monophyletic cluster which was sister to a cluster comprising of sequences of *S. gracilis* and *S. alces*.

In conclusion, this is the first report of molecular characterization of *S. tenella* from India. More studies are required from different agroclimatic zones of India to delineate the actual pattern of phylogeny of *S. tenella* from India. In the present study, two haplotypes were noticed for both 18S rRNA and cox1 genes.

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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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