



Associative Genetic Diversity of *Sarcocystis levinei* Isolates across the Globe

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Abstract: Bubaline sarcocystosis, caused by *Sarcocystis levinei*, is worldwide in distribution. Yet, very limited reports are available across the globe on the phylogenetic aspects of this particular *Sarcocystis* spp. Virtually no literature is available on its molecular aspects from India. Thus, the present study was designed to phylogenetically characterize the various isolates of *S. levinei* based on 18S rRNA and cox 1 mitochondrial genes. A total of 11 isolates of *S. levinei* from Northern India were characterized for 18S rRNA (MG957189-MG957199) and cox 1 (MH255771-MH255781) genes. PCR products were cloned, sequenced, and compared with other sequences across the world. A phylogenetic tree was constructed based on the maximum likelihood (ML) method. Phylogenetic analysis placed *S. levinei* and *S. cruzi* into two monophyletic sister groups. An appreciable amount of genetic variability was noticed between various *S. levinei* sequences for both the gene loci. A total of three haplotypes were noticed for each gene. The generated sequences showed 99.8–100.0% and 99.7–100.0% nucleotide homologies within themselves with regard to the 18S rRNA and cox 1 genes, respectively. Few of the studied sequences showed marked similarity and closeness with the Egyptian sequences than their Indian counterparts. This is the first report of molecular characterization and sequence phylogenetic study of *S. levinei* from India.

Keywords: 18S gene; cox 1 gene; molecular characterization; phylogeny; *Sarcocystis levinei*



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1. Introduction

Bubaline sarcocystosis is caused by four species of *Sarcocystis* genus viz., *S. fusiformis*, and *S. buffalonis* (forming macroscopic sarcocysts), alongside *S. levinei* and *S. sinensis/S. dubeyi* (forming microscopic sarcocysts) [1,2]. There is no valid record of human illness from consumption of *Sarcocystis* spp.-infected buffalo meat; however, reports of human illness in two Chinese volunteers after ingesting *Sarcocystis*-infected meat suggest possible public health significance [1]. Ample work has been reported across the world on characterization studies of *Sarcocystis* spp. affecting different hosts [3–6]. The nuclear (18S ribosomal RNA, 28S ribosomal RNA and Internal Transcribed Spacer-ITS) and mitochondrial cytochrome (cox 1) genes have been used for the genetic characterization of various *Sarcocystis* spp. [7–10].

Sarcocystis levinei is widely reported in the cardiac muscles of buffaloes across the globe [2,11,12]. The parasite has a high prevalence rate of 75% in China, 83% in Iran, 73% in Italy, 74% in Vietnam, and 68% in India [2,12]. But, in spite of its global distribution, very limited work has been conducted on the phylogenetic characterization aspects of *S. levinei* [5]. Even that limited work indicates appreciable genetic variability within its isolates. Although there are ample reports of the distribution of *S. levinei* across India [12–15],

there is no record of phylogenetic characterization of *S. levinei* from India. Of late, the parasite has been thoroughly redescribed [16]. The present study was designed to characterize the Indian isolates of *S. levinei* based on 18SrRNA and cox 1 genes. In addition, the evolutionary phylogeny of *S. levinei*, with regard to other *Sarcocystis* spp. affecting different hosts, was also delineated. Subsequently, the phylogenetic analysis was carried out to determine the placement of Indian isolates in terms of the available *S. levinei* sequences.

2. Results

The sarcocysts were identified as those of *S. levinei* (Figure 1) based on the previously described morphological features [2,16]. The sarcocysts were thin-walled and elongated in shape. A total of 11 sarcocysts were characterized for 18S and cox 1 genes, resulting into 22 sequences (11 apiece). Upon submission to NCBI, accession numbers MG957189–MG957199 were obtained for the 18S gene, while the accession numbers for cox 1 were MH255771–MH255781.



Figure 1. Squashed and methylene-blue-stained preparation of heart tissue showing characteristic elongated sarcocysts.

2.1. Phylogenetic Analysis of 18S rRNA and Cox 1 Gene Sequences of Various *Sarcocystis* spp.

A total of 103 sequences belonging to 50 taxa of *Sarcocystis* spp. were used for 18S rRNA gene analysis, including 11 sequences of *S. levinei* generated from the present study. Since the main objective of our analysis was to compare the phylogenetic placement of various *Sarcocystis* spp. based on the 18S rRNA gene, all sequences/haplotypes of a species were grouped under the name of the respective species. Hence, a total of 45 sequences representing 45 *Sarcocystis* species and 7 sequences of other closely related parasitic species were used. Sequences of 18S rRNA genes were trimmed at both ends so that most sequences started and ended at the homologous nucleotide position, corresponding to positions 161 and 985, respectively, of the *S. bovifelis* sequence. The gene bank sequence of *E. tenella* was used as an out-group species to root the tree for the 18S rRNA gene (Figure 2a).

Likewise, 570 sequences originating from 37 taxa of *Sarcocystis* spp. were used in the analysis of the cox 1 gene, including 11 sequences of the *S. levinei* generated from the present study. However, in order to reduce the 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 300 sequences (haplotypes) were used. Sub-trees formed by two or more sequences/haplotypes of the same species were collapsed, but the quantities of included sequences/haplotypes are given behind the taxon names. The 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 gene sequence KC209690 of *S. bovifelis*. GeneBank sequence HQ702484 of *E. tenella* was used as an out-group species to root the tree for the cox 1 gene (Figure 2b).

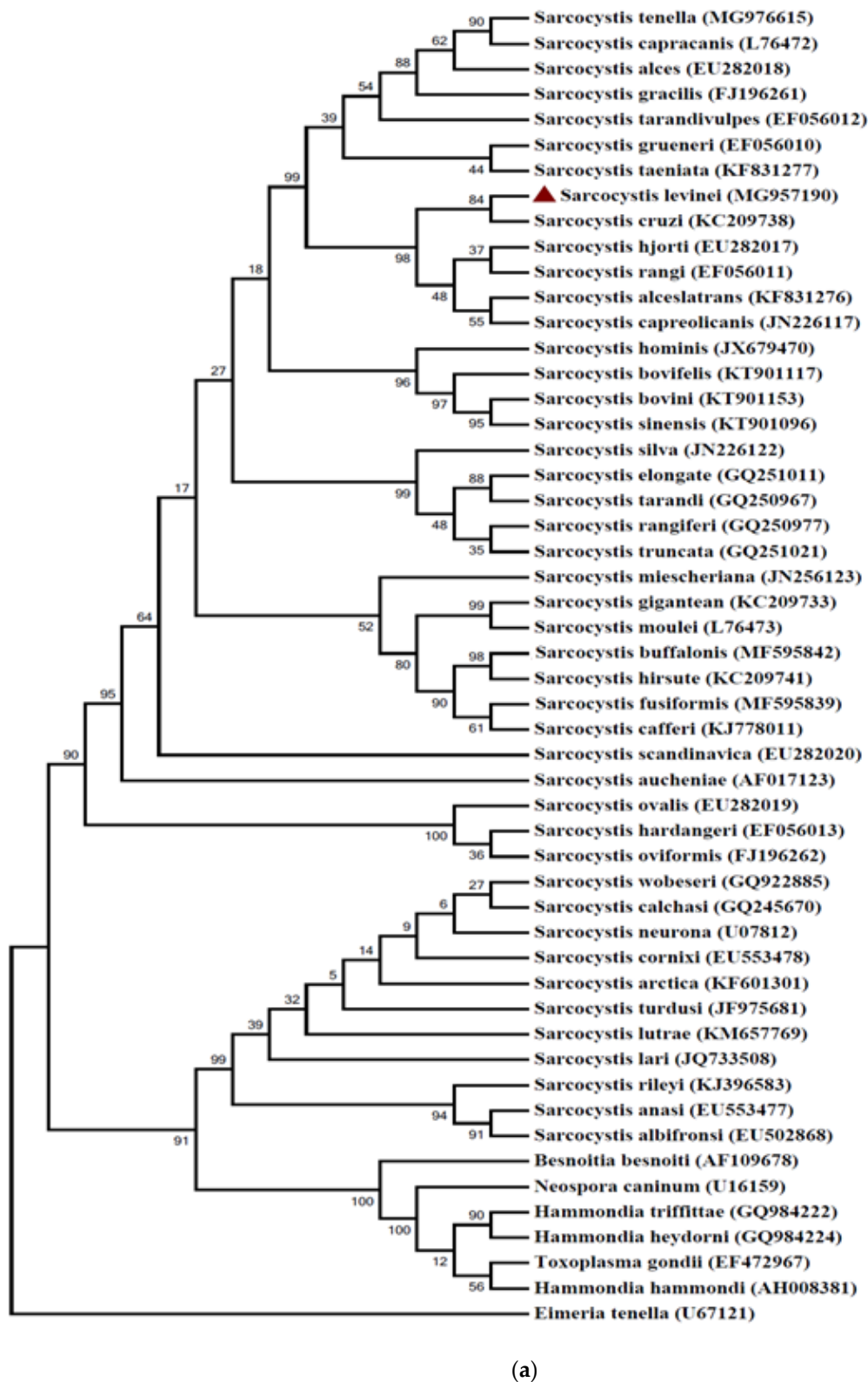


Figure 2. Cont.

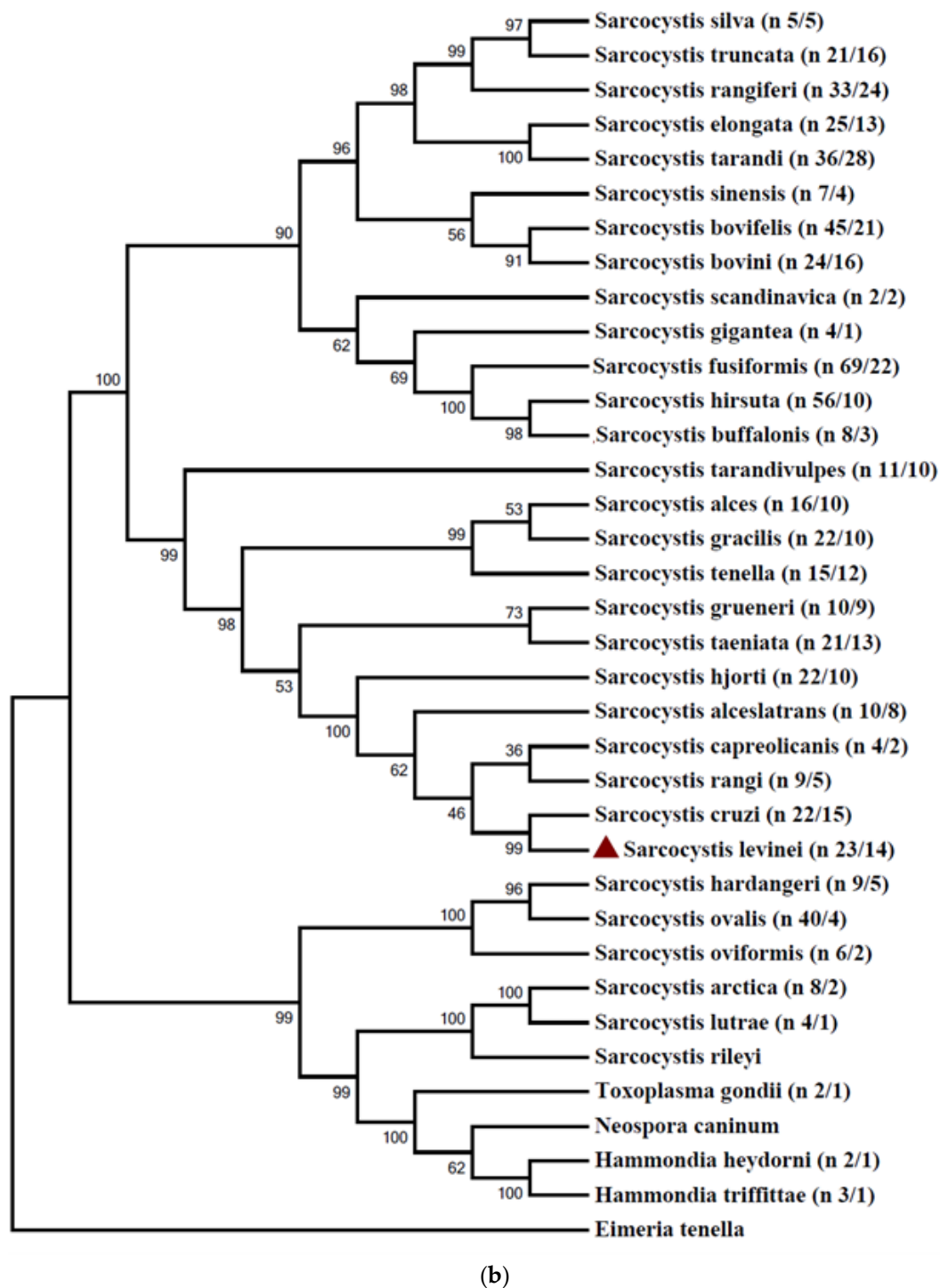


Figure 2. Phylogenetic tree for selected members of Sarcocystidae, inferred using the maximum parsimony method (a) based on partial sequences of 18S and (b) based on partial sequences of *cox 1*. The numbers represent the boot strap values. Sub-trees formed by two or more sequences/haplotypes of the same species were collapsed, but the quantities of included sequences/haplotypes are given behind the taxon names.

2.2. Phylogenetic Analysis and Nucleotide Identity of 18S rRNA and Cox 1 Gene Sequences of the Present Isolates with Other Isolates of *S. levinei*

A total of 11 sarcocysts of *S. levinei* were characterized for the 18S ribosomal gene. These sequences were aligned with other sequences of *S. levinei* available in NCBI, and a phylogenetic tree was drawn using Mega 6 software (Figure 3a). For 18S rRNA, the

length of multiple alignment was 876 bp, and the Tamura 3 parameter was chosen as the best nucleotide evolution model. For cox 1, the length of multiple alignment was 822 bp and the Kimura 2 parameter was chosen as the best nucleotide evolution model. *S. hjorti* was used as the outgroup for both analyses. Only the sequences of *S. levinei* from Egypt were available in NCBI for the comparison and construction of the phylogenetic tree. Likewise, the nucleotide identity of the present sequences with other sequences of *S. levinei* available from India and Egypt was also calculated. A total of five haplotypes were noticed within the studied *S. levinei* sequences. The presented Indian sequences showed 99.7–100.0% genetic identity within themselves. On the other hand, Egyptian isolates of *S. levinei* (KU247914–KU247922) comprised only a single haplotype, which was identical to two isolates of *S. levinei* obtained in the present study (MG957189, MG957194). Eleven cox 1 sequences of *S. levinei* generated in the present study were compared with those of *S. levinei* from Egypt. Overall, 10 haplotypes of *S. levinei* were identified with the cox 1 gene. The presented Indian isolates of the *S. levinei* species numbered 99. A sequence identity of 5–100% was found, whereas, when comparing all available isolates of this *Sarcocystis* species, 98.8–100% sequence identity was calculated. Details of the haplotype number, haplotype diversity, nucleotide diversity, number of segregating sites, and average number of nucleotide differences are represented in tabular form in Table 1.

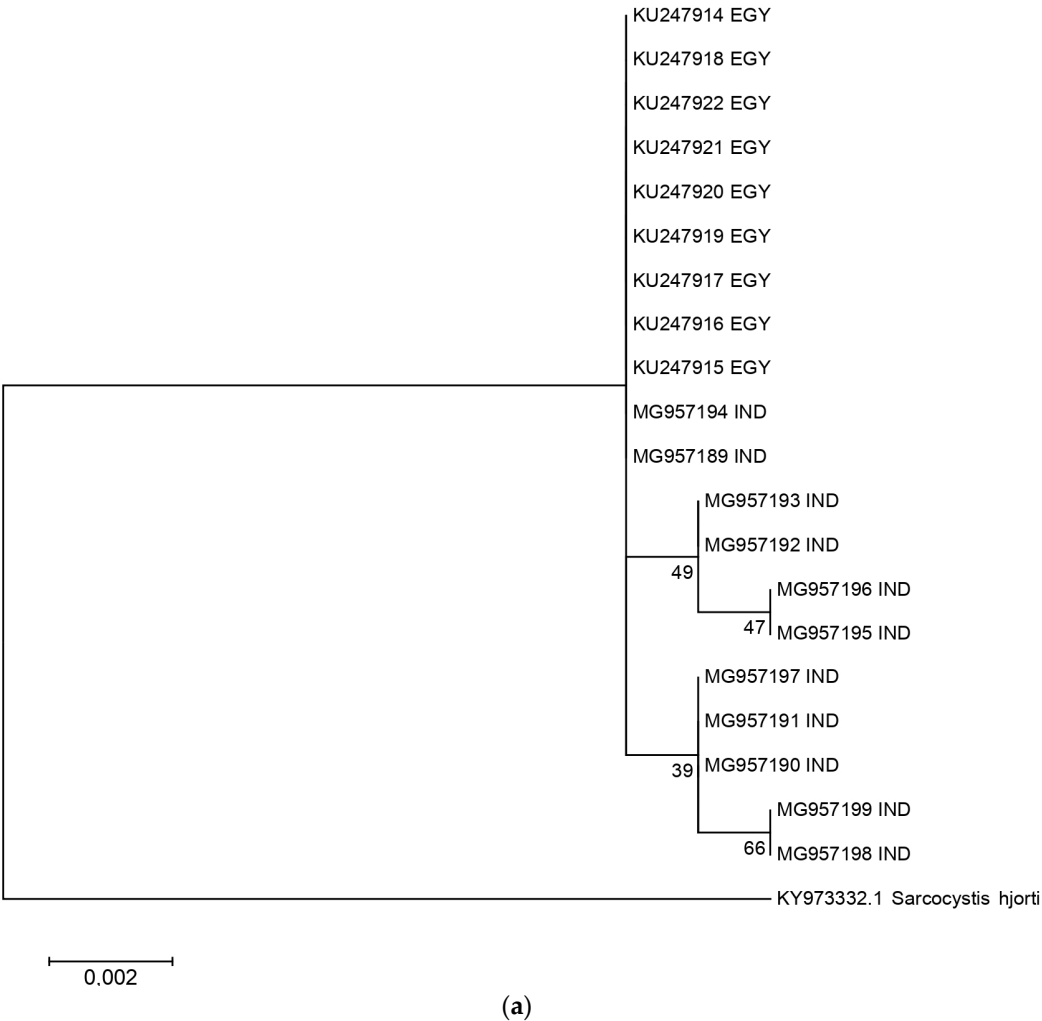


Figure 3. Cont.

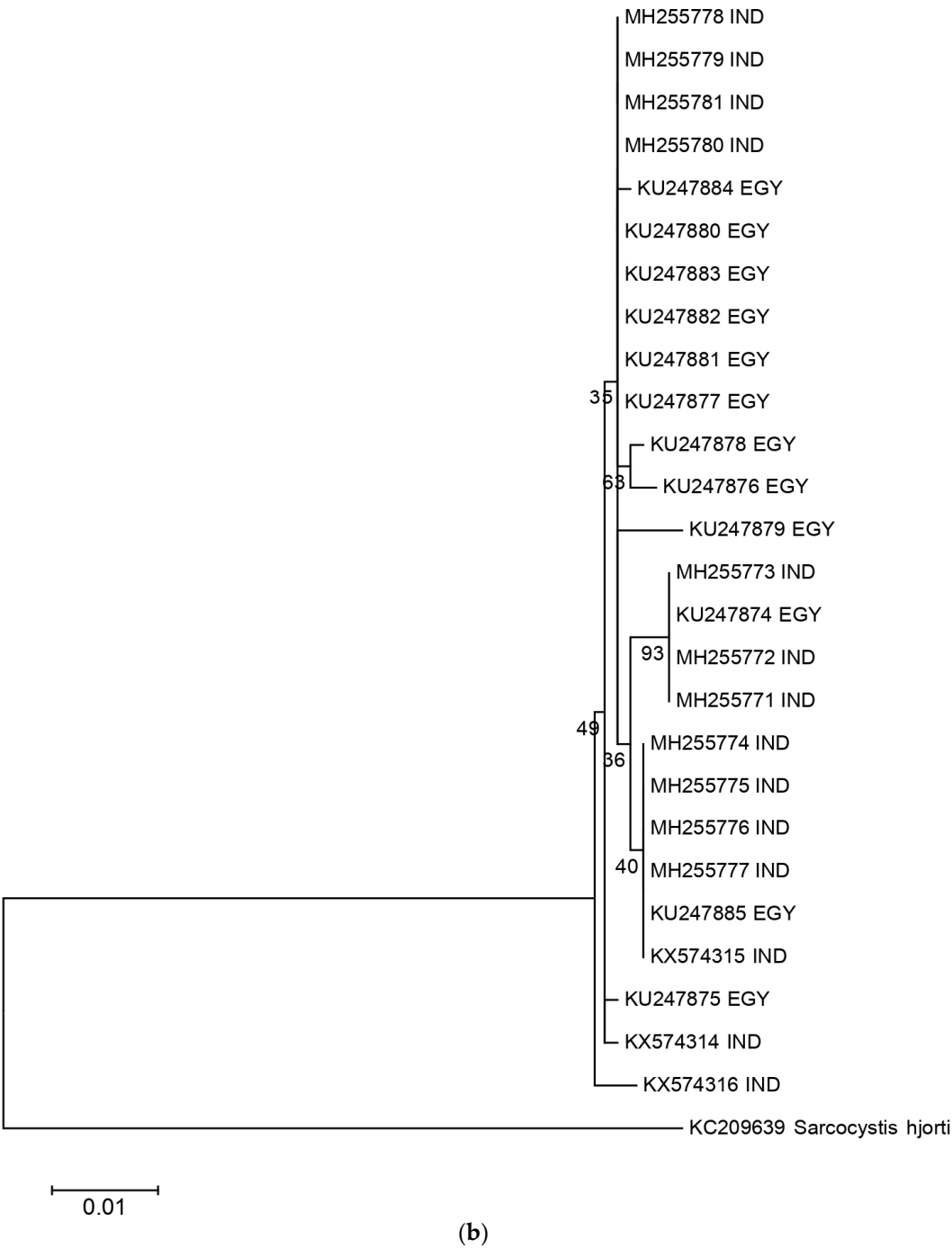


Figure 3. Phylogenetic relationship of *S. levinei* Indian isolates with other isolates across the world, based on the (a) 18S and (b) cox 1 genes. All accession numbers correspond to different *S. levinei* isolates, followed by their country of origin.

Table 1. Comparative study on the sequences of *S. levinei* based on both genes (N_h : number of haplotypes; H_d : haplotype diversity; P_i : nucleotide diversity; S : number of segregating sites; K : average number of nucleotide differences).

Marker	N_h	H_d	P_i	S	K
Cox 1	10	0.825	0.00336	19	2.76308
18S	5	0.679	0.00118	3	1.00526

3. Discussion

Contrary to the general belief that *Sarcocystis* spp. are highly specific for intermediate hosts, a few 18S rRNA gene sequences suggest a considerably high nucleotide identity between *Sarcocystis* spp. affecting cattle with those affecting water buffaloes, and between the species affecting water buffaloes with those affecting African buffaloes [9]. The extensive sequence variation at the 5-end of 18S gene rRNA [17] often leads to interpretations that the morphologically similar *Sarcocystis* spp. from cattle and buffalo can be considered to be the same species [18] which is basically false [5,8,17,19]. Besides cattle and buffaloes, this appreciable amount of intraspecific sequence variations at 5-end of the 18S gene has been reported in many *Sarcocystis* spp. affecting a wide range of hosts [6,20,21]. Furthermore, *Sarcocystis* spp.'s heterogeneity in 18S is also seen in apicomplexan parasites—*Plasmodium*, *Cryptosporidium*, *Babesia*, *Theileria*, and *Eimeria* [22–24]. It is much easier to study 18S-linked intra-isolate variability in *Sarcocystis* spp., owing to the availability of fairly large amount of DNA from a single sarcocyst compared to these apicomplexan parasites [17]; hence, multiple genes can be easily explored from a single source. This intra-isolate heterogeneity involves indels that necessitate the cloning of PCR products before sequencing, as it is very confusing to assign the various sequence variants to a particular species unambiguously until it is confirmed that all of them have originated from a single sarcocyst [17]. Hence, many ambiguities have been reported by researchers owing to the direct sequencing of PCR products involving *Sarcocystis* spp. [4,25,26].

A phylogenetic analysis involving different *Sarcocystis* spp. placed *S. levinei* and *S. cruzi* into a monophyletic group. Certain sequences of *S. levinei* could not be distinguished from those of *S. cruzi*, either at the 18S or 28S rRNA genes [5]. However, the *cox 1* gene was found to be superior to the 18S and 28S rRNA genes with regard to the ability to unambiguously delimit the species [5]. Gjerde et al. [5] reported nucleotide identity *cox 1* sequences of *S. levinei* and *S. cruzi* to be around 92.9–94.0 %. *cox 1* is considered to be superior to the 18S rRNA gene for its ability to separate closely related (recently diverged) and morphologically indistinguishable *Sarcocystis* spp. in ruminants.

The government legislations on slaughter, owing to religious sentiments of the general public in India, limit the number of licensed slaughter houses for large ruminants. Hence, it is not possible to obtain a large amount of material to be collected and transferred to faraway areas for research proposes. Hence, the heart was chosen as the material of choice for the collection of specimens, as *S. levinei* is the only species reported in the cardiac tissue of buffaloes from India [12]. A total of 20 *S. levinei* sequences, including 11 (MG957189–MG957199) generated in the present study, were compared based on the 18S rRNA gene locus. Amongst the present Indian isolates, three haplotypes were noticed. The present Indian isolates showed 99.7–100.0% nucleotide identity, both within themselves and with other isolates available from Egypt. The first molecular characterization of *S. levinei*-like sarcocysts from water buffaloes was performed by Yang et al. [18], and it was confused, at that time, with *S. cruzi* of cattle due to the much higher identity based on the 18S gene. However, Gjerde et al. [5] clarified these differences by comparing multiple genes of both these species. A substantial amount of intra- and/or inter-isolate sequence variation in the 18S rRNA gene has been recorded in several *Sarcocystis* spp. specimens. This is often attributed to such variations and subsequently leads to confusions about the species involved. Thus, the 18S gene alone cannot be used for the genetic characterization of *Sarcocystis* spp. [5,16].

The *cox 1* gene is a promising genetic marker for the differentiation of various *Sarcocystis* spp. Species affecting ruminants [8,11]. This gene seems to be more suitable than the nuclear 18S rRNA gene for the separation of closely related species of *Sarcocystis* spp. Which affect a wide range of ruminants. Amongst the 11 sequences generated from the present study, three haplotypes were noticed. Again, the present isolates formed three clades on the phylogenetic tree, wherein two clades were closer to the sequences of *S. levinei* earlier reported from India (KX574315). However, the third clade was phylogenetically closer to the Egyptian isolates than their Indian counterparts. These sequences shared

99.7–100.0% and 98.8–100.0% nucleotide identity within themselves and with other isolates across the globe, respectively.

4. Materials and Methods

4.1. Sample Collection

Cardiac tissues were collected from buffaloes slaughtered at abattoirs of Mathura, Unnao, and Aligarh, alongside from dead animals brought for post mortem examination at the Department of Pathology, DUVASU, Mathura, India. Selection of the heart for collection of sarcocysts was based on the proven fact that only *S. levinei* has a preference for cardiac tissue, thereby minimizing the chances of picking up any kind of mixed infection. The respective cardiac tissue was observed under a microscope for the presence of sarcocysts following the standard muscle squash technique with methylene blue addition [12]. The sarcocysts were identified based on the morphological features reported in the literature [2,16]. Thereafter, the individual sarcocysts (one per animal) were stored at -20°C .

4.2. DNA Isolation, Primer Selection, and Cloning of 18S rRNA and Cox 1 Genes of *S. levinei*

DNA was isolated from individual sarcocysts using a commercial DNA isolation kit (Qiagen, Germany), following the manufacturer's protocol. The primers for the 18S rRNA and cox 1 genes of *Sarcocystis* spp. were custom-synthesized using the primer sequence described previously [19]. The PCR conditions for both the genes were followed as described previously [6,19]. The amplified products were visualized in ethidium bromide incorporated into 1.25% agarose gel. Water and known positive DNA of *S. tenella* (accession number: MH523439) were used as negative and positive templates (for both the genes, as the primers for both of them were genus-specific). Thereafter, PCR products were purified using gel purification and a DNA clean-up kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Subsequently, the purified products were cloned into CloneJET PCR Cloning Vector (Fermentas, Thermo Fisher Scientific). The recombinant plasmids were harvested (Plasmid purification kit, Fermentas, Thermo Fisher Scientific), and the purified plasmids were outsourced for bidirectional DNA sequencing to Invitrogen Bio Sciences Pvt. Ltd., Gurugram, India, using pJET1.2 universal primers (specific for the CloneJET cloning vector).

4.3. Sequence Analysis and Construction of Phylogenetic Trees

The sequences were submitted into NCBI, and the accession number corresponding to each sequence was obtained. Thereafter, the sequences were analyzed using Mega 6 and Gene tool software. The alignments of the DNA sequences were obtained with the Clustal W algorithm incorporated into the MEGA6 [27]. A gap opening penalty of 10 and gap extension penalties of 0.1 and 0.2 were used for the pairwise and multiple alignments, respectively. The phylogenetic relationship of *S. levinei* with other species of *Sarcocystis*, affecting different hosts, was commutated based on the available nucleotide sequences of the 18S rRNA and cox 1 genes in the NCBI (Figure 2a,b). The respective phylogenetic trees for various isolates of *S. levinei* were constructed using maximum likelihood (ML) (Figure 2a,b). Thereafter, the nucleotide identity between Indian sequences of *S. levinei*, alongside sequences across the world, was also calculated (Figure 3a,b). Phylogeny was tested at 1000 bootstrap replications. For 18S rRNA, the length of multiple alignment was 876 bp and the Tamura 3 parameter was chosen as the best nucleotide evolution model. For cox 1, the length of multiple alignment was 822 bp and the Kimura 2 parameter was chosen as the best nucleotide evolution model. *Sarcocystis hjorti* was used as the outgroup for both analyses. Furthermore, the parameters of intraspecific variation within both analyzed genes are presented in Table 1.

5. Conclusions

The present study is the first detailed report on the phylogenetic characterization of *S. levinei* from India. Before this study, the sequences of *S. levinei* were available only

from Egypt for comparison. Phylogenetics positioned *S. levinei* and *S. cruzi* into two monophyletic sister groups. An appreciable amount of genetic variability was noticed between various *S. levinei* sequences, both at the 18S and cox 1 gene loci. On the basis of the 18S gene, three haplotypes with 99.7–100.0% nucleotide identity were observed. Higher intraspecific variation of *S. levinei* was noticed within cox 1, as 10 haplotypes were identified and the sequence identity was 98.8–100%. The study generated some vital phylogenetic data on this erstwhile-understudied parasite.

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Data Availability Statement: The sequences generated in the present study are available at NCBI portal.

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Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations

18S rRNA	18S ribosomal RNA
28S rRNA	28S ribosomal RNA
ITS	Internal Transcribed Spacer
cox 1	mitochondrial cytochrome 1
DNA	Deoxyribose Nucleic Acid
PCR	Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis

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