Phylogenetic analysis and identification of *Sarcocystis* spp. found in rodents in Peninsular Malaysia

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Background: The tissue specimens used for extraction of DNA in this study were from rodents trapped in four states in Peninsular Malaysia, namely Kedah, Kelantan, Selangor and Johor.

Methods: Histological sections of these rodent muscle tissues stained with hematoxylin and eosin showed infection with *Sarcocystis* spp. Based on these results, the current study was carried out to determine the phylogenetic relationship among the identified *Sarcocystis* spp. in these rodents. The formalin fixed paraffin embedded (FFPE) rodent muscle blocks were subjected to DNA extraction and followed with semi nested PCR targeting 5' and 3' regions of 18S rRNA of *Sarcocystis* spp.

Results: Phylogenetic analysis showed two distinct groups of *Sarcocystis* spp. among the rodents in Peninsular Malaysia. Most of the identified *Sarcocystis* spp. were genetically closely related to *Sarcocystis rodentifelis* and *Sarcocystis muris* and were also observed to be genetically closely related to *Sarcocystis sp.* ex *Columba livia* and *Sarcocystis sp.* cyst type I ex *Anser albifrons*.

Conclusion: Further classification to confirm these *Sarcocystis* spp. was not possible as only partial sequences of 18S rRNA was available and this was insufficient for optimal differentiation.

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Introduction

Sarcocystis spp. are endoparasites that infect a wide range of hosts ranging from mammals, birds to reptiles. *Sarcocystis* spp. require two hosts to complete their life cycle: the definitive and intermediate hosts.[1] Sexual reproduction is only reported in a definitive host which is usually a predator and the asexual reproduction is normally observed in the intermediate host which is usually the prey. *Sarcocystis* spp. infection in humans is usually seen as muscular sarcocystosis, presenting with myalgia, erythematous subcutaneous nodules and fever.[2,3] In this context, humans are likely to be infected opportunistically when they come in contact with intermediate hosts such as rodents, cats and dogs. Rodents have been documented as intermediate hosts for *Sarcocystis singaporensis* and *Sarcocystis nesbitti*.[4] Snakes have been identified as the definitive host for *S. nesbitti*.[4] *Sarcocystis villivillosi*, *S. singaporensis* and *Sarcocystis zamani* have been demonstrated to have a life cycle between rodents and snakes and *Sarcocystis cymruensis* was reported for the first time in Thailand.[5] The definitive hosts for *Sarcocystis sulawesiensi* and *Sarcocystis murinotechis* have not been identified yet, however in this study they found 33% of the caught rats to be infected with *Sarcocystis*.[5] In China rodents were found to be both intermediate and definitive hosts for *S. cynruensis*.[6] In a study by Ambu and co-workers[7], a survey of wild and peri urban rodents was carried out to assess the prevalence of *Sarcocystis* spp. infections in four states in Peninsular Malaysia, namely Kedah, Kelantan, Selangor and Johor. A prevalence rate of 50% *Sarcocystis* spp. was observed in these rodents and this is higher than what was seen in Thailand.[5]

The current study attempted to identify some of the *Sacocystis* spp. that were found in the earlier study by Ambu and co-workers[7] by using the partial rRNA that was extracted from the formalin-fixed paraffin embedded tissue to plot the phylogenetic tree.

Materials and Methods

Formalin-fixed paraffin embedded tissue (FFPET) blocks that were identified as positive for *Sarcocystis* spp. infection using microscopy were used in this study.[7] DNA was extracted from the FFPET samples for the identification of the *Sarcocystis* spp. The DNA was extracted and purified using G-spin™ Total DNA Extraction Mini Kit (Intron Biotechnology, Korea) and the specific -targeted regions of 18S rRNA of the *Sarcocystis* spp. were amplified with semi nested

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PCR using *Pfu* polymerase (i-DNA Biotechnology, Malaysia) according to the manufacturer's protocols. The amplification products were purified and DNA sequencing carried out directly. Primers used for amplification and sequencing of the 18S rRNA are listed in Table 1. Partial nucleotides sequences were analysed and aligned with MEGA 5 prior to phylogenetic analysis. Bootstrapping of 1000 replicates was included in the phylogenetic analysis. Partial 18S rRNAs of the identified *Sarcocystis* spp. were used to plot the phylogenetic tree against known *Sarcocystis* spp. deposited in the GenBank.

Table 1: Primers used for semi-nested PCR amplification of 18S rRNA.

Primer*	Sequence (5'-3')	Amplicon size (bp)	References
A (Forward)	AACCTGGTTGATCCTGCCAGT	963	Medlin *et al.*, 1988[8]
S7 (Forward)	GTAATTCCAGCTCCAATAGCG	380	Fischer and Odening, 1998[9]
3H (Reverse)	GGCAAATGCTTTCGCAGTAG		Yang *et al.*, 2001[10]
S3 (Forward)	TTGTTAAAGACGAACTACTGCG		Fischer and Odening, 1998[9]
S4 (Reverse)	TATCCCCATCACGATGCATAC	629	Fischer and Odening, 1998[9]
B (Reverse)	GATCCTTCTGCAGGTTCACCTAC	875	Medlin *et al.*, 1988[8]

* PCR amplification of 18S by primers set: A, S7 and 3H, generate PCR product located upstream from product amplification by primers set: S3, S4 and B.

Results

In the current study, the amplicon products were 380 bp and 629 bp that targeted the different positions of 18S rRNA. The full length sequence of 18S rRNA could not be amplified in this study. This may be due to the formalin fixation of the tissue and the process involved in de-paraffinising them for the study. The processing could have affected the integrity and intactness of the DNA in the tissue. There were 73 FFPET samples which were identified positive for *Sacrocycstis* spp. with H&E stain. These 73 samples were used for DNA extraction and semi- nested PCR. The PCR amplicons were successfully detected in 24 DNA-extracted FFPE samples. The detection rate by PCR was 32.9% (24/73).

The phylogenetic tree showed two distinct groups of *Sarcocystis* spp. among the rodents in Malaysia. Most of the identified *Sarcocystis* spp. were genetically and closely related to *Sarcocystis rodentifelis* and *Sarcocystis muris* (labeled I in Figures 1 and 2) which indicate rodents as the intermediate hosts. Further classification to confirm these *Sarcocystis* spp. by the phylogenetic tree into either *Sarcocystis rodentifelis* or *Sarcocystis muris* was not possible as only partial sequences of 18S rRNA was obtained from the FFPET samples. For optimal differentiation in molecular taxonomy and classification of the full length of 18S, D2 and D3 regions of 28S and internal transcript spacer-1 between 18S and 28S rRNA sequence will be required.

The remaining *Sarcocystis* spp. in the FFPET samples were also found to be genetically and closely related to *Sarcocystis columbae* and *Sarcocystis* spp. ex *Accipiter nisus* (labeled II in Figures 1 and 2). These two distinct groups were consistently observed among phylogenetic trees plotted with partials 18S rRNA sequences (Figures 1 and 2, respectively). Neighbor-Joining tree was constructed with 1000 replicates of bootstrapping with MEGA5. The accession codes were listed on the left of each reference species of *Sarcocystis sp*. The sample number and place were listed on the left of each *Sarcocystis sp*. identified in rats. Most of the identified *Sarcocystis sp*. were genetically closely related to *Sarcocystis rodentifelis* and *Sarcocystis muris* (labeled I). The remaining identified *Sarcocystis sp*. were genetically closely related to *Sarcocystis columbae* and *Sarcocystis sp*. ex *Accipiter nisus* (labeled II).

Figure 1: Phylogenetic tree of partial 18S rRNA (629 bps) among *Sarcocystis* spp. in Malaysia

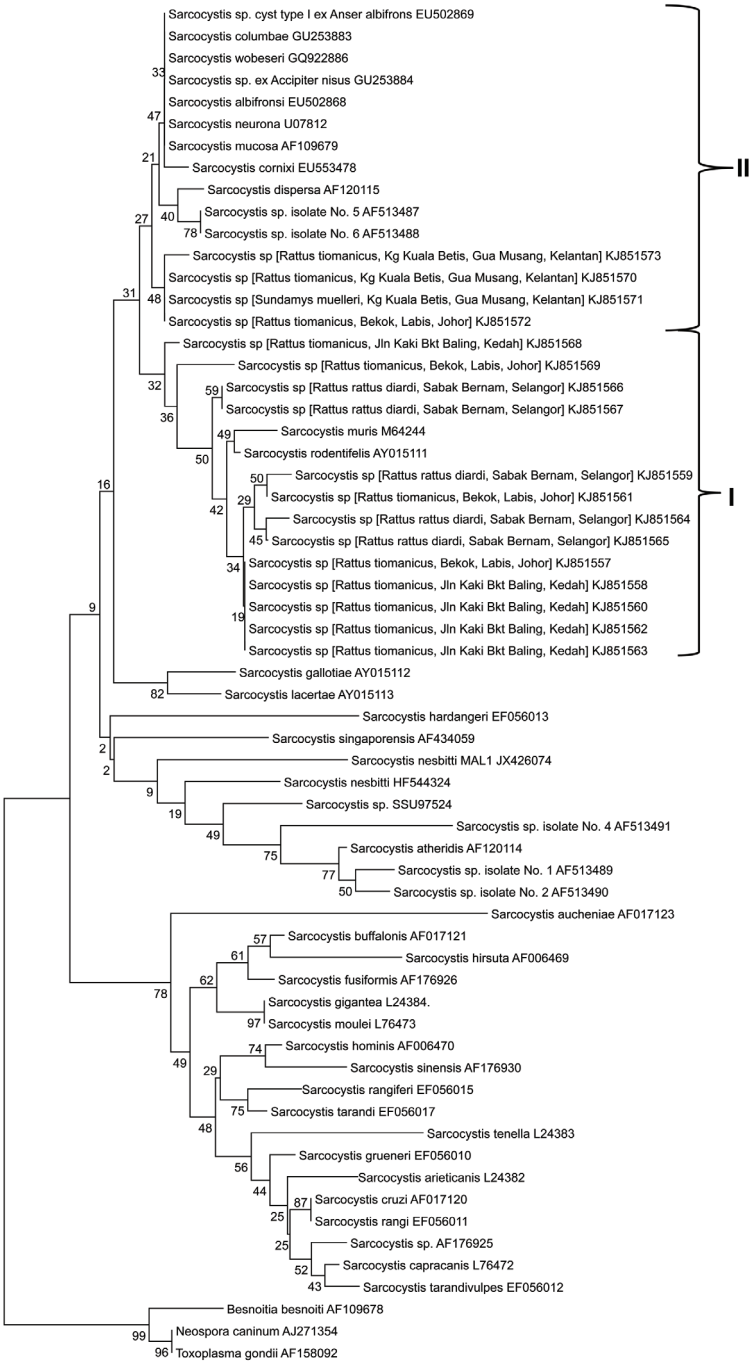
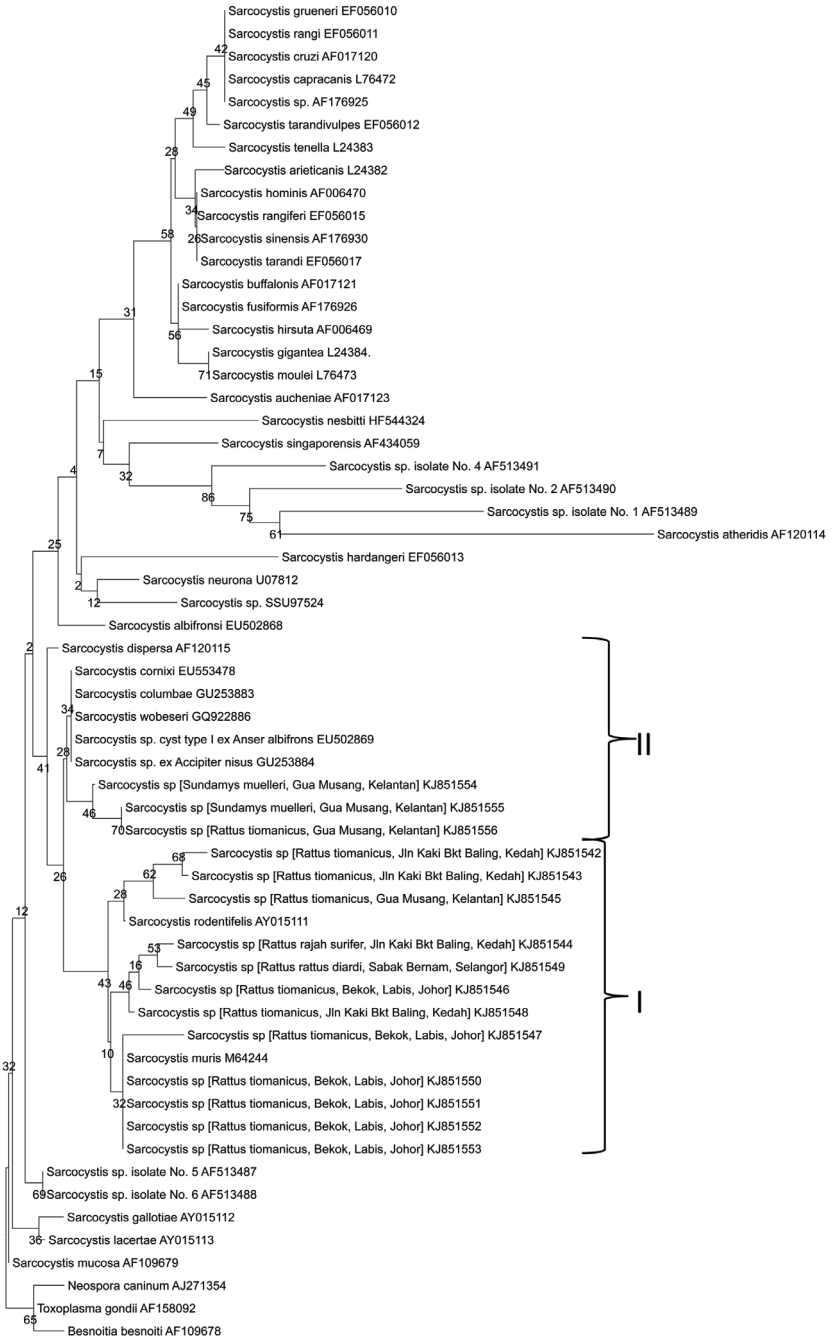


Figure 2: Phylogenetic tree of partial 18S rRNA (380 bps) among *Sarcocystis* spp. in Malaysia



Discussion

Different *Sarcocystis* spp. such as *Sarcocystis singaporensis*, *Sarcocystis villivilliso*, *S. zamani*, *S. sulawesiensi*, *S. murinotechis* and *S. cymruensis* had been detected and identified by microscopy in rodents in China, Thailand, Indonesia and Australia but they were not seen in this study[5,6,11-14]. In China the sarcocysts of *S. cymruensis* retrieved from *Rattus* spp. of wild rats when fed to laboratory rats showed evidence that they can be both intermediate and definitive hosts.[6] In Malaysia a study of faecal specimens of different species of snakes when phylogenetically analysed showed that they were infected with *S. nesbitti*, *S. singaporensis*, *S. Zuoi* and one other unidentified species.[4] Using laboratory rats to elucidate the life cycle of *S. Zuoi*, Hu *et al.* found the King rat snake (*Elaphe carinata*) to be the definitive host.[16] In 1975 Zaman and Colley found *Python reticulatus* to be the definitive host for *Sarcocystis orientalis* in their experimental infections.[17] The discovery of *Sarcocystis* spp. in rodents which are genetically similar to *Sarcocystis columbae* and *Sarcocystis* sp. ex *Accipiter nisus* gives rise to the possibility that rodents are the intermediate host for this species and that it can be transmitted to the definitive hosts such as predatory birds (Goshawk and Sparrowhawk). A laboratory study has shown the successful transmission of *Sarcocystis* infection from an infected Goshawk to mice. The infected mice developed muscle sarcocystosis with the sarcocysts measuring 15-630 X 18-65 µm in length and width, respectively.[15] This finding shows that the measurements of the cysts observed by Ambu and co-researchers in the rat muscles ranging from 139-250 X 50-65 µm[7] are much smaller and belong to a different species. Olias *et al.*[18] found a new highly pathogenic species in the domestic pigeon and the Northern goshawk and identified it as *Sarcocystis calchasi* sp.nov. Further analysis by PCR showed that there were distinct species in the domestic pigeon and the goshawk, *S. calchasi* and another new unidentified *Sarcocystis* species.[19]

In the current study most of the identified *Sarcocystis sp.* were genetically closely related to *S. rodentifelis* and *S. muris*. However there were also other *Sarcocystis* spp. that were genetically closely related to *S. columbae* and *Sarcocystis sp.* ex *Accipiter nisus*.

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