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Research note

Phylogenetic analysis of *Sarcocystis* spp. of mammals and reptiles supports the coevolution of *Sarcocystis* spp. with their final hosts☆

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Abstract

Sequences of the small subunit rRNA genes were obtained for two coccidians, *Sarcocystis dispersa* and an unnamed *Sarcocystis* sp. which parasitise the European barn owl and an African viperid snake as their final host, respectively, and share mouse as their intermediate host. Phylogenetic analysis of the sequence data showed that *Sarcocystis* sp. from the viperid snake is most closely related to another *Sarcocystis* sp. isolated from an American crotalid snake, while *S. dispersa* grouped with other bird-transmitted species. The available dataset failed to resolve the evolutionary relationships among four major branches into which all Sarcocystidae and *Isospora* spp. were split. However, within these branches, the phylogenetic relationships of the majority of analysed members of the genus *Sarcocystis* reflected coevolution with their final, rather than intermediate hosts. © 1999 Australian Society for Parasitology. Published by Elsevier Science Ltd. All rights reserved.

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The family Sarcocystidae contains cyst-forming coccidia which parasitise a wide variety of vertebrates, including humans. The heteroxenous genus *Sarcocystis* is the most numerous of six genera belonging to the family [1]. It represents an attractive group for those who are interested in coevolution of apicomplexan parasites with their hosts. Since ssrRNA sequences are available for a growing number of cyst-forming coccidia, it is becoming possible to improve and refine the current taxonomic system which is based mainly on phenotypic characters, such as morphology of sporocysts, ultrastructural features of the developmental stages and host–parasite

☆ Note: The nucleotide sequences of *Sarcocystis dispersa* and *Sarcocystis* sp. have been deposited in the GenBank™ under the accession numbers AF120115 and AF120114, respectively.

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relationships [1]. The analysis of molecular data can provide information about the phylogenetic relationships of individual *Sarcocystis* spp. Since members of the genus *Sarcocystis* are known to be widespread and sometimes highly pathogenic for animals that serve as their intermediate hosts, understanding the phylogenetic relationships with their final and/or intermediate hosts will have both theoretical and practical implications.

On the basis of an evaluation of multiple biological and morphological characters for members of the major groups of Sporozoea, Barta [2] postulated that in the life-cycle of a heteroxenous coccidium the final host is an ancestral feature, while the intermediate host is a derived one. So far, this postulate has been supported by ssrRNA analysis of *Sarcocystis* spp. transmitted by felids and canids that form two monophyletic groups within the genus [3]. However, an alternative scenario was suggested, according to which the final host has been incorporated either primarily (e.g., *Toxoplasma*) or secondarily (e.g., *Sarcocystis*) into the life-cycle of cyst-forming coccidia [4]. To examine the problem of host–parasite coevolution we have chosen two *Sarcocystis* spp. which share their intermediate host but have a different final host and originate from geographically distant localities.

Sarcocystis sp. is a newly discovered species with a snake–rodent life-cycle and with typical morphological features of the genus. The sporocysts were isolated from faeces of a naturally infected Nitsche's bush viper, *Atheris nitschei* (Serpentes: Viperidae), captured in August 1996 in the vicinity of Kilembe, Ruwenzori Mountains, Uganda (0°14′N, 30°03′E). In the transmission experiments, laboratory mice were found to be susceptible intermediate hosts and developed sarcocysts that were infective for *A. nitschei*. Sporocysts of *S. dispersa*, a *Sarcocystis* sp. of owls which occurs at least in Europe [5] and Australia [6], were obtained from the contents of the intestine of an adult barn owl, *Tyto alba* (Strigiformes) found dead in April 1994 in Mikulov, Southern Moravia, Czech Republic.

The sporocysts of both species were purified by the flotation procedure in Sheather's sugar solution. Approximately 10^3 sporocysts were used to perorally infect outbred ICR laboratory mice, the skeletal muscles of which were found to be infected with cysts after 2 months. Cystozoites of both species were purified from the muscles by tryptic digestion [7], washed repeatedly by resuspension in PBS, and centrifuged at 2500 *g* for 15 min. Total DNA was isolated by a method described elsewhere [8]. Polymerase chain reaction amplifications with oligonucleotides JV1 and JV2 were carried out in reaction mixtures as described earlier [9]. The 35 cycles were 94°C for 1 min, 42°C for 1.5 min, and 74°C for 2 min, with a final extension step of 65°C for 15 min to enhance the addition of A-overhangs. Polymerase chain reaction products of the expected size were isolated from agarose gels, cloned into the pCR vector (Invitrogen), and transformed into *Escherichia coli* XL-1 competent cells (Stratagene). Both strands were sequenced using the Sequenase version 2.0 kit (Amersham). The set of internal primers used was as published previously [9].

The amplified regions of the ssrRNA genes of *Sarcocystis* sp. and *S. dispersa* used for the phylogenetic analysis were 1579-nt and 1540-nt long, respectively. The ssrRNA gene sequences of all cyst-forming coccidia, and those of *Isospora suis*, *Isospora belli*, *Eimeria nieschulzi*, *Cryptosporidium muris*, and *Babesia microti* were included in the analysis. Since an unambiguous alignment is a critical prerequisite for a reliable phylogenetic analysis, we first evaluated the sensitivity of the analysed data to parameters of the alignment process, namely the gap penalties for both pairwise and multiple sequence alignments. To accomplish this, five alignments (primary alignments) were generated by the Clustal algorithm (Windows 32, MegAlign 3.1.7), each by a different set of parameters. Since the primary alignments varied in length (1688–1693 nt) and provided different parsimonious trees, we decided to exclude ambiguously aligned nucleotides from the analysis by Wheeler's "cull" method (W Wheeler, D Gladstein. Malign Version 1.99, 1994). As a result, we obtained a 1310-nt long alignment (amalgamated alignment) which contained only the nucleotides aligned identically in all primary alignments.

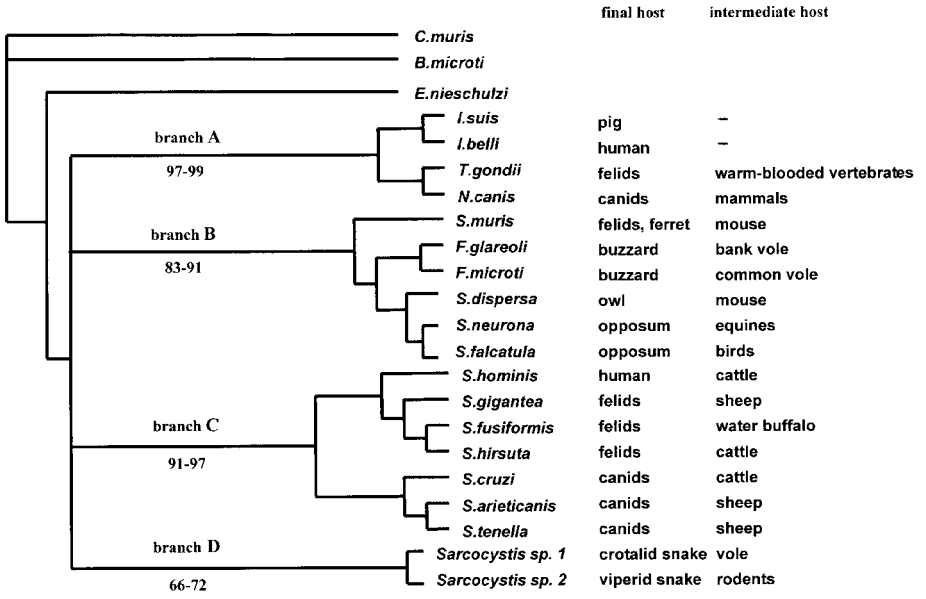

Fig. 1. Consensus tree with plotted final and intermediate hosts. Numbers below each line show the range of bootstrap support in individual primary alignments.

To analyse the crown regions, two additional alignments (group alignments) containing only members of the "B" and "C" branches (see below), respectively, were designed in the same way as described for the entire dataset. All alignments were analysed by the program package PAUP, version 3.1 (DL Swofford. PAUP: phylogenetic analysis using parsimony, version 3.1. Champaign, IL, 1993), with gaps treated as missing data and the Ts/Tv ratio set to 3:1. The branch swapping method equipped with the TBR algorithm was used to search for the most parsimonious tree with alignments containing all taxa. Five-hundred replications by the same algorithm were generated to determine bootstrap values. An exhaustive search was performed with the group alignments.

A strict consensus of the trees obtained by maximum parsimony analysis of the primary alignments revealed a polytomy with four stable groups, which we termed as the "A–D" branches (Fig. 1). The available ssrRNA dataset obviously failed to resolve relationships of these branches, since the same polytomy was obtained by analysis of the amalgamated alignment.

The composition of the "A" branch confirmed the recently described close relationship of heteroxenous genera *Toxoplasma* and *Neospora* with homoxenous *Isospora* spp. [10]. The lack of stability of this branch in the ssrRNA-based trees [11–13] appears to be one of the major sources of polytomy generated by our dataset. The "B" branch embraced a diverse group of species transmitted either by mammals or by birds. The position of *Sarcocystis muris*, previously reported as unstable [9, 11], was resolved by the addition of more species into the analysis and branched invariantly at the base of the "B" branch. *Sarcocystis neurona* has a high homology with *Sarcocystis falcatula*, *S. dispersa* and *Frenkelia* spp. , all the latter species being transmitted by birds. The "C" branch contained *Sarcocystis* spp. which have humans, felids and canids as final hosts. Two *Sarcocystis* spp. circulating between snakes and rodents formed the "D" branch.

In order to demonstrate any correlation between host specificities and phylogenetic positions of the analysed coccidia, we plotted their intermediate and final hosts (Fig. 1). In two out of three *Sarcocystis*-containing branches, the re-

lationships of *Sarcocystis* spp. reflect those of final rather than intermediate hosts. In accordance with previous studies [3], within the "C" branch the pathogenic species transmitted by canids form a monophyletic group to the exclusion of the non-pathogenic felid-transmitted species. Although isolated on different continents from distantly related crotalid and viperid snakes, both snake-transmitted species form a monophyletic branch.

A diverse assembly of species was brought together in the "B" branch. Birds as a final host is a common feature of *S. dispersa* and *Frenkelia* spp., while in the life-cycle of *S. falcatula* birds can serve as intermediate hosts [14] The unusual life-cycle of *S. neurona* is not yet fully understood, but most likely does not include birds [14, 15]. In our trees, relationships of *S. muris* and *Sarcocystis hominis* with other coccidia do not correlate with the phylogenetic position of their final and/or intermediate hosts. Both species constitute early offshoots of the particular branches, which indicates their less stable position in the ssrRNA trees.

Although our results do not provide clear-cut evidence for coevolution of *Sarcocystis* spp. with their hosts, the relationships with final hosts seem to be strong in several cases. Most likely, the evolutionary history of cyst-forming coccidia followed a complex pattern which can perhaps be better understood by analysis of other conserved genes and inclusion of more *Sarcocystis* spp. from different taxonomic groups of hosts and from geographically distant localities.

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