The relationship of *Hammondia hammondi* and *Sarcocystis mucosa* to other heteroxenous cyst-forming coccidia as inferred by phylogenetic analysis of the 18S SSU ribosomal DNA sequence

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SUMMARY

The complete sequence of the 18S small subunit (SSU) ribosomal DNA of *Hammondia hammondi* and *Sarcocystis mucosa* was obtained and compared to SSU rDNA sequences of *Neospora caninum*, *Toxoplasma gondii*, *Besnoitia besnoiti*, 2 species of *Frenkelia*, 3 species of *Isospora*, and 13 species of *Sarcocystis*. Analyses showed that *H. hammondi* and *T. gondii* are monophyletic and that these taxa shared a common ancestor with *N. caninum* and *B. besnoiti*. The weight of evidence shows that *S. mucosa*, *S. neurona*, and *Frenkelia* species form a clade thereby supporting the conclusion that *Sarcocystis* is paraphyletic.

Key words: *Hammondia*, Apicomplexa, phylogenetic analysis.

INTRODUCTION

Due to the paucity of structural features to differentiate among apicomplexan parasites, the sequence of genes encoding slowly evolving ribosomal RNA has been utilized for phylogenetic reconstructions (Johnson *et al*. 1987, 1991; Barta, Jenkins & Danforth, 1991; Gajadhar *et al*. 1991; Gagnon *et al*. 1993; Fenger *et al*. 1994; Holmdahl *et al*. 1994; Ellis *et al*. 1995; Relman *et al*. 1996). Previous studies using SSU rDNA from a wide range of apicomplexan parasites showed that cyst-forming heteroxenous coccidia formed a monophyletic group separate from the homoxenous coccidia, such as *Eimeria* and *Cryptosporidium*. Phylogenetic reconstructions based on alignment of SSU rDNA sequences showed that *T. gondii* and *N. caninum* formed a monophyletic clade distinct from pathogenic and non-pathogenic *Sarcocystis* spp. (Ellis *et al*. 1994; Holmdahl *et al*. 1994; Escalante & Ayala, 1995; Ellis & Morrison, 1995; Jeffries *et al*. 1997). The purpose of this study was to examine 2 hypotheses regarding (1) the relationship of *H. hammondi* to other cyst-forming coccidia and (2) the monophyly of the genus *Sarcocystis*.

H. hammondi is a coccidian parasite that has an obligatory two-host life-cycle (Frenkel & Dubey, 1975; Dubey, 1993). The phylogenetic relationship of this parasite to other cyst-forming coccidia is unknown, but it is thought to be most closely related to *T. gondii* (Frenkel & Dubey, 1975; Sheffield, Melton & Neva, 1976; Melhorn & Frenkel, 1980; Araujo, Dubey & Remington, 1984; Riahi *et al*. 1995, 1998). For instance, both *H. hammondi* and *T. gondii* oocysts are shed by cats after ingestion of tissue cysts present in intermediate hosts such as rodents. In contrast to *Sarcocystis*, metrocytes are not formed in *H. hammondi* nor in *T. gondii*, but bradyzoites within cysts arise from endodyogeny. Sporogony in both *H. hammondi* and *T. gondii* occurs outside the definitive host in contrast to endogenous sporogony observed in pathogenic and non-pathogenic *Sarcocystis* spp. (see Tenter & Johnson, 1997). However, *H. hammondi* and *Sarcocystis* spp. of felids (*S. muris* and *S. gigantea*) share several features that are absent in *T. gondii*. For example, *H. hammondi*, *S. muris*, and *S. gigantea* oocysts are not infectious for cats, only capable of infecting an intermediate host. To determine

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whether *H. hammondi* and *T. gondii* are monophyletic, SSU rDNA sequences from these and related coccidians were compared.

Evidence provided elsewhere has indicated that *Sarcocystis* may be paraphyletic because of the monophyly of some *Sarcocystis* species with *Frenkelia* (Votypka *et al*. 1998). Our research has provided extensive evidence to suggest that *Sarcocystis* is probably composed of at least 3 groups (Holmdahl *et al*. 1999), 1 of which may correspond to the clade sharing a common ancestor with *Frenkelia*. It has also been pointed out that a characteristic of this group is that they have non-ruminants as their intermediate host (Holmdahl *et al*. 1999). *Sarcocystis mucosa* exists as macroscopic sarocysts in the gastrointestinal tract of macropodid marsupials (O'Donoghue *et al*. 1987). It has been suggested that dasyurid marsupials may act as the definitive host in the life-cycle of this organism (Jakes, 1998). Addition of SSU rDNA data from this taxon, may help to support or refute the hypothesis of whether the taxa possessing non-ruminants as intermediate hosts will form a monophyletic group.

MATERIALS AND METHODS

Preparation of H. hammondi *DNA and amplification of 18S rDNA*

H. hammondi oocysts were obtained from faeces of experimental cats fed tissue cysts of mice infected with the H.H-24 strain (Riahi *et al*. 1995). The oocysts were isolated by sucrose gradient centrifugation, sporulated in 2% H_2SO_4 for 2 weeks at room temperature, and then stored at 4 °C. *H. hammondi* oocysts were treated with 20% sodium hypochlorite for 10 min to destroy contaminating microorganisms, washed several times with distilled H_2O, centrifuged and resuspended in sterile H_2O. The oocyst suspension was pipetted dropwise into liquid nitrogen and ground to a fine powder in a sterile mortar and pestle. The extracted oocysts were resuspended in 0·2 M Tris, pH 8·0, 0·1 M EDTA, 0·4 M NaCl containing 1 mg/ml proteinase K and 0·1% SDS and then incubated for 16 h at 50 °C. The DNA suspension was extracted with phenol, phenol–chloroform, and chloroform, precipitated with ethanol, pelleted by centrifugation, and resuspended in sterile 10 mM Tris, pH 8·0, 1 mM EDTA and stored at −20 °C. The SSU rDNA from *H. hammondi* was amplified by PCR using conserved primers as described (Medlin *et al*. 1988) and cloned into the pCRII vector (Invitrogen, San Diego, CA) using techniques provided by the manufacturer. At least 3 clones from 2 different PCR amplifications were subjected to DNA sequencing analysis. Sequencing was performed on plasmid DNA using ^{35}S-dATP and the dideoxy chain termination Sequenase kit (U.S. Biochemical, Cleveland, OH). Primers directed to conserved regions of the SSU rDNA and to *H. hammondi*-specific sequences were employed. The DNA sequence was confirmed by at least 3 separate reactions on both DNA strands.

Preparation of S. mucosa *DNA and amplification of 18S rDNA*

Macroscopic gastrointestinal sarcocysts were obtained from roadkills of Tasmanian Bennetts wallabies (*Macropus rufogriseus*) (O'Donoghue *et al*. 1987; Jakes, 1998). The cysts were lysed in DNA extraction buffer containing 1% SDS, 10 mM Tris, pH 9·0, 100 mM EDTA containing proteinase K (100 μg/ml) at 65 °C for at least 2 h. The released DNA was purified by phenol/chloroform extraction and desalted by ethanol precipitation. The DNA was resuspended in 10 mM Tris, pH 8·0, 0·1 mM EDTA and used for PCR. The SSU rDNA was amplified by universal primers in 4 overlapping fragments (as described by Holmdahl *et al*. 1999) and the PCR products were sequenced directly by cycle sequencing with the aid of an ABI automated sequencer. A consensus sequence for the SSU rDNA was generated from at least 3 sequencing runs from each primer.

Alignment of SSU rDNA sequences and phylogenetic reconstruction

The SSU rDNA sequences of *H. hammondi* (GenBank accession number AF096498) and *S. mucosa* were aligned by clustal W (with default parameters) to SSU rDNA sequences of *T. gondii*, *N. caninum*, *B. besnoiti* (AF109678), *I. felis* (U85705), *I. belli* (U94787), *I. suis* (LSU97523), *S. neurona* (U07812), *S. muris* (M64244), *F. glareoli* (AF009245), *F. microti* (AF009244), *S.* sp. (SSU97524), *S. capracanis* (AF012885), *S. tenella* (L19615), *S. cruzi* (AF017120), *S. arieticanis* (L24382), *S. gigantea* (L24384), *S. moulei* (AF012884), *S. buffalonis* (AF01712), *S. hirsuta* (AF017122), *S. fusiformis* (SFU03071), *S. hominis* (AF006470), *S. aucheniae* (AF017123), *Cyclospora* sp. (U40261), *E. tenella* (U40264), *E. nieschulzi* (U40263) and *E. bovis* (U40264). The alignment was imported into MacClade 3.04 and minor modifications made to the alignment in order to correct clearly ambiguously placed nucleotides.

Consensus SSU rDNA sequences were used for both *T. gondii* and *N. caninum* and were derived from all of the sequence entries available in GenBank for these taxa. The *T. gondii* (or *N. caninum*) sequences were aligned by clustal W and a majority rule procedure was used to produce a consensus by eye using MacClade where different or ambiguous character states were identified. The base found in the majority of the aligned sequences was chosen for the consensus sequence. The phylogenetic relationships between the aligned coccidial

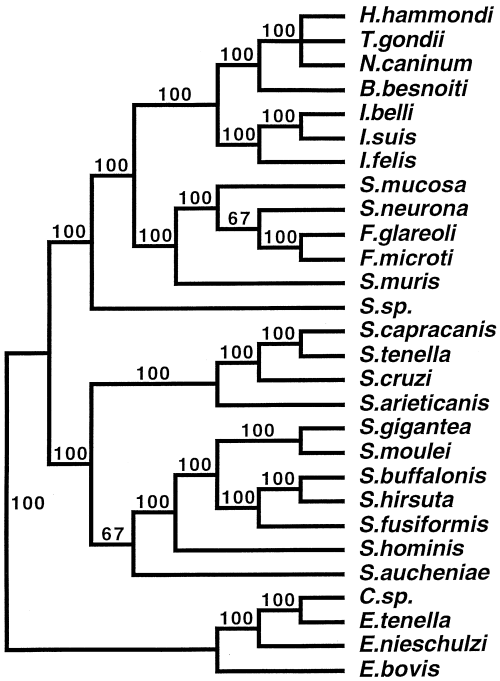

Fig. 1. Phylogenetic relationships among the Sarcocystidae by analysis of SSU rDNA using parsimony. A majority rule consensus tree is shown and the numbers on the branches represent the percentage support for that branch in the 6 most parsimonious trees found.

sequences were reconstructed using distance, maximum likelihood, and parsimony methods. Distance analysis was performed by neighbour-joining using the Kimura 2 parameter model in Treecon (Van de Peer & De Wachter, 1994). Bootstrap analysis was performed as follows – 200 new datasets were randomly generated using eseqboot (Felsenstein, 1985, 1988) on the Australian National Genome Information Service (ANGIS) (Gaeta & Balding, 1997) and converted into a distance matrix using dnadist and the Kimura-2 parameter model. Ednaneighbour was used to generate the trees for the 200 matrices. The program consense was used to generate a majority rule consensus tree (Fig. 1). Maximum likelihood was performed using the ednaml program on ANGIS. Global rearrangements plus 5 random starts were used. Parsimony analysis involved the heuristic search procedure in PAUP 3.1.1. Tree bisection-reconnection plus 10 random starts were used.

RESULTS

A SSU rDNA sequence alignment generated by clustal W containing 28 taxa and 2040 characters was analysed phylogenetically. Parsimony analysis using the heuristic search option yielded 6 most parsimonious trees of 1101 steps (consistency index 0·699; homplasy index 0·301). These trees differed in the relationships between *H. hammondi*, *T. gondii* and *N. caninum*; the relationship of *S. mucosa* and *S. neurona* to *Frenkelia* and the relationship of *S. aucheniae* to the clades containing *S. tenella* and *S. gigantea*. These relationships are best summarized in the form of a majority rule consensus tree (Fig. 1) where the *H. hammondi*, *T. gondii* and *N. caninum* can be seen as a polytomy since the relationships between them are not resolved. *Besnoitia*, as discussed elsewhere is the sister group to these taxa (J. T. Ellis, unpublished observations). In this tree, *S. muris*, *S. mucosa*, *S. neurona* and *Frenkelia* form a monophyletic group that is the sister group to *Isospora*, *B. besnoiti*, *N. caninum*, *H. hammondi* and *T. gondii*, which for convenience we define here as the Toxoplasmatinae. *S. sp.* (from a rattlesnake) is the sister to this combined group.

A distance analysis of the aligned SSU rDNA sequences from the 28 species of coccidia is shown in Table 1. There are few nucleotide differences between the sequences of *T. gondii*, *N. caninum* and *H. hammondi* thus explaining the polytomy observed above. Alignments based on secondary structure showed that SSU-rDNA encoding helical regions of *H. hammondi* and *T. gondii* rRNA were identical and differed from *N. caninum* SSU rDNA by only 3 nucleotides (not shown). In contrast, approximately 100 nucleotide differences were detected between *Sarcocystis* and *H. hammondi*, and approximately 200 base differences between *H. hammondi* and *Eimeria*. Since Ellis & Morrison (1995) and Morrison & Ellis (1997) demonstrated that the strongest phylogenetic signal is located in the helical domains of the SSU rDNA, we conclude that the finding of nucleotide differences between *N. caninum* and *T. gondii*/*H. hammondi* in the helices is support for the conclusion that *H. hammondi* is the sister taxon to *T. gondii*. This observation is consistent with results presented elsewhere using ITS1 and partial LSU rDNA comparisons (Ellis *et al*. 1999) which suggested that *H. hammondi* and *T. gondii* may be monophyletic.

Distance analysis of the data set using neighbour-joining gave a tree (Fig. 2) showing similar relationships to that described by parsimony analysis (Fig. 1). Because of the size of the data set, bootstrap analysis was performed by distance methods. Bootstrapping demonstrated strong support (200/200) for the monophyly of *Sarcocystis*, *Frenkelia*, *Isospora*, *B. besnoiti*, *N. caninum*, *T. gondii* and *H. hammondi*. Strong support (196/200) was also found for the clades of (1) *Sarcocystis* having ruminants as intermediate hosts as observed by others (Holmdahl *et al*. 1999) and (2) the Toxoplasmatinae, thus confirming previous findings (Carreno *et al*. 1998; J. T. Ellis, unpublished observations). Of interest is that *S. mucosa* and *S. neurona* were monophyletic and this conclusion is robust since 153 of 200 bootstraps support this node. This tree differed from parsimony analysis in the position of *S. hominis*, *S. sp.* and also *S. aucheniae*.

Table 1. Nucleotide sequence differences between helical regions of SSU rDNA from the Apicomplexa determined by distance analysis of alignments generated using clustal W

	Hh	Tg	Nc	Bb	Ib	Is	If	Sm	Sn	Fg	Fm	Sm	Ss	Sc	St	Sc	Sa	Sg	Sm	Sh	Sb	Sh	Sf	Sa	Cs	Et	Eb
H. hammondi																											
T. gondii	3																										
N. caninum	4	3																									
B. besnoiti	22	24	21																								
I. belli	32	32	31	37																							
I. suis	41	44	41	51	15																						
I. felis	36	36	38	43	21	25																					
S. mucosa	58	65	57	65	65	80	74																				
S. neurona	65	63	64	66	68	77	74	16																			
F. glareoli	51	48	50	49	55	61	61	17	17																		
F. microti	57	54	56	55	59	66	63	19	19	17																	
S. muris	69	69	68	70	72	82	77	36	34	33	35																
S. sp.	85	87	85	91	88	95	96	77	60	57	61	75															
S. capracanis	115	111	111	114	116	123	121	99	98	89	92	101	129														
S. tenella	106	102	105	103	103	109	111	89	88	78	83	90	107	20													
S. cruzi	107	103	105	105	106	113	113	84	83	77	78	87	119	45	31												
S. arieticanis	119	114	118	116	114	120	120	100	99	90	93	103	132	58	47	56											
S. gigantea	105	104	106	106	116	124	122	88	91	90	91	94	121	111	97	96	113										
S. moulei	109	107	109	110	118	126	126	92	94	93	94	96	127	109	97	95	111	16									
S. hominis	98	95	96	95	98	102	103	82	82	85	83	80	114	75	64	67	77	69	74								
S. buffalonis	116	116	115	115	116	136	125	100	97	97	97	100	145	99	85	86	106	70	67	78							
S. hirsuta	115	112	112	113	117	127	126	98	100	98	100	102	138	104	89	91	111	76	73	82	7						
S. fusiformis	119	117	119	119	122	131	130	97	100	98	98	104	136	102	93	90	110	74	71	76	33	37					
S. aucheniae	128	125	126	122	124	137	138	111	116	103	105	118	154	124	104	106	116	124	121	100	121	125	121				
C. sp.	210	209	207	216	205	211	202	207	203	166	174	209	210	217	205	214	209	229	233	175	232	229	234	231			
E. tenella	211	210	207	214	207	214	203	207	203	169	176	211	218	217	208	215	212	228	231	180	234	231	230	236	53		
E. bovis	200	197	197	201	201	205	197	193	193	155	162	200	208	208	197	208	208	215	216	167	219	216	222	227	63	70	
E. nieschulzi	210	210	207	213	208	216	204	206	202	161	169	203	223	211	203	211	210	219	223	176	225	222	226	229	64	68	60

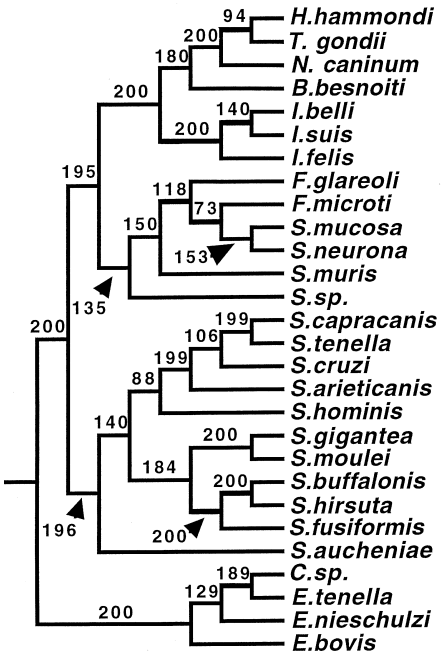

Fig. 2. Phylogenetic relationships among the Sarcocystidae inferred by neighbour-joining and the Kimura 2 parameter model. The bootstrap values represent the support (out of 200) for that branch.

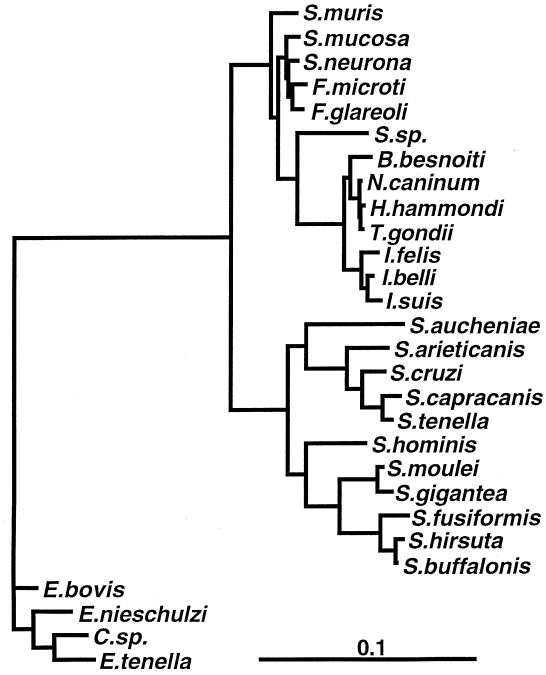

Fig. 3. Phylogenetic relationships among the Sarcocystidae inferred by maximum likelihood analysis. The branch lengths are proportional to the amount of inferred evolutionary change, as shown by the scale bar.

The tree obtained using maximum likelihood (Fig. 3) had a log likelihood of −9218·9. This tree is similar to the consensus majority rule tree obtained from parsimony analysis (Fig. 1). *H. hammondi* is the sister taxon to *T. gondii*. The positions of *S. sp.*, *S. muris* and *S. aucheniae* on the tree are different. *S. sp.* is the sister to the Toxoplasmatinae; *S. muris* is the sister to Toxoplasmatinae and the clade containing the *F. microti*+*F. glareoli* *S. mucosa*+*S. neurona*. The likelihood-ratio test (Felsenstein, 1988) was performed in order to test whether the maximum likelihood tree was any better or worse than the other trees found in these analyses. It indicates that none of these trees are statistically significantly different from each other ($P > 0{\cdot}05$).

DISCUSSION

In this study we set out to examine 2 hypotheses, the relationship between *H. hammondi* and *T. gondii*, and whether *Sarcocystis* species having non-ruminants as an intermediate host form a monophyletic group. Frenkel (1977) divided the Sarcocystidae into 2 subfamilies, Toxoplasmatinae and Sarocystinae. Recent phylogenetic analyses have provided considerable support for this division (J. T. Ellis, unpublished observations). In addition, Eimeridae is considered to be the sister group to the Sarcocystidae. Therefore the approach used in these analyses was the alignment of sequences of the Sarcocystidae to 3 representative sequences of *Eimeria* plus *Cyclospora* (Relman *et al*. 1996).

Using this approach, phylogenetic analyses by distance, maximum likelihood, and parsimony methods gave trees possessing consistent relationships among the taxa. Specifically, the branch leading to *T. gondii*+*H. hammondi*+*N. caninum* is robust, although the 3 taxa yield a polytomy since the amount of variation between the 3 sequences is very small. Our recent analyses of partial LSU rDNA and ITS1 data indicated that *T. gondi* and *H. hammondi* are probably monophyletic (Ellis *et al*. 1999). The analyses from the SSU rDNA presented here are therefore consistent with this conclusion.

Besnoitia, represented in this study by *B. besnoiti*, is the sister group to *N. caninum*, *T. gondii*, and *H. hammondi*. Previous studies on *Besnoitia* of cattle, wildebeest and goats also revealed strong support for this monophyletic group (J. T. Ellis, unpublished observations).

The 3 species of *Isospora* formed a monophyletic group which was the sister to *Besnoitia*+*Hammondia*+*Neospora*+*T. gondii*. This grouping supports the hypothesis for a separate genus within the Sarcocystidae that contains *Isospora* species (Frenkel, 1977). The names *Cystoisospora* or *Levineia* were originally suggested; however, one of the criteria for establishing this genus included heteroxeny which is not fulfilled by the inclusion of homoxenous *Isospora* (*I. suis* and *I. belli*) in this clade.

The analyses of the Sarcocystidae demonstrated strong support for 3 monophyletic groups. These groups are present in all 3 analyses and have a high

bootstrap support in the neighbour-joining tree. They are (1) the Toxoplasmatinae (here defined as *Isospora* + *Besnoitia* + *N. caninum* + *T. gondii* + *H. hammondi*), (2) a monophyletic group of ruminant *Sarcocystis* which contain species forming microcysts and with dogs as their definitive host, and (3) a second monophyletic group of ruminant *Sarcocystis* containing those species forming macrocysts and with cats as their definitive host.

The remaining group of species, containing *Frenkelia*, *S. mucosa*, *S. neurona*, *S. muris* and *S. sp.*, is less robust. The placement of *S. muris* and *S. sp.*, in particular, was unstable. Similar analyses were also performed using sequence alignments based on that described by Van de Peer *et al.* (1997), which defines the complete secondary structure of the SSU rRNA molecule. The results obtained were essentially the same as those described here. In these analyses the position of *S. muris* and *S. sp.* were also unstable. The relatedness of *S. muris* and *S. neurona* is interesting. Although *S. muris* is transmitted via cats to mice, it is most closely related to *S. neurona*. *Sarcocystis falcatula* and *S. neurona* are related organisms utilizing opposum as the definitive host. Although initial phylogenetic studies indicated that there were the same organism (Dame *et al.* 1995; Fenger *et al.* 1995), recent studies have shown that they have unique biological, antigenic, and structural characters (Dubey & Lindsay, 1998; Dubey, Speer & Lindsay, 1998). *S. neurona* causes neurological disease in horses in the Americas and is not infective to birds. Furthermore, *S. neurona* causes neurological disease in immunodeficient mice whereas *S. falcatula* is not infectious to immunodeficient mice (Marsh *et al.* 1997; Dubey & Lindsay, 1998). More data from representative taxa are needed, from *S. falcatula* or from *Sarcocystis* that infect reptiles for example, to resolve the relationships among the branches in this region of the tree.

The lack of resolution in this part of the tree appears to be the result of short branches, indicating a slow evolutionary rate. The likelihood ratio test (Felsenstein, 1988), comparing the maximum likelihood tree with the molecular clock to that without the molecular clock, rejects the molecular clock for these data ($\chi^2 = 68{\cdot}21$, $P < 0{\cdot}001$). The short branch lengths occur in that part of the tree containing *Frenkelia*, *S. neurona*, *S. mucosa* and *S. muris*. Thus there is less informative phylogenetic information among these taxa.

It has been suggested that *Sarcocystis* is paraphyletic and that *Frenkelia* should be incorporated into the genus *Sarcocystis* (Votypka *et al.* 1998). The data presented here show *Frenkelia* + *S. neurona* + *S. mucosa* to be monophyletic and the sister group to the Toxoplasmatinae. If one was to include *Frenkelia* in *Sarcocystis* then it would be necessary to incorporate all the other taxa mentioned into *Sarcocystis* as well. A much more practical recommendation might be to incorporate *S. neurona* + *S. mucosa* + *S. muris* and *Frenkelia* into a new genus, given they potentially show monophyly.

Several hypotheses have been made regarding the evolutional biology and phylogeny of the coccidia (reviewed by Tenter & Johnson, 1997). One idea was that homoxenous coccidia developed from heteroxenous coccidia by simplifying the life-cycle; another was that heteroxenous coccidia evolved from ancestral homoxenous coccidia. Our analysis indicates that the latter hypothesis (suggested by Tadros & Laarman, 1982) is more likely, as it requires only a single origin of the heteroxenous life-style, with a reversal to the homoxenous condition in *Isospora*, whereas the first hypothesis requires 3 separate origins of heteroxeny. Furthermore, all the available information (Holmdahl *et al.* 1999) and the present study suggest that the nature of the intermediate host (ruminant/non-ruminant) represents an important influence on the evolutionary biology of *Sarcocystis*. *Toxoplasma* and *Neospora* have multiple intermediate hosts, and therefore have acquired ruminant hosts independently of *Sarcocystis*.

S. neurona has as its definitive host the Virginia opposum, a marsupial (Dubey & Lindsay, 1998). The close relationship of *S. neurona* to *S. mucosa* is another compelling example supporting the concept of a prehistoric Gondwana with continuous land connections between the present South America and Australia (Veevers, 1991). There is evidence that both these organisms, *S. neurona* from America and *S. mucosa* from Australia, have carnivorous marsupials as their definitive hosts (Dubey & Lindsay, 1998; Jakes, 1998). This concept is further strengthened by the observation that the marsupial genera *Didelphis* and *Dasyrus* are sister groups (Kirsch & Mayer, 1998).

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