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Molecular Phylogenetic Relatedness of *Frenkelia* spp. (Protozoa, Apicomplexa) to *Sarcocystis falcatula* Stiles 1893: Is the Genus *Sarcocystis* Paraphyletic?

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ABSTRACT. The coccidians *Frenkelia microti* and *F. glareoli* (Apicomplexa: Sarcocystidae) form tissue cysts in the brain of small rodents (intermediate hosts) while oocysts are formed in the intestine of final hosts, buzzards of the genus *Buteo*. The inclusion of the small subunit ribosomal RNA gene sequences (SSU rRNA) of both *Frenkelia* species into the SSU rRNA trees of other, tissue cyst-forming coccidia strongly supports paraphyly of the genus *Sarcocystis*. *Frenkelia* spp. exhibit close relatedness to *Sarcocystis falcatula* Stiles 1893, a bird-opossum parasite, recognized under its junior synonym *S. neurona* Dubey et al. 1991, as the causative agent of equine protozoan myeloencephalitis on the American continent. As the definition of the genus *Frenkelia* is based on a plesiomorphic character (affinity to the neural tissue) of supposedly low phylogenetic value, the synonymization of the genus *Frenkelia* with *Sarcocystis* is proposed. This renders the genus *Sarcocystis* monophyletic.

Supplementary key words. Evolution, heteroxenous coccidia, SSU rRNA sequences, life cycle.

TISSUE cyst-forming heteroxenous coccidia (CFC)(Apicomplexa: Coccidia: Sarcocystidae) are defined as parasites circulating between a final host (a carnivore or omnivore) and an intermediate host (usually a herbivore or omnivore). In tissues of the intermediate host cysts with a large number of zoites (cystozoites) are formed destined to infect the final host after being ingested. These organisms are widely distributed and are of paramount importance both in human and animal health. Current classification of the CFC is based on morphological characters of oocysts and sporocysts, fine structure of merogonial stages and host-parasite relationships (final host specificity, tissue specificity in the intermediate hosts and the life cycle)[7].

Presently, six genera of CFC are considered to be valid taxa. They have the following location of cysts in the intermediate host: species of the genus *Hammondia* occur in the skeletal and cardiac muscles, eventually in brain, spleen, lymph nodes, liver and lungs. Representatives of the genus *Besnoitia* develop in various connective tissue cells while *Toxoplasma* and *Neospora* are confined to the brain and the skeletal and cardiac muscles [7]. Members of the by far largest genus *Sarcocystis* produce cysts mostly in the skeletal muscles and less frequently in the nervous tissue [9, 23]. Species of the genus *Frenkelia* have cysts located exclusively in the central nervous system (CNS) [27]. The morphological and life cycle data being insufficient for the analysis of phylogenetic relationships of CFC [28], sequences of the small subunit ribosomal RNA gene (SSU rRNA) have been used for the purpose. The SSU rRNA gene sequences were obtained for several members of the genus *Sarcocystis*, *Toxoplasma* and *Neospora* (reviewed in [29]). Phylogenetic trees inferred from these sequences showed that representatives of the CFC form a monophyletic group, the monoxenic genus *Eimeria* being their sister branch [14, 17, 30].

In this article we extend the phylogenetic analysis of CFC to the genus *Frenkelia*. This genus contains only two species: *Frenkelia microti* (Findlay and Middleton, 1934) Biocca, 1965 occurs in the brain and spinal cord of rodents (most often in voles, genus *Microtus* but also in other small rodents) and in rabbit (*Oryctolagus cuniculus*) under experimental conditions [25]. *F. glareoli* (Erhardová, 1955) Biocca, 1965 develops exclusively in the brain tissue of the bank vole, genus *Clethrionomys*. Gamogony and sporogony take place in the intestine of birds of prey (European, Asian and American species of buzzards, genus *Buteo*) [20, 21, 31] where sporocysts morphologically indistinguishable from those of some other CFC (subfamily Sarcocystinae)[19] are formed. Like in the genus *Sarcocystis* direct transmission of the infection among buzzards by in feces excreted sporocysts is not possible. [3, 24]. If structural and life cycle characters are considered, the genus closest to *Frenkelia* is the genus *Sarcocystis*. The mutual relationship of these two genera is, however, a subject of controversy. On one hand *Frenkelia* has several characters identical with *Sarcocystis* (it is obligatorily heteroxenous; last generation meronts in cysts in the intermediate host form metrocytes which give rise to cystozoites; oocysts sporulate in the final host body). Furthermore, there are some shared antigens and similar cyst wall among *Frenkelia* and *Sarcocystis* [3, 18]. The similarity between *Frenkelia* and *Sarcocystis* led Levine [19] to divide the family Sarcocystidae Poche 1913 into two subfamilies: Sarcocystinae Poche 1913 (with the genera *Sarcocystis* and *Frenkelia*) and Toxoplasmatinae Biocca, 1956 (with the genera *Toxoplasma* and *Besnoitia*). Some authors even suggested synonymization of *Frenkelia* with *Sarcocystis* [3, 18]. On the other hand the strict confinement of cysts to the CNS of intermediate hosts and the commencement of merogony within the liver parenchyma cells represent unique features of the genus *Frenkelia*. This is why other authors considered this genus to be a valid and well-defined taxon [7, 27].

Herein the first data on molecular phylogeny of *Frenkelia* are presented. We show that the inclusion of both *Frenkelia* species into the SSU rRNA coccidia trees strongly supports paraphyly of the genus *Sarcocystis*. *Frenkelia* spp. exhibit a close relatedness to *Sarcocystis falcatula* Stiles, 1893 (syn. *S. neurona* Dubey, Davis, Speer, Bowman, Lahunta, Grantrom, Topper, Hamir, Cummings & Suter, 1991), the causative agent of equine protozoan myeloencephalitis in North and South America [6].

MATERIALS AND METHODS

Parasite diagnosis and isolation. Both *Frenkelia* species were classified according to the shape and size of cysts in the brain and the species of the intermediate host [3]. Approximately 70 pin-head sized cysts containing mature cystozoites of *F. microti* were dissected from the brain of an adult specimen of common vole (*Microtus arvalis*) captured in Milovice, Southern Moravia, Czech Republic in April, 1995. *F. glareoli* cysts were extracted from a heavily infected brain (about 120 cysts) of a bank vole (*Clethrionomys glareolus*) trapped in Česká Skalice, Northern Bohemia, Czech Republic in September 1994. Cysts with remnants of the brain tissue were layered on

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Table 1. Sequence similarity. Above diagonale: percentage of sequence similarities. Below diagonale: percentage of sequence distances.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
1. *C. muris*	***	86.1	78.0	78.2	77.7	79.1	78.1	79.8	80.2	79.9	80.4	80.2	80.1
2. *B. microti*	13.1	***	79.7	78.2	77.5	78.4	77.7	80.5	81.0	81.2	81.4	80.9	81.3
3. *E. nieschulzi*	16.1	14.7	***	82.5	82.3	84.3	81.5	88.0	88.5	88.1	88.3	88.0	88.4
4. *S. arieticanis*	13.9	14.8	11.9	***	93.8	90.2	89.4	86.7	87.1	88.4	88.3	88.1	88.1
5. *S. tenella*	13.3	14.2	11.3	2.2	***	89.9	88.4	86.7	87.0	88.1	88.0	87.8	87.9
6. *S. fusiformis*	13.8	15.6	13.1	5.9	4.8	***	91.3	89.4	89.6	90.9	91.5	91.2	91.1
7. *S. gigantea*	14.3	15.9	13.4	7.1	5.9	4.5	***	86.3	86.7	87.9	88.0	87.7	87.7
8. *T. gondii*	13.9	13.9	10.4	6.9	5.8	6.5	7.2	***	99.5	95.6	95.9	96.0	96.5
9. *N. canis*	13.6	13.5	10.0	6.6	5.7	6.5	7.0	0.3	***	95.8	96.2	96.3	96.6
10. *S. muris*	13.7	12.9	10.1	5.5	4.8	5.5	6.2	3.8	3.8	***	97.9	97.9	97.9
11. *S. falcatula*	13.2	13.1	10.4	5.5	4.7	5.0	6.0	3.4	3.3	1.8	***	98.8	98.9
12. *F. microti*	13.6	13.7	10.5	5.9	5.1	5.4	6.4	3.4	3.4	2.1	1.1	***	99.1
13. *F. glareoli*	13.7	13.1	10.0	5.8	4.9	5.4	6.3	3.0	3.0	1.9	1.0	1.0	***

a continuous sucrose gradient, and left to sediment for 4 h at 4° C. Cysts sedimented at the concentration of 40% were carefully removed, washed 4× in phosphate-buffered saline solution, and subsequently disrupted by several freeze and thaw cycles in liquid nitrogene. Pure merozoites were resuspended in the lysis solution (50 mM Tris, pH 8.0; 1 mM EDTA; 100 mM NaCl, 0.1% sarcosyl) and incubated for 1 h at room temperature. After phenol and phenol-chloroform extractions and ethanol precipitation the air-dried total DNA was resuspended in TE buffer (10 mM Tris, pH 7.0 and 1 mM EDTA).

PCR amplification. SSU rRNA genes were amplified with the oligonucleotides JV-1, 5′ GTATAAGCTTTTATACGGC 3′, and JV-2, 5′ GAATAATTCACCGGAACAC 3′, which anneal to the conserved 5′ and 3′-end regions. Reaction mixtures contained 10 mM Tris, pH 9.0, 1 mM $MgCl_2$, 50 mM KCl, 0.1% Triton, 0.2 mM each of dGTP, dATP, dTTP and dCTP, 0.25 uM of each amplification primer and 2.5 U of Taq DNA polymerase (Fermentas, Lithuania). A Peltier thermal cycler was programmed for 35 cycles of 92° C 1 min, 40° C 40 sec, and 74° C 1 min 30 sec, with final 65° C 15 min extension to enhance the addition of T-overhangs.

Cloning and sequencing. PCR products were analyzed on 0.75% agarose gels stained with ethidium bromide and purified by electroelution. DNA was extracted with phenol-chloroform, ethanol precipitated, ligated into the pT7 Blue vector (Novagen) and transformed into XL-1 *Escherichia coli* competent cells (Stratagene). Both strands were sequenced using the Sequenase 2.0 kit (Amersham-USB) and a set of primers composed of those matching the conserved regions shared between apicomplexans and kinetoplastids (primers S-713, S-828, S-829 of [22]), apicomplexans and helminths (primers 5′2, 3′8, 3′7, 5′6, 3′4 of [5]) and *Frenkelia*-specific primers JV-3 (5′ CTATGCCGACTAGAGATAGG 3′) and JV-4 (5′ CTAGACCTGTCGGCCAAGG 3′).

Data analysis. Two different algorhitms were used in this study to produce alignments, Clustal and Jotun Hein (MegAlign Windows 3.10a; DNASTAR Inc., USA). To establish their affinity to other groups, the SSU rRNA sequences of *F. microti* and *F. glareoli* were first aligned using Clustal method with all apicomplexan SSU rRNA sequences retrieved from the GenBank™. Once the phylogenetic position of these two species within the heteroxenous coccidia was confirmed, we restricted further analysis to this group only. We used 1,567 bp and 1,566 bp long fragments of the SSU rRNA sequences of *F. microti* and *F. glareoli,* respectively, for which homologous fragments are available from other heteroxenous coccidia and *Eimeria nieschulzi, Cryptosporidium muris* and *Babesia microti* as outgroups. Using Clustal algorithm, 1,696 bp long alignment was generated. To obtain the best fitting alignment, several combinations of alignment parameters were tried, and the alignment providing the shortest parsimonious tree was selected. The sequences were further adjusted by eye, particularly in highly variable regions, till the shortest tree was found (alignment I). In order to estimate the influence of different alignment methods on tree topologies, we constructed 1,725 bp long alignment using Jotun Hein algorithm (alignment II). Both alignments are available on request.

While whole alignments were tested by maximum likelihood, for maximum parsimony the sets containing 261 (alignment I) and 269 (alignment II) informative sites were prepared by the "tease" program which removes uninformative sites (Random Cladistics, [26]). Maximum parsimony and maximum likelihood was done using programs Dnapenny, Dnapars, Dnaml and Dnamlk, and the datasets used to compute the bootstrap values were generated by Seqboot (Phylip, version 57c, [13]). Independently, the maximum parsimony and jack knife monophyly index (JMI) were calculated by Hennig86 [12] and Random cladistics programs [26]. In all steps of the analysis by Hennig86 the exhaustive search algorithm (cc-.;ie*) was used.

Nucleotide sequence accession numbers. The SSU rRNA gene sequence data of *F. microti* and *F. glareoli* are available at the GenBank™ under the accession numbers AF009244 and AF009245, respectively.

RESULTS AND DISCUSSION

Phylogenetic analysis of alignments. Amplified region of the SSU rRNA genes of *F. microti* and *F. glareoli* is 1,631 and 1,630 bp long, respectively. Similarity of the most related heteroxenous coccidia has been plotted in Table 1. We began the analysis with the set including all available apicomplexan SSU rRNA sequences. After the branching of both *Frenkelia* species within the CFC branch was established, we restricted our analysis on this group of organisms only. Since it was clearly demonstrated that the topology of trees based on the apicomplexan SSU rRNA sequences is strongly influenced by the type of alignment and phylogenetic method used [10, 11, 28] we have constructed trees based on two different alignments using both maximum parsimony and maximum likelihood methods in order to verify the reliability of trees obtained.

For alignment I, containing 261 informative sites, both maximum parsimony programs provided two equally parsimonious trees. In both trees the genus *Sarcocystis* was split into two branches, one containing only four *Sarcocystis* species ("*Sarcocystis* group"), the other embracing *S. muris, S. falcatula,* and both *Frenkelia* sp. ("*Frenkelia* group"). The trees differed in position of the *Toxoplasma-Neospora* clade. While in the first tree, identical to that obtained by maximum likelihood, the *Toxoplasma-Neospora* clade formed sister group to all other

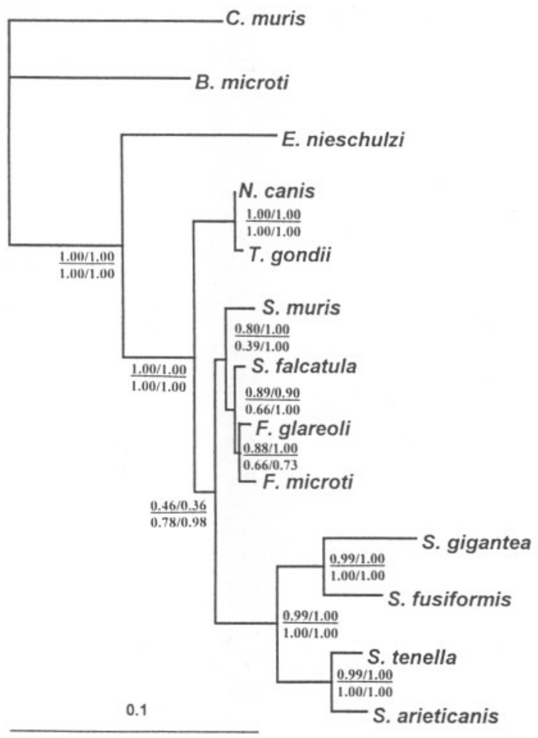

Fig. 1. Tree obtained by maximum parsimony (Dnapenny, Hennig86) and maximum likelihood (Dnaml) programs. The numbers at nodes indicate the bootstrap values/JMI for alignment I (above line) and alignment II (below line).

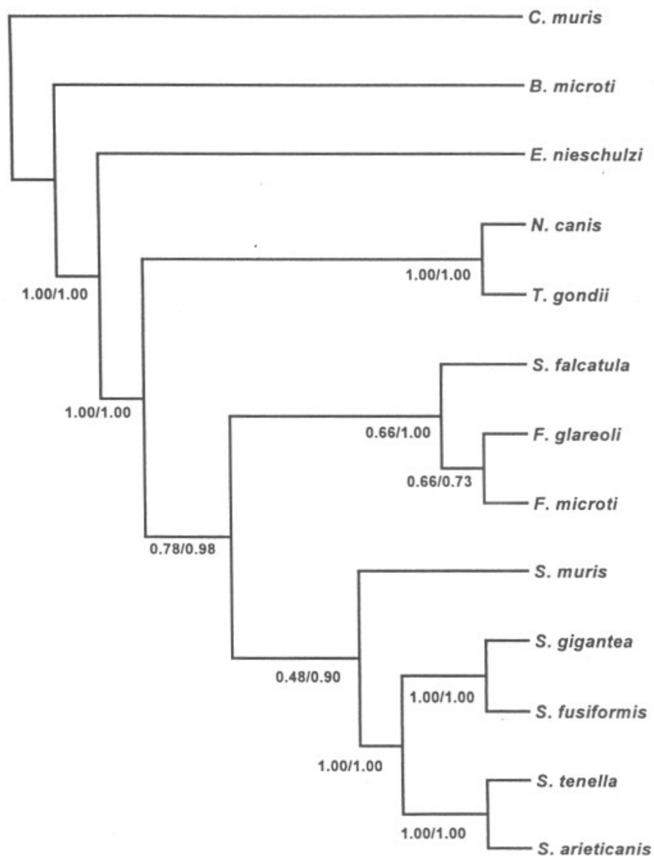

Fig. 2. Tree obtained by Hennig86 maximum parsimony analysis of alignment II. The numbers at nodes indicate bootstrap values/JMI.

CFC (Fig. 1), in the second tree it clustered together with "*Frenkelia* group" (data not shown). General unstability of the *Toxoplasma-Neospora* branch observed in these trees was also reflected by low bootstrap values and JMIs supporting either of both topologies (Fig. 1).

In previous studies based on the SSU rRNA sequences, the genus *Sarcocystis* appeared either monophyletic or paraphyletic in respect to the *Toxoplasma-Neospora* clade. It is well documented that the phylogenetic position of these species is unstable, being strongly influenced by the analyzed data and method used [10 ,17 , 29]. The inclusion of *Frenkelia* SSU rRNA gene sequences in this study renders the genus *Sarcocystis* paraphyletic regardless of the position of the *Toxoplasma-Neospora* clade. However, since the position of the latter clade has important taxonomic and nomenclature implications, we made an attempt to solve this uncertainity using another alignment (alignment II) constructed by Jotun Hein method. The alignment II was 1,725 bp long and contained 269 informative sites. When analyzed by both maximum parsimony and maximum likelihood methods, it provided three different trees depending on the phylogenetic program used. In all trees the *Toxoplasma-Neospora* clade branched off at the base of the CFC in correspondence with the tree shown in Fig. 1 which suggests that the position of this clade as neighbour group to the "*Frenkelia* group" is an artifact caused by the Clustal-based alignment.

Of the two most parsimonious trees produced by Hennig86, one is identical with Fig. 1 while the second tree places *S. muris* at the base of the "*Sarcosystis* group" (Fig. 2). Although this tree is well compatible with conventional classification, more data is needed to verify its validity. In addition, third tree (Fig. 3) was obtained when alignment II was analysed by Phylip parsimony (Dnapenny) and maximum likelihood (Dnaml) methods. This tree is unique among all analyzed trees in that it

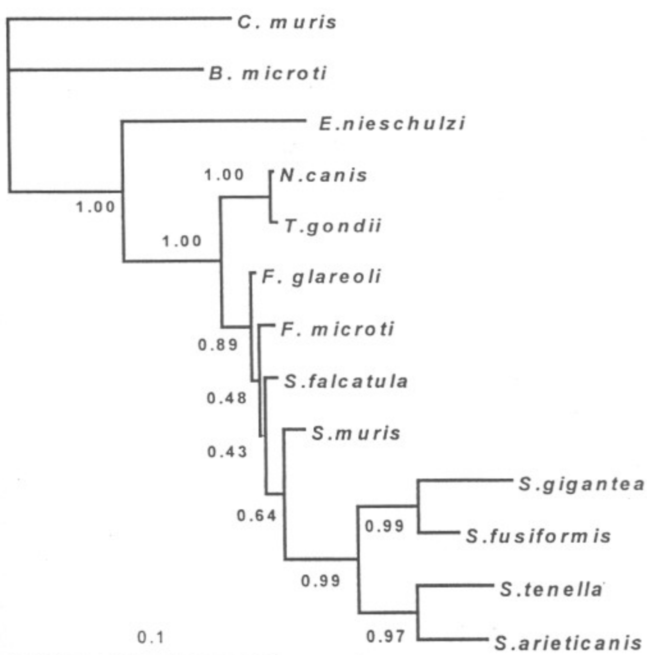

Fig. 3. Tree obtained by maximum parsimony (Dnapenny) and maximum likelihood (Dnaml) analyses of alignment II. The numbers at nodes refer to bootstrap values (Dnapars, 1,000 replicates).

retains the genus *Sarcocystis* monophyletic even after the inclusion of *Frenkelia* species. We assume, however, that the paraphyletic status of the "*Frenkelia* group" in respect to the "*Sarcosystis* group" is an artifact, as evidenced by extremely short branches and low bootstrap values within the former group (Fig. 3). If the concept of molecular clock is taken into consideration (Dnamlk), the topology corresponding to the tree in Fig. 1 is obtained from the same alignment.

Relatedness of *Frenkelia* spp. to *S. falcatula* (syn. *S. neurona*). Rather unexpectedly, *F. microti* and *F. glareoli* appear to be closely related to *S. falcatula* Stiles, 1893 (as redefined by [6]), these three species having an overall sequence homology of the SSU rRNA gene sequences 98.8%—99.1% (Table 1). With the exception of obviously artificial topology shown in Fig. 3, *Frenkelia* sp. and *S. falcatula* constitute well supported monophyletic branch. *S. falcatula* is a bird-opossum parasite which under its junior synonym *S. neurona* Dubey et al., 1991 has been recognized as the etiological agent of equine protozoan myeloencephalitis (EPM) [6]. Horses are considered aberrant rather than natural hosts of the EPM [8]. According to the SSU rRNA gene sequences, *S. falcatula, F. microti* and *F. glareoli* constitute a monophyletic group with high homology among its members. Their relatedness is confirmed by biological characters of these organisms. *S. falcatula* differs from most *Sarcocystis* species by its wide intermediate host range spanning several bird orders (passerine, psittacine, and columbiform birds) [2]. In this respect, *F. microti* behaves similarly, being able to infect mammals of two orders (Rodentia, Lagomorpha). Of consideration is the ability of *S. falcatula* to parasitize neural tissues as documented by its "*S. neurona*" infections in the horse. One can only speculate that similarly the cases of avian myeloencephalitis caused by *Sarcocystis*-like organisms [1, 8, 16, 23] were in fact *S. falcatula* infections. Thus both the wide host range and the affinity to CNS of *S. falcatula* are similar to *Frenkelia* sp.

Presently the genus *Sarcocystis* is considered to be monophyletic by most workers [17, 28, 29]. However, the position of the *Toxoplasma-Neospora* clade (and therefore also the support for monophyly of the genus *Sarcocystis*) is sensitive to the method of sequence alignment and tree-building methods used [10]. The appearance of *Frenkelia* spp. on top of the heteroxenous coccidia tree renders *Sarcocystis* definitely paraphyletic. To avoid the paraphyly of this genus, either *Frenkelia* should be synonymized with *Sarcocystis* (as was suggested by [3, 18]) or *S. falcatula* should be transferred into the genus *Frenkelia.* The wide intermediate host range (contrasting with a strict host specificity of other *Sarcocystis* species), localization of the merogonial development in the CNS, as well as the extremely high sequence homology between the SSU rRNA gene sequences, seem to be in favour of the placement of *S. falcatula* into the genus *Frenkelia* rather than the genus *Sarcocystis.* The wide host range of these parasites might be related to their localization in the CNS, which is an immunoprivileged site [4]. The neural tissue parasitism seems to be a primitive character as it is easier for a parasite to survive in an immunopriviledged site than in other tissues (e.g. muscles) exposed fully to the host defence mechanisms. The plesiomorphic rather than apomorphic nature of neuroaffinity is also supported by the fact that the CFC with affinity for the neural tissue (*T. gondii, N. caninum, Frenkelia* spp., *S. capracanis*) occur in all branches of the heteroxenous coccidia tree. Since primitive (plesiomorphic) characters generally have only a low phylogenetic value and because of the above reasons we propose to synonymize the genus *Frenkelia* with the genus *Sarcocystis.* This would maintain the genus *Sarcocystis* monophyletic.

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