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Phylogenetic relationships of the genus *Frenkelia*: a review of its history and new knowledge gained from comparison of large subunit ribosomal ribonucleic acid gene sequences☆

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Abstract

The different genera currently classified into the family Sarcocystidae include parasites which are of significant medical, veterinary and economic importance. The genus *Sarcocystis* is the largest within the family Sarcocystidae and consists of species which infect a broad range of animals including mammals, birds and reptiles. *Frenkelia*, another genus within this family, consists of parasites that use rodents as intermediate hosts and birds of prey as definitive hosts. Both genera follow an almost identical pattern of life cycle, and their life cycle stages are morphologically very similar. However, the relationship between the two genera remains unresolved because previous analyses of phenotypic characters and of small subunit ribosomal ribonucleic acid gene sequences have questioned the validity of the genus *Frenkelia* or the monophyly of the genus *Sarcocystis* if *Frenkelia* was recognised as a valid genus. We therefore subjected the large subunit ribosomal ribonucleic acid gene sequences of representative taxa in these genera to phylogenetic analyses to ascertain a definitive relationship between the two genera. The full length large subunit ribosomal ribonucleic acid gene sequences obtained were aligned using Clustal W and Dedicated Comparative Sequence Editor secondary structure alignments. The Dedicated Comparative Sequence Editor alignment was then split into two data sets, one including helical regions, and one including non-helical regions, in order to determine the more informative sites. Subsequently, all four alignment data sets were subjected to different tree-building algorithms. All of the analyses produced trees supporting the paraphyly of the genus *Sarcocystis* if *Frenkelia* was recognised as a valid genus and, thus, call for a revision of the current definition of these genera. However, an alternative, more parsimonious and more appropriate solution to the *Sarcocystis*/*Frenkelia* controversy is to synonymise the genus *Frenkelia* with the genus *Sarcocystis*.

Keywords: Sarcocystidae; *Sarcocystis*; *Frenkelia*; Ribosomal DNA; Large subunit ribosomal RNA; Phylogeny; Sequence alignment

☆ Note: Nucleotide sequence data reported in this paper are available in the EMBL, GenBank®, and DDJB databases under the accession numbers U85705-6, AF012883, AF092927, and AF044250-2.

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1. Introduction

Parasites of the phylum Apicomplexa that are classified into the family Sarcocystidae are obligatorily or facultatively heteroxenous coccidia with disporous, tetrazoic oocysts [1]. They are characterised by a sexual phase of development in the intestine of a definitive host and the ability to form tissue cysts in various tissues of an intermediate host. The two hosts follow a predator-prey relationship. There are currently at least six recognised genera (*Sarcocystis*, *Frenkelia*, *Toxoplasma*, *Hammondia*, *Besnoitia*, and *Neospora*) of tissue cyst-forming coccidia [2–5], although the classifications and taxonomies of some of these parasites are controversial [5]. Based on phenotypic characters, the family Sarcocystidae is often subdivided into two subfamilies [1, 3, 6, 7]. The genera *Toxoplasma*, *Hammondia*, *Besnoitia*, and *Neospora* are classified into the subfamily Toxoplasmatinae while *Sarcocystis* and *Frenkelia* are classified into the subfamily Sarcocystinae.

In the Sarcocystinae, the asexual development in the intermediate host first consists of one or several cycles of endopolygeny in various tissues. Endozoites of the terminal generation of endopolygeny initiate the formation of tissue cysts in which two types of reproductive stages (metrocytes and cystozoites) develop by endodyogeny. Mature cysts contain several hundred-thousand cystozoites which do not divide further and are the terminal life cycle stage in the intermediate host. If these cysts are ingested by a definitive host, the cystozoites initiate the sexual phase of the life cycle (gamogony) in subepithelial tissues of the small intestine. Oocyst formation and sporulation also take place in the intestine of the definitive host. Sporulated oocysts or free sporocysts are released into the intestinal lumen and passed into the environment with the faeces [2–5, 8, 9].

This study focusses on taxa representative of the Sarcocystinae, in particular on the genus *Frenkelia* which shares a broad range of phenotypic characters with the genus *Sarcocystis*. Consequently, some authors have questioned the validity of the genus *Frenkelia* [10–14]. By contrast, Rommel [15] questioned the validity of the current distinction between *Sarcocystis* and *Frenkelia* based on the affinity of their tissue cysts to different host tissues and suggested a subdivision of the Sarcocystinae based on other characters, i.e. the size of their cystozoites and the type of their definitive host. While phenotypic characters have been widely used for the classification of tissue cyst-forming coccidia over the last two decades, the reconstruction of their phylogenetic relationships from these characters has been limited because it is not clear which characters are truly homologous and, thus, are phylogenetically informative [5]. As a consequence, it has not yet been possible to establish a robust classification of tissue cyst-forming coccidia that is generally accepted.

More recently, some clarification of the phylogenetic relationships of tissue cyst-forming coccidia has been obtained using phylogenetically informative nucleotide positions of the ssrRNA gene [5, 16, 17], especially those that make up the helices in the secondary structure of the ssrRNA [18], and using the substitution rate of individual positions in an alignment of ssrRNA [19]. However, potential problems associated with the use of a single gene as the only source of data [20] have not been addressed, nor has the problem of scarce informative data been overcome. For these reasons, the phylogenetic relationships of some members of the Sarcocystidae remain unresolved [5, 21]. A large number of informative characters helps to avoid random errors in the data which leads to a more statistically reliable inference [22]. As Olsen [23] stated, "finally there is no substitute for raw data: more information will always yield more reliable phylogenetic inferences", and more specifically, Cavalier-Smith [24] said "because the large ribosomal subunit RNA is twice as long as small subunit rRNA, trees based on this molecule should be even less prone to error".

In the study reported here we have developed a rapid method for PCR amplification and nucleotide sequencing of the full length lsrRNA gene of apicomplexan parasites. In order to avoid any potential artefactual findings we aligned the sequences using Clustal W and second-

ary structure alignments and subjected the data sets to several tree-building algorithms. This allowed us to infer the phylogenetic relationships of *Sarcocystis* and *Frenkelia* species from lsrRNA sequences in order to investigate controversies about their taxonomy and classification that have previously arisen from analyses of phenotypic characters or ssrRNA sequences.

2. Materials and methods

2.1. Genomic deoxyribonucleic acid

Genomic DNA of *Frenkelia microti*, *Frenkelia glareoli*, *Sarcocystis muris*, *Sarcocystis neurona* (SN-2 isolate), *Sarcocystis gigantea*, *Sarcocystis arieticanis*, and *Isospora felis* were obtained as described by us elsewhere [17, 21, 25, 26].

2.2. Polymerase chain reaction amplification

In order to amplify the entire lsrRNA gene of members of the family Sarcocystidae, we aligned the lsrRNA gene sequences of one species from each of 11 genera of protozoa or fungi (*Toxoplasma*, *Theileria*, *Prorocentrum*, *Perkinsus*, *Acanthamoeba*, *Cryptococcus*, *Tetrahymena*, *Trichomonas*, *Tritrichomonas*, *Candida*, and *Saccharomyces*) contained in GenBank™ (accession numbers X75429, L28998, X16108, U07697, X73881, L14067, X54004, M19224, M81842, Z48566, and M19229, respectively). We then searched for potential PCR priming sites conserved in all these taxa, and constructed four forward primers: KL1 (5′ TAC CCG CTG AAC TTA AGC 3′), KL4 (5′ AGC AGG ACG GTG GTC ATG 3′), KL5a (5′ GAC CCT GTT GAG CTT GAC 3′), KL6a (5′ GGA TTG GCT CTG AGG G 3′) and four reverse primers: KL3 (5′ CCA CCA AGA TCT GCA CTA G 3′), KL6b (5′ CCC TCA GAG CCA ATC C 3′), KL2 (5′ ACT TAG AGG CGT TCA GTC 3′), KL5b (5′ GTC AAG CTC AAC AGG GTC 3′) that can be used to amplify overlapping fragments of the lsrRNA gene from nucleotides 24–42 to nucleotides 4337–4354 (murine numbering system). Primer pairs and the approximate length of their target fragments were as follows: KL1/KL3 1500 bp, KL4/KL6b 750 bp, KL5a/KL2 720 bp, KL6a/KL5b 520 bp, KL6a/KL2 1200 bp.

One hundred microlitre reaction volumes were made up of 67 mM Tris–HCl, 16.6 mM ammonium sulphate, 0.45% Triton X-100, 0.2 mg ml^{-1} gelatine, 2.5 mM magnesium chloride, 0.2 mM each of dGTP, dATP, dTTP, and dCTP, 50 pmol of each primer, and 1 U *Taq* polymerase (Promega). Reactions were carried out in a Hybaid thermal cycler. Amplification of fragments KL4/KL6b, KL5a/KL2, and KL6a/KL5b was done using one cycle of 94°C for 3 min, then 31 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by one cycle of 72°C for 5 min. Amplification of fragments KL1/KL3 and KL6a/KL2 was done using one cycle of 93°C for 3 min, 50°C for 2 min, and 72°C for 2 min, then 31 cycles of 93°C for 30 s, 50°C for 2 min, and 72°C for 2 min. Annealing temperatures and extension times were varied according to intensity of the product and presence of non-specific bands. Each of the fragments from each species was amplified three times in order to minimise the effect of PCR-introduced errors.

2.3. Cloning and sequencing

Polymerase chain reaction products were analysed on 1% agarose gels stained with ethidium bromide. Subsequently, products were purified using the QIAquick (Quiagen) PCR purification kit. Deoxyribonucleic acid fragments were then ligated into the pGEM-T vector (Promega) and transformed into DH5α competent cells. One or two positive clones from each of the three PCR reactions were sequenced in forward and reverse directions using the Excell II sequencing kit (Li-Cor) and fluorescently labelled M13 primers. Sequencing reactions were run on automated 4000L Li-Cor sequencers.

2.4. Phylogenetic analysis

The complete lsrRNA gene was sequenced for *F. microti*, *F. glareoli*, *S. muris*, *S. neurona*, *S. gigantea*, *S. arieticanis*, and *I. felis*; and the lsrRNA gene sequences of *Toxoplasma gondii*

and *Eimeria tenella* were obtained from GenBank™ (accession numbers X75429 and AF026388, respectively). The lengths of the full lsrRNA gene sequences varied from 3228 bp in *I. felis* to 3501 bp in *S. arieticanis*.

First, we aligned the full length of the lsrRNA gene sequences using the program ClustalW [27]. The alignment was then subjected to four different tree-building methods including maximum parsimony (PAUP) [28], the distance methods neighbor-joining with Kimura distance and split decomposition with logdet distance (SplitsTree v. 1.0.4) [29], and maximum likelihood (DNAml) [30]. In addition, the sequences were aligned according to their secondary structure by Prof. Rupert De Wachter at Departement Biochemie, Universiteit Antwerpen, Antwerpen, Belgium, using the Dedicated Comparative Sequence Editor (DCSE) program by De Rijk and De Wachter [31]. This alignment was also subjected to the above tree-building algorithms. Subsequently, the latter alignment was split into two data sets, one containing only non-helical regions and one containing helical regions. These two derived data sets were then also used to construct phylogenetic trees using the above strategies.

3. Results

To confirm that the PCR primers were suitable for the amplification of lsrRNA genes from a range of Apicomplexa, we tested them on *S. gigantea*, *T. gondii* (ME49 and RH strains), *Neospora caninum*, *E. tenella*, *Babesia divergens*, *Plasmodium falciparum*, *Perkinsus marinus*, and the closely related dinoflagellate *Crypthecodinium cohnii*. To confirm that the primers did not amplify host or bacterial DNA, we tested them on genomic DNA of mouse, sheep, and *Escherichia coli* (Fig. 1). To then confirm that the amplified products were indeed lsrRNA gene fragments, we probed them with the full lsrRNA gene of *S. gigantea* which had been sequenced first (Fig. 2).

Phylogenetic analysis was carried out for six members of the Sarcocystinae: the two named species of the genus *Frenkelia* (*F. microti* and *F. glareoli*) and four species of the genus *Sarcocystis* (*S. muris*, *S. neurona*, *S. gigantea*, *S. arieticanis*) which were selected because of specific differences in phenotypic characters among them (Table 1). The ingroup also included two other coccidia with disporous, tetrazoic oocysts: a representative of the Toxoplasmatinae (*T. gondii*) and a member of the genus *Isospora* (*I. felis*). A member of the genus *Eimeria* (*E. tenella*) which has a different type of oocyst morphology (tetrasporous, dizoic) was used as an outgroup. Phenotypic characters of these taxa are listed in Table 2.

For the full length sequences aligned by Clustal, the PAUP, neighbor-joining, split decomposition, and maximum likelihood methods all produced trees in which the six members of the Sarcocystinae formed a monophyletic group, to the exclusion of other coccidia with disporous, tetrazoic oocysts (*T. gondii*, *I. felis*). However, the trees did not support the division of the Sarcocystinae into the genera *Sarcocystis* and *Frenkelia* as they are currently defined. Maximum parsimony, neighbor-joining, and split decomposition analyses gave trees with the same topology, in which the *Sarcocystis* species were split onto two branches, one comprising *S. gigantea* and *S. arieticanis*, and the other encompassing *F. microti*, *F. glareoli*, *S. neurona*, and *S. muris* (Fig. 3). The consistency and homoplasy indices for this tree were 0.721 and 0.279, respectively. Maximum likelihood produced a similar tree, but placed *S. muris* on a branch with *S. gigantea* and *S. arieticanis* (Fig. 4). The maximum likelihood value for this tree was −12695.91550.

In the case of the secondary structure alignment of full sequences, all methods gave results similar to those obtained with the full sequences aligned by Clustal. Split decomposition, neighbor-joining, and PAUP analyses all produced trees that had the same topology as the tree shown in Fig. 3; whereas maximum likelihood again produced the tree shown in Fig. 4.

For the secondary structure alignment of only helical regions, the split decomposition, neighbor-joining, and PAUP methods again yielded trees with the same topology as the tree shown in

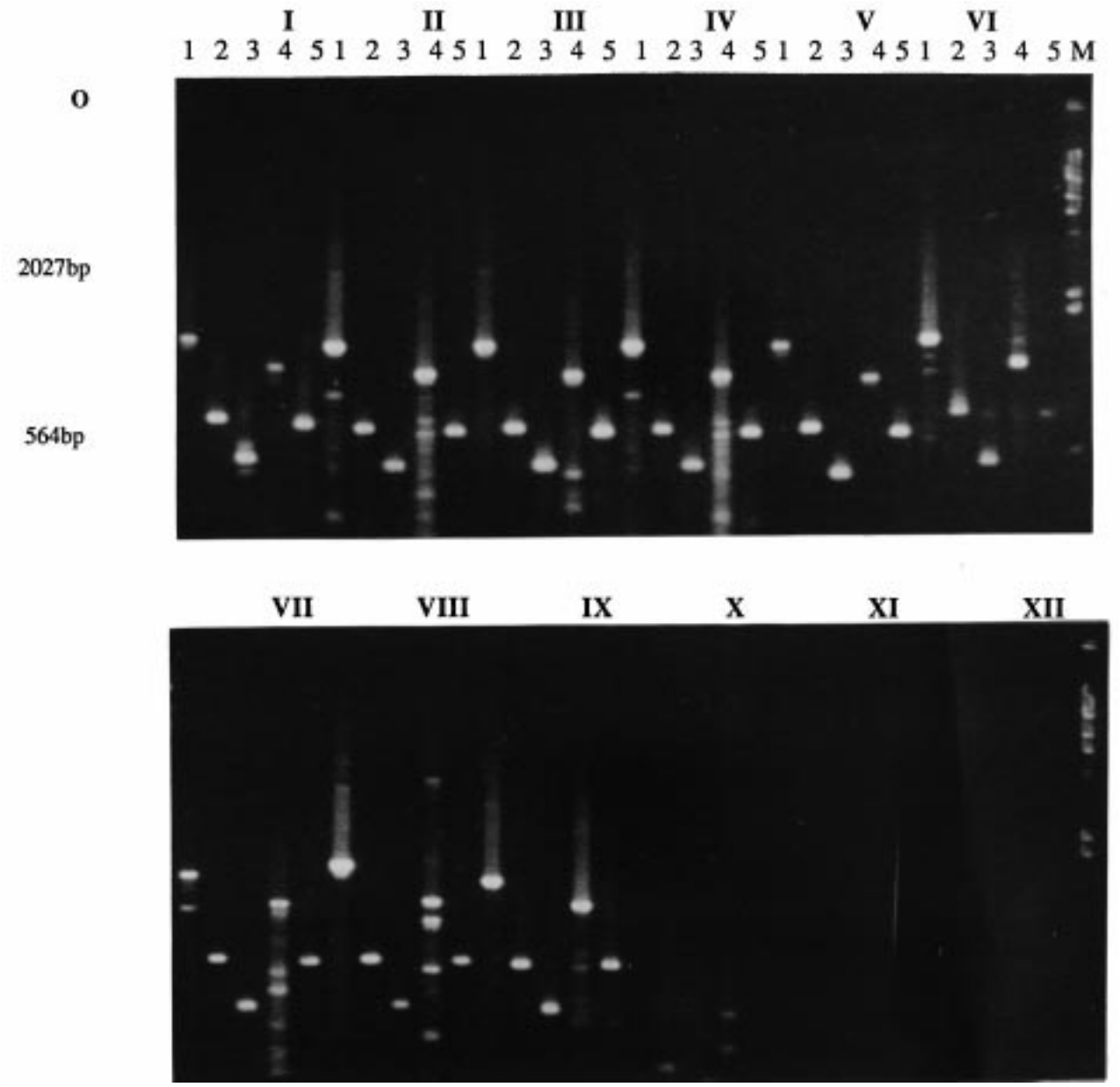

Fig. 1. Five overlapping fragments of the lsrRNA gene (lanes 1–5) obtained by polymerase chain reaction using the following primer pairs: lane 1, KL1/KL3; lane 2, KL4/KL6b; lane 3, KL6a/KL5b; lane 4, KL6a/KL2; lane 5, KL5a/KL2. DNA templates were as follows: **I.** *Sarcocystis gigantea*, **II** *Toxoplasma gondii* RH strain, **III** *Toxoplasma gondii* ME49 strain, **IV** *Neospora caninum*, **V** *Eimeria tenella*, **VI** *Plasmodium falciparum*, **VII** *Babesia divergens*, **VIII** *Perkinsus marinus*, **IX** *Crypthecodinium cohnii*, **X** Mouse, **XI** Sheep, **XII** *Escherichia coli*.

Fig. 3. In this case the tree had a consistency index of 0.724 and a homoplasy index of 0.276. Maximum likelihood gave a different result by placing *S. neurona* on a branch alone, and placing *S. gigantea*, *S. arieticanis*, *S. muris*, *F. microti*, and *F. glareoli* on a sister branch (Fig. 5).

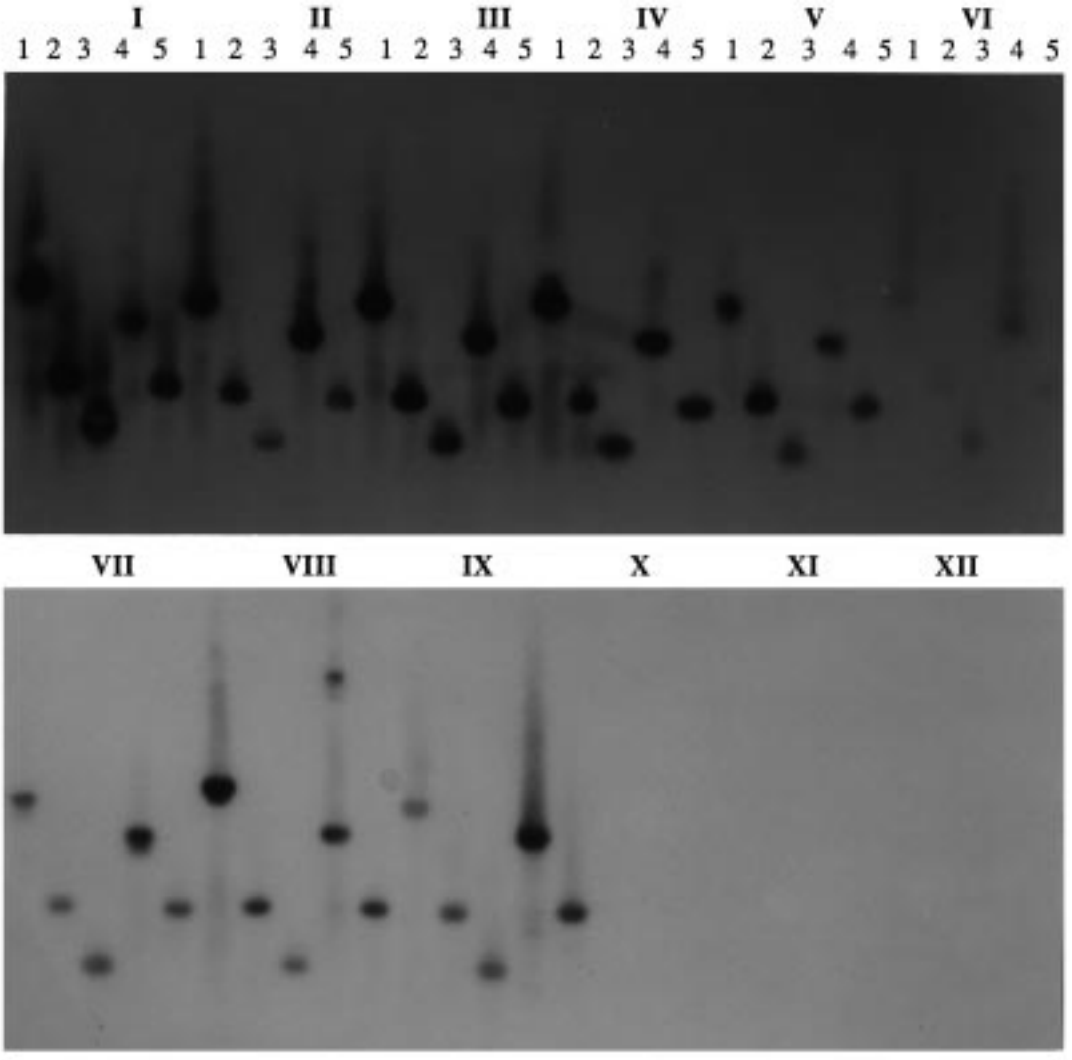

Fig. 2. Southern blot hybridisation of the polymerase chain reaction products shown in Fig. 1. using a *Sarcocystis gigantea* full length lsrRNA gene probe. The sequence of the polymerase chain reaction products and deoxyribonucleic acid templates is the same as in Fig. 1.

The maximum likelihood value for this tree was −4270.72849.

For the secondary structure alignment of only non-helical regions, all of the algorithms created trees with the topology of the tree shown in Fig. 3. Here, the consistency and homoplasy indices were 0.709 and 0.291, respectively, while the maximum likelihood value was −8057.80900.

4. Discussion

The two species of *Frenkelia* recognised to date were first described in the first half of this century. In 1934, Findlay and Middleton [42] described macroscopically visible, lobulated tissue cysts of a protozoan parasite in the brains of field voles, *Microtus agrestis*, in Britain and assigned the name *Toxoplasma microti* to it. In

Table 1
Phenotypic characters of different species currently classified into the genera *Frenkelia* or *Sarcocystis* (subfamily Sarcocystinae)

Character	*S. arieticanis*[a]	*S. gigantea*[b]	*S. muris*[c]	*S. neurona*[d]	*F. microti*[e]	*F. glareoli*[f]
Geographical distribution	world-wide	world-wide	world-wide	North, Central, and South America	Europe, North America, Japan	Europe
Host range						
definitive host	domestic dog	domestic cat	domestic cat, ferret	opossum	common buzzard, red-tailed hawk	common buzzard, rough-legged buzzard
intermediate host	domestic sheep	domestic sheep	house mouse	equines, mice	voles, hamsters, wood and field mice, house mice, rats, chinchilla, rabbit, muskrat?, Norway lemming?, New World porcupine?	red-backed voles
Host specificity						
definitive host	one species of carnivores	one species of carnivores	two families of carnivores	one species of marsupials	one genus of birds of prey	one genus of birds of prey
intermediate host	one species of ruminants	one species of ruminants	one species of rodents	two families of mammals	four families of rodents and one family of lagomorphs	one genus of rodents
Pathogenicity for intermediate host	high	low	intermediate	high	intermediate	intermediate
Vertical transmission in intermediate host	yes	yes	?	?	?	yes
Development in definitive host						
prepatent period	12 days	11–12 days	8–27 days	?	7–8 days	7–9 days
patent period	?	15–53 days	3–81 days	?	35–60 days	7–60 days
size of sporocysts	15–16.5×9.8–10.5 μm	10.5–14.0×8.0–9.7 μm	8.7–11.7×7.5–9.0 μm	7.0–8.0×10.0–12.0 μm	11.7–14.6×8.7–12.0 μm	11.3–13.8×7.8–10.0 μm
Pre-cystic development in						
1st generation endopolygeny	arteries of mesenterium and mesenteric lymph nodes	arterioles and capillaries of lung, kidney and brain	hepatocytes	neural tissue	hepatocytes	hepatocytes, Kupffer cells
2nd generation endopolygeny	capillaries of many organs					

(continued on next page)

Tissue cyst						
location	skeletal and heart muscles	predominantly muscles of oesophagus, larynx, and tongue	skeletal muscles	?	brain	brain, spinal cord
size	≤900 μm	≤10 mm	≤6 mm	?	≤1 mm	≤0.5 mm
shape	cylindrical	subspherical	cylindrical	?	lobulated	subspherical
cyst wall	type 6/7	type 21	type 1	?	type 1	type 1
maturation time	70 days	230 days	76 days	?	~3 months	~3 months
persistence of tissue cysts	up to 105 days p.i.	up to 47 months p.i.	> 200 days	?	up to 3 months p.i.	up to 6 months p.i.
Cystozoite						
size	14.5–15.0×3.0–3.8 μm	10–15×3–4 μm	14–16×4–6 μm	?	8.3–10.6×1.5–2.7 μm	7–9×2–2.7 μm

[a]For further information on this species see [32–34].
[b]For further information on this species see [33–36].
[c]For further information on this species see [33, 37–39].
[d]For further information on this species see [33, 40, 41].
[e]For further information on this species see [42–50].
[f]For further information on this species see [10, 47, 51–58].
? = Character is unknown.

Fig. 3. Phylogenetic tree reconstructed from Clustal or secondary structure alignments of full length lsrRNA gene sequences, and from alignments of helical or non-helical regions only, by maximum parsimony, neighbor-joining, or split decomposition methods as well as by the maximum likelihood method from the alignment of non-helical regions only.

1953, Frenkel [43] discovered the same or a very similar parasite in field mice, *Microtus modestus*, in the USA. However, because its tissue cyst morphology differed from that of *T. gondii* and because it could not be transmitted directly from one rodent host to another, Frenkel [43] concluded that the parasite described by Findlay and Middleton [42] was not a species of *Toxoplasma* and temporarily referred to it as M-organism. In the late 1950s and 1960s, similar tissue cysts of protozoan parasites were described in the brains of many species of the rodent subfamily Microtinae [61]. The generic name *Frenkelia* for M-organisms was erected by Biocca [62] in 1968, and *F. microti* [42, 62] became the type species of the genus. This species forms large, lobulated tissue cysts in the brains of a broad range of intermediate hosts including voles, hamsters, mice,

Table 2
Phenotypic characters of the two genera of Sarcocystinae (*Sarcocystis* and *Frenkelia*) and related genera included in the phylogenetic analysis[a]

Character	*Sarcocystis*	*Frenkelia*	*Toxoplasma*	*Isospora*	*Eimeria*
Oocyst morphology	disporous, tetrazoic	disporous, tetrazoic	disporous, tetrazoic	disporous, tetrazoic	tetrasporous, dizoic
Pattern of life cycle	obligately heteroxenous	obligately heteroxenous	facultatively heteroxenous	homoxenous or facultatively heteroxenous[b]	homoxenous
Type of development					
in definitive host	gamogony and sporogony	gamogony and sporogony	endopolygeny and gamogony	schizogeny[c] and gamogony	schizogeny and gamogony
in environment	none	none	sporogony	sporogony	sporogony
in intermediate host	endopolygeny and endodyogeny	endopolygeny and endodyogeny	endodyogeny	none[d]	./.
Natural route of transmission					
definitive to intermediate host	free sporocysts	free sporocysts	sporulated oocysts	sporulated oocysts[d]	./.
intermediate to definitive host	tissue cysts	tissue cysts	tissue cysts	dormozoites[d]	./.
definitive to definitive host	./.	./.	sporulated oocysts or endozoites	sporulated oocysts	sporulated oocysts
intermediate to intermediate host	./.	./.	tissue cysts or endozoites	./.	./.
Vertical transmission					
in intermediate host	yes	yes	yes	no	./.
Host range					
definitive host	vertebrates	*Buteo* spp.	felids	vertebrates	mostly vertebrates, rarely invertebrates
intermediate host	vertebrates	rodents and lagomorphs	warm-blooded vertebrates	warm-blooded vertebrates[d]	./.
Stages in definitive host					
extraintestinal stages	absent	absent	present	absent or present[b]	absent or present[e]
location of zygote in the intestine	lamina propria	lamina propria	epithelium	epithelium	epithelium or subepithelial sites[b]
duration of patency	weeks	weeks	days	days or weeks[b]	days or weeks[b]
Stages in intermediate host					
location of tissue cyst	striated muscles, sometimes also in neural tissue	central nervous system	many tissues	many tissues	./.
shape of tissue cyst	variable[b]	lobulated or subspherical[b]	subspherical	./.[f]	./.
tissue cyst wall	variable[b]	thin	thin	thin	./.
septa in tissue cyst	present	present	absent	./.	./.
stages within tissue cyst	metrocytes and cystozoites	metrocytes, intermediate cells, and cystozoites	cystozoites	dormozoite	./.
cystozoite	banana-shaped, broad	banana-shaped, broad	crescent-shaped, slender	./.	./.
host cell nucleus	remains unchanged	hypertrophies	remains unchanged	remains unchanged	./.

[a]For further information on these genera see [2–7, 9, 34, 59, 60] and references listed in Table 1. [b]Character is variable within the genus. [c]Character is variable within the genus: schizogony only; schizogony and endodyogeny; schizogony, endodyogeny and endopolygeny. [d]Applies to facultatively heteroxenous *Isospora* species only, character is missing in homoxenous *Isospora* species. [e]Some *Eimeria* species have adapted their life cycle to development in extraintestinal sites. [f]Tissue cysts are usually monozoic. ./. = Character is missing or unknown in the respective taxon.

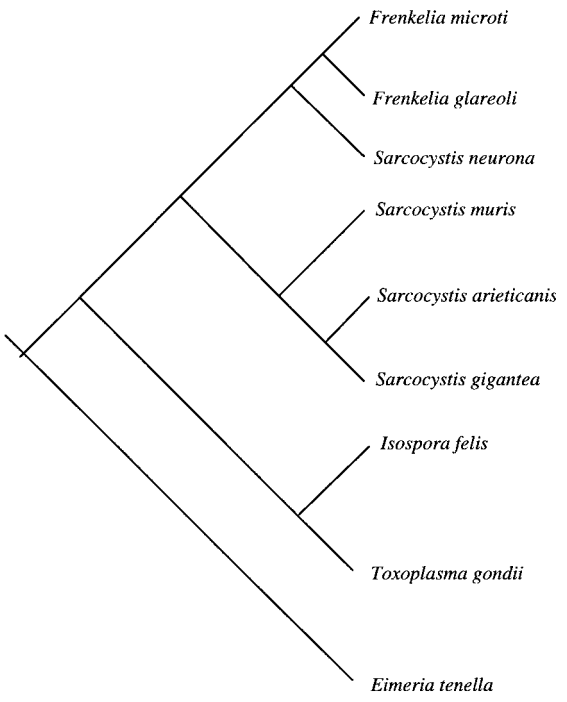

Fig. 4. Phylogenetic tree reconstructed from Clustal or secondary structure alignments of full length lsrRNA gene sequences by maximum likelihood.

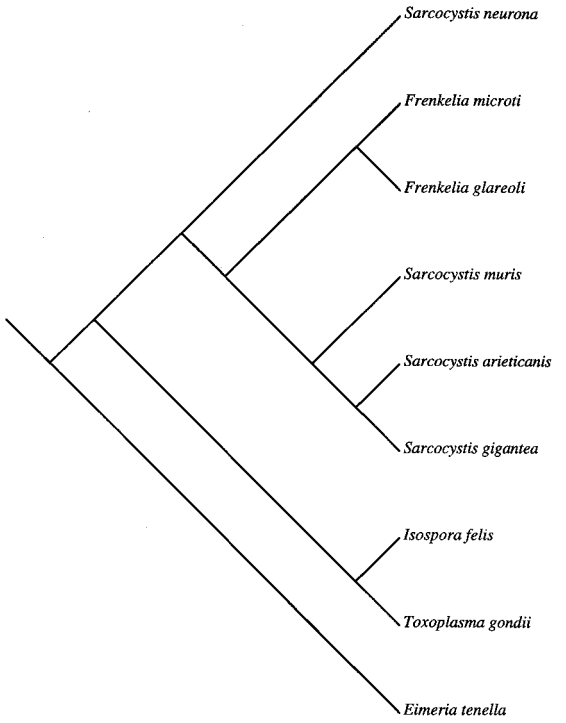

Fig. 5. Phylogenetic tree reconstructed from the alignment of helical regions only by maximum likelihood.

rats, chinchillas, rabbits [44, 45, 47], and probably New World porcupines [63]. However, it is uncertain whether *F. microti* is transmissible to bank voles [44, 45, 47].

Tissue cysts of a protozoan parasite in the brain of a bank vole, *Clethrionomys glareolus*, were first described in 1955 by Erhardová [64] in Czechoslovakia. The parasite was first named *Toxoplasma glareoli*, but was later transferred to the genus *Frenkelia* by Tadros et al. [61]. Thus, *F. glareoli* [64, 61] became the second recognised species of the genus *Frenkelia*. It is sometimes referred to as *Frenkelia clethrionomyobuteonis* [53] or *Frenkelia buteonis* [65, 66] which are synonyms of *F. glareoli*. Unlike *F. microti*, *F. glareoli* forms smaller, subspherical tissue cysts in the brains and spinal cords of its intermediate hosts, which are only red-backed voles, *Clethrionomys* species [39, 54].

The complete life cycles of *F. microti* and *F. glareoli* were elucidated by a series of transmission studies in the late 1970s [44, 46, 53–57, 67]. The common buzzard, *Buteo buteo*, was discovered as a definitive host for both species [44, 53, 55], and more recently, the red-tailed hawk, *Buteo jamaicensis*, and the northern rough-legged buzzard, *Buteo lagopus*, were identified as additional definitive hosts for *F. microti* and *F. glareoli*, respectively [47, 50]. The life cycles of both species of *Frenkelia* consist of two phases of asexual multiplication (endopolygeny and endodyogeny) in the intermediate host, and gamogony and sporogony in the definitive host. *Frenkelia* species are obligatorily heteroxenous, i.e. they are not transmitted horizontally between intermediate or between definitive hosts [47, 55, 60]. However, at least *F. glareoli* may be transmitted vertically in intermediate hosts [58].

Because of similarities between the *Frenkelia* life cycle and that of *Sarcocystis*, Frenkel [1] placed these two genera into the subfamily Sarcocystinae of the family Sarcocystidae.

Phenotypic characters that have been used to define this subfamily, and differentiate it from other members of the Sarcocystidae include the formation of two types of developmental stages (metrocytes and cystozoites) in the tissue cyst, the direct development of cystozoites into gametes (i.e. the absence of a propagative phase in the definitive host), and the endogenous phase of sporogony which results in the release of mature sporocysts into the environment [1, 3, 6, 8, 9, 68–70].

By contrast, the traditional differentiation of *Frenkelia* from *Sarcocystis* has been based only on the location and/or morphology of the asexual stages in the intermediate host [1, 3, 6, 8, 9, 55, 68–71]. In *Frenkelia*, the endopolygenous phase of the life cycle is restricted to the liver of the intermediate host and there is only one generation of endopolygeny [46, 47, 54, 56, 57, 67]. By contrast, in most species of *Sarcocystis* the initial generation of endopolygeny is located in vascular endothelial cells of many organs and is sometimes followed by one or several other generations of endopolygeny in the same or different types of host cells [4, 33]. In addition, tissue cysts of *Frenkelia* are located exclusively in the CNS [44, 46, 54, 56, 67] while those of *Sarcocystis* species have a predilection for skeletal or cardiac muscles [4, 5, 33]. *Frenkelia* as well as *Sarcocystis* multiplies by repeated endodyogeny within the tissue cyst. However, while metrocytes and cystozoites are present in the tissue cysts of both genera, a third cell type, i.e. intermediate cells, has been observed only in cysts of *Frenkelia* [51, 52, 72]. In addition, only *Frenkelia*, and not *Sarcocystis*, is believed to induce hypertrophy of the host cell nucleus [1, 8, 9, 49].

However, while no more species of *Frenkelia* have been described since the 1970s, the genus *Sarcocystis* has been enlarged continuously by the description of new species [14, 59, 73]. It now comprises more than 180 named species and, thus, forms the largest genus of the family Sarcocystidae [5, 6, 14]. Over the last two decades, our knowledge on *Sarcocystis* has increased considerably by comprehensive studies on the life cycles of various species placed into this genus [4, 33, 34]. These studies showed that phenotypic characters previously believed to be characteristic for *Frenkelia* also occur in some species of *Sarcocystis*. For example, in some *Sarcocystis* species of rodents, such as *S. muris*, *Sarcocystis cernae*, *Sarcocystis dispersa*, *Sarcocystis idahoensis* and *Sarcocystis rauschorum*, the initial phase of endopolygeny also takes places in hepatocytes [33, 39]. In addition, while the tissue cysts of most species of *Sarcocystis* are located exclusively or predominantly in muscles, some *Sarcocystis* species such as *Sarcocystis capracanis*, *Sarcocystis cruzi*, *Sarcocystis suihominis*, *Sarcocystis sybillensis*, and *Sarcocystis tenella*, also form cysts in nervous tissue [33, 34]. Moreover, a recent study by Jakob et al. [13] showed that the tissue cysts of *Sarcocystis kirmsei* are located exclusively in the brain of one of its intermediate hosts, the Javan hill mynah, *Gracula religiosa*.

Therefore, the location of the parasite's life cycle stages in the intermediate host is not a valid character for differentiation between *Frenkelia* and *Sarcocystis*. As a consequence the validity of the genus *Frenkelia* has been questioned [10, 12–14, 74]. As early as the 1970s, Kepka and Scholtyseck [72] concluded from EM studies on metrocytes and cystozoites that *Frenkelia* is either synonymous with *Sarcocystis* or is very closely related to it. Based on a comprehensive comparison of the life cycles of *F. glareoli*, *S. cernae*, and *S. dispersa* all of which use rodents as intermediate and birds of prey as definitive hosts, Černá et al. [74] also suggested that *Frenkelia* be synonymised with *Sarcocystis* and concluded that the different location of their tissue cysts in intermediate hosts represents a subgeneric character. Another hypothesis was raised by Rommel [15] who suggested that a division of the Sarcocystinae based on the size of their cystozoites (less than 7 μm or greater than 15 μm) and the type of their definitive host (birds and reptiles, or mammals) would be more accurate than the current distinction between *Frenkelia* and *Sarcocystis* based on the affinity of their tissue cysts to different host tissues. By contrast, other authors suggested that the genus *Frenkelia* be retained because of the unusually

high affinity of the tissue cysts to the CNS of the intermediate host and/or the unique lobulated tissue cyst morphology of its type species, *F. microti* [33, 66]. However, the latter character is variable between the two species of *Frenkelia* and therefore also cannot be used for the definition of the genus.

As conflicting results have been published regarding the phylogenetic relationship of the genera *Frenkelia* and *Sarcocystis* based on ssrRNA genes [21], we carried out phylogenetic analyses of the family Sarcocystidae using lsrRNA gene sequences to compare our findings with those from the ssrRNA data, and resolve the contradictions that have arisen from these data. Two alignment strategies, Clustal and secondary structure alignment, were used and the latter was further split into non-helical and helical alignments. Each of these four alignments was applied to four tree-building algorithms.

In 15 of the 16 trees derived in this study, *S. neurona* was the sister taxon to a monophyletic clade formed by the two *Frenkelia* species. Although the complete life cycle of *S. neurona* is unknown, this parasite resembles the two *Frenkelia* species in having a high affinity for the CNS of its intermediate hosts. The sister relationship of the two *Frenkelia* species with *S. neurona* observed in this study is in accord with a recent phylogenetic analysis of ssrRNA gene sequences by Votýpka et al. [21] who found a close relationship of the *Frenkelia* species with *Sarcocystis falcatula*. Based on partial ssrRNA gene sequence comparison, this parasite was believed to be synonymous with *S. neurona* [75], but has recently been shown to differ from *S. neurona* in biology and ultrastructure [41]. Thus far, the only asexual stages known for *S. neurona* are meronts and merozoites while tissue cysts and its definitive range of natural intermediate hosts are unknown. *Sarcocystis neurona*, *S. falcatula*, and a third unnamed *Sarcocystis* sp. all of which are transmitted by opossums are unusual parasites because they have a wide host range and merogony can last for months in the host and often kill the host [76]. The close phylogenetic relatedness of *S. neurona* with the two *Frenkelia* species and *S. muris* suggests that small mammals may be natural intermediate hosts of *S. neurona*. This finding may facilitate the elucidation of the life cycle of this parasite, in particular the identification of its tissue cyst, which would be an important step not only for the correct taxonomic position of *S. neurona*, but also for the understanding of the epidemiology of the disease caused by this parasite in equines.

Three of the tree-building methods used here (PAUP and the two distance methods) always produced the same tree topology with all four alignment methods. In this tree, the monophyletic group of the two *Frenkelia* species and *S. neurona* formed a clade with *S. muris*, to the exclusion of *S. gigantea* and *S. arieticanis*. *Frenkelia microti* and *F. glareoli* share a range of phenotypic characteristics with *S. muris*. For example, in all three species the endopolygenous phase of the life cycle (one generation only) takes place in hepatocytes of the intermediate host and all three species have a low specificity for either their definitive or their intermediate hosts. By contrast, *S. gigantea* and *S. arieticanis* multiply by endopolygeny in vascular endothelial cells of the intermediate host and both parasites are species-specific for their intermediate as well as their definitive host.

The same tree topology was also obtained from the fourth tree-building algorithm (maximum likelihood) when the secondary structure alignment of non-helical regions was used to infer the tree, while maximum likelihood analysis of the three other alignments produced trees with different topologies. The fact that only maximum likelihood produced trees of different topologies with these alignments may be a result of both the manner in which the algorithm works (working with probabilities rather than distance measurements to find the maximum likelihood that a particular tree will be produced by a certain data set), and that the program may have found more conflicting data in the Clustal and helical alignments as compared with the non-helical alignments. Nevertheless, all of the trees reconstructed from the four different alignments by all four tree-building methods showed monophyly of the Sarcocystinae, but suggested the genus

Sarcocystis to be paraphyletic if the genus *Frenkelia* was regarded valid.

In all of the trees derived in this study, the Sarcocystinae formed a monophyletic group to the exclusion of *T. gondii* and *I. felis*. This result is consistent with previous phylogenetic analyses based on ssrRNA gene sequences which also showed members of the subfamily Sarcocystinae to be a sister group to members of the Toxoplasmatinae and the genus *Isospora* [5, 17]. However, the results obtained in the present analysis of lsrRNA gene sequences as well as the results obtained in the previous analysis of ssrRNA gene sequences [21] are inconsistent with a division of the Sarcocystinae into the genera *Frenkelia* and *Sarcocystis* as they are currently defined. If *Frenkelia* is accepted as a valid genus, then the genus *Sarcocystis* is paraphyletic and needs to be redefined. Clearly, there are distinct lineages within the genus *Sarcocystis* [5, 16, 21, 77, 78], and its nonproportionally larger size compared with other taxa of the Sarcocystidae may call for a division of this genus. However, it is currently not clear what is the extent of genetic diversity among the different lineages of the Sarcocystinae, or between this subfamily and other coccidian taxa. Thus, the question of whether this diversity is large enough to justify a division of the current genus *Sarcocystis* into two or more different genera remains unresolved.

Over the last two decades we have learned that *Frenkelia* and *Sarcocystis* share a broad range of phenotypic characters, such as the lack of asexual reproduction in the definitive host [55], the development of oocysts in the lamina propria of the small intestine of the definitive host [55], a prolonged period of patency (several weeks) in the definitive host [10, 44, 53], an endogenous phase of sporogony [44, 55], a thin, fragile oocyst wall resulting in the release of individual sporocysts into the environment [44, 55], a first phase of asexual development by endopolygeny in the intermediate host [46, 57, 67], a second phase of asexual development by endodyogeny within tissue cysts in the intermediate host [61, 72], the presence of two different developmental stages (metrocytes and cystozoites) in the tissue cyst [46, 61], and the presence of septa within the tissue cyst [54, 61]. These characters distinguish both *Frenkelia* and *Sarcocystis* from genera currently classified into the subfamily Toxoplasmatinae and from the genus *Isospora*. In addition, there is a high antigenic cross-reactivity between *Frenkelia* and *Sarcocystis* [11, 47].

Considering these phenotypic characters and the results obtained from phylogenetic analyses of both ssrRNA and lsrRNA genes, a more parsimonious approach for the classification and taxonomy of tissue cyst-forming coccidia is to classify the current *Frenkelia* and *Sarcocystis* species into the same taxon. In 1976, Tadros and Laarman [10] suggested that all genera of tissue cyst-forming coccidia should be defined on the basis of their oocyst morphology. They synonymised both *Frenkelia* and *Sarcocystis* with their newly erected genus *Endorimospora*, defined to comprise all coccidia with an endogenous phase of sporogony that results in the shedding of mature disporous, tetrazoic oocysts or individual tetrazoic sporocysts. However, this genus was not accepted and the authors later revised this classification because of the general desire to retain the genus *Sarcocystis* [47]. More recently, Odening [14] included the genus *Frenkelia* in a revised list of 189 named species of *Sarcocystis*. He renamed *F. microti* to *Sarcocystis buteonis* [65] and *F. glareoli* to *Sarcocystis glareoli* [64]. Our results strongly support the synonymy of the genus *Frenkelia* with *Sarcocystis* as it is presently defined. However, further studies are required to investigate the phylogenetic relationships of the different lineages within the Sarcocystinae and their relationships with other coccidian taxa. These results will lead to a more accurate classification of the tissue cyst-forming coccidia.

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