ELSEVIER

International Journal for Parasitology 31 (2001) 413–417

INTERNATIONAL Journal for PARASITOLOGY

www.parasitology-online.com

Research note

Multiple origin of the dihomoxenous life cycle in sarcosporidia ☆

J.R. Šlapeta[a,b,*], D. Modrý[a,b], J. Votýpka[b,c], M. Jirků[b,d], B. Koudela[a,b], J. Lukeš[b,d]

[a]*Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, 612 42, Brno, Czech Republic*
[b]*Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic*
[c]*Department of Parasitology, Faculty of Sciences, Charles University, Prague, Czech Republic*
[d]*Faculty of Biology, University of South Bohemia, České Budějovice, Czech Republic*

Received 14 December 2000; received in revised form 16 January 2001; accepted 16 January 2001

Abstract

Although their ssrRNA gene sequences are closely related, the lizard sarcosporidia (Apicomplexa, Sarcocystidae) *Sarcocystis lacertae* and *Sarcocystis gallotiae* posses heteroxenous and dihomoxenous life cycles, respectively. When aligned with available sarcosporidian ssrRNA genes, both species constitute a monophyletic clade that is only distantly related with sarcosporidia that have a viperid snake as their definitive host (*Sarcocystis* sp., *Sarcocystis atheridis*). To test the phyletic status of the dihomoxenous life style, *Sarcocystis rodentifelis* and *Sarcocystis muris*, two dihomoxenous parasites of mammals were included into this study. All studied species group together with former *Frenkelia* spp., *Sarcocystis neurona* and related marsupial and bird sarcosporidia in a monophyletic clade. However, the available dataset supports independent appearance of the dihomoxenous life cycle at least twice during the evolution of the Sarcocystidae.

Keywords: Sarcocystidae; Phylogenetic analysis; Dihomoxeny; Small subunit ribosomal RNA; Life cycle; Evolution

The ssrRNA gene sequences are available for dozens of coccidian species (Apicomplexa, Coccidia) and are widely used for analysing the (in)congruencies between the morphology- and sequence-based taxonomic systems, and possible coevolution of these widespread parasites with their final and/or intermediate hosts (Carreno and Barta, 1999; Doležel et al., 1999; Ellis et al., 1999; Holmdahl et al., 1999; Jenkins et al., 1999; Modrý et al., in press; Mugridge et al., 1999a,b). Moreover, the expansion of the ssrRNA dataset enables analyses of the evolutionary origin of various aspects of coccidian biology.

An extraordinary life cycle has been described in *Sarcocystis* species parasitising the island-dwelling endemic lizards of the genera *Gallotia* (from the Canary Islands) and *Teira* (from Madeira) (Matuschka and Bannert, 1987; Thorpe et al., 1994). These sarcosporidia are transmitted among their hosts by cannibalistic behavior during which the lizards eat each others tail. Such a life cycle, in which even a single specimen can serve as predator (definitive host) or prey (intermediate host) was termed dihomoxenous predator-prey transmission. *Sarcocystis gallotiae*, infecting the lizard *Gallotia galloti* is a typical representative of this group. The development of sarcocysts is confined to *G. galloti* but the specificity of the sexual development is not as strict and oocysts can develop in several lacertid species (Bannert, 1994). In contrast, the life cycle of another lizard sarcosporidium *Sarcocystis lacertae* does not possess dihomoxenic features (J.R.Š, D.M., unpublished results) and is strictly heteroxenous, combining two European reptilian hosts; the lizard *Podarcis muralis* and the colubrid snake *Coronella austriaca* as intermediate and definitive hosts, respectively (Volf et al., 1999).

Sarcocystis rodentifelis is a cyst-forming coccidian parasite, closely related to *Sarcocystis muris*. Its life cycle comprises the Norwegian rat and vole as intermediate and the cat as definitive hosts. Moreover, an alternative development occurs during which the cat (= definitive host) is eliminated from the life cycle and its role is replaced by the development of cystozoites into gamonts and oocysts/sporocysts within the intestine of the rat, an alternative definitive host (Grikieniene et al., 1993). A similar life style was recently described for *S. muris* in mice (Koudela and Modrý, 2000). This type of life cycle is also referred to as dihomoxenous, because of the similarities it shares with the dihomoxenous development of lizard sarcosporidia. Herein,

☆ Nucleotide sequence data reported in this paper for *Sarcocystis lacertae*, *Sarcocystis gallotiae* and *Sarcocystis rodentifelis* are available in the GenBank™ data base under the accession numbers AY015111, AY015112, AY015113, respectively.

* Corresponding author. Tel.: +42-5-4156-2979; fax: +42-5-4924-8841.
E-mail address: slapetaj@vfu.cz (J.R. Šlapeta).

0020-7519/01/$20.00
PII: S0020-7519(01)00127-8

using the ssrRNA sequences we demonstrate that the life histories described above have multiple origin, and that the sarcosporidia parasitising reptiles are polyphyletic.

After the identity of all studied sarcosporidia was proved by an ultrastructural analysis of their cyst wall (data not shown), total DNA was isolated by standard protocols from the following samples: i/cystozoites of *S. rodentifelis* isolated from a vole trapped in the Vilnius district, Lithuania (kindly provided by Jadvyga Grikieniene); ii/*S. gallotiae* from cysts dissected from the infected tails of *G. g. eisentrauti* originating from Northern Tenerife, the Canary Islands; iii/*S. lacertae* from cysts obtained from infected lizards *P. muralis* (Volf et al., 1999). The ssrRNA gene was PCR amplified with the oligonucleotides JV1 and JV2 (Votýpka et al., 1998) and/or ERIB1 and ERIB10 (Barta et al., 1997) that annealed to its conserved 5′ and 3′ ends. The initial denaturation at 95°C for 4 min was followed by 30 cycles at 95°C for 1 min, 50°C for 1 min, 72°C for 1·5 min, and a final extension at 72°C for 10 min. Amplicons were gel-isolated and cloned into the Topo™ TA vector (Invitrogen). Both strands were sequenced on an automated sequencer using BigDye DNA Sequencing Kit (Perkin–Elmer).

Sequences obtained from *S. lacertae*, *S. gallotiae*, and *S. rodentifelis* were 1802, 1591, and 1593 nt long, respectively. ssrRNA genes of a set of coccidian species that cover the presently known biological and morphological diversity of the Sarcocystidae and Eimeriidae were selected for comparison with *Cryptosporidium parvum* as an outgroup. Since both mentioned families are sister groups, an analysis of the relationships within the former family was performed with the Eimeriidae as the only outgroup, thus reducing the risks associated with the use of a too distant outgroup.

The following ssrRNA sequences obtained from the GenBank/EMBL/DDJB databases were adopted in the phylogenetic analysis: *Toxoplasma gondii* consensus sequence from L37415, U12138, L24381, X65508, U03070, U00458, M97703, X68523, X75453, X75429, and X75430; *Neospora caninum* consensus sequence from L23380, U03069, U16159, and U17346; *Hammondia hammondi* AF096498; *Besnoitia besnoiti* AF109678; *Isospora belli* consensus sequence from U94787 and AF10693; *I. ohioensis* AF029303; *I. suis* U97523; *Hyaloklossia lieberkuehni* AF298623; *S. buteonis* (= *Frenkelia microti*) AF009244; *S. glareoli* (= *F. glareoli*) AF009245; *S. dispersa* AF120115; *S. neurona* U07812; *S. mucosa* AF109679; *S. muris* M64244; *S. tenella* L24383; *S. cruzi* AF017120; *S. hirsuta* AF017122; *S. aucheniae* AF017123; *S. atheridis* AF120114; *Sarcocystis* sp. (snake/rodent life cycle) U97524; *Eimeria nieschulzi* U40263; *E. tenella* U40264; *Cyclospora cayetanensis* U40261; *Isospora robini* AF080612; *Caryospora bigenetica* AF060976; *Lankesterella minima* AF080611 and *Cryptosporidium parvum* AF093493.

All retrieved ssrRNA genes were aligned together with their homologues from newly included species using the program Clustal X (Thompson et al., 1997), and the dataset was analysed with a variety of aligning parameters. Sequences that showed monophyly were aligned separately to produce partial alignments, which were then used in the final alignment. This alignment was optimised by eye and ambiguously aligned regions were eliminated. The stability of individual groups and the involvement of systemic error/systemic bias were tested with different numbers of their representatives by the deletion of taxa or groups of taxa and by the elimination of hypervariable residues. Sequence alignments, available on request or at ftp://vfu-www.vfu.cz/slapeta/alignments, were analysed using PAUP* version 4.0b4a (Swofford DL, 1998, PAUP*, Phylogenetic Analysis Using Parsimony (* and other methods), Beta version 4.0b4a. Sinauer Associates, Sunderland, MA, USA) and phylogenetic relationships within obtained trees were analysed using distance, maximum parsimony, maximum likelihood methods and puzzle analysis also implemented in PAUP*. Distance methods were performed using heuristic search with the minimum evolution objective setting and the LogDet matrices. Maximum parsimony analyses were performed using heuristic settings with gaps treated as missing data and the transversion/transition ratios 1:1–3. For maximum likelihood the substitution types 2 or 6 were used. Bootstrap analysis of 1000 replicates for distance method, maximum parsimony, and puzzle were performed. Statistical evaluation of the trees inferred under different topological constraints was performed using Kishino-Hasegawa test performed via PAUP*. The null hypothesis (absence of significant differences between the trees) can be rejected at $P < 0.05$.

The primary alignment of 29 in-group species and the *C. parvum* outgroup was 1707 sites long, corresponding to the residue range 83–1613 of the *T. gondii* ssrRNA gene (Gagnon et al., 1996). After ambiguously aligned regions were eliminated (positions 246–274 and 699–785 that belong to the hypervariable domains V2 and V4, respectively), remaining 1591 sites that included 1166 constant, 198 parsimony-uninformative, and 227 parsimony-informative positions were used for the phylogenetic analyses. Maximum parsimony yielded two trees of 796 steps (CI = 0.7) in which the family Sarcocystidae formed a monophyletic group (Fig. 1). With this method as well as with maximum likelihood, distance methods and puzzle four distinct clades, labelled A to D (according to Doležel et al., 1999), appeared within this group. These clades were well supported, but the support for the internal branches was intermediate and not stable depending on the number of eliminated hypervariable fast-evolving residues and the number of representatives within the clades (data not shown). An analysis with *E. nieschulzi* as an outgroup yielded 118 parsimony- informative sites and, using heuristic search, the length of the tree was 475 steps. The overall topology did not differ from the previous trees.

Next, the analysis was narrowed to clade B that comprised six previously known species and all the newly sequenced ones (Fig. 1 - inset consensus tree). Due to relatively close

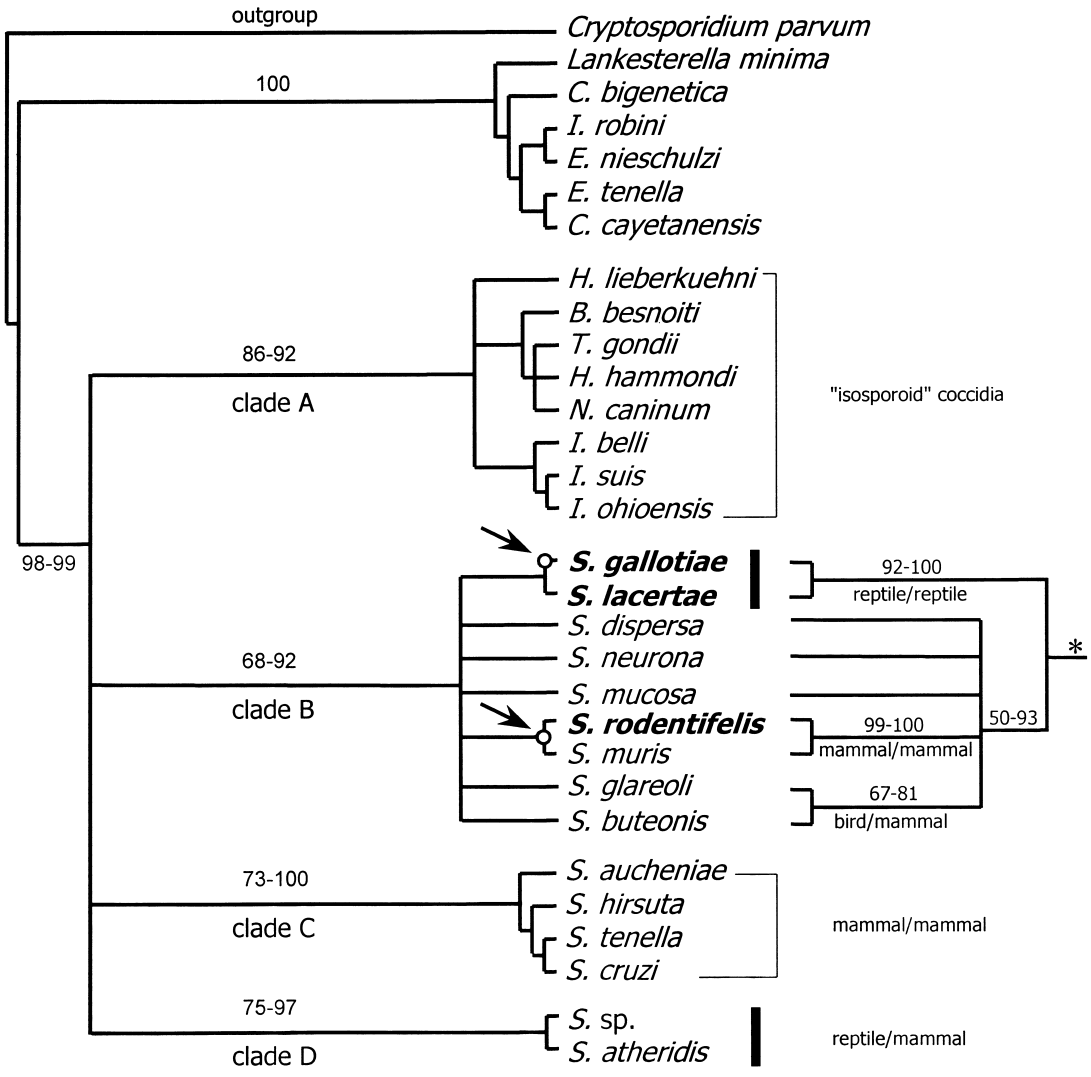

Fig. 1. Phylogenetic relationships among the Sarcocystidae as inferred from the ssrRNA sequences (50% majority rule consensus tree, internal branches in between A–D clades not indicated). Newly included species are in bold. Arrows indicate possible appearances of the dihomoxenous life style. Black bars mark *Sarcocystis* spp. with an ophidian definitive host. The inset consensus tree of the clade B to the right as inferred from the complete ssrRNA sequences rooted (indicated by an asterix) with either *S. atheridis*, *H. lieberkuehni*, *T. gondii* + *H. lieberkuehni*, or *S. cruzi*.

relationships within this clade it was possible to align the entire ssrRNA sequence including the hypervariable domains, which resulted in a more detailed analysis of the 'clade B' species. To root the tree, either *S. atheridis*, *H. lieberkuehni*, *T. gondii* + *H. lieberkuehni*, or *S. cruzi* were chosen, all being the voucher species of the previously recognised four distinct clades within the Sarcocystidae. The alignments contained 1546–1691 aligned sites with gaps included, of which 45–65 were parsimony-informative.

Within clade B three groups with high bootstrap values were identified: the *S. muris* + *S. rodentifelis* group, the *S. buteonis* + *S. glareoli* group, and the *S. lacertae* + *S. gallotiae* group. All analyses suffered a low resolution (often less than 50%) among the above-mentioned groups and other included taxa, that was reflected by low bootstrap values and short branches. The only exception was represented by the well-supported latter group that constituted a sister group to the rest of the 'clade B' species (Fig. 1 - inset consensus tree). Separate analysis of the V4 and V5 domains (corresponding to the region 600–1100 of the *T. gondii* ssrRNA gene) did not alleviate the problem (data not shown).

The tree with monophyletic clades A–D (Fig. 1, unconstrained tree), thus representing polyphyly of reptilian *Sarcocystis* sp. as well as polyphyly of dihomoxenous life cycle had a best likelihood value of $-\ln = 5298$. In contrast, constrained trees either with monophyly of reptilian isolates or monophyly of species with dihomoxenous life cycle had lower likelihood values of $-\ln = 5516$ and $-\ln = 5402$, respectively. According to the Kishino–Hasegawa test, these differences are significant ($P < 0.05$).

As already discussed for medically and veterinary important coccidia of the genera *Neospora*, *Toxoplasma*, and *Hammondia*, the life cycle strategy seems to be a flexible character, which is reflected in a rather facultative status of

the intermediate and/or final hosts. However, the type of the life cycle is still considered by some authors an important feature on the generic level (Frenkel and Dubey, 2000; Mugridge et al., 1999a,b; Odening, 1998). Our results indicate that dihomoxeny evolved independently at least twice: i/in the lineage that includes closely related *S. muris* and *S. rodentifelis*, and ii/in the lineage of *S. gallotiae* after it branched off from the geographically distant but evolutionary related *S. lacertae*. Since *S. lacertae* is, as well as most members of clade B, a strictly heteroxenous species, an ancestral dihomoxeny lost in several lineages does not represent a plausible scenario. Instead, a multiple origin of the dihomoxenous life strategy is a more parsimonious explanation for the observed situation.

When hosts are considered, clade B, to which *S. gallotiae* (lizard/lizard), *S. lacertae* (snake/lizard), and *S. rodentifelis* (cat/rodent) belong, embraces a colorful assembly of former *Frenkelia* spp. (bird/rodent), *S. neurona* (opossum/horse, mouse), *S. dispersa* (owl/mouse), *S. mucosa* (marsupials/? see Jakes, 1998), and *S. muris* (cat/mouse). The composition of this clade and especially the relationships of the newly included species question the degree of coevolution of sarcosporidia with their final hosts. This paradigm is further challenged by the rather unexpected polyphyly of sarcosporidia with an ophidian definitive host. It seems that species parasitising the viperid snakes (*S. atheridis* from Central Africa and *S*. sp. from North America) are rather distantly related to *S. lacertae* that has a European colubrid snake as its definitive host.

Based on the close relationship between the heteroxenous *S. lacertae* and the dihomoxenous *S. gallotiae*, we hypothesise that *S. gallotiae* evolved from an ancestral species that circulated between a colubrid snake and a lizard in the Palearctic region. After being transported to the Canary Islands with rafting lizards, in a new snake-free environment the parasite had to adapt to the lizard/lizard life cycle. However, some level of classical predator-prey heteroxeny (similar to that found in *S. rodentifelis* and *S. muris*) may still be retained, and the ability of *S. gallotiae* to infect snakes as its likely ancient hosts has to be experimentally tested.

Acknowledgements

We thank Dr Jadvyga Grikieniene (Institute of Ecology, Vilnius, Lithuania) for providing the *S. rodentifelis* sporocysts. This study was supported by grants from the Grant Agency of the Czech Academy of Sciences No. A6022903 and the Grant Agency of the Czech Republic No. 524/00/P015.

References

Bannert, B., 1994. Investigation on the host-specificity of dihomoxenous sarcosporidia in the intermediate and definitive host. J. Euk. Microbiol. 41, 183–8.

Barta, J.R., Martin, D.S., Liberator, P.A., Dashkevicz, M., Anderson, J.W., Feighner, S.D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M.C., Danforth, H.D., Ruff, M.D., Profous-Juchelka, H., 1997. Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J. Parasitol. 83, 262–71.

Carreno, R.A., Barta, J.R., 1999. An eimeriid origin of isosporoid coccidia with Stieda bodies as shown by phylogenetic analysis of small subunit ribosomal RNA gene sequences. J. Parasitol. 85, 77–83.

Doležel, D., Koudela, B., Jirků, M., Hypša, V., Oborník, M., Votýpka, J., Modrý, D., Šlapeta, J.R., Lukeš, J., 1999. Phylogenetic analysis of *Sarcocystis* spp. of mammals and reptiles supports the coevolution of *Sarcocystis* spp. with their final hosts. Int. J. Parasitol. 29, 795–8.

Ellis, J.T., Morrison, D.A., Liddell, S., Jenkins, M.C., Mohammed, O.B., Ryce, C., Dubey, J.P., 1999. The genus *Hammondia* is paraphyletic. Parasitology 118, 357–62.

Frenkel, J.K., Dubey, J.P., 2000. The taxonomic importance of obligate heteroxeny: distinction of *Hammondia hammondi* from *Toxoplasma gondii* - another opinion. Parasitol. Res. 86, 783–6.

Gagnon, S., Bourbeau, D., Levesque, R.C., 1996. Secondary structures and features of the 18S, 5.8S and 26S ribosomal RNAs from the Apicomplexan parasite *Toxoplasma gondii*. Gene 173, 129–35.

Grikieniene, J., Arnastauskiene, T., Kutkiene, L., 1993. On some disregarded ways of sarcosporidians circulation and remarks about systematics of the genus *Sarcocystis* Lankester, 1882 with the description of the new species from rodents. Ekologija 1, 16–24 (in Russian with English abstract).

Holmdahl, O.J.M., Morrison, D.A., Ellis, J.T., Huong, L.T.T., 1999. Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNA sequences. Mol. Phyl. Evol. 11, 27–37.

Jakes, K.A., 1998. *Sarcocystis mucosa* in Bennetts wallabies and pademelons from Tasmania. J. Wild. Dis. 34, 594–9.

Jenkins, M.C., Ellis, J.T., Liddell, S., Ryce, C., Munday, B.L., Morrison, D.A., Dubey, J.P., 1999. 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. Parasitology 119, 135–42.

Koudela, B.S., Modrý, D., 2000. *Sarcocystis muris* possesses both diheteroxenous and dihomoxenous characters of life cycle. J. Parasitol. 86, 877–9.

Matuschka, F.R., Bannert, B., 1987. Canibalism and autotomy as predator-prey relationship for monoxenous sarcosporidia. Parasitol. Res. 74, 88–93.

Modrý, D., Šlapeta, J.R., Jirků, M., Oborník, M., Lukeš, J., Koudela, B., in press. Phylogenetic position of a renal coccidium of the European green frogs, '*Isospora*' *lieberkuehni* Labbé, 1894 (Apicomplexa: Sarcocystidae) and the taxonomic implications. Int. J. Syst. Evol. Microbiol.

Mugridge, N.B., Morrison, D.A., Johnson, A.M., Luton, K., Dubey, J.P., Votýpka, J., Tenter, A.M., 1999a. 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. Int. J. Parasitol. 29, 957–72.

Mugridge, N.B., Morrison, D.A., Heckeroth, A.R., Johnson, A.M., Tenter, A.M., 1999b. Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. Int. J. Parasitol. 29, 1545–56.

Odening, K., 1998. The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). Syst. Parasitol. 41, 209–33.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–82.

Thorpe, R.S., McGregor, D.P., Cumming, A.M., Jordan, W.C., 1994. DNA evolution and colonisation sequence of island lizards in relation to geological history – mtDNA RFLP, cytochrome-B, cytochrome-

oxidase, 12S ribosomal-RNA sequence, and nuclear RAPD analysis. Evolution 48, 230–40.

Volf, J., Modrý, D., Koudela, B., Šlapeta, J.R., 1999. Discovery of the life cycle of *Sarcocystis lacertae* Babudieri, 1932 (Apicomplexa: Sarcocystidae), with a species redescription. Folia Parasitol. 46, 257–62.

Votýpka, J., Hypša, V., Jirků, M., Flegr, J., Vávra, J., Lukeš, J., 1998. Molecular phylogenetic relatedness of *Frenkelia* spp. (Protozoa, Apicomplexa) to *Sarcocystis falcatula* Stiles 1893: Is the genus *Sarcocystis* paraphyletic? J. Euk. Microbiol. 45, 137–41.