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Phylogeny and sequence variability of the *Sarcocystis singaporensis* Zaman and Colley, (1975) 1976 ssrDNA

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Abstract The coccidium *Sarcocystis singaporensis* (Apicomplexa: Sarcocystidae) is a cyst-forming parasite with potential as a biological agent for the control of wild populations of rodents in non-native environments. Phylogenetic analysis based on the ssrDNA supports *S. singaporensis* isolates as a sister species to sarcosporidians transmitted between snakes and rodents but an association with the carnivore-ruminant *Sarcocystis* spp. could not be rejected by likelihood ratio tests. Four complete and six partial ssrDNA sequences representing this species are monophyletic in any tree reconstruction method; however, they possess very high pairwise distances of up to 0.053. The obtained sequences suggest the probable existence of at least two divergent paralogous ssrDNAs. Moreover, our results support the co-evolution of lsrDNA and ssrDNA in *S. singaporensis*. The utility of coccidian lsrDNA and ssrDNA for evolutionary studies and their abundance in the primary nucleotide databases is discussed.

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Introduction

Sarcocystis singaporensis (Sarcocystidae: Apicomplexa) has attracted considerable attention in recent years. As an endemic parasite of the South Asian rodents it has potential as a biological agent for the control of wild populations of rodents in non-native environments (Jäkel et al. 1996). It is one of the most intensely studied sarcosporidians since its life cycle, host range and specificity, as well as the ultrastructure of all stages, have been described in detail (Jäkel et al. 1996; Paperna and Martelli 2000). Moreover, the life cycle has been verified under laboratory conditions by several investigators, and the pathways of endogenous development in the intermediate host have been demonstrated in cell cultures in vitro (Jäkel et al. 1999). The host immune response has recently been studied (Jäkel et al. 2001).

Due to the availability of a large set of sequences in databases and the generally good resolution this provides, ssrDNA is a molecule of choice for the reconstruction of coccidian phylogenies. Within the Apicomplexa, coccidian parasites form a monophyletic clade of two sister families (Eimeriidae and Sarcocystidae) distantly related to the *Cryptosporidium* clade (Zhu et al. 2000) with most studies focused on the medically important parasites, such as *Toxoplasma* and *Neospora*. Recently, analysis of the large dataset of available ssrDNA sequences split Sarcocystidae into four distinct clades (labeled A–D), one of which contains solely *Sarcocystis* spp. with a snake-rodent life cycle (Doležel et al. 1999; Jenkins et al. 1999; Mugridge et al. 2000). The dihomoxenous *Sarcocystis* spp. of reptiles and a species with snake-lizard transmission are, however, found in another clade (Šlapeta et al. 2001).

The aim of this study was to determine, using the ssrDNA sequences, the relationship of the “snake (python)/rodent (rat)” species to other sarcosporidians, especially to those with similar final and/or intermediate hosts. In a study that addressed the informational content of the lsrDNA in Sarcocystidae, *S. singaporensis*

and the biologically-related *Sarcocystis zamani* formed a monophyletic clade of snake-rodent species to the exclusion of the other members of the genus (Mugridge et al. 2000). Other species for reptile could not be included in this study because only their *ssrDNA* sequences were known at the time. With the *S. singaporensis* *ssrDNA* sequence available, we have also tested the degree of congruence between the *ssrDNA* and *lsrDNA*-based trees.

Materials and methods

Two different isolates of *S. singaporensis*, GAG3 and GN8 were studied; both were originally collected in Thailand and propagated in a snake-rodent cycle in the laboratory according to established methods (Jäkel et al. 1996). Total DNA was isolated from ethanol-fixed bradyzoites using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR amplification with *Taq* polymerase (Promega) using the JV1-JV2 and ERIB1-ERIB10 primer pairs, cloning and sequencing were performed as described previously (Ślapeta et al. 2001). Briefly, PCR amplicons were gel-isolated and cloned using TOPO TA Cloning Kit (Invitrogen) or pGEM-T Easy Cloning vector (Promega). Five individual colonies for each isolate were sequenced from both directions.

For phylogenetic analysis an alignment of apicomplexan *ssrDNA* sequences, which includes the majority of available taxa with an emphasis on sarcosporidia and life cycle histories, was used (Ślapeta et al. 2001). Using the program Clustal X 1.81 (Thompson et al. 1997) equipped with the profile alignment mode, the following sequences were added to the alignment of Ślapeta et al. (2001): *S. singaporensis*, *Cryptosporidium parvum* L16997, *Cryptosporidium serpentis* AF093500, *Babesia bigemina* X59605, *Theileria equi* Z15105, *Theileria parva* AF013418, *Theileria mutans* AF078815. Additional taxa were included to permit a more extensive outgroup analysis. To reduce systemic bias we tested the influence of different ingroups and outgroups, as well as the effect of the exclusion of highly variable and presumably ambiguous regions on the stability of the obtained trees. In total, 46 *ssrDNA* sequences were analyzed using PAUP*4.0b8 (Swofford DL, 2001, PAUP*, Sinauer, Sunderland, Mass.), by distance, maximum parsimony, and maximum likelihood methods. Gaps were treated as missing data except for pairwise distances calculated for the *S. singaporensis* sequences, which were treated as newstate. The distance method was performed under minimum evolution and distances were made using a general time-reversible measure with rate heterogeneity as the Γ -distribution and proportion of invariant sites (GTR+ Γ +I). Rates of variable sites were assumed to follow the Γ -distribution with the shape parameter $\alpha=0.5$ and the proportion of sites assumed to be invariable was set to 0.4. The maximum likelihood criterion was parameterized in PAUP* according to the tree obtained using the distance method with the corresponding substitution model GTR+ Γ +I. Maximum parsimony trees were constructed by heuristic search with ten random sequence additions. Bootstrap support was calculated for maximum parsimony (300 replicates) and distance method (1,000 replicates). Statistical evaluation of the trees inferred under different topological constraints was performed using the Kishino-Hasegawa and Shimodaira-Hasegawa tests as implemented in PAUP*. Sequence alignments are available on request or are at <ftp://ftp.vfu.cz/slapeta/alignments/>.

Results and discussion

All ten sequences obtained from the two *S. singaporensis* isolates analyzed in this study fall into three categories: (1)

complete *ssrDNA* amplified with the ERIB1 and ERIB10 primers (GN8–5 and GAG3–5, 1,801 and 1,812 bp long, respectively), (2) the 5' and 3' incomplete *ssrDNA* ends amplified with the JV1 and JV2 primers (GN8–2 and GAG3–3, 1,623 and 1,629 bp long, respectively), and (3) partial *ssrDNA* amplified with internal primers covering the highly variable domains (888–1,262 bp long).

Sequences representing the single species *S. singaporensis* possess high pairwise distances, ranging from 0.002–0.01 (2–13 bp), 0.002–0.053 (2–80 bp) and 0.002–0.053 (2–86 bp), for GN8, GAG3 and GN8+GAG3, respectively. The high variability is primarily caused by two sequences, GAG3–1 (AF434055) and GAG3–5 (AF434059), which differ from each other at only two positions. Both sequences contain an unexpectedly high number of variables confined to the conservative *ssrDNA* regions of the alignment. In the GAG3–3 clone, a 7-bp-long region is missing in the V4/E21–3 domain. All available *S. singaporensis* sequences overlap in a 889-bp-long region that contains six parsimony-uninformative and 47 parsimony-informative differences (Table 1). Phylogenetic inference based on this alignment places GAG3–1 and GAG3–5 into a monophyletic clade very distantly related to the remaining GAG3 and GN8 sequences (Fig. 1; inset). These results suggest that two divergent paralogous *ssrDNAs* may exist in our dataset.

Phylogenetic analysis of these sequences supports (>90% bootstrap) the monophyletic character of both *S. singaporensis* isolates, but molecules derived from them do not constitute two separate clades. In addition, using highly divergent GAG3 and GN8 sequences as outgroups to the remaining *S. singaporensis* *ssrDNA* sequences did not provide robust resolution (data not shown). Phylogenetic relationships of other *Sarcocystis* species were analyzed using the complete sequences of GN8–2, GN8–5, GN3–3 and GN3–5. The inclusion or omission of the highly divergent GAG3–5 and GAG3–1 sequences had no effect on the overall results (data not shown). Distance and maximum likelihood methods placed *S. singaporensis* into clade D, which includes the snake-rodent sarcosporidians. Using maximum parsimony *S. singaporensis* appeared with moderate (~65%) bootstrap support as a sister species to clade C (the carnivore-ruminant species). Although in the Kishino-Hasegawa and Shimodaira-Hasegawa tests the association of *S. singaporensis* with the clade D has the best likelihood value, neither of the tests could unambiguously resolve the position of *S. singaporensis* ($P < 0.05$). While the support for clade B was low to moderate, monophyly of clades A and C was generally high (Fig. 1). The within-clade (A–D) resolution varied depending on the tree reconstruction method.

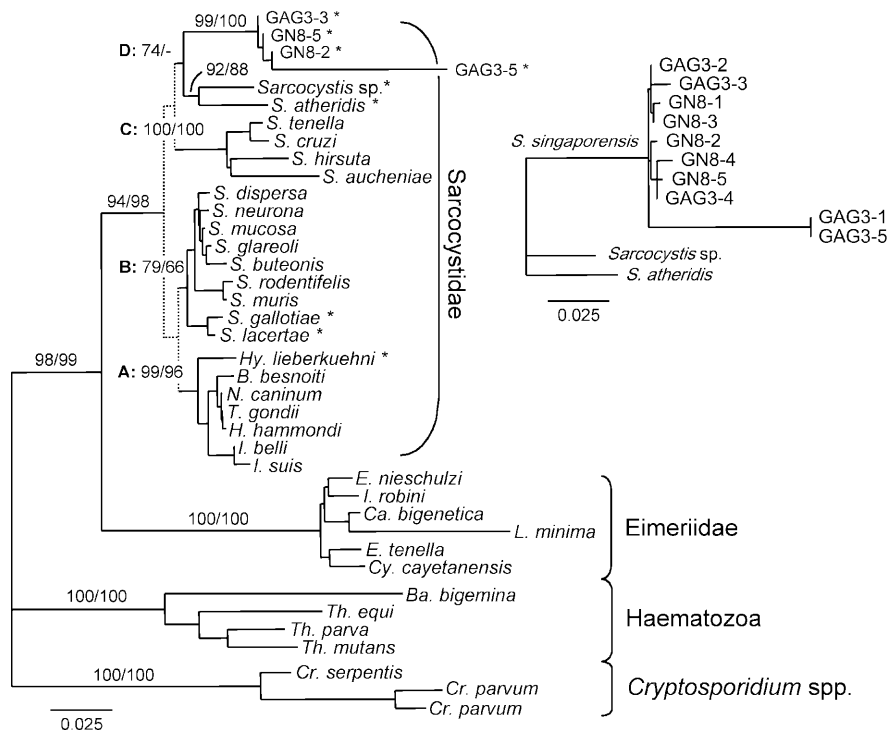
So far, the *ssrDNA* data have favored co-evolution of sarcosporidia parasitizing snakes with their final rather than intermediate host (Doležel et al. 1999). Our results are consistent with this scenario, since *S. singaporensis* from an Asian python forms a sister species to *Sarcocystis atheridis* from an African viper and *Sarcocystis* sp.

Table 1 Variability of *ssrDNA* of *Sarcocystis singaporensis* within partial residues corresponding to V3–V4 domains. Sequences

Nos. to <i>T. gondii</i> model and structural domains	441–V3/16	444–V3/16	485–V3/17	491–V3/17	495–V3/17	512–V3/17	547–V3/19	571–V3/19	587–V3/19	594–V3/19	618–V3/20	651–V4/E21-1	654–V4/E21-1	665–V4/E21-1	702–V4/E21-3
Nos. to ERIB primers	480	483	524	530	534	551	586	610	626	633	657	690	693	704	747
GN8-1	G	C	C	A	T	A	T	G	C	A	T	A	C	T	T
GN8-2	T	C	C	A	A	A	T	G	C	A	T	A	C	G	T
GN8-3	G	C	C	A	T	A	T	G	C	A	T	A	C	G	T
GN8-4	T	C	C	A	A	A	T	G	C	A	T	A	C	T	T
GN8-5	T	C	C	A	T	A	T	G	C	A	T	A	C	G	T
GAG3-1*	T	T	G	G	T	G	T	A	G	A	G	G	T	T	C
GAG3-2	T	C	C	A	T	A	T	G	C	A	T	A	C	G	T
GAG3-3	T	C	C	A	T	A	G	G	C	C	T	A	C	G	C
GAG3-4	T	C	C	A	T	A	T	G	C	A	T	A	C	G	T
GAG3-5	T	T	G	G	T	G	T	A	G	A	G	G	T	T	C

from an American rattlesnake. The branching order may even reflect the fact that the pythons are evolutionarily a more ancient group than the vipers (Heise et al. 1995). *Sarcocystis lacertae* and *Sarcocystis gallotiae* that have lizards as final and/or intermediate hosts remain in clade B, which includes species with a diverse range of hosts (Slapeta et al. 2001). Incongruence in co-evolution, as exemplified by some lizard parasites, may reflect the

existence of a common ancestor with low host specificity that acquired, by prey capture, new definitive and/or intermediate hosts. Resolution within the mixed clade B did not change significantly either in the *lsrDNA*-based trees, regarded to be more informative due to the presence of a high number of variable positions (Mugridge et al. 2000), or when the concatenated (*ssrDNA* and *lsrDNA*) alignments were analyzed (data not shown).



(GAG3-1, GAG3-5) with high number of variables are in *bold*

703-V4/ E21-3	706-7-V4/ E21-3	709-V4/ E21-3	714-6-V4/ E21-3	720-V4/ E21-3	736-7-V4/ E21-3	742-V4/ E21-5	778-81-V4/ E21-6	785-V4/ E21-6	829-30- V4/E21-7	833-V4/ E21-7	883- V4/22	896- V4/23	944- V4/24
749	752-3	755	761-7	770-1	787-8	793	829-832	836	880-1	885	936	949	997
A	GT	G	TAC- CTC	GT	TA	T	TTGT	C	AA	T	T	T	A
A	GT	G	TAC- CTC	GT	TA	C	TTGT	C	CT	T	T	T	A
A	GT	G	TAC- CTC	GT	TA	T	TTGT	C	AA	T	T	T	A
A	GT	A	TAC- CTC	GT	GA	T	TGAT	C	AT	T	T	T	A
A	GT	G	TAC- CTC	GT	TA	T	TGAT	C	CT	T	C	T	A
A	AC	A	CGT	AC	TC	T	CTAC	T	AT	C	T	G	T
A	GT	G	ATT TAC- CTC	GT	TA	T	TTGT	C	AA	T	T	T	A
G	AT	G	-	TT	TA	T	TTGT	C	AA	T	T	T	A
A	GT	G	TAC- CTC	GT	TA	T	TGAT	C	CT	T	T	T	A
A	AC	A	CGT	AC	TC	T	CTAC	T	AT	C	T	G	T
			ATT CTC										

Our analysis supports the co-evolution of the nuclear *ssrRNA* and *lsrRNA* genes.

Phylogenetic analysis provides one of several influential frameworks for the interpretation of biological data. From Table 2, in which the available, up-to-date coccidian rDNA sequences are plotted against the taxonomic groups from which they were derived, it is clear that the *ssrDNA* is the most abundant molecular marker known for 63 species. The set of *lsrDNA* sequences is representative for the Sarcocystidae, while the Eimeriidae are highly underrepresented with only *Eimeria tenella* being sequenced. In several cases, single sequence phylogenies, mainly based on the *ssrDNA*, were insufficient for the inference of correct relationships and more markers should be included, while at the same time the *ssrDNA* represents the most widely available molecular marker for coccidians. Therefore, to study the phylogeny of coccidian taxa (genera or species), *ssrDNA* re-

mains the marker of choice that covers the diversity within this group. In controversial cases, or to differentiate among closely related species such as the groups of *Hammondia/Neospora/Toxoplasma*, *Sarcocystis neu-rona/Sarcocystis falcatula* or *Cryptosporidium* spp. other genes from voucher species must be sequenced (Mugridge et al. 2000; Zhu et al. 2000; Zhao et al. 2001).

The aim of this study was to assess the evolutionary relationships of the model species *S. singaporensis* using the previously unavailable marker – its *ssrDNA*. We

Table 2 Number of *ssrDNA* and *lsrDNA* sequences belonging to defined species with regard to genera and family within the Coccidia. Only those sequences exceeding 1.5 kb and 3.0 kb for the *ssrDNA* and *lsrDNA*, respectively, were included. The number in parentheses indicates number of available sequences with genus and species affiliation plus sequences referred as *species* with no species name (= 4 *Cyclospora* spp./ *Neospora* sp./ *Sarcocystis* sp.). Data were retrieved from the Taxonomy Browser at NCBI server (as of 1 October 2001); no multiple hits (redundancy) of single species were encountered. For details about molecular phylogeny of Eimeriidae genera, see Barta et al. (2001)

Family	Genus	<i>ssrDNA</i>	<i>lsrDNA</i>
Eimeriidae	<i>Isospora</i> sensu stricto	1	–
	<i>Eimeria</i>	29	1
	<i>Cyclospora</i>	4 (8)	–
	<i>Caryospora</i>	1	–
	<i>Lankesterella</i>	1	–
Sarcocystidae	<i>Toxoplasma</i>	1	1
	<i>Neospora</i>	1 (2)	1
	<i>Hammondia</i>	1	2
	<i>Besnoitia</i>	2	1
	<i>Isospora</i> sensu stricto	2	1
	<i>Hyaloklossia</i>	1	–
	<i>Sarcocystis</i>	19 (20)	13
	Total	63 (69)	20

Fig. 1 Phylogenetic tree based on the apicomplexan *ssrDNA* sequences obtained using the minimum evolution criterion. Haematozoa and *Cryptosporidium* sp. were treated as an outgroup; Eimeriidae and Sarcocystidae form sister groups of Coccidia. Within the Sarcocystidae four clades (A–D) are present; however, the relationships within these clades could not be robustly resolved by any alignment optimization or tree reconstruction method used (indicated by dotted lines). *Sarcocystis singaporensis*, as a sister species to *Sarcocystis atheridis* and *Sarcocystis* sp., is also supported by maximum likelihood. Bootstrap values (> 50%) corresponding to major branches are for minimum evolution/maximum parsimony. Species with reptile hosts are indicated by an asterisk. The maximum likelihood tree in the inset represents a phylogram restricted to the *S. singaporensis* sequences; the snake-rodent *Sarcocystis* spp. served as an outgroup. The scales of the trees are identical

sequenced five *ssrDNA* molecules for each *S. singaporensis* isolate and encountered surprisingly high intra-clone variability (Table 1.). Differences among eight out of ten obtained sequences approached ~1% sequence variation, while the ~5% distance of the remaining two sequences (GAG3-1 and GAG3-5) was strikingly high. The eukaryotic genome contains multiple copies of rDNA units (Hillis and Dixon 1991) with a high sequence similarity for a given organism. In the most studied coccidian, *Toxoplasma gondii*, 110 rDNA units exist. Secondary structure modeling of the *T. gondii* *ssrDNA* delineated hypervariable regions, especially the domain V4-E21 (Gagnon et al. 1996). On a set of well-defined strains of *T. gondii*, Luton et al. (1995) encountered a maximum of 1% inter-strain sequence difference in the *ssrDNA*, with no correlation to virulence in mice. In a typical multigene family, exemplified by the multicopy rDNA units, the overall sequence similarity within a single organism is high due to concerted evolution (Buckler et al. 1997; Liao 1999). In *lssrDNA* and *ssrDNA*, intraspecific variability has seldom been reported and a single sequence per species is typically presented. In this respect, *Plasmodium* represents an exception with three stage-specific divergent rDNA paralogs found in its genome (Li et al. 1997). Therefore, the high level of sequence difference among the *ssrDNA* of *S. singaporensis* is of some interest.

There are several plausible explanations for the presence of highly variable *ssrDNA* sequences in our sample:

1. Artifacts caused by *Taq* polymerase. These are usually single-nucleotide substitutions introduced by low fidelity enzymes. Since the nucleotide differences in question include insertions and deletions, which were present in at least two independent clones, this possibility can be excluded.
2. A mixture of different species is present. Although monospecific infection, based on morphological criteria, of laboratory rats with *S. singaporensis* was established in the case of both isolates, this cannot be entirely ruled out without having cloned parasite species (T. Jäkel, pers comm).
3. The presence of divergent rDNA paralogs. Both divergent sequences (GAG3-1 and GAG3-5) constitute very long branches and are highly homoplasious in variable sites. Based on this feature, we propose that there are at least two significantly diverged paralogs of the *ssrDNA* in *S. singaporensis* – the functional copy and the highly divergent copy.

Whether the highly divergent sequence is also functional or represents a pseudogene is not known. Further data on the *ssrDNA* organization and transcription are required to settle this issue. Phylogenetic reconstruction using rDNA is a powerful tool in systematics and taxonomy. Identified paralogs and pseudogenes may provide a better opportunity for outgroup analysis and the resolution of species trees (Buckler et al. 1997) as well as for the interpretation of biological relationships.

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References

- Barta JR, Martin DS, Carreno RA, Siddall ME, Profous-Juchelka H, Hozza M, Powles MA, Sundermann C (2001) Molecular phylogeny of the other tissue coccidia: *Lankesterella* and *Caryospora*. *J Parasitol* 87:121–127
- Buckler ES, Ippolito A, Holtsford TP (1997) The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics* 145:821–832
- Doležel D, Koudela B, Jirk[uring] M, Hypša V, Oborník M, Votýpka J, Modrý D, Šlapeta JR, Lukeš J (1999) Phylogenetic analysis of *Sarcocystis* sp. and *Sarcocystis dispersa* supports the co-evolution of sarcocysts with the final hosts. *Int J Parasitol* 29:795–798
- Gagnon S, Bourbeau D, Levesque RC (1996) Secondary structures and features of the 18S, 5.8S and 26S ribosomal RNAs from the Apicomplexan parasite *Toxoplasma gondii*. *Gene* 173:129–135
- Heise PJ, Maxson LR, Dowling HG, Hedges SB (1995) Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rDNA and 16S rDNA genes. *Mol Biol Evol* 12:259–265
- Hillis DM, Dixon MT (1991) Ribosomal DNA – molecular evolution and phylogenetic inference. *Q Rev Biol* 66:411–453
- Jäkel T, Burgstaller H, Frank W (1996) *Sarcocystis singaporensis*: studies on host specificity, pathogenicity, and potential use as a biocontrol agent of wild rats. *J Parasitol* 82:280–287
- Jäkel T, Archer-Baumann C, Boehmler AM, Sorger I, Henke M, Kliemt D, Mackenstedt U (1999) Identification of a subpopulation of merozoites of *Sarcocystis singaporensis* that invades and partially develops inside muscle cells in vitro. *Parasitology* 118:235–244
- Jäkel T, Khoprasert Y, Kliemt D, Mackenstedt U (2001) Immunoglobulin subclass responses of wild brown rats to *Sarcocystis singaporensis*. *Int J Parasitol* 31:273–283
- Jenkins MC, Ellis JT, Liddell S, Ryce C, Munday BL, Morrison DA, Dubey JP (1999) The relationship of *Hammondia hammondi* and *Sarcocystis mucosa* to other heteroxenous cyst-farming coccidia as inferred by phylogenetic analysis of the 18S SSU ribosomal DNA sequence. *Parasitology* 119:135–142
- Li J, Gutell RR, Damberger SH, Wirtz RA, Kissinger JC, Rogers MJ, Sattabongkot J, McCutchan TF (1997) Regulation and trafficking of three distinct 18S ribosomal RNAs during development of the malaria parasite. *J Mol Biol* 269:203–213
- Liao D (1999) Concerted evolution: molecular and biological implications. *Am J Hum Genet* 64:24–30
- Luton K, Gleeson M, Johnson AM (1995) Ribosomal RNA gene sequence heterogeneity among *Toxoplasma gondii* strains. *Parasitol Res* 81:310–315
- Mugridge NB, Morrison DA, Jäkel T, Heckerroth AR, Tenter AM, Johnson AM (2000) Effects of sequence alignment and structural domains of ribosomal DNA on phylogeny reconstruction for the protozoan family Sarcocystidae. *Mol Biol Evol* 17:1842–1853

- Paperna I, Martelli P (2000). Fine structure of the development of *Sarcocystis singaporensis* in *Python reticulatus* from macrogamont to sporulated oocyst stage. *Parasite* 7:193–200
- Šlapeta JR, Modrý D, Votýpka J, Jirk[uring] M, Koudela B, Lukeš J (2001) Multiple origin of the dihomoxenous life cycle in sarcosporidia. *Int J Parasitol* 31:414–417
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Zhao X, Duszynski DW, Locker ES (2001) Phylogenetic position of *Eimeria antrozoi*, a bat coccidium (Apicomplexa: Eimeriidae) and its relationship to morphologically similar *Eimeria* spp. from bats and rodents based on nuclear 18S and plastid 23S rDNA. *J Parasitol* 87:1120–1123
- Zhu G, Keithly JS, Philippe H (2000) What is the phylogenetic position of *Cryptosporidium*? *Int J Syst Evol Microbiol* 50:1673–1681