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Evolutionary relationships among cyst-forming coccidia *Sarcocystis* spp. (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle

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Abstract

Cyst-forming coccidia of the genus *Sarcocystis* (Alveolata: Apicomplexa: Coccidea) parasitize vertebrates worldwide. Data from the small subunit rRNA genes (SSU) and the D2 domain of the large subunit rRNA genes were used to reconstruct phylogeny for all species in the Sarcocystidae for which sequences are currently available. We have focused on the evolutionary history of species that circulate between snakes as definitive hosts and rodents as intermediate hosts. Trees were reconstructed using maximum parsimony, minimum evolution, maximum likelihood and the bayesian phylogenetics. Our reconstructions support monophyly of Sarcocystidae but fail to robustly resolve the relationship within clades. Using a concatenated dataset of available rDNAs, the ''isosporoid'' coccidia *Neospora*, *Toxoplasma*, *Besnoitia*, *Isospora* and *Hyaloklossia* form a sister group to the monophyletic *Sarcocystis*. Moreover, we show that *Sarcocystis* from arboreal vipers of the genus *Atheris*, which are endemic to the mountain rain forests of the Equatorial Africa, are monophyletic, with sister species parasitizing the desert viper *Pseudocerastes persicus* from the Near East. We report the co-evolution of *Sarcocystis* spp. with their final snake hosts. The geological history of the African continent, mountain ranges, forests and general SSU rDNA rates were used to construct a linearized tree. Possible origin of the heteroxenous life cycle of *Sarcocystis* is discussed.

1. Introduction

Protists of the genus *Sarcocystis* (Alveolata: Apicomplexa: Coccidea) are ubiquitous parasites of vertebrates, causing disease in wild as well as in domesticated animals. Also named sarcosporidia, they were known as ''milky white threads'' from the 19th century. Although predicted already one hundred years ago (Minchin, 1903), the heteroxenous life cycle remained elusive until 1972 (Dubey et al., 1989). In most, if not all members of the genus *Sarcocystis*, the heteroxenous cycle comprises definitive and intermediate hosts and is dependent on the predator–prey relationship.

The genus *Sarcocystis* is speciose with over 200 named species found in mammals, birds, reptiles and fish (Odening, 1998). Many of the species are poorly described with limited data on ultrastructure, host spectrum and the life cycle. Complexity of the life history and host specificity has emerged from the descriptions of several species within a single host, either definitive or intermediate. Besides canine and feline *Sarcocystis* spp. of veterinary importance, several species parasitizing pythons and rodents in South East Asia

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have played an important role in recognizing the importance of ultrastructural features of sarcocysts for the identification of species (Dubey and Odening, 2001; Dubey et al., 1989).

The phylogeny of *Sarcocystis* was first studied for species infecting economically important animals (Tenter and Johnson, 1997). Recently, sarcosporidians are being increasingly explored as useful model organisms for the studies of host–parasite evolution, phylogeny, pathology, with a potential in biological warfare against pests (Doležel et al., 1999; Jäkel et al., 1996; Šlapeta et al., 2001). In this study, we analyze in detail the diversity and biogeography of the *Sarcocystis* isolates from African vipers. Vipers of the African genus *Bitis* are known to serve as definitive hosts for *Sarcocystis dirumpens* (Häfner and Matuschka, 1984; Hoare, 1933) which has an unusually low specificity to its intermediate rodent hosts (Matuschka, 1987). Subcutaneous localization and ultrastructural features of the cyst discriminate *S. hoarensis* which shares the host spectrum with *S. dirumpens* (Matuschka et al., 1987). Furthermore, experimental infections and morphological studies with the isolate obtained from the African tree viper (*Atheris nitschei*) resulted in the description of *S. atheridis* (Šlapeta et al., 1999). The diversity and distribution of African vipers of the genera *Atheris* and *Bitis* (Spawls et al., 2002) and abundance of their *Sarcocystis* isolates have sparked our interest about the co-evolution of these parasites with their hosts.

To address the evolutionary history and diversity of parasites of the genus *Sarcocystis* in vipers, the nuclear rDNA has been selected. Although known to have some limitations, small subunit rDNA (SSU) is generally thought to be a good marker for addressing relationships of apicomplexan species and their history (Morrison and Ellis, 1997; Zhu et al., 2000). Our objectives were to study: (i) the prevalence and distribution of *Sarcocystis* spp. in African vipers; (ii) biology of the isolates: host-specificity using experimental infections, pathology and ultrastructure, to assess the value of biological and morphological characteristics for distinguishing the species; (iii) the usefulness of rDNA sequences for mapping the host–parasite life cycle history in general, and the relationships of known *Sarcocystis* of snakes in particular.

2. Materials and methods

2.1. Study area and isolates

Fecal samples from vipers of the genus *Atheris* and *Bitis* (Serpentes, Viperidae) were collected in Kenya and Tanzania in 1998–2000. Moreover, feces of recently imported specimens from this area, as well as from West Africa, were obtained for examination (Table 1). Species of the genus *Atheris* are described as bush vipers, some being widespread in the African lowland forests (*Atheris squamigera*, *Atheris chlorechis*), others being endemic to humid forests on the foot of the mountains in an altitude of 1500–2000 m up to the bamboo zone (Broadley, 1998; Lawson et al., 2001). *Atheris desaixi* were collected at the type locality in the Mt. Kenya forest near Chuka, Kenya. Unlike *Atheris* spp., *Bitis* spp. are the most common African vipers found throughout sub-Saharan Africa in all types of habitats (Spawls et al., 2002). Fecal samples stored in 2% potassium dichromate were examined using Sheater's sugar flotation technique.

Table 1
Summary of definitive host species, site of origin and morphometry of *Sarcocystis* spp. sporocysts

Host [a]	Locality	Sporocyst, shape index (μm, length/width ration)	Sarcocystis positive/ examined snakes (*n*)	*Sarcocystis* sp.
Atheris nitschei [b]	Uganda, Ruwenzori Mts.	10.4 (10.0–11.0) × 8.0 (7.0–8.5), 1.3 (1.24–1.43)	6/7	*S. atheridis*
Atheris desaixi	Kenya, Mt. Kenya	10.9 (10.0–12.0) × 9.1 (8.5–9.5), 1.19 (1.05–1.35)	4/8	*S.* sp. "I"
Atheris rungweensis	Tanzania, Mt. Rungwe	10.4 (9.5–11.5) × 7.6 (7.0–9.0), 1.37 (1.11–1.57)	1/1	*S.* sp. "II"
Atheris chlorechis	West Africa	10.6 (10.0–11.7) × 9.2 (8.0–10.0), 1.18 (1.15–1.45)	1/3	*S.* sp.
Atheris ceratophora	Tanzania	No	0/6	No
Atheris squamigera	Cameroon	No	0/2	No
Atheris broadleyi	Cameroon	No	0/1	No
Proatheris superciliaris	Tanzania, Malawi	No	0/1	No
Bitis gabonica [c]	Kenya, Tanzania	12.0 (11.5–12.5) × 9.7 (9.0–10.0), 1.17 (1.10–1.30)	1/6	*S.* sp.
Bitis arietans [c]	Kenya, Tanzania	11.7 (11.0–12.5) × 9.4 (9.0–10.0), 1.24 (1.15–1.39)	3/7	*S.* sp. "III"
Bitis nasicornis [c]	West Africa	12.2 (11.5–13.0) × 9.2 (8.0–10.5), 1.28 (1.12–1.42)	1/1	*S.* sp.
Total besides *A. nitschei*			11/36 (30.6%)	
Total with *A. nitschei*			17/43 (42.5%)	

[a] Tribe Atherini comprises genus *Atheris* with 11 species (not listed are: *A. katangensis*, *A. subocularis*, *A. hispida*, and *A. acuminata*) and three terrestrial monospecific genera *Proatheris*, *Montatheris*, and *Adenorhinos* (Broadley, 1998; Lawson et al., 2001).

[b] Data adopted from Šlapeta et al. (1999).

[c] Known to harbor *S. dirumpens* and *S. hoarensis* (Matuschka, 1987).

2.2. Experimental design and sampling procedure for Sarcocystis

For determination of the intermediate host, experimental oral infections of the following rodent species were performed (origin and number of animals are given in parenthesis): laboratory mice (CD-1; 47), laboratory rats (Wistar-Han; 24), multimammate rats *Mastomys* cf. *natalensis* (captive; 2), *Lemniscomys barbarus* (captive; 9) and *Lemniscomys striatus* (captive; 6) (also see Table 2). *L. striatus* were F_1 and F_2 generations originating from the locality of *A. desaixi*. Inoculated rodents (3×10^3–1×10^5 sporocysts) were monitored daily, euthanized and necropsied at different days postinfection (DPI) (for details see Table 2).

In order to extend the dataset, we have included three non-African species: *Sarcocystis* sp. "IV" isolated from feces of the desert viper *Pseudocerastes persicus* from Jordan and experimentally transmitted to jirds and gerbils (*Meriones* and *Gerbillus* spp.) (D.M., unpublished data). *Pseudocerastes persicus* is a sister species to *Atheris* spp. (Herrmann et al., 1999). Two isolates (*Sarcocystis* sp. "V", *Sarcocystis* sp. "VI") represent species found in the striated musculature of European shrews, *Sorex araneus* trapped in 2000 in the Czech Republic (D.M. & J.V., unpublished data).

2.3. Light and electron microscopy

At necropsy, the following tissue samples were collected from each experimental rodent and fixed in 10% buffered formalin: esophagus, stomach, jejunum, ileum, caecum, rectum, brain, lung, liver, kidney, spleen, tongue, heart, diaphragm, muscles of the abdominal wall, brachial muscles, femoral muscles, masseters, upper lip, genital area, and ear. For light microscopy the fixed tissues were processed by standard histological protocols. Paraffin sections were stained with haematoxylin–eosin and Giemsa. Smears of cystozoites were air-dried, fixed with methanol and stained with Giemsa. Muscles containing sarcocysts were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C overnight, postfixed in 1% OsO_4 in the same buffer, dehydrated in a graded series of ethanol and embedded in Durcupan resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 transmission electron microscope.

2.4. DNA isolation, cloning and sequencing

Total DNA from samples stored in 96% ethanol or at −20 °C were isolated using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Concentrated suspensions of cystozoites from either individually dissected sarcocysts or trypsinized infected muscles of the intermediate hosts were used for DNA isolation. Total DNA of *Hyaloklossia lieberkuehni* of the European green frog, *Rana* kl. *esculenta* and *Goussia janae* of the dace, *Leuciscus leuciscus*, was from our previous studies (Jirků et al., 2002; Modrý et al., 2001). PCR amplification with *Taq* polymerase (Promega) was performed using the JV1–JV2 primer pair (Votýpka et al., 1998) for SSU rDNA and CR1–CR2 primer pair (Ellis et al., 1998) for the D2 domain of LSU rDNA (D2 LSU). Amplification consisted of 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 74 °C for 2 min. Amplicons were cloned using the TOPO-TA Cloning Kit (Invitrogen) and three clones were sequenced in both directions on either the ABI or Beckman automatic sequencers. The D2 LSU rDNA amplicons of *Sarcocystis* sp. "V" and *Sarcocystis* sp. "VI" were gel-isolated (Qiagen) and sequenced directly. All sequences were assembled from multiple overlapping regions using the program SeqMan II (DNAStar, Madison, WI, USA), and after removing the amplification primer sequences they were deposited in GenBank (#AF513487–AF513499).

Table 2
Results of experimental infections with *Sarcocystis* spp. isolates and development in rodents

Host of *Sarcocystis* isolate	Sporocysts dose	Intermediate hosts inoculated/sarcocystis positive (DPI examination)				
		Laboratory mouse	Laboratory rat	Multimammate rat	*L. barbarus*	*L. striatus*
A. desaixi	5.000	–	–	–	3/**2** (60)	–
	10.000	3/0 (8, 9, 10) 5/**2** (75)	5/0 (80)	–	1/0 (10)	1/0 (8) 2/**1** (80)
	100.000	5/**4** (80)	–	–	–	–
A. rungweensis	10.000	5/**2** (60) 3/0 (8, 9, 10)	5/0 (60)	–	1/0 (9) 1/0 (65)	1/0 (9) 1/0 (65)
	100.000	3/**2** (80)	–	–	–	–
A. chlorechis	3.000	5/0 (65)	–	–	1/0 (60)	1/0 (60)
B. gabonica	10.000	5/0 (70)	3/0 (60)	–	–	–
B. arietans	10.000	8/0 (60)	8/0 (60)	2/**1** (200)	2/0 (60)	–
B. nasicornis	10.000	5/0 (90)	3/0 (90)	–	–	–

2.5. Molecular phylogeny

Initial sequence alignment of the apicomplexan SSU rDNA sequences was downloaded from The European rRNA Database, where sequences are aligned according to the secondary structure (Van de Peer et al., 2000). Sequences obtained in this study were aligned using the profile mode in the program Clustal X version 1.81 (Thompson et al., 1997). Use of secondary structure pre-aligned sequences is a favorable conservative approximation for the construction of an alignment, since the sequence alignment strategy is known to have important impact on phylogenetic inference (Hickson et al., 2000; Morrison and Ellis, 1997). We excluded all gapped residues to minimize the artifacts caused by problematic positions of indels. Furthermore, the stability of studied groups of sequences and the involvement of systemic bias was tested by the deletion of taxa or whole taxon groups.

The SSU rDNA alignment comprised 55 sequences. Out of these, 34 sequences belong to the Sarcocystidae, represented by all up-to-date available complete or almost complete SSU rDNAs. *Sarcocystis buteonis* and *Sarcocystis glareoli* were originally placed in the genus *Frenkelia* (species *Frenkelia microti* and *Frenkelia glareoli*, respectively) that was recently synonymized with the genus *Sarcocystis* (Odening, 1998). If more than one sequence is available for a species or an isolate (e.g. *Toxoplasma gondii*), only one sequence was considered. The only exception was *Sarcocystis singaporensis*, which is represented by two highly divergent sequences (Šlapeta et al., 2002). The concatenated alignment of SSU rDNA and D2 LSU rDNA has been constructed for 25 species/isolates, with 21 belonging to the Sarcocystidae.

Sequences were analyzed using the program PAUP*4.0b8 (D.L. Swofford, 2001, PAUP*, Sunderland, MA: Sinauer Associates), by distance, maximum parsimony (MP), and maximum likelihood (ML) methods. Distance method was performed under minimum evolution (ME) and distances treated using the LogDet/paralinear model and KHY85 + Γ with $\alpha = 0.5$. Maximum parsimony trees were constructed by 50 random-addition-sequence heuristic search replicates with the NNI swap algorithm. Bootstrap support was calculated for MP (500 replicates) and distance methods (2000 replicates). Weighed MP analysis was performed with the cost of a transversion equal to two and three times the cost of a transition. Since full likelihood searches of larger datasets are prohibitively time consuming, we used the hierarchical likelihood ModelTest version 3.06 (Posada and Crandall, 1998) for optimization of the likelihood search. The ML estimates were reconstructed in PAUP* using heuristic search with the NNI swap algorithm, originally based on the neighbor joining tree and repeated further using ML reconstructed tree. All sequence accession numbers and alignments are available on request or at ftp://ftp.vfu.cz/slapeta/alignments/sarco/.

To further test the evolutionary relationships of Coccidea under consideration, we have used the bayesian analysis as implemented in the program MrBayes, version 2.01 (Huelsenbeck and Ronquist, 2001). It uses the Metropolis coupled Markov chain Monte Carlo search strategy, where with minimal computation time and only on the posterior probability a hypothesis (= tree) is found by sampling the entire posterior probability distribution (= tree space) (Huelsenbeck et al., 2001). The GTR + Γ model of DNA substitution was used with four chains per run and a default temperature set to 0.2. The Markov chain was started from a random tree and run for 1,000,000 generations, sampled every 100 generations. The first 50,000 generations were discarded and the posterior probabilities were calculated by getting a majority rule consensus of the bayesian tree samples in PAUP*.

Phylogenetic relationship of *Sarcocystis* spp. and their final hosts have been evaluated using the program TreeMap 1.0a (Page, 1995), with an algorithm that maximizes the number of co-speciations on superimposed host and parasite trees. Evolutionary events are divided into four categories, which are combined by TreeMap for reconstruction of hypothetical scenarios: co-speciation, duplication, host switch, and sorting events (Page and Charleston, 1998).

Finally, we adopted molecular clock hypothesis to the snake–rodent *Sarcocystis* spp. clade. We have tested the clock and non-clock hypotheses using the likelihood ratio test of PAUP*. The clock model represents the null hypothesis (special case of the more general non-clock model). For χ^2, the degrees of freedom equal s-2, where s is the number of taxa in the tree (Huelsenbeck and Crandall, 1997). The linearized trees for the implementation of calibration points and rates of evolution were performed in MEGA 2.1 (S. Kumar, K. Tamura, I.B. Jakobsen, and M. Nei, 2001, MEGA 2: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, AZ, USA). Incomplete understanding of the clock for nuclear rDNA, as well as the lack of fossil records for coccidian parasites makes the interpretation of dates derived from this hypothesis difficult. However, since the 1980s, there have been trials to use SSU rDNA for the estimation of divergence of eubacteria and eukaryotes (Sogin, 1991). Moreover, Escalante and Ayala (1995) used general evolutionary rate of 0.85% sequence divergence/100 million years (Myr) for divergence of the Apicomplexan parasites based on the entire SSU rDNA, and rate 2 or 4% sequence divergence/100 Myr biased to the hypervariable regions. Furthermore, the following dated events were used for tree calibration: formation of the first land bridges and the early formation of Rift Valley (Oligocene, 35 Myr), and the beginning of intense forest

oscillations (Pliocene, 3 Myr) (Braile et al., 1995; Fjeldså and Lowett, 1997; Graur and Li, 1999; Maley, 1996).

3. Results

3.1. Sarcocystis *spp. in African vipers*

In this study we have examined seven out of 14 known snake species of the tribe Atherini confined to the Equatorial Africa (Broadley, 1998; Lawson et al., 2001). Moreover, 14 specimens belonging to three species of the genus *Bitis* were also surveyed. In total, 36 individual snakes of the subfamily Viperinae were examined, from which 11 individuals (30.6%), representing six viper species, harbored *Sarcocystis* spp. in their feces (Table 1).

Predominantly liberated, sporulated sporocysts of *Sarcocystis* spp. were found in feces, while intact oocysts were rarely seen. The sporocysts of all *Sarcocystis* isolates were colorless and ellipsoidal, with the sporocyst residuum composed of granules ~1 μm in diameter (Table 1).

3.2. Experimental infections and morphology

Sporocysts isolated from the feces of *A. desaixi*, *A. rungweensis*, and *B. arietans* (numbered "I," "II," and "III," respectively) underwent development in experimentally infected rodent species. Despite relatively heavy infections in some cases, none of the inoculated animals showed clinical signs of an acute sarcosporidiosis (Table 2).

The *Sarcocystis* isolates originating from *A. desaixi* *Sarcocystis* sp. "I") and *A. rungweensis* (*Sarcocystis* sp. "II") developed into sarcocysts, which were localized only in the striated musculature. Filliform, whitish and macroscopically almost invisible sarcocysts measured in vivo up to 2 cm in length and 40–80 μm in diameter (80 DPI). Ultrastructurally, the primary cyst wall was 0.5–1.5 μm thick and formed small electron-dense, knob-like protrusions, which were ~100 nm long and ~50 nm wide. Within the sarcocysts, two types of multiplication were observed—endodyogony and endopolygony (data not shown).

For the *Atheris desaixi* isolate, the laboratory white mice and *Lemniscomys barbarus* and *L. striatus* were susceptible. Sarcocysts developed in their esophagus, tongue, diaphragm, muscles of the abdominal wall, brachial muscles, femoral muscles and masseters. The *Atheris rungweensis* isolate was infectious in laboratory white mice, sarcocysts developed in their tongue, diaphragm, muscles of the abdominal wall, brachial muscles, femoral muscles and masseters. The cystozoites measured 7.7 (7–9) × 1.3 (1–1.5) μm and 9.7 (9–10.5) × 2.3 (2–2.5) μm, respectively.

The isolate of *Bitis arietans* (*Sarcocystis* sp. "III") from Tanzania developed subcutaneous sarcocysts in one out of two experimentally infected multimammate rats (Table 2). Spherical thin-walled sarcocysts that measured 600–900 × 800–1000 μm were confined to the upper lip and genital region. Morphology and localization of the cysts favor the conspecificity with *Sarcocystis hoarensis* (Matuschka et al., 1987).

3.3. Phylogenetic relationships within Sarcocystidae

Within sarcosporidians from African vipers we have sequenced the SSU rDNA of *Sarcocystis* sp. "I" (from *A. desaixi*) and *Sarcocystis* sp. "II" (*A. rungweensis*). For these two isolates we were able to find an experimental intermediate host (Table 2; Fig. 1) and study the ultrastructure of the sarcocyst wall. Our decision somewhat limited the analyzed dataset, but on the other hand we analyzed only isolates already morphologically and biologically characterized. Such a caution is dictated by the fact that each viper may serve as a definitive host for several *Sarcocystis* species, and without infection experiments, we could not exclude the presence of more than one *Sarcocystis* species in infected snakes.

The ML was parametrized using ModelTest, the hierarchial LRT selected TrN + I + Γ model, which is a model with unequal rates for transversions and transitions but with equal rates for transversions and different rates for each transition. The reconstruction using the ML analysis found the best tree with monophyletic Coccidea being subdivided into two sister clades—Eimeriidae and Sarcocystidae. The latter splits into four clades (A–D) identified by Doležel et al. (1999), the relationship of which was {A[(B, C)D]}. Within the snake–rodent clade (clade D), the South Asian *S. singaporensis* constitutes an early branch. The African sarcosporidians form a monophyletic clade with *Sarcocystis* sp. "IV" of *Pseudocerastes persicus* from the Middle East as a sister species.

Since ML bootstrap analysis is time consuming, we have performed the bayesian reconstruction (Huelsenbeck et al., 2001) using the GTR + Γ model of DNA substitution. Reconstruction using the bayesian method (Fig. 1) supports an almost identical tree as ML, the only exceptions being minor differences within clade B, but such branches are with low posterior probability.

The distance method using ME determined best tree with the {A[B(C, D)]} relationship. With the exception of clade B, the bootstrap analysis supports all major Sarcocystidae clades. MP analysis and heuristic search found 152 equally parsimonious trees. When the transversions were treated with two and three times the transitions, 18 and 4 trees, respectively, were equally parsimonious. MP reconstruction had difficulty in reconstructing the relationship within the Sarcocystidae clades and the consensus trees supported only the

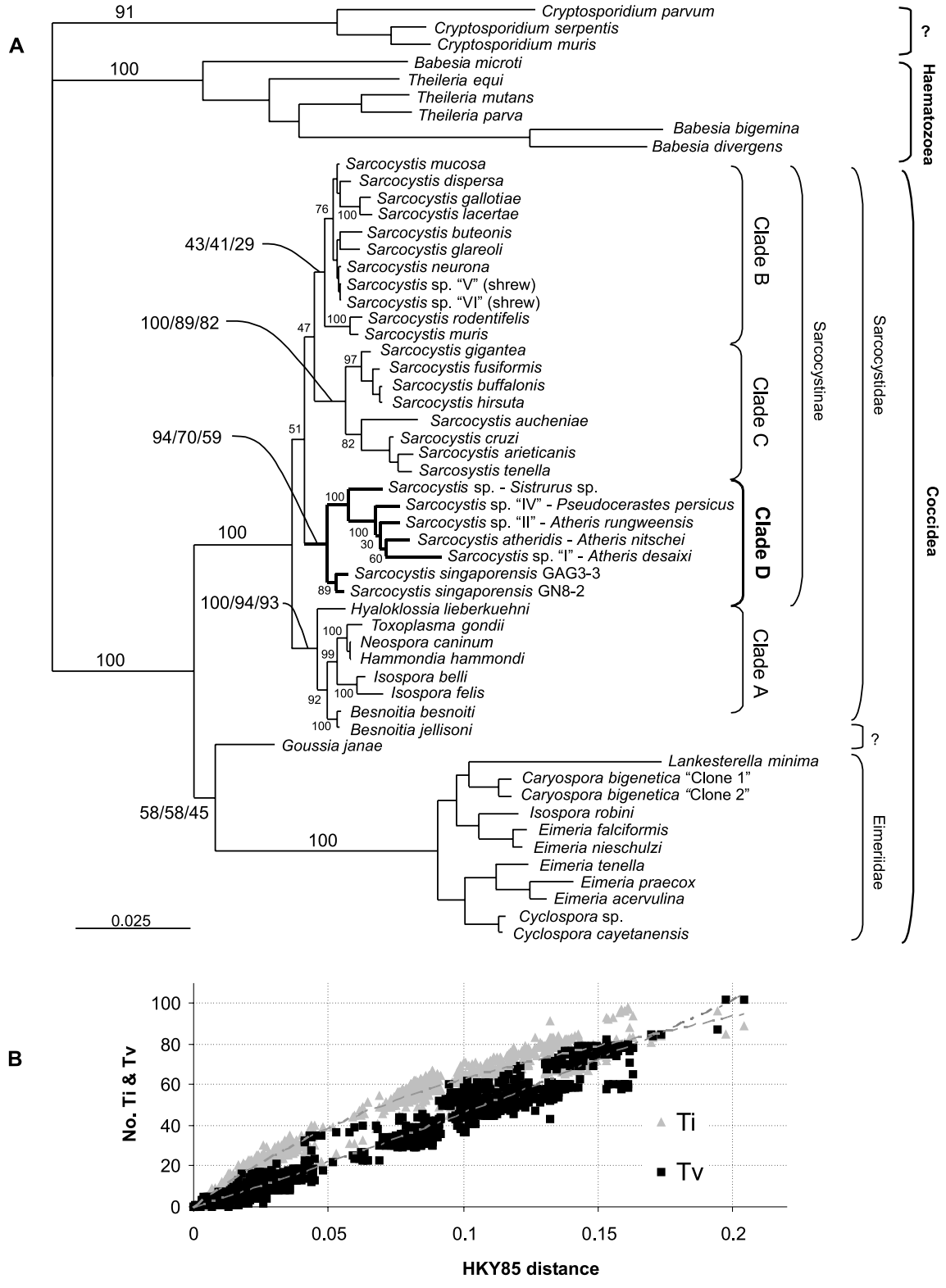


Fig. 1. (A) Phylogram based on the SSU rDNA sequence alignment of Apicomplexa. Tree was reconstructed using the bayesian inference and rooted on *Cryptosporidium* spp. For major nodes and Sarcocystidae the posterior probability values are indicated on branches. The bootstrap values based on minimum evolution and maximum parsimony are also indicated for major clades of the Sarcocystidae. To the right the taxonomic affiliation is presented. Since the classification of *Cryptosporidium* spp. and *Goussia janae* is unsettled, it is marked with a "?" (B) The observed number of transitions (Ti) and transversions (Tv) plotted against HKY85 distances for the SSU rDNA alignment, that was used to construct the above tree.

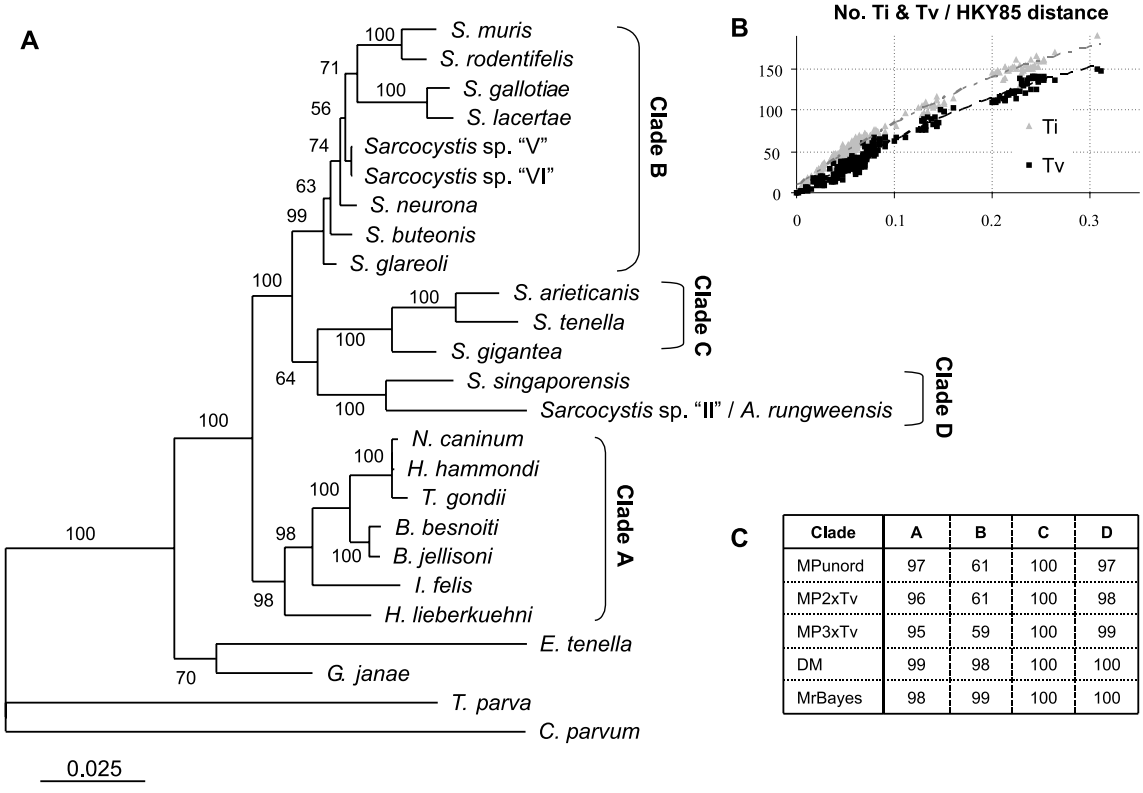
quadritomy (A, B, C, D). To analyze the possible saturation of either transitions or transversions, we have plotted the pairwise transition and transversion to the observed distances (Fig. 1). The transversion curve is almost linear and shows no saturation. The transitions show a higher substitution rate and the plot follows a slightly parabolic appearance (Fig. 1). The highest distances causing such a shape are those of *Lankesterella* vs. the outgroup taxa *Babesia* and *Cryptosporidium*.

To improve the resolution obtained with the SSU rDNA, we sequenced the D2 LSU rDNA for *Goussia janae*, *Hyaloklossia lieberkuehni*, *Sarcocystis lacertae*, *S. gallotiae*, *S. rodentifelis*, *Sarcocystis* sp. "II" (from *A. rungweensis*), and *Sarcocystis* spp. "V" and "VI" (from shrews). When subjected to the ILT test that runs under MP, the combined dataset did not support combination of the two datasets if characters were unordered ($P = 0.02$). If, however, transversions were treated two times transitions, the homogeneity of the two datasets was strongly supported ($P = 0.82$). Subsequently, we have combined the data and paid attention to the nucleotide models used. The saturation of transversions and transitions has not been observed on the plots (Fig. 2; inset graph), since both curves were almost linear. The ModelTest suggested the same TrN + I + Γ model as earlier. The bayesian method supported a branching order similar to that obtained with the SSU rDNA data only (Fig. 2), while support for clade B and the monophyly of Sarcocystidae (B, C, D) increased. Clade A appears as a sister group to Sarcocystidae (Fig. 2, inset table). The ML and MP analyses supported the [C(B, D)] relationship, but both the bayesian and ME methods favored an alternative situation of [B(C, D)].

3.4. Host–parasite co-evolution and divergence dates for the snake–rodent clade

We have evaluated the snake–rodent *Sarcocystis* spp. from clade D using the relationship with their definitive hosts. In order to reconstruct this association, the host tree was plotted against the *Sarcocystis* SSU rDNA tree. The exact search yielded four co-speciations, two duplications, zero host switches and three sorting events. Parasite tree randomization ($n = 10.000$) yielded a mean of only 2.5 co-speciations (SD = 0.8).

The *Sarcocystis* spp. of snakes were analyzed using the molecular clock hypothesis (Fig. 3). A tree constructed using ML (KHY + Γ + I) was tested for clock



Clade	A	B	C	D
MPunord	97	61	100	97
MP2xTv	96	61	100	98
MP3xTv	95	59	100	99
DM	99	98	100	100
MrBayes	98	99	100	100

Fig. 2. (A) Phylogram based on the concatenated SSU and D2 LSU rDNA sequences of Apicomplexa. Tree was reconstructed using the bayesian inference and rooted on *Cryptosporidium parvum* and *Theileria parva*. The posterior probability values are indicated on the branches. (B) The observed number of transitions (Ti) and transversions (Tv) plotted against HKY85 distances for the concatenated SSU and D2 LSU rDNA alignment, that was used to construct the above tree. (C) Table with bootstrap values for maximum parsimony (MPunord), maximum parsimony and the treatment of transitions and transversions 2:1 (MP2xTv) and 3:1 (MP3xTv), distance method (DM) and the bayesian posterior probability (MrBayes). Each value is indicated for clades A–D of the Sarcocystidae.

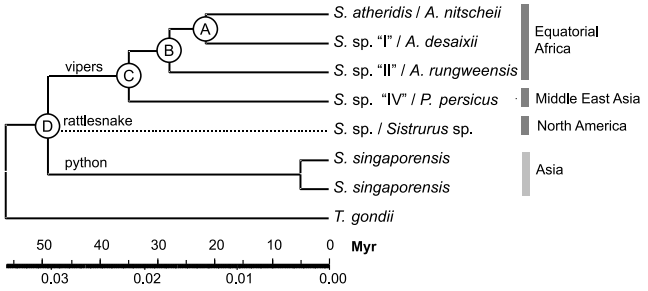


Fig. 3. The linearized tree based on the SSU rDNA sequences of *Sarcocystis* with the snake–rodent life cycle, rooted on *Toxoplasma gondii*. The terminal names are accompanied by the definitive host name and the geographical distribution is indicated to the right. The common names of the hosts are indicated above the branches. The time scale below is based on the calibration of node C to 35 Myr, for details about the node A–D, see Table 3. The dashed branch of an unnamed American *Sarcocystis* sp. (#U97524) with the rattlesnake (*Sistrurus*)–rodent (*Microtus*) life cycle (Carreno et al., 1998) indicates a sequence that did not support the clock-like model.

(−ln = 4446.6) vs. non-clock (−ln = 4427.4) models. The critical value for six degrees of freedom is 12.6, rejecting the null hypothesis and disqualifying the clock-like model. Further analysis selected *Sarcocystis* sp. (#U97524) of *Sistrurus* sp. as a causative agent for the rejection of the clock-like model. Analytical values for the remaining sequences in case of clock and non-clock models were −ln = 4152.6 and −ln = 4146.7, respectively. The critical value was 11.1 for 5 degrees of freedom. The null hypothesis was not rejected, and the clock can therefore by applied.

To calculate the divergence times we used 2 and 0.85% evolutionary rates in 100 Myr (Escalante and Ayala, 1995). In addition, we have evaluated two different scenarios applying different biogeographical calibration points. The first scenario (Fig. 3, node C), using 35 Myr, follows the development of the Afro-Asian land bridges and the beginning of Rift Valley formation in the Oligocene. Furthermore, the viperid snake genera *Atheris* and *Pseudocerastes* are assumed to originate at this time (Lenk et al., 2001). The second scenario assumes that the divergence of the isolates from *Atheris* spp. is of a recent origin, i.e. about 3 Myr in Pliocene (node B). The results of time estimates for different rates, as well as the calibration points, are summarized in Table 3.

4. Discussion

Modern classification of the cyst-forming coccidia (Sarcocystidae) is based on the following criteria: morphology and localization of the developmental stages (merogony, sarcocyst formation), detailed ultrastructure of the primary cyst wall, life cycle, host specificity and the rDNA sequences (Dubey and Odening, 2001; Rosenthal et al., 2001). Here we report on the biology and the evolutionary history of *Sarcocystis* isolates from African vipers. We have reconstructed phylogenetic trees for all available cyst-forming coccidia and identified four major clades. One clade comprised of *Sarcocystis* spp. with a snake–rodent life cycle, including studied *Sarcocystis* from vipers. We tested the co-evolution of *Sarcocystis* with their definitive hosts, which is supported over a random chance. Furthermore, the history of the African continent, mountain ranges, forests and general SSU rDNA rates were used to construct a linearized tree. A possible origin of the heteroxenous life cycle of Sarcocystidae is discussed.

4.1. Sarcocystis *spp. in African bush vipers*

Snakes are known to be common definitive hosts for *Sarcocystis* spp. and several studies reported a high prevalence of sporocysts in the snake's feces (McAllister et al., 1995). There are 13 named *Sarcocystis* spp. with the snake–rodent life cycle (Odening, 1998; Šlapeta et al., 1999). *Sarcocystis* parasitizing snakes are believed to be host specific on the level of the definitive host (Matuschka, 1987), with some exceptions reported by Lindsay et al. (1991) and Paperna and Finkelman (1996). Taking the host specificity into account, both *Sarcocystis* spp. from *A. desaixi* and *A. rungweensis* are closely related to *S. atheridis* from *A. nitschei* (Šlapeta et al., 1999). Additionally, the African species from related viperid genera, namely *S. dirumpens* from *Bitis* spp. and *S. gerbilliechis* from *Echis coloratus* were also considered for comparison. Our isolates developed sarcocysts in mice, while the development of *S. gerbilliechis* was restricted to gerbils (Jäkel, 1995). *Sarcocystis dirumpens* is reported to develop in a wide spectrum of small rodents including rats (Matuschka, 1987), and thus differs from our isolates.

Table 3
Divergence times estimated from SSU rDNA phylogenetic analysis

Evolutionary rate (sequence divergence/Myr)	Time in Myr			
	Node A	Node B	Node C	Node D
Rate = 0.0002 (0.02/100 Myr)	66.9	86.6	108.7	162.3
Rate = 0.000085 (0.0085/100 Myr)	157.4	203.7	255.7	381.8
Calibration of node B and 3 Myr (rate = 0.0058)	2.3	3[a]	3.77	5.6
Calibration of node C and 35 Myr (rate = 0.00062)	21.6	27.9	35[a]	49.9

[a] Used the nodes B and C calibration, respectively.

Comprehensive comparison of primary cyst walls enabled Dubey and colleagues to sort *Sarcocystis* into several groups (Dubey et al., 1989; Dubey and Odening, 2001). Both *Sarcocystis* from *A. desaixi* and *A. rungweensis*, as well as the previously described *S. atheridis* possess the type 1 primary cyst wall, that lacks any advanced morphological features and is considered non-informative for the species diagnosis (Odening, 1998). Apparently, morphological data is insufficient for the differentiation between these isolates and *S. atheridis.*

4.2. Phylogeny of cyst forming coccidia (Sarcocystidae)

The abundance of available sequences favors SSU rDNA to other molecular markers that are available for only a narrow set of organisms within the phylum Apicomplexa (Zhu et al., 2000). We have paid special attention to include all available sequences and to establish an analysis that could be used for the interpretation of biology and life cycle histories of the cyst forming coccidia. The SSU rDNA-based analyses considered 28 available species of Sarcocystidae plus five new sequences. Total evidence analysis included only species/genera for which both the SSU rDNA and the D2 LSU rDNA sequences were available, and such an alignment comprised 25 sequences with 8 newly sequenced D2 LSU rDNA.

Besides traditionally used MP, ME and ML methods, we have also used the bayesian phylogeny method (Huelsenbeck et al., 2001). Although this method did not completely resolve some nodes, the monophyly of the Sarcocystidae, the ''isosporoid'' species (clade A), and the species with canids and felids as definitive hosts (clade C) is highly supported by posterior probability. Monophyly of species, we were especially interested, the snake–rodent species (clade D), is also highly supported in contrast to lower supports provided by traditional methods (Doležel et al., 1999). Neither method improved parameters for clade B (Jenkins et al., 1999; Šlapeta et al., 2001). However, the use of total evidence, herein the concatenation of SSU and D2 LSU rDNA data, generally increased support for the nodes. Our analysis seems to have some advantageous features over the use of SSU rDNA data alone.

4.3. Co-evolution of Sarcocystis *spp. with African vipers and molecular clock*

The co-evolution of Sarcocystidae with their definitive hosts has been repeatedly described (Tenter and Johnson, 1997; Holmdahl et al., 1999; Doležel et al., 1999). An example of feline and canine species infecting ruminants (clade C), Holmdahl et al. (1999) demonstrated a close association between the genotypic groups and phenotypic characteristics, including the definitive and intermediate hosts and the size and type of the primary cyst wall. However, co-evolution with the definitive host seems to be absent for species of clade B (Holmdahl et al., 1999; Jenkins et al., 1999; Mugridge et al., 2000; Šlapeta et al., 2001; Votýpka et al., 1998).

Our analysis brought together *Sarcocystis* from *A. desaixi*, a snake endemic to Mt. Kenya in central Kenya, *A. nitschei*, which is endemic to the Ruwenzori Mts. in western Uganda and *A. rungweensis*, known only from Mt. Rungwe and a few other mountain localities in southeastern Tanzania. Such a sample seemed to be suitable for addressing the question whether the distribution of parasites is a result of co-speciation rather than host switches. We have identified co-evolution by showing that the number of observed co-speciation events exceeds randomization. This makes the co-evolution scenario for the snake–rodent *Sarcocystis* with their hosts the most plausible explanation of the obtained data. Reported four co-speciation and two duplication events are those from which the parasite may have speciated (Page and Charleston, 1998). The duplications are probably a result of the closer relationship of *Sarcocystis* from *A. nitschei* and *A. desaixi*, which does not correlate with a sister relationship of *A. nitschei* and *A. rungweensis* (Broadley, 1998; Lawson et al., 2001). Sorting events are those where the parasite has not evolved (Page and Charleston, 1998). In our case, they may have been caused by changes of the preferred diet of the ancestor hosts, or by the loss of the parasite. Alternatively, there are other *Sarcocystis*, we are not yet aware of.

The tropical rain forest, a habitat of *Atheris* vipers, is known to harbor enormous species diversity (Moritz et al., 2000). The isolated distribution pattern of the East African bush vipers made their coccidian parasites particularly interesting for studies of their evolutionary history. The phylogeny of the vipers in Africa has been studied previously, however, the divergence dates have not been established and many inter-generic relationships remain to be addressed (Lenk et al., 2001). The fossil records indirectly document that Viperidae appeared prior to the Miocene (Szyndlar and Rage, 1999). The present distribution of organisms reflects their historical evolution and can assist in the reconstruction of their historical distribution. Thus, a plausible explanation for the distribution of *Atheris* spp. and their *Sarcocystis* parasites is a refugia model of speciation, that rests on the premise that climatic changes forced rainforest to contract into isolated refugia separated by dry savanna (Fjeldså and Lowett, 1997). The following events had a major impact on the distribution of animals and plants in Africa: (i) relative position of the Equator that reached its present position about 50 Myr ago; (ii) simultaneous movement of the forest towards the south and desertification of the Mediterranean north; (iii) closing of the Tethys Sea and the development of land bridges with Asia (early Miocene–Oligocene, 38–25 Myr); (iv) formation of the East African Rift System (Eocen–Miocen, 40–21 Myr); and,

last but not least, (v) intense forest oscillations (Pliocene, 3 Myr). The mountain ranges that serve as localities for vipers and their *Sarcocystis* parasites appeared with the development of the Rift system; Mt. Kenya in the east (volcanic origin, 2 Myr) and Mt. Rungwe (volcanic origin, 7 Myr) and Ruwenzori Mts. (drift origin, 20 Myr) both in the western Rift (Braile et al., 1995).

Divergence times were estimated using molecular clock, i.e., the proportional substitution between taxa and time. Although the 0.85% as well as 2% per 100 Myr rates have been shown to be useful estimates for evolution of the phylum Apicomplexa (Escalante and Ayala, 1995), it is obviously an overestimation for *Sarcocystis* of snakes, dating the appearance of viperid *Sarcocystis* spp. to > 100 Myr. The origin of vipers is thought to be Asia, from where the vipers invaded Africa via a land bridge across the Tethys Sea in the Tertiary period (Lenk et al., 2001). The sister relationship of the Middle East Asian *Pseudocerastes* isolate with the *Atheris* isolates from Equatorial Africa reflects the collision of Asia and Africa and further supports the Asian origin of vipers. The calibration with the early connection between Africa and Asia (35 Myr) can therefore be considered as relevant. The estimates are in poor correlation with the appearance of the mountain ranges, except with that of the oldest formation of the Ruwenzori Mts. The estimate that uses 3 Myr (the forest oscillation) with the accelerated rate does not represent a plausible explanation. Thus, for our dataset, the molecular clock has only limited value for the estimation of divergence dates. Although, it is possible to conclude that the origin of *Sarcocystis* in vipers correlates with the old emergence of hosts and precedes the Plio-Pleistocene period.

4.4. Origin of the heteroxenous life cycle of the genus Sarcocystis (Sarcocystidae)

Two postulated conflicting hypotheses address the evolution of the heteroxenous life cycle in the past. Landau (1974) proposed that the homoxenous coccidia evolved from the heteroxenous species by a secondary simplification of the life cycle. An alternative scenario assumes that homoxeny predated heteroxeny in coccidia (Tadros and Laarman, 1982). The most recent studies favor the latter hypothesis (Barta, 1989; Escalante and Ayala, 1995; Tenter and Johnson, 1997).

Presented evolutionary trees place the snake–rodent *Sarcocystis* spp. (clade D) as a sister clade to the rest of *Sarcocystis* species. More than 2500 existing snake species are traditionally divided into three presumably monophyletic lineages: Scolecophidia (blind snakes), Henophidia (“primitive” snakes) and Caenophidia (“advanced” snakes) (Heise et al., 1995). The position of their *Sarcocystis* in our tree (Fig. 3) has good correlation with this theory. The pythons (Boidae) belong to the “primitive” lineage, while the viperids (Viperidae) are considered to represent the “advanced” snakes. When one adheres to the co-evolution with the definitive host, then snakes acquired *Sarcocystis* parasites at the time the snake body evolved (~125 Myr, Heise et al., 1995).

Furthermore, careful inspection of sister taxa to the genus *Sarcocystis* and family Sarcocystidae provides further relationships. The two sister branches share homoxenous life style and an aquatic origin: (i) *Hyaloklossia lieberkuehni* from an European green frog, belongs to the Sarcocystidae, where it forms a sister species to the rest of clade A (Modrý et al., 2001) and (ii) *Goussia janae* from a freshwater fish gut represents a distinct lineage between Sarcocystidae and Eimeriidae (Jirků et al., 2002). Both organisms appear to be older sisters to the more advanced species in higher vertebrates, i.e., heteroxenous *Sarcocystis*, and thus in favor of the aquatic/semiaquatic ancestry and homoxenity. If the earliest snakes were aquatic and the terrestrial ones were derived (Scanlon and Lee, 2000) (but conflicting hypotheses exist—Heise et al., 1995), one would suggest that the ancient serpent became first infected by a homoxenous coccidium in its aquatic environment. Nevertheless, such an explanation for the origin of *Sarcocystis* is, at this moment prone to systemic bias due to the lack of many sequences of coccidia from diverse hosts (Perkins et al., 2001). Any new studies aimed at coccidia under-represented or absent from the available dataset (primarily species from the cold-blooded vertebrates and invertebrates) may shed more light on our understanding of the origin of heteroxenity and the evolution of Coccidea.

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