Protist, Vol. 157, 173—183, April 2006
http://www.elsevier.de/protis
Published online date 11 April 2006

Protist

ORIGINAL PAPER

Phylogenetic Analysis of Coccidian Parasites from Invertebrates: Search for Missing Links

Jana Kopečná[a,b], Milan Jirků[a,b], Miroslav Oborník[a,b], Youri S. Tokarev[c], Julius Lukeš[a,b], and David Modrý[a,d,1]

[a]Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic
[b]Faculty of Biology, University of South Bohemia, České Budějovice, Czech Republic
[c]All-Russian Institute for Plant Protection, St. Petersburg, Russian Federation
[d]Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

Submitted September 23, 2005; Accepted February 13, 2006
Monitoring Editor: Frank C. Seeber

Apicomplexan parasites represent one of the most important groups of parasitic unicellular eukaryotes comprising such important human parasites such as *Plasmodium* spp. and *Toxoplasma gondii*. Apicomplexan radiation as well as their adaptation to the parasitic style of life took place before the era of vertebrates. Thus, invertebrates were the first hosts of apicomplexan parasites that switched to vertebrates later in evolution. Despite this fact, apicomplexan parasites of invertebrates, with the exception of gregarines, have so far been ignored in phylogenetic studies. To address this issue, we sequenced the nuclear SSU rRNA genes from the homoxenous apicomplexan parasites of insects *Adelina grylli* and *Adelina dimidiata,* and the heteroxenous *Aggregata octopiana* and *Aggregata eberthii* that are transmitted between cephalopods and crustaceans, and used them for phylogenetic reconstructions. The position of the adelinids as a sister group to *Hepatozoon* spp. within the suborder Adeleorina was stable regardless of the phylogenetic method used. In contrast, both members of the genus *Aggregata* possess highly divergent SSU rRNA genes with an unusual nucleotide composition. Because of this, they form the longest branches in the tree and their position is variable. However, the genus *Aggregata* branches together with adelinids and hepatozoons in most of the analyses, although their position within the scope of this cluster is unstable.

Key words: Apicomplexa; Eucoccidiorida; *Adelina*; *Aggregata*; molecular phylogeny; SSU rDNA.

Introduction

The phylum Apicomplexa comprises important human parasites, such as *Plasmodium* spp., the causative agents of malaria, and *Toxoplasma gondii*, which is widely dispersed even in human populations of developed countries. Therefore, our understanding of the relationships among members of this phylum is important not only from an evolutionary point of view, but also for proper diagnosis and treatment of infections caused by Apicomplexa. One of the main problems influencing the accuracy of current apicomplexan phylogenetic analyses is an inappropriate taxon sampling. While some apicomplexan groups, such as coccidians from vertebrate hosts, are well represented in data sets for phylogenetic reconstructions, their relatives

[1]Corresponding author;
fax 42 541562266
e-mail modryd@vfu.cz (D. Modrý).

doi:10.1016/j.protis.2006.02.005

parasitizing invertebrates have so far been largely ignored (Barta 2001; Jirků et al. 2002; Morrison et al. 2004; Šlapeta et al. 2003).

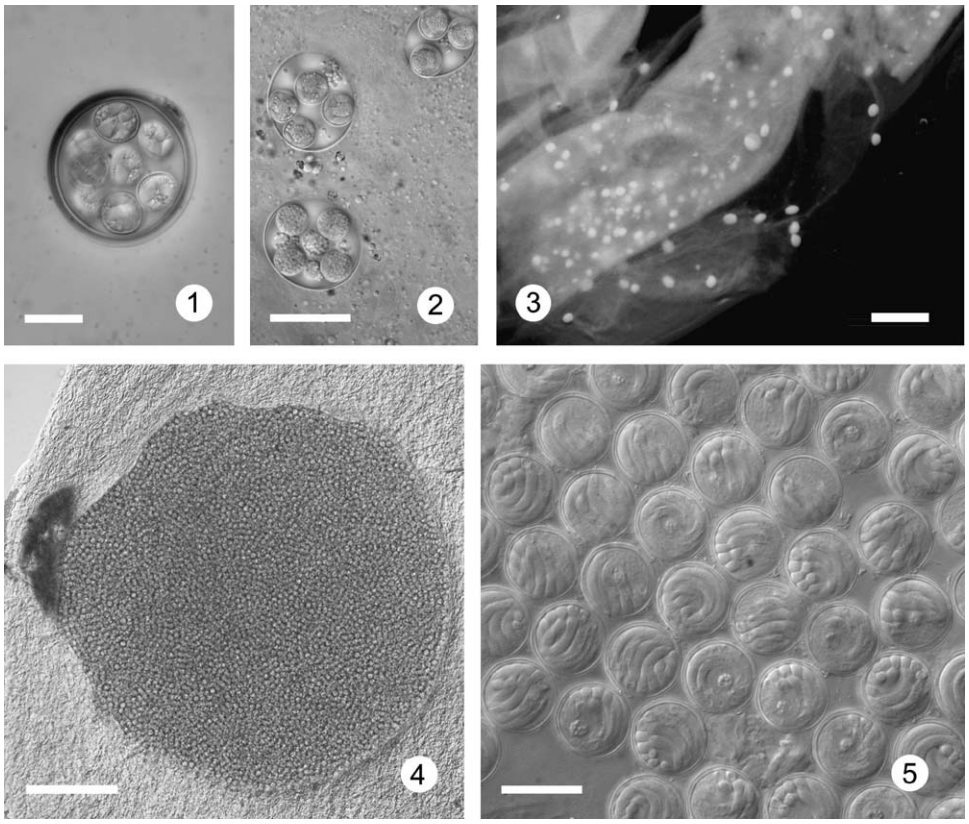
Apicomplexans are intracellular, largely non-flagellated organisms that are classified together with ciliates and dinoflagellates within the Alveolata. Apicomplexans are purely parasitic organisms. Therefore, the transition from the predation to intracellular parasitism is probably the most striking feature in their evolution. It has been suggested that this event occurred approximately at the time of the divergence of dinoflagellates and apicomplexans (Kuvardina et al. 2002; Leander and Keeling 2003, 2004). The time of further radiation of the phylum Apicomplexa is placed in the Precambrian period, ~800 Mya (Escalante and Ayala 1995), when multicellular organisms are supposed to have emerged. It clearly predates the appearance of vertebrates and most likely that of invertebrates as well (Lynch 1999). Since all currently known apicomplexans are obligatory parasites, it is reasonable to assume that the ancestral lineages initially evolved in the invertebrate hosts, probably even before the Cambrian explosion. It is generally accepted that the earliest apicomplexans are archigregarines (Leander et al. 2003). However, further evolution of main lineages within the order Eucoccidiorida is unclear. Any quest for the ancestral forms of the main lineages should therefore be focused on the apicomplexans parasitizing terrestrial and aquatic invertebrate hosts. The absence of molecular data for any representative (!) of Eucoccidiorida from this group of hosts in phylogenetic analyses is alarming, as it may distort or at least conceal the correct relationships between the major apicomplexan groups. Interestingly, vector-transmitted apicomplexans (Haemospororina, Adeleorina and Piroplasmorina) retain the sexual part of their life cycle in the invertebrate hosts, which suggests that these heteroxenous parasites have evolved from monoxenous apicomplexans of invertebrates (Barta 1989). Members of the coccidian genera *Adelina* and *Aggregata*, so far considered unrelated (Barta 2000; Upton 2000), have been isolated from ecologically distant invertebrates, and used in this study. The genus *Adelina* Hesse, 1911 comprises monoxenous apicomplexans usually parasitizing insects. Based on the presence of syzygy in their life cycle, adelinids are traditionally referred to as members of the order Adeleorina, which also comprises heteroxenous haemogregarines alternating between vertebrate and invertebrate hosts (Barta 2000).

The genus *Aggregata* Frenzel, 1885 was established 150 years ago to embrace digenetic parasites that are transmitted between cephalopods and crustaceans (Dobell 1925). Merogonial development occurs in the intestine of crabs and shrimps and proceeds by gamogony and sporogony upon ingestion by a squid or octopus (Gestal et al. 2002; Porchet-Henneré et al. 1981). The octopuses in Naples were so heavily infected that Dobell (1925) complained about the impossibility to experimentally complete the life cycles due to the lack of uninfected definitive hosts. Ever since, various *Aggregata* species have been reported as a very frequent parasite of different cephalopods in the Atlantic Ocean, the North Sea, the Mediterranean, and other places (Gestal et al. 2002; Porchet-Henneré et al. 1981; Sardella and Martonelli 1997). The family Aggregatidae Labbé, grouping *Aggregata* spp. together with the genera *Merocystis* Dakin, 1911 and *Selysia* Dubosq, 1917, is traditionally classified within the suborder Eimeriorina (Dobell 1925; Gestal et al. 2005; Upton 2000).

In a first attempt to fill the gaps in our knowledge about the phylogenetic position of coccidians from invertebrates, we herein present results of phylogenetic analyses of members of the genera *Adelina* and *Aggregata* parasitizing arthropods and molluscs.

Results

Two adelinid species that differ in their endogenous development were selected: *Adelina dimidiata* Schneider, 1875, a typical intestinal parasite of insects (Fig. 1), and *Ad. grylli* Butaeva, 1996 with an extraintestinally located endogenous development (Fig. 2) (Sokolova et al. 1999). Furthermore, two representatives of the genus *Aggregata*, namely *Aggregata octopiana* and *Agg. eberthii*, both from Mediterranean cephalopods were also included in this study. These coccidians are characterized by the formation of large, macroscopically visible oocysts in the viscera of cephalopods (Fig. 3), each containing hundreds of polyzoic sporocysts (Figs 4, 5). To establish the phylogenetic position of the above-mentioned species, we have sequenced their entire SSU rRNA genes. When compared to other available homologs, sequences of the genus *Adelina* were not exceptionally divergent, and their phylogenetic position was very stable in all constructed trees (Figs 7, 8, and data not shown). In contrast, the SSU rRNA genes of both *Aggregata* species had



Figures 1—5. Representative morphology of oocysts of the studied taxa. **1.** Exogenously sporulated oocyst of *Adelina dimidiata* from the feces of *Scolopendra cingulata* (Bar = 30 μm). **2.** In situ sporulated oocysts of *Adelina grylli* from the fat body of *Gryllus bimaculatus* (Bar = 20 μm). **3.** Macroscopic oocysts of *Aggregata octopiana* on the surface of dissected intestine of *Octopus vulgaris* (Bar = 1 cm). **4.** Micrograph of a single oocyst of *Aggregata octopiana* containing thousands of in situ sporulated sporocysts (Bar = 200 μm). **5.** Detail of sporocysts enclosing spirally coiled elongated sporozoites in a head-to-tail position (Bar = 20 μm).

an unbalanced nucleotide composition, shifted towards a very low cytosine content. As shown in Figure 6, the SSU rRNA genes of *Aggregata* spp. contain less than a half of cytosines compared to the same genes in other coccidia. The nucleotide Blast search identified a number of relatively high matches from outside the Apicomplexa, contrary to the discontinuous Megablast, which identified *Ad. bambarooniae* as the most similar sequence. Due to the ambiguity caused by this nucleotide bias, we decided to confirm the origin of amplified SSU rRNA genes from *Aggregata* spp. by Southern hybridization analysis (see Methods for details). The DNA of the related coccidium *Neospora caninum*, newt, and trypanosome were used as controls, while the addition of the *Octopus vulgaris* DNA ensured that the host did not harbor another rare or overlooked parasite that could be the source of amplified DNA. Despite the shortage of *Agg. octopiana* DNA, the blot clearly confirmed that the studied SSU rRNA gene is derived from the oocysts of *Aggregata* (not shown).

For phylogenetic analysis, three different alignments were constructed. Alignment I (1284 nucleotides, see Methods for details) was used to specify the position of the genera *Adelina* and *Aggregata* within alveolates. Although none of the trees inferred from this alignment is highly supported by bootstrap analysis, all major apicomplexan groups appeared in the same positions as in previous analyses (see Fig. 7 and Leander et al. 2003). However, the tree constructed using the maximum likelihood (ML) criterion, differed to some extent from the one based on the LogDet paralinear distances. In particular, both *Aggregata* species either clustered with a very low support with the unidentified apicomplexan parasites of the foraminiferan *Ammonia beccarii* and the mollusc *Tridacna crocea,* or as in the ML tree, they constituted a clade along with the members

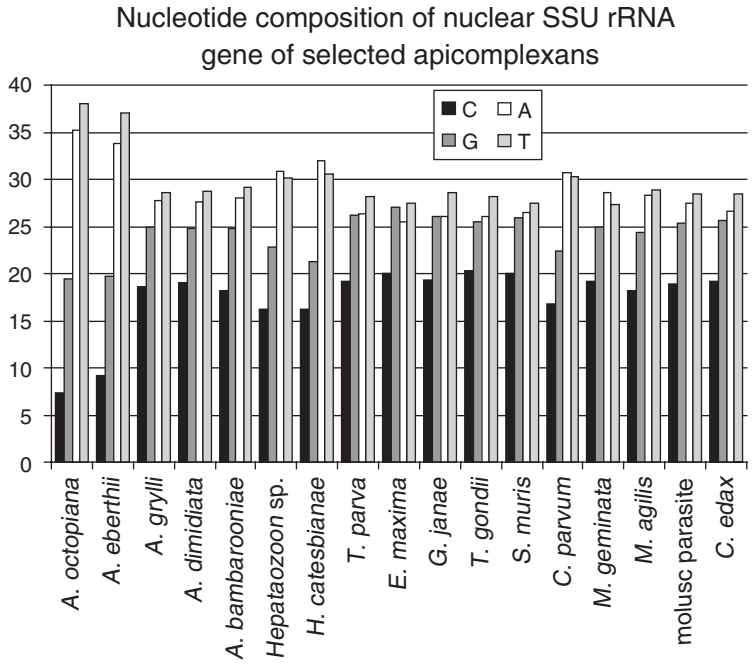


Figure 6. Nucleotide composition of the SSU rRNA gene sequences of selected apicomplexans. Note the extremely low content of cytosine within the *Aggregata* genes.

of Adeleorina, namely *Adelina* and *Hepatozoon* (Fig. 7). Another incongruence of the LogDet-based tree was a joint position of colpodellids with perkinsids on the root of the dinoflagellates (data not shown). In general, the phylogenetic position of the genus *Adelina* as a sister group to the genus *Hepatozoon* was stable regardless of the method of reconstruction used. Molecular phylogenetic analysis thus provides further justification for the existence of the suborder Adeleorina that comprises these two genera. However, classification of Adeleorina within the order Eucoccidiorida is not corroborated by our data. In the large tree based on the alignment I, such a relationship is shown; however, the bootstrap support is marginal (Fig. 7). When the data set (alignment II) was narrowed down to the apicomplexans with colpodellids as outgroup, Adeleorina appeared as a sister group to Piroplasmorida. However, this relationship was not supported by bootstrap analysis, and also the key branches were extremely short.

Since the positions of taxa of interest are not well supported in the large tree, alignments II and III have been constructed that include only apicomplexan sequences with colpodellids used as outgroup. The longest branch representing the parasite from *Am. beccarii* was excluded from further analyses to decrease the risk of possible artifacts. These two alignments were constructed under different gap opening parameters and, when used to construct phylogenetic trees, gave different results only with respect to the position of *Aggregata* species, with no effect on the rest of the tree. The most consistent phylogeny was obtained from alignment II. All the methods used placed the genera *Adelina*, *Hepatozoon*, and *Aggregata* into one cluster (Fig. 7). However, the internal topology varied for different methods of phylogenetic reconstruction. In the ML tree, *Adelina* spp. were ancestral to the genera *Hepatozoon* and *Aggregata*; whereas, the maximum parsimony (MP) analysis placed the genera *Adelina* and *Hepatozoon* as sister groups. In both the ML and MP trees, *Aggregata* spp. appeared on very long branches at the root of the *Hepatozoon* clade. In the LogDet tree, *Aggregata* spp. clustered with the parasite from *Tridacna crocea* at the base of the *Adelina—Hepatozoon* clade; however, this branching is only poorly supported (Fig. 8). The alignment III gives slightly different results when processed using various computational methods. In the LogDet and ML trees, *Aggregata* spp. appear at the root of the *Cryptosporidium* clade; however, the bootstrap trees do not support this topology, placing *Aggregata* at the root of the *Adelina—Hepatozoon* clade (Fig. 8). The same position as in the LogDet

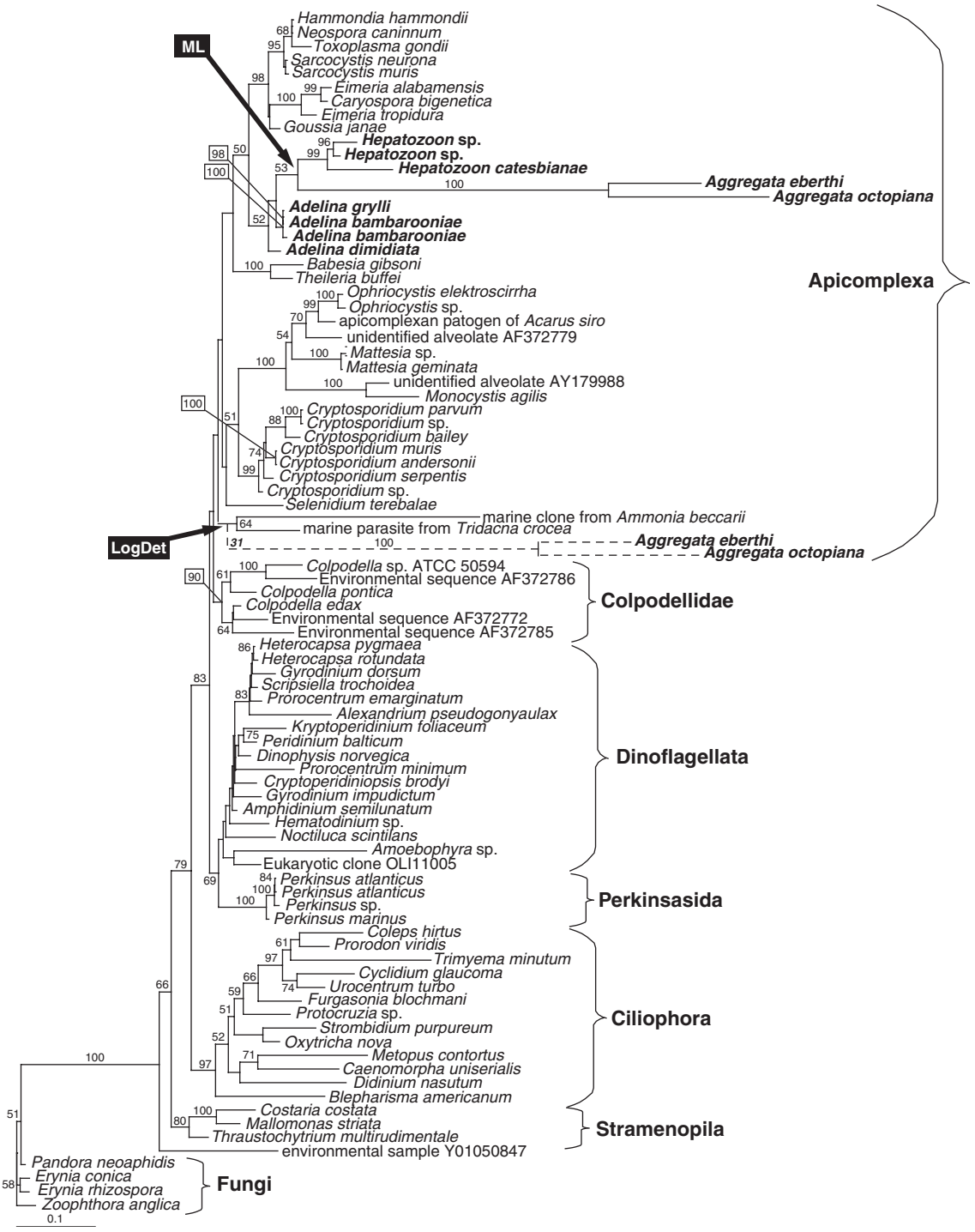


Figure 7. Maximum likelihood tree (loglk $= -21060.62107$) as inferred from the SSU rRNA gene sequences, alignment I (1284 nucleotides from which 466 were constant and 595 parsimony informative). Tree was constructed using GTR model for nucleotide substitutions with discrete γ-distribution in 8+1 categories, with all parameters estimated from the data set (γ shape $= 0.355$, proportion of invariant sites $= 0.000$). ML bootstraps were computed in 300 replicates using the HKY model, one category of sites with all parameters estimated from the data set. Numbers above branches indicate ML bootstraps/LogDet bootstraps (constructed from 818 variable characters, 1000 replicates) higher than 50%, with the exception of selected nodes (numbers in italics). The alternative position of *Aggregata* spp. as obtained by the LogDet method is indicated by dashed branches.

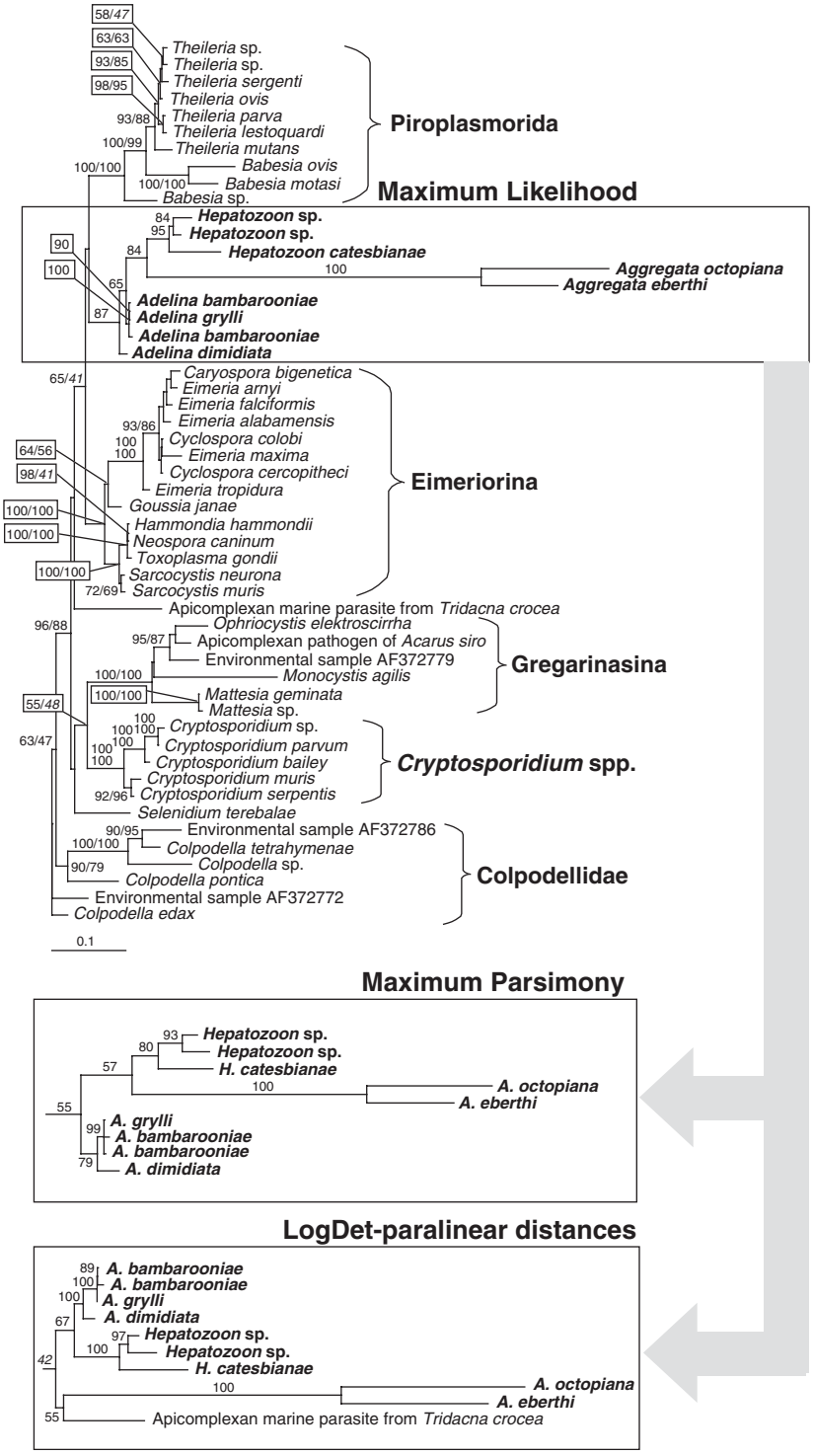


Figure 8. Maximum likelihood phylogenetic tree (loglk $= -13945.27568$) as inferred from the SSU rRNA gene sequences, alignment II (1428 nucleotides from which 564 are constant and 582 parsimony informative). Tree was constructed using the GTR model for nucleotide substitutions with discrete γ distribution in 8+1 categories, with all parameters estimated from data set (γ shape $= 0.375$, proportion of invariant sites $= 0.000$). ML bootstraps were computed in 300 replicates using the HKY model, one category of sites with all parameters estimated from the data set. Numbers above branches indicate ML bootstraps/ LogDet bootstraps (1000 replicates) higher than 50%, with the exception of selected nodes (numbers in italics). Three alternative topologies of the cluster of interest are shown (ML, MP, LogDet) with bootstrap support indicated for each method separately.

bootstrap tree was obtained by the MP method (data not shown).

Discussion

Apicomplexan parasites include many medically and/or economically important pathogens and, as such, attract adequate attention. Since the pioneering sequence-based phylogenetic analyses (Gajadhar et al. 1991), the amount of available molecular data has increased substantially. At present, well-sampled genera within the Eimeriorina include *Sarcocystis*, *Eimeria*, *Isospora*, *Cyclospora*, *Toxoplasma*, and *Neospora*, each represented by dozens of sequenced strains or species. Whereas a plethora of molecular data from these economically important taxa is available, many genera and even several families are still totally missing from the phylogenetic analyses (Jirků et al. 2002; Morrison et al. 2004). These gaps in sampling are mostly due to the relative obscurity, low abundance or presence in unusual environments/hosts of the missing apicomplexans. Yet sequence data from such organisms can reveal important information not only about the coccidian evolution itself, but also about the evolution of parasitism in alveolates. Therefore, obtaining as many representatives of undersampled taxa as possible is necessary.

Monoxenous apicomplexans of the genus *Adelina* are, based on the morphology and life cycles, traditionally classified together with the vector-transmitted haemogregarines within the suborder Adeleorina Léger. The close relationship of *Adelina* and *Hepatozoon*, strongly supported by our results, confirms the legitimacy of this taxonomic unit created almost a hundred years ago (Léger 1911). Moreover, the family Adeleidae Mesnil is well-supported by the SSU rRNA-based trees, in which all available *Adelina* spp. form a monophyletic group. Syzygy is considered to be the main autapomorphic feature of the suborder Adeleorina. This developmental trait represents a unique mode of fertilization, during which gamonts associate prior to the formation of functional gametes and fertilization itself. So far, syzygy was described in all members of the Adeleorina studied in detail, being documented both in monoxenous and heteroxenous taxa (Barta 2000). However, it should be stressed that the process of syzygy and the number and morphology of microgametes (flagellated or aflagellated) vary among various adeleorid genera (Barta 1989; Siddall and Desser 1990; Weiser and Beard 1959).

The traditional classification of coccidia is based on the morphology of oocysts and the number of sporocysts and sporozoites. Additionally, the importance of excystation structures on sporocysts has been stressed (Jirků et al. 2002). The oocysts of adeleid coccidia and haemogregarines, as well as those of *Aggregata*, typically contain several sporocysts, each containing a variable number of sporozoites. Moreover, the sporocysts of both *Adelina* and *Aggregata* excyst via a longitudinal suture in the sporocyst wall, evoking a possible synapomorphic character of these prominent and complex structures. The proximity of *Adelina*, *Hepatozoon*, and *Aggregata* in some of our trees supports this notion. However, the appearance of the genus *Aggregata* within the *Hepatozoon*/*Adelina* clade is inconsistent with the absence of the key developmental feature of the Adeleorina-syzygy, in the Aggregatidae. Already early studies (Dobell 1925) provided evidence that *Aggregata* has numerous elongated bi-flagellated microgametes, analogous to those described in other species of the Eimeriorina (Fig. 10). Confirmed by ultrastructural studies of *Agg. eberthi* (Gestal et al. 2002; Heller 1970), this developmental trait puts a question mark over all topologies placing *Aggregata* spp. inside the above-mentioned clade.

As different topologies have been recovered by various phylogenetic methods, caution has to be exercised when conclusions are drawn from the available sequence data. The affiliation of *Aggregata* spp. with the suborder Adeleorina remains ambiguous. The tree constructed using the LogDet paralinear distances (Fig. 8) shows a topology not necessarily in conflict with the absence of syzygy in the genus *Aggregata,* due to its position at the root of the suborder Adeleorina. Such topology is consistent with the scenario, in which an ancestral state of gamete formation has been retained in *Aggregata*.

Interestingly, members of the genus *Aggregata* have an unusual base composition of their SSU rRNA genes, characterized by an extremely low GC content, unbalanced to the detriment of cytosine, which further complicates their phylogenetic analysis. Apicomplexan parasites, particularly those of the genus *Plasmodium*, are known to possess genomes with extremely low GC content; the malarial parasite *P. falciparum* even has the most AT-rich genome sequenced to date (Gardner et al. 2002). On the other side, genes known from coccidia do not display such exceptional nucleotide compositions. Although they are also AT-rich, the GC content within the SSU rRNA gene usually

does not drop below 42% (see Fig. 6), compared to 36% in *P. falciparum. Aggregata* spp. SSU rRNA genes contain 26% and 28% of GC, which may even represent the lowest GC contents known for this gene so far. When compared to other Apicomplexa, *Aggregata* spp. also display extremely low cytosine content in their SSU rRNA genes: the C content (9% and 7.3% respectively) is far below that in *Plasmodium* (about 15%). Such unusual nucleotide composition substantially influences identification of the sequences as well as computing of their phylogenetic positions. Homology searches using blastn and discontinuous megablast at the NCBI website give quite inconsistent results when *Aggregata* spp. SSU rRNA genes are used as query. Therefore, we deemed it necessary to verify the origin of amplified sequences by Southern blot hybridization. This experimental approach confirmed that both sequences do indeed originate from *Aggregata* spp.

It is well documented that highly divergent sequences may cause phylogenetic artifacts (Bergsten 2005; Johnson et al. 2004; Philippe and Germot 2000). To minimize the risks of the long-branch attraction phenomenon, the longest branches, represented by most gregarines and haemosporidians, were excluded from our analyses. Furthermore, methods such as LogDet and ML with discrete gamma distribution designed to overcome these obstacles were used. In any case, alignment II produced very stable topologies differing only in the internal structure of the clade comprising the genera *Adelina*, *Hepatozoon*, and *Aggregata*. It is necessary to point out that when *Agg. octopiana* and *Agg. eberthi* appeared outside of this clade, they always clustered together with the apicomplexans isolated from the invertebrate hosts. Such a branching order may reflect the fact that apicomplexan parasites of invertebrates also possess relatively divergent sequences.

Apicomplexans are protists typically parasitizing multicellular hosts. It is therefore reasonable to assume that the first radiation of the apicomplexan parasites occurred during the time of emergence of multicellularity in the Precambrian period (Escalante and Ayala 1995), followed by a further major diversification during the Cambrian explosion. Similarly, the evolution of coccidia that affect vertebrates should coincide with the emergence of poikilotherms (Šlapeta et al. 2003). Although any phylogenetic reconstructions of apicomplexans must be distorted by the absence of extinct lineages, a thorough and unbiased sampling will surely improve our understanding of the evolution of these important protistan parasites.

Methods

Organisms: *Adelina dimidiata* Schneider, 1875 was isolated from the centipede *Scolopendra cingulata* Latreille (Chilopoda: Scolopendridae), captured in July 2003 in Melnik, Bulgaria. To obtain sufficient amounts of material, feces of the infected centipede were collected over several weeks during its stay in captivity. Isolated oocysts were macerated in tap water and processed further. Oocysts of *Adelina grylli* Butaeva, 1996 have been obtained from the fat body of experimentally infected crickets *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae).

Aggregata octopiana Schneider, 1875 was isolated from *Octopus vulgaris* Cuvier (Cephalopoda: Octopodidae) captured in May 2004 in the Adriatic Sea, Žuljana, Pelješac Peninsula, Croatia. *Aggregata eberthi* Labbé, 1895 originates from *Sepia officinalis* L. (Cephalopoda: Sepiidae) caught in May 2003 in the same location. In both cases, the macroscopically visible oocysts were carefully dissected from heavily infected intestinal tissue, washed in distilled water, and preserved in 96% ethanol until further processing. The identity of the studied coccidians was confirmed by a thorough morphological evaluation using light microscopy and by comparing the material with original descriptions (Minchin 1903) as well as later taxonomic revisions (Gestal et al. 2002; Sokolova et al. 1999). Micrographs were taken using an Olympus AX 70 microscope equipped with the Nomarski interference contrast microscopy.

DNA extraction, PCR amplification, and sequencing: After release of sporozoites from oocysts, genomic DNA was extracted by pronase E (Sigma) digestion followed by phenol/chloroform extraction and ethanol precipitation as described elsewhere (Maslov et al. 1996). The SSU rRNA gene was amplified using universal eukaryotic primers Eurib1 (ACC TGG TTG ATC CTG CCA G) and reverse Eurib2 (CTT CCG CTG GTT CAC CTA CGG) (Medlin et al. 1988). PCR reactions were carried out in 25 μl with 10 ng DNA, 1 unit Taq-Purple DNA polymerase (TopBio), and 25 pmol of each primer. The amplification program consisted of 30 cycles of 95 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min. PCR products were gel-purified, cloned into the TOPO TA cloning vector (Invitrogen), and both strands were sequenced using a Beckman automatic sequencer.

Sequences: Nucleotide sequences obtained in this study were deposited in GenBank™ under the accession numbers: DQ096835 (*Adelina dimidiata*), DQ096836 (*Adelina grylli*), DQ096837 (*Aggregata octopiana*), and DQ096838 (*Aggregata eberthi*). The following sequences retrieved from GenBank™ were used in our phylogenetic analyses: Apicomplexa (AY490099, AF129883, AF457127, AY334569, AY334568, AY533147, AY533146, AY533144, AY533145, AF081135, AF013418, AY533143, AF081137, AF078815, AB070506, AY196709, AF111185, AF111186, AF291427, U67117, AY613853, AF060975, AF080614, AF324217, M34846, U07812, U17346, AF096498, U00458, AY043206, AF494059, AF494058, AB181504, AF297085, AF130361, AB000912, AF093502, AF093498, AF093494, AF442484, AF093495, AB089285, AY661513, AY278443); Colpodellidae (AF330214, AY449717, AY078092, AY234843); Perkinsasida (AY487833, AF140295, AF252288, X75762); Dinoflagellata (AF274267, AF274266, AF274277, AJ415520, AF022197, AB088302, AF274268, AF231803, AF239261, Y16238, AF080097, AF022197, AF274256, AF286023, AF022200, AF069516); Ciliophora (Z22879, U97111, U97109, U97112, U97108, AJ292526, Z29516, X03948, U57771, AF255357, AF194409, X65150, M97909); Stramenopila (AB022819, U73232, AB022111); Fungi (AF368514, AF368513, AF052405, AF368524); and the environmental samples (AF372779, AY179988, Y01050847, AF372786, AF372772, AF372785, AJ402349, Y01050847).

Southern hybridization: Undigested or *EcoR*I-digested total genomic DNA isolated from *Neospora caninum* (cysts obtained from an experimentally infected mouse), *Trypanosoma brucei* (cultured promastigotes of the strain 29—13), *Octopus vulgaris* (visceral organs of an *Aggregata*-free octopus), *Aggregata octopiana* (oocysts purified as described above), and *Triturus vulgaris* (muscles of an adult newt) was separated on a 0.75% ethidium bromide-stained agarose gel, and blotted overnight at 4 °C. Prior to blotting, the gel was incubated in 0.25 M HCl for 15 min to facilitate the transfer of large molecules. The entire SSU rRNA gene of *Aggregata octopiana* amplified with primers Eurib1 and Eurib2 was labelled with [α-^{32}P] dATP by random priming (HexaLabel kit, Fermentas) and used as probe. Hybridization was carried out overnight in Na-Pi solution (0.25 M NaH_2PO_4/0.25 M Na_2HPO_4/7% SDS/1 mM EDTA) at 65 °C. Blots (Hybond-N; Pharmacia Amersham) were washed extensively at 65 °C in three changes of 3 × SSC/0.1% SDS, 1 × SSC/0.1% SDS, and finally 0.3 × SSC/0.1% SDS, for 20 min each, and the signal was analyzed by a phosphorimager.

Phylogenetic analysis: Three different alignments (I, II, and III) of the small subunit rRNA gene sequences were constructed. First, newly obtained sequences as well as additional apicomplexan sequences available in the GeneBank™ were aligned to the multiple alignment of alveolates published by Leander et al. (2003) using ClustalX (Thompson et al. 1997) (alignment I) and used for phylogenetic analysis. Alignments II and III were obtained by aligning apicomplexan sequences separately by ClustalX with gap opening 10 and 5 respectively. In all alignments, gaps and ambiguously aligned regions were omitted from further analyses. In alignment I, the positions selected by the authors of the original alignment (Leander et al. 2003) have been respected. MP trees were constructed using PAUP 4b10 (Swofford 2000) with 1000 bootstrap replicates. ML trees were computed by PHYML (Guindon and Gascuel 2003) using the GTR model for nucleotide substitutions with discrete γ-distribution in 8+1 categories. All parameters (γ shape and proportion of invariant sites) were estimated from the data set. Multiple data sets (300 replicates) for ML bootstrap analyses were prepared using SeqBoot (PHYLIP 3.6.3., Felsenstein 2001). ML bootstraps were computed using the HKY model for nucleotide substitutions with one category of sites and all parameters (nucleotide frequencies, proportion of invariant sites, and transition/transversion ratio) estimated from the data set. LogDet-paralinear distances as implemented in PAUP 4b10 were used to construct the distance-based trees. LogDet bootstrap analysis was computed from 1000 replicates.

Acknowledgements

We thank Jan Votýpka, Pavla Ptáčková, Julius Lukeš Jr., Katka Modrá, and Martin Kamler for help with collecting the material, and Nedo Hassan and his family for their hospitality in Žuljana. We are indebted to Olympus C&S for their generous support. This study was supported by grants from the Grant Agency of the Czech Republic (524/03/D104), Ministry of Education of the Czech Republic (30-801 and 6007665801), and the Czech Academy of Sciences (Z60220518).

References

Barta JR (1989) Phylogenetic analysis of the class Sporozoea (phyllum Apicomplexa Levine, 1970): evidence for independent evolution of heteroxenous life cycles. J Parasitol **75**: 198—206

Barta JR (2000) Suborder Adeleorina. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, Kansa, pp 305—318

Barta JR (2001) Molecular approaches for inferring evolutionary relationships among protistan parasites. Vet Parasitol **101**: 175—186

Bergsten J (2005) A review of long-branch attraction. Cladistics **21**: 163—193

Dobell C (1925) The life-history and chromosome cycle of *Aggregata eberthi* (Protozoa: Sporozoa: Coccidia). Parasitology **17**: 1—136

Escalante AA, Ayala FJ (1995) Evolutionary origin of *Plasmodium* and other Apicomplexa based on ribosomal-RNA genes. Proc Natl Acad Sci USA **92**: 5793—5797

Felsenstein J (2001). PHYLIP, Phylogeny Inference Package, Version 3.6a3, Department of Genetics* University of Washington, Seattle

Gajadhar AA, Marquardt WC, Hall RJ, Ariztia-Carmona EV, Sogin ML (1991) Ribosomal-RNA sequences of *Sarcocystis muris*, *Theileria annulata*, and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. Mol Biochem Parasitol **45**: 147—154

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DMA, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **419**: 498—511

Gestal C, de la Cadena MP, Pascual S (2002) Malabsorption syndrome observed in the common octopus *Octopus vulgaris* infected with *Aggregata octopiana* (Protista: Apicomplexa). Dis Aquat Org **51**: 61—65

Gestal C, Nigmatullin CM, Hochberg FG, Guerra A, Pascual S (2005) *Aggregata andresi* n.sp (Apicomplexa : Aggregatidae) from the ommastrephid squid *Martialia hyadesi* in the SW Atlantic Ocean and some general remarks on *Aggregata* spp. in cephalopod hosts. Syst Parasitol **60**: 65—73

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol **52**: 696—704

Heller G (1970) Elektronmikroskopische Untersuchungen an *Aggregata eberthi* aus dem Spiraldarm von *Sepia officinalis* (Sporozoa, Coccidia). Z Parasitenkd **33**: 183—193

Jirků M, Modrý D, Šlapeta JR, Koudela B, Lukeš J (2002) The phylogeny of *Goussia* and *Choleoeimeria* (Apicomplexa: Eimeriorina) and the evolution of excystation structures in coccidia. Protist **153**: 380—389

Johnson MD, Tengs T, Oldach DW, Delwiche CF, Stoecker DK (2004) Highly divergent SSU rRNA genes found in the marine ciliates *Myrionecta rubra* and *Mesodinium pulex*. Protist **155**: 347—359

Kuvardina ON, Leander BS, Aleshin VV, Myl'nikov AP, Keeling PJ, Simdyanov TG (2002) The phylogeny of colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. J Eukaryot Microbiol **49**: 498—504

Leander BS, Keeling PJ (2003) Morphostasis in alveolate evolution. Trends Ecol Evol **18**: 395—402

Leander BS, Keeling PJ (2004) Early evolutionary history of dinoflagellates and apicomplexans (Alveolata) as inferred from hsp90 and actin phylogenies. J Phycol **40**: 341—350

Leander BS, Harper JT, Keeling PJ (2003) Molecular phylogeny and surface morphology of marine aseptate gregarines (apicomplexa): *Selenidium* spp and *Lecudina* spp. J Parasitol **89**: 1191—1205

Léger L (1911) *Caryospora simplex*, coccidie monosporée et laclassification des coccidies. Arch Protistenkd **22**: 71—88

Lynch M (1999) The age and relationships of the major animal phyla. Evolution **53**: 319—325

Maslov DA, Lukeš J, Jirků M, Simpson L (1996) Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: Implications for the evolution of parasitism in the trypanosomatid protozoa. Mol Biochem Parasitol **75**: 197—205

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**: 491—499

Minchin EA (1903) Class Sporozoa. In Lankester ER (ed) Treatise on Zoology, Part I. Introduction and Protozoa. Adam & Charles Blask, London

Morrison DA, Bornstein S, Thebo P, Wernery U, Kinne J, Mattsson JG (2004) The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). Int J Parasitol **34**: 501—514

Philippe H, Germot A (2000) Phylogeny of eukaryotes based on ribosomal RNA: Long-branch attraction and models of sequence evolution. J Mol Evol **17**: 830—834

Porchet-Henneré E, Richard A, Ferreira E (1981) Contamination expérimentale in vivo and in vitro par *Aggregata eberthi*: Etude ultrastructurale. J Protozool **28**: 228—239

Sardella NH, Martonelli SR (1997) Occurence of merogony of *Aggregata* Frenzel 1885 (Apicomplexa) in *Pleoticus muelleri* and *Artemesia longinaris* (Crustacea: Natantia) from patagonian waters (Argentina). J Invertebr Pathol **70**: 198—202

Siddall ME, Desser SS (1990) Gametogenesis and sporogonic development of *Haemogregarina balli* (Apicomplexa: Adeleina: Haemogregarinidae) in the leech *Placobdella ornata*. J Protozool **37**: 511—520

Šlapeta JR, Modrý D, Votýpka J, Jirků M, Lukeš J, Koudela B (2003) Evolutionary relationships among cyst-forming coccidia *Sarcocystis* spp (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle. Mol Phylogenet Evol **27**: 464—475

Sokolova YY, Butaeva FG, Dolgikh VV (1999) Light and electron microscopic observation on life cycle stages of *Adelina grylli* Butaeva 1996 (Sporozoa, Adeleidae) from the fat body of the cricket *Gryllus bimaculatus*. Protistology **1**: 34—42

Swofford DL (2000) PAUP* phylogenetic analysis using parsimony (*and other methods). Sinauer Associates Sunderland, MA

Thompson JD, Gibson TJ, Plesniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **24**: 4876—4882

Upton SJ (2000) Suborder Eimeriorina. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence. Kansas, pp 318—339

Weiser J, Beard RL (1959) *Adęlina sericesthis* n. sp., a new coccidian parasite of scarabeid larvae. J Insect Pathol **1**: 99—106