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ORIGINAL PAPER

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Notes on coccidian phylogeny, based on the apicoplast small subunit ribosomal DNA

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Abstract We performed a phylogenetic analysis, based on the partial small subunit rRNA gene (SSU rRNA) sequences from 13 apicoplasts (including new sequences of *Sarcocystis muris* and *Hyaloklossia lieberkuehni*) and 16 other plastids, with cyanobacteria as an outgroup. The apicoplast sequences formed a highly supported monophyletic clade with two distinct clades, representing coccidia and haemosporidia, with coccidia divided into Eimeriidae and Sarcocystidae subclades. We confirmed the phylogenetic position of *H. lieberkuehni* within the Sarcocistidae, as a sister to the *Toxoplasma*/*Neospora* group. The coccidian plastid sequences appear to evolve slowly, while their homologues from haemosporidians are more rapidly evolving. We suggest that the higher evolutionary rate is reflected by the increase in the AT content and the possible reduction of the outer apicoplast membrane in some haemosporidians. Since the apicoplast SSU rRNA gene sequences, when compared with their nuclear homologues, offer a higher number of informative positions, they can be used for phylogenetic inference within the Apicomplexa.

Introduction

Early electron microscopic studies of the apicomplexan parasites in the 1960s revealed the existence of an unusual multimembranous structure. For decades this structure was sporadically mentioned in ultrastructural studies, under various designations, but otherwise remained neglected. Finally, in the 1990s, the multimembranous organelle was identified as a secondary vestigial plastid; and the term apicoplast was coined (McFadden et al. 1997). The apicoplast is an essential component of the apicomplexan cell; and its elimination causes the so-called "delayed death effect" (He et al. 2001).

Although a single origin of the apicoplasts is generally accepted (Denny et al. 1998), their evolutionary history remains uncertain. Phylogenetic analyses based on the apicoplast rRNA genes (Egea and Lang-Unnasch 1995; Zhang et al. 2000) and the gene for nuclear-encoded and plastid-targeted GADPH (Fast et al. 2001) placed apicoplasts within the red plastid lineage, while some other apicoplast genes showed contradictory results (Blanchard and Hicks 1999). Recently, the red peridinin-containing plastid known from dinoflagellates such as *Heterocapsa triquetra* was supposed to be an ancestral red plastid of both dinoflagellates and apicomplexans (Zhang et al. 2000).

The apicoplast sequences are highly divergent when compared with the nuclear genes and thus they offer a relatively high number of phylogenetically informative positions. Since the apicoplast sequences show coevolution with the host genome (Lang-Unasch et al. 1998), they can be used to study phylogenetic relationships within apicomplexan parasites (Gleeson 2000; Zhao and Duszynski 2001). The aim of this study was to test the usability of the apicoplast partial small subunit (SSU) rRNA gene sequences for the apicomplexan phylogeny and to confirm the phylogenetic position of *Hyaloklossia lieberkuehni* previously inferred from the nuclear SSU rRNA gene (Modrý et al. 2001).

Materials and methods

The DNA of *Hyaloklossia lieberkuehni* and *Sarcocystis muris* were obtained from previous studies (Koudela et al. 1999; Modrý et al.

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2001). The plastid SSU rRNA gene was amplified using degenerate oligonucleotides APC1 (CAGCAGCMGCGGTAATAC) and APC2 (ACGGTTACCTTGTTACGACTT), designed on the basis of sequences available in the GenBank. PCR reactions were carried out in 25 µl with 50–100 ng of total DNA, 1 unit Taq polymerase (TaKaRa) and 25 pmol of each primer. The amplification program consisted of 30 cycles of 95 °C for 1 min, 45 °C for 3 min and 72 °C for 50 s. PCR products were gel-purified and cloned and both strands were sequenced on an automatic sequencer. The sequence alignment obtained using the Clustal X program (Thompson et al. 1997) and corrected manually contained the plastid SSU rRNA of *H. lieberkuehni*, *S. muris*, 11 partial apicoplast sequences (for accession numbers see Table 1) and sequences from 16 plastids (AB027236, AF022186, AF041468, AF130038, AF172716–9, M81884, NC001865, U38804, V00159, X12742, X14386, X65101, X75518, Z00044), and a cyanobacterium (D90916). The final alignment, containing 30 sequences, was 894 nucleotides long with 581 variable characters (of which 511 were parsimony-informative) and is available on www.vfu.cz/slapeta/alignments/in the NEXUS format. Phylogenetic analysis was performed using maximum parsimony (MP), with transversions:transitions (TV:TI) weighted 1:1, 3:1 and 5:1, maximum likelihood (ML; ML-HKY85) and distance (ME-K2P, ME-LogDet, ME-K2P-transversion analysis) as implemented in PAUP 4.08b (Swofford 1998).

Results and discussion

In the analyses performed (MP, ML-HKY85, ME-LogDet, ME-K2P), the apicoplast sequences are monophyletic and form two distinct sister clades (Fig. 1). The first clade is composed of the haemosporidian plastids and the second clade contains the apicoplasts of coccidia. The composition of both clades and their close relationship are highly supported by bootstrap analysis (Fig. 1). The tree obtained (Fig. 1) is congruent with previously published phylogenies based on both the nuclear and plastid SSU rRNAs (Lang-Unnasch et al. 1998; Zhu et al. 2000a). The enlargement of the dataset by the inclusion of *Hyaloklossia lieberkuehni* and *Sarcocystis muris* resulted in the appearance within coccidia of the Eimeriidae and Sarcocystidae subclades (Fig. 1). The position of *H. lieberkuehni* as a sister taxon to *Neospora* and *Toxoplasma* has recently been shown, using the nuclear SSU rRNA genes (Modrý et al. 2001) and this provides further evidence for the co-evolution of nuclear and plastid genes. The placement of *S. muris* on the basis of the *Neospora*/*Toxoplasma*/*Hyaloklossia* clade is also congruent with previous studies (e.g. Modrý et al. 2001). Although only partial sequences were analysed (894 nt), they offer 419 parsimony-informative positions within the 13 apicomplexan plastids tested. Such a high number of informative sites provides a dataset with a robustness which is comparable with that of the nuclear homologues which are highly preferred in the inference of coccidian phylogeny. For example, the nuclear SSU rRNA genes of *N. caninum* and *T. gondii* are almost identical, while their plastid homologues differ in more than 20 nucleotides. The plastid SSU rRNA gene sequences therefore represent an informative alternative to the nuclear data, as already referred for ORF470 in *Eimeria* spp (Zhao and Duszynski 2001).

The apicoplast sequences are typically AT-rich. Although the AT content in the SSU rRNA genes in primary plastids does not exceed 50% (*N. tabacum* 43.4% AT, *Porphyra purpurea* 48.4% AT) and is only slightly higher in the secondary plastids (*Guillardia theta* 50.1%, *Euglena gracilis* 58.8% AT), it passes 60% and 70% in coccidian and haemosporidian apicoplasts, respectively (Table 1). It is of interest that the nucleotide composition of the coccidian plastid sequences is different from that of the haemosporidian ones (see Table 1). Such biases in the nucleotide composition are known to cause serious artifacts in the tree topology (Lockhart et al. 1992). To overcome the nucleotide bias, we performed ME-LogDet and TV (ME-K2P, TI omitted) analysis. The topology of the apicoplast cluster obtained using LogDet/paralinear distances (PAUP*) followed those computed by MP and ML methods, while the TV analysis indicated paraphyly of haemosporidians. However, this paraphyletic character was not supported by the bootstrap tree, where a four-way polytomy was created (*Babesia* spp, coccidia, *Hepatozoon catesbianae*, *Plasmodium* spp; data not shown). When the outgroup taxa were limited to *Astasia longa*, *E. gracilis* and *Synechocystis* sp. three different topologies were obtained. The first (MP, ML) was analogous to the previous trees, with apicoplasts divided into two monophyletic clades containing haemosporidians and coccidians, respectively. When the LogDet distances were used for the tree reconstruction, the haemosporidian *H. catesbianae* appeared in the outgroup position

Table 1 Nucleotide composition of partial apicoplast small subunit rRNA gene sequences used for phylogenetic analysis

Organism	Taxonomic position	AT content (%)	Accession number
Babesia bigemina	Haemosporidia	72.53	AF040968
B. bovis	Haemosporidia	71.44	AF040969
Eimeria maxima	Coccidia	64.06	AF040976
E. meleagrimitis	Coccidia	63.21	AF040970
E. tenella	Coccidia	64.76	AF040971
Hepatozoon catesbianae	Haemosporidia	67.61	AF040972
Hyaloklossia lieberkuehni	Coccidia	63.34	AF297120
Neospora caninum	Coccidia	64.29	AF040973
Plasmodium berghei	Haemosporidia	75.50	U79731
P. falciparum	Haemosporidia	76.97	X57167
P. vivax	Haemosporidia	75.45	AF040974
Sarcocystis muris	Coccidia	64.15	AF255924
Toxoplasma gondii	Coccidia	64.01	U28056

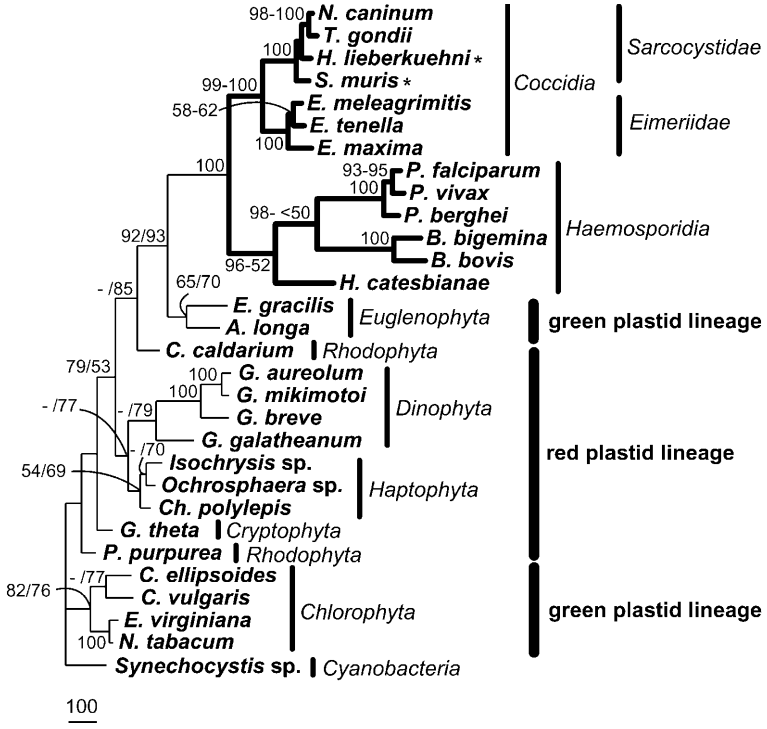

Fig. 1 Maximum parsimony phylogenetic tree, as inferred from partial plastid small subunit rRNA gene sequences. The tree was computed using a 894-nt alignment in which, from 581 variable characters, 511 were parsimony informative. The tree [transversions:transitions (TV:TI) ratio = 1:1] was 1,929 steps long, the consistency index was 0.4925, the rescaled consistency index was 0.3511 and the retention index was 0.7129. Bootstrap supports (1,000 replicates) higher than 50% are indicated. Thick lines indicate the apicoplast clade. The nodal supports within this clade were computed with the TV:TI ratios 1:1, 3:1 and 5:1; and the range of the bootstrap values obtained is shown. Nodal support of the rest of the tree was computed using TV:TI ratios 1:1 and 3:1 respectively; and both values are indicated as *1:1/3:1*. In the whole tree, when bootstraps do not change with the different TV:TI ratios, only one number is shown

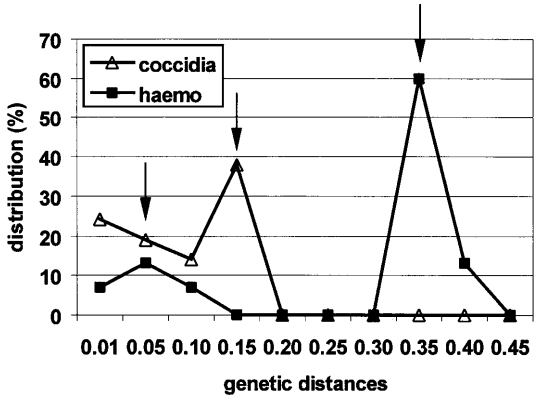

Fig. 2 Distribution of the genetic distances (K2P) within the coccidians (*coccidia*) and haemosporidians (*haemo*). The distribution was computed in the following categories: 0.01–0.05, 0.051–0.10, 0.101–0.15, 0.151–0.20, 0.201–0.25, 0.251–0.30, 0.301–0.35, 0.351–0.40 and 0.401–0.45. Arrows (from the left): the first peak indicates low variability within the genera *Plasmodium* and *Babesia* (*haemo*) and within more closely related coccidians, the second peak shows the major fraction of the genetic distances within the coccidian group (38% of distances in the category 0.151–0.20) and the third peak represents the major fraction of the distances within haemosporidians (60% of distances in the category 0.351–0.40)

against all other apicoplasts. The TV analysis (ME-K2P) placed coccidian plastids in a monophyletic clade, while haemosporidians became their paraphyletic ancestors, with *H. catesbianae* being the most primitive (data not shown). To search for the true topology, we tested these trees using the Shimodaira–Hasegawa likelihood test in PAUP*. The MP/ML tree displaying topology obtained in the initial tree (Fig. 1) was characterized as the best tree, significantly better than the LogDet tree ($P = 0.619$) and the TV tree ($P = 0.152$), thus confirming the existence of two apicoplast lineages.

The sequence data show that there are generally longer branches and a much higher level of variability within the haemosporidian clade, when compared to the coccidia clade. The distribution of genetic distances shows a higher level of variability within the haemosporidians, where 60% of distances falls at 0.35–0.40 while, in the coccidians, the major fraction of distances belongs to the category 0.15–0.20 (Fig. 2). A different rate of evolution also seems to be reflected in other than molecular characteristics. The two distinct clades of Apicomplexa may be characterized by the number of membranes surrounding their plastids. All ultrastructural data for *S. muris* (Hackstein et al. 1995) and *Toxoplasma gondii* (Köhler et al. 1997), together with the results of our analysis of *S. muris* and the fish coccidian *Goussia janae* (data not shown) clearly show the presence of four surrounding membranes. However, in the case of haemosporidians, the available data are

conflicting. Although some authors describe four surrounding membranes in the apicoplast of *Plasmodium falciparum* (McFadden et al. 1997), the conclusions of others are different (Hopkins et al. 1999). We re-evaluated some photographs published previously (Friedhoff and Scholtyseck 1969) and, in *Babesia bigemina*, we could identify three surrounding membranes only. The fact that at least some haemosporidians displaying a higher evolutionary rate may have only three surrounding membranes in their plastids may be explained (as with the ultra-rapidly evolving three-membraned plastids of the dinoflagellates) by a reduction of the outer plastid membrane (Zhang et al. 2000).

The monophyly of apicoplasts and their split into two distinct clades of coccidia and haemosporidia are supported in our analysis. Despite the fact that the apicoplast-like structure was described in the gregarine *Selenidium hollandei* (Schrével 1971), our numerous attempts to amplify the plastid SSU rRNA and other plastid genes from a related species, *Gregarina garnhami*, failed; and Southern hybridizations using apicoplast SSU rRNA probe were also inconclusive (Oborník et al., unpublished data). The existence of the apicoplast in *Cryptosporidium* has already been questioned (Zhu et al. 2000b). Since these apicomplexans were reported to be relatively closely related (Carreno et al. 1999), they could represent a third lineage of highly or totally reduced apicomplexan plastids.

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