International Journal for Parasitology 39 (2009) 1–12

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Invited Review

Evolution of the apicoplast and its hosts: From heterotrophy to autotrophy and back again

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ARTICLE INFO

Article history:
Received 20 May 2008
Received in revised form 23 July 2008
Accepted 25 July 2008

Keywords:
Apicoplast
Apicomplexa
Evolution
Chromera velia
GAPDH

ABSTRACT

The photosynthetic origin of apicomplexan parasites was proposed upon the discovery of a reduced non-photosynthetic plastid termed the apicoplast in their cells. Although it is clear that the apicoplast has evolved through a secondary endosymbiosis, its particular origin within the red or green plastid lineage remains controversial. The recent discovery of *Chromera velia*, the closest known photosynthetic relative to apicomplexan parasites, sheds new light on the evolutionary history of alveolate plastids. Here we review our knowledge on the evolutionary history of Apicomplexa and particularly their plastids, with a focus on the pathway by which they evolved from free-living heterotrophs through photoautotrophs to omnipresent obligatory intracellular parasites. New sequences from *C. velia* (histones H2A, H2B; GAPDH, TufA) and phylogenetic analyses are also presented and discussed here.

1. Introduction

The phylum Apicomplexa traditionally represents a group of obligate parasites that cause serious diseases in animal and human hosts, with malaria being the most devastating human parasitosis. The latter is caused by members of the genus *Plasmodium* and is responsible for 1.1–1.3 million deaths each year. Between 350 and 500 million cases are reported annually and over a billion people are at risk of infection (Tuteja, 2007). Moreover, another apicomplexan parasite of humans, *Toxoplasma gondii*, may be directly or indirectly responsible for the death of thousands of people every year (Flegr, 2007). Apicomplexans of the genera *Sarcocystis*, *Eimeria*, *Babesia* and *Theileria*, to name just a few, cause numerous diseases of domestic animals and other vertebrates, and are thus responsible for substantial economic losses worldwide. Thus these important species are extensively studied. However, the significant hidden diversity of these protists remains virtually ignored. Environmental sequencing (Moreira and Lopéz-García, 2002) and sporadic studies of apicomplexans parasitizing invertebrates (Kopečná et al., 2006; Leander, 2007) have revealed only the tip of an iceberg.

In the traditional taxonomic system, Apicomplexa are defined by possessing the apical complex, an assembly of organelles critical for the parasitic life style, which is located at the anterior apex of the cell (Levine et al., 1980). Another unique feature of the usual apicomplexan cell is the apicoplast, an inconspicuous organelle discovered only relatively recently (McFadden et al., 1996; Köhler et al., 1997). This remnant non-photosynthetic plastid is essential for cell survival, as its disruption leads to the so-called "delayed death effect" (Pfefferkorn et al., 1992; Fichera et al., 1995; He et al., 2001). It was proposed that the apicoplast has evolved through secondary endosymbiosis, similar to the plastids of chromist algae (Cavalier-Smith, 1999). It is believed that during this process, a eukaryotic heterotroph engulfed a eukaryotic alga, which evolved to a plastid surrounded by more than two membranes (e.g. Palmer, 2003). Moreover, alga possessing a secondary plastid can serve as an ancestor of a complex plastid in tertiary endosymbiosis, which frequently and repeatedly led to the appearance of tertiary plastids in dinoflagellates. It was hypothesed that the whole eukaryotic supergroup Chromalveolata is a result of a single endosymbiotic event, in which a heterotrophic eukaryotic host engulfed a rhodophyte alga (Cavalier-Smith, 1999). However, this concept is not fully accepted by the scientific community and other scenarios explaining origins of chromalveolate plastids, such as repeated secondary endosymbioses (Falkowski et al., 2004), tertiary origin of most of the alveolate plastids (Bodyl, 2005) or different composition of eukaryotic supergroups (Burki et al., 2007), have to be taken into account. Throughout its evolutionary history, the engulfed cell was reduced to the extant apicoplast, which so far has been documented in all three main apicomplexan lineages – haemosporidians (e.g. *Plasmodium* and *Leucocytozoon*), coccidians (e.g. *Toxoplasma* and *Eimeria*) and piroplasms (e.g. *Babesia* and *Theileria*), while it seems to have been lost in *Cryptosporidium* spp. (Zhu

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0020-7519/$34.00
doi:10.1016/j.ijpara.2008.07.010

et al., 2000; Xu et al., 2004) and gregarines (Toso and Omoto, 2007). Mainly due to its potential as a prospective target for treatment of malaria (Soldati, 1999), the apicoplast is a subject of extensive research and several excellent reviews have described certain aspects of this organelle (Wilson and Williamson, 1997; Roos et al., 1999; McFadden and Roos, 1999; Maréchal and Cesbron-Delauw, 2001; Wilson, 2002; Foth and McFadden, 2003; Ralph et al., 2004). The main aim of this review is to discuss the evolution of this relic plastid and its host, illuminated recently by the newly described alveolate phylum Chromerida and its only known representative *Chromera velia*, which appears to be the closest available photosynthetic relative to apicomplexan parasites (Moore et al., 2008).

2. Plastid in apicomplexan parasites

From the early 19th century until the first half of the 20th century, apicomplexan parasites were studied only by light microscopy, the resolution of which allowed very limited analysis of intracellular features of these rather tiny cells. This situation changed with the advent of electron microscopy, which revealed not only the nucleus, endoplasmic reticulum (ER), mitochondrion, subpellicular microtubules and apical complex, to name the most prominent cellular structures, but also an obscure multimembraned vesicle or whirl. The function and origin of this inconspicuous structure, usually located between the nucleus and the apical complex, were obscure and it was simply ignored in most schematic figures of the apicomplexan cell published at that time. The era of molecular biology in the study of apicomplexans was initiated by the isolation of extrachromosomal DNA from the avian malarial parasite *Plasmodium lophurae* (Kilejian, 1974). Due to its circular nature and gene content testifying to a prokaryotic origin, this 35 kb-long DNA molecule was considered to be of mitochondrial origin (Kilejian, 1974, 1975). However, another extrachromosomal DNA molecule, not exceeding 6 kb in length, was later isolated from other *Plamodium* species (Suplick et al., 1988; Vaidya et al., 1989; Aldritt et al., 1989; Joseph et al., 1989; Suplick et al., 1990; Feagin, 1992). This linear DNA was subsequently shown to represent the genuine mitochondrial genome of the apicomplexan cell (Suplick et al., 1988; Vaidya et al., 1989). A detailed study of the circular 35 kb-molecule led to the suggestion that it might be of plastid origin (Gardner et al., 1991; Howe, 1992; Williamson et al., 1994; Wilson et al., 1996); however, it took another 5 years to locate it within the cell. Two research groups localised the 35 kb DNA circle to the mysterious multimembraneous structure known from numerous electron microscopy studies, a finding strongly indicative of its plastid origin (McFadden et al., 1996; Köhler et al., 1997).

As a relic plastid, the apicoplast contains one of the smallest known plastid genomes. When compared with the 69 kb-long green secondary plastid from the chlorarachniophyte *Bigelowiella natans* (Rogers et al., 2007), the red secondary plastids ranging in size from 105 to 140 kb (Oudot-Le Secq et al., 2007) or the 190 kb-long red primary plastids (Reith and Munholland, 1995), the apicoplast genome represents only between 18 and 50% of the usual photosynthetic plastid genomes. On the other hand, the peridinin plastids of dinoflagellates, which are putative relatives of the apicoplast, possess even smaller and weirder genomes (Green, 2004). Indeed, a degenerative process has obviously affected photosynthetic organelles throughout their evolutionary history. The massive reduction of the ~3,500 kb cyanobacterial genome to approximately 150 kb-long genomes of primary plastids occurred subsequent to primary endosymbiosis, the engulfment of a photosynthetic bacterium by a eukaryotic heterotroph. It has been estimated that as many as 2,000 genes have been transferred from the engulfed cyanobacterium to the nucleus of the primary host (Martin and Herrmann, 1998; Martin et al., 2002). Moreover, the secondary endosymbiosis that followed the primary event is supposed to have been accompanied by another reduction of the plastid genome. It was well documented in the centric diatom *Thalassiosira pseudonana* that genes can be transferred from the secondary plastid directly to the secondary host nucleus (Armbrust et al., 2004). In particular, genes encoding the psb28 protein were found both in its nuclear and plastid genomes, with the nuclear version possessing an N-terminal targeting pre-sequence necessary to transport the protein into the multimembraneous plastid (Armbrust et al., 2004).

Finally, another wave of plastid genome diminution was triggered by the loss of its photosynthetic function. This phenomenon has been well documented in both primary and secondary plastids. Firstly, the genome of the non-photosynthetic primary plastid from *Epifagus virginiana* was investigated and compared with photosynthetic relatives. The genome contains 70 kbs and only 42 genes from which 38 are components of the gene expression apparatus. When compared with higher plants such as tobacco, the *E. virginiana* plastid genome lost all genes involved in photosynthesis and genes encoding RNA polymerases are also absent (Wolfe et al., 1992). Similarly, the photoautotrophic *Euglena gracilis*, possessing a secondary green plastid, holds a 143 kb-long circular plastid DNA, while its naturally bleached non-photosynthetic mutant *Euglena (Astasia) longa* has retained only a 73 kb-long molecule in its plastid (Gockel and Hachtel, 2000). Although the loss of photosynthesis in the latter euglenid must have been fairly recent, as inferred from the short branches in the nuclear ssrRNA tree (Brosnan et al., 2003), all genes encoding proteins associated with pigmentation and photosynthesis are completely absent in the *E. longa* plastid, with the only exception being *rbc*L (Gockel and Hachtel, 2000). Furthermore, the loss of photosynthesis apparently triggered an extreme shift in the nucleotide composition of the apicomplexan plastids. In some of their genes, such as the ribosomal protein rpl11, the AT content reaches 97% (Oborník et al., 2002a). Sequences biased to such an extent are hardly suitable for phylogenetic analyses, as the danger of artifactual tree topology caused by long branch attraction and nucleotide bias is extremely high (Howe, 1992; Lockhart et al., 1992; Philippe, 2000; Oborník et al., 2002a; Dacks et al., 2002). Similar tendencies to reduce its circular genome were described even in the primary non-photosynthetic plastid of the parasitic alga *Helicosporidium* sp., which does not contain any photosynthetic genes. As the genome of this originally green plastid strongly resembles that of the apicoplast, convergent evolution is held responsible for the current state of both genomes (de Koning and Keeling, 2006).

Throughout their long evolutionary history, the apicomplexan plastids have lost their photosynthetic protein-coding genes in an analogous way as *A. longa* did relatively recently. As a consequence, they lack genes most frequently used to infer plastid phylogeny. The composition of pigment complexes of the photosynthetic ancestor of Apicomplexa is therefore unknown. While the plastid origin of the 35 kb DNA circle and its localisation in a multimembraneous organelle were experimentally confirmed (McFadden et al., 1996; Köhler et al., 1997), numerous phylogenetic analyses failed to identify a lineage from which the apicoplast evolved (Blanchard and Hicks, 1999; Köhler et al., 1997; Oborník et al., 2002a). At the same time, the monophyletic origin of the apicomplexan plastids is generally accepted on the basis of both the genome structure (Denny et al., 1998) and molecular phylogeny (Egea and LangUnnasch, 1995; Lang-Unnasch et al., 1998; Zhang et al., 2000; Oborník et al., 2002b). However, one should keep in mind that even monophyly can be an artifact caused by nucleotide bias or the above-mentioned convergent evolution of the non-photosynthetic plastid genomes (de Koning and Keeling, 2006).

In spite of all these shortcomings, the apicoplast genes have frequently been used for phylogenetic analyses of plastid lineages. Analyses of the ribosomal operon structure and phylogeny based on the rRNA genes strongly support the origin of the apicoplast from within the rhodophyte lineage (McFadden and Waller, 1997; Blanchard and Hicks, 1999; Stoebe and Kowallik, 1999; Zhang et al., 2000).

An alternative view postulating the origin of the apicoplast from a green alga has also been put forward, mainly based on the phylogenetic analysis of the nuclear-encoded cytochrome oxidase subunit 2 (cox2) gene, which is of mitochondrial origin. Importantly, the "green" origin is further supported by a split of this gene into two regions (cox2a and cox2b), a feature shared among the apicomplexans and some chlorophytes and leguminous plants (Funes et al., 2002, 2004). However, Waller and colleagues (2003) strongly opposed this scenario by stating that such organisation is actually predetermined in the mitochondrial genome of related ciliates, where cox2 has already been split into the cox2a and cox2b regions. This observation suggests that the split must have occurred repeatedly in evolution and is unsuitable for tracing the origin of the apicoplast (Waller et al., 2003; Waller and Keeling, 2006). In our opinion, the phylogenetic signal inferred from the rRNA gene sequences and structure of the apicoplast ribosomal operon strongly support affiliation of the apicoplast with the red plastid lineage. Such a scenario is further corroborated by phylogenetic analysis of the nuclear-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GADPH), which suggests a monophyletic origin of the plastids of apicomplexans, dinoflagellates, heterokonts and cryptophytes (Fast et al., 2001; Harper and Keeling, 2003).

Although reduction of the apicoplast also occurred at the level of morphology, ultrastructural features such as the number of surrounding membranes may be informative from the evolutionary perspective. The number of membranes that bind this organelle remains ambiguous, although its fine structure has been studied for the past 35 years. Four surrounding membranes are a hallmark of the apicoplast from the coccidians *T. gondii* (Köhler et al., 1997), *Sarcocystis muris* (Hackstein et al., 1995; Oborník et al., 2002b) and *Goussia janae* (Oborník et al., 2002b), although a recent study based on the three-dimensional (3D) reconstruction of the *T. gondii* apicoplast proposed a spatial alternation of two and four membranes (Köhler, 2005). The number of membranes encircling the apicoplast of the haemosporidian *Plasmodium falciparum* remains controversial as well as some authors observed four membranes (McFadden and Waller, 1997), while others described just three membranes complemented by the inner and outer membranous complexes (Hopkins et al., 1999). It is worth mentioning that the apicoplasts that are likely to be four-membraned (coccidia) and the three-membraned (haemosporidians and piroplasms) form two separate clusters in the plastid phylogeny. Furthermore, they differ in molecular characters such as the non-canonical encoding of tryptophan and perspicuous differences in nucleotide composition (Oborník et al., 2002b).

It is therefore reasonable to assume that the four-membraned structure is ancestral. In such a case, there are no morphologically analogous red plastids found in the extant dinoflagellates, except for those obtained by tertiary endosymbiosis. The green plastids of *Lepidodinium viride* (Watanabe et al., 1990) and *Lepidodinium (Gymnodinium) chlorophorum* (Elbrachter and Schnepf, 1996; Hansen et al., 2007) are probably the only known secondary four-membraned plastids in dinoflagellates, while the remaining dinoflagellate plastids surrounded by four membranes are supposed to have originated via tertiary endosymbiosis (Delwiche and Palmer, 1997; Delwiche, 1999). Yet the green endosymbionts of *L. viride* and *L. chromophorum* are obviously derived from prasinophytes possessing primary plastid of the green plastid lineage. They cannot therefore be taken into account for placing the origin of the apicoplast. However, with a postulation of the three-membraned plastids for *Plasmodium* species, an evolutionary link between the dinoflagellate red (=peridinin) plastids and apicoplasts becomes more plausible. Since in haemosporidians the apicoplast apparently underwent an accelerated evolution compared with their coccidian counterparts, one can propose a reduction of the number of plastid membranes in this apicomplexan lineage. It is reasonable to assume that the ancestral apicomplexan plastid was surrounded by four membranes, as there is no plausible scenario available that would explain an increase in the number of membranes surrounding the organelle.

An assortment of red plastids, likely of different origin, is found in the dinoflagellates, a photosynthetic sister group of the apicomplexans (Delwiche, 1999) (Fig. 1). Their peridinin-pigmented plastid, known to have a unique genomic structure with a number of DNA minicircles (Zhang et al., 1999; Barbrook and Howe, 2000; Green, 2004; Koumandou et al., 2004), probably represents the direct descendant of the original chromalveolate plastid (Zhang et al., 2000). The fucoxanthin-containing plastids are supposed to be tertiary, being derived from haptophytes following their acquisition by the dinoflagellate cell later in evolution (Tengs et al., 2000; Takishita et al., 2004). Moreover, other chromalveolates also served as ancestors of tertiary plastids in dinoflagellates, as exemplified by the genus *Dinophysis*, the tertiary plastid of which evolved through the engulfment of a cryptophyte (Hackett et al., 2003). This view is however disputed by some authors, who consider the plastids of *Dinophysis* to be a non-permanent kleptoplastid (Minnhagen and Janson, 2006). It has also been shown that the dinoflagellates *Kryptoperidinium foliaceum* and *Durinskia baltica* acquired a tertiary plastid by swallowing a diatom. Yet in *K. foliaceum*, the symbiotic process was apparently recent, as a virtually intact diatom cell can be recognised inside the dinoflagellate, with its endosymbiont still containing the mitochondrion and an intact nucleus (McEwan and Keeling, 2004). This enslavement has been established however, since several genes of the endosymbiont have already been transferred to the dinoflagellate nucleus, and their products participate in its metabolism (Patron et al., 2006; Imanian et al., 2007; Imanian and Keeling, 2007) (Fig. 1). In conclusion, sufficient evidence is available to support multiple acquisitions of complex plastids by dinoflagellates in the course of their evolution. This conclusion, however, does not necessarily contradict the chromalveolate hypothesis, as the original chromalveolate plastid could have been replaced by the newly acquired endosymbiont (Fig. 1). We can even speculate that single initial secondary endosymbiosis predisposed chromalveolates to acquire another photosynthetic symbiont later in evolution. The main reason is that after this event, the chromalveolate cell already had a set of nuclear-encoded genes for plastid-targeted proteins and a functional system to import such proteins into the secondary organelle.

3. Photosynthetic ancestry of Apicomplexa

The discovery of the apicoplast was quickly followed by a suggestion that the ancestor of the apicomplexan parasites must have been a free-living photosynthetic alga, possibly a dinoflagellate (McFadden and Waller, 1997). An extensive search for such an alga was undertaken. Only recently however, Robert Moore isolated a morphologically distinct alga among known symbiotic dinoflagellates of the stony coral *Plesiastrea purpurea* and named it *C. velia* (Moore et al., 2008). The only available stage of this novel species is a free-living immotile cell of an oval shape (5–7.0 μm in diameter) and brown colour (Fig. 2). It multiplies by binary division, although two subsequent divisions quite frequently lead to the formation of triads. We speculate that these three mutually attached

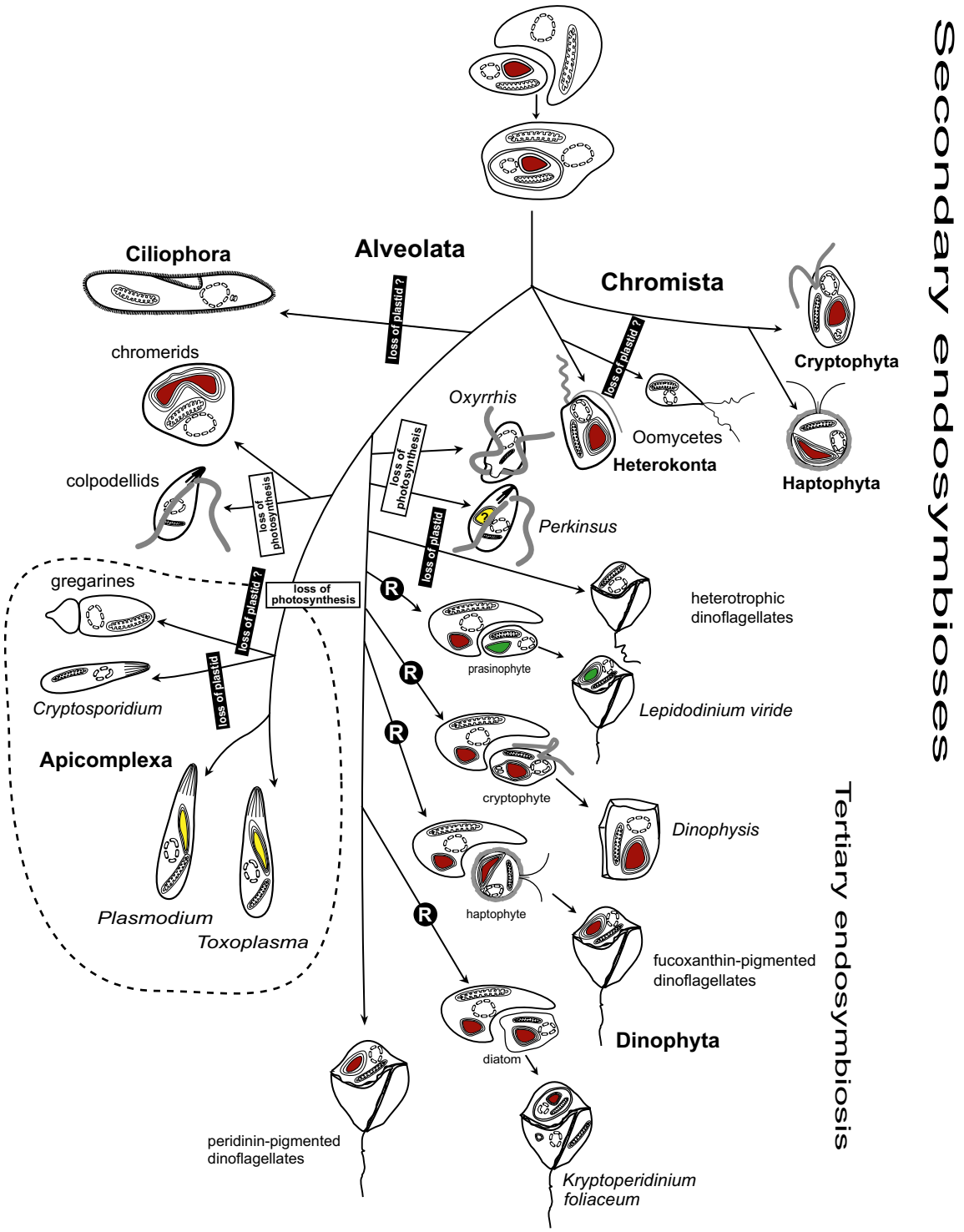

Fig. 1. Evolutionary history of Apicomplexa and other chromalveolates. It is expected that chromalveolates appeared due to a single secondary endosymbiotic event. Their evolution is accompanied by numerous losses and replacements of the plastid, including tertiary endosymbioses by engulfing other photosynthetic chromalveolates. According to our phylogenetic analyses, chromerids branch as early apicomplexans, closely related to the heterotrophic colpodellids. Gregarines appear on the root of the parasitic apicomplexans. R in a black circle indicates plastid replacement.

cells are a product of symmetric binary division, followed by an asymmetric division of only a single daughter cell. *C. velia* contains several ultrastructural features characteristic for alveolates, such as the cortical alveoli (Fig. 3) with an underlying sheet of microtubules, a micropore and, importantly, a large single cone-shaped plastid bound by four membranes (Fig. 4). Inside the plastid, which occupies a significant portion of the cell, conspicuous thylakoid lamellae are arranged in stacks of three or more. The organelle is pigmented by a single chlorophyll *a*, violaxanthin and a novel major carotenoid, which has been assigned an isoform of isofucoxanthin, with a minor component represented by β,β-carotene. A single large mitochondrion with small poorly visible cristae is found in the middle of the cell (Fig. 4). A combination of these features together with phylogenetic analyses (see below) of this alga

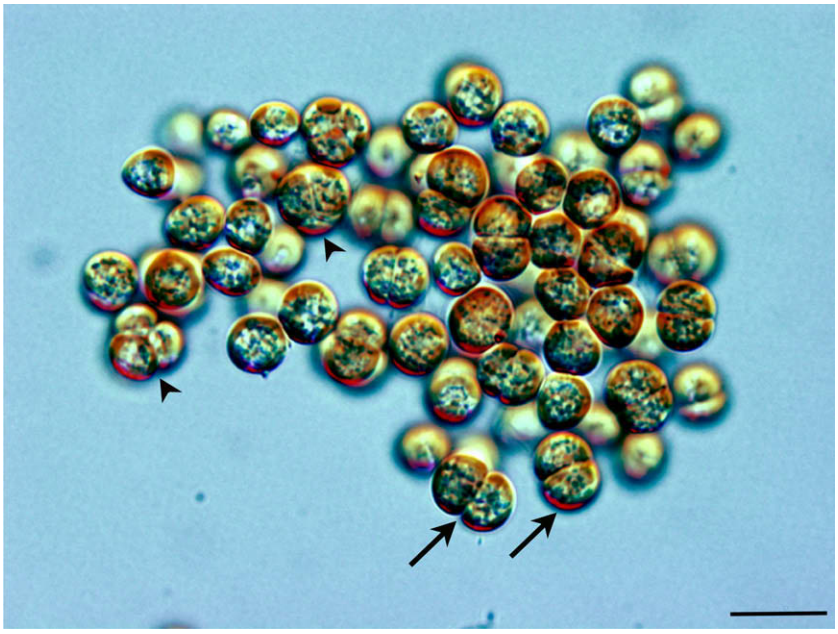

Fig. 2. Light microscopy picture of *Chromera velia* showing individual cells as well as doublets (arrows) and occasional triplets (arrowheads). Scale bar = 10 μm.

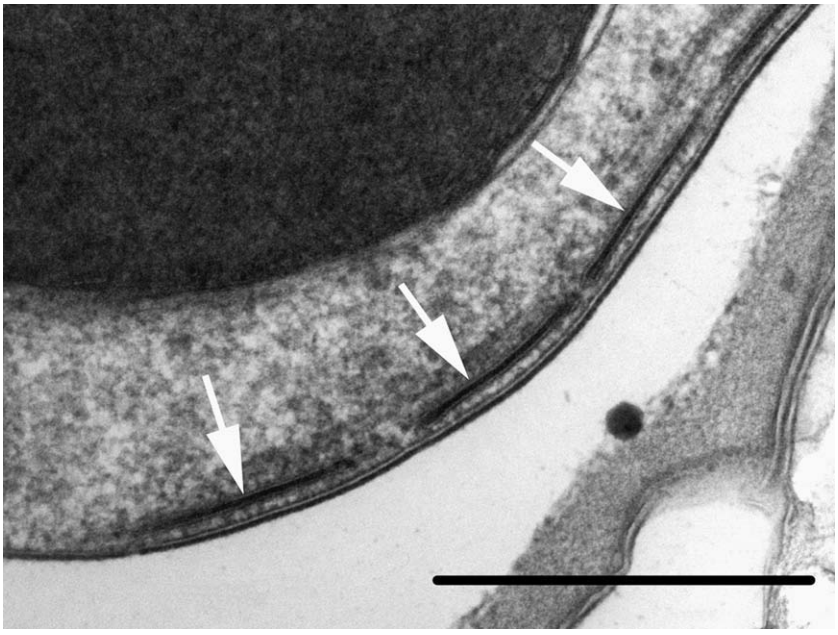

Fig. 3. Transmission electron microscopy picture showing multiple cortical alveoli (arrows) on the periphery of a *Chromera velia* cell. Samples were prepared using the freeze substitution method as described elsewhere (Moore et al., 2008). Scale bar = 1 μm.

led to the establishment of a new phylum within alveolates, named Chromerida after its only known representative (Moore et al., 2008).

The phylogenetic position of *C. velia* was explored using various approaches. rRNA-based phylogenies support its close relationship with either the heterotrophic flagellates of the genus *Colpodella* (nuclear ssrRNA gene), or the apicomplexans (nuclear lsrRNA gene; this gene is not available for colpodellids) (Moore et al., 2008). In addition to these published data, typical histone H2A (EU728805) and H2B (EU728806) genes have been recently discovered in the *C. velia* genome (see Supplementary material), a feature distinguishing it from the dinoflagellates, which have their nuclear DNA uniquely packaged in the apparent absence of histones (Rizzo, 2003; Wong et al., 2003; Chan et al., 2006). Several putative histone genes obtained from the expressed sequence tags libraries of *Crypthecodinium cohnii* (Hackett et al., 2005) differ substantially from their eukaryotic homologues and probably acquired different function(s). Since histones are among the most conservative eukaryotic proteins, they do not contain sufficient phylogenetic information to specify the origin within alveolates. Yet their mere presence in *C. velia* implies that despite its affiliation with alveolates, this alga is certainly not a typical dinoflagellate.

One of the most important findings concerning chromalveolate phylogeny is that all members of this group seem to have substituted, probably by duplication, the original plastid-encoded GAPDH with its nuclear-encoded cytosolic version (Fast et al., 2001;

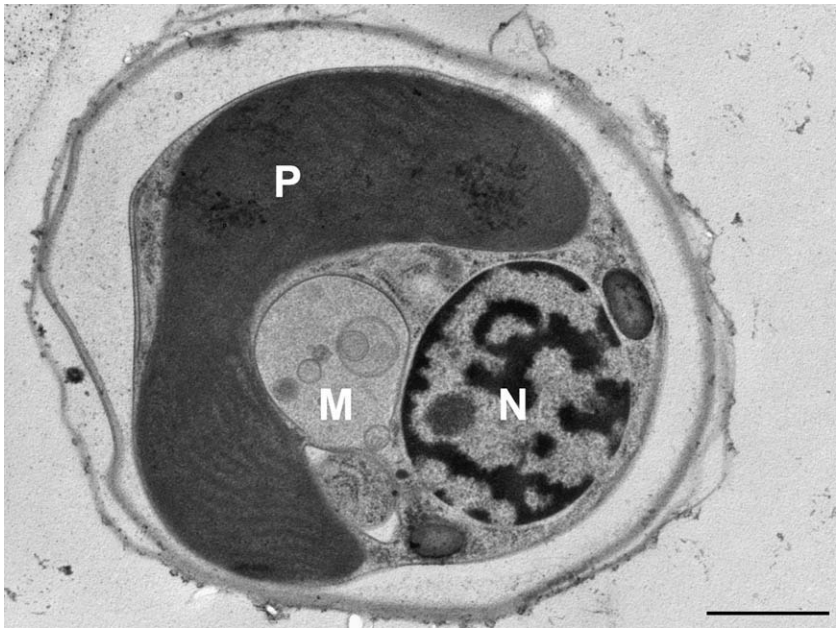

Fig. 4. Transmission electron microscopy of a cross-sectioned *Chromera velia* cell. It shows a large peripheral chloroplast (P) filled with thylakoid lamellae, a central nucleus (N) containing an electron-dense nucleolus and condensed chromatin. The mitochondrion (M) is positioned in the middle of the cell. Samples were prepared as described elsewhere (Moore et al., 2008). Scale bar = 1 μm.

Harper and Keeling, 2003). We have amplified and sequenced both nuclear-encoded GAPDH genes and used the sequences to explore their evolutionary history. Our analysis demonstrates that at least the plastid-targeted chromeran enzyme (EU728804) branches in close proximity to its appropriate apicomplexan GAPDH paralogue. The position of cytosolic GAPDH from *C. velia* (EU728802) remains unresolved (Fig. 5). We can therefore conclude that nuclear genes from *C. velia* sequenced so far, namely the ssrRNA and lsrRNA genes (Moore et al., 2008) and the gene for the plastid-targeted GAPDH (Fig. 5), strongly favour the relationship of this photosynthetic alga with apicomplexan parasites.

The GAPDH tree is also informative concerning other important features of alveolate phylogeny (Fig. 5). In the tree, the genes for nuclear-encoded plastid-targeted GAPDH split into two branches, one of which is composed of genes from peridinin-pigmented dinoflagellates, cryptophytes, stramenopiles and the green plastid-bearing *L. chlorophorum* (Takishita et al., 2008), referred to here as "branch A" (Fig. 5). However, branch B contains genes from *L. chlorophorum*, dinoflagellates with haptophyte-derived plastids, haptophytes, chromerids and apicomplexans (Fig. 5). It has been suggested that the original gene for plastid-targeted GAPDH was, in dinoflagellates possessing haptophyte-derived tertiary plastids, replaced by a counterpart transferred from a tertiary haptophyte endosymbiont through endosymbiotic gene transfer (Takishita et al., 2004). The presence of two distinct genes from the dinoflagellate *L. chlorophorum* is explained by lateral gene transfer (Takishita et al., 2008). Contrary to these suggestions, we propose that presence of the *L. chlorophorum* genes in both branches and the existence of these branches indicate that they may represent out-paralogues originated by an ancient duplication of the plastid-targeted GAPDH that preceded the radiation of chromalveolates. The affiliation of the GAPDH gene from *L. chlorophorum*, which contains a green plastid, with the GAPDH from the red plastid-bearing chromalveolates favours a scenario in which the ancestor of *Lepidodinium* contained the original peridinin-pigmented plastid, which was later replaced by an engulfed prasinophyte. While the chromeran plastid was proposed to share a common ancestry with the peridinin plastids (Moore et al., 2008), it appears here in branch B together with the apicomplexan, haptophytes and dinoflagellates bearing the haptophyte-derived plastid (Fig. 5). Importantly, only the *L. chlorophorum* gene in branch A is, according to in silico prediction (SignalP (Bendtsen et al., 2004), SP = 0.991 + cTP = 0.836), targeted to a complex plastid. The gene from branch B contains at its N-terminus a trans-membrane domain (predicted by Phobius at http://phobius.sbc.su.se/cgi-bin/predict.pl), actually a signal peptide without cleavage site, which is followed by a chloroplast transit peptide (cTP = 0.572). The third *L. chlorophorum* GAPDH gene is putatively mitochondrion-targeted (predicted by TargetP (Emanuelsson et al., 2000)). Thus, sequence motifs suggest three different subcellular locations of GAPDH proteins in *L. chlorophorum*, one in the plastid, another targeted to the ER membrane and the third to the mitochondrion. It has been shown that GAPDH can play other metabolic roles besides glycolysis (Alvarez et al., 2007). It can be involved in signal transduction and intracellular processes (Duclos-Vallee et al., 2002) in maintenance of telomeres in the nuclei of human lung carcinoma cells (Sundararaj et al., 2004). It has been demonstrated to participate in nuclear membrane assembly (Nakagawa et al., 2003) or act as a fusogenic protein in rabbits (Glaser and Gross, 1995). We can speculate that the *L. chlorophorum* GAPDH protein which is, according to in silico predictions, trapped in the ER membrane, acquired a novel function or is targeted to the plastid in a distinct way from the canonical pathway.

The presence of plastid-targeted GAPDH paralogues is supported by the obvious tendency of GAPDH genes to duplicate and form paralogs. In addition to the genes coding for cytosolic, mitochondrial and plastid-targeted GAPDH, other paralogues are also identifiable in the tree: the most basal GAPDH cluster is composed of glycosomal proteins from Euglenids and in addition contains two dinoflagellate sequences. It has been hypothesed that these GAPDH genes have been laterally transferred from Euglenids to some peridinin-pigmented dinoflagellates (Takishita et al., 2003).

Using flow cytometry, we have roughly estimated the size of the *C. velia* nuclear genome to be around 10 Mbp (Fig. 6 and Supplementary material). Even when we take into account the rather low accuracy of the applied method, the size is surprisingly small,

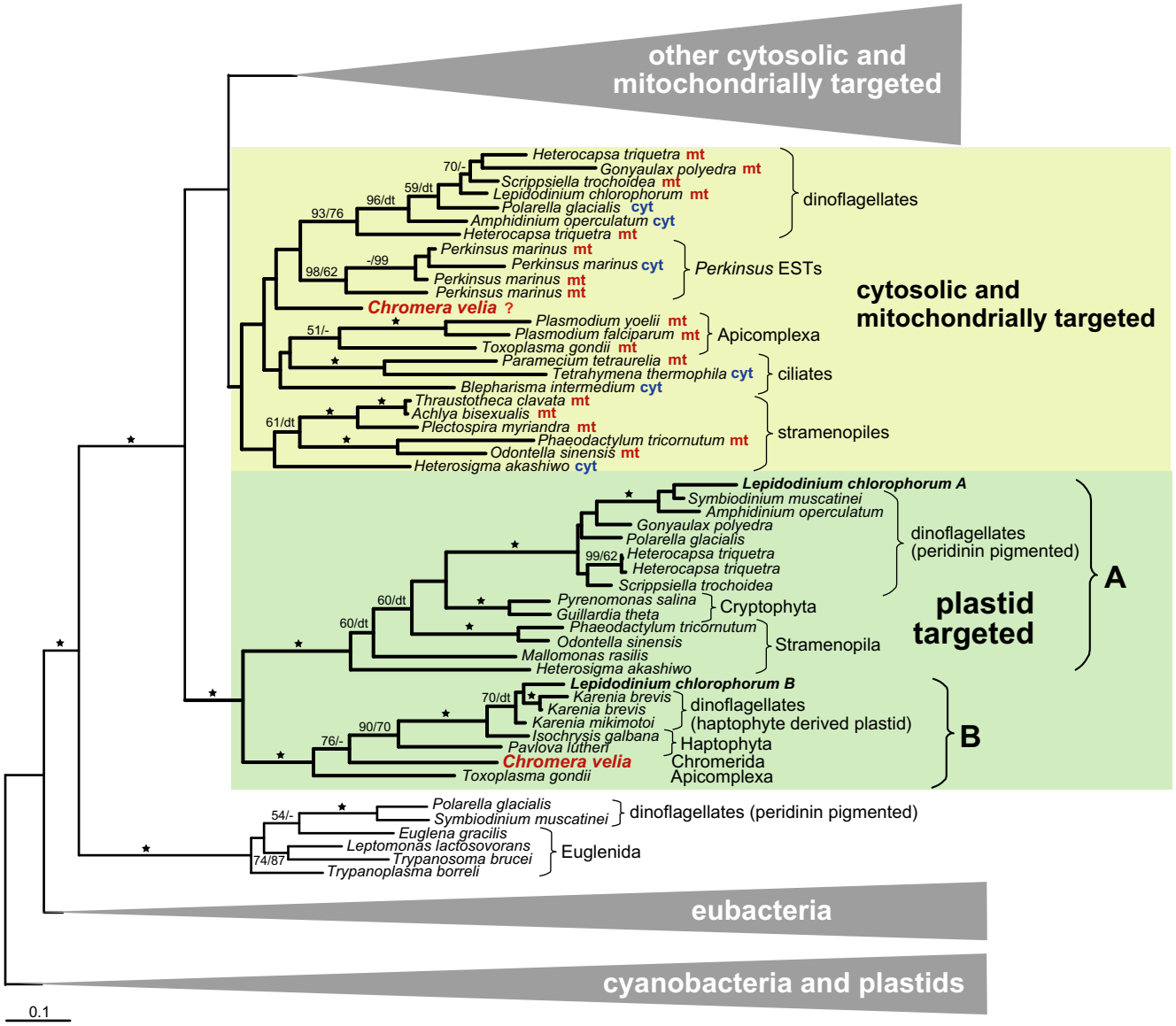

Fig. 5. Maximum likelihood phylogenetic tree inferred from the GAPDH amino-acid sequences. The *Chromera velia* genes were amplified from cDNA (see Supplementary material for details). The phylogenetic position of the cytosolic paralogue of *C. velia* is not well-resolved, while the plastid-targeted version invariably appeared in the proximity of its counterpart from *Toxoplasma gondii*. We enriched the alignment published by Harper and Keeling (2003) with *C. velia* sequences, as well as some other alveolates (see Supplementary material for details). The maximum likelihood (ML) tree was constructed using the PhyML (Guindon and Gascuel, 2003) program, with the substitutional model (WAG+I+Γ) chosen according to PROTTEST (Abascal et al., 2005) results. The neighbour-joining (NJ) tree was constructed using the AsaturA program (van de Peer et al., 2002) designed to deal with saturation of amino acids (see Supplementary material for details). The putative targeting of GAPDH sequences is indicated according to TargetP prediction. Numbers above branches indicate ML/NJ with 200 and 1000 replicates, respectively. Black stars indicate both bootstraps over 90%, dt means different topology in the NJ tree.

representing only about 40% of the *Trypanosoma brucei* genome, which was used for calibration. The genome of *C. velia* would thus be smaller than that of *Plasmodium* spp., *T. gondii* and *Eimeria tenella*, ranging from 23 to 60 Mbp, but probably larger than the genomes of *Cryptosporidium* spp., *Theileria* spp. and *Babesia bovis*, the sizes of which vary between 8 and 9 Mb (Xu et al., 2004; Gardner et al., 2005; Brayton et al., 2007). If this preliminary estimation of the small size of *C. velia* genome is verified by the ongoing genome sequencing project, it would suggest that contrary to other intracellular parasites such as microsporidia (Keeling and Fast, 2002; Keeling and Slamovits, 2005), apicomplexan specialisation for parasitic life style may not be accompanied with a reduction and compaction of the nuclear genome.

One of our major goals is to specify the origin of the chromeran plastid. The plastid ssrRNA gene hints at its common ancestry with the apicoplast, while the *psbA*-based phylogeny prefers, in the absence of the apicomplexan sequences, a relationship with the peridinin-pigmented plastids from dinoflagellates (Moore et al., 2008). In addition to the published phylogenies (Moore et al., 2008), we have constructed trees based on the *tufA* and plastid lsrRNA genes, both of which confirmed the affinity of the chromeran plastid to the apicoplast (Fig. 7 and data not shown). It should be mentioned that *tufA* gene sequences have previously been used to specify the origin of the apicoplast in a fundamental study where authors suggested the origin of the apicoplast is embedded within the green plastid lineage (Köhler et al., 1997). However, even a more enriched dataset does not properly address this question (see Fig. 7). The chromeran plastid *tufA* gene (EU728803) branches together with the apicoplasts as expected, but a cyanobacterial homologue appeared to be the closest relative to the

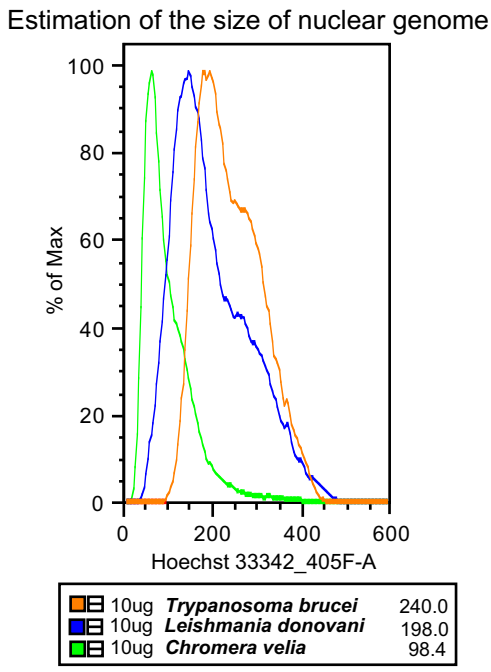

Fig. 6. Estimation of the size of the *Chromera velia* genome, with *Trypanosoma brucei* and *Leishmania donovani* used as controls. The cells were stained with Hoechst 33342 dye and the gene content was measured by flow cytometry (see Supplementary material for details).

apicomplexan-chromeran counterpart instead of primary plastids. However, the most convincing evidence for the *C. velia* plastid sharing ancestry with the apicoplast, lies in the non-canonical coding of tryptophan in the plastid-encoded *psbA* gene. Although all other photosynthetic plastids encode this particular amino acid by the UGG code, along with the coccidian apicoplasts, *C. velia* uses the UGA triplet. While all tryptophans of the *C. velia psbA* gene are specified by UGA, coccidians use both UGG and UGA to encode the amino acid in question at species-specific frequencies. For instance in the *E. tenella* plastid, 26% of tryptophans are encoded by UGA, while in *T. gondii* the use of this non-canonical triplet reaches 60% (Moore et al., 2008). It appears that the ancestor of apicomplexan plastids originally used both canonical and non-canonical coding, the latter being lost in the haemosporidian and piroplasmid lineages.

4. Evolutionary history of Apicomplexa

Due to the absence of fossil remains, the taxonomy and reconstruction of evolutionary history of Apicomplexa have to rely on morphology and molecular phylogeny. There is no doubt this is an ancient phylum. Molecular dating analysis estimates its origin to the period between 600 and 800 million years ago (Escalante and Ayala, 1995; Douzery et al., 2004). Since in those times vertebrates were not yet roaming the earth (Signor, 1994), it is apparent that an originally free-living apicomplexan had begun to parasitize marine invertebrates first, co-evolving with the terrestrial vertebrate hosts much later. The discovery of a free-living, coral-associated photosynthetic predecessor of the obligatory parasitic apicomplexans supports this view (Moore et al., 2008), as does the widespread occurrence of gregarines, which parasitize marine annelids, mollusks and nemerteans, to name just a few of their hosts. As monoxenous parasites with several primitive features, gregarines likely represent an ancestral group of the Apicomplexa (Leander, 2008) (Fig. 1). Indeed, in phylogenetic trees these giants among protists almost invariably form the earliest branch within the core Apicomplexa, usually accompanying the parasitic genus *Cryptosporidium* (Leander et al., 2003).

In classical systems (Levine et al., 1980), the flag character of the phylum Apicomplexa is the apical complex. However, the discovery of the heterotrophic alveolates *Perkinsus* and *Colpodella*, clearly related to the apicomplexans (Kuvardina et al., 2002), challenged this simplistic morphology-based view. Apparently, the presence or absence of a certain ultrastructural feature in extant species is insufficient to illuminate evolutionary history, while molecular phylogenetic approaches are better suited for such an aim. In spite of rather high divergence among apicomplexans, monophyly of the phylum is strongly supported by rRNA- (Barta et al., 1991) and protein-based phylogenies (Leander and Keeling, 2004; Harper et al., 2005). However, these phylogenetic inferences placed *Perkinsus*, a parasite equipped with an apical complex (Sagrista et al., 1996), on the root of the dinoflagellates (Leander and Keeling, 2004; Saldarriaga et al., 2003). Furthermore, *Perkinsus* was recently proposed to possess a non-photosynthetic plastid, reminiscent of the apicoplast (Stelter et al., 2007; Grauvogel et al., 2007). On the other hand, colpodellids, microscopic marine predators that also contain an apical complex but probably lack any plastid, appear in phylogenetic trees in close proximity to the parasitic apicomplexans. As a reflection of this situation, they have been included in the phylum Apicomplexa (Adl et al., 2005).

The phylum in question belongs to a larger group called Alveolata. Along with three traditional phyla (Ciliophora, Apicomplexa and Dinophyta), it includes the newly erected Chromerida. Within alveolates, the heterotrophic ciliates form a basal group, whereas the parasitic apicomplexans and photoautotrophic dinophytes constitute advanced sister groups. Based on the available sequences, chromerids form a sister group to the apicomplexans rather than the dinoflagellates (Moore et al., 2008). If the chromalveolate hypothesis is correct, the chromeran plastid is a direct descendant of the red algal endosymbiont that has been acquired by a common ancestor of chromalveolates about 1.3 billion years ago (Yoon et al., 2004). This ancient endosymbiotic event triggered an outstanding diversification leading to the appearance of a wide variety of photoautotrophs and heterotrophs.

In our opinion, heterotrophy among extant chromalveolates is always a consequence of secondarily lost photosynthetic ability. This event seems to have happened either by a complete loss of the plastid, as is the case of most heterotrophic alveolates (ciliates, oomycetes and some dinoflagellates), or by the loss of photosynthesis in an organelle that has been, however, retained. It is reasonable to assume that the plastid could have been lost only before the host cell delegated essential function(s) to the organelle, thus becoming fully dependent on it. Heterotrophy of basal alveolates is usually interpreted as a major obstacle to the chromalveolate hypothesis (Palmer, 2003; Bodyl, 2005). However, the early-branching alveolates could have lost the endosymbiont (or plastid) shortly after its acquisition, before its metabolism became inseparable from that of the host cell. Another condition for the postulated early elimination of the endosymbiont is the capacity of the host cell to live as a heterotroph. The apparent absence of the apicoplast in the early-branching gregarines and cryptosporidians may be explained in the same vein. All in all, the available data favour a scenario in which alveolates evolved from heterotrophic predators through photoautotrophic algae into heterotrophs during their history over more than 1 billion years and finally into very successful parasites. The ongoing effort to sequence the whole genome of *C. velia* should shed light on some of the key events in this stunning evolutionary pathway.

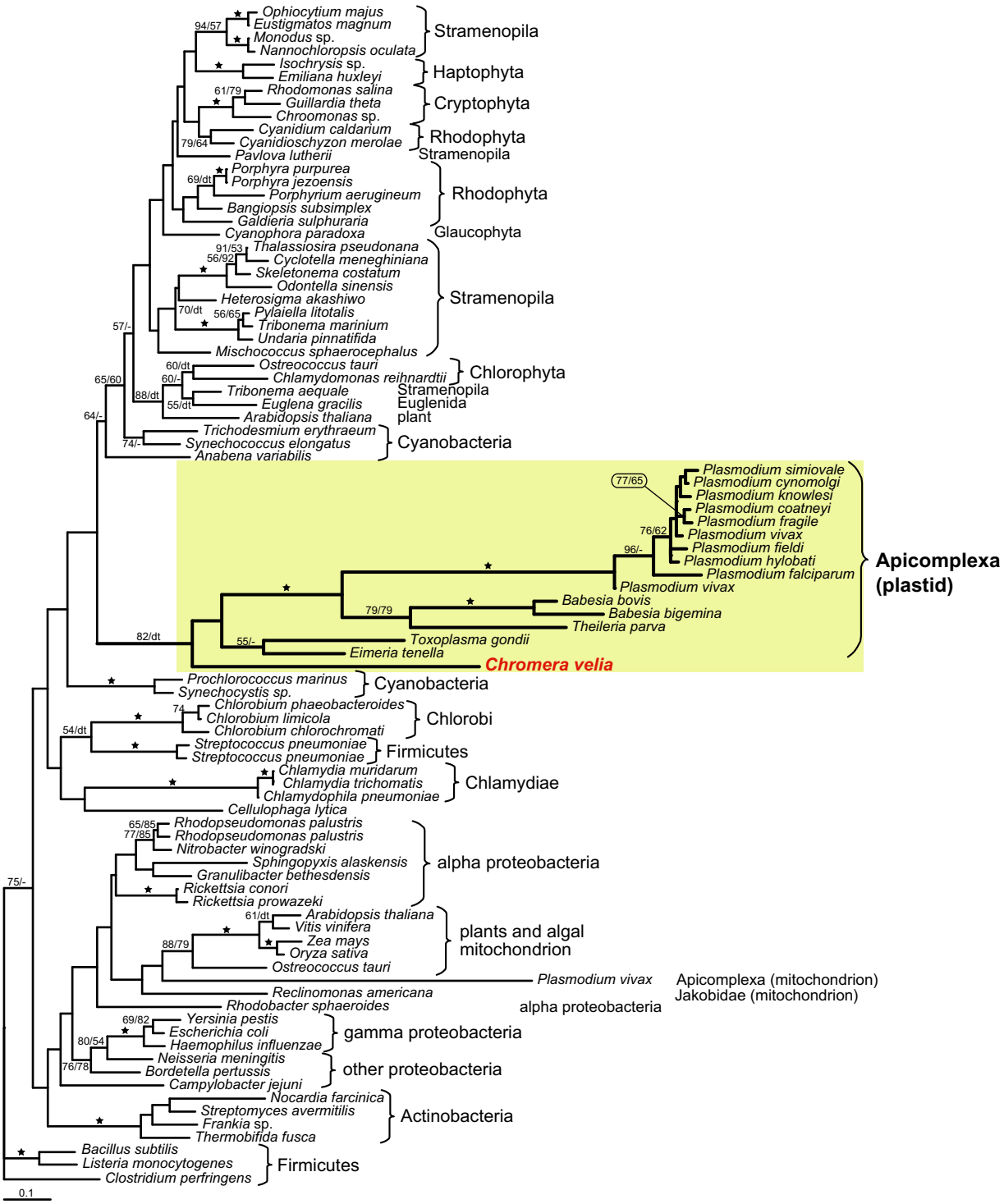

Fig. 7. Maximum likelihood (ML) phylogenetic tree as inferred from *tufA* sequences. The *Chromera velia* sequence was amplified from the total DNA isolated according to Moore et al. (2008). Appropriate homologues are not available from the dinoflagellates. Amino acid sequences of *tufA* gene were aligned using Kalign (Lassmann and Sonnhammer, 2005); ambiguously aligned regions and gaps were excluded from further analysis. The ML tree was constructed using the PhyML (Guindon and Gascuel, 2003) program, with the substitutional model (WAG+I+Γ) chosen according to PROTTEST (Abascal et al., 2005) results. The neighbour joining(NJ) tree was constructed using the AsaturA program (van de Peer et al., 2002) designed to deal with saturation of amino acids (see Supplementary material for details). Numbers above branches indicate ML/NJ with 200 and 1000 replicates, respectively. Black stars indicate both bootstraps over 90%, dt means different topology in the NJ tree (see Supplementary material for details).

Acknowledgements

This work was supported by the Czech Science Foundation Grant No. 206/06/1439, the Ministry of Education of the Czech Republic Grant 6007665801 and Research Plan of the Academy of Sciences of the Czech Republic no: z60220518. We thank Zdeněk Cimburek for help with measuring the size of the *C. velia* genome, Marie Vancová for preparing TEM samples and Hassan Hashimi for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.07.010.

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