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Did the peridinin plastid evolve through tertiary endosymbiosis? A hypothesis

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Most photosynthetic dinoflagellates harbour the peridinin plastid. This plastid is surrounded by three membranes and its characteristic pigments are chlorophyll *c* and the carotenoid peridinin. The evolutionary origin of this peculiar plastid remains controversial and is hotly debated. On the recently published tree of concatenated plastid-encoded proteins, dinoflagellates emerge from within the Chromista (clade containing cryptophytes, heterokonts, and haptophytes) and cluster specifically with Heterokonta. These data inspired a new version of the 'chromalveolate' model, according to which the peridinin plastid evolved by 'descent with modification' from a heterokont-like plastid that had been acquired from a rhodophyte by an ancestral chromalveolate. However, this model of plastid evolution encounters serious obstacles. Firstly, the heterokont plastid is surrounded by four membranes, which means that the ancestral peridinin plastid must have lost one of these primary membranes. However, such a loss could be traumatic, because it could potentially disturb protein import into and/or within the plastid. Secondly, on the phylogenetic tree of Dinoflagellata and Heterokonta, the first to diverge are not plastid, but heterotrophic, aplastidal taxa. Thus, when accepting the single origin of the heterokont and peridinin plastids, we would have to postulate multiple plastid losses, but such a scenario is highly doubtful when the numerous non-photosynthetic functions of plastids and their existence in heterotrophic protists, including parasitic lineages, are considered. Taking these obstacles into account, we suggest an alternative interpretation of the concatenated tree of plastid-encoded proteins. According to our hypothesis, the peridinin plastid evolved from a heterokont alga through tertiary endosymbiosis.

Key words: apicoplast, chromalveolates, endosymbiosis, dinoflagellates, glyceraldehyde-3-phosphate dehydrogenase, plastids, protein targeting

Introduction

Dinoflagellates are unicellular eukaryotic organisms constituting one of the main components of the phytoplankton and microzooplankton of marine and freshwater environments. Characteristic features of dinoflagellates are a nucleus with permanently condensed chromosomes lacking histones, flattened vesicles subtending the plasmalemma, and a flagellar apparatus composed of one transverse and one longitudinal flagellum (van den Hoek *et al*., 1995). Dinoflagellates are true experts in acquiring new plastids during the course of their evolution. In contrast to other photosynthetic groups, they contain several types of plastids acquired from distinct algal groups (for a review see Schnepf & Elbrächter, 1999). For example, the dinoflagellates *Gymnodinium chlorophorum* and *Lepidodinium viride* possess a secondary chlorophyte-derived plastid, whereas *Karenia brevis* and *Karlodinium micrum* acquired their tertiary plastid from a haptophyte. Furthermore, some heterotrophic dinoflagellates regularly capture plastids that are maintained for some time in their cytosol as kleptoplastids. The best-characterized dinoflagellate kleptoplastids are those of *Amphidinium poecilochroum* (Larsen, 1988), *A. latum* (Horiguchi & Pienaar, 1992), *Gymnodinum aeruginosum* (Schnepf *et al*., 1989) and *G. acidotum* (Wilcox & Wedemeyer, 1984). Nevertheless, most dinoflagellates harbour the so-called peridinin plastid, which is believed to be ancestral to all photosynthetic members of the Dinoflagellata (Schnepf & Elbrächter, 1999).

Molecular phylogenetic analyses of nucleus-encoded genes clearly indicate that dinoflagellates are closely related to apicomplexan parasites (Fast *et al*., 2002; Leander & Keeling, 2004). Interestingly, these parasites contain a vestigial plastid known as the apicoplast (reviewed by Foth & McFadden, 2003). In turn, aplastidal ciliates are recovered as sister to the

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Dinoflagellata/Apicomplexa clade (Fast *et al*., 2002; Leander & Keeling, 2004). Dinoflagellata, Apicomplexa, and Ciliata together form the infrakingdom Alveolata. According to the currently popular view, alveolates are closely related to the kingdom Chromista, thus creating a super-assemblage termed the Chromalveolata (Cavalier-Smith, 1999, 2003). Three evolutionary lineages comprise the Chromista: the Cryptophyta, Heterokonta, and Haptophyta. A plastid surrounded by four membranes, derived secondarily from a red alga, is characteristic of photosynthetic representatives of each group (Oliveira & Bhattacharya, 2000; Yoon *et al*., 2002*a*). In support of this red algal origin, cryptophytes still maintain a nucleomorph, the vestigial nucleus of the eukaryotic algal endosymbiont (Douglas *et al*., 2001).

As already mentioned, most photosynthetic dinoflagellates possess the peridinin plastid (Schnepf & Elbrächter, 1999). This plastid is surrounded by three membranes and its thylakoids are arranged in bands of three. Its characteristic pigments are chlorophyll *c* and the carotenoid peridinin (Schnepf & Elbrächter, 1999). The peridinin plastid has a peculiar plastid genome, which is composed of plasmid-like chromosomes known as minicircles (Koumandou *et al*., 2004). These minicircles encode only a handful of core photosynthetic proteins such as atpA, atpB, petB, petD, psaA, psaB or psbA-E. Therefore, almost all dinoflagellate plastid proteins (~2000–3000) are encoded in the nuclear genome (Bachvaroff *et al*., 2004; Hackett *et al*., 2004).

At present, we know sequences of many proteins targeted to the peridinin plastid. Most of them carry tripartite presequences that consist of a signal peptide, a transit peptide, and a stop-transfer sequence (Nassoury *et al*., 2003; Patron *et al*., 2005). Such a presequence structure suggests a complex mechanism of protein import into the peridinin plastid. In accordance with this hypothesis, Nassoury *et al*. (2003) demonstrated that co-translational translocation into the endoplasmic reticulum (ER) is the first step in their targeting. During this process, dinoflagellate plastid proteins are anchored in the membrane and, in this membrane-bound form, they are trafficked to the plastid *via* a pathway involving not only ER but also the Golgi apparatus (Nassoury *et al*., 2003). However, the mechanism for their further translocation across the two inner plastid membranes remains unclear. It is hypothesized that this targeting step resembles the post-translational mechanism of protein import into primary plastids (van Dooren *et al*., 2001), but homologues of Toc and Tic proteins have yet to be identified in dinoflagellates. Thus, the existence of unconventional Toc/Tic-independent pathways is also possible.

Enigmatic evolutionary pathway of the peridinin plastid

Secondary versus *tertiary endosymbiosis and the 'chromalveolate' scenario*

The origin of the peridinin plastid is still controversial and hotly debated. Two contrasting models of its evolutionary pathway have been advanced, a secondary model and a tertiary model (Fig. 1). According to the secondary model, the peridinin plastid evolved from a red alga that could have been engulfed either by myzocytosis or phagocytosis (Fig. 1A; Schnepf & Deichgräber, 1984; Cavalier-Smith, 1999, 2003). In the case of myzocytotic engulfment, this plastid would have been surrounded by three membranes from its origin and its subsequent evolution required no membrane loss (Fig. 1A). With phagocytotic engulfment, its envelope would have been composed of four membranes; this would require the loss of one membrane to arrive at the peridinin plastid (Fig. 1A). The most recent (phagocytotic) version of this secondary model, known as the 'chromalveolate' model, postulates that the peridinin plastid evolved by 'descent with modification' from the same red algal endosymbiont as the apicomplexan plastid and chromist plastids (Cavalier-Smith, 1999, 2003). This model has gained wide acceptance and new data generally are interpreted in the context of its theoretical framework, as will be demonstrated below.

In contrast to the secondary model, a tertiary model assumes that the peridinin plastid evolved from a haptophyte (i.e. an alga already harbouring a secondary rhodophyte-derived plastid), which could also have been engulfed by myzocytosis or phagocytosis (Fig. 1B; Gibbs, 1978; Tengs *et al*., 2000; Bodył, 2005). In the case of myzocytotic engulfment, this plastid would be surrounded by five membranes and two of them would have to be lost (Fig. 1B). With phagocytotic engulfment, its envelope would have been composed of six membranes, which would require a loss of three membranes (Fig. 1B).

Testing of these two models was begun by the Bhattacharya group using plastid-encoded genes. Yoon *et al*. (2002*b*) sequenced *psbA* genes from numerous rhodophytes, dinoflagellates, and haptophytes and included them in phylogenetic analyses. Unexpectedly, dinoflagellates were not the sister group to all chromists, as predicted by the 'chromalveolate' model (Cavalier-Smith, 1999, 2003). Instead, they emerged from within chromists clustering very strongly with haptophytes and the

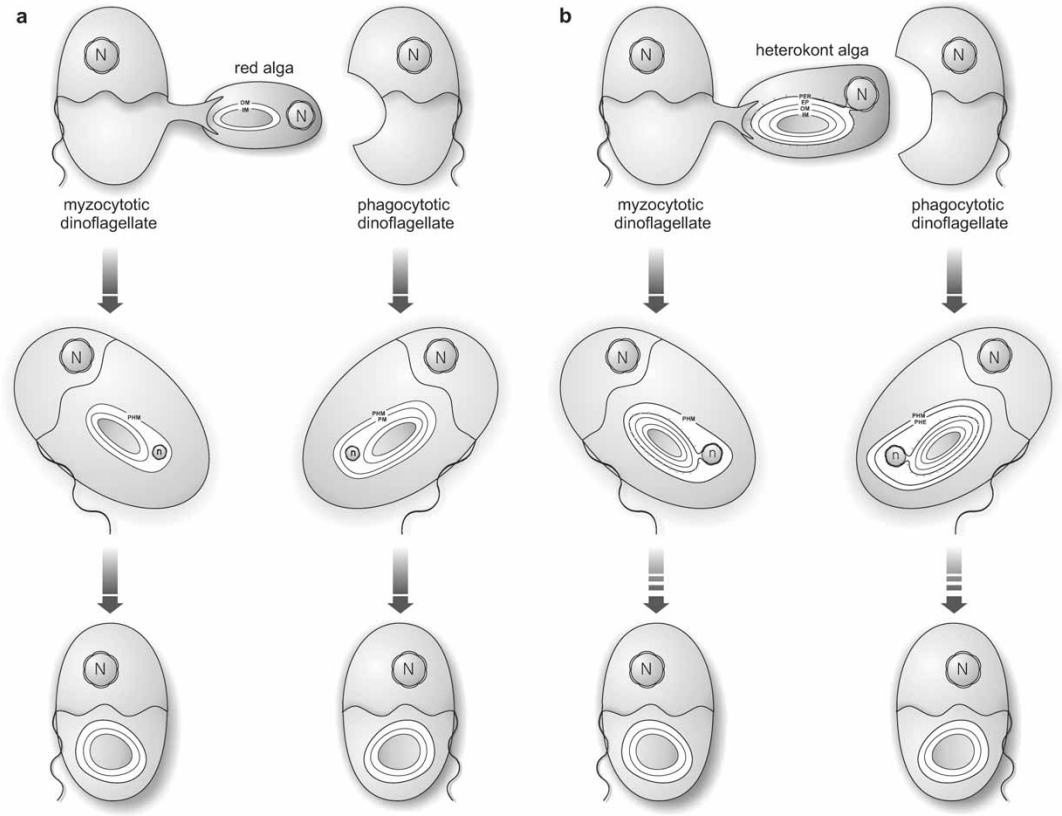

Fig. 1. Models explaining evolutionary origin of the peridinin plastid. (a) According to the secondary model, the peridinin plastid evolved from a red alga that harboured a cyanobacterium-derived plastid surrounded by two membranes: the inner membrane (IM) and the outer membrane (OM). There are two versions of this model: a myzocytotic scenario (left) and phagocytotic scenario (right). In myzocytotic engulfment of the red alga, the peridinin plastid would be surrounded by three membranes: IM, OM, and the phagosomal membrane of the host (PHM). In phagocytotic engulfment, the envelope initially would be composed of four membranes (IM, OM, the endosymbiont plasmalemma (EP), and PHM) and one of them would have to be lost. It is assumed that this membrane was the EP. (b) The tertiary model postulates that the peridinin plastid is derived from a heterokont alga which possessed a red alga-derived plastid surrounded by four membranes: IM, OM, EP, and the plastid endoplasmic reticulum (PER). There is a myzocytotic (left) and phagocytotic (right) version of this model. In myzocytotic engulfment of the heterokont, the peridinin plastid would be initially surrounded by five membranes: IM, OM, EP, PER, and PHM. In phagocytotic engulfment, its envelope would additionally contain the plasmalemma of the heterokont endosymbiont (PHE). This means that the ancestral peridinin plastid must have lost two (myzocytotic scenario) or three (phagocytotic scenario) membranes. Regardless of the scenario followed, it currently is unclear which membranes were eliminated. N: typical eukaryotic nucleus; n: highly reduced eukaryotic nucleus known as the nucleomorph.

dinoflagellates *Karlodinium* and *Karenia*, two taxa shown previously to harbour a tertiary haptophyte-derived plastid termed the fucoxanthin plastid (Tengs *et al*., 2000; Takishita *et al*., 2004, 2005). Although these data appeared to provide strong support for the tertiary, haptophyte origin of the peridinin plastid, Inagaki *et al*. (2004) showed that such a grouping could be artificial, resulting from a similar codon usage in the *psbA* genes of dinoflagellates and haptophytes.

Taking these difficulties into account, Yoon *et al*. (2005) performed further phylogenetic analyses based on plastid protein data. In addition to psbA, they included the amino acid sequences of additional photosynthetic proteins, including psaA, psaB, psbC, and psbD. Interestingly, in trees generated using a concatenated data set of these sequences, dinoflagellates still emerged from within chromists but grouped specifically with heterokonts rather than with haptophytes (Yoon *et al*., 2005). In contrast to their previous interpretation invoking a tertiary endosymbiosis, Yoon *et al*. (2005) then suggested that the peridinin plastid resulted from the same red algal endosymbiosis that created the apicomplexan plastid and chromist plastids (Fig. 2). This scenario clearly resembles the 'chromalveolate' model, but it differs fundamentally from the classical version proposed by Cavalier-Smith (1999, 2003). In the classical 'chromalveolate' model, dinoflagellates, along with other alveolates, represent a sister group to the whole chromist clade, whereas, in the model of Yoon *et al*. (2005), they are a sister to the heterokont lineage only, thereby making the Chromista a paraphyletic clade (Fig. 2). Consequently, this new version of the 'chromalveolate' hypothesis implies that the peridinin plastid evolved vertically

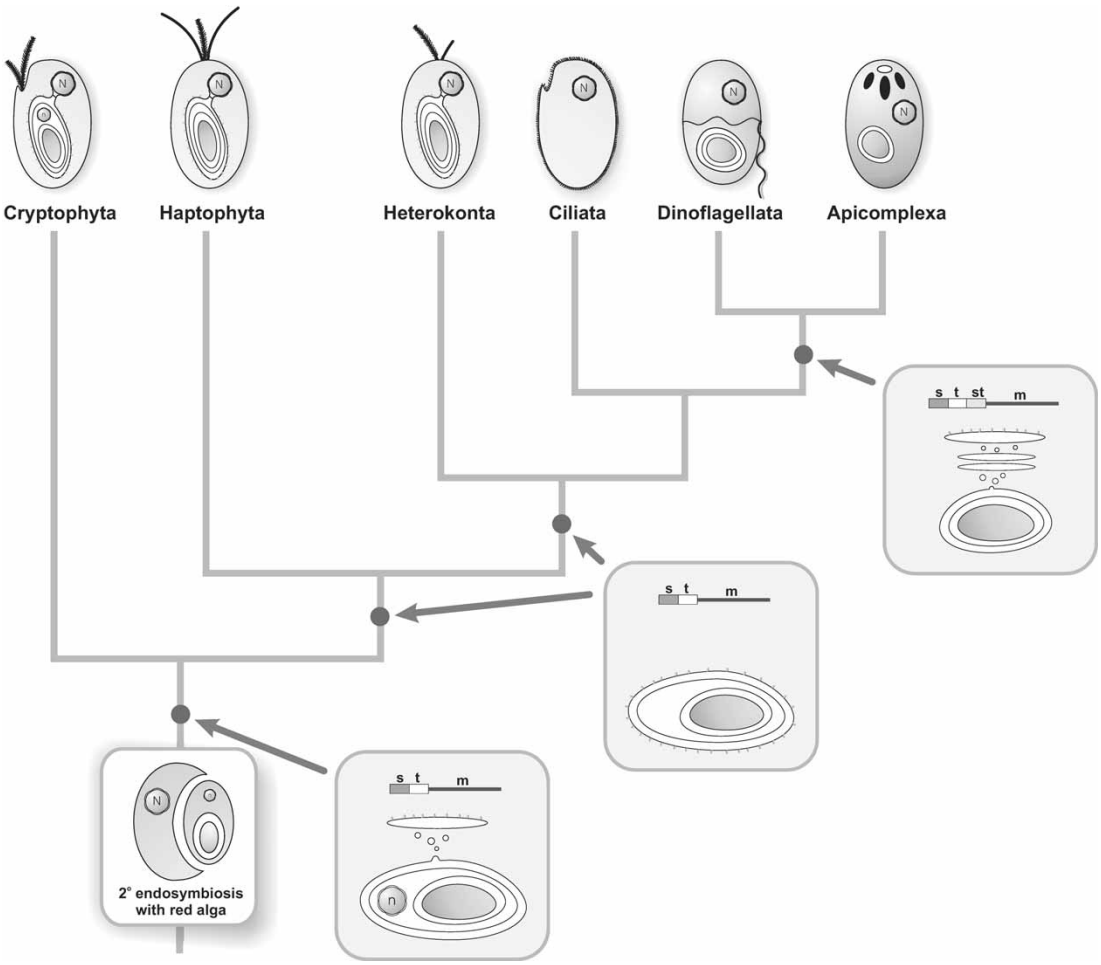

Fig. 2. New version of the 'chromalveolate' model. A single red-algal origin of cryptophyte, haptophyte and heterokont plastids (kingdom of Chromista) as well as dinoflagellate and apicomplexan plastids (infrakingdom of Alveolata) is the main idea of the 'chromalveolate' model. Its classical version assumes that the sister group for Alveolata is the whole chromist clade. In contrast, the new version, proposed by Yoon *et al.* (2005) suggests that the sister group for Alveolata are heterokonts only, thereby making Chromista a paraphyletic clade. Consequently, this model postulates that the peridinin plastid evolved from a heterokont-like plastid by its ultrastructural, biochemical, and genetic modification. Unfortunately, the new evolutionary scenario encounters several obstacles. First, it assumes a complete plastid loss in ciliates, and independent losses in many heterokonts and dinoflagellates. Second, it postulates drastic ultrastructural modifications of the heterokont-like plastid in dinoflagellate and apicomplexan lineages. Third, it implies a thorough rebuilding of the protein import machinery along with its complex targeting signal. As is shown on the schemes to the right of the phylogenetic tree, the targeting machinery of chromist plastids, including that of heterokonts, involves only ER, while their nuclear-encoded proteins carry bipartite presequences composed of a signal peptide (s) followed by a transit peptide (t). At present, chromist plastid proteins are transported directly into the space between the two outer membranes, but initially they were probably targeted in transport vesicles to the plastid. In contrast to chromist plastids, the outer membrane of the peridinin plastid is ribosome-free, and its targeting machinery involves not only the ER but also the Golgi apparatus. Moreover, most of its nuclear-encoded proteins carry tripartite presequences consisting of s, t, and a stop-transfer sequence (st). n: nucleomorph; m: mature protein.

from a heterokont-like plastid through a number of ultrastructural, biochemical, and genetic modifications (Fig. 2). Unfortunately, the necessity of postulating some of these modifications is the Achilles' heel of the scenario proposed by Yoon *et al.* (2005).

Difficulties encountered by the new 'chromalveolate' model and their consequences

The outermost membrane of the heterokont plastid, like that of cryptophyte and haptophyte plastids (Gibbs, 1981), bears ribosomes and exhibits connections with the rough ER (RER) and/or the nuclear envelope (Fig. 2; Apt *et al.*, 2002). This suggests that the membrane is derived from the phagosomal membrane of the host, which fused with the RER resulting in an ER-like membrane known as the plastid ER (PER; Cavalier-Smith, 2003). In contrast to the heterokont plastid, the outermost membrane of the peridinin plastid is smooth and does not have any connections with RER or the nuclear envelope (Fig. 2; Nassoury *et al.*, 2003). Thus, the new

'chromalveolate' scenario for the evolution of the peridinin plastid requires an explanation for these differences in membrane ultrastructure.

The simplest explanation is that the ancestral peridinin plastid, surrounded by four chromist-like membranes, lost the outermost membrane (along with its ribosomes), so that the third ribosome-free membrane became the outermost plastid membrane. Initially, however, such a loss would have disabled import of hundreds of signal peptide-carrying plastid proteins (Nassoury *et al*., 2003; Patron *et al*., 2005) that, according to the 'chromalveolate' hypothesis, must have been encoded in the dinoflagellate nucleus. When these proteins entered the endomembrane system they would not have been able to reach the plastid. Only the origin of a specific class of transport vesicles capable of fusing with the new outer membrane (or a fusion of this membrane with RER) would have made their further import possible.

The classical 'chromalveolate' model postulates that the fusion of the phagosomal membrane with the RER happened only once in an early chromist (Cavalier-Smith, 1999, 2003); one might instead hypothesize, however, that it occurred independently in each chromist lineage (i.e. in cryptophytes, heterokonts, and haptophytes). In this case it could be argued that such a fusion never happened in dinoflagellates. Unfortunately, even this 'rescue scenario' encounters serious obstacles. First, it is more parsimonious to assume that the fusion of the phagosomal membrane with RER occurred only once, especially if we hold to a single origin of chromist plastids. Second, it seems improbable that the ancestral state in dinoflagellates ever involved such a fusion, because the current vesicle-mediated import pathway involves not only ER, but also the Golgi apparatus (Fig. 2; van Dooren *et al*., 2001; Slavicova *et al*., 2005). Presumably, such a fusion was possible in the ancestor of heterokonts (and all chromists), because their ancestral import pathway involved only the ER (Fig. 2). In this case, a membrane mutation disabling budding of plastid-targeted vesicles from ER could lead directly to the fusion of the outermost plastid membrane with ER (for details see Cavalier-Smith, 2003).

Even with this unlikely 'rescue scenario', the version of the 'chromalveolate' model proposed by Yoon *et al*. (2005) encounters serious difficulties resulting from the necessity of postulating the loss of the third membrane (counting from the inside) by the ancestral dinoflagellate plastid (see Cavalier-Smith, 1999, 2003). This membrane, known as the periplastid membrane, is thought to derive from the endosymbiont plasmalemma. Thus, the loss of the periplastid membrane would be a reasonable evolutionary scenario, only if the membrane contained a channel-based translocon for nuclear-encoded plastid proteins (Cavalier-Smith, 1999, 2003; Bodył, 2004). However, the most recent import studies of Kilian & Kroth (2005) suggest that transport vesicles are responsible for the inward protein import in the heterokont plastid. These vesicles pinch off the periplastid membrane and fuse with the outer membrane of the plastid envelope (which corresponds to the endosymbiont plastid), thereby releasing imported proteins into the lumen of this envelope. Consequently, the loss of the periplastid membrane by the ancestral peridinin plastid would have had serious consequences for the further import of proteins into the plastid stroma.

The new 'chromalveolate' model can be rescued once again by the argument that a Toc75 channel (Soll & Schleiff, 2004) existed in the outer membrane of the plastid envelope of the ancestral peridinin plastid, which served to import plastid proteins encoded by the nucleomorph (see also Cavalier-Smith, 2003; Bodył, 2005). In heterokonts, however, the complete loss of the nucleomorph genome had already occurred; their advanced position on the tree obtained by Yoon *et al*. (2005) suggests that the nucleomorph was absent in the common ancestor of Heterokonta and Dinoflagellata (Fig. 2). Thus, it is reasonable to assume that the plastid of this ancestor was devoid of the Toc translocon as well. In agreement with this view, Armbrust *et al*. (2004) and McFadden & van Dooren (2004) were unable to identify the homologues of the Toc75 channel and the Toc159/Toc34 receptors in the completely sequenced genome of the diatom *Thalassiosira pseudonana*. Even if the outer membrane of the plastid envelope of the ancestral peridinin plastid possessed Toc75, it would not necessarily mean that this channel was able to translocate nuclear-encoded plastid proteins, as was clearly demonstrated by the import studies of Wastl & Maier (2000).

Hypothesis: the peridinin plastid has evolved from a heterokont alga through tertiary endosymbiosis

The above arguments compel us to re-interpret the data obtained by Yoon *et al*. (2005), and to propose an alternative evolutionary scenario to either version of the 'chromalveolate' hypothesis. This alternative is offered by the mechanism of tertiary endosymbiosis. Gibbs (1978) had been the first to suggest that the peridinin plastid evolved from a haptophyte, and the scenario was recently discussed in more detail by Bodył (2005) and Bachvaroff *et al*. (2005). However, the concatenated tree of plastid proteins generated by Yoon *et al*. (2005) suggests that the ancestor of the

peridinin plastid could have been a heterokont rather than a haptophyte (Fig. 3). Although the specific clustering of peridinin dinoflagellates with heterokonts on this tree does not have strong bootstrap support, one-sided Kishina-Hasegawa (KH) and approximately unbiased (AU) tests demonstrated that the trees of highest probability were those in which the peridinin plastid grouped with the whole heterokont clade or with one of the heterokont lineages, respectively (Yoon *et al*., 2005). Thus, the phylogenetic analyses of Yoon *et al*. (2005) are an excellent spur for discussion on the evolutionary pathway of this peculiar plastid. Their great advantage is the use of the peridinin plastid as a previously unexplored source of phylogenetic information. It is especially important, because the 'chromalveolate' view on its evolution is mainly based on the phylogenies of nuclear-encoded, plastid-targeted proteins (Fast *et al*., 2001; Patron *et al*., 2004). However, these phylogenies do not unequivocally support the 'chromalveolate' model (see, for example, Kroth *et al*., 2005), as is commonly assumed. We will come back to this problem in our further discussion.

In support of a tertiary origin of the peridinin plastid, on the concatenated protein tree generated by Yoon *et al*. (2005), Dinoflagellata do not form a sister group to the entire Heterokonta, but instead emerge from within this clade. Although this branching pattern has relatively low bootstrap support, an identical topology can be found on the trees of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Takishita *et al*. 2004; see also the later discussion). Furthermore, in AU tests, the trees of highest probability were those in which the peridinin plastid clusters specifically with the diatom *Odontella sinensis* from within the heterokont clade (Yoon *et al*., 2005). In accordance with these findings, Harper & Keeling (2004) demonstrated that the dinoflagellate enolase 2 (found in *Heterocapsa* and *Amphidinium*) falls in a strongly supported group with the diatoms *Thallasiosira pseudonana* and *Pheodactylum tricornutum*. This close relationship between peridinin dinoflagellates and diatoms is exactly what one

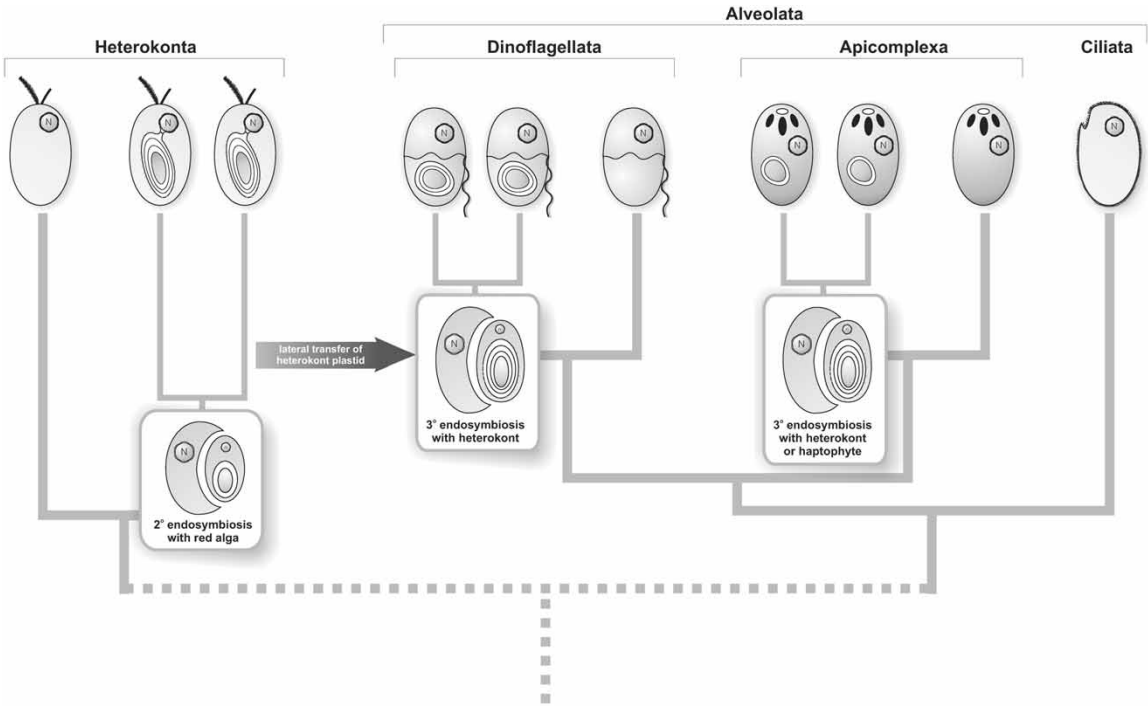

Fig. 3. Hypothetical phylogenetic relationships between the peridinin plastid and the heterokont plastid. The basal positions on the phylogenetic tree of Heterokonta, Dinoflagellata, and Apicomplexa are occupied by plastid-lacking taxa (indicated by bold line). In addition, the sister group for the dinoflagellate/apicomplexan clade are aplastidal ciliates. Thus, the plastid-containing heterokonts, dinoflagellates, and apicomplexans emerge from within heterotrophic, aplastidal groups. A single, common origin of the heterokont and dinoflagellate plastids requires independent plastid losses in ancestral heterokonts, ciliates, and ancestral dinoflagellates. Most available data (discussed in the text) put this evolutionary scenario in doubt. It also remains unclear whether the Heterokonta and Alveolata evolved from a common ancestor (indicated by dashed line). All these problems with the chromalveolate hypothesis disappear, however, if we postulate independent plastid acquisitions by heterotrophic ancestors of heterokonts, dinoflagellates, and apicomplexans. According to this scenario, the heterokont plastid evolved from a red alga through secondary endosymbiosis. The peridinin plastid resulted from tertiary endosymbiosis involving a heterokont alga as the endosymbiont. The apicomplexan plastid also would have been of tertiary origin, but it is unclear if its ancestor was a heterokont or rather a haptophyte.

would expect if the peridinin plastid is derived from a heterokont through tertiary endosymbiosis.

The tree obtained by Yoon *et al*. (2005) alone is not sufficient to conclude that the peridinin plastid evolved from a heterokont through tertiary endosymbiosis. However, together with the other supporting phylogenies cited above, it provides a clear molecular phylogenetic framework for formulating such a hypothesis.

Data and arguments favouring tertiary origin of the peridinin plastid

Weaknesses of the principle of minimizing endosymbiotic events

The main reason to favour a single, secondary origin of the peridinin and heterokont plastids is the principle of minimizing the number of endosymbiotic events. This principle is based on the assumption that the transformation of an endosymbiont into an organelle encounters serious obstacles resulting mainly from the evolution of a complex system for protein import involving proper targeting signals (Cavalier-Smith, 1992, 1999). The principle of minimizing endosymbiotic events has become an inspiration to formulate not only the 'chromalveolate' model (along with its idea of secondary origin of the peridinin plastid), but also the 'cabozoa' model, according to which the chlorarachniophyte plastid and the euglenoid plastid are derived from the same secondary green algal endosymbiosis (for details see Cavalier-Smith, 1999, 2003). There are a number of reasons, however, to doubt the validity of this principle. First, even if we accept all reasonable efforts to minimize such events, there have been no less than ten successful transformations of an endosymbiont into a fully integrated organelle (for a recent discussion see Bodył, 2005). Second, available data clearly support independent endosymbiotic origins for the chlorarachniophyte and euglenoid plastids (Keeling, 2004; Leander, 2004). Third, the genetic richness of red plastids may make them pre-adapted to horizontal transfer through secondary and tertiary endosymbioses (Grzebyk *et al*., 2003). Fourth, all the scenarios based on the principle of minimizing endosymbiotic events postulate non-parsimonious plastid losses (e.g. Cavalier-Smith, 1993, 1999); there is no clear reason to presume that loss of an integrated plastid is more easily accomplished than its gain (see further discussion).

An additional challenge to the principle of minimizing endosymbiotic events is the number of species harbouring photosynthetic endosymbionts that have undergone, or are in the process of undergoing, transformation into a true organelle. A well-documented example of such an organism is the thecate, filose amoeba *Paulinella chromatophora*, which contains photosynthetic organelles resembling cyanelles (Marin *et al*., 2005; Melkonian & Mollenhauer, 2005). According to the most commonly accepted view, the primary endosymbiosis resulting in glaucophyte, rhodophyte and chlorophyte plastids was a unique event (Cavalier-Smith, 2000; Rodriguez-Ezpeleta *et al*., 2005). However, the phylogenetic analyses of Marin *et al*. (2005) clearly demonstrate that *P. chromatophora* acquired its photosynthetic organelles through endosymbiosis independent of the one that created typical primary plastids (see also Rodriguez-Ezpeleta & Philippe, 2006).

At present, we know of a wide array of endosymbiotic associations in which the endosymbiont is a cyanobacterium (e.g. the endosymbiosis between the cyanobacterium *Cyanothece* and the diatom *Rhopalodia*; Prechtl *et al*., 2004), an alga with a primary plastid (e.g. the endosymbiosis between the chlorophyte *Chlorella* and the coelenterate *Hydra*; Habetha & Bosch, 2005) and an alga with a secondary plastid (e.g. the endosymbiosis between the heterokont *Vaucheria* and the mollusc *Elysia*; Rumpho *et al*., 2001; see also Raven, 2002; Okamoto & Inouye, 2005; Foster *et al*., 2006; Kamako & Imamura, 2006). Given the results of recent analyses of *P. chromatophora*, it could be supposed that some of these less well-characterized endosymbionts already represent at least partially integrated organelles with their host cells.

Each new discovery of the successful transformation of an endosymbiont into an organelle further undermines the principle of minimizing endosymbiotic events at all costs, one of the theoretical foundations of current views of plastid evolution. They further provide additional reasons to doubt the 'chromalveolate' hypothesis, along with its idea of secondary origin of the peridinin plastid.

Targeting machineries of the peridinin and heterokont plastids, chlorophyll c*, and diversity of tertiary plastids in dinoflagellates*

Although Yoon *et al*. (2005) suggested a single secondary origin of the peridinin plastid and the heterokont plastid, the already mentioned major differences in their targeting machineries (Fig. 2) favour independent origin of these plastids. If the heterokont and peridinin plastids resulted from the same red algal endosymbiosis, they should have identical targeting machinery as has been argued on many occasions by Cavalier-Smith (1992, 1999). Thus, evidence of very different import pathways, (i.e. that the ER-mediated pathway of protein import into the heterokont plastid and the ER,

Golgi apparatus-mediated pathway of protein import into the peridinin plastid; Fig. 2), are more consistent with independent endosymbiotic events that resulted in distinct targeting machineries (Fig. 3).

It could be postulated that the peridinin plastid evolved by a separate red algal endosymbiosis; but then we would have to assume independent origins of chlorophyll *c*. By proposing a tertiary, heterokont origin (Fig. 3), we overcome this problem; the engulfed alga already possessed chlorophyll *c*.

Additional support for the tertiary origin of the peridinin plastid comes from so-called unusual dinoflagellate plastids. As already discussed, most photosynthetic dinoflagellates possess the peridinin plastid but, in several phylogenetic lineages, atypical plastids have been found (see Introduction). These plastids appear to be derived from both the green and the red evolutionary lineages. The green lineage is represented by the secondary chlorophyte-derived plastid of *Gymnodinium chlorophorum* and *Lepidodinium viride* (Elbrächter & Schnepf, 1996). The red lineage is represented at least by two kinds of plastids: the tertiary haptophyte-derived plastid of *Karenia brevis* and *Karlodinium micrum* (Tengs *et al*., 2000; Takishita *et al*., 2004, 2005), and the tertiary heterokont-derived plastid of *Kryptoperidinium foliaceum* and *Durinskia baltica* (McEwan & Keeling, 2004). A third candidate is the tertiary cryptophyte-derived plastid of dinoflagellates belonging to the genus *Dinophysis* (Schnepf & Elbrächter, 1988; Hackett *et al*., 2003), although it is possible that they represent kleptoplastids (Takishita *et al*., 2002). Even if they are kleptoplastids, available data (e.g. Koike *et al*., 2005) suggest that at least some of them could be on the way to establishing permanent endosymbioses and transforming into true organelles. Taking into account these multiple examples of independent tertiary plastid acquisition among dinoflagellates, it seems likely that the peridinin plastid also originated through tertiary endosymbiosis. In further support of this view, a wide variety of kleptoplastids captured from cryptophytes (Larsen, 1988; Schnepf *et al*., 1989; Horiguchi & Pienaar, 1992) and haptophytes (Koike *et al*., 2005) have also been reported in dinoflagellates.

Similarities between the peridinin plastid and the Karenia/Karlodinum *plastid*

When assessing the relative strengths of the 'chromalveolate' *versus* tertiary origin models, it is difficult to ignore similarities between the peridinin plastid and the fucoxanthin plastid. It is possible that at least some of these result from convergence due to the endosymbiotic acquisition of similar eukaryotic algae by distinct dinoflagellate hosts, leading to comparable patterns of host-symbiont communication and membrane loss by the engulfed algae. Because the fucoxanthin plastid evidently evolved through tertiary endosymbiosis (Tengs *et al*., 2000; Takishita *et al*., 2004, 2005), such similarities may provide additional support for the tertiary origin of the peridinin plastid.

The first similarity between the peridinin plastid and the fucoxanthin plastid concerns the arrangement of thylakoid membranes. In both these plastids, thylakoids are stacked in threes and have a transparent lumen (Schnepf & Elbrächter, 1999; Tengs *et al*., 2000). In itself, this similarity to the heterokont plastid is inconsistent with the earlier version of the 'chromalveolate' hypothesis because thylakoids occur in pairs and have an opaque lumen in the Cryptophyta. In contrast, the tertiary origin model and the Yoon *et al*. (2005) version of the 'chromalveolate' model, are both more parsimonious with the phylogenetic distribution of thylakoid stacking.

The second similarity is less easily reconciled with either version of the 'chromalveolate' hypothesis; it concerns the structure of transit peptides. Patron *et al*. (2005) were the first to demonstrate that peridinin dinoflagellates possess two classes of transit peptides: the first class contains a stop-transfer domain, whereas the second does not. The presence of two types of transit peptides is a unique feature, not found in any other group of eukaryotic algae. However, Waller *et al*. (2006) demonstrated that two classes of transit peptides (one containing the stop-transfer domain and the second devoid of such a domain) occur in the fucoxanthin dinoflagellate *K. micrum*.

A third similarity concerns the number of envelope membranes. Although the number of membranes surrounding the *Karenia*/*Karlodinum* plastid is not entirely clear (e.g. Tengs *et al*., 2000), electron micrographs published by Steidinger *et al*. (1978), Kite & Dodge (1988) and Hansen *et al*. (2000) indicate that, at least in *K. micrum*, *K. brevis* and *Gymnodinium fuscum*, three plastid membranes are present. The existence of a three-membrane envelope in the fucoxanthin plastid is also suggested by the plastid-targeted transit peptides with a stop-transfer domain. To date, such peptides have been found only in the algae containing plastids with three envelope membranes (i.e. peridinin dinoflagellates and euglenoids; van Dooren *et al*., 2001; Slavicova *et al*., 2005).

In recently published phylogenetic trees of several nuclear-encoded plastid proteins (ascorbate peroxidase, phosphoribulokinase, and ATP

synthase gamma), the fucoxanthin dinoflagellate *K. micrum* clustered strongly with the peridinin dinoflagellate *Heterocapsa triquetra* (Patron *et al*., 2006). This finding supports the hypothesis that *K. micrum* plastid proteins are derived not only from the fucoxanthin endosymbiont, but also from the ancestral peridinin plastid endosymbiosis. Therefore, it appears that the plastid of the *Karenia*/*Karlodinium* evolved through replacement of the ancestral peridinin plastid, and through a very similar process of co-evolution with the host cell. The convergent similarities between the fucoxanthin and peridinin plastids, the former almost certainly derived by tertiary origin, suggest that there may be strong selection in dinoflagellates toward specific patterns of structure and communication in establishing a fully integrated host–endosymbiont relationship. Thus, the derived structural features of the peridinin plastid appear to be most consistent with a tertiary endosymbiotic origin.

Serious difficulties in plastid loss

The idea of tertiary origin of the peridinin plastid is strongly supported by the branching patterns of plastid and aplastidal taxa on phylogenetic trees of the Heterokonta and Dinoflagellata. In these trees, the first taxa to branch are heterotrophic, aplastidal taxa (Fig. 3; e.g. Moriya *et al*., 2000; Massana *et al*., 2002; Saldarriaga *et al*., 2003; Leander & Keeling, 2004). Moreover, the sister group to the dinoflagellate/apicomplexan clade is the ciliates (Fig. 3; Fast *et al*., 2002; Leander & Keeling, 2004); to our knowledge, there is no evidence that any member of the ciliates was ever photosynthetic. Thus, when accepting the common, secondary origin of the peridinin and heterokont plastid, a number of independent plastid losses must be postulated. Currently available data clearly do not agree with this hypothesis.

Plastids fulfill vital non-photosynthetic functions including the biosynthesis of isoprenoids, fatty acids, and haem (Ralph *et al*., 2004). Because they are broadly integrated into cellular metabolism, these organelles are maintained by heterotrophic/parasitic forms (e.g. apicomplexan parasites), even if the capacity for photosynthesis is lost (see, for example, Gockel & Hachtel, 2000; Foth & McFadden, 2003; Hoef-Emden, 2005). In fact, it is likely that the primary plastid in the common ancestor of glaucophytes, rhodophytes, and chlorophytes (Cavalier-Smith, 2000; Rodriguez-Ezpeleta *et al*., 2005) already played vital non-photosynthetic functions; at present we know of no case in which a secondarily heterotrophic/parasitic member of one these groups has not retained at least a vestigial plastid (Goff & Coleman, 1995; de Koning & Keeling, 2004; Borza *et al*., 2005; see also parasitic higher plants, Bungard, 2004). Interestingly, such vestigial plastids have been found in the heterotrophic heterokonts *Pteridomonas danica* and *Ciliophrys infusionum* (Sekiguchi *et al*., 2002), thought previously to be examples of complete plastid loss among the later diverging heterokonts (Cavalier-Smith *et al*., 1995). These new findings suggest that the heterokont plastid is involved in vital non-photosynthetic functions, which makes its complete loss very difficult, perhaps impossible. In the light of these data, it is difficult to accept the new 'chromalveolate' hypothesis, according to which the same rhodophyte-derived plastid would have to be lost not only by ancestral heterokonts, but also by ciliates and ancestral dinoflagellates. Thus, it is much more probable that dinoflagellates and heterokonts acquired their plastids by independent endosymbiotic events (Fig. 3).

Supporters of the new and classical versions of the 'chromalveolate' model could, however, argue that plastid losses in early chromist and alveolate lineages were possible because the sole function of the secondary plastid was in carbohydrate metabolism (e.g. Cavalier-Smith, 1993; Cavalier-Smith & Chao, 2006). However, this argument creates a fundamental contradiction for the 'chromalveolate' hypothesis, which presupposes that the ancestors of heterokonts and alveolates possessed a fully integrated rhodophyte-derived plastid, along with its complex import system for nuclear-encoded proteins (see Cavalier-Smith, 1999, 2003). Certainly this must have been the case for plastids supposedly lost in deep branching heterokont and dinoflagellate groups because these lineages are highly derived in the 'chromalveolate' model. Therefore, the argument that these plastids only participated in carbohydrate metabolism does not hold up. If, on the other hand, we accommodate the 'chromalveolate' hypothesis by assuming that the rhodophyte remained only a partially integrated endosymbiont over a prolonged period of evolution, we would have to postulate multiple origins of protein import systems in distinct chromist and alveolate lineages. But this is precisely what the 'chromalveolate' model is designed to avoid. Thus, taking into account the presumably advanced evolutionary stage of the rhodophyte-derived plastid, as well as the metabolic richness already present in primary plastids (Neuhaus & Emes, 2000; Borza *et al*., 2005), it is reasonable to assume that the hypothetical chromalveolate ancestor would have been dependent on non-photosynthetic metabolites/functions of its plastid. Consequently, it is difficult to see how such a plastid could have been lost independently on so many occasions.

Apicomplexan plastid and stability of plastid envelopes

Unlike the peridinin plastid and chromist plastids, the number of envelope membranes of the apicomplexan plastid is still controversial. Hopkins *et al*. (1999) showed evidence that the apicoplast is surrounded by three ribosome-free membranes. In contrast, the electron microscope studies of McFadden & Roos (1999) and Köhler (2005) indicated that the envelope is composed of four and two smooth membranes, respectively. The clear differences in number and structure of the envelope membranes of the peridinin plastid, chromist plastids, and the apicomplexan plastid has important implications for the idea of their common secondary origin. Cell organelles clearly can undergo considerable modifications, even if they are derived from a common ancestor. A widely cited example is the remarkable diversity in the primary plastids found in glaucophytes, rhodophytes, and chlorophytes (Cavalier-Smith, 2000). However, despite the enormous differences found among them, the number of membranes surrounding these plastids has not changed during their long evolution. In view of the hypothetical instability of plastid membranes assumed by the 'chromalveolate' hypothesis, further reduction to one membrane in at least a few evolutionary lineages might be expected. In fact, once established in any given endosymbiotic association, a remarkable stability of envelope membranes is characteristic of not only prokaryote-, but also eukaryote-derived plastids. On the other hand, the vestigial plastids of cryptophytes and heterokonts still maintain four envelope membranes, ribosomes attached to the outermost membrane, and the permanent connection with the nuclear envelope (Sepsenwol, 1973; Belcher & Swale, 1976; Sekiguchi *et al*., 2002).

In contrast to these data, the 'chromalveolate' scenario, particularly the version proposed by Yoon *et al*. (2005), implies that the envelope of the ancestral chromalveolate plastid underwent drastic modifications in the dinoflagellate and/or apicomplexan lineage (Fig. 2). In the apicoplast, these modifications also might involve the loss of receptors for ribosomes (if it is surrounded by four membranes) or the loss of additional membranes (if it is surrounded by two membranes). It is more reasonable to assume that, once the hypothetical chromalveolate envelope was established, along with its complex import system for nuclear-encoded plastid proteins, it would not have undergone further drastic modifications. Any such changes could have been traumatic and deleterious leading potentially to negative selection. Large-scale modifications would have been far more likely had the heterokont plastid been laterally transferred to a different phylogenetic lineage, requiring a major transformation to permit functioning with a new host nuclear genome. Under this scenario it is easier to envision serious modifications, including the loss of existing plastid membranes and the invention of new targeting pathways (Fig. 1B and 3). Although the tertiary model postulates the loss of two or three membranes, such losses would have happened during the process of endosymbiosis, before transformation of the heterokont endosymbiont into a true peridinin plastid fully integrated with the dinoflagellate nuclear genome. It is more likely that the loss of membranes by the heterokont endosymbiont was favoured by selection, precisely because it occurred as part of the development of a new and effective import system, as genes encoding plastid proteins migrated into the dinoflagellate genome.

Phylogenies of nuclear genes and the idea of tertiary origin of the peridinin plastid

As discussed above, the concatenated tree of plastid-encoded proteins generated by Yoon *et al*. (2005) is more compatible with tertiary origin of the peridinin plastid than with the 'chromalveolate' hypothesis. However, a crucial question arises: how to reconcile the idea of tertiary origin of the peridinin plastid with the phylogenies of nuclear rRNAs which stimulated Yoon *et al*. (2005) to propose the new version of the 'chromalveolate' model? On these trees, alveolates cluster specifically with heterokonts (e.g. Cavalier-Smith *et al*., 1994; Ben Ali *et al*., 2001), and an identical superassemblage can be found on the concatenated trees of cytosolic genes obtained by Harper *et al*. (2005). We must remember that, even if this topology reflects true phylogenetic relationships (to confirm this topology we should include more taxa), it does not imply that the peridinin plastid and the heterokont plastid result from a single endosymbiosis. Alveolates and chromists comprise an enormously diverse array of eukaryotic organisms on long and independent evolutionary trajectories. The previously discussed branching pattern of aplastidal taxa on dinoflagellate and heterokont trees, combined with the obstacles to plastid loss, suggest that these plastids evolved through independent endosymbioses (Fig. 3). In support of this hypothesis, the biodiversity of heterotrophic heterokonts and alveolates is much greater than initially supposed. In recent years, many such heterotrophic taxa, representing basal lineages on the phylogenetic trees of Heterokonta and Dinoflagellata, have been discovered

(Lopez-Garcia *et al*., 2001; Massana *et al*., 2002; Stoeck & Epstein, 2003; Slapeta *et al*., 2005). Thus, photosynthetic heterokonts are well enough separated from photosynthetic dinoflagellates, by heterotrophic and aplastidal forms, to assume independent origin of their plastids. Moreover, it is very probable that additional intervening aplastidal lineages once existed, but have died out during numerous geologic extinction episodes (see Stiller *et al*., 2003).

An additional inspiration for Yoon *et al*. (2005) to propose their version of the 'chromalveolate' model were phylogenies of GAPDH sequences. These suggest that the gene for cytosolic GAPDH was duplicated in the chromalveolate ancestor and one of the resulting proteins (after acquisition of the proper presequence) was targeted to the plastid of the rhodophyte endosymbiont, replacing its original GAPDH derived from the cyanobacterial plastid ancestor (Fast *et al*., 2001; Harper & Keeling, 2003). There are a number of reasons, however, to doubt this interpretation (see Bodył, 2005). It is evident that, in chromalveolates (*sensu* Cavalier-Smith), the cytosolic GAPDH was duplicated, but it remains unclear when this duplication took place. Such a duplication could have happened in the chromalveolate ancestor (Fast *et al*., 2001; Harper & Keeling, 2003) or, rather, in only one of the chromist lineages, e.g. in Heterokonta. If the latter, this duplicated gene could be transferred to the dinoflagellate lineage through tertiary endosymbiosis. In support of this hypothesis, dinoflagellates emerge from within the Heterokonta on the GAPDH tree obtained by Takishita *et al*. (2004), making this tree compatible with the results generated by Yoon *et al*. (2005). In fact, there is growing evidence that GAPDH genes themselves have been subject to lateral transfer (both plastidic and cytosolic forms) during protist evolution, including in dinoflagellates (Figge & Cerff, 2001; Qian & Keeling, 2001; Fagan & Hastings, 2002; Takishita *et al*., 2003, 2004).

The need to exercise great caution when reconstructing the evolution of rhodophyte-derived plastids on the basis of nuclear-encoded, plastid-targeted proteins is also suggested by the phylogeny of fructose-1,6-bisphosphate aldolase (FBA). The plastid-targeted FBAs of chromists and alveolates belong to class II and represent type A (Patron *et al*., 2004). It is hypothesized that their ancestral gene originated by a duplication of the cytosolic FBA of class II in the chromalveolate ancestor, thereby providing further evidence for a single origin of the chromist and alveolate plastids (see Keeling, 2004; Keeling *et al*., 2004; Patron *et al*., 2004). However, recent genomic and phylogenetic studies by Kroth *et al*. (2005) pose a challenge to this scenario. Many FBA isoforms of both class I and II were found in rhodophytes and diatoms, and phylogenetic analyses and genome comparisons indicated a significant contribution of horizontal gene transfer (not tandem duplications) to the evolution of these enzymes. Since all alveolates (i.e. dinoflagellates, apicomplexans, and ciliates) contain class I FBAs in their cytosol (Patron *et al*., 2004), it is reasonable to conclude that their cytosolic and plastidic class II FBAs may have been horizontally transferred from a heterokont alga during tertiary endosymbiosis. Interestingly, this tertiary scenario was already considered by Patron *et al*. (2004). In general, gene similarities that could result from endosymbiotic transfer are not reliable characters for inferring host cell phylogenies. The specific cases of GAPDH and FBA sequences arguably favour tertiary endosymbiosis over the 'chromalveolate' hypothesis.

Perspectives

The current paradigm in studies of the evolution of chromist and alveolate plastids is the 'chromalveolate' model. New data are often interpreted within its theoretical framework, even if they are difficult to fit or even are in direct contradiction. We believe that the interpretation offered by Yoon *et al*. (2005) of their tree of concatenated plastid proteins is an example. The arguments presented here offer an alternative to this 'chromalveolate' interpretation. Our hypothesis, that the peridinin plastid evolved from a heterokont alga through tertiary endosymbiosis (Fig. 3), is more consistent with current molecular phylogenetic results, and provides a clearer framework for interpreting the evolution of a number of plastid-related characters.

Further studies are required to highlight the evolutionary pathway of the peridinin plastid based on phylogenies of plastid-encoded proteins; however, these analyses should include many more sequences from heterokonts and dinoflagellates than have been used so far. It will be interesting to see if, on these trees, dinoflagellates continue to emerge from within the heterokont clade. A more reliable phylogenetic tree of Dinoflagellata as a whole would be valuable in reconstructing the evolutionary pathway of the perdinin plastid. It has been postulated that eight independent plastid losses occurred in these protozoans (Saldarriaga *et al*., 2001). As discussed earlier, current data from diverse eukaryotes that have taken up a parasitic/heterotrophic lifestyle secondarily suggest that multiple plastid losses are highly doubtful. Therefore, we should consider alternative evolutionary scenarios, including one that suggests multiple origins of the peridinin plastid (Taylor, 2004).

A key step in understanding the evolutionary history of this peculiar plastid will be an exploration of its non-photosynthetic functions. If the peridinin plastid fulfils vital non-photosynthetic functions, its tertiary (single or multiple) origin will be far more probable than the single secondary origin (with multiple independent losses) postulated by all versions of the 'chromalveolate' hypothesis (Cavalier-Smith, 1999, 2003; Yoon *et al*., 2005).

Further study of the apicoplast may also highlight the evolutionary pathway of the peridinin plastid. Based on electron microscopy, Köhler (2005) proposed that this plastid had a primary origin. Perhaps the common ancestor of alveolates harboured a primary cyanobacterium-derived plastid, which was retained in the Apicomplexa but replaced by a tertiary heterokont-derived plastid in the Dinoflagellata. The complete absence of even vestigial evidence for a plastid in ciliates (Palmer *et al*., 2004), ancestral dinoflagellates (e.g. *Oxyrrhis*) (Saldarriaga *et al*., 2003), and apicomplexans such as *Cryptosporidium* and gregarines (Obornik *et al*., 2002) casts doubt on this hypothesis and suggests a tertiary origin of the apicoplast (Fig. 3). If supported by future data, this scenario would reinforce the importance of tertiary endosymbiosis in the evolution of complex plastid-containing algae.

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