MINIREVIEW
A HYPOTHESIS FOR PLASTID EVOLUTION IN CHROMALVEOLATES[1]

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Four eukaryotic lineages, namely, haptophytes, alveolates, cryptophytes, and heterokonts, contain in most cases photosynthetic and nonphotosynthetic members—the photosynthetic ones with secondary plastids with chl *c* as the main photosynthetic pigment. These four photosynthetic lineages were grouped together on the basis of their pigmentation and called chromalveolates, which is usually understood to imply loss of plastids in the nonphotosynthetic members. Despite the ecological and economic importance of this group of organisms, the phylogenetic relationships among these algae are only partially understood, and the so-called chromalveolate hypothesis is very controversial. This review evaluates the evidence for and against this grouping and summarizes the present understanding of chromalveolate evolution. We also describe a testable hypothesis that is intended to accommodate current knowledge based on plastid and nuclear genomic data, discuss the implications of this model, and comment on areas that require further examination.

Key index words: alveolates; chromalveolate; cryptophyte; dinoflagellate; evolution; haptophyte; heterokont; phylogenetics; plastid

Abbreviations: chl *c*, chlorophyll c; HGT, horizontal gene transfer

Plastid acquisition. Photosynthesis is one of the most critical processes that sustain life on earth. Though different types of phototrophy involving a variety of electron donors have been described in Bacteria and Archaea, only oxygenic photosynthesis is known in eukaryotes. All photosynthetic (i.e., plastid containing) eukaryotes have directly or indirectly gained photosynthesis from a cyanobacterium, acquiring plastids through the processes of primary, secondary, or tertiary endosymbiosis (Guillot and Gibbs 1980a, Gibbs 1981a, Delwiche 1999, Keeling 2004). Primary endosymbiosis is the process by which a cyanobacterium was engulfed and integrated into a nonphotosynthetic eukaryotic host cell. As a result, primary plastids (i.e., those of glaucophytes, land plants, and green and red algae) are surrounded by a double membrane, often thought to be derived from the cyanobacterium (Delwiche 1999, Reumann et al. 1999). However, most algal lineages acquired their plastids in secondary or tertiary endosymbioses. In secondary endosymbiosis, a nonphotosynthetic eukaryotic host cell engulfed a photosynthetic eukaryote, that is, a green or a red alga (Gibbs 1978, 1981a, Guillot and Gibbs 1980a, Delwiche 1999, Keeling 2004). Secondary plastids (i.e., those of euglenoids, chlorarachniophytes, haptophytes, heterokonts, alveolates, and cryptophytes) are surrounded by additional membranes derived from the endosymbiont plasma membrane and host endomembrane system (Gibbs 1981a, Cavalier-Smith 1999, 2002).

Following (or during) each endosymbiotic (primary or secondary) event, the endosymbiont lost the genes required for free-living existence, and hundreds or thousands of endosymbiont genes were transferred to the host nucleus (Martin et al. 2002, Sun et al. 2004, Reyes-Prieto et al. 2006). However, at least in most algae, part of the original endosymbiont genome was maintained as the plastid genome (Palmer and Delwiche 1996, Martin et al. 1998, 2002, Brown 2003). Protein products of plastid-associated nuclear-encoded genes are targeted back to the plastid and imported into the organelle aided by target localization signals and protein-import machinery (Archer and Keegstra 1990, Martin et al. 1998, Cavalier-Smith 1999, 2002). Targeting to primary plastids, such as those of green and red algae, requires a transit peptide to transport the proteins to the plastid across the double membrane (Reumann et al. 1999, Kroth 2002). Recently, more complex and novel routes of protein trafficking have been observed in plants (Jarvis and Robinson 2004, Villarejo et al. 2005, Radhamony and Theg 2006). In the case of secondary plastids, targeting signals consist of a bipartite targeting signal, a

signal and a transit peptide adjacent to one another, directing the protein first to the endomembrane system of the host, and then to the plastid (van Dooren et al. 2001, Cavalier-Smith 2002, Kroth 2002, Ralph et al. 2004). From this it follows that photosynthetic eukaryotes have highly integrated host and endosymbiont genomes that now depend on each other to survive.

Evolutionary relationships among photosynthetic eukaryotes have often been confounded with the relationships among the plastids they harbor (Christensen 1962, Delwiche 1999, Palmer 2003). Once the endosymbiotic origin of plastids was recognized (Margulis 1970, Gibbs 1978, 1981b), relationships among eukaryotic host cells came into question. Algae are regarded today as a polyphyletic clade with respect to the nuclear genome, but current data are insufficient to fully explain the relationships among them. Phylogenetic studies based on concatenated mitochondrial or nuclear genes and sparse taxon sampling were typically able to identify major eukaryotic groups, but could not resolve deep phylogenetic relationships (Van de Peer and De Watcher 1997, Patterson 1999, Baldauf et al. 2000, 2004, Baldauf 2003, Cavalier-Smith 2003, Stechmann and Cavalier-Smith 2003, Keeling 2004, Simpson and Roger 2004). However, six major eukaryotic clades are provisionally recognized today, including Opisthokonta, Amoebozoa, Plantae, Rhizaria, Excavata, and Chromalveolata (Patterson 1999, Nikolaev et al. 2004, Simpson and Roger 2004, Adl et al. 2005, Keeling et al. 2005). These ''supergroups'' are, in some cases, defined primarily on molecular data with strong (e.g., Opisthokonta, Amoebozoa) or weak (e.g., Plantae, Rhizaria) support, while others (e.g., Chromalveolata, Excavata) are based on minimal, if any, support (Burki et al. 2007) and remain highly controversial. Furthermore, several described protist lineages (more than 20) have not yet been assigned to any of these clades (Patterson 1999, Simpson and Patterson 2006). More detailed studies (i.e., increasing taxon and gene sampling) are necessary to assess whether these supergroups are natural or not and how they relate to each other. The supergroup Chromalveolata represents the main focus of this review.

Chromalveolates. During the last decades, chromalveolate lineages have been grouped together, including or excluding some of their members, with the consequent erection of higher taxon names referring to overlapping groupings of taxa. Kingdom Chromista sensu Cavalier-Smith (1989) includes cryptophytes, haptophytes, and heterokonts, based on the presence of mastigonemes (tubular flagellar hairs) and/or the localization of the plastid in the endomembrane system of the host cell. Chromalveolates (Chromista and Alveolata) is a term coined by Cavalier-Smith (1999) to refer to a more inclusive group that embraces all chl *c*–containing algae along with their heterotrophic

relatives (Cavalier-Smith 1999, 2004, Adl et al. 2005), namely, Haptophyta, Cryptophyta, Heterokontophyta (or Stramenopiles), and Alveolata (Dinophyta, Ciliophora, Apicomplexa). Taken together, the chromalveolates constitute a major branch on the tree of life and account for an important fraction of eukaryotic diversity.

Cryptophytes comprise a small group (~200 species) of photosynthetic and heterotrophic organisms that live in marine and freshwater environments. Plastid-containing lineages of cryptophytes appear to be monophyletic to the exclusion of basal heterotrophic taxa from the genus *Goniomonas* (McFadden et al. 1994, Marin et al. 1998). Cryptophyte plastids contain chl *a* and *c*, and phycobiliproteins, the last of which are not arranged in phycobilisomes, as in red algae, but rather are localized in the thylakoid lumen. The plastids were acquired by engulfing a red alga and are surrounded by four membranes, with the outermost membrane continuous with the endoplasmic reticulum (ER) and called the chloroplast ER (CER) (Guillot and Gibbs 1980b, Gibbs 1981a, Oliveira and Bhattacharya 2000). In the periplastidal space (between the inner two and the outer two plastid membranes), cryptophytes still maintain a remnant of the nucleus of the red algal endosymbiont called a ''nucleomorph'' (Guillot and Gibbs 1980a, Douglas et al. 2001).

Most haptophytes are unicellular, photosynthetic eukaryotes occurring mainly in marine environments. There are no well-documented heterotrophic members of this group (Marchant and Thomsen 1994), although many of them are mixotrophic (Andersen 2004). Haptophyte plastids are pigmented with chl *a* and *c*, and two related carotenoid fingerprints, 19′ hexanoyloxyfucoxanthin and 19′ butanoyloxyfucoxanthin (Bijornland and Liaaen-Jensen 1989). The plastids are surrounded by four membranes, from which the outermost membrane is continuous with the ER (CER) (Gibbs 1981a,b).

The phylum Heterokontophyta encompasses an extremely diverse group of protists (~100,000 species) with photosynthetic and heterotrophic members, including parasitic ones (e.g., *Phytophthora infestans*, causative agent of the potato late blight) (Patterson 1999, Andersen 2004, Adl et al. 2005). Early diverging heterokonts, such as bicosoecids, labyrinthulids, and oomycetes, are heterotrophic (Cavalier-Smith and Chao 1996, Karpov et al. 2001), but plastid-related genes have been found in the nuclear genome of oomycetes, suggesting the possibility of a photosynthetic ancestry of this lineage (Andersson and Roger 2002, Robertson and Tartar 2006, Tyler et al. 2006). Photosynthetic members (''Stramenochromes'' sensu Patterson 1999) carry plastids surrounded by four membranes (including CER) that contain chl *a* and *c*, and a diverse range of accessory pigments.

Dinoflagellates, together with ciliates and apicomplexans, are collectively called Alveolates due to the

presence in many of cortical alveoli (a layer of vesicles localized below the plasma membrane) in these three lineages (Cavalier-Smith 1991). Ciliates are heterotrophic and aplastidic, while apicomplexans are parasitic and contain a reduced plastid, called the apicoplast, surrounded by four membranes (Köhler et al. 1997, Foth and McFadden 2003). Most current evidence supports a red algal ancestry of the apicoplast and a close relationship with peridinin-containing dinoflagellate plastids (Blanchard and Hicks 1999, Foth and McFadden 2003, Waller and Keeling 2006, Waller et al. 2006). Dinoflagellates are biflagellate protists with diverse ecological roles and nutritional strategies: photosynthetic, mixotrophic, predatory, and parasitic (Schnepf and Elbrächter 1999, Hackett et al. 2004a). Most photosynthetic dinoflagellates (hereafter called peridinin-dinoflagellates) have plastids that are surrounded by three membranes and contain chl a and c, and peridinin as the major photosynthetic pigments. Many, if not all, peridinin-dinoflagellates appear to lack a conventional plastid genome and have instead several small circular DNA molecules, typically $\sim$2–3 kbp, containing 0, 1, or 2 plastid genes, with a total of fewer than 20 genes and RNAs identified so far (Zhang et al. 1999, Barbrook and Howe 2000, Hiller 2001, Barbrook et al. 2006, Nelson et al. 2007). Peridinin-containing plastids are also peculiar in the use of a nuclear-encoded form II RUBISCO of a type found elsewhere only in anoxygenic phototrophs instead of a plastid-encoded form Ib RUBISCO, as in all other red-algal derived plastids from haptophytes, cryptophytes, and heterokonts (Morse et al. 1995, Delwiche and Palmer 1996).

Historically, the four photosynthetic chromalveolate lineages (cryptophytes, haptophytes, heterokonts, and dinoflagellates) have been grouped together based on their common pigmentation (Christensen 1962, 1989). However, when ultrastructural and molecular studies revealed that plastids were endosymbiotic cyanobacteria that could potentially be acquired in independent events, the value of plastid characters as phylogenetically informative features (for the organism) came into question, and these four lineages were more often treated separately. Today, the evolutionary relationships among cryptophytes, haptophytes, heterokonts, and alveolates are still controversial, but multiple studies are starting to shed some light.

Evolution of photosynthetic eukaryotes. A model describing the evolution of a significant number of photosynthetic eukaryotes is emerging based on work done by several laboratories (Fig. 1). A single primary endosymbiotic event probably gave rise to the three primary-plastid-containing lineages, collectively called Plantae (or Archaeplastida): green algae (including land plants), red algae (Rhodophyta), and probably glaucophytes (Cavalier-Smith 1998, Moreira et al. 2000, Adl et al. 2005, Rodriguez-Ezpeleta et al. 2005, Weber et al. 2006, Reyes-Prieto and Bhattacharya 2007, but see Nozaki et al. 2003, 2007, Nozaki 2005). Two main innovations occurred in the common ancestor of Plantae, including the
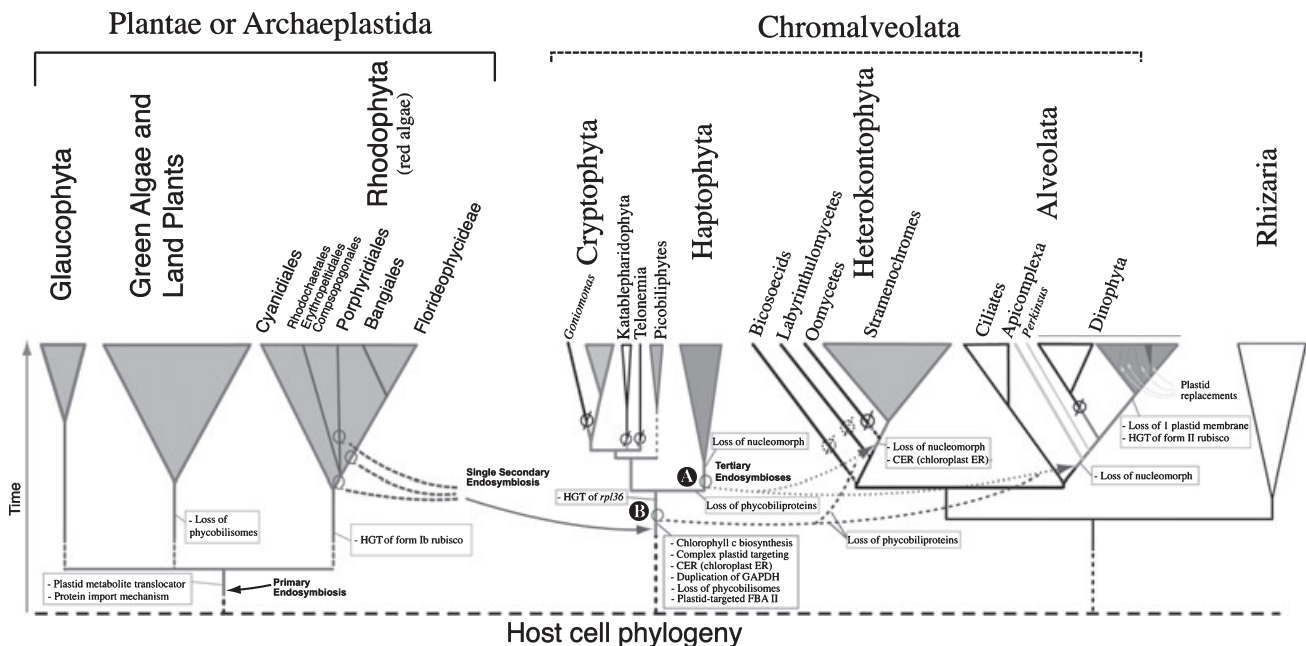

FIG. 1. Proposed model of evolution of chromalveolate lineages. Dashed lines indicate relationships with weak support or uncertain characters. Crossed circles represent loss of photosynthesis and putative plastid losses in heterotrophic lineages. Filled triangles (clades) indicate photosynthetic members; open triangles, heterotrophic ones. Plastid-related characters are mapped on the phylogram. A and B represent two alternative evolutionary hypotheses discussed in the text.

origin of a plastid metabolite translocator necessary for establishment of the organelle (Weber et al. 2006), and the development of a protein import mechanism required for the retargeting of proteins encoded by cyanobacterial genes transferred to the host nucleus (Cavalier-Smith 2002, Steiner and Loffelhardt 2002). Recent studies based on nuclear molecular data support the monophyly of Plantae, although relationships among these lineages are unclear (Burger et al. 1999, Moreira et al. 2000, Keeling 2004, Sanchez-Puerta et al. 2004, Rodriguez-Ezpeleta et al. 2005, Weber et al. 2006). In congruence with the putative monophyly of the Plantae, plastids from glaucophytes and red and green algae descend from a common ancestor in cyanobacteria (Delwiche et al. 1995).

At least three secondary endosymbiotic events are recognized today. Two endosymbioses involve the engulfment of a green alga by an ancestral euglenoid and a chlorarachniophyte in two separate events (Delwiche 1999, Keeling 2004, Rogers et al. 2007) (not shown in Fig. 1). A third one involves the engulfment of a red alga (Fig. 1), which ultimately gave rise to the plastids of the four chl *c*–containing eukaryotic lineages (i.e., chromalveolates; Cavalier-Smith 1999, Delwiche 1999, Bhattacharya et al. 2003, Keeling 2004). Whether chromalveolates are a monophyletic group derived from a plastid-containing ancestor continues to be the subject of much discussion. Recently, a series of relevant studies have been published, changing the field rapidly. Here, we will evaluate the evidence for chromalveolate relationships (both plastid and host relationships) and present a testable hypothesis based on current data.

Assessing evolutionary relationships among plastids from chromalveolates. Strong evidence exists for the monophyly of chl *c*–containing plastids in chromalveolates and their association with red algal plastids (Ishida and Green 2002, Yoon et al. 2002, 2005, Bachvaroff et al. 2005, Shalchian-Tabrizi et al. 2006c, Khan et al. 2007, Sanchez-Puerta et al. 2007a). Furthermore, independent evidence for the monophyly of the chl *c* plastids comes from plastid-targeted nuclear-encoded genes (Fast et al. 2001, Harper and Keeling 2003, Patron et al. 2004, Petersen et al. 2006, Teich et al. 2007). However, relationships among plastids from haptophytes, cryptophytes, heterokonts, and dinoflagellates remain poorly understood.

Plastid evolution has been studied on the basis of a high number of genes given the feasibility of sequencing complete plastid genomes, although taxon sampling has generally been lacking (Martin et al. 1998, 2002, Hagopian et al. 2004, Bachvaroff et al. 2005, Sanchez-Puerta et al. 2007a). Some studies, however, have included a denser taxon sampling and only one or a few plastid-associated genes (Ishida and Green 2002, Yoon et al. 2002, 2005). Neither approach has been successful in elucidating all plastid relationships, probably due to the speed and antiquity of many of the key events, heterogeneous evolutionary rates across genes and taxa, compositional bias, low taxon sampling (even in the largest studies), and putative multiple endosymbioses. In addition, assessment of plastid relationships based on plastid-associated nuclear-encoded genes has been problematic due to confounding cases of horizontal gene transfer (Hackett et al. 2004b, Li et al. 2006, Petersen et al. 2006, Reyes-Prieto et al. 2007).

Most phylogenetic studies based on plastid genes agree on the early diverging position of the cryptophyte plastids. Cryptophyte plastids are often found to be sister to all other chl *c*–containing plastids, with moderate to high bootstrap support (Yoon et al. 2002, 2005, Bachvaroff et al. 2005, Shalchian-Tabrizi et al. 2006c, Khan et al. 2007, Rogers et al. 2007, Sanchez-Puerta et al. 2007a). In contrast, a few phylogenetic analyses showed variable support for a sister relationship between cryptophyte and haptophyte plastids (Rice and Palmer 2006, Iida et al. 2007, Khan et al. 2007, Sanchez-Puerta et al. 2007a). The replacement of a native plastid gene (*rpl36*) by a laterally transferred bacterial copy of this gene in cryptophyte and haptophyte plastid genomes also suggests that their plastids may be closely related (Rice and Palmer 2006). These conflicting results may be due to the limitations of phylogenetic analysis (including problems due to heterotachy, long branch attraction, sampling and systematic errors), additional cases of horizontal gene transfer, or multiple endosymbiotic events leading to plastid acquisition among chromalveolates (see below).

Relationships among heterokont, haptophyte, and peridinin-dinoflagellate plastids are highly unstable in phylogenetic analyses, probably in large part due to the accelerated rate of evolution of peridinin-dinoflagellate plastid sequences (Zhang et al. 2000, Bachvaroff et al. 2006). Several studies showed a sister relationship of the haptophyte and peridinin-dinoflagellate plastids with variable support (Bachvaroff et al. 2005, Shalchian-Tabrizi et al. 2006c, Sanchez-Puerta et al. 2007a), while others found a weak relationship of heterokont and peridinin-dinoflagellate plastids (Durnford et al. 1999, Inagaki et al. 2004, Yoon et al. 2005, Inagaki and Roger 2006). Overall, studies reveal a complex set of relationships among chromalveolate plastids, which are only partially understood.

Adding to the complexity, peridinin-containing plastids have been replaced in several dinoflagellates with other plastid types, including some acquired from green algae, haptophytes, cryptophytes, or diatoms in independent secondary or tertiary endosymbiotic events (Dodge 1975, Chesnick et al. 1997, Schnepf and Elbrächter 1999, Saldarriaga et al. 2001, Tengs et al. 2001, Shalchian-Tabrizi et al. 2006b, Imanian et al. 2007). Atypically pigmented dinoflagellates contain plastid-encoded form I

RUBISCO derived from the novel endosymbiont, although it is possible that they have also retained form II RUBISCO in their nuclear genome. Most of these atypically pigmented dinoflagellates have received relatively little attention, and in most cases, the details of interactions between the endosymbiont and nucleus are poorly known (Cavalier-Smith 1999, Nosenko et al. 2006, Patron et al. 2006). The most prominent group of anomalously pigmented dino-flagellates are those containing haptophyte-derived tertiary plastids, which include such important organisms as the red-tide alga *Karenia brevis* (Tengs et al. 2000, Nosenko et al. 2006). Preliminary studies of protein trafficking in haptophyte-containing dino-flagellates suggested a protein-targeting mechanism different to that functioning in peridinin-containing dinoflagellates (Patron et al. 2006). Further work needs to be done on other anomalously pigmented dinoflagellates to assess if a protein transport system has been established, and if so, whether a novel system was developed or the same protein import mechanism is maintained.

Assessing evolutionary relationships among chromalveolate host cells. Knowledge of chl *c* plastid relationships conveys only partial information regarding the number of endosymbiotic events that took place in chromalveolate evolution, or the relationships among their host genomes. Several models of host cell evolution are congruent with a monophyletic chl *c* plastid clade (Cavalier-Smith 1999, Bachvaroff et al. 2005, Bodyl 2005, Bodyl and Moszczynski 2006, Sanchez-Puerta et al. 2007a). To piece the story together, relationships among host cell genomes also need to be analyzed, using mitochondrial genes or nucleus-encoded genes that are not plastid derived.

Several attempts to resolve deep eukaryotic relationships yield unresolved trees, in particular, for chromalveolate lineages that group weakly with different lineages depending on the methodology, molecular marker, and taxa included (Bhattacharya et al. 1995, Van de Peer and De Watcher 1997, Baldauf et al. 2000, Stibitz et al. 2000, Stechmann and Cavalier-Smith 2003, Sanchez-Puerta et al. 2004). Lately, more resolved phylogenies using larger data sets and more complex analytical methods have been obtained (see below).

A sister relationship of heterokonts and alveolates has been reported from several phylogenetic analyses based on independent molecular data sets (Van de Peer and De Watcher 1997, Fast et al. 2002, Harper et al. 2005). Surprisingly, recent phylogenetic analyses found a well-supported relationship of alveolates, heterokonts, and members of the supergroup Rhizaria (Burki et al. 2007, Hackett et al. 2007, Not et al. 2007). Rhizaria encompasses a wide diversity of parasitic or free-living unicellular eukaryotes (Simpson and Patterson 2006). Most members of Rhizaria are heterotrophic, with the exception of chlorarachniophytes, which contain

secondary plastids acquired from green algae in a separate endosymbiotic event (Rogers et al. 2007). In addition, what appears to be a completely independent primary endosymbiosis has been described involving a cyanobacterium and the rhizarian filose amoeba *Paulinella chromatophora* (Kies 1974, Marin et al. 2005, Yoon et al. 2006); however, additional work is needed to fully characterize this symbiotic relationship (Bodyl et al. 2007, Reyes-Prieto et al. 2007).

Cryptophyte and haptophyte lineages have been more difficult to place phylogenetically and have been often individually associated with a variety of taxa with low support (Bhattacharya et al. 1993, Medlin et al. 1997, Tengs et al. 2000, Stechmann and Cavalier-Smith 2003). Recent phylogenetic analyses based on nuclear genes showed a sibling relationship of haptophytes and cryptophytes with moderate to strong support (Harper et al. 2005, Burki et al. 2007, Hackett et al. 2007, Patron et al. 2007). In addition, two newly erected heterotrophic protist phyla (Kathablepharida and Telonemia) have been associated with cryptophytes based on molecular data (Okamoto and Inouye 2005b, Shalchian-Tabrizi et al. 2006a). Interestingly, a possible ongoing secondary endosymbiosis between a katablepharid *Hatena arenicola* and a green alga has been described (Okamoto and Inouye 2005a, 2006), although further studies are needed to better understand the nature of the endosymbiotic relationship. Furthermore, a new lineage of photosynthetic eukaryotes, picobiliphytes, has recently been discovered by environmental sequencing and weakly positioned as a sister group to cryptophytes (Not et al. 2007). This new group of photosynthetic picoplankton contains a plastid-like structure with phycobiliproteins, and presumably a nucleomorph (Not et al. 2007); however, these data need to be further confirmed. The clade formed by cryptophytes, *Telonema*, picobiliphytes, katablepharids, and haptophytes has not been affiliated to any other group of eukaryotes with certainty.

Piecing together the evolutionary history of Chromalveolata. Despite the big challenges due to scattered and equivocal data, a provisional model of chromalveolate evolution can be outlined combining observations from host and endosymbiont studies (Fig. 1). This model represents a collection of interrelated hypotheses, each of which can (and should) be individually tested. We propose a single secondary endosymbiosis with a red alga during the evolution of cryptophytes and haptophytes, and one or two tertiary endosymbiotic events (excluding the exceptional plastid replacements described in some dinoflagellate lineages) during the evolution of heterokonts and alveolates. Under the proposed hypothesis, chromalveolates are not necessarily monophyletic and are only possibly paraphyletic (with respect to Rhizaria). Recently, Burki et al. (2007) postulated that chromalveolate lineages

might have acquired their plastids in a few independent secondary endosymbioses with red algae. This hypothesis is not consistent with the evidence given by analyses based on the nuclear-encoded plastid-targeted genes GAPDH and FBA, which show that chromalveolate lineages (and not red algae) share a duplication of the *gapdh* gene (Fast et al. 2001, Harper and Keeling 2003) and a particular type of FBA gene (Patron et al. 2004). In addition, separate plastid acquisition in 'chromalveolates' from red algae would also invoke independent evolution of chl *c*. What follows is a working hypothesis for the evolution of chromalveolate lineages that is consistent in most ways with current data.

In a single secondary endosymbiotic event, the common ancestor of cryptophytes + haptophytes engulfed a unicellular red alga and retained it as a secondary plastid (Fig. 1). This endosymbiosis took place after the divergence of members of the Cyanidiales (Rhodophyta) and before the split between Bangiales and Florideophycideae (Yoon et al. 2002, 2004, Sanchez-Puerta et al. 2007a). One study suggests a member of the red algal order Porphyridiales as the ancestor of the chl *c* plastids (Shalchian-Tabrizi et al. 2006c). Better taxon sampling within the Rhodophyta is needed to establish conclusively the closest living ancestor of chromalveolate plastids, and it is important to bear in mind that key red algal lineages may have since gone extinct.

A number of evolutionary changes occurred in the organelles and host cells following endosymbiosis. Some of the most relevant plastid-related evolutionary events include development of a targeting system for complex plastids (surrounded by more than two membranes), fusion of the outermost membrane (i.e., the food vacuole) with the ER to form the CER, and biosynthesis of chl *c*. In addition, under this model, a copy of the gene *rpl36* was laterally transferred from a bacterium to the plastid genome of the common ancestor of cryptophytes and haptophytes, replacing the native *rpl36* gene (Rice and Palmer 2006). As a result, it is likely that two copies of the gene *rpl36* (one foreign, one native) coexisted in the population (not in a single plastid genome) for some time, until the native copy was presumably lost in haptophytes and cryptophytes (Hackett et al. 2007, see below).

After the divergence of haptophytes, the sister clade diversified into four main lineages: cryptophytes, katablepharids, telonemids, and possibly picobiliphytes. Secondary loss of photosynthesis (and maybe plastids) occurred in *Goniomonas*, katablepharids, and *Telonema*. Haptophytes lost the phycobiliproteins and also the nucleomorph and might have retained the native copy of *rpl36* for some time as a polymorphism within the plastid population. It is possible that some lineages of haptophytes retained only the native *rpl36* as a result of lineage sorting. So far, the single haptophyte

examined, *Emiliania huxleyi*, contains only the foreign copy of *rpl36* in its plastid genome (Sanchez-Puerta et al. 2005, Rice and Palmer 2006). Additional *rpl36* data on a broad range of haptophytes would help clarify this scenario.

Plastid acquisition in the common ancestor of heterokonts and alveolates may have occurred after the divergence of Rhizaria. Under this model, a single endosymbiotic event took place in the ancestor of heterokonts + alveolates. Alternatively, two separate endosymbioses could have occurred independently in heterokonts and alveolates (Fig. 1, see below). It is difficult to estimate the timing of this event in the evolutionary history of heterokonts given the lack of data from early diverging lineages of heterokonts, such as bicosoecids and labyrinthulids. Under this scenario, the other endosymbiosis would have occurred in the common ancestor of apicomplexans and dinoflagellates to the exclusion of ciliates.

Whatever the timing of their acquisition, identifying the source of the plastid of heterokonts and alveolates is difficult. One possibility is that a haptophyte was engulfed in one (or two) tertiary endosymbioses (Fig. 1, hypothesis A). This endosymbiotic haptophyte might have contained only the native copy of *rpl36* and not the foreign one as a result of lineage sorting. This model of plastid evolution is consistent with chromalveolate plastid relationships observed in most phylogenies based on plastid genes. Alternatively, heterokonts and alveolates could have engulfed (in one or two separate events) a photosynthetic ancestor of cryptophytes and haptophytes, previous to the horizontal transfer of *rpl36* (Fig. 1, hypothesis B). Under this second scenario, heterokonts and alveolates must have lost the phycobiliproteins independently from haptophytes. This hypothesis conflicts with most phylogenetic analyses based on plastid genes (Yoon et al. 2002, 2005, Bachvaroff et al. 2005, Shalchian-Tabrizi et al. 2006c, Khan et al. 2007, Rogers et al. 2007, Sanchez-Puerta et al. 2007a), albeit with some exceptions (Rice and Palmer 2006, Iida et al. 2007, Khan et al. 2007, Sanchez-Puerta et al. 2007a). Under either hypothesis, heterokonts and alveolates lost the nucleomorph after plastid gain (Fig. 1).

Plastid acquisitions were followed by loss of photosynthetic ability (and plastid altogether in some cases) in several lineages, including oomycetes, heterotrophic dinoflagellates, ciliates, and apicomplexans. In agreement with this hypothesis, plastid-associated genes have been found in the nuclear genome of oomycetes (Andersson and Roger 2002, Robertson and Tartar 2006, Tyler et al. 2006), heterotrophic dinoflagellates (Sanchez-Puerta et al. 2007b, Stelter et al. 2007), and even the remnant of a plastid in apicomplexans (Köhler et al. 1997, Foth and McFadden 2003) and the dinoflagellate *Perkinsus* (Teles-Grilo et al. 2007). In contrast, no trace of

the ancestral presence of a plastid has yet been reported for ciliates, even though the complete nuclear genome of the ciliate *Tetrahymena* has been sequenced (Eisen et al. 2006). However, it is important to mention that only the macronucleus (not the micronucleus) of this ciliate has been examined. The relationship and coadaptation of host and endosymbiont are extremely intimate and involve several key metabolic pathways beyond photosynthesis; these are often retained in secondarily nonphotosynthetic lineages. In this context, it would be surprising if ciliates had altogether dispensed with the metabolic functions carried out by the organelle or organelle-derived genes after losing their plastids. For this reason, we favor two independent tertiary endosymbioses in the evolution of heterokonts and alveolates (Fig. 1). In this scenario, ciliates would have never contained a plastid, and few plastid-derived genes would be expected in their nuclear genome.

One important concern about the proposed evolutionary model refers to the loss of plastid membranes after tertiary endosymbiosis. A tertiary endosymbiotic event would theoretically lead to a plastid surrounded by six membranes (four from the original secondary plastid present in the endosymbiont, plus the endosymbiont plasma membrane and one derived from the host vacuole). However, plastids from heterokonts and apicomplexans contain four membranes, and peridinin-dinoflagellates only three. It has been questioned how the number of plastid membranes could be reduced and how likely this evolutionary event is (Cavalier-Smith 1999, 2002). Evidence from a separate, unambiguous case of tertiary endosymbiosis indicates that membranes can be lost or fused, although the mechanism remains unknown. In this independent ''natural experiment,'' a dinoflagellate replaced its peridinin-plastid by engulfing a haptophyte and retaining it as a tertiary plastid surrounded today by only four membranes (Dodge 1975, Tengs et al. 2000). In addition, it is unclear how complex and unique is the fusion of the plastid outermost membrane with the ER (to form the CER) that occurs in cryptophytes, haptophytes, and heterokonts, and what is the likelihood of evolving independently in the ancestor of cryptophytes + haptophytes and in heterokonts, or to be lost (e.g., in dinoflagellates). Because a typical food vacuole occupies a topological position in the cell comparable to that of the ER, fusion of these structures is not necessarily surprising.

Another relevant issue refers to the complexity and consequent likelihood of multiple independent plastid acquisitions. Alternative evolutionary hypotheses to the one presented here have been proposed (Cavalier-Smith 1999, Bachvaroff et al. 2005, Nozaki 2005, Bodyl and Moszczynski 2006, Petersen et al. 2006, Hackett et al. 2007, Nozaki et al. 2007, Patron et al. 2007, Sanchez-Puerta et al. 2007a, Teich et al.

2007). Many of these hypotheses rely partly on the assumption that complex processes, such as establishment of an organelle (particularly, the development of a targeting mechanism), are not likely to originate twice in evolution, indicating that certain evolutionary innovations must have arisen a minimal number of times (Cavalier-Smith 1999). This is nominally a parsimonious argument, but recent studies indicate that developing a targeting system in complex plastids is not as unlikely as once speculated (Bodyl and Moszczynski 2006, Reyes-Prieto et al. 2007, Teich et al. 2007). Furthermore, the chromalveolate hypothesis (and slight variants of it) propose that the common ancestor of chromalveolates (and perhaps Rhizaria) would have acquired a plastid in a single secondary endosymbiotic event by engulfing a unicellular red alga (Cavalier-Smith 1999, Hackett et al. 2007, Patron et al. 2007). However, there are few data that directly support a common ancestry of all of the chromalveolate lineages (including Rhizaria or not), and plastid relationships are often not congruent with current host phylogeny.

In conclusion, several alternative evolutionary hypotheses would be consistent with the current evolutionary evidence. We propose a model of algal evolution that accommodates the conflicting relationships of chromalveolate plastids and their host cells. To test the interrelated hypotheses, additional sampling of important lineages (e.g., early divergent heterokonts, additional members of Haptophyta, sister groups to cryptophytes, and other yet to be discovered algal lineages), together with overall increase in molecular data, would continue to clear the path toward understanding chromalveolate evolution and the intriguing development of key evolutionary events. In particular, assessing the presence of plastid-derived genes in the nuclear genome of members of Rhizaria, additional ciliate species, and early divergent heterokonts would help distinguish whether the plastids of heterokonts and alveolates were acquired in independent events or in a single endosymbiosis in their common ancestor. Furthermore, examining the presence/absence of foreign and native *rpl36* in several haptophytes, as well as heterokonts and dinoflagellates, would be useful to test the hypothesis that we postulate here (see above). Increasing the taxon sampling in plastid phylogenies should help us understand the conflicts observed in current phylogenetic analyses. Also, the predicted presence of a duplicated *gapdh* gene, a plastid-targeted *fbaII* gene, a foreign *rpl36* gene, and CER in phycobiliphytes could be tested. Lastly, new ultrastructural work in the context of modern phylogenetic information, particularly examining the presence and characteristics of CER (Gibbs 1981a,b) in a broader range of chromalveolates, will help us understand the complexity and evolution of this cellular structure.

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, R. O., Barta, J. R., Bowser, S. S., et al. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* 52:399–451.

Andersen, R. A. 2004. Biology and systematics of heterokont and haptophyte algae. *Am. J. Bot.* 91:1508–22.

Andersson, J. O. & Roger, A. J. 2002. A cyanobacterial gene in nonphotosynthetic protists – an early chloroplast acquisition in eukaryotes? *Curr. Biol.* 12:115–9.

Archer, E. & Keegstra, K. 1990. Current views on chloroplast protein import and hypotheses on the origin of the transport mechanisms. *J. Bioenerg. Biomembr.* 22:789–810.

Bachvaroff, T. R., Sanchez-Puerta, M. V. & Delwiche, C. F. 2005. Chlorophyll *c* containing plastid relationships based on analyses of a multi-gene dataset with all four chromalveolate lineages. *Mol. Biol. Evol.* 22:1–11.

Bachvaroff, T. R., Sanchez-Puerta, M. V. & Delwiche, C. F. 2006. Rate variation as a function of gene origin in plastid-derived genes of peridinin-containing dinoflagellates. *J. Mol. Evol.* 62:42–52.

Baldauf, S. F. 2003. The deep roots of eukaryotes. *Science* 300:1703–6.

Baldauf, S. F., Bhattacharya, D., Cockrill, J., Hugenholtz, P., Pawlowski, J. & Simpson, A. 2004. The tree of life. *In* Cracraft, J. & Donoghue, M. [Eds.] *Assembling the Tree of Life.* Oxford University Press, New York, pp. 43–75.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290:972–6.

Barbrook, A. & Howe, C. 2000. Minicircular plastid DNA in the dinoflagellate *Amphidinium operculatum. Mol. Gen. Genet.* 263:152–8.

Barbrook, A. C., Santucci, N., Plenderleith, L. J., Hiller, R. G. & Howe, C. J. 2006. Comparative analysis of dinoflagellate chloroplast genomes reveals rRNA and tRNA genes. *BMC Genomics* 7:297.

Bhattacharya, D., Helmchen, T., Bibeau, C. & Melkonian, M. 1995. Comparisons of nuclear-encoded small-subunit ribosomal RNAs reveal the evolutionary position of the Glaucocystophyta. *Mol. Biol. Evol.* 12:415–20.

Bhattacharya, D., Stickel, S. K. & Sogin, M. L. 1993. Isolation and molecular phylogenetic analysis of actin-coding regions from *Emiliania huxleyi*, a prymnesiophyte alga, by reverse transcriptase and PCR methods. *Mol. Biol. Evol.* 10:689–703.

Bhattacharya, D., Yoon, H. S. & Hackett, J. D. 2003. Photosynthetic eukaryotes unite: endosymbiosis connects the dots. *BioEssays* 26:50–60.

Bijornland, T. & Liaaen-Jensen, S. 1989. Distribution patterns of carotenoid in relation to chromophyte phylogeny and systematics. *In* Green, J. C., Leadbeater, B. S. C. & Diver, W. [Eds.] *The Chromophyte Algae: Problems and Perspectives.* Clarendon Press, Oxford, UK, pp. 37–60.

Blanchard, J. & Hicks, J. 1999. The non-photosynthetic plastid in malarial parasites and other apicomplexans is derived from outside the green plastid lineage. *J. Eukaryot. Microbiol.* 46:367–75.

Bodyl, A. 2005. Do plastid-related characters support the chromalveolate hypothesis? *J. Phycol.* 41:712–9.

Bodyl, A., Mackiewicz, P. & Stiller, J. W. 2007. The intracellular cyanobacteria of *Paulinella chromatophora*: endosymbionts or organelles? *Trends Microbiol.* 15:295–6.

Bodyl, A. & Moszczynski, K. 2006. Did the peridinin plastid evolve through tertiary endosymbiosis? A hypothesis. *Eur. J. Phycol.* 41:435–48.

Brown, J. R. 2003. Ancient horizontal gene transfer. *Nat. Rev.* 4:121–32.

Burger, G., Saint-Louis, D., Gray, M. W. & Lang, B. F. 1999. Complete sequence of the mitochondrial DNA of the red alga *Porphyra purpurea*: cyanobacterial introns and shared ancestry of red and green algae. *Plant Cell* 11:1675–94.

Burki, F., Shalchian-Tabrizi, K., Minge, M., Skjaeveland, A., Nikolaev, S., Jakobsen, K. S. & Pawlowski, J. 2007. Phylogenomics reshuffles the eukaryotic supergroups. *PLoS ONE* 8:790.

Cavalier-Smith, T. 1989. The kingdom Chromista. *In* Green, J., Leadbeater, B. S. C. & Diver, W. [Eds.] *The Chromophyte Algae: Problems and Perspectives.* Clarendon Press, Oxford, UK, pp. 381–407.

Cavalier-Smith, T. 1991. Cell diversification in heterotrophic flagellates. *In* Patterson, D. & Larsen, J. [Eds.] *The Biology of Free Living Heterotrophic Flagellates.* Oxford University Press, New York, pp. 113–31.

Cavalier-Smith, T. 1998. A revised six-kingdom system of life. *Biol. Rev.* 73:203–66.

Cavalier-Smith, T. 1999. Principles of protein and lipid targeting in secondary symbiogenesis: euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. *J. Eukaryot. Microbiol.* 46:347–66.

Cavalier-Smith, T. 2002. Genomic reduction and evolution of novel membranes and protein-targeting machinery in eukaryote-eukaryote chimaeras (meta-algae). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358:109–34.

Cavalier-Smith, T. 2003. Protist phylogeny and the high level classification of Protozoa. *Eur. J. Protistol.* 39:338–48.

Cavalier-Smith, T. 2004. Only six kingdoms of life. *Proc. R. Soc. Lond. B Biol. Sci.* 271:1251–62.

Cavalier-Smith, T. & Chao, E. 1996. 18S rRNA sequence of *Heterosigma carterae* (Raphidophyceae), and the phylogeny of heterokont algae (Ochrophyta). *Phycologia* 35:500–10.

Chesnick, J. M., Kooistra, W. H. C. F., Wellbrock, U. & Medlin, L. K. 1997. Ribosomal RNA analysis indicates a benthic pennate diatom ancestry for the endosymbionts of the dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrhophyta). *J. Eukaryot. Microbiol.* 44:314–20.

Christensen, T. 1962. II. Systematisk Botanik, 2. Alger. *In* Bocher, T., Lange, M. & Sorensen, T. [Eds.] *Botanik.* Munksgaard, Copenhagen, pp. 1–178.

Christensen, T. 1989. The Chromophyta, past and present. *In* Green, J. C., Leadbeater, B. S. C. & Diver, W. [Eds.] *The Chromophyte Algae: Problems and Perspectives.* Clarendon Press, Oxford, UK, pp. 1–12.

Delwiche, C. F. 1999. Tracing the thread of plastid diversity through the tapestry of life. *Am. Nat.* 154:S164–77.

Delwiche, C. F., Kuhsel, M. & Palmer, J. D. 1995. Phylogenetic analysis of *tufA* sequences indicates a cyanobacterial origin of all plastids. *Mol. Phylogenet. Evol.* 4:110–28.

Delwiche, C. F. & Palmer, J. D. 1996. Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. *Mol. Biol. Evol.* 13:873–82.

Dodge, J. D. 1975. A survey of chloroplast ultrastructure in the dinophyceae. *Phycologia* 14:253–63.

van Dooren, G. G., Schwartzbach, S. D., Osafune, T. & McFadden, G. I. 2001. Translocation of proteins across the multiple membranes of complex plastids. *Biochim. Biophys. Acta* 1541:34–53.

Douglas, S., Zauner, S., Fraunholz, M., Beaton, M., Penny, S., Deng, L.-T., Wu, X., Reith, M., Cavalier-Smith, T. & Maier, U.-G. 2001. The highly reduced genome of an enslaved algal nucleus. *Nature* 410:1091–6.

Durnford, D., Deane, J., Tan, S., McFadden, G. I., Gantt, E. & Green, B. 1999. A phylogenetic assessment of the eukaryotic light-harvesting antenna protein, with implications for plastid evolution. *J. Mol. Evol.* 48:59–68.

Eisen, J. A., Coyne, R. S., Wu, M., Wu, D., Thiagarajan, M., Wortman, J. R., Badger, J. H., et al. 2006. Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. *PLoS Biol.* 4:286.

Fast, N. M., Kissinger, J. C., Roos, D. S. & Keeling, P. J. 2001. Nuclear-encoded, plastid-targeted genes suggest a single

common origin for apicomplexan and dinoflagellate plastids. *Mol. Biol. Evol.* 18:418–26.

Fast, N. M., Xue, L., Bingham, S. & Keeling, P. J. 2002. Re-examining alveolate evolution using multiple protein molecular phylogenies. *J. Eukaryot. Microbiol.* 49:30–7.

Foth, B. J. & McFadden, G. I. 2003. The apicoplast: a plastid in *Plasmodium falciparum* and other apicomplexan parasites. *Int. Rev. Cytol.* 224:57–110.

Gibbs, S. 1978. The chloroplasts of *Euglena* may have evolved from symbiotic green algae. *Can. J. Bot.* 56:2883–9.

Gibbs, S. 1981a. The chloroplast endoplasmic reticulum: structure, function, and evolutionary significance. *Int. Rev. Cytol.* 72: 49–99.

Gibbs, S. 1981b. The chloroplast of some algal groups may have evolved from endosymbiotic eukaryotic algae. *Ann. N. Y. Acad. Sci.* 361:193–208.

Guillot, M. & Gibbs, S. 1980a. Evidence that the chloroplast and nucleomorph of cryptomonads are remnants of a eukaryotic symbiont. *J. Cell Biol.* 87:186.

Guillot, M. & Gibbs, S. 1980b. The cryptomonad nucleomorph: its ultrastructure and evolutionary significance. *J. Phycol.* 16:558–68.

Hackett, J. D., Anderson, D., Erdner, D. & Bhattacharya, D. 2004a. Dinoflagellates: a remarkable evolutionary experiment. *Am. J. Bot.* 91:1523–34.

Hackett, J. D., Yoon, H. S., Li, S., Reyes-Prieto, A., Rummele, S. E. & Bhattacharya, D. 2007. Phylogenomic analysis supports the monophyly of cryptophytes and haptophytes and the association of 'Rhizaria' with chromalveolates. *Mol. Biol. Evol.* 24:1702–13.

Hackett, J. D., Yoon, H. S., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Nosenko, T. & Bhattacharya, D. 2004b. Migration of the plastid genome to the nucleus in a peridinin dinoflagellate. *Curr. Biol.* 14:213–8.

Hagopian, J., Reis, M., Kitajima, J., Bhattacharya, D. & Oliveira, M. 2004. Comparative analysis of the complete plastid genome sequence of the red alga *Gracilaria tenuistipitata* var. *liui* provides insights into the evolution of rhodoplasts and their relationship to other plastids. *J. Mol. Evol.* 59:464–77.

Harper, J. T. & Keeling, P. J. 2003. Nucleus-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicates a single origin for chromalveolate plastids. *Mol. Biol. Evol.* 20:1730–5.

Harper, J. T., Waanders, E. & Keeling, P. J. 2005. On the monophyly of chromoalveolates using a six-protein phylogeny of eukaryotes. *Int. J. Syst. Evol. Microbiol.* 55:487–96.

Hiller, R. 2001. Empty minicircles and *petB/atpA* and *psbD/psbE* genes in tandem in *Amphidinium carterae* plastid DNA. *FEBS Lett.* 505:449–52.

Iida, K., Takishita, K., Ohshima, K. & Inagaki, Y. 2007. Assessing the monophyly of chlorophyll-*c* containing plastids by multi-gene phylogenies under the unlinked model conditions. *Mol. Phylogenet. Evol.* 45:227–38.

Imanian, B., Carpenter, K. J. & Keeling, P. J. 2007. Mitochondrial genome of a tertiary endosymbiont retains genes for electron transport proteins. *J. Eukaryot. Microbiol.* 54:146–53.

Inagaki, Y. & Roger, A. J. 2006. Phylogenetic estimation under codon models can be biased by codon usage heterogeneity. *Mol. Phylogenet. Evol.* 40:428–34.

Inagaki, Y., Simpson, A., Dacks, J. & Roger, A. J. 2004. Phylogenetic artifacts can be caused by leucine, serine, and arginine codon usage heterogeneity: dinoflagellate plastid origins as a case study. *Syst. Biol.* 53:582–93.

Ishida, K. I. & Green, B. R. 2002. Second- and third-hand chloroplasts in dinoflagellates: phylogeny of oxygen-evolving enhancer1 (*PsbO*) protein reveals replacement of a nuclear-encoded plastid gene by that of a haptophyte tertiary endosymbiont. *Proc. Natl. Acad. Sci. U. S. A.* 99:9294–9.

Jarvis, P. & Robinson, C. 2004. Mechanisms of protein import and routing in chloroplasts. *Curr. Biol.* 14:1064–77.

Karpov, S., Sogin, M. L. & Silberman, J. 2001. Rootlet homology, taxonomy, and phylogeny of bicosoecids based on 18S rRNA gene sequences. *Protistology* 2:34–47.

Keeling, P. J. 2004. Diversity and evolutionary history of plastids and their hosts. *Am. J. Bot.* 91:1481–93.

Keeling, P. J., Burger, G., Durnford, D. G., Lang, B. F., Lee, R., Pearlman, R., Roger, A. J. & Gray, M. W. 2005. The tree of eukaryotes. *Trends Ecol. Evol.* 20:670–6.

Khan, H., Parks, N., Kozera, C., Curtis, B. A., Parsons, B., Bowman, S. & Archibald, J. M. 2007. Plastid genome sequence of the cryptophyte alga *Rhodomonas salina* CCMP1319: lateral transfer of putative DNA replication machinery and a test of chromist plastid phylogeny. *Mol. Biol. Evol.* 24:1832–42.

Kies, L. 1974. Electron microscopical investigations on *Paulinella chromatophora* Lauterborn, a thecamoeba containing blue-green endosymbionts (Cyanelles) (author's transl.). *Protoplasma* 80:69–89.

Köhler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R., Palmer, J. D. & Roos, D. S. 1997. A plastid of probable green algal origin in apicomplexan parasites. *Science* 275:1485–9.

Kroth, P. 2002. Protein transport into secondary plastids and the evolution of primary and secondary plastids. *Int. Rev. Cytol.* 221:191–255.

Li, S., Nosenko, T., Hackett, J. D. & Bhattacharya, D. 2006. Phylogenomic analysis identifies red algal genes of endosymbiotic origin in the chromalveolates. *Mol. Biol. Evol.* 23:663–74.

Marchant, H. & Thomsen, H. A. 1994. Haptophytes in polar waters. *In* Green, J. & Leadbeater, B. S. C. [Eds.] *The Haptophyte Algae.* Clarendon Press, Oxford, UK, pp. 209–28.

Margulis, L. 1970. *Origin of Eucaryotic Cells.* Yale University Press, New Haven, Connecticut, 349 pp.

Marin, B., Klinberg, M. & Melkonian, M. 1998. Phylogenetic relationships among the Cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. *Protist* 149:265–76.

Marin, B., Nowack, E. & Melkonian, M. 2005. A plastid in the making: evidence for a second primary endosymbiosis. *Protist* 156:425–32.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, H. & Penny, D. 2002. Evolutionary analysis of *Arabidopsis*, cyanobacterial and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 99:12246–51.

Martin, W., Stoebe, B., Goremykin, V., Hansmann, S., Hasegawa, M. & Kowallik, K. V. 1998. Gene transfer to the nucleus and the evolution of chloroplasts. *Nature* 393:162–5.

McFadden, G. I., Gilson, P. & Hill, D. 1994. *Goniomonas*: rRNA sequences indicate that this phagotrophic flagellate is a close relative of the host component of cryptomonads. *Eur. J. Phycol.* 29:29–32.

Medlin, L. K., Kooistra, W. H. C. F., Potter, D., Saunders, G. W. & Andersen, R. A. 1997. Phylogenetic relationships of the 'golden algae' (haptophytes, heterokonts, chromophytes) and their plastids. *Plant Syst. Evol.* 11:S187–219.

Moreira, D., Le Guyader, H. & Philippe, H. 2000. The origin of red algae and the evolution of chloroplasts. *Nature* 405:69–72.

Morse, D., Salois, P., Markovic, P. & Hastings, J. 1995. A nuclear-encoded form II Rubisco in dinoflagellates. *Science* 268:1622–4.

Nelson, M. J., Dang, Y., Filek, E., Zhang, Z., Yu, V. W., Ishida, K. & Green, B. R. 2007. Identification and transcription of transfer RNA genes in dinoflagellate plastid minicircles. *Gene* 392:291–8.

Nikolaev, S., Berney, C., Fahrni, J., Bolivar, I., Polet, S., Mylnikov, A., Aleshin, V., Petrov, N. & Pawlowski, J. 2004. The twilight of Heliozoa and rise of Rhizaria, an emerging supergroup of amoeboid eukaryotes. *Proc. Natl. Acad. Sci. U. S. A.* 101:8066–71.

Nosenko, T., Lidie, K., Van Dolah, F., Lindquist, E., Cheng, J. & Bhattacharya, D. 2006. Chimeric plastid proteome in the Florida "red tide" dinoflagellate *Karenia brevis. Mol. Biol. Evol.* 23:2026–38.

Not, F., Valentin, K., Romari, K., Lovejoy, C., Massana, R., Tobe, K., Vaulot, D. & Medlin, L. K. 2007. Picobiliphytes: a marine

picoplanktonic algal group with unknown affinities to other eukaryotes. *Science* 315:253–5.

Nozaki, H. 2005. A new scenario of plastid evolution: plastid primary endosymbiosis before the divergence of the ''Plantae,'' emended. *J. Plant. Res.* 118:247–55.

Nozaki, H., Iseki, M., Hasegawa, M., Misawa, K., Nakada, T., Sasaki, N. & Watanabe, M. 2007. Phylogeny of primary photosynthetic eukaryotes as deduced from slowly evolving nuclear genes. *Mol. Biol. Evol.* 24:1592–5.

Nozaki, H., Matsuzaki, M., Takahara, M., Misumi, O., Kuroiwa, H., Hasegawa, M., Shin-i, T., Kohara, Y., Ogasawara, N. & Kuroiwa, T. 2003. The phylogenetic position of red algae revealed by multiple nuclear genes from mitochondria-containing eukaryotes and an alternative hypothesis on the origin of plastids. *J. Mol. Evol.* 56:485–97.

Okamoto, N. & Inouye, I. 2005a. A secondary symbiosis in progress? *Science* 310:287.

Okamoto, N. & Inouye, I. 2005b. The katablepharids are a distant sister group of the Cryptophyta: a proposal for Katablepharidophyta divisio nova/Kathablepharida phylum novum based on SSU rDNA and beta-tubulin phylogeny. *Protist* 156:163–79.

Okamoto, N. & Inouye, I. 2006. *Hatena arenicola* gen. et sp. nov., a katablepharid undergoing probable plastid acquisition. *Protist* 157:401–19.

Oliveira, M. & Bhattacharya, D. 2000. Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. *Am. J. Bot.* 87:482–92.

Palmer, J. D. 2003. The symbiotic birth and spread of plastids; how many times and whodunit? *J. Phycol.* 39:4–11.

Palmer, J. D. & Delwiche, C. F. 1996. Second-hand chloroplasts and the case of the disappearing nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 93:7432–5.

Patron, N. J., Inagaki, Y. & Keeling, P. J. 2007. Multiple gene phylogenies support the monophyly of cryptomonad and haptophyte host lineages. *Curr. Biol.* 17:887–91.

Patron, N., Rogers, M. & Keeling, P. J. 2004. Gene replacement of fructose 1,6 bisphosphate aldolase supports the hypothesis of a single photosynthetic ancestor of chromalveolates. *Eukaryot. Cell* 3:1169–75.

Patron, N. J., Waller, R. F. & Keeling, P. J. 2006. A tertiary plastid uses genes from two endosymbionts. *J. Mol. Biol.* 357:1373–82.

Patterson, D. 1999. The diversity of eukaryotes. *Am. Nat.* 65:S96–124.

Petersen, J., Teich, R., Brinkmann, H. & Cerff, R. 2006. A ''green'' phosphoribulokinase incomplex algae with red plastids: evidence for a single secondary endosymbiosis leading to haptophytes, cryptophytes, heterokonts, and dinoflagellates. *J. Mol. Evol.* 62:143–57.

Radhamony, R. & Theg, S. 2006. Evidence for an ER to Golgi to chloroplast protein transport pathway. *Trends Cell Biol.* 16:385–7.

Ralph, S. A., Foth, B. J., Hall, N. & McFadden, G. I. 2004. Evolutionary pressures on apicoplast transit peptides. *Mol. Biol. Evol.* 21:2183–94.

Reumann, S., Davila-Aponte, J. & Keegstra, K. 1999. The evolutionary origin of the protein-translocating channel of chloroplastic envelope membranes: identification of a cyanobacterial homolog. *Proc. Natl. Acad. Sci. U. S. A.* 96:784–9.

Reyes-Prieto, A. & Bhattacharya, D. 2007. Phylogeny of Calvin cycle enzymes supports Plantae monophyly. *Mol. Phylogenet. Evol.* 45:384–91.

Reyes-Prieto, A., Hackett, J. D., Soares, M. B., Bonaldo, M. F. & Bhattacharya, D. 2006. Cyanobacterial contribution to algal nuclear genomes is primarily limited to plastid functions. *Curr. Biol.* 16:2320–5.

Reyes-Prieto, A., Weber, A. P. & Bhattacharya, D. 2007. The origin and establishment of the plastid in algae and plants. *Annu. Rev. Genet.* 41:147–68.

Rice, D. & Palmer, J. D. 2006. An exceptional horizontal gene transfer in plastids: gene replacement by a distant bacterial paralog and evidence that haptophyte and cryptophyte plastids are sisters. *BMC Biol.* 4:31.

Robertson, D. & Tartar, A. 2006. Evolution of glutamine synthetase in heterokonts: evidence for endosymbiotic gene transfer and the early evolution of photosynthesis. *Mol. Biol. Evol.* 23:1048–55.

Rodriguez-Ezpeleta, N., Brinkmann, H., Burey, S., Roure, B., Burger, G., Loffelhardt, W., Bohnert, H., Phillipe, H. & Lang, B. F. 2005. Monophyly of primary photosynthetic eukaryotes: green plants, red algae, and glaucophytes. *Curr. Biol.* 15:1325–30.

Rogers, M. B., Gilson, P. R., Su, V., McFadden, G. I. & Keeling, P. J. 2007. The complete chloroplast genome of the chlorarachniophyte *Bigelowiella natans*: evidence for independent origins of chlorarachniophyte and euglenid secondary endosymbionts. *Mol. Biol. Evol.* 24:54–62.

Saldarriaga, J. F., Taylor, F., Keeling, P. J. & Cavalier-Smith, T. 2001. Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacements. *J. Mol. Evol.* 53:204–13.

Sanchez-Puerta, M. V., Bachvaroff, T. R. & Delwiche, C. F. 2004. The complete mitochondrial genome sequence of the haptophyte *Emiliania huxleyi* and its relation to heterokonts. *DNA Res.* 11:1–10.

Sanchez-Puerta, M. V., Bachvaroff, T. R. & Delwiche, C. F. 2005. The complete plastid genome sequence of the haptophyte *Emiliania huxleyi*: a comparison to other plastid genomes. *DNA Res.* 12:151–6.

Sanchez-Puerta, M. V., Bachvaroff, T. R. & Delwiche, C. F. 2007a. Sorting wheat from chaff in multi-gene analyses of chlorophyll-c containing plastids. *Mol. Phylogenet. Evol.* 44:885–97.

Sanchez-Puerta, M. V., Lippmeier, J. C., Apt, K. E. & Delwiche, C. F. 2007b. Plastid genes in a non-photosynthetic dinoflagellate. *Protist* 158:105–17.

Schnepf, E. & Elbrächter, M. 1999. Dinophyte chloroplasts and phylogeny – a review. *Grana* 38:81–97.

Shalchian-Tabrizi, K., Eikrem, W., Klaveness, D., Vaulot, D., Minge, M. A., LeGall, F., Romari, K., et al. 2006a. *Telonemia*, a new protist phylum with affinity to chromist lineages. *Proc. R. Soc. Lond. B Biol. Sci.* 273:1833–42.

Shalchian-Tabrizi, K., Minge, M., Cavalier-Smith, T., Nedreklepp, J., Klaveness, D. & Jakobsen, K. S. 2006b. Combined heat shock protein 90 and ribosomal RNA sequence phylogeny supports multiple replacements of dinoflagellate plastids. *J. Eukaryot. Microbiol.* 53:217–24.

Shalchian-Tabrizi, K., Skanseng, M., Ronquist, F., Klaveness, D., Bachvaroff, T. R., Delwiche, C. F., Botnen, A., Tengs, T. & Jakobsen, K. S. 2006c. Heterotachy processes in rhodophyte-derived second-hand plastid genes: implications for addressing the origin and evolution of dinoflagellate plastids. *Mol. Biol. Evol.* 23:1504–15.

Simpson, A. & Patterson, D. J. 2006. Current perspectives on high-level groupings of protists. *In* Katz, L. A. & Bhattacharya, D. [Eds.] *Genomics and Evolution of Microbial Eukaryotes*. Oxford University Press, New York, pp. 7–30.

Simpson, A. & Roger, A. J. 2004. The real kingdoms of eukaryotes. *Curr. Biol.* 14:693–6.

Stechmann, A. & Cavalier-Smith, T. 2003. Phylogenetic analysis of eukaryotes using heat-shock protein Hsp90. *J. Mol. Evol.* 57:408–19.

Steiner, J. & Loffelhardt, W. 2002. Protein import into cyanelles. *Trends Plant Sci.* 7:72–7.

Stelter, K., El-Sayed, N. M. & Seeber, F. 2007. The expression of a plant-type ferredoxin redox system provides molecular evidence for a plastid in the early dinoflagellate *Perkinsus marinus*. *Protist* 158:119–30.

Stibitz, T., Keeling, P. J. & Bhattacharya, D. 2000. Symbiotic origin of a novel actin gene in the cryptophyte *Pyrenomonas helgolandii*. *Mol. Biol. Evol.* 17:1731–8.

Sun, Q., Emanuelsson, O. & van Wijk, K. 2004. Analysis of curated and predicted plastid subproteomes of *Arabidopsis*. Subcellular compartmentalization leads to distinctive proteome properties. *Plant Physiol.* 135:723–34.

Teich, R., Zauner, S., Baurain, D., Brinkmann, H. & Petersen, J. 2007. Origin and distribution of Calvin cycle fructose and sedoheptulose bisphosphatases in Plantae and complex algae:

a secondary origin of complex red plastids and subsequent propagation via tertiary endosymbioses. *Protist* 158:263–76.

Teles-Grilo, M., Tato-Costa, J., Duarte, S., Maia, A., Casal, G. & Azevedo, C. 2007. Is there a plastid in *Perkinsus atlanticus* (Phylum Perkinzoa)? *Eur. J. Protistol.* 43:163–7.

Tengs, T., Bowers, H. A., Ziman, A. P., Stoecker, D. K. & Oldach, D. W. 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. *Mol. Ecol.* 10:515–23.

Tengs, T., Dahlberg, O. J., Shalchian-Tabrizi, K., Klaveness, D., Rudi, K., Delwiche, C. F. & Jakobsen, K. S. 2000. Phylogenetic analyses indicate that the 19′ hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17:718–29.

Tyler, B. M., Tripathy, S., Zhang, X., Dehal, P., Jiang, R. H. Y., Aerts, A., Arredondo, F. D., et al. 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. *Science* 313:1261–6.

Van de Peer, Y. & De Watcher, R. 1997. Evolutionary relationships among eukaryotic crown taxa taking into account site-to-site variation in 18S rRNA. *J. Mol. Evol.* 45:619–30.

Villarejo, A., Burén, S., Larsson, S., Déjardin, A., Monné, M., Rudhe, C., Karlsson, J., et al. 2005. Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. *Nat. Cell Biol.* 7:1224–31.

Waller, R. F. & Keeling, P. J. 2006. Alveolate and chlorophycean mitochondrial *cox2* genes split twice independently. *Gene* 383:33–7.

Waller, R., Patron, N. & Keeling, P. J. 2006. Phylogenetic history of plastid-targeted proteins in the peridinin-containing dinoflagellate *Heterocapsa triquetra*. *Int. J. Syst. Evol. Microbiol.* 56:1439–47.

Weber, A., Linka, M. & Bhattacharya, D. 2006. Single, ancient origin of a plastid metabolite translocator family in Plantae from an endomembrane-derived ancestor. *Eukaryot. Cell* 5:609–12.

Yoon, H. S., Hackett, J. D., Ciniglia, C., Pinto, G. & Bhattacharya, D. 2004. A molecular timeline for the origin of photosynthetic eukaryotes. *Mol. Biol. Evol.* 21:809–18.

Yoon, H. S., Hackett, J. D., Pinto, G. & Bhattacharya, D. 2002. The single, ancient origin of chromist plastids. *Proc. Natl. Acad. Sci. U. S. A.* 99:15507–12.

Yoon, H. S., Hackett, J. D., Van Dolah, F., Nosenko, T., Lidie, K. & Bhattacharya, D. 2005. Tertiary endosymbiosis driven genome evolution in dinoflagellate algae. *Mol. Biol. Evol.* 22:1299–308.

Yoon, H. S., Reyes-Prieto, A., Melkonian, M. & Bhattacharya, D. 2006. Minimal plastid genome evolution in the *Paulinella* endosymbiont. *Curr. Biol.* 16:R670–2.

Zhang, Z., Green, B. R. & Cavalier-Smith, T. 1999. Single gene circles in dinoflagellate chloroplast genomes. *Nature* 400:155–9.

Zhang, Z., Green, B. R. & Cavalier-Smith, T. 2000. Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. *J. Mol. Evol.* 51:26–40.