

Mini review

The origin and spread of eukaryotic photosynthesis: evolving views in light of genomics

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Abstract

Plants and algae acquired photosynthesis through the assimilation of a prokaryotic endosymbiont related to the ancestors of modern-day cyanobacteria. This landmark event, known as the primary endosymbiotic origin of plastids, is generally thought to have occurred only once during the history of eukaryotes and to have given rise to the plastids of green algae, land plants, red algae and glaucophyte algae through vertical evolution. Plastids have also spread horizontally across the tree of eukaryotes by "secondary" endosymbioses involving heterotrophic host eukaryotes and both green and red algal endosymbionts. Here I provide an overview of current research in the area of plastid evolution, focusing on the latest advances in the field of algal comparative genomics. Recent genome-scale analyses of both photosynthetic and non-photosynthetic eukaryotes have provided fresh new insight into the pattern and process of secondary endosymbiosis, although it is still not possible to discern with confidence the number of endosymbiotic events that gave rise to the known spectrum of eukaryotic phototrophs. In fact, with more genomic data has come the intriguing possibility that the nuclear genomes of some secondary plastid-containing algae are a mosaic of genes derived from multiple endosymbioses, adding yet another layer of complexity to the convoluted evolutionary history of these fascinating organisms.

Keywords: algae; chloroplasts; endosymbiosis; evolution; gene transfer; genomics; plastid.

Introduction

The last decade of research in comparative genomics has transformed our understanding of eukaryotic evolution. Nowhere have these advances been more significant than for photosynthetic eukaryotes and their plastids (chloroplasts). Hundreds of plastid genomes have now been sequenced, and as a result of the huge technological advances that made it possible to sequence the human genome (Lander et al. 2001, Venter et al. 2001), the number of completely sequenced nucle-

ar genomes has increased dramatically and will continue to do so for the foreseeable future. As of January 2008, 65 nuclear genomes have already been published and >400 complete genome sequencing projects are underway (Liolios et al. 2008), many of them targeting photosynthetic organisms. The first eukaryotic phototroph to have its nuclear genome completely sequenced was the land plant *Arabidopsis thaliana* Heynhold (The *Arabidopsis* Genome Initiative, 2000), an achievement of considerable benefit to the scores of researchers focused on this model organism. The list has since grown to include the black cottonwood *Populus trichocarpa* Torr. et A. Gray (Tuskan et al. 2006), the moss *Physcomyrella patens* (Hedw.) Bruch et Schimp. (Rensing et al. 2008) and a variety of unicellular algae, including *Chlamydomonas reinhardtii* P.A. Dangeard (Merchant et al. 2007), *Ostreococcus tauri* C. Courties et M.-J. Chrétiennot-Dinet (Derelle et al. 2006) and *Cyanidioschyzon merolae* P. De Luca, R. Taddei et L. Varano (Matsuzaki et al. 2004).

While analyses of the plastid and nuclear genomes of these and many other plants and algae have made it possible to sketch a basic picture of plastid diversity and evolution (Delwiche et al. 2004), many important questions about the spread of photosynthesis in eukaryotes remain unanswered. Modern-day plastid-containing organisms exhibit a tremendous amount of genetic, biochemical and cell biological diversity, in large part due to the fact that on multiple occasions plastids have been passed between distantly related eukaryotic hosts. In this article I present an overview of current hypotheses about the origin and evolution of photosynthesis in eukaryotes from the perspective of comparative genomics. Significant progress is being made, though it will be some time before a consensus on the most fundamental aspects of secondary plastid evolution is reached.

The birth and spread of plastids

The endosymbiotic origin of plastids is now the material of textbooks. A large and diverse body of ultrastructural, biochemical and molecular data has shown beyond all reasonable doubt that plastids – the light-harvesting organelles of plants and algae – are the descendants of once free-living cyanobacteria that took up permanent residence inside a eukaryotic host (Gray and Spencer 1996, Graham and Wilcox 2000, Reyes-Prieto et al. 2007; Figure 1A and B), probably more than a billion years ago (Yoon et al. 2004). During the transition from endosymbiont to organelle, most of the cyanobacterial genome was lost or transferred to the host nuclear genome (Martin et al. 1998, Timmis et al. 2004) and the host cell evolved a protein import apparatus for targeting nucleus-

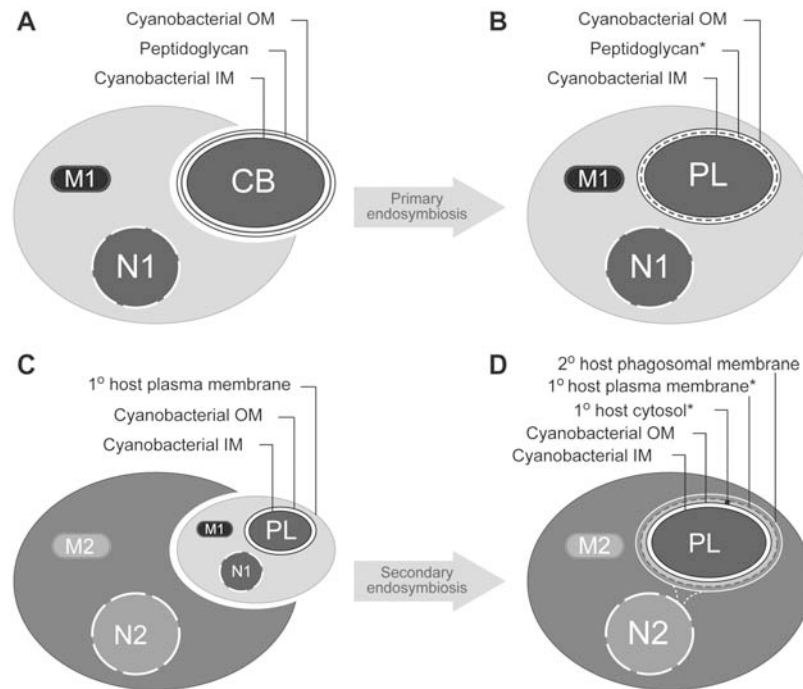


Figure 1 Primary and secondary endosymbiotic origin of plastids.

(A) Primary endosymbiosis. Diagram depicts the engulfment of a photosynthetic cyanobacterium by a non-photosynthetic, heterotrophic eukaryote. (B) Primary plastid. The basic features of a primary plastid-containing eukaryote with two plastid membranes and, in the case of glaucophyte algae, retention of a peptidoglycan layer. (C) Secondary endosymbiosis. This process involves the uptake of a primary plastid-containing alga by a non-photosynthetic host eukaryote. (D) Secondary plastid-containing eukaryote with 3 or 4 plastid membranes; the outermost membrane is thought to be derived from the phagosomal membrane of the secondary eukaryotic host cell. In the three-membrane secondary plastids of euglenids and peridinin-containing dinoflagellates, the plasma membrane of the primary host eukaryote is believed to have been lost. In cryptophyte and chlorarachniophyte algae, the space between the inner and outer pairs of plastid membranes still houses the primary host cell nucleus, referred to as the nucleomorph. In cryptophytes, haptophytes and stramenopiles the outermost plastid membrane is contiguous with the nuclear envelope. CB, cyanobacterium; PL, plastid; OM, outer membrane; IM, inner membrane; N, nucleus; M, mitochondrion.

encoded, endosymbiont-derived gene products back to their compartment of origin (McFadden 1999, Soll and Schleiff 2004). Evidence for a single “primary” endosymbiotic origin of all plastids comes from consideration of plastid genome structure, content and phylogeny (e.g., Stoebe and Kowallik 1999, Turner et al. 1999, Howe et al. 2003), as well as the nature of the plastid protein import apparatus in diverse algal species (McFadden 1999, McFadden and van Dooren 2004). Three modern-day eukaryotic lineages, red algae, glaucophyte algae and green algae (and their land plant descendants), possess double membrane-bound plastids (Table 1 and Figure 1) that are generally believed to have evolved vertically since the original primary endosymbiosis (Moreira et al. 2000, Palmer 2003, Rodriguez-Ezpeleta et al. 2005) (Table 1), although as will be discussed below, this is an open question.

Beyond red, green and glaucophyte algae, additional endosymbioses must be invoked to explain the presence of plastids in all other photosynthetic eukaryotes. Considered within an evolutionary framework, algae are “patchily” distributed across the eukaryotic tree (Keeling 2004, Keeling et al. 2005) and while canonical plastids probably evolved only once, it is clear that on more than one occasion they have moved from primary plastid-bearing eukaryotes to heterotrophic eukaryotic hosts by “secondary” endosymbiosis (Delwiche 1999, McFadden

2001, Archibald and Keeling 2005). This process (Figure 1C and D) results in a plastid with additional membranes (usually 3 or 4) and in some species, the retention of the “smoking gun” of secondary endosymbiosis, the remnant nucleus of the engulfed algal cell (Gilson 2001, Archibald 2007). As is the case in primary endosymbiosis, the integration of secondary endosymbiont and host involves massive amounts of intracellular gene transfer – in this case with both the plastid and nuclear genomes as donors – and additional evolutionary tinkering to allow the products of transferred genes to be re-imported back to the organelle (McFadden 1999, Ishida 2005, Gould et al. 2006). The situation is even more complex in dinoflagellate algae, some of which have replaced their secondary plastids with those of another secondary plastid-containing alga in what is referred to as “tertiary” endosymbiosis (Chesnick et al. 1996, Tengs et al. 2000, Hackett et al. 2004). In essence, secondary and tertiary plastid-containing algae are the microscopic equivalent of Russian nesting dolls and, as a result, are exceedingly complex at the genetic level. In addition to the dinoflagellates, which are notorious for their ability to form devastating algal blooms, examples of secondary plastid-containing lineages are the cryptophytes, haptophytes [e.g., the ecologically significant coccolithophore *Emiliania huxleyi* (Lohmann) W.H. Hay et H. Mohler], stramenopiles (e.g., giant kelp and diatoms), euglenids (e.g.,

Table 1 Characteristics of primary and secondary plastids.¹

Lineage	Putative origin	Pigmentation	Membranes	Cellular location
Glaucophytes	1°	Chl <i>a</i> phycobiliproteins	2 ²	Cytosol
Red algae	1°	Chl <i>a</i> phycobiliproteins	2	Cytosol
Green algae+land plants	1°	Chl <i>a+b</i> zeaxanthin	2	Cytosol
Cryptophytes ³	2° (red)	Chl <i>a+c</i> phycobiliproteins Alloxanthin	4	Lumen of host RER ⁴
Haptophytes	2° (red)	Chl <i>a+c</i> fucoxanthin	4	Lumen of host RER ⁴
Stramenopiles	2° (red)	Chl <i>a+c</i> fucoxanthin	4	Lumen of host RER ⁴
Dinoflagellates ⁵	2° (red)	Chl <i>a+c</i> peridinin	3 ⁵	Cytosol ⁶
Apicomplexans	2° (red)	None (non-photosynthetic)	4	Cytosol ⁶
Euglenids	2° (green)	Chl <i>a+b</i> diadinoxanthin	3	Cytosol ⁶
Chlorarachniophytes ³	2° (green)	Chl <i>a+b</i> violaxanthin	4	Cytosol ⁶

¹ Data taken primarily from Graham and Wilcox (2000), Larkum et al. (2007) and references therein.

² The glaucophyte plastid possesses a layer of peptidoglycan between its inner and outer membranes, as presumably existed in the cyanobacterial progenitor of plastids.

³ The cryptophytes and chlorarachniophytes are unusual in that the nucleus of their red and green algal endosymbionts persists in highly degenerate form called a nucleomorph. In both lineages, the nucleomorph is located in the space between the inner and outer pairs of plastid membranes, which is derived from the remnant cytosol of the primary algal host cell and sometimes referred to as the periplastid space.

⁴ The outermost plastid membrane in cryptophytes, haptophytes and most stramenopiles (heterokonts) is continuous with the outer membrane of the secondary host nucleus. The plastid thus physically resides within the lumen of the rough endoplasmic reticulum, an arrangement sometimes referred to as the chloroplast ER.

⁵ Only ~50% of known dinoflagellate species are photosynthetic. Those that are usually possess a peridinin-pigmented plastid, although some dinoflagellates have also replaced this organelle with plastids acquired from haptophytes and diatoms (tertiary endosymbiosis) or green algae (serial secondary endosymbiosis). Plastid membrane number varies depending on the plastid type. Refer to Hackett et al. (2004) for a comprehensive overview of the intricacies of dinoflagellate plastid evolution.

⁶ The plastids in these lineages are not physically connected to the host cell endoplasmic reticulum, although they are surrounded by additional membranes and thus enveloped by a region of endomembrane lumen of unknown origin.

Chl, chlorophyll; RER, rough endoplasmic reticulum.

Euglena gracilis Klebs) and chlorarachniophytes. These also include the apicomplexans, secondarily non-photosynthetic pathogens such as the malaria parasite *Plasmodium* that harbor a remnant plastid dubbed the “apicoplast” (McFadden and Waller 1997, Waller and McFadden 2005). Table 1 provides a summary of the main lineages of photosynthetic eukaryotes and the salient features of their plastids.

Endosymbiotic uncertainty

How does one determine whether the plastids of any two photosynthetic eukaryotes are of independent origin or the result of a single endosymbiotic event in their common ancestor? In the era of molecular phylogenetics, the acid test is to establish whether or not trees inferred from both plastid and host nuclear genes are congruent with one another and consistent with non-sequence-based data (e.g., ultrastructure, plastid pigmentation). The challenges associated with applying this seemingly straightforward methodology will not be elaborated upon here: interested readers are referred to a recent article by Larkum et al. (2007), which provides a lucid overview of the types of data that have been used to address the question of plastid monophyly or polyphyly and the assumptions inherent therein. Suffice it to say, determining the number of endosymbioses that have given rise to the known spectrum of plastid-bearing eukaryotes has proven to be a daunting task, with the various hypotheses evolving steadily in response to new data and analytical methods.

The view endorsed by most researchers in the field is that primary plastids evolved only once in a common ancestor shared by green algae (and plants), red algae and glaucophytes. Consistent with the above-mentioned criteria, phylogenies of plastid, mitochondrial and nuclear genes generally show these three lineages to form a monophyletic group (Moreira et al. 2000, Palmer 2003, Rodriguez-Ezpeleta et al. 2005, Reyes-Prieto et al. 2007), although it is important to note that the evolutionary signal present in some genes (e.g., *RPB1* and *EF2*) is enigmatic and has led some to question the notion of primary plastid monophyly (Stiller and Hall 1997, Stiller et al. 2001, Stiller 2007). In the case of secondary plastid-containing organisms, a minimum of two independent endosymbioses must be inferred to account for the lineages listed in Table 1. This is because all secondary plastids belong to one of two evolutionarily distinct subtypes, those that are derived from red algal endosymbionts and contain chlorophyll *a+c*, and those with chlorophyll *a+b*-containing plastids of green algal ancestry. The euglenids harbor plastids that belong to the latter category, as do the chlorarachniophyte algae. Cavalier-Smith (1999) proposed that the plastids in these two groups are the product of a single, ancient endosymbiosis involving a green alga in their common ancestor. However, the fact that euglenids and chlorarachniophytes belong to two different eukaryotic “supergroups”, the Excavata and Rhizaria, respectively (Keeling et al. 2005), and are nested within non-photosynthetic lineages, has led most researchers to doubt this hypothesis (e.g., Leander 2004). Recent phylogenetic investigation of the plastid genomes of euglenids and chlorarachniophytes (Rogers et al. 2007)

as well as the nucleus-encoded gene *psbO* (Takahashi et al. 2007) cast further doubt on this idea, suggesting that while both plastids are clearly green algal in origin, they are likely the product of independent secondary endosymbioses.

In the case of secondary plastids of red algal ancestry, the situation is much more complicated – and controversial. Hypotheses explaining the origin of red secondary plastids are of two basic sorts, those that attempt to minimize the number of endosymbiotic events and those that posit multiple independent assimilations of red algal endosymbionts. Both have their merits and shortcomings. The “chromalveolate hypothesis” of Cavalier-Smith (1999) posits a single engulfment of a red algal endosymbiont in the common ancestor of *all* lineages that currently harbor a red secondary plastid, these being the chromists (cryptophytes, haptophytes and many stramenopiles) and plastid-bearing alveolates (dinoflagellates and apicomplexans) (Table 1). The rationale for appealing to parsimony is that, at the genetic level, secondary endosymbiosis would seem to be exceedingly difficult, requiring (i) the transfer of thousands of genes from the primary endosymbiont nucleus to that of the secondary host (recall that most of these genes were already transferred once during the primary endosymbiotic origin of plastids), (ii) the evolution of a plastid protein import system, and (iii) the acquisition of coding sequence at the 5' end of each of these genes capable of producing an amino-terminal topogenic signal that will target the protein back to the plastid using the newly-evolved import apparatus.

While the chromalveolate hypothesis is parsimonious from the perspective of endosymbiont integration, its most troubling corollary is that it demands numerous instances of plastid loss and/or loss of photosynthesis. For example, stramenopiles such as oomycetes, labyrinthulids and thraustochytrids are non-photosynthetic and do not (as far as we know) possess a plastid or any remnant thereof. Yet under the chromalveolate hypothesis, these organisms evolved from plastid-bearing ancestors. The ciliates are a large and exclusively non-photosynthetic lineage belonging to the alveolates to which the same disturbing logic must be applied. Those uncomfortable with the idea of plastid loss endorse the alternative view, i.e., that endosymbiosis is, all things considered, relatively easy and that multiple independent endosymbioses (either secondary or tertiary) involving evolutionarily distinct host eukaryotes and different (although possibly closely related) endosymbionts is a better explanation for the distribution of secondary plastids (Bodyl 2005). Proponents of this view suggest that non-photosynthetic eukaryotes related to plastid-bearing chromalveolate taxa, such as the basal cryptophyte *Goniomonas*, many dinoflagellates and all known ciliates are primitively non-photosynthetic – they do not have a plastid today and their ancestors never had one.

As noted above, the dinoflagellates are unique in the diversity of plastid types they possess and have clearly undergone multiple endosymbioses, a fact that has been used to support the notion that endosymbiosis is common (Bodyl 2005). However, while these organisms are certainly the undisputed champions of plastid acquisi-

tion, it is significant that in cases such as the haptophyte plastid-containing dinoflagellate *Karlodinium micrum* (B. Leadbeater et J.D. Dodge) J. Larsen, the evidence suggests that these additional endosymbioses are plastid *replacements* and not *de novo* acquisitions (Patron et al. 2006, Shalchian-Tabrizi et al. 2006b). Plastid replacement is presumably much easier than evolving a secondary plastid “from scratch”, due to the pre-existence of a protein import apparatus and thousands of nucleus-encoded genes for plastid-targeted proteins.

How difficult is plastid loss? Critics of the chromalveolate hypothesis (e.g., Falkowski et al. 2004, Bodyl 2005) have argued that it is extremely difficult and may in fact be impossible, pointing to the fact that even highly derived intracellular parasites such as the apicomplexan *Plasmodium* still retain a plastid, which now has nothing to do with photosynthesis but serves as the site of isoprenoid and fatty acid biosynthesis (Waller and McFadden 2005). This is certainly the case, but recent data have shown convincingly that plastid loss can happen. The complete nuclear genome of the non-photosynthetic oomycete *Phytophthora* harbors scores of genes of red algal ancestry (Tyler et al. 2006) even though the organism does not possess a plastid, and genes of putative algal origin have been found in the genome of the apicomplexan parasite *Cryptosporidium* (Huang et al. 2004). Recently, molecular evidence for a plastid has been uncovered in the non-photosynthetic dinoflagellates *Cryptocodinium cohnii* Seligo (Sanchez-Puerta 2007) and *Oxyrrhis marina* Dujardin (Slamovits and Keeling 2008). A remnant plastid (Teles-Grilo et al. 2007) and plastid-type isoprenoid biosynthesis genes (Grauvogel and Petersen 2007) have also been identified in the oyster parasite *Perkinsus*, a “basal” dinoflagellate. The old adage “absence of evidence is not evidence for absence” certainly applies when it comes to remnant plastids.

Comparative genomics

So what do genome sequences have to say about the chromalveolate hypothesis? Early single-locus phylogenies of plastid-encoded genes from cryptophytes, haptophytes and stramenopiles, together with their homologs in red algae (Daugbjerg and Andersen 1997, Oliveira and Bhattacharya 2000, Müller et al. 2001), did not cluster chromist sequences as a monophyletic group and were interpreted as evidence for the independent endosymbiotic origin of their plastids. While nuclear small subunit ribosomal RNA (SSU rRNA) phylogenies pointed to the same conclusion (Bhattacharya et al. 1995), analysis of a five-gene plastid dataset produced a robust monophyletic chromist plastid clade and was heralded as evidence for “the single, ancient origin of chromist plastids” (Yoon et al. 2002). More recent analyses of larger concatenated plastid protein datasets (e.g., Khan et al. 2007, Rogers et al. 2007) have been less compelling: statistical support for chromist monophyly is generally weak when sophisticated phylogenetic methods are used (Iida et al. 2007, Khan et al. 2007), and inclusion of the divergent sequences of dinoflagellate algae raises

concerns about tree-reconstruction artifacts (Bachvaroff et al. 2005, Iida et al. 2007). Furthermore, the apicomplexans cannot be included in such analyses, as their remnant plastids have been stripped of all genes related to photosynthesis (and ciliates do not possess a plastid). In sum, while these results are consistent with chromist monophyly and a single origin for chlorophyll *c*-containing plastids (Table 1), they are inconclusive in that plastid phylogenies alone do not bear on the question of chromalveolate host monophyly. An exciting recent development has been the discovery of *Chromera velia* R.B. Moore, M. Oborník, J. Janou, T. Chrudimský, M. Vancová, D.H. Green, S.W. Wright, N.W. Davies, C.J.S. Bolch, K. Heimann, J. Iapeta, O. Hoegh-Guldberg, J.M. Logsdon et D.A. Carter, a free-living, chlorophyll *a*-containing phototroph that appears to be specifically related to apicomplexan parasites (Moore et al. 2008). As such, this organism could represent a photosynthetic “missing link” between dinoflagellates and apicomplexans and thus shed light on the nature of their common ancestor (Keeling 2008).

Initial analyses of nuclear genes provided tantalizing evidence in support of the chromalveolate hypothesis. Photosynthetic eukaryotes possess two evolutionarily distinct isoforms of the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one targeted to the plastid, the other servicing the host cytosol. Remarkably, the plastid-targeted enzyme in chromists, dinoflagellates and alveolates was found to be the product of a duplicate of the *cytosolic* version of the gene, not the canonical cyanobacterial homolog, as in primary plastid-containing algae (Fast et al. 2001, Harper and Keeling 2003). A similar situation exists for the fructose-1,6-bisphosphate aldolase (FBA) gene of chromalveolates (Patron et al. 2004), with the general conclusion being that such “endosymbiotic gene replacements” are rare and thus serve as discrete characters uniting the organisms that possess them, in this case, the chromalveolate taxa. The GAPDH and FBA results are potentially significant, but critics point out that while these genes are nucleus-encoded, they are still *plastid-associated* and could well have been inherited in a non-vertical fashion (Bodyl 2005), something for which GAPDH genes are notorious (Qian and Keeling 2001, Fagan and Hastings 2002, Takishita et al. 2003).

Concatenated gene phylogenies and the “supergroup shuffle”

An emerging theme in molecular systematics is the use of concatenated sequence datasets – genes or proteins are strung together and analyzed as a single entity in the hope of increasing phylogenetic signal and improving resolution of deep evolutionary divergences (Delsuc et al. 2005). As the nuclear genomes of chromalveolate taxa continue to be probed more deeply, such methodologies now make it possible to test the hypothesis of chromalveolate host monophyly once-and-for-all using large, multi-gene datasets of house-keeping genes that are entirely unrelated to the plastid and photosynthesis. The results have provided new – and completely unexpected

– insight into the evolutionary history of chromalveolate taxa and, more generally, the process of plastid gain and loss in eukaryotic evolution.

Initial attempts at assessing chromalveolate monophyly with concatenated nuclear genes suggested a relationship between stramenopiles and alveolates (Harper et al. 2005), and haptophytes and cryptophytes, though curiously, not between the three chromist lineages themselves (Harper et al. 2005). Three recent and independent studies have now upped the ante considerably. Analyzing a set of no fewer than 102 proteins, Patron et al. (2007) resolved a robust relationship between two of the three chromist lineages, cryptophytes and haptophytes, as did Burki et al. (2007), and the same result was also obtained by Hackett et al. (2007). Most unexpectedly, the latter two studies identified a specific evolutionary connection between members of the supergroup Rhizaria (to which the green algal secondary plastid-containing chlorarachniophytes belong) and the alveolates (Burki et al. 2007, Hackett et al. 2007), either specifically to stramenopiles or as sister to stramenopiles + alveolates. It is significant that, unlike the plastid gene phylogenies summarized above, these analyses include representatives from all main chromalveolate lineages, including dinoflagellates, ciliates and apicomplexans, and thus do not suffer from the nagging uncertainties so often associated with “missing data”.

How should we interpret these new observations? Regardless of one’s view on plastid evolution, the analyses represent a significant advance from the perspective of global eukaryotic systematics, as they suggest some sort of merger between two of the six increasingly recognized (though actively debated) “supergroups”, viz., chromalveolates and Rhizaria (Keeling et al. 2005, Parfrey et al. 2006). However, it is the way in which the two groups have come together – with the stramenopiles showing a specific affinity for Rhizaria and alveolates and not the other chlorophyll-*c*-containing lineages – that influences how we interpret the impact of secondary endosymbiosis in eukaryotic evolution. There are two main possibilities, both dependent on the eventual placement of the cryptophyte-haptophyte clade relative to stramenopiles, alveolates and Rhizaria, something that is not yet clear (Burki et al. 2007, Hackett et al. 2007). On the one hand, it is conceivable that a truly ancient secondary endosymbiosis occurred in a common ancestor shared between those lineages currently designated as chromalveolates and rhizarians, a scenario that is even more convoluted from the perspective of plastid loss than the chromalveolate hypothesis itself. This is because apart from the enigmatic and recently debated protist *Paulinella chromatophora* Lauterborn, which has recently acquired photosynthesis by engulfing a *Synechococcus*-type cyanobacterium (Marin et al. 2005, Archibald 2006, Theissen and Martin 2006, Yoon et al. 2006, Nowack et al. 2008), the chlorarachniophytes are the *only* phototrophic members of the entire group (Cavalier-Smith and Chao 2003, Nikolaev et al. 2004), and they possess a secondary plastid demonstrably of green algal origin (Rogers et al. 2007). Nevertheless, recent EST-based surveys have uncovered several metabolic enzymes that are consistent with this possibility: both

stramenopiles and haptophytes possess an unusual non-cyanobacterial, plastid-targeted homolog of ribulose-5-phosphate-3 epimerase (Rogers et al. 2007) that was previously identified only in the chlorarachniophyte *Bigelowiella natans* Moestrup (Archibald et al. 2003). The same study found a suspiciously similar transketolase gene in *B. natans* and a broad selection of chromalveolates (dinoflagellates, stramenopiles, haptophytes and cryptophytes) that is related to the bacterial group Chlamydiales (Rogers et al. 2007). To be sure, no single gene is likely to tell the whole story, but it will be interesting to see whether additional rare genomic features uniting chromalveolates and Rhizaria are uncovered as more genomic data become available.

Alternatively, sequential waves of secondary and tertiary endosymbioses involving both red and green algal endosymbionts could explain the distribution of plastids in chromalveolates and Rhizaria, parsimonious from the perspective of plastid loss but not plastid gain. Similar ideas have already been proposed to explain the distribution of chromalveolate plastids based on phylogenies of nucleus-encoded, plastid-targeted proteins (e.g., Bodyl 2006, Li et al. 2006, Nosenko et al. 2006). Of course, all this assumes that the latest large-scale phylogenies (Burki et al. 2007, Hackett et al. 2007, Patron et al. 2007) are relaying the actual evolutionary history of the host cell components of the plastid-bearing lineages in question, something that will need to be confirmed with additional studies, even larger datasets and deeper sampling at the genus and species level.

Whereto now (and how)?

Within the next few years, at least one complete nuclear genome sequence will be available from each of the primary and secondary plastid-containing lineages, excluding the dinoflagellates for which a reasonable amount of EST data already exists. Combined with a much broader sampling of genome sequences from diverse red and green algae, rigorous examination of these genomes will allow even more comprehensive and taxon-rich multi-gene phylogenies to be constructed, as well as provide additional evolutionarily informative characters, such as gene fusions and endosymbiotic gene replacements. However, gene replacements are only useful as informative characters when recognized as such. In the context of secondary endosymbiosis, the engulfed algal cell possesses a nuclear genome whose suite of genes for plastid-targeted proteins is transferred to the secondary host nucleus, but there is no reason to assume that the thousands of genes for non-plastid-related cellular processes are immune to transfer (Archibald 2005). Indeed, there are indications that endosymbiotic gene transfers and, possibly, replacements involving eukaryotic core house-keeping genes have occurred in the context of secondary endosymbiosis (Stibitz et al. 2000, Tanifuji et al. 2006, Ahmadinejad et al. 2007, Hackett et al. 2007), not to mention the potentially significant impact of lateral gene transfer (LGT) in organisms that have a habit of engulfing other microbes (Doolittle 1998, Archibald et al. 2003, Andersson 2005). A particularly striking instance of

eukaryote-eukaryote LGT is the alpha-tubulin genes of the excavate protist *Andalucia*, which appear to be derived from a relative of modern-day diplomonads (Simpson et al. 2008). This example indicates that even the most evolutionarily conserved and widely used phylogenetic markers can be inherited in a non-vertical fashion and, left undetected, the potential for such genes to deleteriously impact the results of concatenated phylogenies is enormous. If the nuclear genomes of secondary plastid-containing eukaryotes are indeed a mosaic of genes derived from multiple endosymbioses, and endosymbiotic gene replacement has been a significant factor in the evolution of their host nuclear genomes, this raises important questions about our ability to resolve the deepest branches of the tree of life when they include photosynthetic organisms and their non-photosynthetic relatives (Lane and Archibald 2008). It is now more important than ever to fine-tune and further develop methods for identifying incongruent phylogenetic signals in concatenated datasets.

At any rate, most would agree that together with improved “phylogenomic” methods, more sequence data from key taxa are what is needed most. Fortunately, with “next generation” sequencing technologies such as pyrosequencing taking hold (Schuster 2008) and “whole genome amplification” methods becoming increasingly useful (Spits et al. 2006), complete genomes from even the most obscure and intractable eukaryotic microbes will soon be available. Near the top of the list are the katablepharids and telonemids, two non-photosynthetic lineages that appear to be specifically related to cryptophyte algae (Okamoto and Inouye 2005, Shalchian-Tabrizi et al. 2006a). Given that cryptophytes and haptophytes appear to be each other’s closest relatives (Burki et al. 2007, Hackett et al. 2007, Patron et al. 2007), the presence of a remnant plastid and/or genes of red algal origin in the nuclear genomes of katablepharids and telonemids would push a red algal secondary endosymbiosis back to a common ancestor shared by all four groups. Another potentially significant lineage is the “picobiliphytes”, a newly-discovered group allied with cryptophytes and katablepharids in nuclear SSU rRNA phylogenies, and with a photosynthetic body reminiscent of a plastid and possibly a nucleomorph (Not et al. 2007, Cuvelier et al. 2008). Finally, a concerted effort to obtain genomic data from representative lineages at the base of the dinoflagellates and apicomplexans, including *Colpodella*, *Oxyrrhis* and gregarines (Leander and Keeling 2003) will go a long way to addressing the long-standing controversies about the origin and evolution of the apicoplast and the nature of the common ancestor of these two groups. The genome of the dinoflagellate-like oyster parasite *Perkinsus* is, in fact, already being sequenced (<http://www.tigr.org/tdb/e2k1/pmg/>) and, together with more genomic data from *Chromera velia* (Moore et al. 2008), will be very useful in this regard. Paradoxically, detailed analysis of the nuclear genomes of non-photosynthetic eukaryotes has the potential to provide some of the most profound insights in the tempo and mode of plastid evolution. Identifying and studying the genetic remnants of past endosymbioses in such genomes should keep molecular evolutionists busy for years to come.

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