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Chromalveolates and the Evolution of Plastids by Secondary Endosymbiosis[1]

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ABSTRACT. The establishment of a new plastid organelle by secondary endosymbiosis represents a series of events of massive complexity, and yet we know it has taken place multiple times because both green and red algae have been taken up by other eukaryotic lineages. Exactly how many times these events have succeeded, however, has been a matter of debate that significantly impacts how we view plastid evolution, protein targeting, and eukaryotic relationships. On the green side it is now largely accepted that two independent events led to plastids of euglenids and chlorarachniophytes. How many times red algae have been taken up is less clear, because there are many more lineages with red alga-derived plastids (cryptomonads, haptophytes, heterokonts, dinoflagellates and apicomplexa) and the relationships between these lineages are less clear. Ten years ago, Cavalier-Smith proposed that these plastids were all derived from a single endosymbiosis, an idea that was dubbed the chromalveolate hypothesis. No one observation has yet supported the chromalveolate hypothesis as a whole, but molecular data from plastid-encoded and plastid-targeted proteins have provided strong support for several components of the overall hypothesis, and evidence for cryptic plastids and new photosynthetic lineages (e.g. *Chromera*) have transformed our view of plastid distribution within the group. Collectively, these data are most easily reconciled with a single origin of the chromalveolate plastids, although the phylogeny of chromalveolate host lineages (and potentially Rhizaria) remain to be reconciled with this plastid data.

Key Words. Photosynthesis, phylogeny, protein targeting, supergroup.

THE CHROMALVEOLATE HYPOTHESIS—AN IDEA ABOUT PLASTID EVOLUTION

Plastid evolution. It is by now a familiar story that plastids and mitochondria arose by the endosymbiotic uptake of a cyanobacterium and proteobacterium, respectively, and that these were progressively reduced and integrated with their new host resulting in the highly specialized organelles we know today (Gray, Burger, and Lang 1999, 2001; Keeling 2004; Palmer 2003). Unlike mitochondria, which appear to have originated in the common ancestor of all known extant eukaryotic lineages, the plastid arose sometime later within a well-defined subgroup of eukaryotes, known as Plantae or Archaeplastida. This group comprises three major algal groups, glaucophytes, red algae and green algae, and their plant descendents. These groups all contain what is called a primary plastid, bounded by two membranes and thought to have originated from a single endosymbiotic event in their common ancestor (McFadden 2001; Rodriguez-Ezpeleta et al. 2005).

This relatively restricted distribution of the photosynthetic organelle also allows for another major difference between plastids and mitochondria: that plastids move from one lineage to another. Such movement has never been documented in mitochondria, perhaps because all lineages already have one, so why bother? A few lineages have substantially reduced their mitochondria to anaerobic mitosomes or hydrogenosomes (Williams and Keeling 2003). Such organisms could in theory acquire a new mitochondrion by secondary means, but it is likely such an acquisition would be of limited use in their anaerobic or microaerobic habitats, and also rare because they seldom encounter (or eat) organisms with aerobic mitochondria.

With plastids, however, most of eukaryotic diversity lacks them and the immediate advantage to acquiring photosynthesis is clear in many habitats. Plastids have accordingly been secondarily acquired several times through the uptake of a primary alga by a second eukaryote, and the retention of its plastid by this new host (Keeling 2004; Lane and Archibald 2008). Both green and red algal plastids have been moved in this way, and the level of integration between host and symbiont spans a wide range, from casual and temporary associations to organelles that are fully integrated with their host at the cell and genetic level.

Because both green and red algae have been involved in the secondary endosymbiotic origin of new algal lineages, we know that these events have taken place more than once in parallel, but exactly how many times has been a matter of great debate. On the green side, there are two lineages with fully integrated secondary plastids of green algal origin, euglenids and chlorarachniophytes. It has been proposed that these plastids trace back to a single common secondary endosymbiosis, the Cabozoa hypothesis (Cavalier-Smith 1999). However, analysis of the phylogeny of their plastid-encoded proteins suggests instead that these plastids originated twice independently (Rogers et al. 2007). On the red side, the evidence is more complex. In part, this is due to the much greater diversity of eukaryotes with secondary plastids derived from red algae and the increasingly complex relationship of these lineages with their non-photosynthetic cousins.

Given the great diversity of red secondary plastids, the knowledge that many of the algae that contained them had close relatives that were non-photosynthetic, and the scarcity of data from most of these groups, one might conclude that it would be reckless to propose a single common origin for these plastids. Nevertheless, in 1999 Cavalier-Smith (Cavalier-Smith 1999) proposed exactly that, and since that time the so-called chromalveolate hypothesis has been a focal point in the study of plastid evolution.

The chromalveolate hypothesis. Before evaluating the evidence for and against a hypothesis, it is useful to restate the hypothesis, in particular in this case because the exact nature of the chromalveolate hypothesis has been misrepresented in one important way. The hypothesis is built around the process of endosymbiosis and, more precisely, the difficulty in establishing a protein targeting system in a nascent plastid. The number of plastid origins by secondary endosymbiosis, it states, should be limited in evolutionary schemes because this limits the number of complex events (establishment of targeting systems and targeting information) needed to explain plastid diversity (Cavalier-Smith 1999). Specifically, the main point of the chromalveolate hypothesis posits that a single secondary endosymbiosis with a red alga gave rise to a plastid ancestor of all chromalveolates. A corollary of this is that the host lineages are related, but this is not the central thesis, nor was this idea the driving force behind

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the hypothesis. This is of critical importance because the central thesis of common plastid origin is not challenged by the finding that other lineages lacking this plastid fall within the chromalveolates in host phylogenies. Indeed, one view would be that nuclear gene phylogenies cannot actually disprove the chromalveolate hypothesis but only suggest that other lineages may also have descended from the original chromalveolate ancestor. In reality, nuclear gene phylogenies could be so difficult to reconcile with plastid data that they effectively do disprove the hypothesis, but this would only realistically be the case if ''chromalveolates'' are widely separated in well-supported trees. This has become extremely relevant in recent years, with the debate over the relationship between chromalveolates and rhizarians. The opposite extreme would be that you cannot prove the chromalveolate hypothesis with plastid data, because plastids can move between lineages. This view is weakened by the total absence of tertiary endosymbioses outside a few dinoflagellates.

WHAT ARE CHROMALVEOLATES?

The chromalveolates encompass a wide diversity of lineages with radically different nutritional modes, cell types, and structures. It includes some of the most diverse and well-studied protist groups, so that it has been estimated that over 50% of all formally described protists are chromalveolates (Cavalier-Smith 2004). There are six major lineages in the group and many small lineages or genera of uncertain evolutionary placement (Fig. 1).

Cryptomonads. Cryptomonads are common freshwater and marine flagellates, nearly all of which are photosynthetic (Kugrens, Lee, and Hill 2000). They are primarily known for their retention of a relict nucleus of their red algal symbiont, called a nucleomorph, along with its plastid (Archibald 2007). They are the only chromalveolate lineage demonstrated to have retained this nucleus (the only other case being the green algal symbiont of chlorarachniophytes). Their plastid is surrounded by four membranes, with the nucleomorph between an outer and inner pair. The outermost membrane is continuous with the host rough endoplasmic reticulum (RER) and the outer membrane of the nucleus. Their plastids have biliproteins in the thylakoid lumen, which have been lost in other chromalveolates. The single genus considered to lack a plastid is *Goniomonas*, from which no evidence of a plastid or nucleomorph has been observed by electron microscopy.

Haptophytes. Haptophytes are abundant primary producers in marine environments, some forming large blooms and one subgroup covering their cells with distinctive plates or coccothiths (Green and Jordan 2000). Virtually all known haptophytes are photosynthetic, and possess four membrane-bounded plastids where the outer membrane is continuous with the host RER and outer membrane of the nucleus.

Stramenopiles. Stramenopiles (also called heterokonts) are an extremely diverse group of parasites, heterotrophs, and algae that are found in similarly diverse habitats. They are generally unified by the possession of two unequal flagella, one with tripartite tubular hairs, and have been shown to form a monophyletic group in many molecular phylogenetic analyses. The non-photosynthetic stramenopiles (e.g. oomycetes, bicosoecids, opalinids, labyrinthulomycetes, and others) form between two to potentially several independent lineages whose phylogenetic relationships are not clear (Cavalier-Smith and Chao 2006). The photosynthetic stramenopiles (e.g. diatoms, brown algae, chrysophytes, synurophytes, raphidiophytes, and others) form a monophyletic lineage (collectively the ochrophytes), within which the branching order is also not certain (Ben Ali et al. 2002; Cavalier-Smith and Chao 2006). Plastids in photosynthetic stramenopiles are surrounded by four membranes and, as with cryptomonads and haptophytes, the outer membrane is continuous with the host RER and outer membrane of the nucleus.

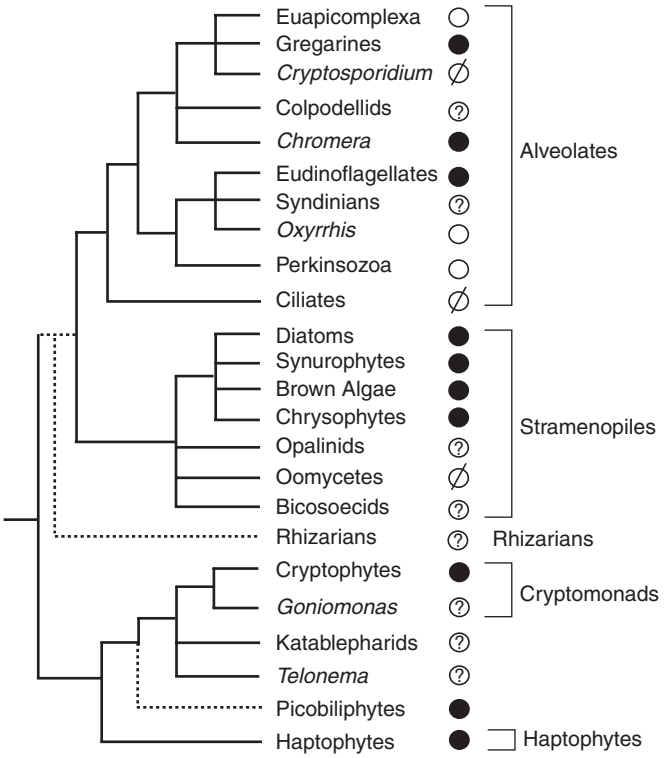

Fig. **1.** Schematic tree outlining the current hypotheses of chromalveolate relationships. Many relationships between chromalveolate subgroups are now well supported. Regions of the tree for which no consistent relationships have emerged are indicated by polytomies (e.g. lineages at the base of apicomplexa and dinoflagellates, and the branching order of most subgroups of stramenopiles). Other more tenuous relationships are indicated by dashed lines. The monophyly of alveolates and stramenopiles is consistently found, but needs further evidence. The relationship between Rhizaria and subgroups of chromalveolates is an emerging observation of great interest that needs to be further refined and would be much stronger if other supporting characters were found. The picobiliphytes have been found to be related to cryptomonads in small subunit rRNA phylogenies, but in the same trees no relationship between cryptomonads and haptophytes was found, so the exact position of this group remains uncertain. *Telonema* has also been found to be related to cryptomonads in HSP90 phylogeny and also in large multi-gene phylogenies, so its position appears to be resolved now.

Ciliates. Ciliates are a very large and well-studied group of non-photosynthetic parasites, symbionts, and heterotrophs that are defined by the possession of dimorphic nuclei (germline micronucleus and somatic macronucleus), the presence of many short flagella (cilia) anchored by characteristic fibers, and conjugation as the sexual process (Lynn 2008). No plastid has been identified in any ciliate, although some have kleptoplasts (Johnson et al. 2007). Ciliates, together with the dinoflagellates and apicomplexans, are members of the alveolates.

Dinoflagellates. Dinoflagellates are a common and widespread group of parasitic, heterotrophic, or photosynthetic protists distinguished by flagellar structures and an unique set of nuclear/chromosomal characters collectively called the dinokaryon (Dodge and Lee 2000). About half the described species are photosynthetic. The majority of these possess a three-membrane plastid distinguished by the pigment peridinin and no connection to the host ER. A few lineages also have other types of plastid that are derived from other primary or secondary algae through additional tertiary or serial secondary endosymbiotic events (Keeling 2004). Many of the non-photosynthetic lineages are

clearly derived from photosynthetic ancestors by a relatively recent loss of photosynthesis, but some of the earliest-diverging dinoflagellate lineages also lack plastids (Saldarriaga et al. 2001). Dinoflagellates, together with the ciliates and apicomplexans, are members of the alveolates.

Apicomplexa. Apicomplexans are all parasites, and nearly all obligate intracellular parasites of animals (Perkins et al. 2000). They are responsible for many significant diseases, in particular malaria. They are distinguished by the apical complex, a suite of structures used in the infection process. A non-photosynthetic plastid bounded by four membranes, the outermost of which is smooth and lacks any clear connection to the host ER, has now been identified and well studied in many species (Ralph et al. 2004). However, the earliest-diverging lineages either seem to lack a plastid (*Cryptosporidium*) or at least no evidence for one has been found (gregarines). Apicomplexans, together with the dinoflagellates and ciliates, are members of the alveolates.

Other lineages within the chromalveolates. There are a number of smaller lineages now known to be related to some subset of the chromalveolates (Fig. 1). These include groups of predominantly heterotrophic predators such as katablepharids, *Oxyrrhis*, *Telonema*, and colpodellids, parasites such as syndinians and perkinsids (Kuvardina et al. 2002; Leander and Keeling 2003; Okamoto and Inouye 2006; Saldarriaga et al. 2003; Shalchian-Tabrizi et al. 2006), as well as photosynthetic algae such as picobiliphytes (or biliphytes), and *Chromera* (Cuvelier et al. 2008; Moore et al. 2008; Not et al. 2007). Many of these will be discussed below in the context of the distribution of plastids.

RELATIONSHIPS BETWEEN CHROMALVEOLATE GROUPS

In addition to phylogenetic evidence for the chromalveolates as a whole, it is essential to review the data-supporting relationships between subgroups. The reason for this is that there is no single data set that specifically unites all chromalveolates. Instead, the view that they are related is based on assembling different kinds of data that unite various subsets of the group. Sometimes these data unite a couple of chromalveolate lineages, and sometimes there is evidence for a relationship between most members of the group. Examining this network of data as a whole, the support for various subgroups overlaps in a way most consistent with the monophyly of all chromalveolates (Fig. 2). In this section, the evidence for two major subgroups is summarized, and in the next section, evidence for the monophyly of chromalveolates as a whole is summarized.

Alveolates and stramenopiles. The alveolates are one of the best-supported major assemblages of protists, if not the best supported. They are united by morphological characteristics, most conspicuously the alveoli—membranous sacs below the plasma membrane—for which they are named. They are also well supported by a great number of molecular phylogenetic studies, so that the monophyly of alveolates has not been seriously questioned in quite some time (Fig. 2). Within the group, there is also strong support from molecular phylogenies of individual or concatenated genes for a sister relationship between dinoflagellates and apicomplexa to the exclusion of ciliates (Burki et al. 2007; Burki, Shalchian-Tabrizi, and Pawlowski 2008; Fast et al. 2001; Hackett et al. 2007; Patron, Inagaki, and Keeling 2007; Van de Peer, van der Auwera, and DeWacher 1996; Wolters 1991).

Within alveolates there is also now strong support from many molecular analyses for the perkinsids being the deepest-branching sisters to the dinoflagellate lineage (Leander and Keeling 2004; Saldarriaga et al. 2003). Multi-gene phylogenies also support the early divergence of *Oxyrrhis*, while protein insertion data place this genus after *Perkinsus* (Leander and Keeling 2004), and small

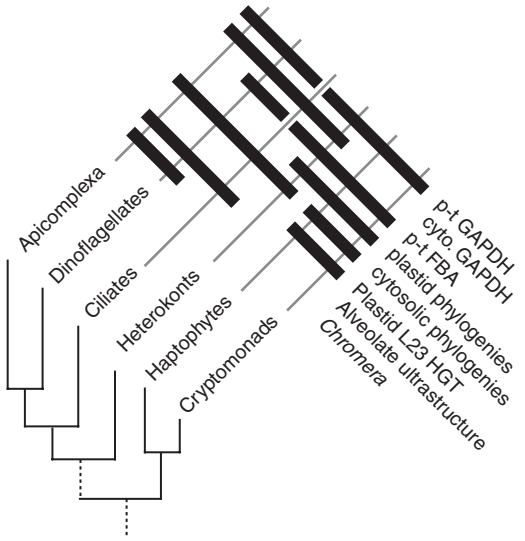

Fig. **2.** Simplified tree of chromalveolates, summarizing how molecular evidence for the hypothesis is distributed across the major subgroups of chromalveolates. There is no one piece of evidence or analysis that unambiguously unites the entire group, but if one considers all the evidence uniting various subgroups of the plastid and cytosolic lineages to one another, the entire group is supported by one or more kinds of data. Evidence bars that are broken between groups means that the evidence does not support the union of those groups (e.g. cytosolic phylogenies), whereas lines that are broken around a group indicates that evidence is simply missing from that group (e.g. plastid glyceraldehydes-3-phosphate dehydrogenase from ciliates). The morphological characteristics that unite alveolates are also included because it is particularly strong and consistent, and also completely consistent with molecular analyses.

subunit (SSU) rRNA phylogeny supports the early divergence of syndinians, but the relative order of these is not known because different genes have been used for each. In the apicomplexan lineage, gregarines and *Cryptosporidium* have been demonstrated repeatedly to have been early-diverging members of the group (Leander 2008). Colpodellids and *Chromera* have also been suggested to be early-diverging sisters to the apicomplexan lineage: in the case of colpodellids this is based on SSU rRNA alone and not yet well supported (Kuvardina et al. 2002), but in the case of *Chromera* this position is better supported by independent phylogenies based on several genes (Moore et al. 2008).

Alveolates as a whole have been consistently shown by many molecular phylogenies to be the sister group to stramenopiles (analyses that include rhizarians are discussed below), including several large-scale analyses with many concatenated genes from many taxa (Burki et al. 2007, 2008; Hackett et al. 2007; Patron, Inagaki, and Keeling 2007; Rodriguez-Ezpeleta et al. 2005; Simpson, Inagaki, and Roger 2006). They also share an insertion in the cytosolic homologue of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fast et al. 2001) (Fig. 2).

Cryptomonads, haptophytes, and relatives. The phylogenetic positions of cryptomonads and haptophytes have both been highly contentious, but a very strong case can now be made that they are closely related to one another (Fig. 2), and possibly part of a very large and diverse lineage that also includes katablepharids, picobiliphytes, and *Telonema*. A relationship between cryptomonads and haptophytes is seen in a few single-gene phylogenies (Harper, Waanders, and Keeling 2005), but also in all multigene phylogenies where they are represented (Burki et al. 2007, 2008; Hackett et al. 2007; Patron et al. 2007). They also share a unique horizontal gene transfer event to their plastids, where ribosomal protein 28 (rpl28) has been replaced by a paralogous bac-

terial gene (Rice and Palmer 2006), altogether making a very compelling case for a close relationship of both host and plastid lineages. Katablepharids have also been convincingly shown to be the sister group to cryptomonads in SSU rRNA phylogeny (Okamoto and Inouye 2005), and weaker evidence from SSU rRNA trees suggests picobiliphytes may also branch with cryptomonads (Cuvelier et al. 2008; Not et al. 2007). *Telonema* is a ubiquitous but monogeneric group of unknown origin, but combined data from HSP90 and SSU rRNA suggests it too is related to the cryptomonads (Shalchian-Tabrizi et al. 2006), and recent multigene analyses have, further, strongly supported this (Shalchian-Tabrizi, pers. commun.). Altogether, the newly recognized (and unnamed) group of cryptomonads, haptophytes, picobiliphytes, katablempharis, and telonemids is one of considerable diversity.

PHYLOGENETIC EVIDENCE FOR AND AGAINST THE CHROMALVEOLATE HYPOTHESIS

From the plastid lineage. Unfortunately, gathering evidence to test the chromalveolate hypothesis has not been as simple as sequencing plastid genomes and constructing phylogenies based on their genes, largely due to the nature of alveolate plastid genomes. The apicomplexan plastid genome is bereft of all genes relating to photosynthesis, because that function has been lost (Williams and Keeling 2003), while the peridinin plastids of dinoflagellates have a massively reduced genome due to large-scale movement of genes to the nucleus (Bachvaroff et al. 2004; Green 2004; Hackett et al. 2004). Ironically, nearly all the genes retained as aberrant minicircles in dinoflagellate plastid genomes relate to photosynthesis (Zhang, Green, and Cavalier-Smith 1999); so there are few comparisons possible with apicomplexan plastid genomes (Keeling 2008). In contrast, the stramenopile, haptophyte, and cryptomonad plastid genomes are relatively normal in their structure and content, and large multigene phylogenies using these data support the monophyly of these three groups (Hagopian et al. 2004; Khan et al. 2007; Yoon et al. 2002) (Fig. 2). Two qualifications on this are that red algal plastid genomes are not very well sampled, and that the inclusion of certain fast-evolving genes disrupts this monophyly (Hagopian et al. 2004; Khan et al. 2007).

Plastid gene phylogeny is generally more ambiguous on the relationships between cryptomonads, haptophytes, and stramenopiles, but the acquisition of *rpl32* by horizontal gene transfer in cryptomonads and haptophytes (Rice and Palmer 2006) provides strong evidence that they are sisters to the exclusion of stramenopiles, which is consistent with analyses of their nuclear lineages as well (Hackett et al. 2007; Patron et al. 2007).

The evolutionary history of the plastid lineage is most unambiguously reconstructed using the plastid genome, but most of the genes for plastid proteins are actually encoded in the nucleus. In the simplest case, these moved from the cyanobacterium to the ancestor of the red algal nucleus, and in chromalveolates then moved again to the nucleus of the ancestral secondary host (Keeling 2008). In theory, these genes should still represent plastid evolution, but the various movements make their interpretation slightly more complex. Two such genes nevertheless provide strong evidence for a single origin of chromalveolate plastids, ironically because neither seems to have followed the expected evolutionary path. Fructose-6-phosphate aldolase (FBA) and GAPDH are both involved in glycolysis and the Calvin cycle. Ancestrally, plants and algae would therefore be expected to have two copies of each in their nuclear genomes: a phylogenetically eukaryotic cytosolic copy and a phylogenetically cyanobacterial plastid-targeted copy. This is true for GAPDH, except for the chromalveolates, where the cytosolic copy appears to have duplicated and one copy has taken over the plastid-targeted function (there is no plastid GAPDH in ciliates) (Fast et al. 2001; Harper and Keeling 2003). In the case of FBA, such a duplication and takeover took place long ago in the ancestor of red and green algae (Gross et al. 1999), but once again chromalveolates are different because they have acquired a distinct and non-homologous type of FBA, which has itself duplicated so one copy functions in the cytosol and the other is targeted to the plastid (Patron et al. 2004). In both cases these observations are straightforward if chromalveolate plastids originated in a single common endosymbiosis: these acquisitions, duplications, and re-targeting events took place once in the common ancestor of chromalveolates (Fig. 2). If one postulates multiple origins of chromalveolate plastids, no such simple explanation is possible, because the plastid-targeted GAPDH and FBA genes from chromalveolates are strongly related to one another, there is no way to reconcile their shared possession of these unusual genes without evoking lateral gene transfer between lineages with independently derived red algal plastids, or transfer of the whole plastid and many nuclear genes targeted to them (Fast et al. 2001; Harper and Keeling 2003; Patron et al. 2004). These are formally possible, and would be fascinating events, but there is currently no evidence to favour this complex explanation over the simpler interpretation that these events took place in a common ancestor.

From the host (cytoplasmic) lineage. With evidence from plastid data emerging to support the chromalveolate hypothesis, there has been a great deal of interest in determining the relationships of the host lineages as well. Individual gene phylogenies do not unite all chromalveolates. Generally, cryptomonads and haptophytes branch separately from alveolates and stramenopiles, although there is little or no support separating them in most analyses (e.g. see Harper et al. 2005). The generation of large-scale sequence data through whole genomes or expressed sequence tag (EST) projects has now been completed for at least one member of each of the main lineages of chromalveolates. Multigene phylogenies of nuclear genes derived from these data have so far strongly supported the alveolates, generally recovered the alveolates and stramenopiles, and most recently also strongly supported the monophyly of the cryptomonads and haptophytes (Burki et al. 2007, 2008; Hackett et al. 2007; Patron et al. 2007). However, these analyses have so far failed entirely to support the chromalveolates as a whole. While this obviously does not support the chromalveolate hypothesis, it is probably premature to argue against it either because in most such analyses there is no support for the separation of cryptomonads and haptophytes from the other chromalveolates, and when alternate topologies are compared, the monophyly of chromalveolates cannot be rejected, suggesting that the amount of data available is insufficient to test the relationship (Patron et al. 2007). If future analyses definitively demonstrate that the cryptomonads and haptophytes are not closely related to the other chromalveolates, reconciling such a relationship with the data from plastid-targeted proteins like GAPDH and FBA (see above sections) will be difficult.

A CHROMALVEOLATE-RHIZARIAN LINK: WHAT WOULD THIS MEAN FOR PLASTID EVOLUTION?

Multigene phylogenies of the cytosolic lineage have also suggested relationship between a subset of chromalveolates and rhizarians (Burki et al. 2007, 2008; Hackett et al. 2007). Rhizaria is a supergroup in its own right, comprising a morphologically diverse selection of protists, including the major lineages radiolaria, foraminiferans, and cercozoans. It has only recently been widely accepted as a monophyletic group, almost entirely due to strong and consistent results from molecular phylogenies (Nikolaev et al. 2004). Indeed, its members are so diverse that there is no morphological feature that unambiguously distinguishes the group, although reticulpodia are a common feature in many lineages. Individual analyses showed no supported position for rhizarians,

and the first small concatenated analyses showed them to be weakly related to cryptomonads and haptophytes (Harper, Waanders and Keeling 2005). Large-scale EST projects from several rhizarians have now allowed their inclusion into large multigene phylogenetic analyses. In these trees, rhizarians have showed a specific affinity to the alveolates and stramenopiles (Burki et al. 2007, 2008; Hackett et al. 2007). Originally it was suggested rhizarians were sisters to the strameopiles (Burki et al. 2007), but support for this relationship has since eroded and most analyses now show them sister to alveolates and stramenopiles (although topology comparisons sometimes fail to reject alternatives). Moreover, in the most recent analyses, the cryptomonad/haptophyte lineage are the sister group to the alveolate/stramenopile/rhizarian lineage (Burki et al. 2008; Hackett et al. 2007): in analyses where they fall elsewhere it is never with convincing support.

The possibility that rhizrians fall within the chromalveolates is exciting for a number of reasons, and if born out by future data and analyses it may be a first glance at a higher-order structure of the tree of eukaryotes. It would be valuable, for example, to analyze the individual trees making up these large analyses to determine if the data are consistently supporting this conclusion or only a subset of the genes. It would also be interesting if other, non-phylogenetic, characters could be brought to bear on the issue, such as the horizontal gene transfer of *rpl28* uniting cryptomonads and haptophytes or the plastid-targeted genes uniting chromalveolates as a whole. Intriguingly, one such case may exist. The insertion that unites cytosolic GAPDH genes of alveolates and stramenopiles (Fast and Keeling 2001) is also found in the cytosolic GAPDH of the chlorarachniophyte rhizarian *Bigelowiella natans* (PJK., unpubl. data). GAPDH evolution is complex, so by itself this is not very compelling, but if similar stories emerge it will greatly strengthen the case for a chromalveolate–rhizarian relationship.

Even taken at face value, however, a relationship with rhizarians does not actually challenge the central thesis that the chromalveolate plastids originated in a single common endosymbiosis, although it does affect how we might interpret the subsequent evolution of that plastid (assuming other data do not reject that central thesis). The question arises, can the single origin of chromalveolates plastids and the possibility that rhizaria branch within chromalveolates be brought together into a reasonable model? The simplest model that accounts for all the data currently available would suggest that rhizarians are essentially another non-photosynthetic lineage within the chromalveolates (several others exist, see next section), either having lost the plastid or, intriguingly, potentially retained it in some as yet undiscovered form in some lineages (Burki et al. 2007, 2008; Hackett et al. 2007). From this one would predict they should retain some relicts of this ancestry, but there is little evidence for or against this. The chlorarachniophytes again offer a tempting possibility. They are photosynthetic rhizarians, but acquired a secondary green algal plastid independently of the chromalveolate event, so their nucleus-encoded plastid-targeted proteins are expected to be green algal. However, many plastid-targeted genes in the genome of the chlorarachniophyte *B. natans* are of red descent (Archibald et al. 2003). While these could be interpreted as relicts of an ancient red plastid in the context of a chromalveolate/rhizarian relationship, it also has many plastid-targeted proteins derived from other lineages aside from the lineage from which the plastid is apparently derived (i.e. other green lineages or bacteria: [Archibald et al. 2003]). One would have to plead different cases for different genes to suggest the red genes are ancestral relicts. More importantly, *B. natans* is nested well within the rhizarian lineage (Moreira et al. 2007; Nikolaev et al. 2004), so one would also have to speculate that relict plastid proteins were maintained in the nuclear lineage through a long period of non-photosynthetic evolution, only to be later targeted to the new green algal plastid. This does not seem very plausible, and would suggest other non-photosynthetic rhizarians should still have these genes too. So far none has been found. Altogether, the original explanation for these genes, recent horizontal gene transfer, perhaps through phagotrophy (Archibald et al. 2003), remains the most simple.

CRYPTIC PLASTIDS AND RELICT PLASTID GENES IN NON-PHOTOSYNTHETIC CHROMALVEOLATES

The limited distribution of photosynthetic lineages within the chromalveolates, and particularly the number of cases where photosynthetic chromalveolates are sister to one or more non-photosynthetic lineages, superficially suggests multiple independent plastid gains is more likely than a single ancestral origin (Delwiche and Palmer 1997; Palmer and Delwiche 1998). I say superficial because it is very important to distinguish between the lack of a plastid and the lack of photosynthesis when making such an argument, and this is difficult to do in practice. Plastids have lost photosynthesis many times in many lineages, and such cryptic, non-photosynthetic plastids can be very difficult to detect (Williams and Keeling 2003). The majority of non-photosynthetic chromalveolates are not actually known to lack a plastid, the possibility has simply never been examined directly. Indeed, until the chromalveolate hypothesis was proposed, there was no reason to suspect they might harbor a cryptic one.

The importance of this distinction has been dramatically illustrated by the emerging evidence for a plastid or plastid ancestry in many of these non-photosynthetic lineages. The apicomplexan plastid was the first of these to be found and also acted as an important catalyst for the examination of other non-photosynthetic lineages. This ''apicoplast'' has now been found in representatives of several major groups, its function, protein import mechanism, and cell biology have been thoroughly investigated in several species (Ralph et al. 2004; Wilson 2002). More recently, data have emerged for a plastid organelle in *Perkinsus atlanticus* (Teles-Grilo et al. 2007), and genes for plastid-derived proteins characterized in *Perkinsus marinus* (Stelter, el-Sayed, and Seeber 2007). Similarly, eight plastid-derived genes were characterized in *Oxyrrhis marina*, and at least four of these have N-terminal leaders suggesting they are targeted to a still unidentified organelle (Slamovits and Keeling 2008).

In other lineages, there is no indication that a plastid exists in the cell, but the genome retains clues that the organelle was once there and has been lost. The most compelling case comes from the basal apicomplexan *Cryptosporidium*. No plastid structure or genome has been found (Zhu, Marchewka, and Keithly 2000), and the complete genome encodes no plastid-targeted proteins (Abrahamsen et al. 2004; Xu et al. 2004). However, searching the genome for cases of horizontal gene transfer did reveal genes that appear to be derived from the red algal ancestor of the plastid (Huang et al. 2004). The symbiont provenance of these genes is significantly bolstered by the demonstration through *Chromera* that the ancestor of apicomplexans and dinoflagellates possessed a plastid (Moore et al. 2008). A similar case has also been made for the oomycete *Phytophthora* (Tyler et al. 2006) and most recently the ciliates *Tetrahymena* and *Paramecium* (Reyes-Prieto, Moustafa, and Bhattacharya 2008). In both cases, the complete genome reveals no obvious class of plastid-targeted protein, but they do contain genes phylogenetically related to plastid-derived genes in other algae, which are interpreted as relicts of a now lost plastid.

In all non-photosynthetic chromalveolate lineages where significant sequence data are now available, there is either evidence for a plastid or at least relict plastid-derived genes. Moreover, recent advances in reconstructing the phylogenetic relationships between certain subgroups and other means of

demonstrating relatedness of plastid lineages have pin-pointed two ancestral nodes within chromalveolates where very strong evidence from plastid and cytosolic data concurs that a plastid must have existed. These are the ancestor of apicomplexans and dinoflagellates (Keeling 2008; Moore et al. 2008), and the ancestor of cryptomonads and haptophytes (Burki et al. 2007; Hackett et al. 2007; Patron et al. 2007). Even in the absence of direct evidence, the null hypothesis for all non-photosynthetic and plastid-lacking members within these clades (e.g. gregarines, colpodellids, syndinians, katablepharids) now has to be that they are derived from plastid-bearing ancestors. This means that there are only two chromalveolate groups, ciliates and non-photosynthetic stramenopiles, for which we do not have very strong evidence for a plastid-bearing ancestor, and even these are only questionable so long as the evidence for a single origin of all chromalveolate plastids remains questionable. Because examples of both have already been shown to contain relict plastid-derived genes (*Tetrahymena* and *Phytopthora*), it seems reasonable to conclude that the ancestors of these groups might well have possessed a plastid as well. Even in the most conservative interpretation, our current understanding of the distribution of plastids and plastid relicts within chromalveolates is no longer a reason to be skeptical of an early common origin of the organelle. Going even further, some of these lineages can now be said to be the most unambiguous examples of plastid loss in all eukaryotes, in particular *Cryptosporidium*, and therefore models for what seems to be a very rare process.

CONCLUSIONS: WHAT CAN WE SAY WITH CONFIDENCE?

The validity of the chromalveolate hypothesis remains a highly contentious issue. This is not surprising given the huge diversity of eukaryotes involved and the age of the events being tested. Nevertheless, in under a decade the concept has gone from largely being dismissed to being the hypothesis to ''beat'' when explaining the origins of these organisms, and one that permeates many large-scale schemes of eukaryotic evolution (Adl et al. 2005; Keeling et al. 2005; Lane and Archibald 2008; Simpson and Roger 2002). Providing more evidence for or against the overall unity of the chromalveolates remains a major task, one that has three distinct components that are obvious at this time. First, large-scale multigene phylogenetic analyses of nuclear data will certainly continue to be investigated, and whether or not these analyses converge on a model that either supports or directly challenges the hypothesis is one of the outstanding questions. Secondly, whether future analyses of plastid data continue to support the hypothesis or not will be critical, especially if plastid data could be extended to include representatives of all chromalveolate groups in a single analysis. Related to this, the continued examination of non-photosynthetic lineages for plastid relicts will clarify the real distribution of plastids in the ancestors of chromalveolate groups. Third, the discovery of *Chromera* showed us just how transformative one organism at a critical juncture of the tree can be, so the discovery and characterization of new lineages is bound to play a role in further refining the hypothesis as a whole, or subdivisions of it.

Having outlined the points of weakness, it would be positive to end on a list of things that we can now conclude.

Cryptomonads and haptophytes. The phylogenetic evidence for the sister relationship between cryptomonads and haptophytes is now very strong. The relationship is supported by all large multigene analyses of the host lineage, even relatively small ones (Burki et al. 2007; Hackett et al. 2007; Harper et al. 2005; Patron et al. 2007). A close relationship of their plastids is also supported by the shared transfer of *rpl36* (Rice and Palmer 2006). This means that the ancestor of these lineages, katablepharids, and perhaps also picobiliphytes, and *Telonema* contained a plastid (and a nucleomorph).

Apicomplexa and dinoflagellates. The apicomplexa and dinoflagellates have been proposed to be sisters since the first molecular analyses that included both groups (Wolters 1991), and the support continues to be high in large multigene analyses. The sister relationship of their plastid has been one of the most hotly debated subjects of the chromalveolate hypothesis because they are hard to compare and because of the erroneous but widely credited assertion that the apicoplast is derived from a green alga (Wilson 2002). The characteristics of the *Chromera* host and plastid now appear to neatly unite these two groups, and provids compelling evidence that their common ancestor did contain a plastid (Keeling 2008; Moore et al. 2008). This means that the ancestors of colpodellids, gregarines, *Cryptosporidium*, perkinsids, *Oxyrrhis*, syndinians, and all other non-photosynthetic dinoflagellates also had a plastid.

Alveolates and stramenopiles. The sister relationship of alveolates and stramenopiles has been consistently seen in many single-gene trees (Van de Peer and De Wachter 1997), and also in the great majority of large multigene analyses. It was briefly challenged by an early analysis of chromalveolates and rhizarians because rhizarians were specifically related to stramenopiles (Burki et al. 2007), but this relationship has not been supported in other analyses (Burki et al. 2008; Hackett et al. 2007). This remains a fairly strong group, but not as strong as either of the above subgroups because it lacks supporting data from the plastid lineage.

Chromalveolates as a whole. Based on the available data, there are really only two plausible possibilities for the origin of chromalveolates: either they are monophyletic (possibly including rhizarians), or they are divided into two independently originating groups comprising alveolates and stramenopiles, and cryptomonads and haptophytes. The evidence from plastid-targeted proteins and plastid genomes for a common origin of all chromalveolates unites these two subgroups (Fast et al. 2001; Hagopian et al. 2004; Harper and Keeling 2003; Khan et al. 2007; Yoon et al. 2002), and has not been discredited as yet, despite further sampling and analysis. In fact the evidence for monophyly of heterokont, haptophyte and cryptomonad plastid sequences is improving. This therefore remains a strong block of data supporting the chromalveolate hypothesis as a whole. The possibility that the ancestors of other lineages (e.g. rhizarians) also possessed this plastid is not evidence against the chromalveolate hypothesis, instead the hypothesis provides a way to interpret such evidence (i.e. such findings do not challenge the hypothesis so much as they extend it).

As for the host lineage, there are few data that currently argue against the chromalveolate hypothesis with any support. Some single-gene trees suggest cryptomonads and/or haptophytes go elsewhere in the tree, which is evidence against the hypothesis at face value, although most of it is undermined by the demonstration that these two lineages are related to one another. However, the great majority of analyses that do not actively support the chromalveolate hypothesis simply fail to do so, as opposed to actively pointing to some alternative hypothesis. This is an important distinction because failing to resolve a relationship is quite different from showing that it is false.

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