MINIREVIEW

Do Red and Green Make Brown?: Perspectives on Plastid Acquisitions within Chromalveolates

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The chromalveolate “supergroup” is of key interest in contemporary phycology, as it contains the overwhelming majority of extant algal species, including several phyla of key importance to oceanic net primary productivity such as diatoms, kelps, and dinoflagellates. There is also intense current interest in the exploitation of these algae for industrial purposes, such as biodiesel production. However, the evolution of the constituent species, and in particular the origin and radiation of the chloroplast genomes, remains poorly understood. In this review, we discuss current theories of the origins of the extant red alga-derived chloroplast lineages in the chromalveolates and the potential ramifications of the recent discovery of large numbers of green algal genes in chromalveolate genomes. We consider that the best explanation for this is that chromalveolates historically possessed a cryptic green algal endosymbiont that was subsequently replaced by a red algal chloroplast. We consider how changing selective pressures acting on ancient chromalveolate lineages may have selectively favored the serial endosymbioses of green and red algae and whether a complex endosymbiotic history facilitated the rise of chromalveolates to their current position of ecological prominence.

Algae are emerging as being of key interest in contemporary biological research. As the principal primary producers in oceanic and freshwater communities, algae support the development of complex food webs and biodiverse communities and are responsible for the net flux of nearly 2 gigatons of carbon per year from the atmosphere to the lithosphere, an amount equivalent to or higher than that of tropical rainforests (24, 68, 122). Understanding why specific algal lineages are more ecologically prominent than others may provide valuable insight into the stability of these ecosystems, particularly as some of the most important taxa are believed to be sensitive to changes in atmospheric and oceanic climates (42, 49), so that phytoplankton community composition is predicted to change considerably in response to current and future climate (28, 31, 44). In addition, algae are morphologically and physiologically diverse, ranging from microscopic single-celled diatoms and prasinophytes smaller than some bacteria to forests of giant kelps, and differing in their photosynthetic pigments, hence red, green, and brown algae, among others (Fig. 1). The enormous array of biological and biochemical characteristics presented by algae offers great opportunities for exploitation across a wide range of technologies, for example, in the production of biodiesel, industrial chemicals, and even nanotechnologies such as microchips (58, 71). This variety offers challenges too, and a much better understanding of the biochemical properties of different algal groups and their chloroplast lineages, which are intimately related to their evolutionary histories, will be required to aid in the identification and culturing of candidate species.

In this review, we explore the evolutionary history of the chromalveolates, a group of algae that includes major ecologically important lineages, such as diatoms, dinoflagellates, and haptophytes. We will consider both the nuclear lineages and their constituent chloroplasts, which are believed to have been derived from the secondary endosymbiosis of a red alga, and we will evaluate the recent hypothesis that chromalveolates historically possessed a green algal endosymbiont (81). We suggest that a complex evolutionary history wherein an ancient, green alga-derived chloroplast was replaced by serial transfer of a red alga-derived chloroplast between specific chromalveolate lineages would explain the observation and distribution of red and green alga-derived genes in extant chromalveolates. We will conclude by exploring whether the serial endosymbioses of green and red algae may explain the ecological prominence of extant chromalveolates.

A SYMPHONY OF RED, GREEN, AND BROWN—THE DIVERSITY OF ALGAE

By the term “algae,” the authors refer to any eukaryotes that possess chloroplasts, other than land plants (embryophytes). Chloroplast lineages are scattered across several of the major assemblies of eukaryotes currently defined by phylogenetic analysis (Fig. 2). Extant chloroplasts have not been identified within the opisthokonts or Amoebozoa, and only one photosynthetic lineage has been identified within the Excavates. The last common ancestors of these three “supergroups” of eukaryotes almost certainly did not contain chloroplasts: therefore, the last common ancestor of all extant eukaryotes was likewise nonphotosynthetic, and chloroplasts were acquired more recently by specific eukaryotic lineages. Chloroplasts
Red-derived chloroplasts, chloroplasts play important roles in global ecology, particularly biotic acquisition (91, 111). Symbionts that may represent early intermediates in endosymbiotic protist lineages have been reported to harbor green algal chloroplasts which were lost either prior or subsequent to secondary loss events. Several recent genomic studies have identified large-scale gene acquisition events in various eukaryotes; there is compelling genetic evidence, for example, that dinoflagellates that contain green algal or tertiary endosymbiotic chloroplasts which were lost either prior or subsequent to secondary loss events are not restricted to nonphotosynthetic lineages, such as the heterotrophic apicomplexa (52, 79). Furthermore, two novel putatively photosynthetic alveolates—Chromera velia and the species CCMP3155—have been identified and appear to be close relatives of the dinoflagellates and apicomplexa (52, 79). These nonphotosynthetic lineages are believed to have descended from photosynthetic ancestors and secondarily lost photosynthetic capacity while retaining hallmarks of their photosynthetic past such as nonpigmented chloroplast analogues or photosynthetic alveolates (52, 79).

The taxonomic distribution of algae is further complicated by the presence of several nonphotosynthetic taxa within otherwise photosynthetic lineages, such as the heterotrophic Euglenid Astasia longa, several nonphotosynthetic plants such as Rafflesia (corpse flower), and—of perhaps greatest interest to contemporary biologists—the apicomplexa (123). These nonphotosynthetic lineages are believed to have descended from photosynthetic ancestors and secondarily lost photosynthetic capacity while retaining hallmarks of their photosynthetic past such as nonpigmented chloroplast analogues or nuclear genes acquired from an ancient chloroplast. These secondary loss events are not restricted to nonphotosynthetic eukaryotes; there is compelling genetic evidence, for example, that dinoflagellates that contain green algal or tertiary endosymbiotic lineages originally possessed secondary red alga-derived chloroplasts which were lost either prior or subsequent to endosymbiosis of the extant chloroplast lineage (83, 95, 126). More controversial are hypothesized cryptic endosymbiotic events where a chloroplast lineage has been proposed to have been acquired but secondarily lost by all extant descendants, such that nothing remains other than genes transferred into the host nucleus. Several recent genomic studies have identified large-scale gene acquisition events in various eukaryotes.
that might be consistent with cryptic endosymbioses (47, 82, 99, 112), but in the absence of extant relatives containing orthologous chloroplasts, it is difficult to determine whether these are indeed the footprints of ancient chloroplasts or the result of nonendosymbiotic lateral gene transfer between different eukaryotic lineages. However, even discounting cryptic endosymbiotic events, the scattered distribution of algae across the eukaryotes must have arisen from multiple primary, secondary, and tertiary endosymbioses, together with chloroplast loss events (Fig. 2).

This complex phylogenetic distribution raises two key questions about algal evolution. First, when and how were chloroplasts acquired by different algal lineages: whether algae originated by a relatively small number of ancient endosymbioses, followed by many secondary loss events in their extant nonphotosynthetic relatives or whether there were large numbers of recent independent endosymbioses following their divergence from the closest nonphotosynthetic relatives. In particular, the precise origins of secondary red alga-derived chloroplasts in chromalveolates are heavily debated. Second, as befits their diverse evolutionary history, algae occupy a wide range of ecological niches but planetary ecosystems are dominated by
just a few groups, so an important question is why some algae are more ecologically common and cosmopolitan than others.

THE CHROMALVEOLATES—A KINGDOM OR NOT?

It has long been proposed, from ultrastructural and morphological similarities, that various combinations of cryptomonads, haptophytes, stramenopiles, and the three principal alveolate lineages might be closely related (17, 124). Several of these relationships found weak support from early molecular phylogenies of nuclear genes, principally 18S rRNA (18, 34, 76). All four phyla were first grouped together by Cavalier-Smith (15), who proposed the elegant and ingenious “chromalveolate hypothesis” that secondary, red alga-derived chloroplasts were acquired once via an ancestral secondary endosymbiosis by a common ancestor of the Chromalveolates, a “kingdom” composed of all four groups.

More recent phylogenies drawn from data sets consisting of multiple, in some cases more than 100, concatenated nuclear genes have complicated and challenged the concept of the chromalveolates. First, several entirely nonphotosynthetic lineages which were previously of unknown position or believed to belong elsewhere in the tree of eukaryotes have robustly been positioned within the chromalveolates. The cryptomonads and haptophytes have been shown to group with three nonphotosynthetic phyla (the telonemids, kathablepharids, and centrohelids; Fig. 2), to form a single clade termed the Hacrobia or “CCTH clade,” characterized by the shared presence of bacterium-derived rpl36 in haptophyte and cryptomonad chloroplast genomes (11, 90, 100). More surprisingly, the rhizaria, a diverse assembly of amoeboid eukaryotes, including cercozoans and foraminiferans, have turned out to be closely related to the stramenopiles and alveolates. These three phyla have been grouped as the “SAR clade” or Harosa and share the synapomorphy (a feature conserved across, and unique to, a particular phylogenetic group, such that it is diagnostically of members of that group) of a novel duplicated isoform of Rab GTPase (12, 13, 16, 26). From a taxonomic perspective, only a minority of the major recognized chromalveolate lineages are, in fact, photosynthetic (1, 123) (Fig. 2).

More critically, recent studies have cast doubt on the monophyly of the Chromalveolate kingdom, even considering the expanded concepts of the CCTH and SAR clades. Although some multigene studies robustly support a sister group relationship between the nuclear lineages of these clades (12, 41, 85, 86), other analyses recover alternative topologies, such as an exclusive sister group relationship between the CCTH clade and archaeplastids (12, 93), while a small number weakly support positions for the CCTH clade within the archaeplastids, as a sister group to the red (43) or green (7, 92) algae. Any one of these variant topologies implicitly invalidates the Chromalveolate kingdom as a taxonomic concept.

Resolving the branching relationships between the archaeplastids and the CCTH and SAR clades is a challenging task, as a number of stochastic and systemic effects such as long-branch attraction, short-branch exclusion, and biased gene sampling may distort the topologies obtained (30, 51, 103, 109). Notably, in some of the above studies, the initial relationships recovered between “chromalveolate” taxa were shown to be unstable following the removal of fast-evolving sequences or species from the phylogenetic data sets (43, 88), consistent with these relationships being artifactual. To some extent, these problems may be reduced by using specifically slow-evolving genes or species but are ultimately likely unavoidable given the ancient divergence of chromalveolate lineages (8, 125). In addition, even in slow-evolving data sets, genes acquired from red alga-derived endosymbionts may lead to artifactual phylogenetic associations. Recently, Baurain et al (7) utilized an innovative “variable length bootstrap” strategy, calculating the amount of information required to recover strong support for a given topology, calibrated to account for divergence date and gene transfers from red algal endosymbiont lineages. Using this approach, they failed to recover support for the monophyly of cryptomonad, haptophyte, and stramenopile nuclear lineages. However, the same criteria have yet to be applied to a test of the monophyly of CCTH clade and archaeplastid nuclear genomes, and it is possible that the lack of support recovered is due to additional factors, e.g., large-scale lateral gene transfer into specific chromalveolates from a donor lineage other than the red algae.

Ultimately, more work will be required to determine whether the “chromalveolates” are indeed monophyletic. The extent and effect of lateral gene transfer into chromalveolates from non-red lineages must be quantified before it will be possible to confirm or refute alternative phylogenetic relationships for chromalveolate nuclear genomes. In addition, the evolutionary relationships recovered in phylogenetic studies must be substantiated by discrete and unambiguous synapomorphies across the chromalveolates or between the CCTH clade and archaeplastids. These features may be ultrastructural, novel gene duplications, in accordance with the SAR clade Rab GTPase, or lateral gene transfers, similar to the CCTH clade rpl36. Until this information is uncovered, the most straightforward hypothesis is for a monophyletic origin of chromalveolates and therefore that the CCTH and SAR clades are sister groups.

TAKEN AS RED—THE ORIGIN OF CHROMALVEOLATE CHLOROPLASTS

Even if the chromalveolates do turn out to be monophyletic, the validity of an ancient secondary red algal endosymbiosis is still open to debate. Given the current taxonomic composition of the CCTH and SAR clades, it is difficult to determine whether an ancient endosymbiosis, followed by multiple independent loss events, is more parsimonious than a scenario where chloroplasts were acquired independently by different photosynthetic chromalveolates. Critically, in contrast to nuclear gene phylogenies, there is strong evidence for the monophyly of chromalveolate chloroplasts (7, 52, 60); hence, if chloroplasts were acquired independently by different chromalveolate lineages, they must have been transferred between different chromalveolate lineages by tertiary endosymbiosis rather than acquired via multiple independent secondary endosymbioses. Sanchez-Puerta and Delwiche (105) and Bodily et al (9) have proposed serial endosymbiotic models wherein secondary red algal chloroplasts were originally acquired by an ancestor of the CCTH clade and then transferred laterally by tertiary endosymbiosis into the SAR clade. Under these scenarios, many of the nonphotosynthetic chromalveolate lineages (e.g., cen-
trohelids and rhizaria) diverged prior to the endosymbiotic event, in other words, never acquired a chloroplast in the first place.

Multiple lines of evidence are needed for a single ancestral endosymbiotic origin of chromalveolate chloroplasts to be validated. First, nuclear and chloroplast phylogenies of the chromalveolates must be congruent. If the CCTH clade indeed turns out to be a sister group to the archaeplastids in nuclear gene phylogenies, it would automatically disprove the chromalveolate hypothesis. In addition, if phylogenies of chloroplast genes result in a topology different from that of nuclear genes, this would specifically point to tertiary, internal endosymbiotic events. While some chloroplast phylogenies neatly assign chromalveolates to the CCTH and SAR clades (52, 60), others suggest alternative groupings, such as a close relationship between haptophyte and SAR clade plastids to the exclusion of cryptomonads (22, 55, 64, 104, 125), which could provide evidence for a tertiary endosymbiotic acquisition of a haptophyte ancestor by an early member of the SAR clade. Even if future nuclear and chloroplast gene phylogenies support the monophyly of the chloralveolates and the CCTH and SAR clades, an internal tertiary endosymbiosis could have occurred, for example, after the divergence of the CCTH and SAR clades, but prior to the radiation of the constituent photosynthetic phyla.

More compelling evidence for the chromalveolate hypothesis would be provided by finding “footprints” of secondary chloroplast loss in nonphotosynthetic chromalveolates. This idea is well established for apicomplexa, which retain a nonphotosynthetic “apicoplast” that is bound by four membranes, retains a genome, and is engaged in metabolism and protein synthesis characteristic of extant chloroplasts (32, 65). A photosynthetic origin of the apicoplast has been cemented by the phylogenetic grouping of the apicomplexa with the photosynthetic alveolates Chromera velia and CCMP3155 (46, 72). There is similar genetic and ultrastructural evidence for remnant chloroplast-derived organelles in perkinsids, nonphotosynthetic relatives of the dinoflagellates (53, 72, 117). In the absence of identifiable chloroplast-derived organelles in nonphotosynthetic chromalveolates, red alga-derived genes indicate an ancient chloroplast acquisition, but they may be the result of recent lateral gene transfers or random phylogenetic “noise.” Genomic surveys of the oomycetes Phytophthora ramorum, P. sojae, and Pythium ultimatum, believed to be the closest relatives of photosynthetic stramenopiles (101), have recovered only small numbers of well-supported red alga-derived genes, inconsistent with an endosymbiotic origin (62, 110).

The best evidence in favor of an ancient red algal endosymbiosis would be the presence of a nuclear, chloroplast-targeted gene synapomorphy across the chromalveolates, which could be the product of an ancient endosymbiosis. For example, photosynthetic chromalveolates utilize a chloroplast-targeted isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that arose from the duplication of the endogenous host-derived, cytoplasm-targeted gene (29) and a chloroplast-targeted isoform of fructose bisphosphate aldolase (FBA) that was laterally acquired from a bacterial donor (94). These chloroplast-targeted isoforms might even be retained in nonphotosynthetic chromalveolates that had historically contained chloroplasts, as following chloroplast loss, they would be targeted to the cytoplasm and could be retained over the original cytoplasmic isoforms. However, these synapomorphies may also arise from recent lateral gene transfers within the chromalveolates. This has been explicitly demonstrated by recent GAPDH phylogenies which suggest that the chloroplast-targeted isoform has been transferred at least twice between chromalveolates (89, 115).

On balance, the phylogenetic incongruities between chromalveolate chloroplast and nuclear lineages and the limited evidence of chloroplast loss in some nonphotosynthetic chromalveolates argue against the chromalveolate hypothesis of a single ancestral red algal endosymbiosis, but there is as yet no conclusive answer to when and how red chloroplast lineages originated in the chromalveolates. This will be clarified by genome sequencing of nonphotosynthetic representatives of the rhizaria and the CCTH clade to determine whether red algal genes and other synapomorphies are present in these lineages. However, we also believe that the evidence for and against alternative hypotheses of the origin of chromalveolate chloroplasts should be explored further. For example, a serial chloroplast transfer between haptophytes and the SAR clade might be identifiable not only from chloroplast gene phylogenies but also from specific gene synapomorphies not found in other photosynthetic chromalveolates. In particular, if there were a large number of genes shared exclusively between the haptophytes and the SAR clade, it is much more likely that they would be the result of a tertiary endosymbiosis than massive, independent gene loss events elsewhere within the chromalveolates.

GOING GREEN—EVIDENCE FOR ANCIENT GREEN ANCESTORS

Recently, discussions of the evolutionary history of chromalveolate algae have been compounded by the discovery of a number of green alga-derived genes in chromalveolates. These include green algal forms of phosphoribulokinase, chlorophyll a synthase, ferredoxin:NADPH reductase, chloroplast signal processing peptidase, the ammonium transporter AMT1, and five enzymes involved in carotenoid biosynthesis (21, 33, 64, 74, 83, 97). Genome analyses of the diatoms Thalassiosira pseudonana (5) and Phaeodactylum tricornutum (10) have recovered substantially more genes of putative green than red origin. The means by which these “green genes” originated was addressed by Moustafa et al (81) via a series of single-gene phylogenies that identified over 1,700 genes of specific green algal affinity in both T. pseudonana and P. tricornutum, of which approximately 500 specifically grouped with prasinophytes, compared to only 400 to 450 genes of red algal affinity, leading the authors to make the bold assertion that these genes were the “footprint” of an ancient cryptic green algal endosymbiont acquired by a common ancestor of the chromalveolates and then replaced by the extant red chloroplast lineages.

The green gene data set presented by Moustafa et al (81), while exciting, is problematic. Most critically, out of the >1,700 putative green genes, only 442 were identified from phylogenies containing red algal sequences, because the only complete red algal genome sequence available is that of Cyanidioschyzon merolae, which is highly reduced, containing ap-
TABLE 1. Total numbers of green and red genes recovered by Moustafa et al. (81) that encode functional protein classes well conserved in C. merolae

| Functional class<sup>a</sup> | Pr/Tp<sup>b</sup> |
|-----------------------------|-----------------|
|                            | Green | Red |
| Ribosome associated<sup>c</sup> | 13/15 | 10/14 |
| tRNA associated<sup>d</sup>    | 7/4   | 14/12 |
| DNA replication<sup>e</sup>    | 5/3   | 3/3  |
| Nuclear structural<sup>f</sup> | 5/8   | 1/0  |
| Endomembrane trafficking<sup>g</sup> | 9/5   | 0/1  |
| Protein folding<sup>h</sup>    | 22/22 | 11/11 |
| Protein degradation<sup>i</sup> | 18/28 | 12/14 |
| Amino acid metabolism<sup>j</sup> | 20/18 | 4/13 |

<sup>a</sup> Functional classes likely to be relatively unreduced in the C. merolae genome were identified from reference 78; gene numbers were quantified by keyword searches across the red and green data sets in reference 81.

<sup>b</sup> Ribosome structural/ribosome binding.

<sup>c</sup> Principally aminoacyl-tRNA synthetase/ligase.

<sup>d</sup> DNA nuclelease/polymerase/ligase/glyrase/topoisomerase.

<sup>e</sup> Histone/chromosome/centromere/nucleosome structural proteins. Excludes histone-modifying and chromatin-remodeling enzymes.

<sup>f</sup> SNARE/coatomer/sec/rib proteins. Includes biosynthesis proteins.

<sup>g</sup> Hsp/bip/calreticulin/calnexin/ERp24/ERp57/protein and peptidyl isomerase/other chaperone/chaperomin.

<sup>h</sup> Peptidase/protease/proteinase. Includes inhibitors.

<sup>i</sup> All amino acid related (e.g., alanine/alanyl related), with the exception of chaperones, peptidases, tRNA ligases, sugar and prenyltransferases, and amino acid-specific protein kinases and phosphatases.

<sup>j</sup> Pt, genes identified in P. tricornutum; Tp, genes identified in T. pseudonana.

proximately half as many protein-coding genes as either of the diatoms (5, 10, 73, 88). A recent BLAST study (19) of over 60,000 expressed sequence tags from the red algae *Porphyridium cruentum* and *Calliathornia tuberculosa* identified nearly 350 genes that supported archaeplastid monophyly but lacked an identifiable homologue in the C. merolae genome. The addition of sequences from less reduced red algal genomes may similarly uncover novel red genes in chromalveolate genomes, many of which may come from the green gene data set. In our opinion, however, this is unlikely to remove the green genes altogether or to reduce the number significantly, as the C. merolae genome appears to have retained most of the genes shared by red algae and chromalveolates; even including sequences from *P. cruentum* and *C. tuberculosa*, only approximately 750 candidate red genes are identifiable from chromalveolates (19). Furthermore, while the C. merolae genome is extremely reduced, it contains greater proportions of some genes, e.g., those involved in translation, DNA replication, and protein and amino acid biosynthesis, than other sequenced photosynthetic eukaryotes (73, 78), yet we could visually identify a consistently green signal even in these relatively unreduced functional gene families across both species (Table 1). We consider that even in a most extreme scenario, a consistent and strong green signal should still emerge.

Sequence divergence and lateral gene transfer may present more significant problems to the green gene data set. Sequences from C. merolae are known to be phylogenetically unstable, to the extent that many published eukaryotic phylogenies eschew them for sequences from other, less-fast-evolving red algae (7, 86, 103). Moustafa et al (81) applied a cutoff of 75% likelihood support when assigning genes to a red or green origin, but it is possible that in doing so they rejected a large number of genuine but more divergent red alga-derived genes or equally identified artifactual green genes due to long branch exclusion of C. merolae sequences. In addition, even if the green phylogenetic associations observed are genuine, they may contain genes transferred from the chromalveolates into specific green algal lineages; this is well supported for one specific isoform of prasinophyte phytoene synthase (33, 118). In total, the green gene data set contains only 144 genes for which (i) a red algal sequence is available and (ii) red algae turn out to be phylogenetically at the base of a well-supported green algal/chromalveolate clade (i.e., are unlikely to be due to red algal sequence divergence or lateral gene transfer from chromalveolates into specific green algal lineages). Nonetheless, this number is well in excess of the number of red algal signals identified in ciliates or oomycetes (62, 99, 121), indicating that these genes represent a large-scale transfer event from the green algae into the diatoms.

With this in mind, we propose four scenarios for the acquisition of green genes by diatom lineages (Fig. 3). In scenario A, green genes were donated to the diatoms via repeated lateral transfer events. This is consistent with the observation that the green genes belong phylogenetically with several different green algal lineages (81). However, this would presumably have led to the acquisition of a random assortment of genes by diatoms, whereas the green gene data set recovered is relatively enriched in mitochondrial and chloroplast-targeted genes. In addition, while the green genes vastly outnumber red genes, they are distributed over a narrower proportional range of gene families (80), consistent with their having been predominantly ancient acquisitions that had subsequently diversified by gene duplication rather than a functionally diverse array of recent lateral acquisitions. The green gene heritage of diatoms is therefore most likely to have arisen from a combination of recent lateral gene transfers and an endosymbiotic event.

The phylogenetic conservation of the green gene data set provides insight into when this endosymbiosis occurred. Although a greater proportion of the green genes recovered in diatoms were also identified in other stramenopiles (pelagophytes and oomycetes) (62, 81) than in other chromalveolate taxa, significant numbers of green genes were recovered in all of the other chromalveolate taxa studied, including over 50 in representatives of the apicomplexa and ciliates (81). If these green genes are genuine, they would specifically support an ancient green algal endosymbiosis prior to the radiation of the chromalveolates (Fig. 3, scenario B), as opposed to a recent endosymbiosis within the stramenopiles (scenario C). An ancient green endosymbiosis has major implications for chromalveolate evolutionary history. First, it would strongly support chromalveolate monophyly and could explain the frequent phylogenetic placement of the CCTH clade as a sister taxon to the archaeplastids. Second, it could potentially explain the contradictory evidence of an ancient endosymbiosis and recent internal transfer of red algal plastids. An ancient green endosymbiosis could, for example, have resulted in the origin of synapomorphies such as the chloroplast-targeted isoforms of FBA and GAPDH, which would be retained following the later acquisition of a red algal plastid lineage.

Most significantly, more green algal genes recovered in diatoms (>400) were specifically found in the haptophyte *Emiliania huxleyi* than any of the alveolate phyla, despite the much
closer relationships between stramenopile and alveolate nuclear lineages and the relative sizes of the haptophyte and alveolate gene datasets used in the analyses (80, 81). Cuvelier et al (22) have recently identified a large number of green algal genes in an uncultured microscopic haptophyte distantly related to *E. huxleyi*, implying that many of these genes are conserved across the whole of the haptophytes. These genes might provide discrete evidence for a tertiary haptophyte endosymbiosis by a stramenopile ancestor (scenario D) and support the recent dispersal of red lineage chloroplasts by tertiary endosymbiosis.

Taking all these points into consideration, we present a theoretical model of chromalveolate plastid evolution where an ancient green endosymbiont was replaced by the invasion of a red endosymbiont via an ancient member of the CCTH clade (Fig. 4). This model would explain the relative incongruities between chromalveolate chloroplast and nuclear phylogenies and also explain the conservatism of gene synapomorphies supporting an ancient endosymbiosis, such as GAPDH, with the lack of evidence for a red chloroplast history in some nonphotosynthetic chromalveolates. Clearly, this hypothesis requires testing, in the first instance by the incorporation of additional complete red algal genomes into phylogenetic analyses. In addition, the effects of red sequence divergence should be addressed by conducting single-gene phylogenies containing only slow-evolving sites and/or quantifying the number of
green genes in phylogenetic data sets containing representa-
tives of all three archaeplastid phyla. The effects of chromal-
veolate-to-green lateral gene transfer could be controlled by
rejecting any phylogenies in which the green algae are
polyphyletic. By this means, a convincing and robust green
gene data set would be obtained which could be used to assess
the validity of the model, for example, to determine whether
genes from broad samples of chromalveolates group mono-
phyletically within the green algae for these phylogenies, con-
sistent with an ancient endosymbiotic origin. If red chloroplasts
were acquired via several recent endosymbioses, chromalveo-
late taxa that never acquired a red lineage—principally the
rhizaria and possibly the ciliates—might contain significant
numbers of conserved green genes but not red genes. Finally,
if red chloroplasts spread through the chromalveolates via tertiary endosymbioses between the CCTH and SAR clades, one might expect to find green genes specifically conserved between haptophytes and stramenopiles but not dinoflagellates or similarly between haptophytes and dinoflagellates but not stramenopiles. Ultimately, a much wider range of sequences from red algae, rhizaria, and other currently undersampled chromalveolates will be required for these analyses to be statistically viable.

THE COLORFUL HISTORY OF CHROMALVEOLATE CHLOROPLASTS—INTEGRATING EVOLUTION AND FUNCTION

The model of chromalveolate evolution presented in Fig. 4 explicitly assumes that an ancient green endosymbiont was universally replaced in chromalveolates by a red alga-derived chloroplast. If this occurred after the radiation of the chromalveolates, it must be asked why red chloroplasts were taken up by every extant photosynthetic chromalveolate lineage while no chromalveolates retaining an ancestral, prasinophyte-derived endosymbiont have yet been identified. In addition, while a much wider range of sequences from red algae, rhizaria, and other currently undersampled chromalveolates will be required for these analyses to be statistically viable.

The “portable plastid” hypothesis (40) proposes that algae with red-derived chloroplasts are advantaged over algae with green-derived chloroplasts because they contain relatively larger genomes than green algal chloroplasts and specifically retain a number of genes involved in regulating photosynthesis and metabolism that allow them to integrate readily with host metabolic environments. Although an enlarged chloroplast genome would be selectively advantageous to chromalveolates, e.g., due to more efficient redox regulation of gene expression (4), the chloroplast genomes of diatoms and dinoflagellates are significantly more reduced than those of red algae (10, 46), and it is unlikely that chloroplast genome size alone explains the selective advantage of the red chloroplast lineage. In fact, many of the proposed endosymbiont-derived genes in chromalveolate genomes appear to be derived from the endosymbiont nucleus (22, 54, 80), and genes transferred from the nuclear genomes of red algal endosymbionts may have also contributed to the selective advantage of early chromalveolates.

Notably, the chromalveolates emerge in fossil assemblies from a geological period with a distinctive planetary climate. Atmospheric carbon dioxide levels appear to have fallen and oxygen levels to have risen across the last 600 million years. This is believed to have been due to the colonization of land by plants and specifically the evolution of vascular plants (i.e., lycophytes, ferns, gymnosperms, and angiosperms; Fig. 5, points 1 to 4), which increased soil weathering and drawdown of atmospheric CO2. This reached an extreme in the Permian following the diversification of vascular plants, with atmospheric carbon dioxide decreasing to close to contemporary
levels and oxygen levels peaking at somewhere between 25 and 35% of the total composition (48, 61). In addition, the Per- 
imian-Triassic boundary is marked by a profound reduction in 
pecies diversity, including the loss of ~50% of the marine 
auna and 50 to 80% of the terrestrial plant species, and fur- 
ther major extinctions occurring at the ends of the Triassic and 
the Cretaceous (75, 96). Chromalveolates containing red chlor- 
oplast lineages may have risen to dominance by persisting 
through these extinction events and invading the niches va- 
cated by competitors less adaptable to the new environmental 
conditions.

In low-CO2 paleoclimates such as the late Permian, one 
selectively advantageous feature would be a higher photosyn- 
specificity for CO2 over O2. This could be facilitated by 
investment in carbon-concentrating mechanisms, which con- 
centrate CO2 around the active site of RubisCO (36), or by 
isofoms of RubisCO with higher substrate specificities for 
CO2 over O2, such as the ID form of RubisCO found in red 
algal chloroplasts compared to the green algal IB form of 
RubisCO (6). Moreover, such algae would be competitively 
advantaged in nutrient-limited environments, as their require- 
ments for light-harvesting and electron transport proteins and 
would be streamlined, and therefore they would have a lower 
requirement for trace elements such as iron essential for the 
synthesis of photosystem proteins and known to be growth 
limiting for extant algal communities (27, 69, 120). Notably, 
red algae and chromalveolates tend to contain much lower 
levels of iron, copper, and zinc than green algae, which might 
be indicative of greater resource efficiency (98). Furthermore, 
recent studies of various diatoms have suggested that the tran- 
scriptional profiles and net photosynthetic rates of cells grown 
under elevated CO2 are similar to those of cells grown under 
ambient conditions, whereas variation in other environmental 
factors such as light and nitrate abundance effect much greater 
physiological and transcriptomic changes (14, 37, 67, 119). This 
implies that diatoms at least are not substantially limited by the 
current composition of the atmosphere.

If ancient chromalveolates were selectively advantaged due 
to the greater resource efficiency engendered by the acquisi- 
tion of a red algal chloroplast, one would expect that red algae 
would likewise have radiated and risen to dominance in a 
post-Permian environment. However, extant red algae are less 
species rich and many of the most ecologically prominent ex- 
tant red algae, e.g., Coralliniales, only began to diversify much 
more recently, during the Cretaceous or later (3, 27, 108). We 
suggest that additional factors specific to chromalveolates may 
have conferred a selective advantage. One possibility is that a 
complex endosymbiotic heritage equipped early chromalve- 
lates with an enlarged gene pool of endogenous green- and 
red-derived genes so that they were more able to expand into 
niches previously occupied by green algae in what has else- 
where been termed the “shopping bag model” of algal evolu- 
tion (38, 59). For example, the green alga-derived chromalveo- 
late isoform of phosphoribulokinase has unusually low activity 
in all of the taxa studied (66, 97). This is significant in that 
chromalveolates lack CP12, a circadian regulator of PRK 
found in both red and green algae (39, 66), and in the absence 
of the negative regulator, the lower-activity isoform may have 
been selected over a higher-activity, red orthologue. Con- 
versely, individuals that retained the higher-specificity red iso-
form of RubisCO may have been selectively advantaged over 
others that lost this gene early during endosymbiosis. Although 
many of the red and green genes found in extant chromalveo- 
lates are likely to have been retained under neutral selection, 
it is tempting to speculate that some chromalveolate green 
genes were selected due to the greater “endosymbiotic hybrid 
vigor” of early chromalveolates.

CONCLUSIONS

The original endosymbiotic definition of the chromalveo- 
lates, unifying chromists and alveolates on the grounds of an 
ancestral secondary red algal endosymbiosis, has been chal- 
lenged by recent developments in eukaryotic taxonomy. The 
evidence for chromalveolate monophyly and an ancestral red 
algal endosymbiosis is inconclusive, and alternative models 
such as a close relationship between the CCTH clade and archaeplastids and the lateral spread of red lineage chloro- 
plasts through the chromalveolates by tertiary endosymbiosis 
may prove to be better supported. Moreover, the identification 
of large numbers of green genes in chromalveolates suggests 
that an ancient chromalveolate ancestor contained a green 
algal endosymbiont. If this conclusion withstands more rigor- 
ous phylogenetic analysis, it implies a subsequent replacement 
of the green chloroplast by a red algal endosymbiont in all 
extant photosynthetic chromalveolates (Fig. 4). This model has 
far-reaching implications in the study of chromalveolate evo-
lution by providing an alternative defining synapomorphy for 
chromalveolates and explaining why some chromalveolate lin- 
eages group phylogenetically with archaeplastids.

The idea that an ancient green endosymbiont was replaced 
via repeated secondary and tertiary endosymbioses of a red 
algae-derived chloroplast is consistent with changes in the se- 
lective pressures acting on algae during a protracted period of 
low atmospheric CO2. Furthermore, an “endosymbiotic mo- 
saic” of red, green, and chromalveolate-derived genes could 
have provided the genetic diversity to allow extant chromal- 
veolates to dominate post-Permian algal communities. Many 
of these hypotheses will be resolved through broader, more 
balanced taxonomic sampling in phylogenetic data sets and 
from current genome sequencing projects such as that of the 
photosynthetic apicomplexan relative Chromera velia, the 
model rhizarian Bigelowiella natans, the relatively slow-evolv- 
ing red algae Chromidrus crispus and Porphyra yezoensis, and 
the glaucophyte Cyanophora paradoxa (45). In parallel, it will be 
essential to gain a better understanding of the physiological 
properties of different endosymbiont lineages, including 
whether the red chloroplast lineage mitigates CO2 and nu- 
trient limitation and whether the retention of specific green 
genes confers enhanced selective fitness on chromalveo- 
lates. This could include comparative studies, such as inves-
tigation of the kinetic properties of homologous enzymes 
from different endosymbiotic lineages (6, 66) or identifica- 
tion of genes that are differentially expressed in particular 
species under stress conditions using deep-sequencing ap- 
proaches (53, 67). If these features can be linked to ob- 
served changes in phytoplankton community structure 
following environmental perturbation (31, 44, 69), the evo-
lutionary diversity of algae may be placed into a functional 
context. Ultimately, exploring a relationship between atmo-
spheric chemistry and changes observed in the algal fossil record may provide valuable insight into how extant algae will respond physiologically, ecologically, and selectively to the continued anthropogenic effects on the planetary cli-

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