The Number, Speed, and Impact of Plastid Endosymbioses in Eukaryotic Evolution

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Abstract

Plastids (chloroplasts) have long been recognized to have originated by endosymbiosis of a cyanobacterium, but their subsequent evolutionary history has proved complex because they have also moved between eukaryotes during additional rounds of secondary and tertiary endosymbioses. Much of this history has been revealed by genomic analyses, but some debates remain unresolved, in particular those relating to secondary red plastids of the chromalveolates, especially cryptomonads. Here, I examine several fundamental questions and assumptions about endosymbiosis and plastid evolution, including the number of endosymbiotic events needed to explain plastid diversity, whether the genetic contribution of the endosymbionts to the host genome goes far beyond plastid-targeted genes, and whether organelle origins are best viewed as a singular transition involving one symbiont or as a gradual transition involving a long line of transient food/symbionts. I also discuss a possible link between transporters and the evolution of protein targeting in organelle integration.

Contents

INTRODUCTION

It is a common misperception that evolution is going somewhere in particular, when instead much about the history of life has been complicated and unpredictable. The evolutionary history of plastids—particularly what we have learned in the past 10 years through advances as diverse as improved high-throughput sequencing and a growing reappreciation of species discovery and natural history—is a good example: Major transitions in evolution do include adaptations of remarkable importance, but they also include reversals, redundancy and waste, improbable complexities, and often more chance than necessity.

Plastid: generic term for organelles derived from endosymbiosis of a cyanobacterium, including chloroplasts, leukoplasts, rhodoplasts, apicoplasts, etc.

Many of the complexities of plastid evolution stem from the fact that it is also a story of symbiosis. That plastids arose through the endosymbiotic uptake of a cyanobacterium is now past any serious debate. This initial endosymbiosis was one of the more important events in the evolution of life; by itself, however, it explains only a fraction of plastid diversity because plastids then spread endosymbiotically to other eukaryotes. Here, I briefly review the main endosymbiotic events that led to the current diversity of plastids, including some of the more contentious outstanding questions, but also focus on the actual process of endosymbiosis and the implications of different models of plastid origin in plant and algal genomes.

THE ENDOSYMBIOTIC BUILDING BLOCKS OF PLASTID EVOLUTION

The single most confounding factor in plastid evolution is the simple fact that, although plastids may all be closely related, the algae that contain them are not (**Figure 1**). This is because plastid evolution has included multiple layers of endosymbiotic events.

Primary endosymbiosis, the ultimate source of photosynthesis in eukaryotes, refers to the uptake and retention of a cyanobacterium by a eukaryote. The plastids of land plants and green algae, red algae, and glaucocytophyte algae all derive from primary endosymbiosis (**Figure 2**). Much debate has led to a consensus that primary plastids originated from a common ancestor. This issue is not covered here because it has been discussed in detail elsewhere (62, 84) (see also sidebar, *Paulinella*).

Primary endosymbiosis led to a plastid bounded by two membranes. It is difficult to trace the history of membranes through this process, but based on the presence of certain lipids and membrane proteins (in particular

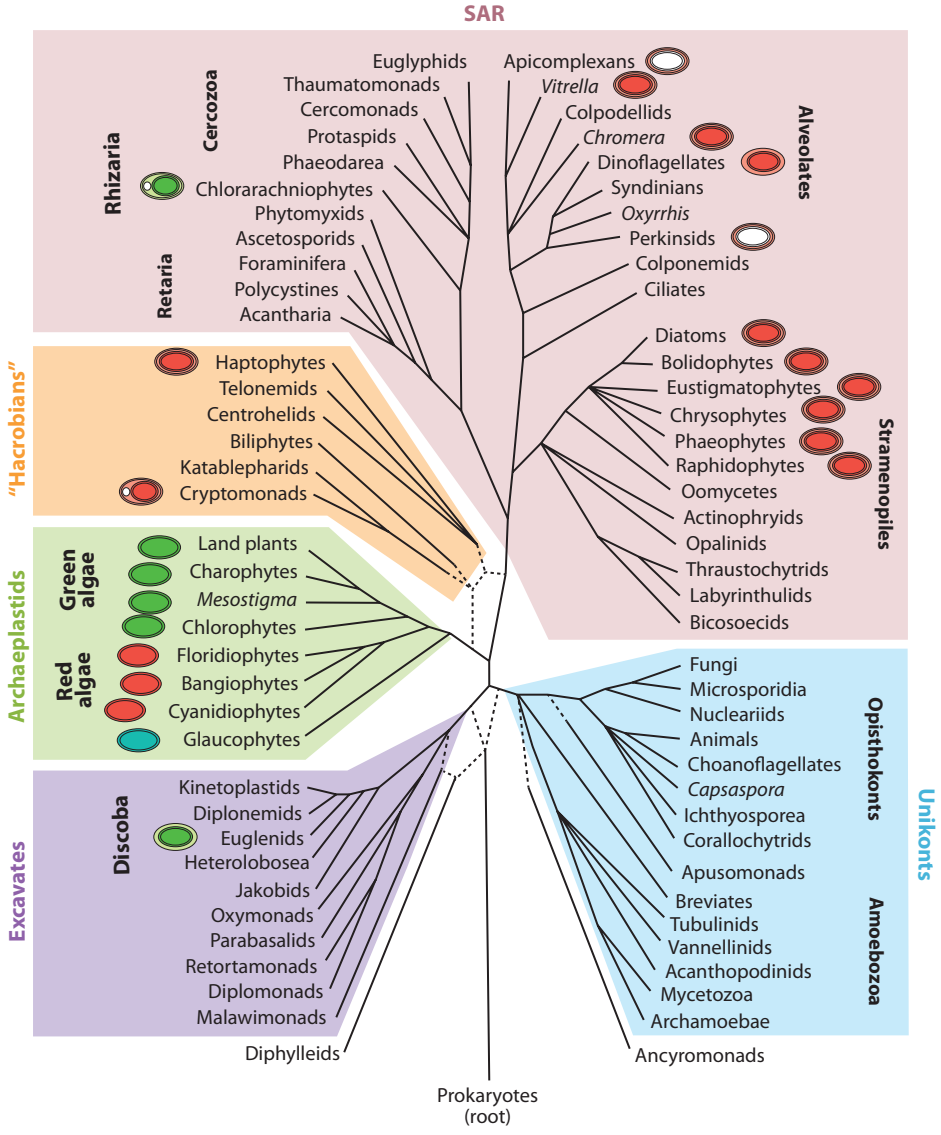


Figure 1

Tree of eukaryotes with plastid lineages. This hypothesis for relationships between major eukaryotic lineages is a synthesis of many studies based on large phylogenomic analyses or rare genomic characteristics. Dotted lines denote particularly contentious relationships, and in some cases two conflicting possibilities are shown. Colored boxes denote supergroups, including the more disputed hacrobians, which are shown because there is not yet a viable alternative. Chromalveolates would be equivalent to the Hacrobia and SAR (stramenopile-alveolate-rhizarian) groups combined. The membrane structure and origin of plastids (where known) are shown at the periphery. The primary plastids with two membranes are found in the archaeplastids (*green*, *red*, *and cyan*), and a second independent primary plastid is found in the rhizarian euglyphid *Paulinella* (see sidebar, *Paulinella*). Secondary plastids are found in SAR organisms, cryptomonads and haptophytes (both hacrobian), and euglenid excavates. The secondary plastids show the membrane number and whether nucleomorphs are present, and the color indicates whether they are derived from green or red algae. Plastids in apicomplexans and perkinsids are derived from a red alga, but are shown in white to indicate they are now all nonphotosynthetic.

PAULINELLA

The long debate over whether plastids arose from a single primary endosymbiosis has focused almost exclusively on the plastids of glaucophytes, red algae, and green algae, but one strange and fascinating amoeba has much to say on this issue. *Paulinella chromatophora* was characterized more than a century ago (85, 100). It is a euglyphid testate amoeba (a shelled rhizarian amoeba) but is different from its kin in possessing two bean-shaped photosynthetic compartments called chromatophores. Chromatophores are vertically inherited and tied to host division (75, 76). These were recognized as functionally like plastids but more closely resemble the cyanobacterium *Synechococcus* (75, 76), which was borne out by molecular analyses (90). The key question of whether the chromatophore was genetically integrated with its host (e.g., are there nucleus-encoded chromatophore-targeted proteins?) was suggested by the absence of certain genes from the chromatophore genome (113), and recent studies suggest that protein targeting may well occur (106, 112, 114). If this is conclusively demonstrated, then there is no argument to not refer to this as a second independent origin of plastids.

Primary endosymbiosis: plastid origin in which a eukaryote takes up a photosynthetic cyanobacterium, resulting in a primary plastid

Transit peptide: N-terminal protein extension that is recognized by the TIC/TOC complexes for posttranslational protein import into plastids

Secondary endosymbiosis: plastid origin in which a eukaryote takes up another eukaryote already containing a primary plastid, resulting in a secondary plastid

beta barrel proteins, such as Toc75) (69, 134), it is most likely that the two membranes of primary plastids correspond to the two membranes of the gram-negative cyanobacterial cell (21). Assuming the endosymbiont was taken up by phagocytosis, this in turn suggests that the phagosomal membrane that would have originally surrounded the new endosymbiont was lost, which was likely an extremely important event in the transition from "food" to "organelle." This transition is often erroneously represented in textbook diagrams of endosymbiosis, which show the cyanobacterium with a single membrane and the outer membrane of the plastid deriving from the phagosome (e.g., 39).

The dominant mode of evolution in the symbiont in the early stages of the integration was reduction, and many genes that were unnecessary in the new intracellular environment were simply lost. However, many other genes were transferred from the symbiont to the host nucleus (25). Once expressed, the products of some of these genes were targeted back to the integrating organelle in a process nearly always mediated by an N-terminal extension called a transit peptide, which is recognized by protein complexes in the outer and inner membranes of the plastid called TIC and TOC (for translocon inner-membrane chloroplast and translocon outer-membrane chloroplast, respectively) (136). Based on the observed presence of *Chlamydia*-like genes in plant and algal lineages, it has also been suggested that the ancestor of the host harbored another bacterial symbiont related to Chlamydiales (63, 105, 142), but there are many interpretations of these data.

Up to this point, the origin of plastids proceeded much as we imagine the origin of mitochondria did, with one important exception: Whereas mitochondria originated very early in eukaryotic evolution and the resulting organelle is still found in all known eukaryotic lineages (132, 148), plastids originated somewhat later. This has one important implication, namely that some eukaryotes had plastids and others did not, which is significant because it creates conditions favoring the spread of plastids between lineages, a process called secondary endosymbiosis. In this case, a primary alga is itself swallowed by another eukaryote and then reduced and integrated within its new host in ways superficially similar to primary endosymbiosis (74, 97). There is, however, one difference that proves critical for how the integration proceeds, which is that the phagosomal membrane surrounding the secondary plastid is not lost, leading to plastids with four membranes: the two original plastid membranes, the plasma membrane of the endosymbiotic alga, and the phagosomal membrane of the secondary host (96). In two lineages, the dinoflagellates and the euglenids, one membrane has been lost (most likely the third from the inside, corresponding to the plasma membrane of the algal endosymbiont, because targeting most obviously requires the other three membranes), resulting in three membranes (23, 107, 137).

As with the primary endosymbiont, many genes were lost from the secondary

endosymbiont and many others were transferred to the host nucleus. Because most genes for plastid-related proteins had already been relocated to the primary algal nucleus, in secondary algae genes for plastid-targeted proteins have generally moved and their products have been retargeted twice (3, 4, 27, 50). However, the retention of the phagosomal membrane around secondary plastids puts secondary plastids on a completely different footing with their host: Primary plastids are in the cytoplasm, but secondary plastids are topologically within the lumen of the endomembrane system. Accordingly, nucleus-encoded plastid-targeted proteins in secondary algae have a bipartite targeting system. Plastid-targeting leaders are composed of a signal peptide (which targets the proteins to the endomembrane system) followed by a transit peptide–like sequence (which targets them to the plastid) (2, 82, 137, 146).

MULTIPLE SECONDARY ENDOSYMBIOSES—BUT HOW MANY?

Secondary endosymbiosis is known to have taken place more than once, but the exact number of secondary endosymbiotic events remains contentious. We know that it occurred multiple times because both green and red algae have been taken up in secondary endosymbiotic events (**Figure 2**) (74, 81, 97). From an evolutionary perspective, this is fortuitous because it allows us to compare the effects of multiple independent secondary endosymbiotic events to differentiate general principles from isolated events. In the case of green algal symbionts, there is now broad consensus from all available evidence that the two lineages with green secondary plastids—the euglenids and the chlorarachniophytes—acquired these plastids independently. Indeed, phylogenetic trees based on whole plastid genomes also show that they are not specifically related within the green algae (126), and the hosts are also phylogenetically distant: Euglenids are related to trypanosomes in Excavata, whereas chlorarachniophytes are cercozoan amoebae in Rhizaria (**Figure 1**) (9, 73, 80, 87). The membrane structures of these green plastids are also different: Euglenid plastids have three membranes, whereas chlorarachniophyte plastids have four and also retain a relict green algal nucleus called a nucleomorph (43), which is absent in euglenids.

The evolution of red algal secondary plastids is more contentious, mostly because the host phylogeny is still unsettled but also because there are more lineages and likely more ancient events to consider. Red secondary plastids are found in cryptomonads (which also contain a red nucleomorph), haptophytes, stramenopiles, and dinoflagellates, all of which are also characterized by the unique presence of chlorophyll *c* (22, 71). Cryptomonad, haptophyte, and stramenopile plastids also share a common structure with four membranes, the outermost of which is connected to the endomembrane and the nuclear envelope (23, 24), but dinoflagellate plastids are bounded by three membranes (107). The last group with a red algal plastid is the apicomplexans, a lineage of obligate intracellular parasites including important pathogens (e.g., *Plasmodium* and *Toxoplasma*). The discovery of the four-membrane apicomplexan plastid nearly 20 years ago (83, 98, 150, 151) and the ensuing debate over whether it derived from a green alga or a red alga (150) catalyzed advances in the field by accelerating comparative plastid genomics. This debate was ultimately resolved by the discovery of new lineages of still-photosynthetic apicomplexan relatives (68, 103) (see below), underscoring the importance of continuing field-based species discovery.

The debate over even this most basic feature of apicomplexan plastid evolution is a relatively simple problem compared with the larger issue of how many endosymbiotic events are needed to explain the whole diversity of red secondary plastids. In its current form, this debate centers on two independent but potentially compatible hypotheses: the hacrobian hypothesis and the chromalveolate hypothesis. Although both are extremely contentious, they serve a useful purpose as null hypotheses.

Bipartite targeting peptide: leader on proteins targeted to secondary plastids, consisting of a signal peptide followed by a transit peptide

Signal peptide: N-terminal protein extension that is recognized by the signal recognition particle and cotranslationally inserted into the endomembrane system

THE HACROBIAN HYPOTHESIS

The core point of the hacrobian hypothesis (116) is that two major algal lineages, the cryptomonads and the haptophytes, share a common ancestor, as do their plastids. This idea stemmed from the shared unique presence of a horizontally transferred rpl36 in the plastids of cryptomonads and haptophytes (125) together with phylogenomic analysis that united these lineages in both nuclear and plastid trees (49, 68, 119). These data appeared to offer a well-supported and consistent view, but this concept has since become more complex.

First, a number of heterotrophic lineages were added to this hypothetical grouping based on nuclear phylogenomic analyses (16, 18): the katablepharids, telonemids, centrohelids, and biliphytes [the biliphytes, or picobiliphytes, were originally described as a photosynthetic taxon (111), but single-cell genomic data now suggest that they lack plastids (155)]. The strength of these associations is variable, with only the katablepharids being unquestionably related to cryptomonads (18, 77, 116). The absence of plastids in at least some of these lineages (18, 155) complicates the simple view that the hacrobian plastids are monophyletic. Second, recent phylogenomic analyses of nuclear genes have called the monophyly of the host lineages into question. Specifically, and in contrast to initial analyses that supported the hacrobians as a group (49, 119), recent analyses split them into two subgroups: the cryptomonads (together with the katablepharids and perhaps the biliphytes) and the haptophytes (perhaps together with telonemids and centrohelids), with the cryptomonads branching with archaeplastids without significant statistical support (18). This conflicts with plastid phylogenies, which put cryptomonads and haptophytes together with strong support (68).

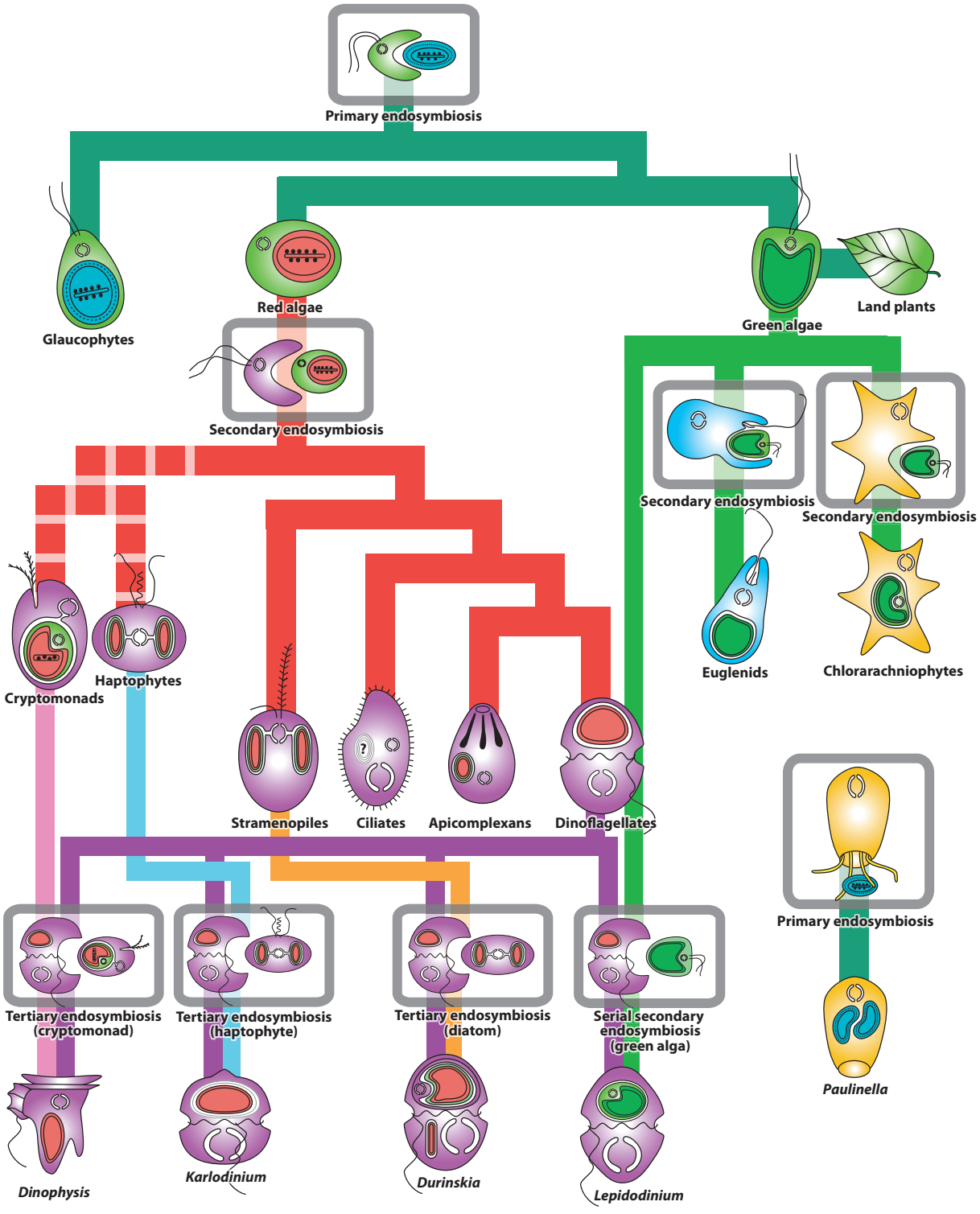
Altogether, the hacrobian hypothesis remains unresolved, and—getting back to the core issue of the common ancestry of cryptomonads and haptophytes and their plastids—the implications of potentially different signals from plastid and nuclear sequences require further investigation. All of this focuses attention on the position of the cryptomonads as one of the greater mysteries in the tree of eukaryotes (18).

THE CHROMALVEOLATE HYPOTHESIS

The chromalveolate hypothesis refers to the idea that all red secondary plastids can be traced back to a single endosymbiotic event (23). In practice, this has been tested mostly by examining whether all lineages with red algal secondary plastids (and all lineages demonstrated to be closely related to any of them) are closely related in both plastid and nuclear gene trees. Although this is a good first approach, a better test is to examine whether their plastid-targeting systems are homologous, as was done to test the monophyly of mitochondria and hydrogenosomes (122).

Figure 2

Schematic depicting major endosymbiotic events in plastid evolution. At the top, primary plastids originated through the endosymbiotic integration of a cyanobacterium, resulting in two-membrane plastids in glaucophytes (which retain the peptidoglycan wall between the two plastid membranes), red algae, green algae, and their close relatives, the land plants. Green algae were integrated in two independent secondary endosymbioses, giving rise to plastid-bearing euglenids and chlorarachniophytes (*middle right*). The origin of red algal secondary plastids (*middle left*) is more contentious, in particular regarding whether the cryptomonad and haptophyte plastids (*dashed red lines*) originated from the same endosymbiosis as those of stramenopiles and alveolates (the latter consisting of ciliates, apicomplexans, and dinoflagellates). An additional round of endosymbiosis occurred in dinoflagellates (*bottom*). Serial secondary endosymbiosis involved the integration of another primary plastid (a green alga), resulting in *Lepidodinium*. Tertiary endosymbiosis involved the integration of another secondary alga. The greatest degree of integration involves a haptophyte, but other putative tertiary plastids were acquired from cryptomonads and diatoms. Independent of this entire history, another primary endosymbiotic origin of plastids in the euglyphid testate amoeba *Paulinella* appears to be under way involving a cyanobacterium related to *Synechococcus* (*bottom right*).



The chromalveolate hypothesis has proved to be extraordinarily provocative, but in polarizing the field it has also provoked a good deal of constructive work on the evolution of algal diversity. Many in the field have never accepted the monophyly of these plastids; however, it is useful to discuss both from a historical perspective and because it has emerged as the null hypothesis (like it or not, this group has appeared in most "tree of eukaryotes" diagrams in the past decade, with **Figure 1** being an exception). Moreover, even with much skepticism, there is no single well-supported alternative, and most criticism stems from data failing to support the hypothesis rather than actively supporting an alternative.

The Plastid Lineage and the Chromalveolate Hypothesis

The chromalveolate group was initially proposed to reduce the number of secondary endosymbiotic events as much as possible (23). The plastids also uniquely share the presence of chlorophyll *c*, and cryptomonad, haptophyte, and stramenopile plastids share a strikingly similar membrane topology in relation to the endomembrane and nuclear envelope (22, 24). From molecular data, trees based on whole plastid genomes have generally united cryptomonads, haptophytes, and stramenopiles, especially when analyzing genes related to photosynthesis, which tend to be more conserved (51, 68, 126). But it has been nearly impossible to evaluate how the alveolates (dinoflagellates and apicomplexans) fit into this tree because of the strange nature of their plastid genomes. Apicomplexans, as intracellular parasites, unsurprisingly have highly reduced plastid genomes that lack photosynthesis-related genes (78, 151). Dinoflagellates have even stranger genomes, having moved most of their genes to the nucleus and converted the rest of their genome to small minicircles (47, 156). The upshot is that apicomplexans and dinoflagellates have almost no genes in common, and neither group's plastids are readily comparable to those of other groups.

This dead end was initially sidestepped by analyzing nucleus-encoded genes for plastid-targeted proteins, which served as stand-ins for the plastids themselves. Two enzymes provided the first molecular support for the inclusion of alveolate plastids: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-bisphosphate aldolase (FBA) (35, 54, 120). The phylogenetic null hypothesis for chromalveolate plastid-targeted proteins would be that they are related to red algal plastid-targeted homologs that themselves derived from cyanobacteria. However, chromalveolate GAPDH and FBA show different phylogenetic patterns. Plastid-targeted GAPDH derived from a gene duplication of an ancestral cytosolic copy (35, 54), and plastid FBA in chromalveolates derived from a class of FBA nonhomologous to that found in plastids of green and red algae (120). The history of GAPDH and FBA within the chromalveolate groups is more complex than originally thought (1, 139), however, so how these characteristics relate to the evolution of the organisms is no longer so clear.

A somewhat more satisfying approach became possible in recent years owing to the discovery of new algal lineages that have provided critical new plastid data. A survey of dinoflagellate symbionts of coral isolated two new photosynthetic species, and when their phylogenetic position was determined, they were found to be deep-branching relatives of apicomplexans (103, 115). The plastid genomes of these organisms, *Chromera* and *Vitrella*, were completely sequenced and found to represent a much less reduced and derived state than those of either apicomplexans or dinoflagellates (68). Indeed, these plastids bring together features of both groups (including some nucleus-encoded characteristics, like the proteobacteria-derived RuBisCO formerly restricted to dinoflagellates) and have fewer oddities than any other known alveolate plastid (68). Accordingly, these genomes allowed the first direct test of the common ancestry of alveolate plastids and those of other chromalveolates, and analyses supported the union of alveolate and stramenopile plastids

but only weakly supported the chromalveolates as a whole (68).

It is also noteworthy that the analysis of *Chromera* and *Vitrella* effectively ended one of the most long-standing and confounding debates surrounding the chromalveolates: the red versus green origin of the apicomplexan plastid. Unsurprisingly, the discovery that parasitic apicomplexans have a plastid initiated a debate on what it was doing there. What was surprising was how easy it was to explain its functions (38, 123) but how difficult it was to explain its origins. Although it was generally accepted that the apicoplast originated by secondary endosymbiosis (99, 150), there were conflicting hypotheses as to whether this was a red alga, and possibly related to dinoflagellate plastids (42, 149), or a green alga, arising from an independent endosymbiosis (41, 78). As noted above, apicomplexan and dinoflagellate plastid genomes are effectively impossible to directly compare, so this deadlock was only really resolved by the discovery of *Chromera* and *Vitrella*. Both phylogenetic and character analyses of these genomes together with those of either apicomplexans or dinoflagellates individually have shown beyond any serious doubt that the apicoplast is indeed derived from the same red alga as the dinoflagellate plastid (68).

The Host Lineage and the Chromalveolate Hypothesis

Testing the chromalveolate hypothesis using data from nuclear genomes has proved even more difficult. Based on early single-gene trees (144), concatenations of small numbers of genes (55), and the distribution of the Rab1a GTPase (31), the only consistent feature was the monophyly of the alveolates and stramenopiles. With the advent of large phylogenomic analyses, hundreds of nuclear genes were brought to bear on the question, and one major advance quickly emerged: the inclusion of Rhizaria. Rhizaria is a large group of primarily amoebae and amoeboflagellates of stunning diversity—so diverse, in fact, that the supergroup was established solely on the grounds of molecular phylogenetic data (19, 108) because no unique and common morphological characteristic has been described. The first phylogenomic analyses including rhizarian representatives showed a strong relationship to the alveolates and stramenopiles (19, 20, 49), and this relationship has remained consistent in subsequent studies that include many more data from many additional rhizarian taxa (14, 16–18). Some variations in exactly how these three groups are related have been observed, but the most strongly supported analyses indicate that alveolates and stramenopiles are sisters, with rhizarians branching at their base. This group has not been formally named but is widely known as the SAR (stramenopile-alveolate-rhizarian) clade.

The chromalveolate hypothesis is not substantially altered by the inclusion of rhizarians, because it simply requires one more loss of an ancestral red algal plastid (see below); however, phylogenomic analyses of nuclear data from hacrobian taxa have challenged the hypothesis. As noted above, the first phylogenomic analyses of nuclear data initially strongly supported a union of cryptomonads and haptophytes but did not resolve their relationship with other chromalveolates. Since then, the monophyly of the hacrobians has also been challenged by nuclear gene data (see above), but, more critically, so has their relationship to chromalveolates: In some analyses, haptophytes go with SAR but cryptomonads do not (18), whereas in other analyses neither goes with SAR (7). This debate remains unresolved, because alternative positions have also failed to gain any support, but it can be said that large-scale phylogenomic data from the nuclear lineage fail to support the chromalveolate hypothesis.

WHEN IS AN ENDOSYMBIOSIS HYPOTHESIS PROVEN? THE COMMON ANCESTRY OF THE ALVEOLATE-STRAMENOPILE PLASTIDS

The alveolate-stramenopile group that is supported by all analyses of plastid and cytosolic

data (see above) captures most of the diversity and controversy of the chromalveolate hypothesis. For example, if one accepts that the demonstrably related secondary red plastids of these demonstrably related host lineages share a common endosymbiotic ancestry, then the ancestors of major nonphotosynthetic lineages like ciliates (alveolates) and oomycetes (stramenopiles) must have had a plastid. Indeed, many of the surprising implications of the chromalveolate hypothesis are already manifest in this reduced, "chromalveolate-light" version of the hypothesis, but this relationship and its implications are not widely discussed and the implications are not entirely accepted. This leads to the question, what would it take to prove a common ancestral endosymbiotic origin of such plastids?

Given the consistency of plastid and host phylogenies, there are few specific reasons to question the common ancestry of alveolate-stramenopile plastids. But one issue that does require discussion is the abundance of plastid-lacking lineages within alveolates and stramenopiles and the fact that they appear to be clustered basal to plastid-containing lineages (e.g., ciliates at the "base" of alveolates and oomycetes at the "base" of stramenopiles). This can be interpreted as evidence for two independent plastid origins late in the evolution of each lineage (10, 34). However, any interpretation hinges on a complete understanding of two issues: the distribution itself and the likelihood of events that lead to the distribution.

At first glance, the distribution seems obvious—organisms either have plastids or do not. But this is not so simple, because cryptic plastids have been found in a variety of lineages [e.g., perkinsids and apicomplexans (37, 98, 140, 151)]. The best-studied aplastidal alveolates and stramenopiles [e.g., the best-studied ciliates and oomycetes (30, 142)] really do lack plastids, but most others have not been investigated in enough detail to be sure. At the same time, when interpreting the distribution it is important to think about the number of *events*, and not the number of *organisms* with or without plastids. Many ciliates may lack plastids, but this could represent a single event of plastid loss, and as such is no more unlikely than a plastid loss from a single species.

Examining the distribution of plastid loss events (or putative plastid loss, given that we mostly cannot distinguish loss from severe reduction) on the alveolate-stramenopile tree also reveals that the notion that they are basal is questionable. In the stramenopiles, plastid loss events within plastid-containing lineages are known (as is the photosynthesis loss event), and the number of these events is perhaps equal to the number needed to explain the basal lineages (8, 23, 45, 101, 131). In alveolates the situation is even more unbalanced: Ciliates are not really basal to apicomplexans and dinoflagellates so much as sister to them (**Figure 1**), and plastid loss has happened several times in the lineages leading to apicomplexans (e.g., *Cryptosporidium* and *Colpodella*) and many more times in the dinoflagellates (128). With alveolates and stramenopiles related in both plastid and host trees, why the one additional loss event to account for ciliates should be given any special weight is unclear.

Interpreting the distribution of events also requires weighting the likelihood of those events, but we suffer from a near-complete lack of understanding about the process of plastid loss. Although there has been a great deal of speculation about how plastids originate, little concrete has been said about why or how they are lost or what traces might be left behind. Moreover, there are few cases where we can be certain of the two prerequisites for concluding that plastid loss occurred: that an organism's ancestor had a plastid and that that organism now does not—one or both of which are often murky questions.

Lastly, if one accepts the phylogenetic relationship between both host and plastid components of the alveolates and stramenopiles, then what is the alternative to concluding that their common ancestor had the same plastid? The only alternative model that is consistent with the data at face value is that the plastid originated in one lineage and was subsequently transferred to the other by a process called

tertiary endosymbiosis, which is discussed below.

TERTIARY AND SERIAL SECONDARY ENDOSYMBIOSIS

Tertiary and serial secondary endosymbioses add yet more layers of associations after secondary endosymbiosis. Both are known only in dinoflagellates, which ancestrally had a red secondary plastid, but a few lineages seemingly lost or reduced this plastid and either took up another secondary alga to form a tertiary plastid or took up another primary alga to form a serial secondary plastid.

Serial secondary endosymbiosis is generally thought to have occurred only once, represented by the dinoflagellate genus *Lepidodinium*, where the ancestral red algal secondary plastid has been replaced by a green algal one (147). The *Lepidodinium* plastid genome confirms its ancestry from a chlorophyte, and analysis of its nucleus-encoded plastid-targeted genes shows that it acquired genes from a variety of sources (94, 102).

Truly unambiguous tertiary endosymbiosis is only really known in one lineage of dinoflagellates, represented by the genera *Karenia* and *Karlodinium*, which have lost their ancestral plastid and acquired a new one from a haptophyte (141). The haptophyte is completely reduced so that all that remains is the plastid, but unfortunately the structure of the plastid is poorly known, so the number and nature of the membranes are uncertain. Neither is the nature of protein targeting known, but, intriguingly, the targeting peptides include a signal peptide (indicating the plastid is in the endomembrane system) followed by a sequence with little or nothing in common with any known transit peptide (121). It appears that this system uses a targeting mechanism with at least some unique elements, and it would be a worthwhile system to use in studying possible alternative solutions to the challenges of protein targeting.

Two other dinoflagellate lineages have undergone similar processes, but neither is quite as straightforward. *Dinophysis* has what may be a tertiary plastid derived from a cryptomonad (130), but there remains some debate as to whether it is totally integrated or a transient residue of feeding called a kleptoplast (48, 138). A different situation is again found in another lineage of dinoflagellates, represented by *Kryptoperidinium* and *Durinskia*, which have acquired a plastid from diatoms (26, 29). There is no question that these are permanent and completely integrated into the dinoflagellate host cell cycle, but in this case the diatom retained its own nucleus and even intact mitochondria (29, 65). It is unclear whether there is any integration at the level of gene transfer and protein targeting; if so, they defy how organelles are most commonly defined.

Although tertiary endosymbiosis may be evolutionarily rare and restricted to a handful of dinoflagellate lineages, it is nonetheless a potentially important process for our understanding of organelle integration. This is the case because tertiary endosymbionts offer two practical advantages as model systems. First, because the events took place relatively recently, they might offer intermediate stages in the integration process, and fewer clues will have been erased by time, as is inevitable with more ancient endosymbioses. Second, the phylogenetic identities of both hosts and endosymbionts are well known (26, 66, 67, 141). This is important in allowing the unambiguous identification of endosymbiont-derived genes, and in testing hypotheses about the endosymbiont's genetic contribution and the order of events that take place during the integration process (see below).

A better understanding of known cases of tertiary endosymbiosis is also potentially important to explain incongruences between plastid and nuclear gene phylogenies. We currently have an unsatisfying situation where one gets the sense that many people working in the field believe that certain secondary plastids might really be cryptic tertiary plastids, but there are strikingly few cases where this has been specifically spelled out in the literature, and even those attempt to explain different plastids for different reasons (5, 12, 154). These hypotheses generally stem not from actively contradictory

phylogenies but rather from phylogenies that fail to resolve the position of the host lineage. Tertiary endosymbiosis has also never been observed outside of dinoflagellates, and even there it is rare, so extending it to other lineages should be based on observed similarities. However, what we do know about the nature of tertiary plastids is inconsistent with what we know about secondary plastids: For example, tertiary plastids do not appear to possess plastid membrane structures or plastid-targeting systems like those of secondary plastids (29, 121).

So although cryptic tertiary endosymbiosis remains a formal possibility to be explored with actual data, it is not a quick fix. Known tertiary plastids need to be studied in much greater detail, and direct evidence for contradictions between plastid and host phylogenies that point to a specific tertiary event needs to be tested. Most important, the evolutionary origins of plastid-targeting systems should be explored in much more detail. Neither phylogenies of host genes nor those of plastid genes can formally disprove the monophyly or polyphyly of chromalveolates: Phylogenies that favor cryptic tertiary endosymbioses can infinitely account for monophyletic plastids, whereas those that favor plastid loss can infinitely account for polyphyletic (or paraphyletic) hosts. Targeting systems might offer the clearest data on the origins of diverse plastids.

THE PROCESS OF ENDOSYMBIOSIS AND PLASTID ORIGINS

It is easy to say plastids originated by "endosymbiosis," but we seldom ask what that really means. There are many possible ways to integrate two cells, and as is often the case with evolution, the order of events in a transition is critical to the details of its outcome. Below, I question several common assumptions and their implications.

How Much Does an Endosymbiont Contribute to Its Host?

It has long been clear that the genetic contribution of an endosymbiont to its host goes beyond just genes for proteins that are targeted back to the plastid (e.g., 93), but exactly how significant this additional contribution may be is a major question. This issue becomes more complex in secondary (and tertiary) endosymbioses, because nonplastid genes ultimately derived from the cyanobacterial symbiont may have been acquired in addition to genes from the endosymbiont (green or red algal) nuclear lineage. Understanding this process is key to both how we understand the process of endosymbiosis and how we reconstruct its evolutionary history. This is because the existence of significant numbers of such endosymbiotic genes in the host nuclear genomes have been not only inferred but also used as evidence for a number of evolutionary scenarios that fall broadly into two categories: that known endosymbiotic events may be older than they seem, and that other cryptic endosymbiotic events also took place. These are reviewed in the next two sections.

How old are known secondary symbioses?

It has been argued that a number of nonphotosynthetic lineages were once photosynthetic, based on the inferred presence of nucleus-encoded genes believed to be derived from a now-lost or cryptic symbiont (37, 95, 124, 133, 142). Typically, the nonphotosynthetic lineage is closely related to an algal group, so the argument is not for a previously unrecognized endosymbiosis but rather for a known endosymbiosis having taken place earlier than previously thought. It is also important to note that these genes are typically not argued to function in an existing plastid, or even to have once functioned in a plastid; instead, they are thought to be simple genes with some phylogenetic affinity to cyanobacteria, plastids, or the nuclear lineage of a secondary symbiont (i.e., "red" or "green").

One of the first such suggestions was that the largely parasitic kinetoplastids (e.g., *Trypanosoma*) had once contained the green algal–derived plastid still found in their sister group, the euglenids (53, 92). This was based on the conclusion that genes in trypanosomes showed some kind of relationship to "green" genes in phylogenetic reconstructions.

However, closer inspection of most of these genes showed that they did not support this conclusion (i.e., the trypanosome gene was not actually closely related to a green homolog) or were misleading owing to poor sampling (32, 127). In addition, this ancient origin of the green plastid was not supported by the biology and distribution of plastids within the euglenid lineage, which strongly suggested that the plastid was acquired relatively recently, probably owing to the evolution of eukaryotrophy occurring within the diversification of euglenids rather than before (86).

The discredited idea that trypanosomes once contained a plastid should be kept in mind when interpreting such phylogenetic data, because a number of similar cases have been made for various chromalveolate subgroups. As noted above, the chromalveolates include a number of nonphotosynthetic lineages. Within the framework of the chromalveolate hypothesis (or the "chromalveolate-light" hypothesis), the ancestor of these lineages once contained a red algal plastid. In what appeared to be a dramatic consequence of this symbiosis, the first genome of an oomycete was reported to contain more than 700 symbiont-derived genes (142), a substantial proportion of the genome. The first analysis of a ciliate genome concluded that no significant number of plastid genes were to be found in *Tetrahymena* (30); however, a reanalysis concluded that *Tetrahymena* and *Paramecium* do in fact contain symbiont-derived genes, although only 16 were identified (124). Similarly, genomes of the apicomplexan *Cryptosporidium* and the nonphotosynthetic dinoflagellate *Crypthecodinium* were found to contain genes phylogenetically inferred to be derived from the red lineage (64, 129).

Two other cases—the deep-branching and nonphotosynthetic dinoflagellate relatives *Perkinsus* and *Oxyrrhis*—stand out because in both genera, putatively plastid-derived genes were found but were also suggested to be targeted to cryptic plastids. In the case of *Oxyrrhis*, this was based on the observation that some genes that were phylogenetically related to plastid-targeted genes in other dinoflagellates still retained the distinctive N-terminal plastid-targeting leaders (133). In *Perkinsus*, genes encoding N-terminal leaders were also identified (37, 46, 95), but protein products of some genes have also been localized to discrete membrane-bounded structures—altogether constituting strong evidence for the maintenance of a relict plastid, which in this case appears to be involved in isoprenoid biosynthesis (37, 46, 95).

The strength of evidence for these conclusions varies dramatically, and unfortunately, the most interesting are supported by the weakest evidence. In lineages where their phylogenetic position already makes a strong case for an ancestral plastid, the evidence for relict plastid genes is relatively strong. For example, in *Perkinsus*, where there is now even evidence for the organelle, and in *Oxyrrhis* and *Crypthecodinium*, where the genes in question are actually related to plastid-targeted homologs, a plastid-containing ancestor can justifiably be concluded. In the major nonphotosynthetic oomycete and ciliate lineages, however, the genes in question are generally not obviously related to plastid function. If these genes are to be interpreted in the context of the chromalveolate symbiosis, then there is a critical assumption with implications that are seldom recognized: specifically, that the red algal ancestor donated nucleus-encoded cytosolic proteins to its new host. The implication of this is that such transfers must have happened in the early stages of the symbiosis, and the genes would have acquired a function that led to their retention in the ancestor of all chromalveolates. Accordingly, genes that are truly red algal relicts of the chromalveolate symbiosis should be widespread within extant chromalveolates, including the photosynthetic ones. In other words, if the red genes in these nonphotosynthetic lineages are compared, many should be the same genes, they should be related to one another, and they should have homologs in other chromalveolate groups. Alternatively, a lack of significant overlap in the red genes identified in different lineages would suggest that the current pattern is unrelated to the possible presence of an ancestral endosymbiont. Remarkably, however, these

results have not been examined as a whole, so the degree of overlap is unknown.

All this leads to the question, how many genes does it take to distinguish between horizontal gene transfer and endosymbiosis? Most of the phylogenies that have been suggested to support an influx of genes from secondary endosymbiont nuclei to the host nuclei (apart from those that encode proteins actually targeted to the plastid) appear to be based more on phylogenetic noise than on signal, and where detailed analyses have been done, relatively few putatively endosymbiont-derived genes remain (15, 28). Without more information on the overall proportion of genes from these genomes that appear to be phylogenetically incongruent with the known position of the host, it is impossible to say whether these genes represent a legitimate spike in phylogenetic signal indicative of some relict of the endosymbiotic event, or background noise from unresolved phylogenies and low-level horizontal gene transfer.

Were there other cryptic secondary endosymbioses? There is a second issue that has emerged from similar data, which is the question of whether there were older cryptic endosymbiotic events that took place prior to the establishment of extant plastids in photosynthetic lineages. Specifically, a number of analyses have suggested that the red plastid in chromalveolates was preceded by a green one, which has since been lost except for some relict nuclear genes. This idea goes back to early genomic surveys of plastid-containing chromalveolates where genes for a number of plastid-targeted proteins were found to be phylogenetically green rather than red (40, 50, 109, 145). At the same time, phylogenomic analyses began to reveal the now-accepted relationship between rhizarians, alveolates, and stramenopiles (see above). This is significant because chlorarachniophytes are rhizarians, and they have a secondary plastid of green algal origin that contains many genes for plastid-targeted proteins derived from the red lineage (3). Ultimately, whole-genome analyses of diatoms concluded that a large contingent of green genes were encoded in the diatom genome (104), and similar results were found for a photosynthetic relative of apicomplexans, *Chromera*, where the phylogenetic signals for green and red genes were concluded to be approximately equal, at 250 genes each (152).

Once again, however, if these green genes in chromalveolates were derived from an ancient cryptic green plastid, we would expect the various chromalveolate lineages to retain many of the same green genes today, but this has not been demonstrated. Most important, however, a careful reanalysis of the green genes has also suggested that the contribution may be dramatically overestimated by automatic phylogenetic analyses. In the case of the diatoms, reanalysis of the 1,700 reported green genes showed that only a handful could be confidently attributed to the green lineage (28). The remainder were found to be poorly resolved, missing key taxa (such as red algae), or more probably a result of horizontal gene transfer. Similarly, in the case of *Chromera*, the original study pointed out that the green signal is best interpreted as the manifestation of artifacts due to many more data being available for green lineages than red (152); a reanalysis using a slightly different automated search strategy and manual inspection of the trees went even further and concluded that only 23 genes were clearly red and 9 clearly green (15). Overall, the data supporting a cryptic green plastid in the evolution of chromalveolates are rapidly diminishing. Indeed, at this point, the data are indistinguishable from a small number of horizontal gene transfer events, leading to the prediction that similar levels of foreign genes that are phylogenetically affiliated with other lineages will also be found in these genomes.

Which Comes First, Gene Transfer or Cellular Fixation?

Although we all might agree that endosymbiosis requires that a symbiont be ingested, be permanently retained by the host, transfer genes to the host, and evolve a protein-targeting system, digging into the specifics shows that the order

in which these events take place is not so obvious. Though generally unstated, the textbook view of endosymbiosis also implies that plastids originate rather suddenly—a heterotroph ingests an algal cell but fails to digest it. Such autotrophy-by-indigestion might be shocking for both parties, so it is reasonable to compare the implications of the order of events to see what else might result in a fair description of endosymbiosis. Below are two of several possibilities, in an attempt to show how changing the hypothesized order of events changes both the nature of the transformation and our expectations about how the cells are affected.

Endosymbiosis as a rapid transition. In this model, a heterotrophic predator engulfs a photosynthetic prey cell, but rather than being digested, the autotroph is retained and survives within the heterotroph. This singular event sets off a long-term transformation of both cells, but primarily of the autotroph. One could imagine it adapting to its intracellular environment by dividing with its host cell, losing functions that are now redundant or unnecessary (like motility), and evolving transporters to exchange nutrients and energy with the host. Over time, genes flowing from the endosymbiont to the host (perhaps by lysis, if there are multiple symbionts within a host) find their way into the nucleus and are incorporated into nuclear chromosomes and expressed on cytosolic ribosomes.

The most complex step in such a scheme would be the establishment of a protein-targeting system. In primary endosymbionts, this corresponds to transit peptides and the TIC/TOC system; the origin of this system is obscure, but it appears that some parts come from the host and others from the endosymbiont (6, 70, 88). In secondary endosymbionts, the second half of the protein-targeting system (based on transit peptides and the TIC/TOC system) is already in place in the endosymbiont. The first step requires sorting to the endomembrane mediated by a signal peptide, a system already in place in the host. The major hurdle in secondary endosymbiosis would therefore be to link these two targeting systems together, which requires sorting proteins that are already in the endomembrane system specifically to the plastid. It is now clear that in all secondary plastids, this step is dependent on information encoded in the transit peptide (2, 44, 57, 59, 60, 79, 82) and some protein complex that mediates passage across the third membrane. In chromalveolates, the protein complex is the symbiont-specific ERAD-like machinery (SELMA) system, which is derived from the endoplasmic reticulum–associated degradation (ERAD) complex (13, 36, 56, 135). At least in chlorarachniophytes, some other solution seems to have been found, because no plastid-specific SELMA-like system is encoded in the genome (58). It is also unclear how the loss of the third membrane in euglenids and dinoflagellates would have affected this step, and their transit peptides are notably different.

The existence of a system to specifically import proteins from the cytosol to the endosymbiont sets up an evolutionary ratchet because it allows an increasing number of proteins to be targeted relatively easily. Even if the rate of transfer from symbiont to host remains unchanged, the rate at which host-encoded genes make endosymbiont-encoded homologs functionally redundant would increase dramatically simply because there is a mechanism to target the products of transferred genes back to the organelle. At the genomic level, the expected outcome of this order of events is that the host nucleus contains a large number of symbiont-derived genes, all of which should share a common phylogenetic signal tracing back to a single endosymbiont.

Endosymbiosis as a slower transition and the importance of transporters: the targeting-ratchet model. Although there is nothing impossible about the above order of events, subtle changes that lead to different expectations are just as plausible, or arguably more so. Consider, for example, the implications of establishing protein targeting *before* the endosymbiont is permanently fixed in the host. In this model (**Figure 3**), a grazing heterotroph begins to transiently retain photosynthetic

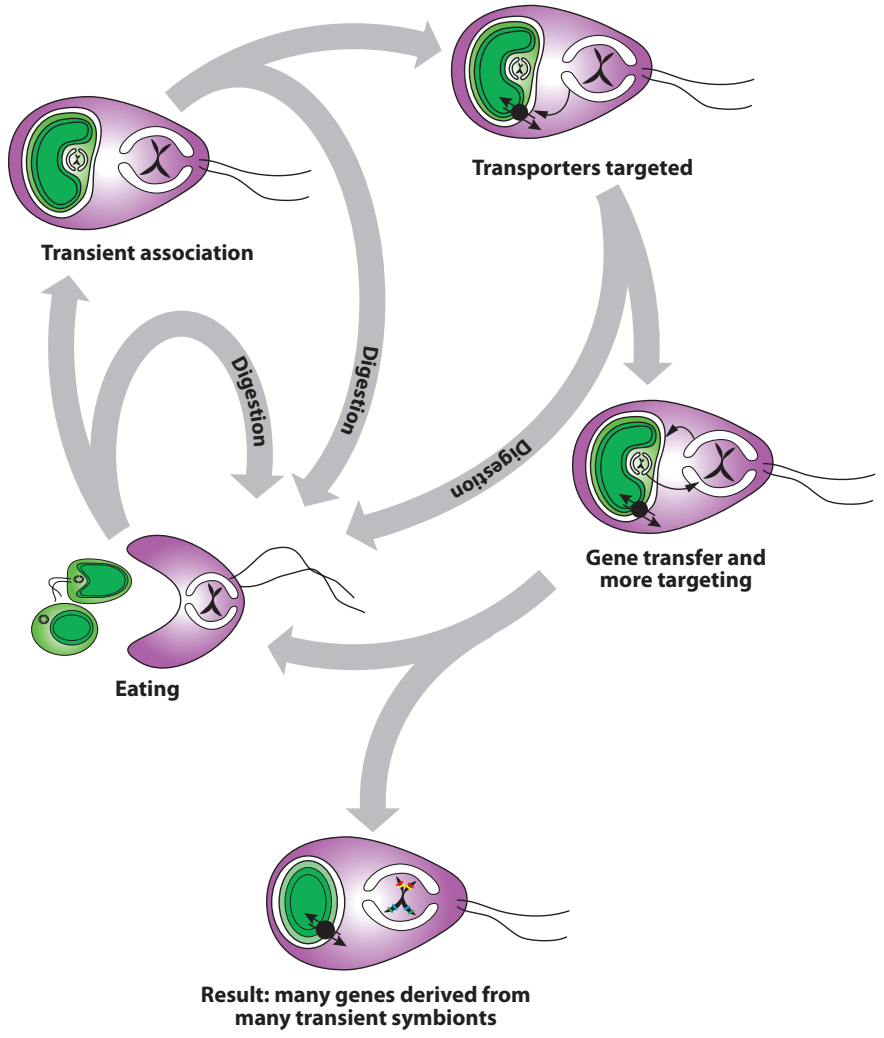


Figure 3

A targeting-ratchet model for the endosymbiotic origin of plastids as a long-term cyclic association with photosynthetic food. This illustration shows a secondary endosymbiosis, but a similar model could be developed for primary or tertiary endosymbiosis. In the cyclic portion (*top*), a heterotrophic predator (*purple*) eats and digests various algal prey (*bottom left*). Over time, the host predator begins to transiently retain prey cells before digesting them (*top left*). For longer associations to benefit the host, it would need to acquire energy from photosynthesis without digesting the prey. This could be achieved by specifically targeting transporter proteins to membranes of the transient symbionts, allowing nonlethal energy and nutrient uptake (*top right*). The existence of this targeting creates a ratchet, allowing an increasing number of proteins to be targeted to transient symbionts, which in turn allows proteins encoded by genes acquired from symbionts to be targeted to subsequent symbionts (*bottom right*), driving the integration process. Ultimately, the retention period lengthens to the point that one particular food alga (or many within a population) is never digested and goes on to become the plastid organelle. However, the history of gene transfer and targeting from transient symbionts means that "algal" genes in the host are not necessarily all derived from the same symbiont as the plastid (indicated by the many colored genes on the host chromosome), even if they encode plastid-targeted proteins.

prey before digesting them [as is known to occur in nature (33, 52, 72, 89)]. Over time, the retention period could increase as the host develops control over signaling degradation. But if the predator has no means of extracting the energy and nutrients of its meal without digesting it, then there is no advantage to increasing the retention time. To reap the full benefits of prolonging such an association, it would need transporters that could extract the energy and nutrients from the transient symbiont without digesting it.

The importance of transporters in the origin of plastids has been recognized (143) but is typically overshadowed by the importance of protein targeting (11, 23, 91). Both are important, but their evolutionary origins might also be tied to one another: If the predator can target transporters to its prey, it would not only have greater, nonlethal access to the energy produced by photosynthesis, but would also set up a powerful evolutionary ratchet where the acquisition of additional genes from transient symbionts could provide positive feedback if the targeting of their products prolongs the useful retention of and/or control over symbionts. In other words, targeting would ratchet the system toward fixation, but fixation does not necessarily ratchet toward establishing targeting. This cycle could continue with transient symbionts remaining in the cytoplasm for increasing periods of time before being digested and with the collection of host-encoded genes for symbiont-targeted proteins growing in the nucleus, until the process is ultimately fixed when some prey cell is never digested and becomes the organelle.

This targeting-ratchet model shares several steps in common with Larkum's "shopping bag" model for plastid evolution (62, 84) and shares Tyra's emphasis on the early importance of transporters in plastid origins (143). But the evolutionary link between targeting and transporters (which can be applied to primary or secondary plastids, or to mitochondria, for that matter) and the ratchet it creates early in the process provide a plausible first push toward genetic integration.

The order of events also has other implications at the cellular and genomic levels. At the cellular level, it suggests that the plastids within an algal lineage might derive from different symbionts. In practice, this would be difficult to recognize unless very different kinds of algae were fixed in a related lineage of hosts, but there is one intriguing case. The diatom-derived tertiary plastids are constrained to a small group of related dinoflagellates, but molecular phylogenies have shown that the plastids are derived from both pennate and centric diatoms. They might have been serial replacements (61) but might also have been fixed independently in different but closely related diatom-eating dinoflagellate hosts.

At the genomic level, it is possible that the genes for plastid-targeted proteins within a single cell need not all be derived from the same lineages as the organelle to which they are targeted [as pointed out in the shopping bag model (62, 84)]. If the host has a narrow prey preference, then the distinction might be impossible to discern, but if the host is grazing on a wide variety of prey, then genes for plastid-targeted proteins would be expected to be derived from an equally wide variety of sources. Interestingly, most secondary algal lineages have now been observed to contain a substantial number of genes for plastid-targeted proteins that are clearly not derived from the same lineage as the plastid itself (3, 40, 50, 109, 110). Generally this is attributed to a single cryptic endosymbiosis, or more realistically to horizontal gene transfer. Indeed, it is probably impossible to entirely distinguish the process outlined above from horizontal gene transfer, but it is worth noting that random horizontal gene transfer should continue throughout the evolution of a given lineage, so we should see such genes shared by all members of a lineage in some cases (an ancient origin) but also narrowly distributed in other cases (a more recent origin). Either way, components of key plastid pathways come from different lineages, but whether these were overlaid on ancestrally homogeneous pathways by horizontal gene transfer or represent a gradual building up of the plastid proteome from a slow

and cyclic integration of different prey cells is an interesting question to ponder. At the same time, it is worth remembering that each primary, secondary, and tertiary plastid origin is a unique event, so different models might best explain different plastid endosymbioses.

CONCLUDING REMARKS: WHAT ARE THE NEXT OBVIOUS QUESTIONS?

Investigation of the complex evolutionary history of plastids has benefited hugely from successive technical revolutions that have made sequence analysis of diverse lineages at the genomic level possible. Indeed, we may even be approaching the point where we have substantially characterized the data available to us from representatives of all major algal lineages. However, if recent years have revealed any blind spots in this field, it is our ignorance of the natural diversity of these lineages, and particularly the oddballs that branch close to them. The discovery of photosynthetic relatives of apicomplexans (103), for example, has allowed formerly impossible leaps forward in our understanding of this chapter in plastid evolution. Equally illuminating new nonphotosynthetic relatives of algal lineages have also been identified (111, 116–118, 153, 155). Although less appreciated, each of these discoveries has tremendous potential to upset our established views of plastid evolution. At this point, the greatest potential for transformative change is from a renaissance of natural history and species discovery.

SUMMARY POINTS

1. Plastids, or chloroplasts, originated by the endosymbiotic uptake of a cyanobacterium leading to primary plastids (like those in plants and green and red algae) but then spread to other eukaryotic lineages through progressive rounds of secondary and even tertiary endosymbiosis.
2. The evolutionary history of these subsequent endosymbiotic events is becoming clearer, but there is still controversy over the number of times secondary red algal plastids originated.
3. During endosymbiosis, many genes are transferred from the symbiont to its new host, but the extent of this contribution (beyond genes for plastid proteins) is unclear. Recent claims for substantial numbers of "algal" genes in host nuclei now appear to be the result of sampling biases and difficulties with automated phylogenetic analyses.
4. In one model of plastid origins, protein targeting evolves early in the process to target transporters to transient photosynthetic symbionts; once established, it creates a gene transfer ratchet that leads to progressive integration and finally fixation of the organelle in the cell.

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Annual Review of Plant Biology
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Contents

Indexes

Errata

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