

Tracing the Thread of Plastid Diversity through the Tapestry of Life

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ABSTRACT: Plastids (chloroplasts) are endosymbiotic organelles derived from previously free-living cyanobacteria. They are dependent on their host cell to the degree that the majority of the proteins expressed in the plastid are encoded in the nuclear genome of the host cell, and it is this genetic dependency that distinguishes organelles from obligate endosymbionts. Reduction in the size of the plastid genome has occurred via gene loss, substitution of nuclear genes, and gene transfer. The plastids of Chlorophyta and plants, Rhodophyta, and Glaucocystophyta are primary plastids (i.e., derived directly from a cyanobacterium). These three lineages may or may not be descended from a single endosymbiotic event. All other lineages of plastids have acquired their plastids by secondary (or tertiary) endosymbiosis, in which a eukaryote already equipped with plastids is preyed upon by a second eukaryote. Considerable gene transfer has occurred among genomes and, at times, between organisms. The eukaryotic crown group Alveolata has a particularly complex history of plastid acquisition.

Keywords: plastid, chloroplast, endosymbiosis, phylogeny, rubisco, apicomplexa.

Photosynthesis in eukaryotes is carried out by plastids, which are endosymbiotic organelles derived from cyanobacteria (Mereschkowsky 1905). The most familiar kind of plastid is the plant plastid, but these represent only a tiny portion of the plastid diversity found among other photosynthetic eukaryotes (see Van den Hoek et al. 1995 for an overview of the algae). A great diversity of algal plastids is evident by any measure, including structure, pigmentation, gene content, and DNA sequence divergence. Plastids are also the site for a number of biochemical tasks other than photosynthesis, including fatty acid and amino acid biosynthesis, and not all are pigmented (Taiz

and Zeiger 1991). Hence, the general term “plastid” is more appropriate for the purposes of this article than is “chloroplast,” which refers to photosynthetic plastids, particularly those of green algae and plants. The derivation of plastids from cyanobacteria is not in serious question, although, through force of habit, this is often referred to as the endosymbiotic hypothesis. But while the cyanobacterial origin of plastids is well established (Delwiche et al. 1995), fundamental questions in plastid evolution, including the number of independent origins of plastids, the mechanisms by which the plastid was permanently incorporated into the host cell, and the relationships among different plastid lineages, remain active areas of study (Delwiche et al. 1995; Gray and Spencer 1996; Van de Peer et al. 1996; Douglas 1998; Martin et al. 1998; Palmer and Delwiche 1998).

The most dramatic feature distinguishing plastids from free-living cyanobacteria is the tremendous reduction in the size and gene content of plastid genomes (Martin and Herrmann 1998; Palmer and Delwiche 1998). While the genome of the cyanobacterium *Synechocystis* PCC 6803 is 3,573 kb and contains about 3,200 genes (Kaneko et al. 1996), that of the plastid of the red alga *Porphyra purpurea* is only 191 kb and contains roughly 250 genes (Reith and Munholland 1995). The genomes of plastids on the green lineage that includes those of land plants are even more reduced; the liverwort *Marchantia polymorpha* has a plastid genome of 121 kb and contains 120 recognized open reading frames (Ohyama et al. 1986; Shinozaki et al. 1986). This is comparable to—or somewhat more complex than—the plastid genomes of other land plants. It is this reduction in the plastid genome, and the concomitant absolute dependency of the plastid on the host cell, that distinguishes an endosymbiotic organelle from an obligate endosymbiont (Cavalier-Smith and Lee 1985). The plastid is not, however, as highly reduced in terms of protein complexity as the reduction in the size of the plastid genome would suggest (Emes and Tobin 1993). Estimates of the number of proteins in plastids range from 500 to 5,000—in any case, a number that greatly exceeds the complexity of any known plastid genome (Martin and

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Herrmann 1998). Those plastid proteins not encoded in the plastid genome are encoded in the nuclear genome and must be targeted to the plastid.

Three mechanisms underlie this reduction of the plastid genome; gene loss, substitution, and transfer. First, in the case of gene loss, genes that no longer confer a selective advantage in the endosymbiotic environment may be lost outright. A probable example of such a loss is that of the cyanobacterial cell wall, which is entirely lacking in most plastid lineages (but see comments on the plastids in glaucocystophytes in "Three Lineages of Primary Plastids").

In the second case, that of gene substitution, a gene originally resident in the nuclear genome and expressed in the cytosol may also have its product targeted to the plastid. In such a case, the plastid and cytosolic versions of the protein are closely related and may be identical and transcribed from a single locus, and, consequently, they are difficult to trace individually. For this reason, relatively little information is available about this mechanism of gene replacement, but it is likely to have had a significant role in plastid evolution (Martin and Schnarrenberger 1997).

The final mechanism for reduction of the plastid genome is gene transfer. In this case, a plastid gene probably first undergoes a duplication event that results in a second copy of the gene resident in the nuclear genome, a process that is in at least some cases RNA mediated (Nugent and Palmer 1991). If the gene product from the nuclear copy is then targeted into the plastid, the result is two redundant loci, one in the plastid genome and one in the nuclear genome. Because these loci are redundant, selection will act to maintain one functional copy of the gene, but it is likely that one of the two will eventually undergo deleterious mutations and ultimately be lost entirely. If this occurs in the plastid genome, the gene has been transferred from the plastid to the nuclear genome. Note that such peripatetic genes are not limited to the nuclear genome; for example, sequences originally of plastid origin have also been detected in the mitochondrial genome of plants (Nugent and Palmer 1988).

For a plastid gene to undergo successful transfer from the plastid to the nuclear genome, it is essential that the gene product be targeted back to the plastid. This is effected by transit peptides, polypeptide leaders that direct the gene product to the appropriate compartment. Plastids and mitochondria have distinct transit peptides, such that gene products are selectively targeted to one or the other endosymbiotic organelle. But, remarkably, the transit peptides of both red algal and land plant plastids seem to be functionally interchangeable (Apt et al. 1993; Jakowitsch et al. 1996). The two membranes of the primary plastid have often been interpreted as corresponding to a single bacterial membrane and a food vacuolar membrane. However, Cavalier-Smith (1987) has suggested that these two

membranes could also represent the two membranes of the gram-negative cyanobacterial cell. This view is bolstered by the recent determination that Toc75, a key component of the plant plastid's protein import apparatus, is homologous to a cyanobacterial secretory protein, both localized to the outer plastid envelope (reported in two papers submitted within 5 d of each other: Bölder et al. 1998; Reuman et al. 1999). It is presumably possible for changes to occur in the localization of membrane proteins. However, the elaborate membrane structures present in some algae, particularly those with secondary plastids, implies that such changes do not occur very easily. Thus, the location of Toc75 may be a clue to the origin of the two membranes of the primary plastid. Toc75 seems to be an essential protein and is present in all fully sequenced gram-negative bacterial genomes to date, which suggests that this would be a useful protein to examine in Rhodophyta, Glaucocystophyta, and other algal groups.

The precise content of plastid genomes depends on the lineage. Following the sequencing in the late 1980s of the plastid genomes of *Nicotiana tabacum* (tobacco) and *M. polymorpha* (a liverwort), it was widely assumed that all plastids would have roughly the same set of just over 100 genes. This view of plastids as genetically uniform entities with genomes reduced to a bare minimum of genes has been displaced as information about the plastids of diverse algal groups has become available (reviewed in Palmer and Delwiche 1998). The plastids of red algae and those of glaucocystophytes are substantially larger and encode genes for a wider range of cellular functions than do those of green algae and plants. Although the genomes of all photosynthetic plastids contain key genes involved with gene expression and photosynthesis, the genomes of green algal plastids contain few genes that are not involved with one of these processes. By contrast, the red algae, as evidenced by *P. purpurea*, have plastid genes involved with several biosynthetic pathways, nitrogen assimilation, protein transport and processing, and metabolic regulation; the *Cyanophora paradoxa* plastid genome is similarly elaborate and adds to its repertoire genes for biosynthesis of both NAD⁺ and the peptidoglycan wall that is distinctive to glaucocystophyte plastids. Whether there is a functional difference between genes encoded in the plastid and those encoded in the nucleus but targeted to the plastid is not known. A useful survey of plastid gene content is presented by Stoebe et al. (1998).

Three Lineages of Primary Plastids

The genetic interplay between nucleus and plastid is complex enough in plants, red algae, and glaucocystophytes, but in other groups of algae there is an added level of complexity. Many groups of algae have acquired their plas-

tids via secondary endosymbiosis, where a eukaryote already equipped with plastids took up permanent residence inside a second eukaryotic cell (fig. 1). The result is a set of nested cellular compartments, one inside the other, and information about the evolutionary history of the organism can be gleaned from study of these membranes and their function. Which lineages, then, have primary plastids, and which have acquired their plastids via secondary endosymbiosis? Current evidence suggests that there are but three lineages of primary plastids, found in green algae and plants, red algae, and glaucocystophytes (Delwiche and Palmer 1997; Martin et al. 1998; Palmer and Delwiche 1998).

The Glaucocystophyte Lineage

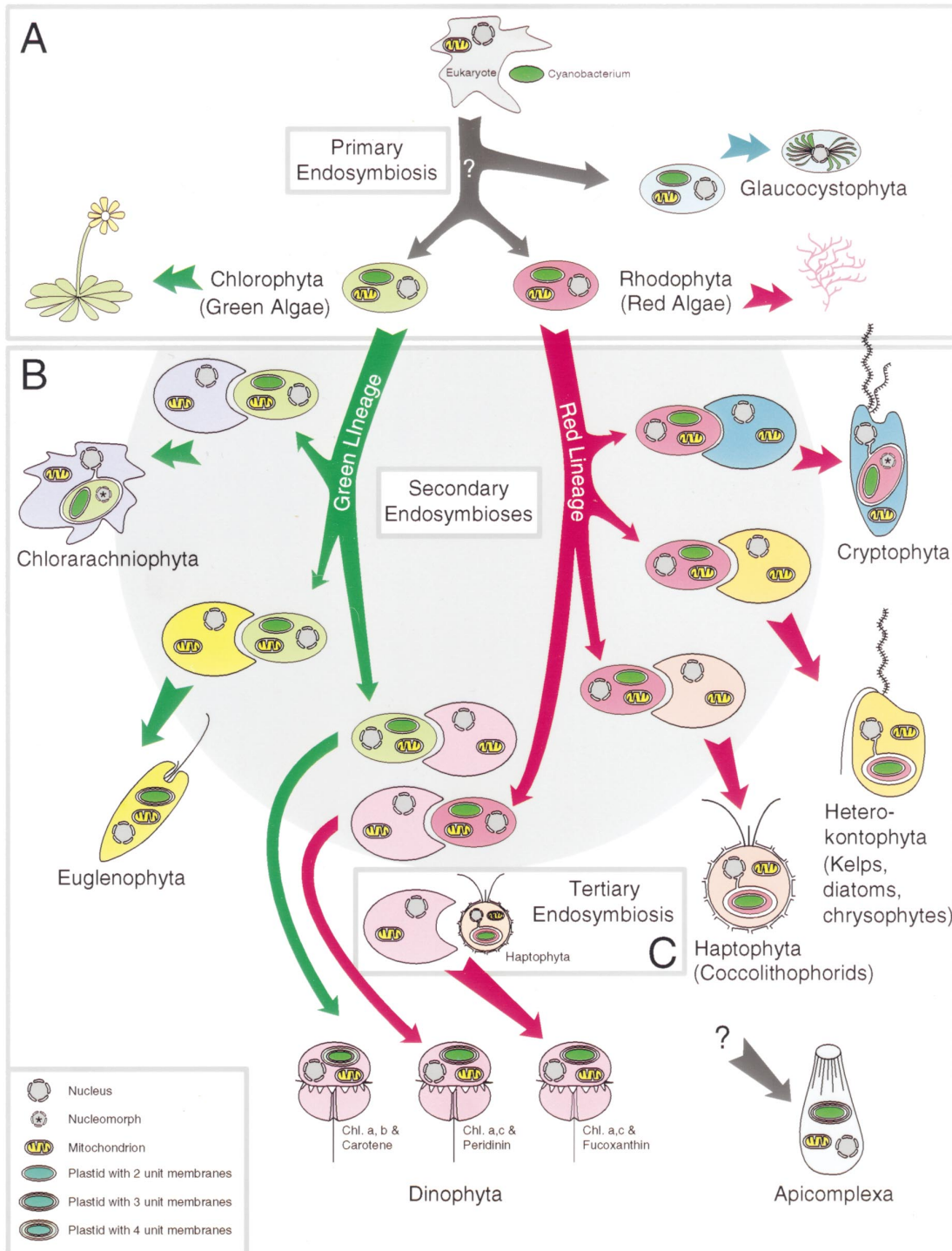
The Glaucocystophyta are a small group of relatively uncommon freshwater, unicellular algae that are of little economic importance, and they are often neglected even by specialists. It has become apparent, however, that this group occupies a key position in the evolution of plastids. Unlike other plastids, the plastids of glaucocystophytes retain the remnant of a gram-negative bacterial cell wall of the type found in cyanobacteria (Löffelhardt and Bohnert 1994). With a thin peptidoglycan cell wall and cyanobacterium-like pigmentation, the glaucocystophyte plastid clearly shows its cyanobacterial ancestry, and it has caused confusion for many years; even today, several high-quality textbooks incorrectly refer to the plastid of the glaucocystophyte *Cyanophora paradoxa* as an unreduced cyanobacterium. In fact, the *C. paradoxa* plastid genome shows the same reduction as other plastids when compared to free-living cyanobacteria (it is 136 kb and contains 191 genes; Stirewalt et al. 1995). The peptidoglycan cell wall of the plastid is thus a feature retained from their free-living cyanobacterial ancestor. In this context, the Glaucocystophyta are remarkable only for their retention of an ancestral character present in neither green nor red plastids. If primary plastids are monophyletic (i.e., they are all derived from a single common plastid ancestor), then it would be gratifying if the glaucocystophyte plastid were the outgroup to all other primary plastids, for this would call for only a single loss of the plastid wall.

Indeed, although the relationships among the primary plastid lineages remain uncertain, several molecular phylogenetic analyses have placed the glaucocystophyte plastid as an outgroup to other primary plastids (Helmchen et al. 1995; Gray and Spencer 1996; Martin et al. 1998). If this is correct, and if plastids are monophyletic (see "Counting Endosymbiotic Events"), then the glaucocystophytes would be an extremely important group for an understanding of the evolution of photosynthetic eukaryotes. Because the Glaucocystophyta were originally grouped on

the basis of their plastid characters (many of which are plesiomorphies; i.e., ancestral features shared with cyanobacteria), it is not surprising that some taxa were artifactually included in this group. Such is the case with *Glaucosphaera vacuolata*, which lacks a plastid cell wall, and in molecular phylogenetic analyses groups unequivocally with red algae (Bhattacharya and Schmidt 1997). No certain case of a secondary plastid derived from the glaucocystophytes is known. One candidate would be *Paulinella chromatophora*, an amoeboid protist with glaucocystophyte-like plastids. However, this organism remains quite poorly understood, and the features linking its plastids with those of the Glaucocystophyta are essentially cyanobacterial characters (Bhattacharya et al. 1995; Bhattacharya and Schmidt 1997).

The Green Lineage

The most familiar plastids are those of plants and green algae (Chlorophyta). These constitute the second lineage of primary plastids. Green algae have an old fossil record, extending back well over a billion years, perhaps much longer (Tappan 1980; Knoll 1992). The green algae are structurally diverse and occur in a wide range of habitats, from marine and fresh waters to soils, leaf surfaces, and snow. Their plastids are surrounded by two membranes and are pigmented by chlorophylls *a* and *b* (a few also have chlorophyll *c*), with relatively small quantities of several carotenoids (Van den Hoek 1995). It was this similarity to the pigments of plants that led to the inference that the ancestors of land plants (i.e., embryophytes) would be among the green algae (Bower 1908), and it is now clear that, phylogenetically, plants are a group of green algae adapted to life on land (Graham et al. 1991; Huss and Kranz 1997; Chapman et al. 1998; Qiu and Palmer 1999). Because plants are derived from among green algae, they are treated here as green algae unless noted otherwise. Although they have produced a few surprises, molecular phylogenetic studies of green algal phylogeny have generally been compatible with other phylogenetic information, including ultrastructural data (Friedl 1997; Chapman et al. 1998). Thus, the simple membrane system surrounding the plastid, the congruence of phylogenies based on nuclear and organellar genes, and the antiquity of the green algae in the fossil record all indicate that the green algal plastid is of primary origin (but see Stiller and Hall 1997 and the discussion in Delwiche and Palmer 1997). Secondary plastids derived from the green lineage are found in the Chlorarachniophyta and the Euglenophyta.



The Red Lineage

The plastids of the red algae (Rhodophyta) constitute the third primary plastid lineage. Like the green algae, the red algae are an ancient group in the fossil record, and some of the oldest fossils interpreted as being of eukaryotic origin are often referred to the red algae, although clearly these organisms were very different from any extant alga (Tappan 1980; Han and Runnegar 1992; Knoll 1992). The red algae are more restricted in distribution than green algae, being limited primarily to coastal marine waters. A few are freshwater, but almost all are aquatic and live attached to a substrate, thus limiting them to a relatively narrow band of coastal and inland waters. Like those of green algae, the plastids of red algae are surrounded by two membranes. However, they are pigmented with chlorophyll *a* and phycobiliproteins, which are organized into phycobilisomes. Phycobilisomes are relatively large light-harvesting pigment/protein complexes that are water-soluble and attached to the surface of the thylakoid membrane (Gantt 1981). Thylakoids with phycobilisomes do not form stacks like those in other plastids, and consequently the plastids of red algae (and glaucocystophytes) bear an obvious ultrastructural resemblance to cyanobacteria.

A number of algal groups have secondary plastids derived from those of red algae, including several with distinctive pigmentation. The cryptomonads (Cryptophyta) were the first group in which secondary plastids were recognized (Gibbs 1993), on the basis of their complex membrane structure. Like red algae, they have phycobiliproteins, but these are distributed in the intrathylakoidal space rather than in the phycobilisomes found in red algae, Glaucocystophyta, and Cyanobacteria. In addition, cryptomonads possess a second type of chlorophyll, chlorophyll *c*, which is found in the remaining red lineage plastids. These groups—which include the Heterokontophyta (including kelps, diatoms, chrysophytes, and related

groups), Haptophyta (the coccolithophorids), and probably those dinoflagellates pigmented with peridinin—have chlorophylls *a* and *c*, along with a variety of carotenoids, for pigmentation (Van den Hoek et al. 1995). Stacked thylakoids are found in those lineages (including the cryptomonads) that lack phycobilisomes. The derivation of chlorophyll *c* containing plastids from the red algal lineage is still somewhat conjectural, but recent analyses of both gene sequences and gene content are consistent with this conclusion (Delwiche et al. 1995; Van de Peer et al. 1996; Douglas and Penny 1999).

Endosymbiosis: Primary and Secondary and Tertiary, Oh My!

The recognition that secondary endosymbiosis played a major part in plastid evolution (fig. 1) stemmed from ultrastructural studies of cryptomonads, red-pigmented unicells that are remarkable in that they retain a second, highly reduced, eukaryotic nucleus (called a nucleomorph) within the secondary endosymbiont (Gibbs 1962). The secondary endosymbiont of cryptomonads is thought to have been a red alga and persists only as a periplastidal space bounded by a dual-membrane system termed the “chloroplast endoplasmic reticulum,” or CER (Greenwood et al. 1977; Gibbs 1981). The outer envelope of the CER is continuous with the nuclear envelope and probably corresponds to a host food vacuole, while the inner membrane—surrounding both nucleomorph and primary plastid—probably corresponds to the original eukaryotic endosymbiont cell membrane. The primary plastid itself, like other primary plastids, is bound by two membranes. Thus, the plastid stroma is separated from the host cytosol by four membranes.

A similar situation is seen in *Chlorarachnion reptans*, an unusual network-building amoeboid alga that has green-pigmented plastids. The Chlorarachniophyta, like the

Figure 1: One hypothesis for endosymbiotic events in the evolution of plastids. Plastids were derived from free-living cyanobacteria a relatively small number of times. This figure assumes a single origin of plastids, but this remains uncertain, as does the precise relationship among the primary plastid lineages. *A*, Three algal lineages with primary plastids can be identified, each marked by its distinctive plastid type: the red algae (Rhodophyta), green algae (Chlorophyta), and Glaucocystophyta. *B*, Subsequently, a number of secondary endosymbiotic events resulted in the acquisition of plastids via predation of a plastid-containing eukaryote by a second eukaryote. The pigmentation, ultrastructure, and genome of the plastid reflect the primary plastid lineage to which it belongs rather than the properties of the host lineage. In the case of the cryptomonads (Cryptophyta) and Chlorarachniophyta, the primary plastids—from the red lineage and green lineage, respectively—are enclosed in a cellular compartment that also contains a reduced eukaryotic nucleus, the nucleomorph. In most other taxa with secondary plastids, the endosymbiont is further reduced, no nucleomorph is present, and there is little evidence of the secondary origin of the plastids other than the properties of the plastid itself and, typically, extra membranes surrounding the plastid. The Dinophyta have a particularly complicated history of plastid acquisition and have plastids of several different types. Three of these are shown here. In *Lepidodinium*, the plastids are derived from the green lineage and have associated nucleomorph-like structures. In most photosynthetic dinoflagellates, the plastids are derived from the red lineage and include the accessory pigment peridinin. *C*, Finally, in a tertiary endosymbiotic event, three species have acquired fucoxanthin-pigmented plastids from a haptophyte. The recently discovered plastids of apicomplexan parasites are unpigmented, and their evolutionary acquisition remains under investigation. A more detailed discussion of these plastid lineages is presented by Palmer and Delwiche (1998).

Cryptophyta, have the complex membrane structure of the chloroplast endoplasmic reticulum and a tiny nucleomorph. Because of the green pigmentation and ultrastructure of the plastid, the secondary endosymbiont of chlorarachniophytes was thought to be a green alga (Hibberd and Norris 1984; Ludwig and Gibbs 1989). This conclusion has been supported by more recent molecular phylogenetic studies (McFadden and Gilson 1995; McFadden et al. 1995, 1997). Although *C. reptans* was for many years a very poorly understood organism, this excellent work has confirmed the interpretation (based on ultrastructural work) that its plastid is the product of a secondary endosymbiosis.

As with primary plastids, the secondary plastids have undergone a tremendous reduction in the size and complexity of their genomes. In the case of *C. reptans*, the nucleomorph genome, although almost certainly derived from the typical eukaryotic nucleus of a green alga, has been reduced to just 380 kb and is encoded on three small chromosomes between 95 and 145 kb in size. A substantial portion of this genome has been sequenced, and, like the plastid genome, it is highly compact and composed largely of housekeeping genes, and it seems likely the genome is present largely to maintain a few key genes. Even the introns are very small; most are precisely 19 nt in length (Gilson and McFadden 1996). Secondary plastids with nucleomorphs are also found in the cryptomonads, and their nucleomorphs have undergone a similar reduction in size and complexity (Douglas et al. 1991; Hofmann et al. 1994). In chlorarachniophytes and cryptomonads, there are at least four genomes in play: those of the plastid, nucleomorph, nucleus, and cytosolic mitochondrion. The relationship among these genomes can be quite complex; in the case of cryptomonads, the genes *groEL* and *cpn60*, two members of a gene family, are apportioned to the plastid and nucleomorph genomes, respectively (Wastl et al. 1998). In addition, while the residual secondary endosymbiont lacks mitochondria, it is likely that some genes of endosymbiont mitochondrial ancestry persist in either the nucleomorph or nuclear genome. From the great reduction of the nucleomorph genome, it can be inferred that genes from this genome have probably been transferred to the nuclear genome, but the true measure of transfers among the various genomes remains to be determined.

Given the theme of nucleomorph genome reduction in the cryptomonads and chlorarachniophytes, one might ask whether a secondary endosymbiont could entirely lose its nuclear genome. Indeed, this seems to have occurred several times. In heterokonts—including brown algae and diatoms among others—there is a complex membrane system surrounding the plastid that strongly resembles the CER of cryptomonads but lacks a nucleomorph. Gibbs (1981) interpreted this feature as a secondary endosym-

biont that had completely lost its nucleus. This conclusion has been supported by molecular phylogenetic studies, which place the plastids of heterokonts as close relatives of those of red algae and cryptomonads (Douglas et al. 1991), while the host cells are relatively distantly related.

In other taxa, such as the Euglenophyta and some dinoflagellates, the plastids are surrounded by three membranes but otherwise carry little structural evidence of their secondary origin (Gibbs 1978). The host cells of euglenoids are related to the protozoan group Kinetoplastida (Cavalier-Smith 1993), and yet their plastids closely resemble those of green algae (Gibbs 1978). Molecular phylogenetic analyses of genes from plastids of the euglenoids have been equivocal, with analyses of rRNA genes often placing them among red lineage plastids. It seems likely, however, that this is an analytical artifact, as the euglenoid rRNA is highly divergent from other sequences and has a strong base compositional bias (Lockhart et al. 1992; Van de Peer and De Wachter 1997). Phylogenetic analysis of the plastid gene *tufA* does place euglenoid plastids on the green lineage (Delwiche et al. 1995). This conclusion was supported by the innovative whole-genome analysis of Martin et al. (1998), which used a small number of taxa but a very large number of characters. In addition to gene sequence analyses, several features of plastid gene content and arrangement place the euglenophyte plastid on the green lineage (see Gray and Spencer 1996).

A secondary origin of euglenoid plastids is also suggested by the manner in which nuclear-encoded gene products are imported. In *Euglena gracilis*, plastid-destined polypeptides are targeted by a bipartite transit peptide that resembles both a secretory peptide and a typical plastid peptide (Chan et al. 1990; Kishore et al. 1993). Recent work has also demonstrated similar transit peptides in *Chlorarachnion reptans* and shown that these peptides target the protein first to the endoplasmic reticulum, and subsequently to the plastid (G. McFadden, personal communication). Such transit peptides have also been demonstrated for nuclear-encoded, plastid-targeted peptides in the apicomplexan parasites *Toxoplasma gondii* and *Plasmodium falciparum* (Waller et al. 1998). In an ingenious series of deletion experiments with fusion proteins, this group has also demonstrated that the two portions of the transit peptide function as had been predicted on structural grounds; the first portion of the transit peptide targets into the endoplasmic reticulum but is not sufficient for targeting to the plastid. At the same time, the second portion of the transit sequence leads to accumulation in the cytosol unless full function of the transit peptide is restored by addition of a secretory sequence (D. Roos, personal communication).

It has been suggested that the number of membranes in secondary plastids reflects the feeding mechanism by

which they were acquired, with phagocytosis leading to a four-membraned structure and myzocytosis, a feeding mechanism that does not involve intake of the prey cell membrane, giving rise to triple-membraned plastids (Schnepf and Deichgräber 1984; Schnepf et al. 1985; Van den Hoek et al 1995; Palmer and Delwiche 1998). This concept requires further investigation, but it is noteworthy that myzocytosis is known to occur in dinoflagellates and at least one euglenoid, *Peranema* (Triemer 1997).

Remarkably, there are also tertiary plastids. Chesnick et al. (1996) verified with molecular methods that the endosymbiont of *Peridinium foliaceum* is a diatom, as had been believed on the basis of ultrastructural studies (Dodge 1971). Because the diatom plastid—along with those of other heterokonts—is itself a secondary plastid, this is a candidate for tertiary endosymbiosis. However, in *P. foliaceum* a second eukaryotic nucleus is associated with the plastid, and unlike the Cryptophyta and Chlorarachniophyta, this nucleus is not obviously reduced in size or genetic function. A similar situation is present in *Gymnodinium acidotum*, which retains a cryptomonad for long periods of time, although there is evidence that in this case the endosymbiont is not permanent (Zhang et al. 1982; Wilcox and Wedemayer 1985; Farmer and Roberts 1990). In both of these cases, it is unclear that the endosymbiont has the reduced genome and genetic dependence on the host cell that is characteristic of plastids and mitochondria (Cavalier-Smith and Lee 1985). The best case for a true tertiary plastid is in the fucoxanthin-containing dinoflagellates *Gymnodinium breve*, *Gymnodinium galatheanum*, and *Gyrodinium aureolum*. In these taxa the plastids have both a pigmentation and ultrastructure reminiscent of the plastids of haptophytes (or “coccolithophorids”), a group of marine algae with calcareous scales that have secondary plastids. A recent molecular phylogenetic study has confirmed that these plastids are derived from haptophytes (T. Tengs, O. J. Dahlberg, K. Shalchian-Tabrizi, C. Delwiche, D. Klaveness, K. Rudi, and K. S. Jakobsen, unpublished manuscript), and as there is no evidence of a nucleomorph or other endosymbiont nucleus, these plastids seem to be true tertiary organelles.

Rubisco in the Red Primary Plastid Lineage

Swapping of genes has not been limited to exchanges among the compartments of the eukaryotic cell. While most plastid genes indicate that all primary plastids are derived from cyanobacteria (and may be monophyletic in origin), the genes *rbcL* and *rbcS*, which encode the critical carbon-fixing enzyme rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) present a markedly different picture. Early analyses of rubisco genes from red algae and

those taxa with secondary plastids derived from red algae showed that these taxa have a rubisco that more closely resembles the rubisco of certain proteobacteria than it does that of cyanobacteria (reviewed in Delwiche and Palmer 1996). Proteobacteria are an important and metabolically diverse group of bacteria that include the familiar *Escherichia coli* as well as many genera of photosynthetic bacteria such as *Rhodospseudomonas*, *Chromatium*, and *Ectothiorhodospira*. Photosynthetic proteobacteria capture light energy with a photosystem II–like mechanism and use rubisco to fix carbon, but unlike the cyanobacteria and plastids, they are unable to use water as an electron donor and do not generate oxygen during photosynthesis (Imhoff 1995). As a consequence, many proteobacterial rubiscos are relatively sensitive to oxygen (Tabita 1995), so the presence of a proteobacterial rubisco in oxygenic phototrophs is surprising.

To understand the implications of the kind of rubisco found in red lineage plastids, it is first necessary to be familiar with the members of this gene family. Several kinds of rubisco have been identified (fig. 2). An ancient gene duplication and subsequent divergence is thought to have given rise to the structurally distinct form I rubisco (composed of eight small and eight large subunits), found in both proteobacteria and cyanobacteria, and form II rubisco (two large subunits only), found only in proteobacteria (and, remarkably, in dinoflagellates). It has been postulated that the dimeric form II enzyme is the ancestral type, and this view has found some support with the identification of rubisco sequences in archaeal genomes that seem likely to be dimeric, although the archaeal rubisco sequences show similarities with both form I and form II rubiscos (data not shown). Form I rubisco is itself classified into several groups. Type IB is found in cyanobacteria and the plastids of green algae and glaucocystophytes, while the relatively closely related type IA is found in some proteobacteria and cyanobacteria. A second cluster of rubisco types is made up of type ID, found in the plastids of red lineage plastids, and type IC, found in some proteobacteria (Tabita 1995; Delwiche and Palmer 1996). Relatively few rubisco sequences have been determined from bacteria, and it is possible that additional types will be identified.

The phylogeny of these rubisco genes is difficult to reconcile with the phylogeny of the organisms in which they are found. Delwiche and Palmer (1996) identified several incongruities between the rubisco phylogeny and bacterial phylogeny as inferred from six separate genes and from this concluded that the rubisco phylogeny is incompatible with an aggregate “organismal” phylogeny. Several mechanisms could explain such apparent incongruities. First, the phylogenetic analyses used to infer individual gene histories could be in error. However, while this can explain some incongruities, others are so dramatic that analytical

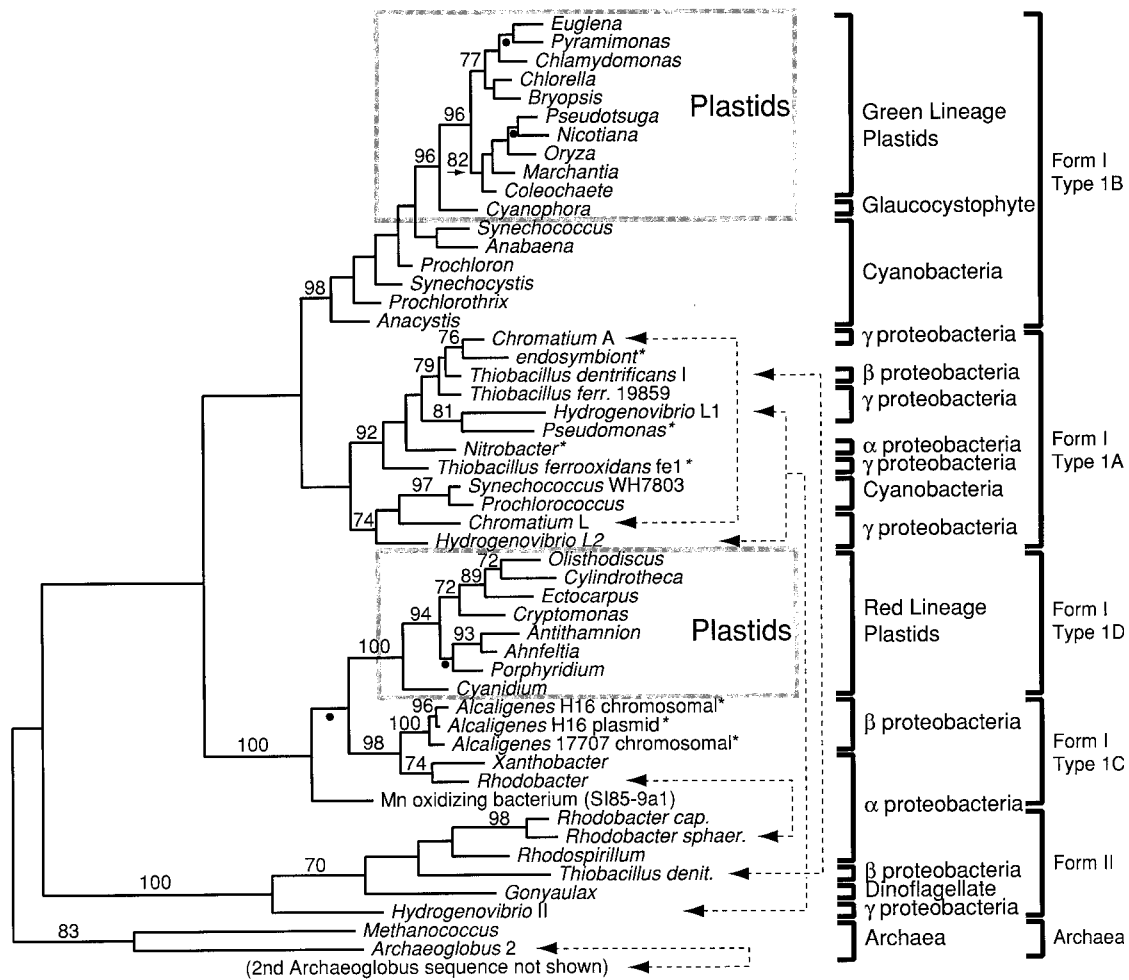


Figure 2: Horizontal gene transfer and duplication of rubisco genes, showing incongruity between rubisco phylogeny and classifications based on other genes. Several such incongruities exist; the most obvious is the division of plastids into two groups despite the fact that several lines of evidence place all plastids among cyanobacteria. On the left is a phylogenetic tree of rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) genes from bacteria and archaea, as well as from representative plastids, determined by parsimony analysis of amino acid data. Indicated to the right is an “organismal” classification based on SSU rRNA and supported by multiple other genes, as well as a classification of rubisco genes based on their structure and catalytic properties (Tabita 1995). Arrows connected by dotted lines in the center of the figure indicate multiple rubisco genes from a single organism’s genome. The tree shown is one of 24 equally parsimonious trees of length 2,924 character state changes; branches that collapse in the strict consensus of all 24 equally parsimonious trees are indicated by a bullet. Bootstrap values of 70% or greater are given above the branch. Bootstrap analysis provides a measure of how consistently the data support the indicated feature of the tree. Asterisks indicate taxa for which SSU rRNA sequences are not available from the same strain. A more detailed discussion of a similar analysis (although excluding the archaeal sequences) is presented by Delwiche and Palmer (1996).

artifact is clearly not an adequate explanation. This is the case with rubisco, where form IA and IB rubiscos share <60% amino acid identity with form IC and ID sequences but have >70% amino acid identity within these pairs (Delwiche and Palmer 1996). Second, many genes exist in gene families as a product of gene duplications, with subsequent divergence and specialization of the duplicated genes. Such paralogous sequences, if mistaken for or-

thologs, can create considerable confusion. Rubisco genes clearly form a complex gene family; there is no doubt that substantial gene duplication has occurred (fig. 2). Martin et al. (1998, p. 163) make an important point when they speculate that “*rbcl* genes ... are related by duplication.” However, it is highly unlikely that duplication alone can explain the entire phylogeny. Particularly interesting is the fact that no cyanobacteria have been identified that have

type IC or ID rubisco, as would be expected if red lineage plastids acquired their rubisco directly from their cyanobacterial ancestor (Delwiche and Palmer 1996). Thus, horizontal gene transfer is the best mechanism to explain at least some features of the rubisco phylogeny.

A key issue, then, is whether these transfers involved large regions of the genome with multiple genes moving simultaneously or a gradual accretion of individual genes. Doolittle (1998) has pointed out that predators are subject to a constant influx of DNA from their prey and has argued that this could create an evolutionary ratchet that would lead to the gradual replacement of predator genes with those of their prey. If multiple genes are transferred simultaneously, then one would expect to find several suites of genes with phylogenies that are congruent within the suite but incongruent among suites. By contrast, if a gradual mechanism is at play, then one would expect to find multiple genes with congruent phylogenies and occasional genes with phylogenies that are consistent neither with the bulk of the genome nor with each other. This latter model seems more appropriate for the rubisco phylogeny, as there seems to be considerable congruence among plastid genes, with rubisco the clear exception (Delwiche and Palmer 1996).

Counting Endosymbiotic Events: Are Primary Plastids Monophyletic?

Much of the preceding discussion implies—but is not directly dependent on—the assumption that primary plastids are monophyletic in origin. There are contradictory data on this subject, and a monophyletic origin of plastids is by no means certain. The once appealing hypothesis that green-pigmented cyanobacteria (the “prochlorophytes”) gave rise to green plastids has not been supported by molecular phylogenetic analyses (Palenik and Haselkorn 1992; Urbach et al. 1992), but no other cyanobacterial lineage has been identified as closely related to plastids. Rather, plastids seem to be an ancient lineage among cyanobacteria, a view that is also easier to reconcile with the fossil record (Turner 1997). There is good evidence that the glaucocystophyte, green, and red lineages are the three primary plastid lineages, with plastids in other groups derived secondarily from these lineages (Palmer and Delwiche 1998; Turner 1997; Martin et al. 1998). If the three primary plastid lineages are monophyletic, this would imply both that all plastids are the product of a single endosymbiotic event and that the host cells themselves constitute a monophyletic group. From this it would be predicted that molecular phylogenies based on genes from the plastid, mitochondrial, and nuclear genomes should all produce congruent phylogenies with the Glaucocys-

tophyta, red algae, and green algae as sister taxa. This could be further complicated by the presence of intervening lineages that have secondarily lost plastids, although it should be noted that outright loss of plastids is rare in both red and green algae. Mitochondrial and plastid genes (with the exception of rubisco) do generally show the three groups with primary plastids as a monophyletic group, while data from the nuclear genome are more equivocal (reviewed in Palmer and Delwiche 1998). The situation is complicated by the fact that phylogenetic methods in current use can be misled if the mode of sequence evolution is different on different parts of the tree (Lockhart et al. 1998). This is a serious concern, as one could reasonably suppose that there was something about life as an endosymbiotic organelle that led to similar selective pressures on independently acquired endosymbionts. Thus, there is a plausible scenario by which multiple plastid genes could erroneously indicate plastid monophyly. This would not, however, account for monophyly of taxa with plastids in analyses based on mitochondrial genes, and congruence among phylogenetic analyses based on genes from different genomes is consequently very important.

One important area for development will be analysis of additional genes from the nuclear genome. Even now, relatively few genes have been sequenced from enough of the appropriate taxa to test monophyly of the groups with primary plastids. SSU rRNA, by far the most widely sampled gene, does not support plastid monophyly, with virtually all analyses separating the Rhodophyta and Chlorophyta. Neither, however, do rRNA analyses strongly support any competing hypothesis (reviewed in Palmer and Delwiche 1998). Fortunately, with the current explosion of DNA sequencing projects, the prospects for new sources of information are good (Bhattacharya and Schmidt 1997; Brown and Doolittle 1997; Medlin et al. 1997).

Another possibility, that the three primary plastid lineages independently acquired plastids from closely related cyanobacteria, is very difficult to evaluate by phylogenetic analysis of sequence data. Congruent phylogenies based on nuclear genes that showed monophyly could provide strong circumstantial evidence, but only properties that are a consequence of endosymbiosis itself can provide direct evidence of polyphyly in this case. Two properties that have been examined in this context are the gene content in the reduced genomes of plastids and the transit peptides by which nuclear-encoded genes are targeted to the plastid. When the plastid genome is viewed as the product of a progressive reduction from that of free-living cyanobacteria, there are marked similarities in the gene content of these different plastid genomes. All but eight of 191 genes in the *Cyanophora paradoxa* plastid DNA are also present in the *Porphyra purpurea* plastid genome, as are all but 20

of the 120 genes in the *Marchantia polymorpha* plastid (Stoebe et al. 1998).

Plastids in the Alveolata: A Microcosm of Plastid Evolution

The eukaryotic crown group Alveolata, which includes the ciliates, apicomplexans (which are nearly all obligate parasites of animals), and dinoflagellates, has a remarkable variety of plastid associations. The dinoflagellates are often thought of as photosynthetic organisms. In fact, about half of all dinoflagellates are photosynthetic, the majority of which rely on a plastid that is surrounded by three membranes and pigmented with chlorophylls *a*, *c*, and peridinin, but several other types of plastids are known (Dodge 1975; Delwiche and Palmer 1997; Palmer and Delwiche 1998). To date, the peridinin-type plastid of dinoflagellates remains almost completely unstudied by molecular methods. In collaboration with the K. S. Jakobsen laboratory in Oslo, Norway, we have recently succeeded in isolating putative plastid genes from the peridinin-containing dinoflagellate *Gymnodinium sanguineum* (C. F. Delwiche, T. Bachvaroff, T. Tengs, unpublished data). These sequences are highly AT-rich and divergent from other homologues, and we feel that it will be necessary to use information from several genes to perform reliable phylogenetic analyses. A key question is whether the peridinin-type plastid, which seems to be scattered rather arbitrarily among dinoflagellates, was present in the common ancestor of all extant dinoflagellates.

In addition to the peridinin-type plastid, there are several other plastid types found in various dinoflagellates, with the degree of incorporation in the host cell ranging from minimal to fully heritable organelles. As noted earlier, *Gymnodinium galatheanum*, *Gyrodinium aureolum*, and *Gymnodinium breve* have tertiary plastids acquired from a haptophyte (Tengs et al., unpublished manuscript). Another remarkable case is seen in *Lepidodinium viride*, which has green-pigmented plastids surrounded by four membranes, with tiny vesicles in the space between the inner and outer pair of membranes that may correspond to a nucleomorph or mitochondria. What makes *L. viride* particularly interesting is that it also expresses on its cell surface scales that show a remarkable similarity to the scales of prasinophycean green algae, suggesting that the genes for these scales may have been acquired from the endosymbiotic alga (Watanabe et al. 1987, 1990; Van den Hoek et al. 1995; Palmer and Delwiche 1998). If this is the case, *L. viride* may be among the most flamboyant cases of chimerism yet identified. Nor does this exhaust the catalog of dinoflagellate plastid diversity, although in several dinoflagellates with anomalous pigmentation, it is unclear whether the plastids are heritable organelles belonging to

the dinoflagellate or were temporarily sequestered from prey.

The phylogenetic affinities of the peridinin-type plastid of dinoflagellates is of particular interest because of the recent identification of a plastid in apicomplexan parasites (Köhler et al. 1997). In the mid-1990s, it became clear that there were two extranuclear genomes in apicomplexan parasites (the tremendously important pathogens responsible for malaria, toxoplasmosis, and a variety of serious diseases of livestock and other animals). One of these genomes is a 35-kb element that was initially confused with a mitochondrial genome but that had a gene content and organization unlike any known mitochondrial genome. In fact, the organization of this genome resembled that of a highly reduced plastid genome, and my coauthors and I were able to show in 1997 that the 35-kb genome is located in a membrane-bound spherical organelle and falls in phylogenetic analyses solidly within the plastids (Köhler et al. 1997). In all probability, this reduced and nonphotosynthetic plastid is of secondary origin, but it is not known from what primary plastid lineage it is derived.

Because apicomplexans and dinoflagellates are closely related, one obvious possibility is that the apicomplexan plastid is homologous to the peridinin-containing plastid of dinoflagellates. Köhler et al. (1997) concluded that there was no a priori reason to believe that it was related to the plastids of dinoflagellates and indicated that the plastid may be derived from the green lineage. A different view has been presented on the basis of gene content and arrangement in the plastid genome, which are more consistent with a nongreen origin of the apicomplexan plastid (McFadden and Waller 1997; Blanchard and Hicks 1998; Leitsch et al. 1999). These analyses are complicated by the difficulty of determining homology in the highly divergent *Plasmodium falciparum* plastid, but they are at least as compelling as single-gene analyses. The key to resolving the evolutionary origin of the apicomplexan plastid will be integration of information from multiple sources, particularly analyses of a greater length of sequence, and gene content studies that include data from dinoflagellate plastids.

It has been shown that the apicomplexan plastid is essential for parasite survival and can serve as a drug target (Fichera and Roos 1997). Because animals do not have plastid genomes, there seems to be a high potential for developing treatments targeted to this organelle that have relatively low toxicity to the host organism. Thus, the recognition of a previously unknown endosymbiotic organelle and an understanding of the genetic complexity of the eukaryotic cell have opened up new avenues for treatment of some of the world's most deadly and expensive diseases.

The ciliates have long been known to acquire temporary plastids ("kleptochloroplasts") from their prey, and in a

few cases these plastids may persist for some time (Tomas and Cox 1973). Thus, the Alveolata as a whole show a remarkable diversity of plastid associations, ranging from the temporary borrowing of plastids common in ciliates to the nonphotosynthetic but essential plastids of apicomplexans to the rainbow of plastids in photosynthetic dinoflagellates. It is not clear what properties of the Alveolata make them particularly predisposed to acquire secondary plastids, but it seems likely that further study of plastid acquisition in this group would lead to a better understanding of the mechanisms and selective pressures that lead to endosymbiosis. It is particularly interesting to contemplate that among the Alveolata that have temporary associations with plastids, there may be mechanisms in place that facilitate retention of plastids for a period of time. Such mechanisms have been implied in the sea slug *Elysia chlorotica* (Pierce et al. 1996) and if present complicate the view of endosymbiosis as a single event. Perhaps the ability of the nucleus to provide sustenance for the plastid began even before there was any permanent association between the plastid and the host.

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