Chapter 3

The Evolutionary History of Plastids: A Molecular Phylogenetic Perspective

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CONTENTS

Abstract

Plastids, the light-harvesting organelles of photosynthetic eukaryotes, are derived from an ancient symbiosis between a eukaryote and a cyanobacterium. This process is called primary endosymbiosis, and accounts for plastids in glaucocystophytes, red algae, green algae and land plants. All other plastid-containing eukaryotes acquired their plastids from either a red or a green alga by secondary endosymbiosis, in which a eukaryotic cell swallows a second phototrophic eukaryote and retains its photosynthetic machinery. Secondary endosymbiosis accounts for the plastids found in most of the diversity of eukaryotic algae, including the chlorarachniophytes, euglenids, cryptomonads, haptophytes, heterokonts, dinoflagellates and apicomplexan parasites. This chapter discusses the changing views with respect to the origin and evolution of plastid-containing organisms, with an emphasis on the molecular phylogenetic evidence bearing on the number of primary and secondary endosymbioses that have occurred during eukaryotic evolution.

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3.1 Introduction

The origin of photosynthesis was one of the major turning points in eukaryotic evolution. Beyond forming the foundation of most marine, freshwater and terrestrial ecosystems, the diversification of plants and algae has impacted most aspects of eukaryotic diversity and evolution in some way. Both the origin and the spread of photosynthesis in eukaryotes have complicated histories: the history of our knowledge on these subjects, and also, as it turns out, the evolutionary history of photosynthetic organelles themselves are unexpectedly complex. It is now widely accepted that the photosynthetic organelles of eukaryotes (plastids or chloroplasts) are derived from once free-living cyanobacteria. The similarities between plastids and cyanobacteria had been noted as early as the end of the 19th century (Schimper, 1883), and in 1905, Mereschkowsky formally proposed that the organelle was derived from a prokaryotic cell (Mereschkowsky, 1905). Nearly a century later, a wealth of ultrastructural, biochemical and molecular data now support this conclusion.

Although the prokaryotic nature of plastids is now patently obvious, many questions remain as to how the two cells integrated, what kinds of cells were involved in the endosymbiotic partnership and when and how many times these events took place. Part of the difficulty in resolving these questions lies in the extraordinary diversity of extant photosynthetic eukaryotes. Plastids are found in organisms ranging from benthic and planktonic unicells to soil microorganisms, 30-m kelps on the ocean floor and the trees and other plants that inhabit dry land. Plastid-bearing organisms are not only morphologically diverse but also heterogeneous with respect to their mode of nutrition: many are strict phototrophs, others maintain both phototrophic and heterotrophic lifestyles and still others are nonphotosynthetic parasites or predators. Because of this diversity, inferring the evolutionary origins of plastids and tracing their history among modern-day photosynthetic organisms have proven difficult. The situation has been further confounded by plastids having spread laterally among unrelated eukaryotic lineages. This process, called secondary endosymbiosis, has generated the bulk of algal biodiversity.

This chapter discusses the origin and evolution of primary and secondary plastids. In particular, it focuses on molecular phylogenetic evidence bearing on the question of the number of times each of these processes has occurred during eukaryotic evolution and the nature of the cells involved.

3.2 Primary Plastids

Oxidative photosynthesis first evolved in the ancestor of cyanobacteria, a ubiquitous and diverse group of photosynthetic prokaryotes. Several prokaryotic lineages use some form of photosynthesis, but the system found in cyanobacteria is distinctive as it uses two consecutive light-harnessing photosystems (Photosystems I and II) and oxygen as the terminal electron acceptor (Blankenship, 1994). The origin of this very powerful system was a critical event in evolution: it not only resulted in the drastic alteration of the earth's atmosphere by forming substantial quantities of free oxygen gas but also set the stage for photosynthesis in eukaryotes.

As diverse and abundant as the cyanobacteria are, they still account for only a small fraction of primary production at present. The vast majority of primary production is carried out by eukaryotic phototrophs (plants and algae) that acquired their photosynthetic apparatus by engulfing and retaining a cyanobacterium. This process, illustrated in Figure 3.1, began with a phagotrophic eukaryote swallowing a cyanobacterium, likely intended to be a meal for the predator (Figure 3.1a). Rather than being digested, however, the cyanobacterium was retained in the host cell, where it continued to photosynthesize (Figure 3.1b). Initially, the host probably took up endosymbionts transiently for a short-term gain of some

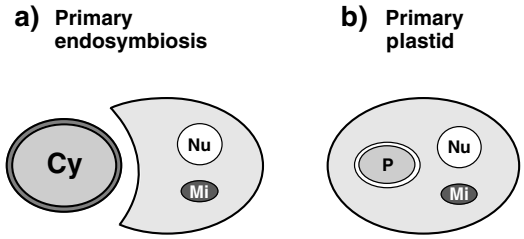

FIGURE 3.1 The process of primary endosymbiosis. (a) Primary endosymbiosis involves the uptake of a cyanobacterium by a phagotrophic eukaryote. (b) Primary-plastid-containing organism with two membranes surrounding its photosynthetic apparatus. The plastid resides in the cytosol and its two membranes are derived from the inner and outer membranes of the Gram-negative bacterium (see text). Abbreviations: Cy, cyanobacterium; Nu, nucleus; Mi, mitochondrion; P, plastid.

free carbohydrate, but at some point this partnership was fixed. The process of phagocytosis would result in a three-membrane-bound inclusion: two membranes from the Gram-negative cyanobacterium and the phagosomal membrane of the host. However, primary plastids are bound by two membranes only and the protein composition of these membranes (Jarvis and Soll, 2001) suggests that they correspond to the inner and outer membranes of the cyanobacterium. It has been suggested therefore that the endosymbiont was not digested because the phagosomal membrane ruptured, leaving the endosymbiont relatively safe in the host cytosol. This might be the case, but it is also possible that a prolonged series of transient endosymbioses allowed the host to adapt to the maintenance of an endosymbiont, and that the fixation of the partnership was finally ensured by some other event.

Whatever the initial forces promoting the fixation of the endosymbiosis, the cyanobacterium and host ultimately became mutually dependent by genetic integration. While an endosymbiont lives within its host there is constant potential for endosymbiont genes to move to the host genome. If these genes are integrated into the host chromosomes, transcribed and translated, and their protein products somehow transported back to the endosymbiont, then the original endosymbiont-encoded copies of the gene are redundant and can be lost without deleterious effect. Once a mechanism is in place to ensure that a class of proteins is targeted to the endosymbiont, it opens the door to further gene migrations, and the endosymbiont becomes increasingly dependent on the host for its full complement of proteins. Although this process seems staggeringly unlikely, it must have happened thousands of times. All plastids retain a genome, but in all cases it is a mere shadow of the original cyanobacterial genome and only encodes a small fraction of the proteins needed to maintain the organelle. The majority of plastid proteins are encoded in the nuclear genomes of plants and algae. In most cases, these genes are clearly of cyanobacterial origin, but have been transferred to the nuclear genome. [A recent analysis of the genome of the flowering plant *Arabidopsis* estimates that ca. 4500 genes are derived from the plastid endosymbiont, and about half of these encode proteins that appear to be targeted to the plastid (Martin et al., 2002)]. These proteins are expressed using the host transcription and translational apparatus and are posttranslationally targeted to the plastid. This targeting relies on an N-terminal extension on each of these proteins, called a transit peptide. The transit peptide is the flag that tells the cell that the protein belongs in the plastid, and it is transported across the two plastid membranes by a complex protein translocation system embedded on the outer and inner membranes.

On the other side of the equation, the host might also become dependent on the endosymbiont. Photosynthesis is clearly an enviable trait to maintain, but it can be lost,

and this has occurred in several plants and a large number of algal lineages. However, the endosymbiont was not merely a photosynthetic machine; initially it was a biochemically complex cell. As it integrated with the host, a few pathways not related to photosynthesis were also retained by the plastid, and it is possible that the corresponding pathways were lost in the host, making the plastid indispensable. Indeed, plastids of plants and algae have been documented to carry out diverse biochemical roles, such as biosynthesis of amino acids, fatty acids, isoprenoids and heme, in addition to photosynthesis and various biosynthetic pathways related to photosynthesis. Whether these pathways have been partially or fully lost in various hosts is unclear, but it is clear that each makes absolute loss of the plastid (as opposed to loss of photosynthesis, which is relatively common) a very difficult undertaking. In no case has plastid loss been unambiguously documented, although nonphotosynthetic plastids can be very difficult to detect, and it is hard to say what kind of evidence would be necessary to prove that a plastid was absent.

3.3 One Origin

In spite of the huge variety in morphology, biochemistry and pigment content of modern plastids, it has become clear that they all evolved from a single endosymbiotic event. This was not always thought to be the case. The diversity of plastid types has always left some suspicion that plastids arose multiple times independently by several endosymbioses involving different cyanobacteria. The recognition that plastids were being passed around among otherwise-unrelated eukaryotes by secondary endosymbiosis (see later) simplified this problem to a great extent as it revealed that several algal lineages derived plastids from red or green algae. With a full understanding of the extent of secondary endosymbiosis, it was clear that only three lineages harbor plastids derived from primary endosymbiotic events with a cyanobacterium: red algae, green algae and glaucocystophytes. Molecular data immediately supported the notion that the plastids harbored in all three lineages were closely related to one another to the exclusion of all cyanobacteria. Phylogenies based on genes encoding plastid small subunit ribosomal RNA (SSUrRNA) and elongation factor Tu (EF-Tu), and an intron found in plastid $tRNA^{Leu}$, supported the monophyly of these primary plastids (e.g., Besendahl et al., 2000; Delwiche et al., 1995; Helmchen et al., 1995). In addition to these phylogenetic studies, there are reports that plastids possess a novel class of three-helix light-harvesting antennae proteins (Durnford et al., 1999) and plastid genomes share a number of unique characteristics, most importantly the presence of an inverted repeat structure consisting of the rRNA operon and tRNAs and the organization of ribosomal protein operons (McFadden and Waller, 1997; Stoebe and Kowallik, 1999). This evidence notwithstanding, phylogenies based on genes from the host nuclear genomes appeared to challenge the monophyly of plastids. The first gene trees (based on SSUrRNA) that included sequences from red, green and glaucocystophyte algae did not show them to be a monophyletic group (Bhattacharya et al., 1995a), leading to various hypotheses regarding independent plastid origins. This was also the case with a few protein-coding genes, including β-tubulin and actin (in some analyses; Bhattacharya and Weber, 1997; Keeling et al., 1999). Although these trees did not support the monophyly of primary algae, they did not really argue against it either, because there was virtually no support for the position of these lineages. Several weak gene trees failing to support a group do not equate to strong evidence against the group. What is needed is a strong gene tree supporting some other relationship. One gene phylogeny apparently did support an alternative story: analysis of the largest subunit of RNA polymerase II (RPB1) showed strong statistical support for a separation between red and green algae (Stiller and Hall, 1997). This was used to support a model of plastid evolution in which only one of red, green or glaucocystophyte algae contained a primary plastid, and the other two contained secondary plastids, which had

simply reduced their membrane compliment to two. This is very difficult to imagine in light of the protein-trafficking system in secondary plastids (see later).

Subsequently, other genes have been analyzed individually and in combination, and altogether the case for multiple independent plastid origins has evaporated. Several nuclear genes have shown a weak relationship between red and green algae, including enolase; actin (in some analyses); α-tubulin; hsp70; hsp90; α and β subunits of vacuolar ATPase; and a combined analysis of α- and β-tubulins, EF-1α and actin (Archibald et al., 2001; Baldauf et al., 2000; Keeling, 2001; Keeling et al., 1999; Keeling and Palmer, 2001; Moreira et al., 2000; Stibitz et al., 2000). Moreover, the phylogeny of translation elongation factor 2 (EF-2) was shown to support the monophyly of red and green algae with very high statistical support, as was a combined analysis of 13 nuclear-encoded cytosolic genes (Moreira et al., 2000). Conversely, a reanalysis of the RPB1 gene failed to reject the possibility of a monophyletic red and green algae (Moreira et al., 2000). Recent analyses of RPB1 have also shown the red and green algae to branch together, albeit weakly (D. Longet, JMA, PJK and J. Pawlowski, unpublished data). For glaucocystophytes, there is little molecular data from nuclear genes, but some genes show them branching with the red or green algae, or both (e.g., Stibitz et al., 2000), and two multigene analyses also concluded that the red and green algae were closely related to the glaucocystophytes (Baldauf et al., 2000; Moreira et al., 2000). Altogether, the consistent picture coming from plastid genes, mitochondrial genes and nuclear genes is the same: red, green and glaucocystophyte algae share a common ancestor, and the origin of primary plastids can reasonably be inferred to trace back to a single endosymbiotic event.

3.4 Three Lineages

At present, primary plastids are found in only three lineages of eukaryotes: the glaucocystophytes, the red algae and the green algae (and their land plant relatives). Land plants and green algae are a pervasive and dominant group of eukaryotes. Green algae are found in almost every aquatic and marine environment known and are an extremely diverse and specious group. The green algal lineage is deeply divided into two major subgroups, the chlorophytes and streptophytes, and land plants evolved from within the streptophyte clade. Green algae and land plants are characterized by the presence of chlorophylls *a* and *b* in their plastid. Red algae are also a very diverse and specious group. They are predominantly marine but are also present in freshwater environments, and some red algae inhabit relatively hostile environments such as high heat or high salinity. Certain red algae have evolved a complex form of multicellularity not unlike plants in many respects. Red algal plastids are also distinctive as they contain chlorophyll *a* and phycobilins, the latter being accessory pigments associated with structures called phycobilisomes that are visible by electron microscopy on the outside of the inner plastid membrane. Glaucocystophytes are a relatively small group of algae, with only three genera and a handful of species. The glaucocystophytes also contain chlorophyll *a* and phycobilins, but their plastid is dramatically distinguished from other primary plastids in that it has retained the peptidoglycan wall between the inner and outer membranes.

The peptidoglycan wall of the cyanobacterial endosymbiont has been lost in red and green algae, and this has led to speculation that the glaucocystophytes might be the descendents of an early stage of the primary endosymbiosis (Herdman and Stanier, 1977). However, every other possible relationship between the three primary algal lineages has been proposed based on different lines of evidence (Cavalier-Smith, 1982; Delwiche et al., 1995; Kowallik, 1997; Martin et al., 1998; Valentin and Zetsche, 1990). Initially, molecular phylogenies with data from all three groups shed little light on this problem, as many phylogenies failed to even unite the three lineages (see previously), whereas others showed little consistency in

the relative order of the three clades. In phylogenies based on plastid EF-Tu, green algae are sister to a red algal–glaucocystophyte clade (Delwiche et al., 1995). In contrast, rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) phylogenies show that red algae contain one type of rubisco, whereas glaucocystophytes and green algae share another distantly related type (Delwiche and Palmer, 1996; Valentin and Zetsche, 1990). The molecule most extensively applied to this question is plastid SSUrRNA, and phylogenies based on this gene generally show glaucocystophytes branching before green and red algae (Helmchen et al., 1995; Turner et al., 1999). However, different analyses result in different topologies and varying levels of support (Nelissen et al., 1995), leaving the matter open to speculation.

One approach that has been used to resolve these relationships with greater certainty is to analyze very large data sets of concatenated genes. Recent analyses of plastid-encoded genes identified glaucocystophytes as the earliest primary-plastid-containing lineage, and in most phylogenies this position is well supported (Martin et al., 1998, 2002). Even with this amount of data, however, maximum likelihood analysis fails to unambiguously resolve the relative position of glaucocystophytes to green and red algae. This region of the tree is clearly recalcitrant to phylogenetic reconstruction, but for the most part, existing data seem to support the notion that glaucocystophytes are the deepest lineage of primary-plastid-containing eukaryotes.

3.5 Secondary Plastids

Although all plastids appear to be the product of a single primary endosymbiosis, primary-plastid-containing organisms account for only a fraction of eukaryotic photosynthetic diversity. A vast array of algae have acquired plastids through a process called secondary endosymbiosis, in which a phagotrophic eukaryote engulfs a eukaryotic cell with a primary plastid and retains its photosynthetic machinery (Figure 3.2a,b). Secondary (or complex) plastids differ from primary plastids in several important respects. First, and as a natural consequence of eukaryotic phagocytosis, secondary plastids are characterized by the presence of additional membranes. Whereas primary plastids have a double-membrane envelope, secondary plastids have three or four membranes. The outermost membrane corresponds to the phagosomal membrane of the secondary host cell and the third membrane of four-membrane plastids is thought to be derived from the plasma membrane of the algal endosymbiont (McFadden, 1999). Secondary plastids therefore reside within the lumen of the host cell's endomembrane system, unlike primary plastids, which are in the cytosol. Second, the differences in membrane structure between primary and secondary plastids result in different mechanisms for targeting proteins to the organelle. The vast majority of plastid proteins in plants and algae are encoded in the nucleus. These proteins are translated in the cytosol and imported into the plastid via a transit peptide. In algae with secondary plastids, the proteins must cross one or more additional membranes, for which they make use of the signal-peptide secretion system. Plastid proteins are first targeted to the endomembrane system by using a signal peptide, then localized to the plastid (by an unknown mechanism) and finally sent across the remaining two membranes by a standard transit peptide, as in primary plastids (Cavalier-Smith, 1999; Ishida et al., 2000; Kishore et al., 1993; McFadden 1999).

The space between the second and third membranes of four-membrane plastids corresponds to the remnant cytosol of the engulfed algal cell. In two different algal groups, this cellular compartment harbors the "smoking gun" of the process of secondary endosymbiosis: the relict nucleus of the eukaryotic endosymbiont. First identified as a double-membrane-bound body in early microscopic studies of cryptomonads (Greenwood et al., 1977), the nucleomorph was later discovered in the same compartment of chlorarachniophyte algae (Hibberd and Norris, 1984) and was found to contain DNA (Ludwig and Gibbs, 1989).

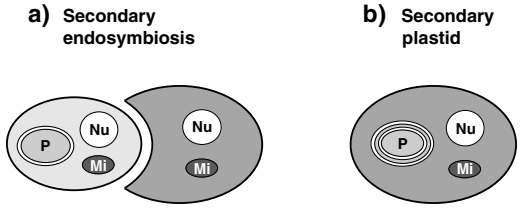

FIGURE 3.2 The general features and end product of secondary endosymbiosis. (a) Secondary endosymbiosis involves a phagotrophic, nonphotosynthetic eukaryote engulfing a primary-plastid-containing eukaryote. (b) A secondary-plastid-containing organism. The plastid resides within the endomembrane system of the host cell and is bound by additional membranes, typically four. The outermost (fourth) membrane corresponds to the phagosomal membrane of the secondary host, and the third membrane is derived from the plasma membrane of the engulfed algal cell (see text). Abbreviations: Cy, cyanobacterium; Nu, nucleus; Mi, mitochondrion; P, plastid.

Nucleomorphs have since been shown to be genuine eukaryotic nuclei, although with highly reduced, AT-rich and generally divergent genomes (Douglas et al., 2001; Gilson et al., 1997; Gilson and McFadden, 1996). Nucleomorph genome sequencing projects (Douglas et al., 2001; Gilson and McFadden, 2002) have revealed that large numbers of genes encoding plastid proteins have been transferred from the nucleomorph to the host nucleus in cryptomonads and chlorarachniophytes. This process has presumably gone to completion in all other algae that possess secondary plastids but lack nucleomorphs.

3.6 Red and Green Endosymbionts

The most obvious division among secondary plastids is between those derived from red or green algal endosymbionts. Two distinct groups of algae have green algal secondary plastids: euglenids and chlorarachniophytes. Euglenids are a common group of flagellates in both freshwater and marine environments, whereas the chlorarachniophytes are a more rare class of amoeboflagellate algae characterized by long and sometimes reticulating pseudopodia. A green algal origin for their respective plastids seemed likely given their photosynthetic pigment composition: both contain the signature pigmentation of green algae and land plants — chlorophylls *a* and *b*. This has since been confirmed by molecular data. Phylogenies of plastid-, and, in the case of the chlorarachniophytes, nucleomorph-encoded genes demonstrate that both plastids are derived from green algae (e.g., Durnford et al., 1999; Ishida et al., 1997, 1999; McFadden et al., 1995; Turmel et al., 1999). In neither case, however, has a particular green algal lineage been identified as the unambiguous source of the organelle. Although phylogenies of chloroplast EF-Tu have suggested that the chlorarachniophyte endosymbiont was an ulvophycean green alga (Ishida et al., 1997), nucleomorph SSUrRNA trees have been used to argue that the endosymbiont was an ulvophyte (Ishida et al., 1999) or, alternatively, a trebouxiophyte green alga (Van de Peer et al., 1996). The use of nucleomorph-encoded genes has been hampered by the fact that they are typically fast evolving (Gilson and McFadden, 2002) and therefore prone to misplacement in molecular phylogenies. Phylogenetic trees inferred from plastid SSUrRNA and the large subunit of rubisco have placed the chlorarachniophytes basal to the chlorophytes (to which the ulvophytes and trebouxiophytes belong), the streptophytes and the euglenids (McFadden et al., 1995). To complicate matters further, the chlorarachniophytes have recently been shown to possess a gene encoding the metabolic enzyme enolase that shows affinity to streptophyte enolases, a result that is supported by several homologous amino acid insertions (Keeling and Palmer,

2001). The origin of the chlorarachniophyte endosymbiont within green algae is thus at present an open question.

The exact origin of the euglenid plastid is similarly ambiguous. The plastid genome of *Euglena gracilis* has been completely sequenced (Hallick et al., 1993) and shares many features with other green algal plastid genomes. However, no specific group has been singled out as a close relative of the endosymbiont, beyond the fact that it was likely a member of the Chlorophyta (Turmel et al., 1999). The plastids in euglenids and chlorarachniophytes are surrounded by three and four membranes, respectively, and the two groups are generally considered to have acquired their plastids independently (see later).

By comparison, a much larger number of algal groups possess secondary plastids derived from red algae. The cryptomonads are an abundant group of flagellated unicells that are significant in possessing, together with the chlorarachniophytes, a nucleomorph. The nucleomorph genome of the cryptomonad *Guillardia theta* has been completely sequenced and is a mere 551 kb in size, partitioned among three similarly sized chromosomes (Douglas et al., 2001). The heterokonts (or stramenopiles) and haptophytes are two very important algal groups that together make up a large fraction of the earth's aquatic photosynthesizers. From a cell biological perspective, the cryptomonad, haptophyte and heterokont plastids are unique in that the outermost membrane surrounding the plastid is continuous with the endoplasmic reticulum (ER) and the outer membrane of the nuclear envelope (Gibbs, 1981). Molecular evidence for a red algal origin for their secondary plastids is extensive and involves consideration of conserved features of plastid genome organization (Douglas and Penny, 1999) as well as phylogenetic analyses of plastid and nucleomorph rRNAs and several proteins (e.g., Archibald et al., 2001; Daugbjerg and Andersen, 1997; Van de Peer and De Wachter, 1997; Van der Auwera et al., 1998). The phylogenies of the large and small subunits of rubisco have been particularly informative. In contrast to green algae, chlorarachniophytes and euglenids, which contain a rubisco enzyme derived from cyanobacteria, red algae contain a distantly related proteobacterial type of rubisco that appears to have been acquired via lateral gene transfer (Delwiche, 1999; Delwiche and Palmer, 1996). Significantly, the cryptomonads, haptophytes and heterokonts contain the proteobacterial or red algal form of rubisco.

The dinoflagellates and apicomplexan parasites, which, together with ciliates, make up a higher-order group of eukaryotes referred to as the alveolates (Wolters, 1991), also appear to harbor plastids derived from red algae. In the case of the apicomplexans, the presence of a plastid was most unexpected (McFadden et al., 1996; Wilson et al., 1996), because these organisms are nonphotosynthetic, intracellular parasites of animals. Determining the source of the relict organelle has been difficult, in large part because all of the genes encoding proteins directly involved in photosynthesis have been lost, and the ones that remain are extremely divergent. Although early phylogenies based on EF-Tu suggested that the apicomplexan plastid was derived from a green alga (Köhler et al., 1997), a number of observations on the gene content of the genome and phylogenetic analyses based on other genes suggested that the plastid was red algal (Williamson et al., 1994; McFadden and Waller, 1997; Blanchard et al ., 1999; Gardner et al., 2002). More recently, a green algal origin for the apicomplexan plastid has been suggested based on characteristics of the mitochondrial protein COXII. In nearly all eukaryotes, COXII is found as a single protein encoded in the mitochondrial genome, but apicomplexa and certain green algal COXIIs are split into two pieces encoded by the nuclear genes *cox2a* and *cox2b* (Funes et al., 2002). Interestingly, Funes et al. show that the split genes in apicomplexa are closely related to their split homologues in green algae, leading to the conclusion that apicomplexa inherited these split genes from a green algal endosymbiont that gave rise to the plastid (Funes et al., 2002). However, these authors did not analyze ciliate COXIIs, which are critical because ciliates are close relatives of apicomplexa. When these genes

are included in the phylogeny, the ciliates and apicomplexa form a clade to the exclusion of green algae (unpublished observations). Moreover, the ciliate proteins contain a very large (up to 300 amino acids) insertion in exactly the same position as the split in the apicomplexan and green algal homologues, which seems unlikely to be a coincidence. It will be interesting to investigate the nature of dinoflagellate COXIIs (none are known as yet), but considering all the information available at present, there is no evolutionary link between the COXIIs of apicomplexa and green algae.

In general, the overall characteristics of the apicomplexan plastid genome seem more consistent with a red algal origin. Analyses of plastid gene sequences from dinoflagellates support a red ancestry for their plastid (Takishita and Uchida, 1999; Zhang et al., 1999) and also suggest that the dinoflagellate and apicomplexan plastids are specifically related (Zhang et al., 2000; see later), consistent with the known relationship between the two host cells. With the exception of the dinoflagellate plastid, which is bound by three membranes, all other red algal secondary plastids are surrounded by four membranes. The cryptomonads, haptophytes, heterokonts and dinoflagellates are distinctive in that their plastids contain the secondary photosynthetic pigment chlorophyll *c*. (The Apicomplexa are nonphotosynthetic and thus lack pigmentation.) Table 3.1 summarizes the full spectrum of primary- and secondary-plastid-containing organisms and the general features of their plastids.

3.7 How Many Secondary Endosymbioses?

The fact that modern-day algae harbor secondary plastids derived from both red and green algal endosymbionts necessitates that secondary endosymbiosis has occurred at least twice during eukaryotic evolution. However, the morphological and biochemical diversity of secondary-plastid-containing algae, particularly among those with red algal plastids, is such that it has been difficult to determine the exact number of events. Depending on the type of data considered, estimates have ranged from two to as many as seven separate secondary endosymbioses (Cavalier-Smith, 1999; Delwiche and Palmer, 1997). Fortunately, recent molecular data have begun to clarify this controversial issue.

TABLE 3.1 General Characteristics of Primary and Secondary Plastids

Lineage	*Plastid Type*	*Plastid Membranes*	*Pigments*
Green algae	1°	2	Chlorophyll a + b
Red algae	1°	2	Chlorophyll a, phycobilins
Glaucocystophytes	1°	2[a]	Chlorophyll a, phycobilins
Chlorarachniophytes[b]	2° (green)	4	Chlorophyll a + b
Euglenids	2° (green)	3	Chlorophyll a + b
Cryptomonads[b]	2° (red)	4	Chlorophyll a + c, phycobilins
Heterokonts	2° (red)	4	Chlorophyll a + c
Haptophytes	2° (red)	4	Chlorophyll a + c
Dinoflagellates	2° (red)	3	Chlorophyll a + c, peridinin
Apicomplexa	2° (red)	4	N/A[c]

[a] Glaucocystophyte plastids are also bound by a peptidoglycan layer between the two membranes.

[b] Groups that have retained the nucleus of the algal endosymbiont (the nucleomorph).

[c] Apicomplexan parasites are nonphotosynthetic; their plastids contain no light-harvesting pigments.

With respect to the green plastids of chlorarachniophytes and euglenids, although it has been suggested that their photosynthetic organelles trace back to a single secondary endosymbiotic event (Cavalier-Smith, 1999, 2000), the most widely held view is that they were acquired separately from different green algae. This is based on a general lack of morphological similarity shared between the host cells (Hibberd and Norris, 1984) and on both their hosts and plastids appearing unrelated in molecular phylogenies (Bhattacharya et al., 1995b; Ishida et al., 1997; Keeling, 2001; McFadden et al., 1995). The host component of the chlorarachniophyte algae is a member of the Cercozoa, a large assemblage of protists that includes the euglyphid amoebae, cercomonad flagellates and plasmodiophorid plant pathogens. The Cercozoa are an extremely diverse lineage, and they have only recently been recognized as a monophyletic group based on molecular phylogenies (Bhattacharya et al., 1995b; Cavalier-Smith, 1998; Cavalier-Smith and Chao, 1997; Keeling et al., 1998). Cercozoa have more recently been suggested to be related to the Foraminifera, a group of nonphotosynthetic, pseudopod-forming, predominantly testate protists, based on phylogenies of actin (Keeling, 2001) and a shared insertion in their polyubiquitin genes (Archibald et al., 2002). The euglenid host, on the other hand, belongs to the Euglenozoa, a large eukaryotic group that also includes kinetoplastids and diplonemids. Within the Euglenozoa, the photosynthetic euglenids (such as *Euglena*) are clearly nested within heterotrophic lineages (Preisfeld et al., 2001) and the inferred series of cytoskeletal transformations in the group indicates that phototrophy is a relatively recent adaptation within euglenozoan evolution (Leander et al., 2001a,b). At present, the data is most consistent with the hypothesis that the euglenid and chlorarachniophyte host cells are not specifically related and that their plastids are of independent origin.

The origin and evolution of red secondary plastids have been more difficult to discern. A large number of biochemical and ultrastructural features are suggestive of a specific relationship between some or all chlorophyll *a* + *c*-containing algae and the possibility that their plastids share a common origin. In particular, the heterokonts, haptophytes and cryptomonads possess four-membrane plastids that reside in the lumen of the ER. Cavalier-Smith (1981, 1982) placed these organisms together in the kingdom Chromista on the basis of this characteristic, arguing that fusion of the outermost plastid membrane with the outer nuclear envelope is improbable and therefore likely occurred only once in their common ancestor (Cavalier-Smith, 1986). The tubular mastigonemes (hairs) on the flagella of both heterokonts and cryptomonads have been argued to be homologous (Cavalier-Smith, 1986), and with respect to the heterokonts and haptophytes, each contain fucoxanthin and chrysolaminaran. Starch is present in both cryptomonads and dinoflagellates (stored in the cytosol of dinoflagellates but in the periplastid space in cryptomonads), and, finally, heterokonts, haptophytes and dinoflagellates each have three thylakoids per stack in their respective plastids as well as tubular mitochondrial cristae (cryptomonads have flat cristae). Of the four lineages with chlorophyll *a* + *c*-pigmented plastids, the heterokonts and haptophytes are most similar from a morphological and biochemical perspective, and have historically been grouped together as chromophyte algae (Andersen, 1991).

Despite these similarities, early molecular studies painted a different picture. Phylogenetic analyses of rubisco and plastid SSUrRNA showed that cryptomonads, heterokonts and haptophytes did not form a monophyletic group within the red algae (Daugbjerg and Andersen, 1997; Medlin et al., 1995; Müller et al., 2001; Oliveira and Bhattacharya, 2000). Nuclear SSUrRNA trees also failed to unite the host components of the three groups (Bhattacharya et al., 1995a), and together these results supported the idea that the haptophyte, heterokont and cryptomonad plastids were derived from separate endosymbiotic events. However, more recent molecular data are more in line with morphological and biochemical considerations and a single origin for their plastids. A comprehensive analysis of a concatenated dataset containing five plastid-encoded genes (SSUrRNA, *psa*A, *psb*A,

*rbc*L and *tuf*A) showed the cryptomonads, heterokonts and haptophytes to be a highly supported monophyletic group (Yoon et al., 2002b).

The dinoflagellates and apicomplexans have been particularly difficult to fit into the puzzle of plastid origins. Until recently, no gene sequences were available from dinoflagellate plastid genomes and there has been considerable debate on whether the relict apicomplexan plastid is of green or red algal origin (Blanchard and Hicks, 1999; Köhler et al., 1997; McFadden and Waller, 1997). Several dinoflagellate plastid genes have now been characterized (Takishita and Uchida, 1999; Zhang et al., 1999). Curiously, these genes are encoded on single-gene minicircles, unlike all other known plastid genomes (Zhang et al., 1999). In molecular phylogenies of rRNA, dinoflagellate and apicomplexan sequences branch together, suggesting that their plastids share a common ancestry (Zhang et al., 2000). However, this result has been interpreted with caution: both the dinoflagellate and the apicomplexan genes are divergent and AT rich, leaving open the possibility that their affinity in phylogenetic trees is due to methodological artifact.

More recently, analysis of the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has provided additional support for the hypothesis that the dinoflagellate and apicomplexan plastids are related and, significantly, suggests that the red algal plastids of heterokonts, cryptomonads and alveolates share a single origin. All algae and plants have two nuclear-encoded forms of GAPDH: one that functions in the plastid and another that functions in the cytosol. As expected, the GAPDH isoform targeted to the primary plastids of red and green algae is related to the GAPDH found in cyanobacteria. In stark contrast, the dinoflagellate, apicomplexan, heterokont and cryptomonad plastid GAPDH is not cyanobacterial, but is related to the eukaryotic cytosolic isoform (Fast et al., 2001). Apparently, the gene encoding the cytosolic GAPDH was duplicated, acquired the information necessary to target its protein product to the plastid and took over the role of the cyanobacterial homologue. This process is known as endosymbiotic gene replacement (Martin and Schnarrenberger, 1997), and in the case of GAPDH the data suggest that the replacement took place only once, in a common ancestor shared by apicomplexa, dinoflagellates, heterokonts and probably cryptomonads (Fast et al., 2001). Curiously, the dinoflagellate *Pyrocystis* possesses a cyanobacterial-like GAPDH that is closely related to that of *Euglena* (Fagan and Hastings, 2002); the origin of this gene is unclear. Nevertheless, the overall picture of red secondary plastid evolution painted by GAPDH is increasingly supported by analyses of host molecules. Phylogenetic analyses of nuclear-encoded rRNA suggest that the alveolates and heterokonts are a monophyletic group (Ben Ali et al., 2001; Van de Peer and De Wachter, 1997), a result that is also found in analyses of protein-coding genes (Baldauf et al., 2000).

Figure 3.3 summarizes the current consensus for the evolutionary history of primary and secondary plastids. Following a single endosymbiotic event between a heterotrophic eukaryote and a cyanobacterium, three primary-plastid-containing groups diverged from one another: the glaucocystophytes, the red algae and the green algae. Two secondary endosymbioses involving different hosts and different green algae gave rise to the euglenids and chlorarachniophytes, and a single secondary endosymbiosis occurred between a red alga and an ancestor of the cryptomonads, heterokonts, haptophytes and alveolates. All algae containing secondary plastids of red algal origin thus appear to comprise a eukaryotic supergroup dubbed as the chromalveolates (Cavalier-Smith, 1999).

3.8 Loss of Photosynthesis: How Common Is It?

The chromalveolate theory of plastid evolution has important ramifications for the evolution and cell biology of a large number of important eukaryotic lineages. Perhaps most significant is the fact that it invokes extensive loss of photosynthesis. Within alveolates, only approximately half of known dinoflagellate species are photosynthetic (Taylor, 1987), and the

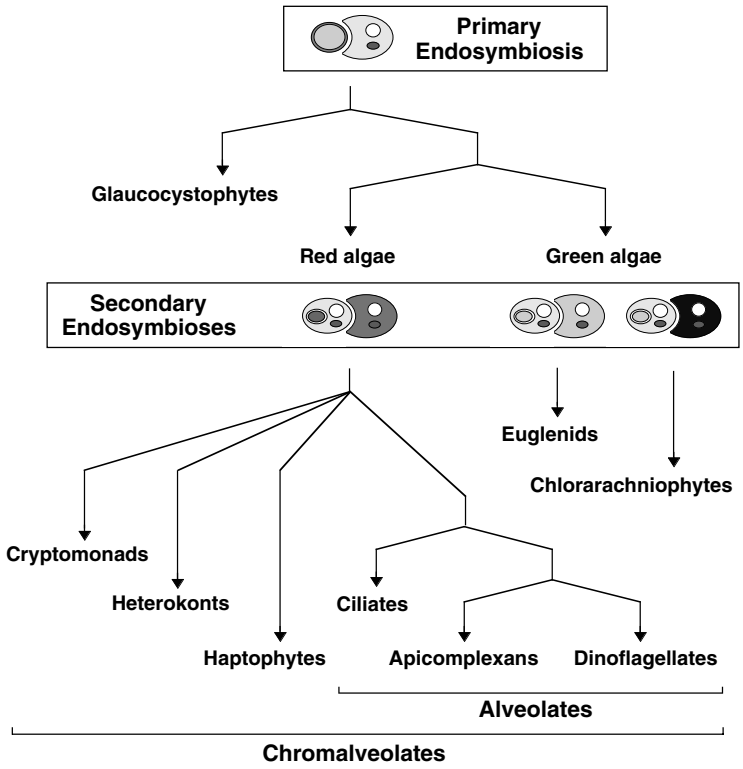

FIGURE 3.3 One scheme for the evolution of photosynthetic eukaryotes by primary and secondary endosymbiosis. A single primary endosymbiosis between a phagotrophic eukaryote and a cyanobacterium (top) gave rise to the three primary-plastid-containing lineages: glaucocystophytes, red algae and green algae. Subsequent to the diversification of these three groups, three secondary endosymbiotic events took place. Two separate endosymbioses involving different hosts and different green algae led to the chlorarachniophytes and the photosynthetic members of the euglenids. A single, ancient secondary endosymbiotic event between a phagotrophic eukaryote and a red alga spawned a lineage from which the cryptomonads, heterokonts, haptophytes, ciliates, apicomplexans and dinoflagellates evolved. The ciliates and apicomplexans are entirely nonphotosynthetic and loss of photosynthesis is rampant in dinoflagellates and heterokonts (see text).

ciliates, a major group of protist predators, are an entirely nonphotosynthetic lineage. If the heterokont and alveolate common ancestor had a plastid, then ciliates must have evolved from a plastid-containing organism, and might still have a plastid or plastid-derived genes (Fast et al., 2001). The heterokonts themselves also contain a large number of nonphotosynthetic members, many of which branch basal to photosynthetic lineages in phylogenetic trees. This pattern has previously been interpreted as evidence for the idea that the group as a whole was ancestrally nonphotosynthetic, and that a plastid (and photosynthesis) was acquired at a later point in the evolution of the heterokonts (Leipe et al., 1994). However, loss of photosynthesis has been invoked for some heterokonts (e.g., Cavalier-Smith et al., 1995), and a plastid-derived 6-phosphogluconate dehydrogenase gene has recently been identified in the nonphotosynthetic heterokont plant pathogen *Phytophthera infestans* (Andersson and Roger 2002), suggesting that the group as a whole had a photosynthetic ancestor. Within dinoflagellates, a recent analysis of nuclear SSUrRNA genes allowed inference of eight independent instances of loss of photosynthesis (Saldarriaga et al., 2001), a

number that is sure to grow as more nonphotosynthetic lineages are sampled. Loss of photosynthesis — which should be distinguished from outright plastid loss — now appears to be far more common than previously appreciated (Archibald and Keeling, 2002; Cavalier-Smith, 1999).

3.9 Tertiary Endosymbiosis, Serial Secondary Endosymbiosis

The story of plastid evolution does not end with secondary endosymbiosis. When one considers the history of plastids within the dinoflagellates, an extraordinarily diverse and ecologically significant lineage, it is clear that the situation is even more complex. Most photosynthetic dinoflagellates contain three-membrane plastids with peridinin and chlorophylls *a* and *c*. Some dinoflagellates, however, have replaced their peridinin-containing plastids with an unrelated one through a process known as tertiary endosymbiosis. Remarkably, three of the five red secondary-plastid-containing lineages have had their plastids "stolen" by at least one dinoflagellate group. The endosymbiont of *Peridinium balticum* and *P. foliacium* is a diatom (heterokont) (Chesnick et al., 1997), that of *Gymnodinium acidotum* a cryptomonad (Farmer and Roberts, 1990; Wilcox and Wedemayer, 1985) and *Gymnodinium* and *Gyrodinium* harbor plastids of haptophyte origin (Tengs et al., 2000). It has recently been suggested that the standard peridinin plastid in dinoflagellates is itself derived from a haptophyte via tertiary endosymbiosis, on the basis of *psa*A, *psb*A and *rbc*L (large subunit of rubisco) gene phylogenies (Yoon et al., 2002a). Similarly, the apicomplexan plastid has also recently been suggested to be a product of a plastid replacement (Palmer, 2003), based on the apparent conflict between data suggesting that it is derived from a red alga (Fast et al., 2001; McFadden and Waller, 1997) and data suggesting it is derived from a green alga (Funes et al., 2002; Köhler et al., 1997). Although neither case can be proven or disproven definitively, the strongest evidence at present suggests that the plastid is red in nature and no such replacement took place. Comparing the full sequence of the *Plasmodium* genome (Gardner et al., 2002) with other chromalveolate genomes to come will doubtless settle this debate.

Still other dinoflagellates appear to have replaced their ancestral secondary plastid with a primary plastid in what is, strictly speaking, a serial secondary endosymbiosis. *Lepidodinium viride* has a chlorophyll *a* + *b*-containing plastid that appears to be derived from a green alga (Watanabe et al., 1990). The degree to which the various tertiary and serial secondary endosymbionts and plastids have integrated with their dinoflagellate hosts varies from transient endosymbioses to fully integrated and reduced organelles (Delwiche, 1999).

It is not clear why dinoflagellates have on the one hand repeatedly lost photosynthesis and on the other substituted their peridinin-containing plastids with plastids from other algae. However, the answer might lie, at least in part, in the fact that many dinoflagellates are mixotrophs: they practice both heterotrophy and phototrophy. The maintenance of a dual mode of nutrition might allows plastids (and photosynthesis) to come and go. Phagocytosis provides not only a constant supply of potential replacement plastids from prey algae but also an alternative energy source in instances where photosynthesis is insufficient. Regardless of the reasons for such rampant plastid shuffling, tertiary endosymbiosis adds another layer of complexity to an already complex array of nuclear- and plastid-encoded proteins that service the dinoflagellate plastid. In many instances, the nuclear-encoded, plastid-targeted proteins that functioned in the peridinin plastid can simply be recycled and imported into the newly acquired organelle. However, it is also possible that the genes encoding such proteins will be transferred from the endosymbiont nucleus to that of the host before it is lost, replacing the host copy. This has recently been demonstrated. In the dinoflagellate *Karenia brevis* (= *Gymnodinium breve*), the original nuclear-encoded oxygen-evolving enhancer 1 (PsbO) plastid protein from the peridinin-type plastid has been replaced

by a haptophyte version that came in with the tertiary endosymbiont (Ishida and Green, 2002). Tertiary plastids are therefore evolutionary chimaeras in terms of their complement of plastid proteins.

3.10 A Second Primary Endosymbiosis?

Endosymbiosis has played a very important role in several of the critical steps in eukaryotic evolution. However, the process is often difficult to study because it is by nature a relatively rare and catastrophic event that often leads to sweeping changes in the host and endosymbiont. These changes erase many of the traces of how the event took place, or even who was involved. This is one reason why the process of secondary endosymbiosis is attractive to study: because secondary plastids have evolved multiple times independently, one can compare the results of different events and determine what some of the general principles of the process might be. In the case of the original primary plastid (and the origin of mitochondria), this powerful comparative approach is lost because the organelle arose from a unique endosymbiotic partnership, and hence there is nothing with which to compare the process — or is there?

One very interesting but very poorly understood organism that can provide a wealth of comparative information on the origin of plastids is the filose amoeba *Paulinella chromatophora*. Filose amoebae are a group of testate, or shelled, amoebae that are part of the Cercozoa, the same large and diverse lineage to which the chlorarachniophytes belong (although, within Cercozoa, *Paulinella* and other filose amoebae are not closely related to chlorarachniophytes). *P. chromatophora* is of interest because it might represent an independent origin of plastids. Each *P. chromatophora* cell harbors two kidney-shaped endosymbionts that have been shown by light microscopy and ultrastructural studies to be cyanobacteria (Kies, 1974). Interestingly, the endosymbionts could not be cultivated outside the host, and are known to divide synchronously with host cell division. (Each daughter of host division gets an endosymbiont, which then divides once to reconstitute the natural compliment of two endosymbionts.) The cyanobacteria have been suggested to retain their peptidoglycan wall and reside within a host vacuole (Kies, 1974); however, it is not exactly clear how many membranes actually surround the endosymbionts. *P. chromatophora* has not been observed to feed, and digestive vacuoles have never been observed (Kies, 1974), suggesting that the endosymbionts satisfy the energy requirements of the cell. Indeed, early biochemical observations indicated that the cyanobacteria are actively photosynthetic, and that photosynthate is transferred to the host where it is converted into macromolecules (Kies and Kremer, 1979). The cyanobacteria do not resemble extant plastids, suggesting that they are not derived from a secondary endosymbiosis, but instead appear to resemble *Synechococcus* (Kies, 1974), implying that they have been derived from an independent endosymbiosis. Interestingly, another species of *Paulinella*, *P. ovalis*, is nearly identical to *P. chromatophora* except that it is nonphotosynthetic and lacks any evidence of the endosymbionts. *P. ovalis* is phagotrophic, contains a clear digestive vacuole and feeds on *Synechococcus* (Johnson et al., 1988). One can see a possible evolutionary trajectory wherein a heterotrophic amoeba feeding on unicellular cyanobacteria captured a prey cell, and for some reason failed to digest it, resulting in something like *P. chromatophora* (Johnson et al., 1988). Judging from the similarity between *P. ovalis* and *P. chromatophora*, this event must have taken place very recently, so *P. chromatophora* would appear to be an ideal organism in which to study the early stages of a possible primary endosymbiosis and compare the features of this partnership with modern and fully developed plastids.

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References

Andersen, R. A. (1991) The cytoskeleton of chromophyte algae. *Protoplasma* 164: 143–159.

Andersson, J. O. and Roger, A. J. (2002) A cyanobacterial gene in nonphotosynthetic protists: An early chloroplast acquisition in eukaryotes? *Curr. Biol.* 12: 115–119.

Archibald, J. M., Cavalier-Smith, T., Maier, U. and Douglas, S. (2001) Molecular chaperones encoded by a reduced nucleus- the cryptomonad nucleomorph. *J. Mol. Evol.* 52: 490–501.

Archibald, J. M. and Keeling, P. J. (2002) Recycled plastids: A green movement in eukaryotic evolution. *Trends Genet.* 18: 577–584.

Archibald, J. M., Longet, D., Pawlowski, J. and Keeling, P. J. (2003) A novel polyubiquitin structure in Cercozoa and Foraminifera: Evidence for a new eukaryotic supergroup. *Mol. Biol. Evol.* 20: 62–66.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. and Doolittle, W. F. (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290: 972–977.

Ben Ali A., De Baere, R., Van der Auwera,G., De Wachter, R and Van de Peer, Y. (2001) Phylogenetic relationships among algae based on complete large-subunit rRNA sequences. *Int. J. Syst. Evol. Microbiol.* 51: 737–749.

Besendahl, A., Qiu, Y. L., Lee, J., Palmer, J.D and Bhattacharya, D. (2000) The cyanobacterial origin and vertical transmission of the plastid tRNA(Leu) group-I intron. *Curr. Genet.* 37: 12–23.

Bhattacharya, D., Helmchen, T., Bibeau, C. and Melkonian, M. (1995a) Comparison of nuclear-encoded small-subunit ribosomal RNAs reveal the evolutionary position of the Glaucocystophyta. *Mol. Biol. Evol.* 12: 415–420.

Bhattacharya, D., Helmchen, T. and Melkonian, M. (1995b) Molecular evolutionary analyses of nuclear-encoded small subunit ribosomal RNA identify an independent rhizopod lineage containing the Euglyphidae and the Chlorarachniophyta. *J. Euk. Microbiol.* 42:64–68.

Bhattacharya, D. and Weber, K. (1997) The actin gene of the glaucocystophyte *Cyanophora paradoxa*: Analysis of the coding region and introns, and an actin phylogeny of eukaryotes. *Curr. Genet.* 31: 439–446.

Blanchard J. L. and Hicks J. S. (1999) The non-photosynthetic plastid in malarial parasites and other apicomplexans is derived from outside the green plastid lineage. *J. Euk. Microbiol.* 46: 367–375.

Blankenship, R. E. (1994) Protein structure, electron transfer and evolution of prokaryotic photosynthetic reaction centers. *Ant. Van Leeuwen.* 65: 311–329.

Cavalier-Smith, T. (1981) Eukaryote kingdoms: Seven or nine? *Biosystems* 14: 461–481.

Cavalier-Smith, T. (1982) The origins of plastids. *Biol. J. Linn. Soc.* 17: 289–306.

Cavalier-Smith, T. (1986) The kingdom Chromista: Origin and systematics. *Progr. Phycol. Res.* 4: 309–347.

Cavalier-Smith, T. (1998) A revised six-kingdom system of life. *Biol. Rev. Camb. Philos. Soc.* 73: 203–266.

Cavalier-Smith, T. (1999) Principles of protein and lipid targeting in secondary symbiogenesis: Euglenoid, dinoflagellate, and sporozoan plastid origins and the eukaryote family tree. *J. Euk. Microbiol.* 46: 347–366.

Cavalier-Smith, T. (2000) Membrane heredity and early chloroplast evolution. *Trends Plant Sci.* 5: 174–182.

Cavalier-Smith, T. and Chao, E. E. (1997) Sarcomonad ribosomal RNA sequences, rhizopod phylogeny, and the origin of euglyphid amoebae. *Arch. Protistenkd.* 147: 227–236.

Cavalier-Smith, T., Chao, E. E. and Allsopp, M. T. E. P. (1995) Ribosomal RNA evidence for chloroplast loss within heterokonta: Pedinellid relationships and a revised classification of ochristan algae. *Arch. Protistenkd.* 145: 209–220.

Chesnick, J. M., Hooistra, W. H., Wellbrock, U. and Medlin, L. K. (1997) Ribosomal RNA analysis indicates a benthic pennate diatom ancestry for the endosymbionts of the dinoflagellates *Peridinium foliaceum* and *Peridinium balticum* (Pyrrhophyta). *J. Euk. Microbiol.* 44: 314–320.

Daugbjerg, N and Andersen, R. A. (1997) Phylogenetic analyses of the *rbcL* sequences from haptophytes and heterokont algae suggest their chloroplasts are unrelated. *Mol. Biol. Evol.* 14: 1242–1251.

Delwiche, C. F. (1999) Tracing the thread of plastid diversity through the tapestry of life. *Am. Nat.* 154 (suppl.): S164–S177.

Delwiche, C. F., Kuhsel, M. and Palmer, J. D. (1995) Phylogenetic analysis of *tuf*A sequences indicates a cyanobacterial origin of all plastids. *Mol. Phylogenet. Evol.* 4: 110–128.

Delwiche, C. F. and Palmer, J. D. (1996) Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. *Mol. Biol. Evol.* 13: 873–882.

Delwiche, C. F. and Palmer, J. D. (1997) The origin of plastids and their spread via secondary endosymbiosis. In *Origins of Algae and Their Plastids* (Bhattacharya, D., Ed.). Springer-Verlag, Wien, pp. 53–86.

Douglas, S. E. and Penny, S. L. (1999) The plastid genome from the cryptomonad alga, *Guillardia theta*: Complete sequence and conserved synteny groups confirm its common ancestry with red algae. *J. Mol. Evol.* 48: 236–244.

Douglas, S. E., Zauner S., Fraunholz, M., Beaton, M., Penny S., Deng, L., Wu X., Reith, M., Cavalier-Smith, T. and Maier, U. -G. (2001) The highly reduced genome of an enslaved algal nucleus. *Nature* 410: 1091–1096.

Durnford, D. G., Deane, J. A., Tan, S., McFadden, G. I., Gantt, E. and Green, B. R. (1999) A phylogenetic assessment of the eukaryotic light-harvesting antenna proteins, with implications for plastid evolution. *J. Mol. Evol.* 48: 59–68.

Fagan, T. M. and Hastings, J. W. (2002) Phylogenetic analysis indicates multiple origins of chloroplast glyceraldehyde-3-phosphate dehydrogenase genes in dinoflagellates. *Mol. Biol. Evol.* 19: 1203–1207.

Farmer, M. A. and Roberts, K. R. (1990) Organelle loss in the endosymbiont of *Gymnodinium acidotum* (Dinophyceae). *Protoplasma* 153: 178–185.

Fast, N. M., Kissinger, J. C., Roos, D. S. and Keeling, P. J. (2001) Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol. Biol. Evol.* 18: 418–426.

Funes, S., Davidson, E., Reyes-Prieto, A., Magallón, S., Herion, P., King, M. P. and Gonzalez-Halphen, D. (2002) Apicomplexan split cox2 genes indicate a green algal apicoplast ancestor. *Science* 298: 2155.

Gardner, M. J., Goldman, N., Barnett, P., Moore, P. W., Rangachari, K., Strath, M., Whyte, A. Williamson, D. H. and Wilson, R. J. (1994) Phylogenetic analysis of the *rpo*B gene from the plastid-like DNA of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 66: 221–231.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J, Angiuoli, S., Pertea, M., Allen J., Selengut, J, Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G.M., Mungall C., Venter, J.C., Carucci, D.J., Hoffman, S.L., Newbold, C., Davis, R. W., Fraser, C.M. and Barrell, B. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419: 498–511.

Gibbs, S. P. (1981) The chloroplast endoplasmic reticulum: structure, function, and evolutionary significance. *Int. Rev. Cytol.* 72: 49–99.

Gilson, P. R., Maier, U. G. and McFadden, G. I. (1997) Size isn't everything: Lessons in genetic miniaturisation from nucleomorphs. *Curr. Opin. Genet. Dev.* 7: 800–806.

Gilson, P. R. and McFadden, G. I. (1996) The miniaturized nuclear genome of a eukaryotic endosymbiont contains genes that overlap, genes that are cotranscribed, and the smallest known spliceosomal introns. *Proc. Natl. Acad. Sci. USA* 93: 7737–7742.

Gilson, P. R. and McFadden, G. I. (2002) Jam packed genomes: A preliminary, comparative analysis of nucleomorphs. *Genetica* 115: 13–28.

Greenwood, A. D., Griffiths, H. B. and Santore, U. J. (1977) Chloroplasts and cell compartments in Cryptophyceae. *Br. Phycol. J.* 12: 119.

Hallick, R. B., Hong, L., Drager, R. G., Favreau, M. R., Monfort A., Orsat, B., Spielmann, A. and Stutz, E. (1993) Complete sequence of *Euglena gracilis* chloroplast DNA. *Nucleic Acids Res.* 21: 3537–3544.

Helmchen, T. A., Bhattacharya, D. and Melkonian, M (1995) Analyses of ribosomal RNA sequences from glaucocystophyte cyanelles provide new insights into the evolutionary relationships of plastids. *J. Mol. Evol.* 41: 203–210.

Herdman, M. and Stanier, R. (1977) The cyanelle: Chloroplast or endosymbiotic procaryote? *FEMS Microbiol. Lett.* 1: 7–12.

Hibberd, D. J. and Norris, R. E. (1984) Cytology and ultrastructure of *Chlorarachnion reptans* (Chlorarachniophyta divisio nova, Chlorarachniophyceae classis nova). *J. Phycol.* 20: 310–330.

Ishida, K., Cao Y., Hasegawa, M., Okada, N. and Hara, Y. (1997) The origin of chlorarachniophyte plastids, as inferred from phylogenetic comparisons of amino acid sequences of EF-Tu. *J. Mol. Evol.* 45: 682–687.

Ishida, K. and Green, B. R. (2002) Second- and third-hand chloroplasts in dinoflagellates: Phylogeny of oxygen-evolving enhancer 1 (PsbO) protein reveals replacement of a nuclear-encoded plastid gene by that of a haptophyte tertiary endosymbiont. *Proc. Natl. Acad. Sci. USA* 99: 9294–9299.

Ishida, K., Green, B. R. and Cavalier-Smith, T. (1999) Diversification of a chimaeric algal group, the chlorarachniophytes: Phylogeny of nuclear and nucleomorph small-subunit rRNA genes. *Mol. Biol. Evol.* 16: 321–331.

Ishida, K., Green, B. R. and Cavalier-Smith, T. (2000) Endomembrane structure and the protein targeting pathway in *Heterosigma akashiwo* (Raphidophyceae, Chromista). *J. Phycol.* 36: 1135–1144.

Jarvis, P. and Soll, J. (2001) Toc, Tic, and chloroplast protein import. *Biochim. Biophys. Acta* 1541: 64–79.

Johnson, P. W., Hargraves, P. E. and Sieburth, J. M. (1988) Ultrastructure and ecology of *Calycomonas ovalis* Wulff, 1919 (Chrysophyceae) and its redescription as a testate rhizopod, *Paulinella ovalis* n. comb. (Filosea: Euglyphina). *J. Protozool.* 35: 618–626.

Keeling, P. J. (2001) Foraminifera and Cercozoa are related in actin phylogeny: Two orphans find a home? *Mol. Biol. Evol.* 18: 1551–1557.

Keeling, P. J., Deane, J. A., Hink-Schauer, C., Douglas, S. E., Maier. U. G. and McFadden, G. I. (1999) The secondary endosymbiont of the cryptomonad *Guillardia theta* contains alpha, beta-, and gamma-tubulin genes. *Mol. Biol. Evol.* 16: 1308–1313.

Keeling, P. J., Deane, J. A. and McFadden, G. I. (1998) The phylogenetic position of alpha- and beta-tubulins from the *Chlorarachnion* host and *Cercomonas* (Cercozoa). *J. Euk. Microbiol.* 45: 561–570.

Keeling, P. J. and Palmer, J. D. (2001) Lateral transfer at the gene and subgenic levels in the evolution of eukaryotic enolase. *Proc. Natl. Acad. Sci. USA* 98: 10745–10750.

Kies, L. (1974) Elektronenmikroskopische Untersuchungen an *Paulinella chromatophora* Lauterborn, einer Thekamöbe mit blau-grünen Endosymbionten (Cyanellen). *Protoplasma* 80: 69–89.

Kies, L. and Kremer, B. P. (1979) Function of cyanelles in the Tecamoeba *Paulinella chromatophora*. *Naturewissenschaften* 66: 578–579.

Kishore, R., Muchhal, U. S. and Schwartzbach, S. D. (1993) The presequence of *Euglena* LHCPII, a cytoplasmically synthesized chloroplast protein, contains a functional endoplasmic reticulum-targeting domain. *Proc. Natl. Acad. Sci. USA* 90: 11845–11849.

Köhler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R. J. M., Palmer, J. D. and Roos, D. S. (1997) A plastid of probable green algal origin in apicomplexan parasites. *Science* 275: 1485–1489.

Kowallik, K. V. (1997) Origin and evolution of chloroplasts: Current status and future perspectives. In *Eukaryotism and Symbiosis: Intertaxonic Combination versus Symbiotic Adaptation* (Schenk, H. E., Herrmann, R. G., Jeon, K. W., Müller, N. E. and Schwemmler, W., Eds.), Springer, Berlin, pp. 3–23.

Leander, B. S., Triemer, R. E. and Farmer, M. A. (2001a) Character evolution in heterotrophic euglenids. *Eur. J. Protistol.* 37: 337-356.

Leander, B. S., Witek, R. P. and Farmer, M. A. (2001b) Trends in the evolution of the euglenid pellicle. *Evolution* 55: 2215–2235.

Leipe, D. D. and Wainright, P. O., Gunderson, J. H., Porter, D., Patterson, D. J., Valois, F., Himmerich. S. and Sogin, M. L. (1994) The stramenopiles from a molecular perspective: 16S-like rRNA sequences from *Labyrinthuloides minuta* and *Cafeteria roenbergensis*. *Phycologia* 33: 369–377.

Ludwig, M. and Gibbs, S. P. (1989) Evidence that nucleomorphs of *Chlorarachnion reptans* (Chlorarachniophyceae) are vestigial nuclei: Morphology, division and DNA-DAPI fluorescence. *J. Phycol.* 25: 385–394.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., Leister, D., Stoebe, B., Hasegawa, M. and Penny, D. (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. USA* 99: 12246–12251.

Martin, W. and Schnarrenberger, C. (1997) The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: A case study of functional redundancy in ancient pathways through endosymbiosis. *Curr. Genet.* 32: 1–18.

Martin, W., Stoebe, B., Goremykin, V., Hansmann, S., Hasegawa, M., and Kowallik, K. V. (1998) Gene transfer to the nucleus and the evolution of chloroplasts. *Nature* 393: 162–165.

McFadden, G. I. (1999) Plastids and protein targeting. *J. Euk. Microbiol.* 46: 339–346.

McFadden, G. I., Gilson, P. R. and Waller, R. F. (1995) Molecular phylogeny of chlorarachniophytes based on plastid rRNA and *rbcL* sequences. *Arch. Protistenkd.* 145: 231–239.

McFadden, G. I., Reith, M., Munholland, J. and Lang-Unnasch, N. (1996) Plastid in human parasites. *Nature* 381: 482.

McFadden, G. I. And Waller, R. F. (1997) Plastids in parasites of humans. *Bioessays* 19: 1033–1040.

Medlin, L. K., Cooper, A., Hill, C., Wrieden, S. and Wellbrock, U. (1995) Phylogenetic position of the Chromista plastids based on small subunit rRNA coding regions. *Curr. Genet.* 28: 560–565.

Mereschkowsky, C. (1905) Über Natur und Ursprung der Chromatophoren im Pflanzenreiche. *Biol. Centralbl.* 25:593–604. English translation in Martin,W. and Kowallik, K. V. (1999) Annotated English translation of Mereschkowsky's 1905 paper "Über Natur und Ursprung der Chromatophoren im Pflanzenreiche." *Eur. J. Phycol.* 34: 287–295.

Moreira, D., Le Guyader, H. and Phillippe, H. (2000) The origin of red algae and the evolution of chloroplasts. *Nature* 405: 69–72.

Müller, K. M., Oliveira, M. C., Sheath, R. G. and Bhattacharya, D, (2001) Ribosomal DNA phylogeny of the Bangiophycidae (Rhodophyta) and the origin of secondary plastids. *Am. J. Bot.* 88: 1390–1400.

Nelissen, B., Van de Peer, Y., Wilmotte, A. and De Wachter, R. (1995) An early origin of plastids within the cyanobacterial divergence is suggested by evolutionary trees based on complete 16S rRNA sequences. *Mol. Biol. Evol.* 12: 1166–1173.

Oliveira, M. C. and Bhattacharya D. (2000) Phylogeny of the Bangiophycidae (Rhodophyta) and the secondary endosymbiotic origin of algal plastids. *Am. J. Bot.* 87: 482–492.

Palmer, J. D. (2003) The symbiotic birth and spread of plastids: How many times and whodunnit? *J. Phycol.* 39, 4–11.

Preisfeld, A., Busse, I., Klingberg, M., Talke S. and Ruppel, H. G. (2001) Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the Rhabdomonadales (Euglenozoa). *Int. J. Syst. Evol. Microbiol.* 51: 751–758.

Saldarriaga, J. F., Taylor, F. J. R., Keeling, P. J. and Cavalier-Smith, T. (2001) Dinoflagellate nuclear SSUrRNA phylogeny suggests multiple plastid losses and replacements. *J. Mol. Evol.* 53: 204–213.

Schimper, A. F. W. (1883) Ueber die Entwickelung der Chlorophyllkörner und Farbkörper. *Bot. Zeit.* 41: 105–114, 121–131, 137–146, 153–162.

Stibitz, T. B., Keeling, P. J. and Bhattacharya, D. (2000) Symbiotic origin of a novel actin gene in the cryptophyte *Pyrenomonas helgolandii*. *Mol. Biol. Evol.* 17: 1731–1738.

Stiller, J. W. and Hall, B. D. (1997) The origin of red algae: implications for plastid evolution. *Proc. Natl. Acad. Sci. USA* 94: 4520–4525.

Stoebe, B. and Kowallik, K. V. (1999) Gene-cluster analysis in chloroplast genomics. *Trends Genet.* 15: 344–347.

Takishita, K. and Uchida, A. (1999) Molecular cloning and nucleotide sequence analysis of *psb*A from the dinoflagellates: Origin of the dinoflagellate plastid. *Phycol. Res.* 47: 207–216.

Taylor, F. J. R. (Ed.) (1987) *The Biology of Dinoflagellates*, Blackwell Scientific Publications, Oxford.

Tengs, T., Dahlberg, O. J., Shalchian-Tabrizi, K., Klaveness, D., Rudi, K., Delwiche, C. F. and Jakobsen, K.S. (2000) Phylogenetic analyses indicate that the 19'hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17: 718–729.

Turmel, M., Otis, C. and Lemieux, C. (1999) The complete chloroplast DNA sequence of the green alga *Nephroselmis olivacea*: insights into the architecture of ancestral chloroplast genomes. *Proc. Natl. Acad. Sci. USA* 96: 10248–10253.

Turner, S., Pryer, K. M., Miao, V. P. and Palmer, J. D. (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Euk. Microbiol.* 46: 327–338.

Valentin, K. and Zetsche, K. (1990) Nucleotide sequence of the gene for the large subunit of Rubisco from *Cyanophora paradoxa:* Phylogenetic implications. *Curr. Genet.* 18: 199–202.

Van de Peer, Y. and De Wachter, R. (1997) Evolutionary relationships among eukaryotic crown taxa taking into account site-to-site variation in 18S rRNA. *J. Mol. Evol.* 45: 619–630.

Van de Peer, Y., Rensing, S. A. and Maier, U.-G. (1996) Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. *Proc. Natl. Acad. Sci. USA* 93: 7732–7736.

Van der Auwera, G., Hofmann, C. J. B., De Rijk, P., De Wachter, R. (1998) The origin of red algae and cryptomonad nucleomorphs: A comparative phylogeny based on small and large subunit rRNA sequences of *Palmaria palmata*, *Gracilaria verrucosa*, and the *Guillardia theta* nucleomorph. *Mol. Phylogenet. Evol.* 10: 333–342.

Watanabe, M. M., Suda, S., Inouye, I., Sawaguchi, I. and Chihara, M. (1990) *Lepidodinium viride* gen et sp. nov. (Gymnodiniales, Dinophyta), a green dinoflagellate with a chlorophyll *a*- and *b*-containing endosymbiont. *J. Phycol.* 26: 741–751.

Wilcox L. W. and Wedemayer G. J. (1985) Dinoflagellate with blue-green chloroplasts derived from an endosymbiotic eukaryote. *Science* 227: 192–194.

Williamson, DH., Gardner, M.J., Preiser, P., Moore, D.J., Rangachari, K. and Wilson, R.J. (1994) The evolutionary origin of the 35 kb circular DNA of *Plasmodium falciparum*: new evidence supports a possible rhodophyte ancestry. *Mol. Gen. Genet*. 243: 249–252.

Wilson, R. J. M. I., Denny P. W., Preiser, D.J., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W. and Williamson, D.H. (1996) Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol*. 261: 155–172.

Wolters, J. (1991) The troublesome parasites: Molecular and morphological evidence that Apicomplexa belong to the dinoflagellate-ciliate clade. *Biosystems* 25: 75–84.

Yoon, H. S., Hackett, J.D. and Bhattacharya, D. (2002a) A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *Proc. Natl. Acad. Sci. USA* 99: 11724–11729.

Yoon, H.S., Hackett, J.D., Pinto, G. and Bhattacharya, D. (2002b) A single, ancient origin of the plastid in the Chromista. *Proc. Natl. Acad. Sci. USA* 99: 15507–15512.

Zhang, Z., Green, B.R. and Cavalier-Smith, T. (1999) Single gene circles in dinoflagellate chloroplast genomes. *Nature* 400: 155–159.

Zhang, Z., Green, B.R. and Cavalier-Smith, T. (2000) Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: A possible common origin for sporozoan and dinoflagellate plastids. *J. Mol. Evol*. 51: 26–40.