21 Lie, Y.S. and Macdonald, P.M. (1999) Apontic binds the translational repressor bruno and is implicated in regulation of *oskar* mRNA translation. *Development* 126, 1129–1138
22 Wang, C. *et al.* (1994) Genetics of nanos localization in *Drosophila*. *Dev. Dyn.* 199, 103–115
23 Bergsten, S.E. and Gavis, E.R. (1999) Role for mRNA localization in translational activation but not spatial restriction of *nanos* RNA. *Development* 126, 659–669
24 Smibert, C.A. *et al.* (1996) Smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo. *Genes Dev.* 10, 2600–2609
25 Dahanukar, A. and Wharton, R.P. (1996) The Nanos gradient in *Drosophila* embryos is generated by translational regulation. *Genes Dev.* 10, 2610–2620
26 Gavis, E.R. *et al.* (1996) A conserved 90 nucleotide element mediates translational repression of *nanos* RNA. *Development* 122, 2791–2800
27 Smibert, C.A. *et al.* (1999) Smaug, a novel and conserved protein, contributes to repression of *nanos* mRNA translation *in vitro*. *RNA* 5, 1535–1547
28 Dahanukar, A. *et al.* (1999) Smaug, a novel RNA-binding protein that operates a translational switch in *Drosophila*. *Mol. Cell* 4, 209–218
29 Kim, C.A. *et al.* (2002) The SAM domain of polyhomeotic forms a helical polymer. *Nat. Struct. Biol.* 9, 453–457
30 Clark, I.E. *et al.* (2000) Synthesis of the posterior determinant Nanos is spatially restricted by a novel cotranslational regulatory mechanism. *Curr. Biol.* 10, 1311–1314
31 Crucs, S. *et al.* (2000) Overlapping but distinct RNA elements control repression and activation of *nanos* translation. *Mol. Cell* 5, 457–467
32 Murata, Y. and Wharton, R.P. (1995) Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in *Drosophila* embryos. *Cell* 80, 747–756
33 Wharton, R.P. *et al.* (1998) The Pumilio RNA-binding domain is also a translational regulator. *Mol. Cell* 1, 863–872
34 Barker, D.D. *et al.* (1992) Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes Dev.* 6, 2312–2326
35 Zhang, B. *et al.* (1997) A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germline. *Nature* 390, 477–484
36 Edwards, T.A. *et al.* (2001) Structure of Pumilio reveals similarity between RNA and peptide binding motifs. *Cell* 105, 281–289
37 Wang, X. *et al.* (2001) Crystal structure of a Pumilio homology domain. *Mol. Cell* 7, 855–865
38 Wang, X. *et al.* (2002) Modular recognition of RNA by a human pumilio-homology domain. *Cell* 110, 501–512
39 Sonoda, J. and Wharton, R.P. (1999) Recruitment of Nanos to hunchback mRNA by Pumilio. *Genes Dev.* 13, 2704–2712
40 Sonoda, J. and Wharton, R.P. (2001) *Drosophila* brain tumor is a translational repressor. *Genes Dev.* 15, 762–773
41 Slack, F.J. and Ruvkun, G. (1998) A novel repeat domain that is often associated with RING finger and B- box motifs. *Trends Biochem. Sci.* 23, 474–475
42 Wharton, R.P. and Struhl, G. (1991) RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen *nanos*. *Cell* 67, 955–967
43 Chagnovich, D. and Lehmann, R. (2001) Poly(A)-independent regulation of maternal hunchback translation in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11359–11364
44 Muckenthaler, M. *et al.* (1998) IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol. Cell* 2, 383–388
45 Ostareck, D.H. *et al.* (1997) mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3′ end. *Cell* 89, 597–606

Recycled plastids: a ‘green movement’ in eukaryotic evolution

John M. Archibald and Patrick J. Keeling

Secondary endosymbiosis is the process that drives the spread of plastids (chloroplasts) from one eukaryote to another. The number of times that this has occurred and the kinds of cells involved are now becoming clear. Reconstructions of plastid history using molecular data suggest that secondary endosymbiosis is very rare and that perhaps as few as three endosymbioses have resulted in a large proportion of algal diversity. The significance of these events extends beyond photosynthesis, however, because non-photosynthetic organisms such as ciliates appear to have evolved from photosynthetic ancestors and could still harbor plastid-derived genes or relict plastids.

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It is now well established that plastids, the light-harvesting organelles of photosynthetic eukaryotes, are the product of an ancient symbiosis between a eukaryote and a cyanobacterium. Together with the endosymbiosis that gave rise to mitochondria, the origin of plastids ranks as one of the most significant events in the evolution of the eukaryotic cell, because it gave rise to all phototrophic eukaryotes.

Studies based on gene sequences encoded in the mitochondria [1], plastids [2–4] and nuclei [5,6] of plants and algae reveal that all plastids ultimately trace back to a single endosymbiotic event with a cyanobacterium, called the primary endosymbiosis (Box 1). However, primary-plastid-containing plants and algae only account for a fraction of photosynthetic biodiversity: many eukaryotic algae acquired photosynthesis through secondary endosymbiosis, and it is these algae upon which we focus here.

In secondary endosymbiosis, a eukaryote obtained a plastid by engulfing a phototrophic eukaryote with a primary plastid and retaining its photosynthetic apparatus. In effect, plastids have spread laterally between distantly related eukaryotic lineages by being eaten but not digested. The array of algae produced by this process is extremely diverse so, even though this has been an important force in eukaryotic evolution, it has been difficult to determine the number of times plastids have moved between eukaryotes, or even the nature of the cells involved. This article focuses on recent molecular data bearing on this issue, which suggest that secondary endosymbiosis has been a relatively rare event in the evolution of eukaryotic cells, and that many non-photosynthetic eukaryotes might have descended from plastid-bearing ancestors.

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Box 1. Endosymbiotic origin of plastids

The idea that plastids might be derived from endosymbiotic prokaryotes was first introduced in the 1800s and is now practically uncontested because it is supported by many types of extremely compelling evidence. Early arguments for an endosymbiotic origin of plastids were based on microscopic observations, the pigments contained within the plastid and the kinds of drugs that inhibit their metabolism. However, the discovery that plastids contained DNA led to some of the most convincing evidence for their prokaryotic ancestry. All plastids contain a small genome, which encodes many genes that show a close phylogenetic relationship to cyanobacteria [a]. In addition, certain aspects of the gene order and genome organization are also conserved between plastids and cyanobacteria. This is best illustrated by the ribosomal-protein 'superoperon', which is a fusion of the S10, *spc* and α operons (and the *str* operon in certain algae) [b]. This organization is found exclusively in cyanobacteria and plastids. Although the evidence that plastids originated from cyanobacteria by endosymbiosis is now extremely strong, the exact nature of the cyanobacterial endosymbiont remains highly contentious because plastids show no obvious similarity or phylogenetic relationship to any one kind of cyanobacterium.

References

a Gray, M.W. and Spencer, D.F. (1996) Organellar evolution. In *Evolution of Microbial Life* (Vol. 54) (Roberts, D.M. *et al.*, eds), pp. 109–126, Cambridge University Press
b McFadden, G.I. and Waller, R.F. (1997) Plastids in parasites of humans. *BioEssays* 19, 1033–1040

Second-hand plastids

Early work on photosynthetic eukaryotes led to the identification of two fundamentally different types of plastid. Primary (or simple) plastids are surrounded by two membranes and are found in red algae, green algae, land plants and glaucocystophytes (Box 2). Primary plastids descend vertically from the original endosymbiosis with a cyanobacterium, and their membranes correspond to the inner and outer membranes of its Gram-negative envelope (Fig. 1a,b). The plastids of glaucocystophytes have also retained the peptidoglycan cell wall that is characteristic of their Gram-negative cyanobacterial ancestors, a feature that has been lost in all other plastids.

Secondary (or complex) plastids are present in all other algae and are characterized by the presence of additional membranes. The plastids of heterokonts, haptophytes, apicomplexa, cryptomonads and chlorarachniophytes are bound by four membranes, whereas those of euglenids and dinoflagellates are surrounded by three membranes. Initially enigmatic, these additional plastid membranes are now known to be a natural consequence of secondary endosymbiosis. In four-membrane plastids, the third membrane surrounding the organelle is derived from the plasma membrane of the endosymbiont, and the fourth membrane corresponds to the phagosomal membrane of the secondary host (Fig. 1c,d) [7]. From a cell-biological perspective, the most significant difference between primary and secondary plastids is the fact that primary plastids reside within the cytosol of the host, whereas secondary plastids reside within the lumen of the endomembrane system. This is most striking in cryptomonads, haptophytes and heterokonts, in which the outermost plastid membrane is physically continuous with the endoplasmic reticulum (ER) and outer envelope of the nucleus [8].

At the level of genes and proteins, the differences between the membranes of primary and secondary plastids have significant repercussions in gene transfer and protein trafficking. In all algae, most plastid proteins are encoded by nuclear genes and the proteins are targeted to primary plastids post-translationally using a transit peptide. However, nuclear-encoded proteins targeted to secondary plastids must traverse one or more additional membranes. They achieve this by making use of the

Box 2. Glossary of algal diversity

The diversity of algal groups is sometimes confusing, owing in large part to the fact that the 'algae' are not all related to one another, as a result of secondary endosymbiosis. Here, we provide a quick primer, with some features of each of the major lineages discussed in this article.

Green algae (and plants) (e.g. *Chlamydomonas*): Plants evolved from green algae and are very similar to them in many respects. Both are extremely abundant, morphologically diverse, successful lineages.

Red algae (e.g. *Porphyra*): Very abundant, diverse group ranging from microscopic balls to large multicellular seaweeds. Some of the large seaweeds are used to produce carbohydrates such as carrageenan or to make the nori used to wrap sushi.

Glaucocystophytes (e.g. *Cyanophora*): A little-studied group of algae with a primary plastid. Most remarkable because their plastid is the only one that has retained the peptidoglycan wall between its two membranes.

Chlorarachniophytes (e.g. *Chlorarachnion*): Relatively rare marine amoeboflagellate algae with green secondary plastids. Best known because the secondary endosymbiont has retained its nucleus (called a nucleomorph) and a miniature genome.

Euglenids (e.g. *Euglena*): Common algae in marine and freshwater environments with a green secondary plastid. Known for their peculiar movement and as close relatives of the parasitic trypanosomes.

Cryptomonads (e.g. *Guillardia*): Common algae with a red secondary plastid. Best known because, along with chlorarachniophytes, they have retained a nucleomorph. The complete sequence of a cryptomonad nucleomorph genome is now known and is a model of reduction and compaction.

Haptophytes (e.g. *Emiliania*): Common, ecologically important algae with a red secondary plastid. Many haptophytes are covered in elaborate calcareous scales called coccoliths, which are a primary component of chalk sediments such as the white cliffs of Dover.

Heterokonts (e.g. *Laminaria, Phytophthora*): A very diverse group that includes many photosynthetic forms (e.g. kelps and diatoms) and non-photosynthetic forms (e.g. oomycetes such as the potato-late-blight agent). Photosynthetic types have a red secondary plastid, and evidence now suggests that the entire group is derived from a photosynthetic ancestor.

Dinoflagellates (e.g. *Amphidinium*): Very common group with a red secondary plastid. Best known for causing 'red tides' and toxic shellfish poisoning, but also very important ecologically.

Apicomplexa (e.g. *Plasmodium, Cryptosporidium*): A very diverse group, all of which are obligate intracellular parasites. They cause many medically and commercially significant diseases, notably malaria. Recently found to contain a plastid, now known to be a red secondary plastid.

Ciliates (e.g. *Paramecium*): A completely non-photosynthetic group of protists that are characterized by the presence of cilia or short flagella in large numbers. They are very important microbial predators that have never been found to contain a plastid, but evidence now suggests that they might be derived from photosynthetic ancestors.

Alveolates: A group consisting of ciliates, apicomplexa and dinoflagellates.

Chromalveolates: Group consisting of cryptomonads, heterokonts, haptophytes and alveolates.

signal-peptide secretion system: the proteins are first co-translationally targeted to the ER using a signal peptide, then diverted to the plastid by a sorting system that remains uncharacterized, and finally traverse the two inner plastid membranes using a standard transit peptide [7,9–11]. All secondary plastids seem to use the same general strategy for plastid-protein trafficking, but there are few data about whether various groups have evolved this system independently or in common, because the details of how the system works in these organisms are poorly understood.

Although the presence of four membranes is consistent with the simplest model of secondary endosymbiosis, the evolution of three-membrane plastids in euglenids and dinoflagellates is somewhat more controversial (Box 3). Several scenarios have been proposed to explain their origin [12,13] but one particularly interesting suggestion is that differences in membrane number reflect plastids originating from different feeding mechanisms. Typical eukaryotic phagocytosis results in the entire food cell being engulfed in a host vacuole, and hence in a four-membrane plastid (Fig. 1). By contrast, the relatively rare process of myzocytosis does not involve ingestion of the plasma membrane of the prey cell, only its contents. Such a mechanism could, in theory, explain the evolution of plastids bound by three membranes [14]. However, this idea suffers from one crucial problem: the integration of host and endosymbiont is a complex process involving the transfer of hundreds of genes to the nucleus and the acquisition of a sophisticated protein targeting machinery. It is difficult to fathom how an endosymbiont with no plasma membrane could divide and segregate its nucleus and other essential components during the many generations necessary to carry out this genetic and cellular integration successfully. A much simpler explanation for the three-membrane topology of the euglenid and dinoflagellate plastids is that one membrane has been lost. As mentioned previously, targeting of nuclear-encoded proteins to secondary plastids occurs via the secretory pathway, including that of proteins targeted to three-membrane plastids of euglenids and apparently also dinoflagellates. For such a targeting system to work in three-membrane plastids, the outermost membrane would have to be derived from the phagosomal membrane of the host cell, which suggests that the plasma membrane of the endosymbiont was lost in euglenid and dinoflagellate plastids (Fig. 1d).

In four-membrane plastids, the region between the second and third membranes, called the periplastid space, is derived from the cytosol of the primary host cell (Fig. 1d). In two different algal lineages, this space retains the most intriguing and compelling evidence for the process of secondary endosymbiosis: the remnant nucleus of the endosymbiont. Early ultrastructural studies of cryptomonads revealed the presence of a small double-membrane-bound body in the periplastid space [15]. A similar structure was later found in the same cellular compartment of the chlorarachniophytes [16]. These organelles, dubbed

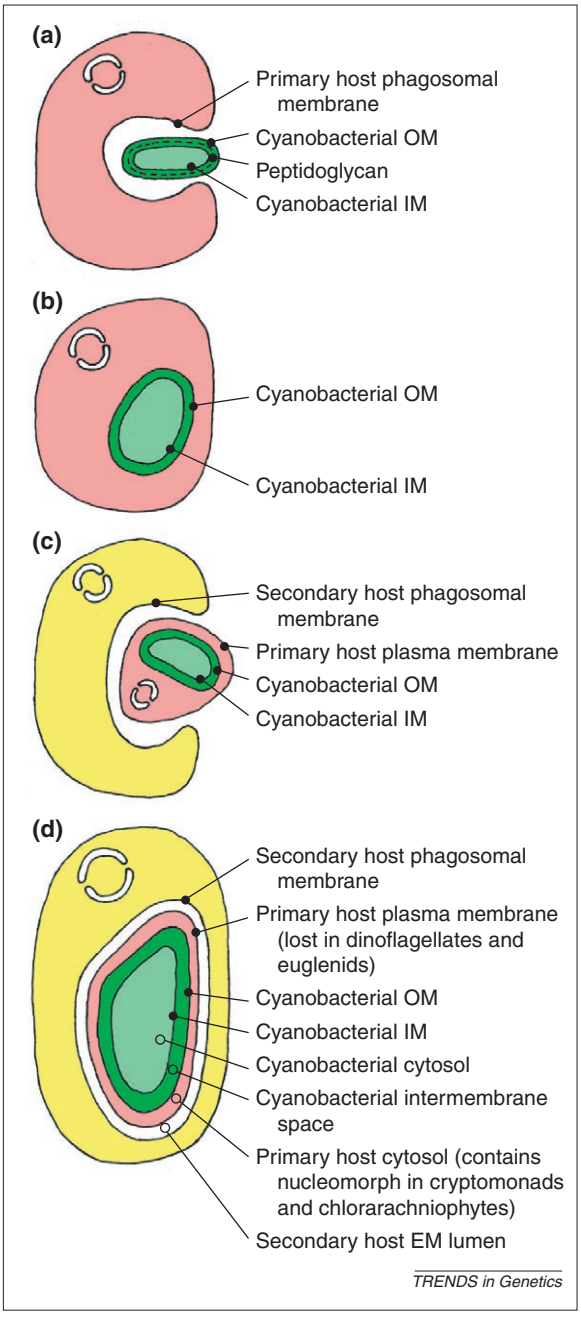

Fig. 1. Primary and secondary endosymbiosis. (a,b) Primary endosymbiosis (a) between heterotrophic eukaryote (red) and cyanobacterium (green) to form a primary plastid where the primary host phagosomal membrane has been lost (b). This process was followed by the diversification of glaucocystophytes, red algae and green algae (including plants). In red and green algae, the cyanobacterial peptidoglycan wall has been lost. (c,d) Secondary endosymbiosis (c) between a second heterotrophic eukaryote (yellow) and the primary alga (red with green plastid) to form a secondary plastid (d). This process has resulted in the evolution of many algal groups, including chlorarachniophytes, euglenids, cryptomonads, heterokonts, haptophytes, apicomplexa and dinoflagellates. All membranes of primary and secondary plastids, and their original sources are labeled. The original sources of cellular compartments resulting from secondary endosymbiosis are also shown. Abbreviations: EM, endomembrane; IM, inner membrane; OM, outer membrane.

Box 3. Tertiary endosymbiosis

Most photosynthetic dinoflagellates contain a three-membrane plastid with the characteristic pigment peridinin. However, some dinoflagellates have substituted their peridinin-containing plastids with a new one in a process referred to as tertiary endosymbiosis. Dinoflagellates are known to have acquired tertiary plastids from cryptomonads, heterokonts and haptophytes, as well as a second secondary plastid from a green alga [a,b]. Dinoflagellates are truly the algal experts at plastid acquisition, raising an intriguing question: has a dinoflagellate ever substituted its peridinin-containing plastid with another peridinin-containing plastid from an unrelated dinoflagellate?

References

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

b Tengs, T. *et al.* (2000) Phylogenetic analyses indicate that the 19′hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. *Mol. Biol. Evol.* 17, 718–729

'nucleomorphs', have since been confirmed to be bona fide – albeit highly reduced – nuclei. In both cryptomonads and chlorarachniophytes, the nucleomorph genome is extremely small (~551 kb and 380 kb, respectively) and the gene sequences encoded in these genomes are very divergent and AT biased [17,18]. Interestingly, the two genomes share some superficial similarities in their organization: both contain three linear chromosomes with rRNA operons in all six sub-telomeric regions. Both are compacted with short intergenic spaces and cryptomonads have few introns, whereas chlorarachniophytes have many introns that have been reduced in size [17,18]. The nucleomorph genome of the cryptomonad *Guillardia theta* is now completely sequenced, and one of the most surprising features of this genome is that only 30 of the 511 identified genes encode proteins targeted to the plastid [18]. Most of the genes encoding plastid proteins have moved from the nucleomorph to the host nucleus of *G. theta*, as have all plastid protein-coding genes in organisms with secondary plastids that lack nucleomorphs. Why are the vestigial nuclei retained in cryptomonads and chlorarachniophytes but not other lineages? It is not certain, but we are observing either a stage in an ongoing process of reduction or, perhaps more likely, the endpoint of a process that has been frozen in these organisms for unknown reasons.

In addition to the relatively well characterized transfer of genes encoding plastid-targeted proteins, the integration of both primary and secondary endosymbionts also appears to promote a second, less-well-characterized, class of gene transfers. Plant and algal nuclear genomes contain an unknown number of cyanobacterium-derived genes whose protein products are functionally unrelated to the modern organelle. This phenomenon, called 'endosymbiotic gene replacement' [19], has recently been suggested to extend to secondary endosymbiosis. Chlorarachniophyte and cryptomonad host nuclear genomes encode enolase genes that are derived from nuclear genomes of green and red algae, respectively [20], although the *Chlorarachnion* gene is derived from a type of green alga not typically thought to be related to the endosymbiont. It has been suggested that these genes might have originated in the degenerating nuclei of the endosymbionts during the process of genetic integration, potentially revealing a new effect of secondary endosymbiosis: facilitating eukaryote-to-eukaryote lateral gene transfer.

How many secondary endosymbioses?

With the realization that secondary-plastid-containing algae constitute a large proportion of the diversity of photosynthetic eukaryotes comes an important question: how often have these mergers happened? The integration of endosymbiont and host is an immensely complex series of events that has a formidable effect on both host and endosymbiont. It involves massive transfers of DNA between genomes, the development of a sophisticated protein-targeting machinery and a substantial reorganization of core and secondary metabolism. Untangling these events and understanding their effects on eukaryotic evolution requires fundamental knowledge of which algal lineages arose from the same endosymbiotic partnerships and which arose independently. However, this question has generated different estimates depending upon the kind of evidence considered [2,9,21,22]. Fortunately, a clearer picture is now emerging from a synthesis of biochemical and morphological evidence with molecular data.

Green endosymbionts

The deepest and most obvious division among secondary plastids is between those derived from green algae and those derived from red algae, a distinction long recognized from pigmentation. All photosynthetic eukaryotes (and cyanobacteria) use chlorophyll *a* as their main light-harvesting pigment, but differ in the distribution of various accessory pigments. Primary plastids of green algae and their land-plant relatives contain chlorophyll *a* and *b*, whereas red algae and glaucocystophytes contain chlorophyll *a* and phycobilin pigments. Plastids of euglenids and chlorarachniophytes are unique among secondary plastids in that they have the characteristic green algal complement of chlorophyll *a* and *b*, suggesting that their plastids might be derived from green algae. This conclusion is now supported by a wealth of molecular data.

The *Euglena* plastid genome has been completely sequenced [23] and, although it contains several interesting and unique features such as an abundance of group-II and group-III introns, it has many characteristics of a green-algal plastid genome, and many phylogenetic trees of plastid genes support this conclusion. In the case of *Chlorarachnion*, molecular phylogenetic data from both plastid and nucleomorph genomes also support a green-algal origin for the endosymbiont, although many nucleomorph genes are so divergent that their green roots were originally difficult to discern [24–26]. Although it has been suggested that euglenid and

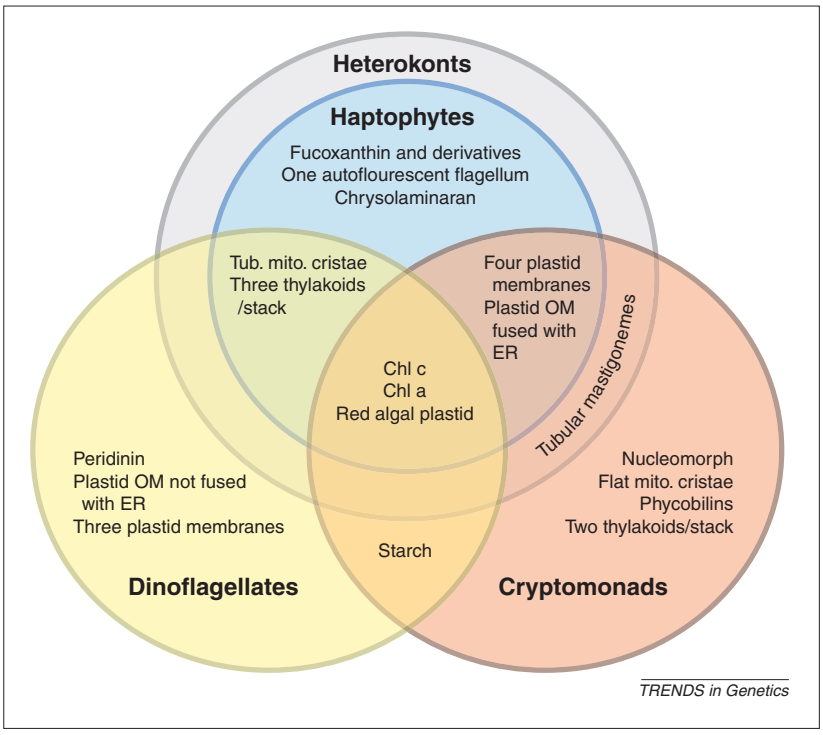

Fig. 2. Venn diagram of the pigment composition and significant biochemical and cell-biological features of organisms that have secondary plastids of red-algal origin. Abbreviations: Chl, chlorophyll; ER, endoplasmic reticulum; mito., mitochondrial; OM, outer membrane; Tub., tubular. Note that (1) fucoxanthin carotenoids are quite diverse in haptophytes and heterokonts, (2) starch is stored in the cytosol of dinoflagellates, but in the periplastid space of cryptomonads.

chlorarachniophyte plastids are the product of a single secondary endosymbiosis [9,27], there is currently no evidence supporting this. The host cells lack significant structural similarity [16] and phylogenies based on nuclear and plastid genes show no support for a specific relationship between euglenid and chlorarachniophyte hosts or plastids [24,28–30], altogether suggesting that these lineages represent two independent endosymbiotic events involving different hosts and different green algae.

Red endosymbionts

In contrast to green endosymbionts, the situation with red endosymbionts remains quite complicated, in part because of the greater diversity they represent. A range of data, especially molecular phylogenies based on plastid and cryptomonad nucleomorph genes, and conserved features of plastid genome organization, have now conclusively shown that the plastids of heterokonts, haptophytes, cryptomonads, dinoflagellates and apicomplexan parasites are all derived from red algae [3,21,31–37]. Apicomplexan plastids are non-photosynthetic and accordingly have no pigments, but all other red-algal secondary plastids contain a unique combination of chlorophylls *a* and *c*, whereas cryptomonads also contain phycobilins. Among eukaryotes, chlorophyll *c* is unique to these algae [although chlorophyll-*c*-like pigments have been found in a few other isolated cases (e.g. Ref. [38])], suggesting that all organisms with this chlorophyll might be directly related. Indeed, several biochemical and ultrastructural features suggest a relationship between some or all chlorophyll *a*+*c*-containing organisms (Fig. 2). Of the four lineages with chlorophyll-*a*+*c*-pigmented plastids, heterokonts and haptophytes are most similar from an ultrastructural and biochemical perspective, sharing fucoxanthin and fucoxanthin-like carotenoids, a single autofluorescent flagellum, and chrysolaminaran stored in cytoplasmic vacuoles, characteristics that once led to their classification together [39].

Although these data are suggestive, this picture is not without wrinkles. Most significantly, a common origin of these plastids implies that both plastid and host lineages should be demonstrably related, but early molecular data appeared to contradict such a relationship. The sequences of haptophyte, heterokont and cryptomonad plastid SSU rRNA and Rubisco have been examined extensively, and typically do not form a single group in phylogenetic analyses [21,34,40]. From the host lineage, phylogenies of nuclear SSU rRNA have also failed to show such a relationship [41], and this has been interpreted as additional support for several independent endosymbioses involving red algae. Recently, however, an analysis of five concatenated plastid genes showed strong support for a monophyletic group consisting of haptophytes, heterokonts and cryptomonads (D. Bhattacharya, pers. commun.), tipping the scales decidedly in favor of a single origin for the plastids of these organisms.

At the same time as the relationships among cryptomonads, heterokonts and haptophytes were being debated, another line of inquiry developed that has altered our view of eukaryotic evolution considerably. It has long been known that apicomplexa and dinoflagellates are close relatives (together with ciliates, making up the alveolates) [42]. Accordingly, when a cryptic plastid was discovered in apicomplexa [43,44], it immediately sparked a heated debate about whether apicomplexan and dinoflagellate plastids share a common origin, a debate heightened by uncertainty about whether the apicomplexan plastid was derived from a red or green alga [45–47]. Until very recently, however, no dinoflagellate plastid sequences were available to test this hypothesis. Several dinoflagellate plastid genes have now been characterized and, unexpectedly, found to reside on small single-gene minicircles, unlike all other known plastid DNAs [48]. Phylogenetic analyses of these genes not only confirmed a red-algal origin for the dinoflagellate plastid [48,49] but also suggested a specific relationship between the plastids of dinoflagellates and apicomplexa [36]. These data are, however, plagued by the fact that both the dinoflagellate and apicomplexan plastid genes are extraordinarily divergent and AT rich. Such divergent, biased sequences tend to cluster together in phylogenetic trees regardless of their true evolutionary history, making it impossible to rule out a methodological artifact [36]. Nevertheless, the simplest interpretation is that apicomplexan and

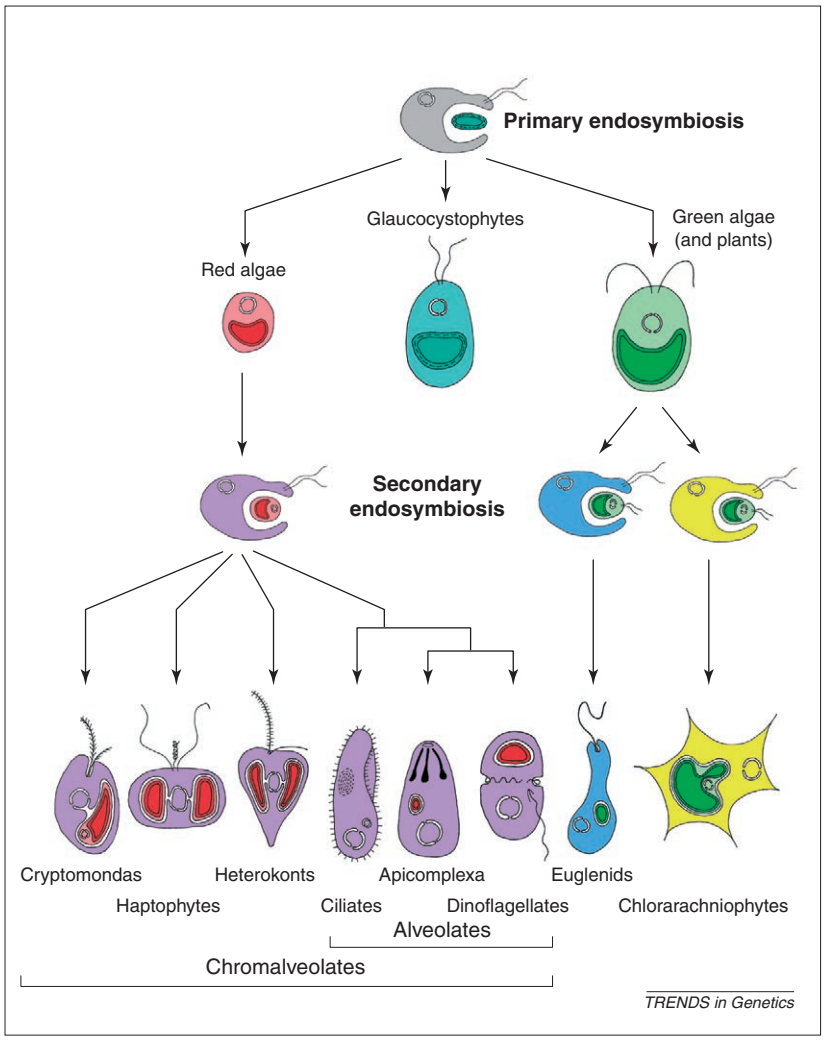

Fig. 3. A scheme for the origin and evolution of all plastids by primary and secondary endosymbiosis. A single primary endosymbiosis between an unknown heterotrophic eukaryote (gray) and a cyanobacterium led to the three primary-plastid-bearing lineages (top). Two secondary endosymbiotic events involving two different green algae and unrelated hosts led to euglenids (blue) and chlorarachniophytes (yellow). A single endosymbiosis between a red alga and a heterotrophic host led to all remaining eukaryotic algae (purple). Loss of photosynthesis is pervasive in several of these lineages and, in the ciliates, the entire lineage is non-photosynthetic. In these lineages it is not known whether plastids have been lost or whether cryptic plastids persist.

dinoflagellate plastids are the product of a single secondary endosymbiosis with a red alga.

This tentative conclusion has now been significantly bolstered by the analysis of a nuclear-encoded, plastid-targeted protein. Photosynthetic eukaryotes have two different nuclear-encoded forms of the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one cytosolic and the other plastid-targeted. The primary plastid-targeted homolog in red and green algae is, as expected, related to cyanobacterial GAPDH (as is the plastid GAPDH in euglenids). By contrast, the secondary-plastid-targeted GAPDH sequences in apicomplexa, dinoflagellates, heterokonts and cryptomonads have been found to be derived specifically from eukaryotic cytosolic GAPDH homologs, not from cyanobacterial homologs [37]. One interesting exception to this has been found: a single dinoflagellate has been shown to have a cyanobacterial-like plastid GAPDH, closely related to that of *Euglena* [50]. How this gene ended up in this dinoflagellate is not known but, in general, it appears that the cyanobacterial plastid-targeted GAPDH gene from the endosymbiotic red-algal nucleus was not transferred to the secondary-host nucleus during the integration of the endosymbiont. Instead, the secondary host's cytosolic GAPDH gene duplicated and acquired a plastid-targeting leader, and its protein product took over the role of the cyanobacterial enzyme in the plastid [37].

As mentioned previously, endosymbiotic gene replacements such as this have been well characterized [19] but they are not very common. In addition, the plastid-targeted GAPDH genes from apicomplexa, dinoflagellates, heterokonts and cryptomonads all form an extremely well-supported cluster in GAPDH phylogeny and are weakly related to the cytosolic GAPDHs from these same organisms (plus ciliates, and with the exception of the cryptomonads), as one would expect if this gene replacement occurred in the common ancestor of apicomplexa, dinoflagellates and heterokonts, and probably also of cryptomonads [37].

Further evidence supporting this conclusion is accumulating from molecular phylogenies of the hosts. As mentioned previously, apicomplexa, dinoflagellates and ciliates are together known as alveolates. Now, a specific relationship between alveolates and heterokonts is supported by analyses of nuclear rRNA [32,51] and various protein-coding genes, individually and in combination [5]. Although this does not account for cryptomonads and haptophytes (for which we have very little molecular information), it should be stressed that there is no evidence that they are not related to this emerging group. Weak phylogenies that do not associate groups of organisms are by no means equivalent to demonstrating that they are in fact unrelated. Indeed, all of the current evidence seems to indicate that these lineages form a single supergroup, which has been hypothesized previously, and dubbed the chromalveolates [9]. From this single, ancient endosymbiosis stemmed a large portion of eukaryotic biodiversity. The full diversity of eukaryotic algae can accordingly be explained by four endosymbiotic events (Fig. 3): a single primary endosymbiosis and three secondary endosymbioses, one involving a red alga and two involving different green algae.

Cryptomonads: the fly in the ointment or the key to the puzzle?

Although available data suggest a single origin of chromalveolate plastids, the cryptomonads are the weakest link in the chain of evidence. Cryptomonads are least often seen to group with other chromalveolates in molecular trees (e.g. Refs [37,41]). Furthermore, the proposed endosymbiotic replacement of the cryptomonad enolase gene has not affected alveolates [20]. This could be an indication that this

enolase came from some other red alga or that cryptomonads actually acquired their secondary plastid independently, but there is another possibility: that cryptomonads are the earliest lineage of chromalveolates. If this was the case, the enolase gene replacement might have occurred early in the evolution of chromalveolates, but after the cryptomonad lineage split from other chromalveolates. This is consistent with the distribution of the enolase-gene replacement and the relatively weak results from molecular phylogenetics. It has been argued that cryptomonads share specific traits with haptophytes and heterokonts (e.g. Ref. [9]), but they also retain several 'primitive' features such as the presence of the nucleomorph and phycobilins. These features might have been present in the common ancestor of cryptomonads and other chromalveolates and only lost after the divergence of cryptomonads. If this is so then cryptomonads are again a focal point of plastid evolution because they descend from an early stage of this key evolutionary event.

Plastid reduction and loss: an emerging theme

One reason why it has been difficult to determine the evolution of plastids is that we do not understand the process of plastid loss, making it impossible to evaluate different hypotheses of plastid origins. An auxiliary issue is the difficulty of actually proving that a plastid has been lost, as opposed to the loss of photosynthesis. Loss of photosynthesis has been documented in many lineages and, in nearly all cases in which it has been carefully examined, the plastid has been retained. However, most non-photosynthetic lineages have not been carefully scrutinized for the presence of a plastid and, indeed, why should they be? Until the discovery of the apicomplexan plastid and the more-recent evidence that secondary plastids pre-dated the divergence of heterokonts and alveolates, there was little reason to look for such an organelle in these organisms. That has now changed.

A single origin for chromalveolate plastids has far-reaching implications for the evolution of several major eukaryotic groups previously given little notice in discussions of plastids and photosynthesis. For example, if the alveolate and heterokont plastids originated in their common ancestor then the non-photosynthetic ciliates must have evolved from plastid-bearing organisms and might still contain a plastid (Fig. 3). The same is true for many other non-photosynthetic alveolates and heterokonts. Indeed, it has already been suggested that some non-photosynthetic heterokonts evolved from photosynthetic ancestors (e.g. Ref. [52]). Further support for this idea comes from the recent identification of an apparently plastid-derived 6-phosphogluconate dehydrogenase gene in the non-photosynthetic heterokont plant pathogen *Phytophthora infestans* [53]. Interestingly, this *Phytophthora* gene lacks a targeting peptide and therefore appears to operate in the cytosol, which in turn raises the question of whether *Phytophthora* retains a plastid or only a few genes derived from an ancestral plastid. These questions might be the tip of an iceberg, because molecular phylogenetics now predicts that plastid-derived genes should be found in many non-photosynthetic groups. If this prediction bears fruit, it will be intriguing to see how these plastids have been lost or re-tooled during evolution, because these are processes about which we know very little. Indeed, even the number of times photosynthesis has been lost in chromalveolates is unclear, owing to ambiguities surrounding the relationships within the group [54–56]. It must be a relatively common process, however, because a recent analysis of dinoflagellate phylogeny discerned at least eight independent losses of photosynthesis in this group alone, and furthermore suggested that this number will increase significantly when data from more non-photosynthetic dinoflagellates are available [57].

Future directions

Unambiguously determining the evolutionary history of plastids and their host lineages will require additional corroborating evidence. However, it will certainly be a milestone that not only has a profound effect on our understanding of the major events in eukaryotic evolution but also yields considerable predictive power. An early, common origin of all chromalveolate plastids hints at the possible presence of molecular relicts of plastids, or perhaps even intact organelles, in organisms such as ciliates and many other non-photosynthetic alveolates and heterokonts. To search for evidence of these cryptic organelles, we might take a cue from the now-well-characterized plastid of apicomplexa. Two major functions of this organelle are isoprenoid and fatty acid biosynthesis [58]. This explains why the plastid has been retained in apicomplexa but also shows that the integration of these plastid pathways into the host metabolism was an extremely ancient event, probably occurring soon after the endosymbiosis took place. Other relict plastids in other non-photosynthetic lineages could therefore retain these pathways, and we might even specifically seek out the fatty acid and isoprenoid biosynthetic enzymes in organisms such as ciliates and oomycetes to determine whether a plastid is still present.

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References

1 Burger, G. *et al.* (1999) Complete sequence of the mitochondrial DNA of the red alga *Porphyra purpurea*. Cyanobacterial introns and shared ancestry of red and green algae. *Plant Cell* 11, 1675–1694
2 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.), pp. 53–86, Springer-Verlag
3 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
4 Turmel, M. *et al.* (1999) The complete chloroplast DNA sequence of the green alga *Nephroselmis olivacea*: insights into the architecture of

ancestral chloroplast genomes. *Proc. Natl. Acad. Sci. U. S. A.* 96, 10248–10253
5 Baldauf, S.L. *et al.* (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290, 972–977
6 Moreira, D. *et al.* (2000) The origin of red algae and the evolution of chloroplasts. *Nature* 405, 69–72
7 McFadden, G.I. (1999) Plastids and protein targeting. *J. Eukaryot. Microbiol.* 46, 339–346
8 Gibbs, S.P. (1981) The chloroplast endoplasmic reticulum: structure, function, and evolutionary significance. *Int. Rev. Cytol.* 72, 49–99
9 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. Eukaryot. Microbiol.* 46, 347–366
10 Ishida, K. *et al.* (2000) Endomembrane structure and the protein targeting pathway in *Heterosigma akashiwo* (Raphidophyceae, Chromista). *J. Phycol.* 36, 1135–1144
11 Kishore, R. *et al.* (1993) The presequence of *Euglena* LHCPII, a cytoplasmically synthesized chloroplast protein, contains a functional endoplasmic reticulum-targeting domain. *Proc. Natl. Acad. Sci. U. S. A.* 90, 11845–11849
12 Gibbs, S.P. (1978) The chloroplasts of *Euglena* may have evolved from symbiotic green algae. *Can. J. Bot.* 56, 2883–2889
13 Cavalier-Smith, T. (1982) The origins of plastids. *Biol. J. Linn. Soc. London* 17, 289–306
14 Schnepf, E. and Deichgraber, G. (1984) 'Myzocytosis', a kind of endocytosis with implications to compartmentalization in endosymbiosis. *Naturwissenschaften* 71, 218–219
15 Greenwood, A.D. *et al.* (1977) Chloroplasts and cell compartments in Cryptophyceae. *Br. Phycol. J.* 12, 119
16 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
17 Gilson, P.R. *et al.* (1997) Size isn't everything: lessons in genetic miniaturisation from nucleomorphs. *Curr. Opin. Genet. Dev.* 7, 800–806
18 Douglas, S.E. *et al.* (2001) The highly reduced genome of an enslaved algal nucleus. *Nature* 410, 1091–1096
19 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
20 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. U. S. A.* 98, 10745–10750
21 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
22 Delwiche, C.F. (1999) Tracing the thread of plastid diversity through the tapestry of life. *Am. Nat.* 154 (Suppl.), S164–S177
23 Hallick, R.B. *et al.* (1993) Complete sequence of *Euglena gracilis* chloroplast DNA. *Nucleic Acids Res.* 21, 3537–3544
24 McFadden, G.I. *et al.* (1995) Molecular phylogeny of chlorarachniophytes based on plastid rRNA and *rbcL* sequences. *Arch. Protistenkd.* 145, 231–239
25 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. U. S. A.* 93, 7737–7742
26 Van de Peer, Y. *et al.* (1996) Substitution rate calibration of small subunit ribosomal RNA identifies chlorarachniophyte endosymbionts as remnants of green algae. *Proc. Natl. Acad. Sci. U. S. A.* 93, 7732–7736
27 Cavalier-Smith, T. (2000) Membrane heredity and early chloroplast evolution. *Trends Plant Sci.* 5, 174–182
28 Bhattacharya, D. *et al.* (1995) Molecular evolutionary analyses of nuclear-encoded small subunit ribosomal RNA identify an independent rhizopod lineage containing the Euglyphidae and the Chlorarachniophyta. *J. Eukaryot. Microbiol.* 42, 64–68
29 Keeling, P.J. (2001) Foraminifera and Cercozoa are related in actin phylogeny: two orphans find a home? *Mol. Biol. Evol.* 18, 1551–1557
30 Ishida, K. *et al.* (1997) The origin of chlorarachniophyte plastids, as inferred from phylogenetic comparisons of amino acid sequences of EF-Tu. *J. Mol. Evol.* 45, 682–687
31 Archibald, J.M. *et al.* (2001) Molecular chaperones encoded by a reduced nucleus – the cryptomonad nucleomorph. *J. Mol. Evol.* 52, 490–501
32 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
33 Van der Auwera, G. *et al.* (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. Phylog. Evol.* 10, 333–342
34 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
35 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
36 Zhang, Z. *et al.* (2000) Phylogeny of ultra-rapidly evolving dinoflagellate chloroplast genes: a possible common origin for sporozoan and dinoflagellate plastids. *J. Mol. Evol.* 51, 26–40
37 Fast, N.M. *et al.* (2001) Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol. Biol. Evol.* 18, 418–426
38 Larkum, A.W.D. *et al.* (1994) Light-harvesting chlorophyll c-like pigment in *Prochloron*. *Proc. Natl. Acad. Sci. U. S. A.* 91, 679–683
39 Andersen, R.A. (1991) The cytoskeleton of chromophyte algae. *Protoplasma* 164, 143–159
40 Muller, K.M. *et al.* (2001) Ribosomal DNA phylogeny of the Bangiophycidae (Rhodophyta) and the origin of secondary plastids. *Am. J. Bot.* 88, 1390–1400
41 Bhattacharya, D. *et al.* (1995) Comparison of nuclear-encoded small-subunit ribosomal RNAs reveal the evolutionary position of the glaucocystophyta. *Mol. Biol. Evol.* 12, 415–420
42 Wolters, J. (1991) The troublesome parasites: molecular and morphological evidence that Apicomplexa belong to the dinoflagellate-ciliate clade. *Biosystems* 25, 75–84
43 McFadden, G.I. *et al.* (1996) Plastid in human parasites. *Nature* 381, 482
44 Wilson, R.J.M.I. *et al.* (1996) Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol.* 261, 155–172
45 Köhler, S. *et al.* (1997) A plastid of probable green algal origin in apicomplexan parasites. *Science* 275, 1485–1489
46 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. Eukaryot. Microbiol.* 46, 367–375
47 McFadden, G.I. and Waller, R.F. (1997) Plastids in parasites of humans. *BioEssays* 19, 1033–1040
48 Zhang, Z. *et al.* (1999) Single gene circles in dinoflagellate chloroplast genomes. *Nature* 400, 155–159
49 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
50 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
51 Ben Ali, A. *et al.* (2001) Phylogenetic relationships among algae based on complete large-subunit rRNA sequences. *Int. J. Syst. Evol. Microbiol.* 51, 737–749
52 Cavalier-Smith, T. *et al.* (1995) Ribosomal RNA evidence for chloroplast loss within heterokonta: pedinellid relationships and a revised classification of ochristan algae. *Arch. Protistenkd.* 145, 209–220
53 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
54 Van de Peer, Y. *et al.* (1996) The evolution of stramenopiles and alveolates as derived by 'substitution rate calibration' of small ribosomal subunit RNA. *J. Mol. Evol.* 42, 201–210
55 Van der Auwera, G. *et al.* (1995) The phylogeny of the hypochytriomycota as deduced from ribosomal RNA sequences of *Hypochytrium catenoides*. *Mol. Biol. Evol.* 12, 671–678
56 McFadden, G.I. *et al.* (1994) *Goniomonas*: rRNA sequences indicate that this phagotrophic flagellate is a close relative of the host component of cryptomonads. *Eur. J. Phycol.* 29, 29–32
57 Saldarriaga, J.F. *et al.* (2001) Dinoflagellate nuclear SSU rRNA phylogeny suggests multiple plastid losses and replacements. *J. Mol. Evol.* 53, 204–213
58 Gleeson, M.T. (2000) The plastid in Apicomplexa: what use is it? *Int. J. Parasitol.* 30, 1053–1070