

## MINIREVIEW

### THE SYMBIOTIC BIRTH AND SPREAD OF PLASTIDS: HOW MANY TIMES AND WHODUNIT?

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**I discuss the evidence for a single origin of primary plastids in the context of a paper in this issue challenging this view, and I review recent evidence concerning the number of secondary plastid endosymbioses and the controversy over whether the relic plastid of apicomplexans is of red or green algal origin. A broad consensus has developed that the plastids of green algae, red algae, and glaucophytes arose from the same primary, cyanobacterial endosymbiosis. Although the analyses in this issue by Stiller and colleagues firmly undermine one of many sources of data, gene content similarities among plastid genomes used to argue for a monophyletic origin of primary plastids, the overall evidence still clearly favors monophyly. Nonetheless, this issue should not be considered settled and new data should be sought from better sampling of cyanobacteria and glaucophytes, from sequenced nuclear genomes, and from careful analysis of such key features as the plastid import apparatus. With respect to the number of secondary plastid symbioses, it is completely unclear as to whether the secondary plastids of euglenophytes and chlorarachniophytes arose by the same or two different algal endosymbioses. Recent analyses of certain plastid and nuclear genes support the chromalveolate hypothesis of Cavalier-Smith, namely, that the plastids of heterokonts, haptophytes, cryptophytes, dinoflagellates, and apicomplexans all arose from a common endosymbiosis involving a red alga. However, another recent paper presents intriguing conflicting data on this score for one of these groups—apicomplexans—arguing instead that they acquired their plastids from green algae.**

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There is nothing new under the sun, well almost. Most major evolutionary innovations during the three billion years plus of life on our planet have happened more than once, often many times. Multicellularity has evolved many times (Lang et al. 2002). Land animals have gone back to the ocean, or taken wing, several times. Vision of one kind or another has evolved a few dozen times. In plants, C<sub>4</sub> and CAM photosynthesis have evolved many times, as have a parasitic, nonphotosynthetic lifestyle, and so on.

“How many times did it happen?” is a major, fundamental question in the evolution of the eukaryotic cell and its organelles, one that is intricately and im-

portantly related to a correct understanding of eukaryotic phylogeny. A fuller form of this question is: How many different times have genetically integrated organelles arisen through prokaryotic/eukaryotic endosymbiosis and then been transmitted through secondary and tertiary eukaryotic/eukaryotic symbiosis, and through what combinations of prokaryotic and eukaryotic partners? This question has multiple layers to it. First, how many different organelles have an endosymbiotic origin? This question is now pretty much answered: the chloroplast and the mitochondrion are indisputably of endosymbiotic origin (see McFadden 2001 for a trenchant historical treatment of research on plastid origins), whereas there is a resounding lack of evidence for two candidate organelles (Cavalier-Smith 1987, Hall et al. 1989) — the peroxisome and the basal body/centriole — and instead, increasing reason to think that they are of autogenous origin. Second, how many times did chloroplasts arise by primary (cyanobacterial/eukaryotic) endosymbiosis? (There is little doubt that the mitochondrion arose only once, early on if not at the base of eukaryotic evolution). This question is the subject of a paper in this issue by Stiller et al. (2003), who challenge the widespread view that primary plastids arose only once. Third, how many times have primary plastids been introduced into other lineages of eukaryotes by secondary (eukaryotic/eukaryotic) endosymbiosis, and who have the partners been? The recent publication of several key papers bearing on these two questions makes it timely to discuss certain aspects of plastid secondary symbiosis.

#### ONE OR MORE PRIMARY ORIGINS OF PLASTIDS?

There is universal consensus that all well-recognized types of primary plastid-containing organisms fall into three groups, each clearly monophyletic: the green algae (including, of course, land plants), red algae and glaucophytes (Fig. 1). There is also broad consensus, based on many lines of evidence, that all three of these lineages “probably” trace back to the same cyanobacterial endosymbiosis; that is, primary plastids arose once and only once. I say “probably”, because some authors regard the issue as settled and others see a need for more evidence (Bhattacharya and Medlin 1995, 1998, Delwiche and Palmer 1997, Palmer and Delwiche, 1998, Delwiche 1999, Palmer 2000, Moreira and Philippe 2001, Cavalier-Smith 2002a,b, 2003). Enter John Stiller and colleagues. In 1997, Stiller and Hall published nuclear gene phylogenies (for *RBPI*, which encodes the largest subunit of RNA polymerase II) in which red algae emerge early in eukaryotic evolution relative to a diverse clade com-

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prising green algae, animals, fungi, and *Acanthamoeba*. This led them to conclude that there is a fundamental conflict between data from plastid genomes, which imply a monophyletic origin of red and green plastids, and from nuclear genes, which suggest that the host nuclear lineages of red and green algae are not related. Stiller and Hall (1997) then advanced three scenarios to reconcile this perceived conflict (see Delwiche and Palmer 1997 for a critical evaluation of their arguments). In a series of follow-up studies (Stiller and Hall 1998, 2002, Stiller et al. 2001), Stiller and colleagues have extended their phylogenetic sampling of the RBP1 gene, reaching the same conclusion as in 1997. In a paper appearing in this issue, they now directly question various gene phylogenies and miscellaneous characters used to adduce a single origin of primary plastids.

Stiller et al. (2003) have carried out analyses to address one of these features, the striking similarity in gene content among all major plastid lineages. Stiller et al. regard this as “one of the most influential sources of data leading to this consensus” (for plastid monophyly), but here I must take issue. Although Kowallik (1994) certainly argued strongly that plastid gene content similarity must be the result of plastid monophyly, most other authors have regarded this as simply one of many lines of evidence for plastid monophyly, generally ranking it less important than evidence from gene phylogenies, plastid gene order, and plastid targeting (Delwiche and Palmer 1997, Bhattacharya and Medlin 1998, McFadden 2001).

Stiller et al. (2003) focus their analyses on two of the major sets of genes in plastid genomes, for ribosomal proteins and tRNAs, choosing them because they allow inclusion of a mitochondrial genome as a control for similarity due to convergent evolution. Their statistical analyses lead them to conclude that there is no more similarity in ribosomal protein and tRNA gene content among plastid genomes than between plastids and mitochondria. They interpret this as reflecting a “dominant impact of convergent evolution on gene content”. To illustrate how these strongly convergent forces invalidate the use of gene losses in phylogenetic inference, they show that when ribosomal protein and/or tRNA genes are used as presence/absence characters to construct “gene-loss” trees, one gets completely nonsensical trees in which green plastids group not with other plastids but with the mitochondrion of *Reclinomonas*!

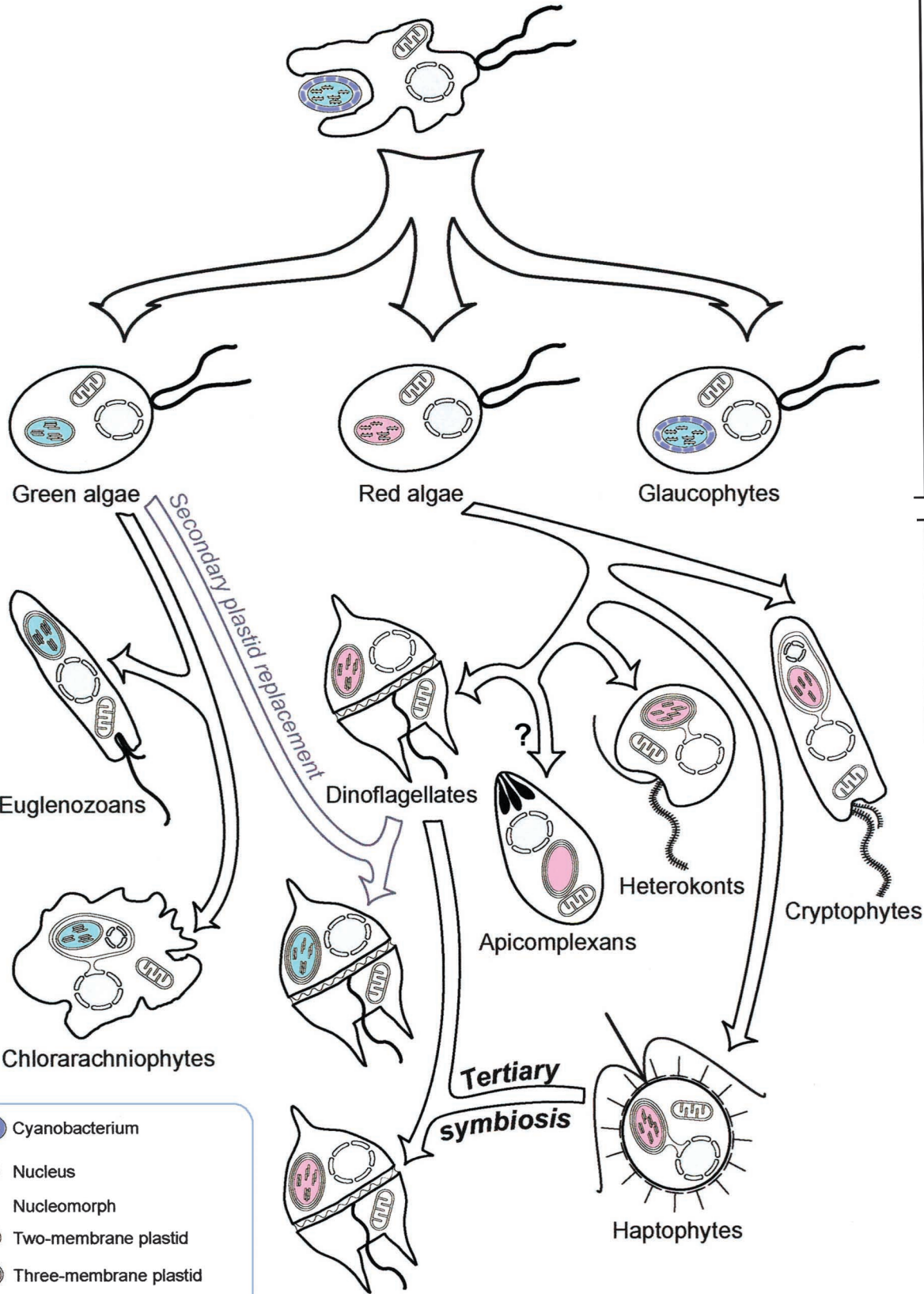
While I think Stiller et al. (2003) have overemphasized the influence of the gene content data, I nonetheless find their analyses and conclusions both convincing and also important. They represent a fresh approach, complementary to the whole-plastid genome analyses of Martin et al. (1998, 2002), to make crystal-clear the message that certain sets of plastid genes are lost over and over again. This would lead to plastid genomes of similar gene content through parallel and convergent evolution rather than common ancestry, and indicates that gene content is in general an unreliable character for plastid phylogeny.








In their discussion, Stiller et al. (2003) go on to evaluate and question three other lines of evidence used over the years to infer monophyly of primary plastids. They wonder, as I first did 10 years ago (Palmer 1993), whether two distinctive gene clusters (Reith and Munholland 1993, Stoebe and Kowallik 1999) that are uniquely shared among all three lineages of primary plastids relative to all examined cyanobacteria might be the product of convergent evolution, rather than constituting good evidence in favor of plastid monophyly as is commonly thought.

Second, Stiller et al. (2003) seem to accept the substantial body of evidence from phylogenetic analyses of plastid genes that primary plastids form a monophyletic clade relative to examined cyanobacteria (see Delwiche and Palmer 1997 for review, also see Turner et al. 1999). [Recent analyses of 23S rRNA sequences with extensive cyanobacterial sampling also strongly support this conclusion (S. Turner, K. M. Pryer, J. D. Palmer, unpublished data); however, the whole-plastid genome analyses of Martin et al. (1998, 2002) simply do not bear on this issue, because they include but a single cyanobacterium, yet have been incorrectly interpreted by several authors (e.g., Douglas 1998, Turmel et al. 1999, Sato 2001, Kroth 2002, Stoebe and Maier 2002), but not Martin et al. themselves, to imply a single origin of plastids.] However, Stiller et al. (2003) also point out that this result does not necessarily rule out multiple endosymbioses, for example, from closely related cyanobacteria, or even from not-so-closely related ones, since plastids thus far do not ally with any particular group of cyanobacteria. This point has been made before (Delwiche and Palmer 1997, Turner 1997, Delwiche 1999, Howe et al. 2003), although perhaps not so fully as by Stiller et al. (2003), and it should always be kept in mind. It should drive an increased sampling of cyanobacteria in future studies, especially a search for cyanobacteria that are particularly closely related to plastids. At the same time, it is undeniable that the best interpretation of the plastid sequence data is that in fact there was only a single primary endosymbiosis.

Finally, Stiller et al. (2003) question the phylogenetic significance of the much-ballyhooed findings that a number of different transit peptides are able to complement protein import function across different primary lineages of plastids (reviewed in McFadden 2001, Kroth 2002, Steiner and Löffelhardt 2002). While admitting that this result is certainly consistent with a monophyletic origin of the plastid import machinery and thus of plastids themselves, they also point out that it could simply reflect an ancestral, conserved function (rather than a derived function uniting plastid types) and cite a number of studies in which precursor polypeptides have been found to mistarget between plastids and mitochondria of distantly related organisms (see Stiller et al. 2003 for references). More compellingly, they call for careful comparative study of the actual import machinery across the three primary lineages. These are all good points. Recent studies of the plastid import apparatus

PRIMARY SYMBIOSIS  
SECONDARY SYMBIOSIS



-  Cyanobacterium
-  Nucleus
-  Nucleomorph
-  Two-membrane plastid
-  Three-membrane plastid
-  Four-membrane plastid
-  Thylakoid with phycobilisomes

do in fact appear to support plastid monophyly by identifying two components of the import apparatus (*tic22* and stromal processing protease) that seem to be shared and derived by green and red plastids relative to cyanobacteria (Douglas et al. 2001, Gardner et al. 2002, G. I. McFadden, personal communication). But this is clearly just a start, and a much deeper understanding of ancestral and potentially derived features of the import apparatus across the great diversity of plastid types is certainly needed.

So, where does all this leave us with respect to the number of cyanobacterial origins of plastids? First, it should be pointed out that there are several other datasets, not covered by Stiller et al. (2003), that also support a single origin of primary plastids. The presence of a large, rRNA-encoding inverted repeat may well be a shared derived feature uniting all plastid lineages (Turmel et al. 1999), but there are also reasons to question the phylogenetic significance of this character (Delwiche and Palmer, 1997). Stronger evidence comes from two sets of genes, one of which should be regarded as a direct, plastid-lineage character and the other as a character of host nuclear lineages. The former are the triple-helix chlorophyll-binding, light-harvesting antenna proteins, which appear to be a post-endosymbiotic invention that is shared by red- and green-derived plastids (but not yet characterized in glaucophytes; cyanobacteria have also not been extensively surveyed for their potential presence) (Wolfe et al. 1994, Durnford et al. 1999). The latter is the gene for cytoplasmic elongation factor G/2, which strongly unites red and green algae in all molecular phylogenies constructed to date (Moreira et al. 2000, Stiller et al. 2001; glaucophytes are again unexamined).

Second, so far, I have only considered the evidence commonly used to argue in favor of primary plastid monophyly. But what is the actual evidence *against* plastid monophyly and how strong is it? The only molecular phylogenetic evidence used to argue for a potentially separate origin of red and green plastids is the nuclear *RPBI* gene of Stiller and colleagues, but the relevance of this dataset to this issue is quite questionable. Moreira et al. (2000) used a closer outgroup to analyze a taxonomically expanded set of *RPBI* sequences than that analyzed by Stiller and Hall (1997), in which statistically significant support was found for an early branching of red algae apart from green algae, animals, and fungi, and found that this separation was no longer significantly supported. Moreover, subsequent *RPBI* analy-

ses by Stiller et al. (Stiller et al. 2001, Stiller and Hall 2002) have generally failed to find significant support for this separation; only when certain long-branched sequences are removed is even moderate support for this placement found (Stiller et al. 2001). No support, even with long branch taxa removed, for a separation of red and green algae was also reported by Dacks et al. (2002) in their most recent *RPBI* analyses, while other recent *RPBI* analyses (P. Keeling, personal communication) actually recover, albeit with weak support, a monophyletic red and green algal clade.

Overall, then, while Stiller and colleagues continue to play a useful devils-advocate role in this controversy, the overall weight of evidence continues, as it has for many years, to strongly favor a monophyletic origin of primary plastids. At the same time, the issue should not be considered settled. Because it is such a fundamentally important issue, one hopes that new sources of data will be brought to bear on it. Glaucophytes are poorly sampled, and even the one "token" glaucophyte, *Cyanopora paradoxa*, is understudied relative to green and red algae. As already mentioned, cyanobacterial sampling in plastid gene phylogenies needs to be improved substantially. Now that several cyanobacterial genomes have been sequenced, the time has come to systematically analyze cyanobacterial and plastid genomes for signatures of either plastid monophyly or polyphyly, and then to survey these traits comprehensively across the broad diversity of cyanobacteria and plastids to see what tales they have to tell. As discussed earlier and by Stiller et al. (2003), careful examination of potentially derived similarities of the plastid import machinery across primary plastids should be quite instructive, and there may be other such features of plastid genetic and metabolic machineries worth exploring (Sato 2001). Ultimately, enough whole genome sequences should become available, both on the cyanobacterial/endosymbiont side and on the nuclear/host side (mitochondrial genomes may not be up to the task; Stiller et al. 2001) as to effectively settle the issue.

#### HOW MANY SECONDARY SYMBIOSES?

Seven major groups of eukaryotes (Fig. 1) have clearly acquired their plastids by the process of secondary endosymbiosis, in which a presumptively non-photosynthetic eukaryote engulfed and enslaved a photosynthetic (or at least plastid-containing) eukaryote and retained both its plastid and many of the hundreds if not thousands of plastid-derived genes that

FIG. 1. Primary and secondary symbiosis and the diversity of algae and their plastids. Three groups (green algae, red algae, and glaucophytes) probably derive directly from a single, common primary endosymbiosis between a cyanobacterium and a phagotrophic eukaryote. Euglenozoans (euglenophytes) and Chlorarachniophytes acquired green plastids by secondary symbiosis; whether this happened separately or as a common event is completely unclear at present. A single secondary symbiosis of a red algal may well have given rise to all five secondary plastid types of the "chromalveolates", namely apicomplexans (but see text for current controversy) and dinoflagellates (both alveolates), and heterokonts, cryptophytes, and haptophytes (all chromists). In certain dinoflagellates, processes of secondary replacement and tertiary symbiosis have led to the replacement of their red-algal plastids with either green algal or haptophyte plastids, respectively. Note that some secondary plastids (chlorarachniophytes and cryptophytes) have retained their endosymbiont nucleus (the nucleomorph), and that the number and topology of plastid membranes are variable. This figure is modified from Moreira and Philippe (2001) with permission from Elsevier Science, Copyright (2001).

were already present in the endosymbiont's nucleus (for review, see Cavalier-Smith 1999, 2003, McFadden 2001, Archibald and Keeling 2002). There are two defining features of secondary plastids. First, they contain one or two extra membranes surrounding the plastid (for a total of three or four membranes; Fig. 1). Second, and a consequence of the first, they use complex, bipartite transit peptides to import cytoplasmically synthesized proteins, the first part a secretory signal directing the protein through the ER-derived membrane that forms the outermost shell of these complex plastids, and the second part a classic transit peptide ferrying the protein through the two inner membranes of primary symbiotic origin (McFadden, 1999, Kroth 2002, Cavalier-Smith 2003). Fascinatingly, two of the seven groups of secondary plastids contain a residual, "bonsai" endosymbiont nucleus (termed a "nucleomorph") located between the inner and outer pairs of plastid membranes, whereas the endosymbiont nucleus seems to have completely disappeared in the other five groups (Gilson and McFadden 1997, Cavalier-Smith 2003). Even more remarkably, the two nucleomorph genomes, one of green algal origin (in chlorarachniophytes) and the other of red algal origin (in cryptophytes), have converged upon eerily similarly reduced and compacted organizations and gene contents (Gilson and McFadden 1996, Douglas et al. 2001, Cavalier-Smith 2003).

The number of secondary symbioses for these seven groups is quite unclear and in certain respects controversial, much more so really than for primary symbiosis. Cavalier-Smith (1982, 2003) has long argued that secondary plastid symbiosis is a very difficult and rare process, quite possibly having occurred only twice, once via green algal enslavement leading to chlorarachniophytes and euglenophytes, and once via red algal uptake leading to the other five lineages of secondary plastids (see Fig. 1). Conversely, I and others (Bhattacharya and Medlin 1995, 1998, Delwiche and Palmer 1997, Palmer and Delwiche 1998) have suggested that each of the seven secondary lineages may have acquired their plastids through an independent secondary symbiosis. This controversy is still completely unsettled on the green side. Cavalier-Smith himself has wavered back and forth on this issue (compare Cavalier-Smith 1999, 2002a,b, 2003), and there is little in the way of significant phylogenetic information from plastid, mitochondrial, or nuclear genomes, or from features of the plastid import apparatus. Even worse, plastid genes of euglenophytes and chlorarachniophytes (e.g., Oliveira and Bhattacharya 2000) tend to be anomalously divergent in sequence and base composition and thus susceptible to long-branch artifacts of phylogenetic reconstruction. The question of common versus independent secondary origins of the euglenophyte and chlorarachniophyte plastids will probably remain unsettled until 1) a well-resolved phylogeny of the nucleus is in hand, 2) nuclear genomes of the many nonphotosynthetic (and probably non-plastid-containing) relatives allied to each lineage (and which must have once contained a plastid if

there were but a single green secondary symbiosis) are carefully inspected for genes of green algal origin, and 3) the import machineries of these two secondary green lineages are carefully analyzed for any shared derived features that could reflect a common origin.

On the red side, however, major advances have recently been made. Two recent papers lend considerable support to Cavalier-Smith's "chromalveolate" hypothesis (Cavalier-Smith 1999), which posits that the red- or putatively-red- (see next section) derived plastids of cryptophytes, heterokonts, and haptophytes (all members of his kingdom Chromista) and of dinoflagellates and apicomplexans (members of kingdom Alveolata) all trace back to a single common secondary symbiosis, with the many nonphotosynthetic members of this superkingdom reflecting repeated loss of photosynthesis and probably plastids too. Fast et al. (2001) concluded that four of these five groups, including dinoflagellates and apicomplexans, have all replaced their nuclear-encoded plastid glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of red algal origin with a plastid-targeted GAPDH putatively derived by duplication of a host GAPDH gene of cytosolic function. This result is now strengthened by the unpublished finding (J. T. Harper and P. J. Keeling, personal communication) that the one chromalveolate group (haptophytes) not examined by Fast et al. (2001) also contains this same uniquely derived GAPDH gene. These findings strongly support the chromalveolate hypothesis of a single secondary symbiosis on the red side, although the case from this particular gene will be even stronger if upon systematic examination all red algae are shown to lack it (the phylogenetic connection in GAPDH trees of the putatively related chromalveolate genes for plastid and cytosolic GAPDH is tenuous; also uncertain is the relationship of these two sets of genes to the only two cytosolic GAPDH genes characterized from red algae).

In a complementary study, Yoon et al. (2002a) adduce strong support from a five-gene plastid dataset for a single secondary origin of the plastid genomes of the three chromist lineages: cryptophytes, heterokonts, and haptophytes. This is the first clear, direct (i.e. plastid genome-based) evidence for chromist monophyly, and is a welcome relief after a number of single-gene and few-gene analyses (including ones from the same laboratory, Oliveira and Bhattacharya 2000, Yoon et al. 2002b) that are generally inconclusive on this issue, if not tending to favor multiple separate secondary origins. The apparent resolution by Yoon et al. (2002a) in favor of chromist monophyly speaks to the importance of sampling multiple genes in phylogenetic analyses. At the same time, this carries the danger, especially in trendy "whole-genome" analyses with oft-inadequate taxonomic sampling, of leading to strongly supported, but erroneous groupings resulting from systematic biases in the evolution of certain of the few sampled genomes. This is probably part of the explanation for the strongly supported conflict between the multi-gene trees with reasonable taxonomic sampling of Yoon et al. (2002a), which

strongly imply chromist monophyly, and the whole-plastid genome trees with scanty sampling of Martin et al. (2002), which strongly imply separate origins of the plastids of cryptomonads and heterokonts. Another part of the explanation may be the inclusion of rapidly evolving genes, such as those encoding ribosomal proteins, in the whole-genome analyses (D. Bhattacharya, personal communication), whereas Yoon et al. (2002a) used only slowly evolving genes that are probably better suited to such deep phylogenetic questions. I predict that with better taxonomic sampling and perhaps a more judicious choice of genes, the “whole” plastid-genome analyses will converge on the chromist monophyly results of Yoon et al. (2002a).

Taken together, the GAPDH (Fast et al. 2001) and plastid multigene (Yoon et al. 2002a) datasets cover all five groups of putative chromalveolates and reinforce each other in providing the first good evidence for a monophyletic origin via secondary symbiosis of the plastids of these five diverse groups. Furthermore, two recent multigene nuclear phylogenies also support, albeit at a low level statistically, the chromalveolate hypothesis (Baldauf et al. 2000, Baptiste et al. 2002). This symbiosis is a fundamental event in eukaryotic evolution, having given rise to the photosynthetic common ancestor of a protistan superassemblage: the chromalveolates. If further studies confirm the chromalveolate hypothesis, then, as elaborated by Cavalier-Smith (2002b, 2003), one has to recognize many, many losses of photosynthesis (and probably the plastid too) in diverse lineages of non-photosynthetic chromists and alveolates, including such relatively well-studied groups as ciliates and oomycetes. Indeed, Andersson and Roger (2002) have recently identified a gene in one oomycete that is consistent with a photosynthetic ancestry for this group. Genome projects should clearly reject or confirm that ciliates, oomycetes, etc. are of photosynthetic ancestry, and in so doing provide a definitive test of the chromalveolate hypothesis. If the current evidence holds, then it will be exciting to see whether any of these lineages, such as ciliates, might even contain a heretofore undetected cryptic plastid, as recognized some 12 years ago in Apicomplexans (Wilson et al. 1991) and analogous to the only recently recognized cryptic mitochondria (sometimes termed “cryptons”) in such “amitochondrial” lineages as microsporidia (Williams et al. 2002) and *Entamoeba* (Mai et al. 1999, Tovar et al. 1999).

The Russian doll game of a cell (= plastid) within a cell within a cell, etc. does not stop at secondary symbiosis. Cases of tertiary symbiosis have now been identified, all involving dinoflagellates, in which plastids themselves of secondary origin are acquired through yet another bout of symbiosis (Fig. 1). Another fascinating twist to the story of serial plastid symbiosis occurs in certain other dinoflagellates, in which their red algal-derived plastid is replaced by a plastid of green algal origin in a process termed “secondary replacement” (Fig. 1). Various constraints permit discussion of these intriguing cases and their controversial aspects, but the interested reader should consult

Chesnick et al. (1997), Inagaki et al. (2000), Tengs et al. (2000), Saldarriaga et al. (2001), Ishida and Green (2002), and Yoon et al. (2002b).

#### THE APICOPLAST: GREEN OR RED IN ORIGIN?

Although the green versus red ancestry of six of the groups of secondary plastids is undisputed, the ancestry of the seventh, the nonphotosynthetic apicoplast of the parasite phylum Apicomplexan, is controversial. This controversy would almost certainly not exist if the apicoplast genome were not such a reduced and divergent genome, with only a relatively few genes left, most of which are so rapidly evolving and base compositionally biased as to be virtually worthless for phylogenetic analysis. For example, plastid rRNA phylogenies have been suggested to support, albeit with reservations by the authors (Zhang et al. 2000), a common origin of the apicoplast and stereotypical (i.e. peridinin-containing) plastids of clearly red origin, but I find these analyses unreliable due to the extraordinary branch lengths of the apicomplexan and dinoflagellate sequences and the obvious artifacts in their region of the trees. Analysis of *tufA*, the least divergent and most phylogenetically promising of these relict genes, suggests, albeit only weakly, a green algal origin of the apicoplast (Köhler et al. 1997). Similarities in gene content and gene order, however, favor a red algal origin (Williamson et al. 1994, McFadden and Waller 1997, Blanchard and Hicks 1999), as does the sisterhood of dinoflagellates, whose ancestral plastid type is clearly of red origin, and apicomplexans in nuclear gene phylogenies. However, we have already seen that gene content is in many ways unreliable for inferring plastid phylogeny (see earlier), and convergence in gene order cannot be dismissed in such a severely reduced genome as in *Plasmodium*. The best evidence, therefore, in favor of a red origin of the apicoplast comes from the recent GAPDH study of Fast et al. (2001) discussed earlier.

Although the above evidence strongly favors a red algal origin of the apicoplast, provocative data have very recently been published that demand that a green algal origin of the apicoplast should still be taken seriously. The *cox2* gene is known to be a mitochondrial gene in all respiring eukaryotes except certain legumes (where it is present intact in the nucleus; Adams et al. 1999) and certain green algae, where it is present as two chromosomally separate ([http://www.biology.duke.edu/chlamy\\_genome/nuclear\\_maps.html](http://www.biology.duke.edu/chlamy_genome/nuclear_maps.html)), fragmented genes reflecting a gene fission event uniquely found only in the nucleus and mitochondrion of this algal clade (Perez-Martinez et al. 2001). Remarkably, Funes et al. (2002) have now identified the “same” fission pair of split *cox2* genes, again residing on different nuclear chromosomes (Gardner et al. 2002), in diverse apicomplexans. Phylogenetic analysis supports the conjecture that these two apicomplexan *cox2* genes are of green algal origin (Funes et al. 2002). But what really counts is their location and split nature; given the rarity of *cox2* gene transfer and fission, it is quite unlikely that apicomplexans and green algae have experienced

independent fission (at what seems to be the same location) and double gene transfer.

Accepting therefore that the apicomplexan *cox2* genes arose from green algae (and it is clear that things could not have gone in the other direction, because certain green algae contain transition stages that unambiguously identify them as the donors; Funes et al. 2002), how can we reconcile the *cox2* data that indicate a green algal origin of the apicoplast with the GAPDH data (Fast et al. 2001) that imply a red algal origin? Lateral gene transfer, outside the context of secondary symbiosis, could be invoked for either set of genes, although this is a bit unwieldy for both. This is because for *cox2* one would have to invoke a double gene transfer (of *cox2a* and *cox2b*), and for GAPDH one would have to postulate at least two transfers of the same gene; for example, from heterokonts to both apicomplexans and dinoflagellates (see Fig. 3 of Fast et al. 2001). Could both genes be right with respect to the plastid ancestry of apicomplexans? Might they bespeak a history of two successive red and green secondary symbioses in these colorless parasites? A chromalveolate/red algal ancestry of apicomplexans as implied by GAPDH phylogeny could be entirely correct, and so too could *cox2*-implied green algal ancestry, if this red plastid had been lost at the base of apicomplexans and in effect replaced by a green algal plastid (and set of nuclear genes). Dinoflagellates already provide multiple clear precedents for replacement of secondary red plastids by other plastids, including by a green plastid in *Lepidodinium* (Saldarriaga et al. 2001), so why not the same in apicomplexans too? Figuring out which, if any, of these three scenarios to explain the GAPDH/*cox2* red/green "conflict" is correct will require a lot more data, and careful analyses. Fortunately, much, perhaps all of the necessary data is now in hand, waiting to be mined, thanks to the recent sequencing of the *Plasmodium* nuclear genome, some 10% of which encodes apicoplast proteins (Gardner et al. 2002).

In conclusion, these are heady, exciting days for enthusiasts of plastid phylogeny and symbiosis. New insights, and surprises, into the number and nature of plastid symbioses are coming quickly, and with the onslaught of genomic sequences really just beginning, a robust picture of plastid phylogeny should soon emerge. A rigorous phylogenetic framework should, in turn, set the stage for in-depth study of the genetic and biochemical evolution of those major features of plastid molecular biology and metabolism that are fundamentally related to or affected by their symbiotic origin and partnership within the eukaryotic cell, such as the machineries for protein import, plastid division, DNA replication, and transcription.

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