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Update on Evolution

Algal Phylogeny and the Origin of Land Plants[1]

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The green algae and land plants form a monophyletic lineage (the chlorophytes) that contains both protistan and higher taxa (Graham, 1996). An important issue regarding the evolution of this green lineage that still remains in question is the identity of the green algal (i.e. flagellate) ancestor of land plants. Modern molecular phylogenetic data provide the framework for reconstructing this evolutionary history and for asking deeper questions about the origin of the genetic inventions that have played a role in the radiation of the green lineage, a group that contains nearly all levels of vegetative morphology, from single cells to filaments to well-organized colonies to complex terrestrial plants.

The green lineage is, however, only one example of photosynthetic taxa that have successfully colonized our planet. A much greater diversity of plastid-containing organisms is defined by the various other forms of algae. The algae include the green algal relatives of land plants and a diverse collection of single-celled and multicellular taxa such as the heterokonts, rhodophytes (red algae), cryptophytes, chlorarachniophytes, dinoflagellates, and haptophytes. Understanding the interrelationships and origins of these lineages is an interesting problem in evolutionary biology, not only because the algae contain the dominant primary producers on this planet, but also because uncovering the ancestry of their plastids offers the possibility to gain insights into the many facets of endosymbiosis, such as endosymbiont genome reduction and gene transfer to the host nucleus (Gilson and McFadden, 1996). Present knowledge argues overwhelmingly for a cyanobacterial origin of all algal plastids, with stable incorporation of many of the endosymbiont's genes in the host genome (Bhattacharya and Medlin, 1995). These and other recent data concerning the origins of algae and their plastids form a starting point from which the origin of the green lineage can be better understood.

THE ALGAE ARE A DIVERSE GROUP OF EUKARYOTES

The algae, which can be loosely defined as photosynthetic eukaryotes/protists excluding the land plants, have a bewildering array of cell morphologies and life cycles and live in a multitude of habitats. The major lineages of the algae are the Chlorophyta (green algae), Rhodophyta (red algae), Glaucocystophyta, Euglenophyta, Chlorarachniophyta, Heterokonta, Haptophyta, Cryptophyta, and the dinoflagellates (within the Alveolata). The latter four groups have been loosely termed the chromophyte algae because they contain chlorophyll *a* and *c* and various xanthophylls that make them appear yellow or brown. The algae include not only the world's largest protists, the kelps (*Macrocystis* spp. in the Heterokonta, which may be up to 30 m in length), but also many bacteria-sized (1–5 μm) coccoid taxa (e.g. *Chlorella* spp. and *Micromonas* spp. in the Chlorophyta and *Pelagomonas* spp. in the Heterokonta).

Many tiny, single-celled algae live within a complex exoskeleton made of $CaCO_3$ or silica, which accumulates over time in deep sea deposits in the world's oceans (coccolithophorids, diatoms). The fossil remains of these algae are routinely used for paleoclimatic reconstructions and to predict climate change. Algae have played critical roles in ecological studies of aquatic (e.g. kelp forests in northern California) and terrestrial ecosystems and have been used as model protists in physiological and biochemical studies (e.g. *Chlamydomonas reinhardtii* in the Chlorophyta) and have been the cause of many fundamental questions in biology because of their diverse and complex life histories. In addition, algae have had a long history in the food (e.g. nori, wakame) and drug (e.g. agar-agar, carrageenan, alginic acid) industries. The motile stage of most algae have two (or more) flagellae, but some lineages lost this character once during their early evolution (e.g. Rhodophyta) or multiple times in different members relatively late in evolution (Trebouxiophyceae, Chlorophyta). Readers are referred to descriptive/systematic treatments such as in Van den Hoek et al. (1995) for detailed information regarding the different algal groups.

When phycologists were first faced with the daunting problem of understanding the interrelationships of the different algal groups, they realized that it would be difficult to find a set of reliable characters that could form the basis of this classification. The distinct morphology of many lineages such as the red algae (e.g. the complete lack of flagellated stages) allowed their separation from other algae and recognition as an independent natural lineage, but the interrelationship of this group (as with many other algal groups) with other eukaryotes remained unclear.

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Many advances were made possible with the advent of electron microscopy, which allowed the detailed study of algal cell ultrastructure and led to hypotheses about the phylogeny of these taxa (Mattox and Stewart, 1984). One of the most important concepts that came out of these studies was the recognition that vegetative cell morphology forms a poor basis for a natural classification. The plasticity of forms has led to the misclassification of species and overclassification of ecotypes and has contributed to confusion in algal taxonomy. It is now widely recognized that aspects of the motile/reproductive cell ultrastructure, such as the morphology of the flagellae, flagellar roots, and basal bodies, form a far more reliable basis for taxonomic classification because these characters are stable over evolutionary time (Friedl, 1997). One problem with using morphological characters for creating phylogenies arises from the difficulties in assigning phylogenetic values to certain key characters (e.g. ultrastructure of flagellar hairs) in distantly related groups that either vary substantially in morphology or are of questionable homology (character state shared through common ancestry).

Further attempts to classify the algae during the 1960s and 1970s were based on ultrastructural features of plastids of the major groups. These data led to the recognition of two fundamentally different types of plastids: the simple plastids in the chlorophytes, rhodophytes, and glaucocystophytes, which have two bounding membranes, and the complex plastids (Sitte, 1993) in virtually all other algae (Fig. 1), which have three or more bounding membranes. The simple plastids are thought to retain the two membranes of the engulfed cyanobacterium resulting from a primary endosymbiotic event.

Algae with plastids bounded by three or four membranes have gained this organelle from the engulfment of an existing alga (secondary endosymbiosis; Gibbs, 1993). In the case of plastids with four membranes, the third membrane is presumably the plasmalemma of the engulfed eukaryote, whereas the fourth is the phagosomal membrane of the host cell. Three-membraned complex plastids such as those in the euglenophytes and in most dinoflagellates appear to have resulted from the loss of one of the outer membranes. The number of plastid membranes has turned out to be a good marker for primary and secondary endosymbiotic events and in combination with sequence-based phylogenies has clarified the origin of most algal plastids (Delwiche and Palmer, 1997).

PLASTID ORIGIN DEFINES ALGAL ORIGIN

The molecular phylogenetic studies have superceded the ultrastructure-based classification schemes and have shown that the morphological diversity of the algae results in fact from their polyphyletic origins within the eukaryotic tree of life (Bhattacharya and Medlin, 1995; Stiller and Hall, 1997). The algae are an artificial group that includes some taxa that are more closely related to other nonphotosynthetic protists (e.g. chlorarachniophytes to filose amoebae, euglenophytes to kinetoplastids, heterokont algae to labyrinthulomycetes) than to other algae. A schematic view of the tree of life based on rDNA sequence comparisons is shown in Figure 2. The important features of this tree are (a) the three domains of life (Archaea, Bacteria, Eukarya), (b) the divergence of a number of protist groups (Microsporidia, Trichomonads) relatively deep within the eukaryotic domain, (c) the occurrence of a near-simultaneous origin of many eukaryotic groups within the so-called crown-group radiation, and (d) the enigmatic position of the photosynthetic euglenophytes as the earliest algal divergence.

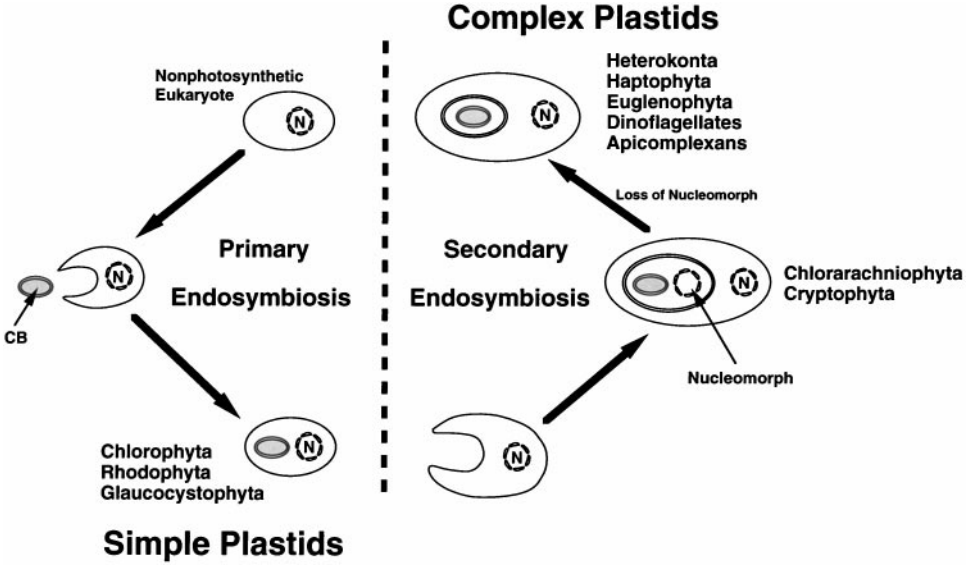

Figure 1. Origins of simple and complex plastids via primary and secondary endosymbioses. Algae containing simple and complex plastids are listed alongside the cells. The chloroplast ER (continuity of the outer plastid membrane and the outer membrane of the nuclear envelope) found in some complex plastid-containing algae is not shown. N, Nucleus; CB, cyanobacterium.

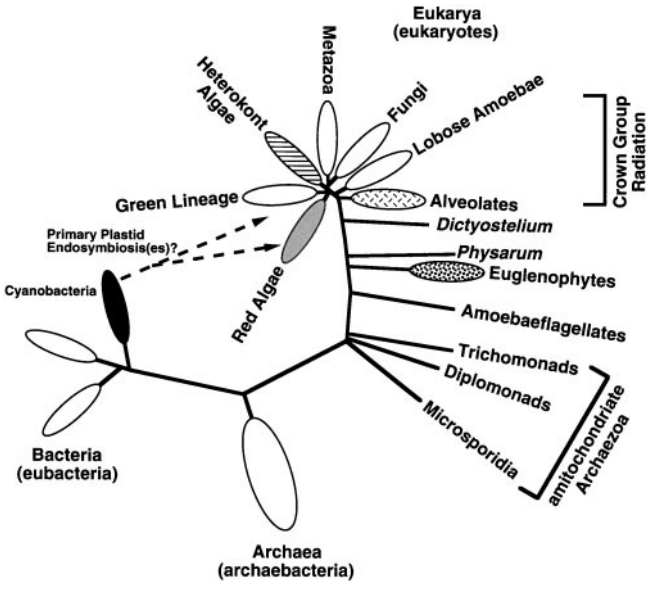

Figure 2. Small subunit rDNA phylogeny showing the three domains of life with emphasis on the phylogeny of the eukaryotes. This tree has been adapted from Sogin et al. (1996). The branch lengths are approximate evolutionary distances and show only the relative positions of the taxa. Photosynthetic taxa are shown in gray or stippled fields.

A more detailed phylogenetic analysis of the crown group radiation is shown in Figure 3A. Here we present a phylogeny of the host cells (Fig. 3A) and of the plastids that are found in these host cells (Fig. 3B). These analyses allow us to deal separately with the origins of the hosts and the endosymbionts and then, by comparison of the tree topologies, to reach conclusions about the number of primary and secondary endosymbiotic events that explain the origin of the algae. The plastid tree, for example, shows that there are three closely related major lineages of plastids: i.e. the simple plastids of the chlorophytes, rhodophytes, and glaucocystophytes (the latter are termed cyanelles), which diverge nearly simultaneously from each other and are a monophyletic sister group of the cyanobacteria. This result is consistent with a primary endosymbiotic origin of these plastids from a cyanobacterial ancestor. Phylogenetic analyses of additional plastid genes such as *tuf*A (Köhler et al., 1997), *atpB* (Douglas and Murphy, 1994), *rpo*C1 (Palenik and Swift, 1996), and *psb*A (Hess et al., 1995) also provide moderate to strong support for plastid monophyly. The phylogenetic data do not, however, allow us to distinguish between the endosymbiosis of a single cyanobacterium or of several closely related cyanobacteria in the common ancestor(s) of the chlorophytes, rhodophytes, and glaucocystophytes.

The hypothesis that all simple plastids trace their ancestry to a single origin is supported by great similarities in plastid genome content and organization (e.g. *psbB/N/H* and *atp/rps/rpo* gene clusters, Douglas, 1992). Because gene order is variable among the cyanobacteria, this suggests that if these organelles are of separate origins then massive convergence must have occurred independently in each plastid lineage to result in the present gene maps. Furthermore, immunological studies have shown that the light-harvesting antenna complex proteins of PSI are related in green and red algae but not in the cyanobacteria. Finally, the ability of plastid transit peptides from the glaucocystophyte alga *Cyanophora paradoxa* to direct efficiently the targeting of nuclear-encoded plastid proteins into land-plant plastids (and vice versa) suggests that these organelles share a common import pathway (for review, see Delwiche and Palmer, 1997). In spite of this evidence, the search for the cyanobacterial ancestor(s) for the different simple-plastid types, if such taxa exist, remains one of the most important areas of future research in algal evolution.

In this regard, the discovery of the Prochlorophyta (Lewin, 1976) deserves mention in any discussion of plastid endosymbiosis. These cyanobacteria contain chlorophyll *a* and *b* and closely spaced thylakoids similar to those of the green algae. They provided the first opportunity to test the hypothesis of a separate primary endosymbiotic origin of the green algal plastid. Other known cyanobacteria were presumably the source of the red algal and glaucocystophyte simple plastids, since these all contain only chlorophyll *a* and phycobilins. The expectation of a missing link within the prochlorophytes was unfulfilled because phylogenetic analyses have shown that the prochlorophytes are a paraphyletic group within the cyanobacteria and that none of its members shares a direct common ancestry with the monophyletic green algal plastid lineage (Palenik and Haselkorn, 1992; Urbach et al., 1992). The paraphyletic origin of the prochlorophytes also suggests that chlorophyll *b* has had multiple origins within the cyanobacteria.

In light of the above data, do we then find a monophyletic origin of the host cells of the simple-plastid-containing algae in Figure 3A? Until now, no rDNA phylogeny has been able to show conclusively that chlorophytes, rhodophytes, and glaucocystophytes form a monophyletic grouping in the host cell trees. However, there are preliminary data from actin sequence comparisons that support this scenario (Bhattacharya and Weber, 1997) and limited RNA polymerase II (*RPB1*) data that do not (Stiller and Hall, 1997). Comparison of the *cox3* gene in red and green algal mitochondria support the monophyly of these groups, although glaucocystophyte sequences have not yet been included in these analyses (Boyen et al., 1994). Clearly, additional taxa must be included in these protein-sequence phylogenies to test the monophyly of all simple-plastid-containing algae. The most parsimonious scenario of a single origin of all simple-plastid-containing algae from an ancestral protoalga remains open to question. What, then, about the origin of complex plastids?

Here the molecular sequences provide clear and compelling results about secondary endosymbiosis. First, it is important to see in the host cell tree that some algal groups (e.g. heterokonts, cryptophytes, chlorarachniophytes) form secondarily photosynthetic groups that trace their ancestries to nonphotosynthetic ancestors (Fig. 3A). The apicomplexan relatives of ciliates and dinoflagellates (*Prorocentrum micans* within the alveolate lineage) contain a 35-kb, circular, extrachromosomal DNA that has also been identified as a reduced plastid genome. However, unlike most

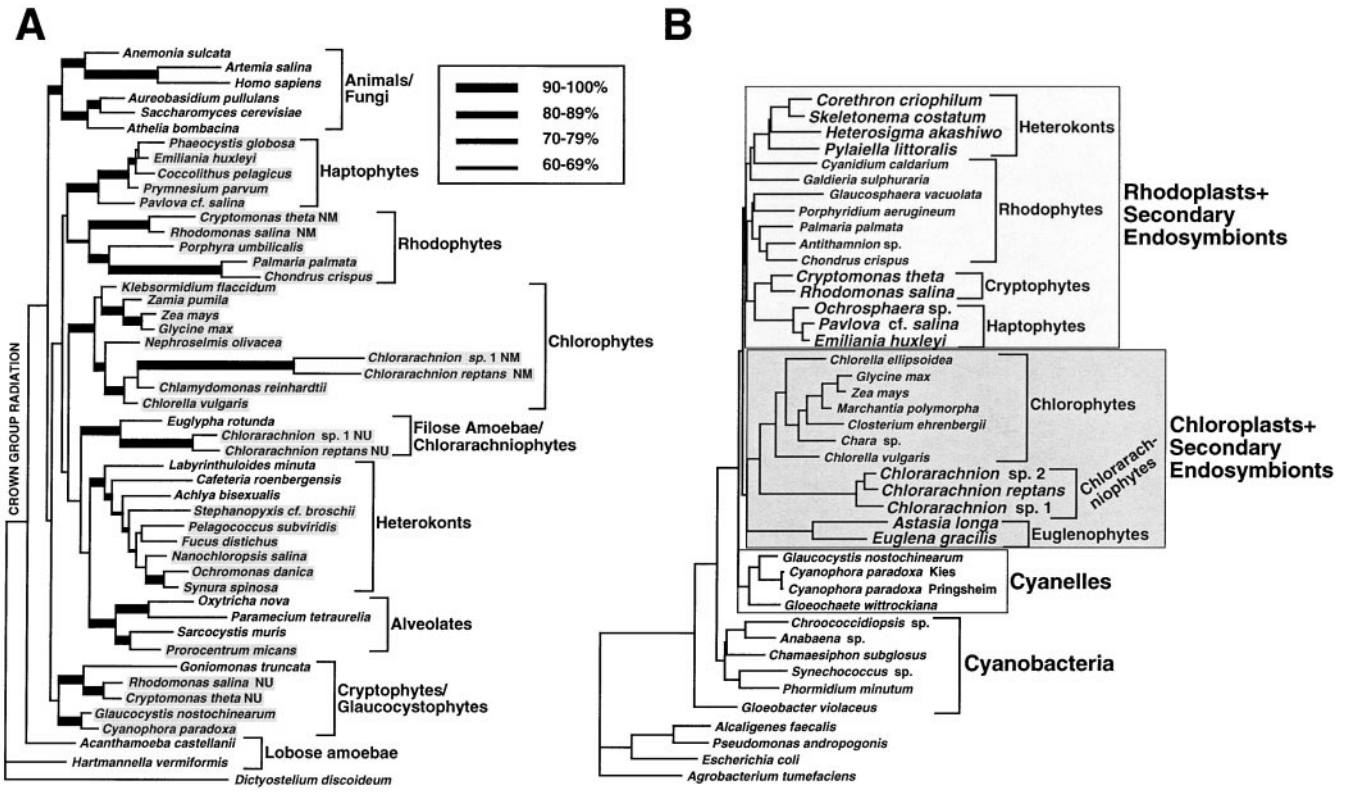

Figure 3. Small subunit rDNA phylogenies. A, Host cell (nuclear) phylogeny constructed with the maximum likelihood method. Results of a bootstrap neighbor-joining distance analysis with a Kimura matrix as input (100 replications) are shown as branch nodes of differing thicknesses (see box to the right in A). NM, Gene from the nucleomorph or vestigial nucleus within the plastid; NU, gene from the nucleus of the same organism. Photosynthetic taxa are shown in the gray field. This tree is rooted within the branch leading to *Dictyostelium discoideum*. Bootstrap values greater than 70% are normally interpreted as providing support for the groupings to the right of these values. B, Plastid phylogeny constructed with the neighbor-joining method with a LogDet matrix as input. The three simple plastid lineages (plus the complex plastids derived from these algae) have been boxed and are shown in the gray fields. The complex plastids are shown in large typeface. This tree is rooted within the branch leading to *Agrobacterium tumefaciens*. For further details about these trees, see Bhattacharya and Medlin (1995).

dinoflagellates, this plastid has four membranes and has likely resulted from the secondary endosymbiosis of a green alga (Köhler et al., 1997). The apicomplexans, including the malaria parasite *Plasmodium falciparum* can therefore be thought of as the most peculiar algae. In addition, as shown in Figure 3B, all known complex plastid rDNA sequences form sister groups to or are embedded within the different simple-plastid lineages. These data argue convincingly for the origin of complex plastids from secondary endosymbioses involving existing red and green algae.

The four-membraned plastids of the cryptophytes, heterokonts, and haptophytes have all arisen from separate secondary endosymbioses involving red algae. Likewise, the three- and four-membraned plastids of the euglenophytes and chlorarachniophytes, respectively, trace their origins within the green algal lineage. These results are supported by a number of ultrastructural and biochemical characters (e.g. all chloroplasts and secondary endosymbionts from this lineage contain chlorophyll *a* and *b*). The presence of photosynthetic euglenoids can therefore be most easily explained by the secondary endosymbiosis of an existing green alga into this relatively early diverging protist group, rather than by a close phylogenetic relationship between euglenophytes and the green algae. Although bootstrap values are not shown in the LogDet tree in Figure 3B, another method, transversion analysis, provides corroborative evidence for this topology (Van de Peer et al., 1996). The use of methods that correct for nucleotide bias, such as LogDet transformation, are necessary for the reconstruction of plastid rDNA phylogenies due to the varying nucleotide contents of this gene in different plastid genomes (Bhattacharya and Medlin, 1995; Van de Peer et al., 1996).

More convincing evidence for secondary endosymbiosis comes from the finding of remnants of the nuclear genome of the algal symbionts in the periplastidial compartment between the second and third membranes of the complex plastids of the cryptophyte and chlorarachniophyte algae. This nucleomorph DNA has been analyzed and supports further the algal origin of these plastids. See, for example, the grouping of the chlorarachniophyte nucleomorph rDNA sequences with those of the green algae (Fig. 2A; Van de Peer et al., 1996).

Complete genome sequencing of the small, linear nucleomorph chromosomes identified in the cryptophytes and chlorarachniophytes promises to provide many new insights into the process of genome reduction (e.g. the existence of mini-spliceosomal introns of 19–20 nucleotides and an average spacer length of 65 nucleotides) that must occur following secondary endosymbiosis of an alga (Gilson and McFadden, 1996). The chlorarachniophyte nucleomorph genome consists of three linear chromosomes with a total size of 380 kb. The lack of nucleomorph DNA in the heterokont, haptophyte, and euglenophyte plastids is most easily interpreted as the complete reduction of these genomes by the host cell. However, among the dinoflagellates are binucleate species, in which the intact endosymbiont nucleus remains within the host dinoflagellate. Rubisco sequence comparisons have identified, for example, the origin of the nondinokaryon nucleus in *Peridinium foliaceum* as an advanced diatom genus (Chesnick et al., 1996).

Taken together, the phylogenetic analyses of algal hosts and plastids shows that there is good support for a monophyletic origin of the simple plastids from a cyanobacterial endosymbiont, although the host cell trees have not yet been able to prove conclusively this scenario. Secondary endosymbiosis has played a dominant role in the origin(s) of algae, since many previously nonphotosynthetic taxa have become algae after the uptake of an existing photosynthetic eukaryote (Bhattacharya, 1997). The dinoflagellates contain a large variety of yet unexplored complex plastids that will likely provide many further examples of independent secondary endosymbioses in algal evolution. Future research on secondary endosymbiosis should focus on uncovering the origins of the complex plastids in dinoflagellate taxa such as *Lepidodinium viride*, *Dinophysis* spp., and *Gyrodinium aureolum*, which appear to contain plastids of chlorophyte, cryptophyte, and haptophyte origins, respectively (Bhattacharya and Medlin, 1995).

However, secondary endosymbiosis is not always the result of a previously nonphotosynthetic protist engulfing an alga since the cryptophytes share a common ancestry with the simple plastid (cyanelle)-containing glaucocystophytes (Fig. 3A). In this lineage, the common ancestor of the cryptophytes was likely photosynthetic (i.e. contained a cyanelle). This alga lost its plastid (e.g. *Goniomonas truncata*) and later replaced it with that from a red alga. It is hypothesized that the uptake of a secondary endosymbiont may be simplified if the nuclear-encoded plastid proteins with existing transit sequences already exist in the host cell nucleus and that these genes can be reutilized after the uptake of a new symbiont that is phylogenetically closely related to the original endosymbiont (Häuber et al., 1994). The host cell would then require only the invention of a modified transit sequence to allow entrance into the now four-membraned plastid. This hypothesis would be supported by the finding of remnant genes encoding plastid proteins in the nuclear genome of *G. truncata*.

The results and hypotheses described above show that the green algae are only one of many photosynthetic groups that have evolved multiple times on our planet. The green lineage contains a simple plastid that traces its origin to a primary endosymbiosis and has as a possible sister group other simple, plastid-containing algae such as the rhodophytes and glaucocystophytes. Characters that together distinguish the green lineage from all other eukaryotes are a two-membraned plastid containing chlorophyll *a* and *b* and stacked thylakoids with intraplastidial starch storage and a stellate structure in the flagellar transition region. In the next section the phylogeny of the green lineage will be presented in more detail.

FROM GREEN ALGAE TO LAND PLANTS

The results of approximately 25 years of electron-microscopic analyses of members of the green lineage have resulted in a number of hypotheses regarding the origin and diversification of these taxa. The most important of these is based on the observation that two fundamentally different types of microtubule organization are found within the green lineage during cytokinesis. The first, termed a phycoplast, is characterized by the collapse of the spindle apparatus after mitosis, with the microtubules oriented in the same direction as the plane of cell division. The second, termed a phragmoplast, is characterized by the development of a persistent telophase spindle and a cleavage furrow, with the microtubules oriented at right angles to the plane of cell division. That charophytes and land plants have a phragmoplast type of cell division, whereas chlorophytes, trebouxiophytes, and some members of the ulvophytes have a phycoplast type of cell division led to the division of the green algae and land plants into two distinct groups based on this cytokinetic character (Mattox and Stewart, 1984; Fig. 4).

Features of the motile cells were also found to be largely in accordance with this scheme and have played a central role in the resolution of evolutionary relationships of members of the green lineage. Of particular importance for the distinction of the charophytes from other members of the green lineage are the usual presence of square-shaped scales on the surface of biflagellate, asymmetric cells with a lateral/subapical flagellar insertion, and the presence of multilayered structures associated with the flagellar roots. The molecular sequence data from nuclear- (Bhattacharya and Ehlting, 1995; Kranz and Huss, 1996; Friedl, 1997), plastid- (Manhart, 1994), and mitochondria-encoded (Malek et al., 1996) genes also support a sister-group relationship between charophytes and land plants. In addition, several structural markers such as the presence of group II introns in two different tRNA genes of all land plants, *Coleochaete* spp., and *Nitella* spp. (Charales), which are found in only one of these genes in *Spirogyra* spp. (Zygnematales) and in no chlorophytes, suggests that the charophytes are directly related to the land plants, with *Chara/Nitella* spp. being more closely related than *Spirogyra* spp. (Manhart and Palmer, 1990). In light of these data the monophyly of charophyte green algae and land plants may be considered to be established within the literature.

An intriguing question that still remains to be verified pertains to the ancestry of the charophytes/land plants, because the monophyly of the green lineage is consistent with a flagellate ancestry of this group. The discovery of a

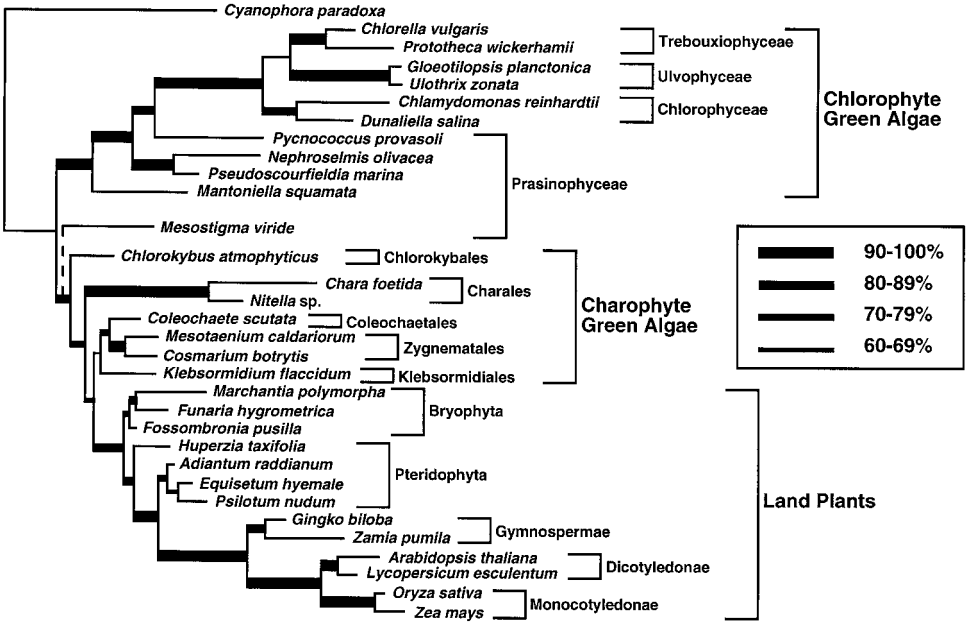

Figure 4. Small subunit rDNA phylogeny of the green lineage. This tree has been constructed with a weighted maximum parsimony method, and the results of bootstap analyses (200 replications) are shown as branch nodes of differing thicknesses (see box on the right). The likely position of divergence of the prasinophyte *M. viride* is shown with a broken line. The phylogeny is rooted within the branch leading to the rDNA sequence of the glaucocystophyte *C. paradoxa*.

group of unicellular taxa (the Prasinophyceae) that have both their body and flagella covered with square-shaped, nonmineralized scales and have parallel basal bodies and a depression or groove from which the flagella arise offered a likely source for this missing link. The typical scales of the prasinophytes are also found in the flagellate stages of the Charophyceae and the Ulvophyceae but not in other eukaryotes (Melkonian and Surek, 1995).

Analyses of small subunit rDNA sequences have demonstrated that the prasinophytes are a paraphyletic group that arise as multiple independent lineages at the base of the radiation of the chlorophytes, ulvophytes, and trebouxiophytes (Fig. 4). One member of the Prasinophyceae, *Mesostigma viride*, has been positioned with low bootstrap support at the base of the charophytes/land plants in a rDNA analysis (Melkonian and Surek, 1995). This relationship was predicted earlier from the unique cell ultrastructure of *M. viride*. It is the only prasinophyte to lack flagellar hair scales, and its cell body is extremely compressed along the anterior-posterior axis. Until now, no clear positioning within the green algae was possible because of the equivocal nature of the morphological data. Both *M. viride* and the charophytes share the feature of two multilayered structures located in the identical orientation to the flagellar roots.

In summary, there is now some evidence for a prasinophyte ancestry for the charophytes, which themselves are the ancestors of the bryophytes, ferns, gymnosperms, and angiosperms (Fig. 4). Fossil evidence shows that the land plants have existed for approximately 450 to 470 million years (Gray et al., 1982). This assemblage has therefore seen its members evolve from a single-celled alga similar to *M. viride* to the charophytes, the most complex green algae, with some members (Charales) reaching a size of 2 to 30 cm, to the bryophytes and then to the other land plants. To gain insights into the genetic developments that have led stepwise to the origin of land plants we are analyzing actin-coding regions to see whether duplications of this important cytoskeletal gene family may have accompanied the origin of multicellularity in the green lineage (Bhattacharya and Ehlting, 1995).

Actin exists as a constitutively expressed single-copy gene in all green algae except the ulvophytes, which appear to have undergone independent gene duplications. The charophytes also contain single-copy actin genes. The present data show actin gene duplications to appear first within the ferns, which are positioned as the sister group to the complex flowering plant actin gene families. Future research will also focus on resolving the origin of coding regions within the green algae and charophytes involved in morphogenesis, such as the MADS box genes, to gain further insights into the evolution of the green lineage. It is likely that the evolution of multicellularity within plants has followed another plan than that in animals (for review, see Meyerowitz, 1997) and that phylogeny reconstruction can play an important role in creating a logical framework for understanding the basis for plant organismal evolution.

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