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Origin and early evolution of chloroplasts

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Abstract. Photosynthetic organisms have played a significant role as primary producers and ultimate sources of the atmospheric oxygen through geologic time. How they have evolved is one of the key questions in the study of the biological and environmental history of the Earth. Modern algal chloroplasts (plastids) are greatly diversified in morphology and pigmentation. Because the paleontological record can never be complete, a multidisciplinary approach is essential to reveal the pattern, timing, and mechanism of chloroplast evolution. Several independent lines of evidence show that all chloroplasts derived from a single endosymbiotic cyanobacterium and spread in different eukaryotic taxa via multiple secondary endosymbioses. With the advent of the genomic era, comparative genomics has been employed to reveal the course of their evolution. Yet, the study of non-model organisms remains important to understand how today's diverse life has evolved, such as in the case of uniquely pigmented prochlorophytes. Paleontological records may provide constraints on the timing of the primary and secondary endosymbiotic events.

Key words: algae, chloroplasts, cyanobacteria, endosymbiosis, evolution, molecular phylogeny, Precambrian microfossils

Introduction

Photosynthesis is a physicochemical process in which plants, algae and photosynthetic bacteria use light energy to drive the synthesis of organic compounds. Photosynthesis in plants, algae and cyanobacteria involves the reduction of CO_2 to reduced organic carbon and the removal of electrons from H_2O, resulting in the release of O_2 as a byproduct (oxygenic photosynthesis). Other bacteria use light energy to produce organic compounds without generating O_2 (anoxygenic photosynthesis). The energy and reduced carbon provided by photosynthesis are required for the survival of virtually all life on earth. On the other hand, O_2 released by oxygenic photosynthesis has changed the Earth's surface from anoxic to oxic. The shift in the environmental oxidation state had a big impact on organismic metabolic systems. Photosynthetic organisms indeed have played a pivotal role in the development of the Earth system and how they have evolved is one of the key questions in the study of biological and environmental history.

Chloroplasts are organelles of eukaryotic algae and land plants, in which oxygenic photosynthesis is carried out. It has been suggested that chloroplasts were derived from a cyanobacterial endosymbiont and have spread among different eukaryotic taxa by subsequent endosymbiotic events (Figure 1) (Delwiche and Palmer, 1997; Douglas, 1998; McFadden, 2001; Palmer, 2003; Bhattacharya *et al.*, 2003). The idea of the endosymbiotic origin of chloroplasts can be traced back to the early 20th century. Mereschowsky (1905; for English translation, see Martin and Kowallik, 1999) first regarded cyanobacteria as chloroplast ancestors and suggested that differently pigmented bacteria had given rise to the present diversity of algae as a result of multiple symbiotic events (Mereschowsky, 1910). The idea was revived as the symbiotic theory, that is, that eukaryotic organelles such as chloroplasts and mitochondria originated from previously free-living prokaryotes that were stabilized as permanent intracellular elements within primitive eukaryotic cells (Margulis, 1970).

Fossil records provide direct evidence of ancient life. However, geological records are always affected by incomplete preservation and a multidisciplinary approach is required to look into early biological history. In the past few decades, molecular phylogeny has become a powerful tool to elucidate biological evolution. Thanks to the accumulation of genome sequence data, the history of life has been further unveiled by comparative genomics.

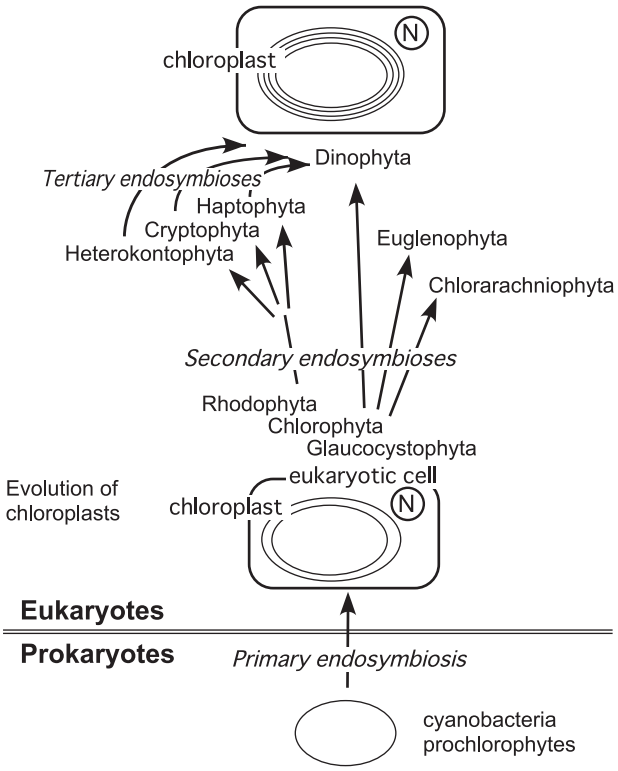

Figure 1. The evolutionary relationships among cyanobacteria/prochlorophytes and eukaryotic algae. Solid arrows indicate primary, secondary or tertiary endosymbioses. N represents a nucleus. The peptidoglycan wall of glaucophytes is not shown. The chloroplasts of the euglenoids and some dinoflagellates have three membranes. The cryptophytes and chlorarachniophyte chloroplasts retain a nucleomorph between two inner and two outer membranes.

The purpose of this review is to bring about a better understanding of the origin and early evolution of chloroplasts, especially of primary endosymbiotic origin, by linking molecular-biological and paleontological studies. The author employs the research on prochlorophytes as an example to describe how the molecular-biological approach may throw light on the history of early photosynthetic organisms.

Chloroplast diversification via endosymbioses

Modern algae contain chloroplasts diversified greatly in morphology and pigmentation (Table 1). Accordingly, these features have been regarded as important characteristics in the study of their evolution as well as in their classification.

The structure of chloroplast envelope membranes has been linked to their evolutionary history (Whatley, 1981). The chloroplasts of three algal groups, Chlorophyta, Rhodophyta, and Glaucophyta, are surrounded by two envelope membranes and are considered to have descended directly from the primary endosymbiotic event between a cyanobacterium and a non-photosynthetic host eukaryote. The glaucophyte chloroplasts are unique in structure: they retain a gram-negative-bacteria-like wall known as a peptidoglycan wall between the two chloroplast membranes (Bhattacharya and Schmidt, 1997). The chloroplasts of the other algal taxa are surrounded by three membranes (Euglenophyta and a part of Dinophyta) or four (Heterokontophyta, Cryptophyta, Haptophyta, and Chlorarachniophyta). Such chloroplasts have been suggested to be the results of secondary endosymbiotic events, in which previously non-photosynthetic eukaryotes obtained chloroplasts by engulfing a eukaryotic alga already equipped with chloroplasts. One piece of strong evidence for the secondary symbiosis hypothesis is the presence of nucleomorphs in cryptophytes and chlorarachniophytes (Douglas, 1998; Maier *et al.*, 2000). The nucleomorph is a remnant of the primary-host nucleus in the periplastidal compartment between the two inner and the two outer chloroplast envelope membranes. The two inner membranes are considered to be comparable to the two surrounding membranes of the primary chloroplasts, while the outer two, often designated as the chloroplast endoplasmic reticulum, may have derived from the cell membrane of the primary host and the food vacuole of the secondary host (Gibbs, 1981; McFadden, 1999). Phylogenetic analyses have suggested that chlorarachniophyte chloroplasts originated from the green algal lineage, while cryptophytes originated from the red algal one (van der Peer *et al.*, 1996; Durnford *et al.*, 1999), consistent with their photosynthetic pigment commonality (Table 1).

Heterokontophytes, haptophytes, and cryptophytes use chlorophyll *c* as an accessory pigment (chromophyte algae). They have been suggested to have a common red-algal origin and were classified in the kingdom Chromista (Cavalier-Smith, 1981). Cavalier-Smith (1999) further proposed that the Chromista is a sister group of the Alveolata, comprising dinoflagellates, parasitic apicomplexans, which bear non-photosynthetic plastids (apicoplasts), and no-plastid-bearing ciliates (the chromalveolate hypothesis).

A genome-base phylogeny of 41 protein-coding genes sampled from 15 complete chloroplast genome sequences indicated that the Cryptophyta and the Heterokontophyta have two independent secondary-endosymbiotic origins (Martin *et al.*, 2002); however, the analysis did not include taxonomically diverse

Table 1. The chloroplast structure and pigmentation of the oxygenic photosynthetic organisms. (a) Some dinoflagellates are known to contain chloroplasts of tertiary endosymbiotic origin. (b) A peptidoglycan cell wall remains between the two chloroplast membranes. (c) A nucleomorph is located between two outer and two inner membranes. (d) Some dinoflagellate chloroplasts have different structure and/or pigmentation. (e) Only major photosynthetic pigments are shown. Chl: chlorophyll. PB: phycobilins. F: fucoxanthin. PD: peridinins. (f) *P. marinus* contains both divinyl and monovinyl chlorophyll.

		Division	Class	Common names	Number of chloroplast membranes	Pigments (e)
Prokaryota	—	**Cyanophyta**		blue-green algae	—	Chl a, PB
	—	**Prochlorophyta**			—	Chl *a*, Chl *b* (f)
Eukaryota	Primary-endosymbiotic chloroplasts	**Glaucophyta**			2 (b)	Chl *a*, PB
		Rhodophyta		red algae	2	Chl *a*, PB
		Chlorophyta		green algae, land plants	2	Chl *a*, Chl *b*
	Secondary-endosymbiotic chloroplasts (a)	**Heterokontophyta**	Bacillariophyceae	diatoms	4	Chl *a*, Chl c_1, c_2, c_3, F
			Eustigmatophyceae		4	Chl *a*
			Xanthophyceae	yellow-green algae	4	Chl *a*, Chl c_1, c_2
			Phaeophyceae	brown algae	4	Chl *a*, Chl c_1, c_2, c_3, F
			Raphidophyceae		4	Chl *a*, Chl c_1, c_2
			Chrysophyceae	golden algae	4	Chl *a*, Chl c_1, c_2, F
		Cryptophyta			4	Chl *a*, Chl c_2, PB
		Haptophyta			4 (c)	Chl *a*, Chl c_1, c_2, F
		Chlorarachniophyta			4	Chl *a*, Chl *b*
		Euglenophyta			3	Chl *a*, Chl *b*
		Dinophyta		dinoflagellates	3 (d)	Chl *a*, Chl c_2, PD

algal groups due to limitation of available genomic information. Yoon *et al.* (2002) investigated phylogenetic relationships among the three chromist groups and various red algae by using a concatenated sequence of five plastid-encoded genes. Their analysis clearly showed that the heterokontophyte, haptophyte, and cryptophyte chloroplasts had descended from a single red-algal ancestor. The Chromista monophyly was also supported by unique replacement of plastid-targeted proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and fructose-1,6-bisphosphate aldolase (FBA), in chromoalveolates (Fast *et al.*, 2001; Harper and Keeling, 2003; Patron *et al.*, 2004). However, a recent phylogenetic analysis using six cytosolic protein sequences suggests that cryptophytes and haptophytes form a weakly supported cluster apart from a monophyletic clade of the other four chromoalveolate groups (Harper *et al.*, 2005). The chromalveolate hypothesis still awaits further verification.

The Euglenophyta and Chlorarachniophyta, like the Chlorophyta, contain chlorophyll *b* as an accessory pigment. Molecular analyses have shown that their chloroplasts may have come from the chlorophyte lineage (van de Peer *et al.*, 1996; Durnford *et al.*, 1999). To minimize the number of independent endosymbiotic events, Cavalier-Smith (1999, 2000) advanced the suggestion that the chlorophyll *b*-containing chloroplasts of euglenoids and chlorarachniophytes might have derived from a single secondary endosymbiosis. However, morphological and molecular-phylogenetic data have not shown support for their monophyly and the two groups may be the result of independent endosymbiotic events (Archibald and Keeling, 2002).

The evolution of dinoflagellate chloroplasts is more complicated. About half of known living dinoflagellate species are heterotrophic, not photosynthetic (Gaines & Elbrachter, 1987). The most common dinoflagellate chloroplasts are three-membrane-bound and contain

peridinin as an accessory pigment. Notably, their genome structure is very different from other algae. Ordinary chloroplasts contain a circular genome comprising 50 to 200 genes. In contrast, the genes of the peridinin-containing chloroplasts are located on minicircles, most of which contain a single gene (Zhang *et al.*, 1999; Green, 2004). Phylogenetic analyses using chloroplast-encoded genes have shown that the peridinin-containing dinoflagellate chloroplasts evolved from red algae (Takishita and Uchida, 1999; Zhang *et al.*, 1999, 2000). Besides peridinin-containing species, there also exist several types of dinoflagellate containing other photosynthetic pigments, such as chlorophyll *b*, fucoxanthin, and phycobilins (Dodge, 1989). These chloroplasts have been suggested to have multiple endosymbiotic origins from prasinophyte green algae (Watanabe *et al.*, 1987), diatoms (Chesnick *et al.*, 1997), cryptophytes (Takishita *et al.*, 2002; Hacket *et al.*, 2003), or haptophytes (Tengs *et al.*, 2000; Ishida and Green, 2002). The chloroplasts that arose by engulfing diatoms, haptophytes or cryptophytes have evolved via ''tertiary'' endosymbiotic events. Noteworthily, chloroplasts of some dinoflagellates (e.g., two-membrane-bound chloroplasts of *Dinophysis*) could be kleptoplasts, which are temporary chloroplasts taken from eukaryotic algae (for discussion, Hackett *et al.*, 2004).

Monophyletic origin of chloroplasts

The algal taxa containing secondary-endosymbiotic chloroplasts have been tied with one of the three primary-chloroplast groups. How about the primary-endosymbiotic chloroplasts? Do glaucophytes, rhodophytes and chlorophytes have a common cyanobacterial ancestor, or multiple independent origins? Several lines of evidence have shown strong support for a monophyletic origin of all chloroplasts (for reviews, Delwiche and Palmer, 1997; Douglas, 1998; McFadden, 2001; Palmer, 2003).

One line of evidence for chloroplast monophyly lies in the photosynthetic apparatus. Most eukaryotic algae are known to contain proteins of the light-harvesting chlorophyll protein complex (LHC) superfamily as chlorophyll-binding proteins, whereas cyanobacteria and prochlorophytes do not have the LHC (Green *et al.*, 1994) but rather contain the high-light-inducible protein (HLIP), which is similar to the LHCs and a possible progenitor of the eukaryotic LHCs (Dolganov *et al.*, 1995). A protein of the LHC family has been identified in the photosystem I from two red algae (Wolf *et al.*, 1994; Marquardt *et al.*, 2001) but has not been detected in the Glaucophyta by an immunological method (Koike *et al.*, 2000), indicating that the ancestral LHC may have arisen before the divergence of the Rhodophyta and Chlorophyta.

Monophyly of the chloroplasts has been examined extensively by analyses of chloroplast-encoded genes. The phylogenies were mostly inferred from a single chloroplast gene (e.g., 16S rRNA, *tuf*A, *atp*B, *rpo*C1 and *psb*A) and have shown good support for chloroplast monophyly (for a review, see Delwich & Palmer, 1997; for 16S rRNA phylogenies, see Turner *et al.*, 1999; Wilmotte and Herdman, 2001). Cyanobacterial and chlorophast genome sequencing projects have provided a large set of data, which can be used for constructing a multigene phylogeny. Genome-base analyses have been performed to investigate the evolution of chloroplast genome contents (Martin *et al.*, 1998, 2002), but did not verify the chloroplast monophyly because only one cyanobacterium was included in the trees.

If the chloroplasts were derived from a single endosymbiotic event, all phylogenies based on the chloroplast, mitochondrial, and nuclear genes should group the three algal lineages of the primary chloroplasts, i.e., glaucophytes, rhodophytes and chlorophytes (Delwiche and Palmer, 1997).

Hundreds of mitochondrial genome sequences have been determined to date, but nearly 90% are of animals and half of the rest are of fungi (for detailed information, visit the websites of the GenomeNet at www.genome.jp or of the Organelle Genome Megasequencing Program (OGMP) at http://megasun.bch.umontreal.ca/ogmp/). Complete mitochondrial genome sequences are available for 16 chlorophytes (10 plants and 6 green algae), 3 rhodophytes, and a haptophyte, a cryptophyte, and four heterokonts (two golden algae and two brown algae). Although their genome size varies from the 6 Kbp of a malaria parasite to the 2400 Kbp of a land plant (average $\sim$ 30 Kbp) (Gray *et al.*, 2004), most mitochondria contain the genes for the cytochrome oxydase subunits 1 to 3 (*cox1–3*) and for cytochrome *b* (*cob*) in common (Leblanc *et al.*, 1997). Phylogenetic analysis of cox3 (Boyen *et al.*, 1994) and that of the concatenated protein sequences of *cox1–3* and *cob* (Lang *et al.*, 1999) has supported the rhodophyte-chlorophyte sisterhood.

Molecular phylogenies inferred from a single nuclear marker such as rRNAs and genes for the proteins β-tubulin, TPI (trisephosphate isomerase), EF-1α, GAPDH, and actin have presented insufficient, or sometimes inconclusive, support for the monophyly of the three primary chloroplasts (for a review, see Delwiche and Palmer, 1997). Multiple-gene analyses using the nuclear-encoded EF-2 (elongation factor 2) and a

concatenated protein sequence of 13 nuclear genes have shown confident support for the sisterhood of rhodophytes and chlorophytes, but the calculation did not include glaucophytes (Moreira *et al.*, 2000). Recently, phylogenetic analysis of multiple nuclear genes including glaucophytes has been performed, providing strong support for the single endosymbiotic origin of glaucophytes, rhodophytes and chlorophytes (Rodriguez-Ezpeleta *et al.*, 2005).

Prochlorophytes tell another story of the chloroplast progenitor

It is now almost proven that all the algal chloroplasts are derived from a single cyanobacterial ancestor. What kind of cyanobacterium gave rise to the first chloroplast? Phylogenetic analyses of cyanobacterial and chloroplast 16S rRNA sequences indicated that the chloroplast cluster seemed to have a relatively deep position but did not specifically link with a specific living cyanobacterium (Nelissen *et al.*, 1995; Turner *et al.*, 1999; Wilmotte and Herdman, 2001). No other genes have been studied as much as 16S rRNA and molecular phylogeny has not provided further information on the chloroplast ancestor. But study of the unusually pigmented oxygenic photosynthetic prokaryotes known as prochlorophytes has shown a new aspect of the chloroplast progenitor.

The origin of chloroplasts had been linked with cyanobacteria, because they were the only bacteria known to perform oxygenic photosynthesis. Such an origin may fit well in the case of the chloroplasts of rhodophytes and glaucophytes because they, like cyanobacteria, contain phycobilins as accessory pigments. But how did the current diversity of the algal photosynthetic pigments evolve?

Three scenarios have been presented to explain the evolution of differently pigmented chloroplasts. Raven (1970) proposed that multiple primary-endosymbiotic events might have given rise to modern algal diversity, by taking up prokaryotes with different pigment compositions, i.e., chlorophyll *a* and one of the following accessory pigments, phycobilins, chlorophyll *b* or chlorophyll *c*. In this scenario, the phycobilin-containing chloroplasts of rhodophytes, glaucophytes and cryptophytes might have derived from cyanobacteria, while the chloroplasts of chlorophytes and euglenoids originated from hypothetical ''green prokaryotes'' bearing chlorophyll *b*, and those of phaeophytes, chrysophytes, xanthophytes and dinoflagellates from ''yellow prokaryotes'' containing chlorophyll *c* (Figure 2A).

When a new type of oxygenic photosynthetic organ-

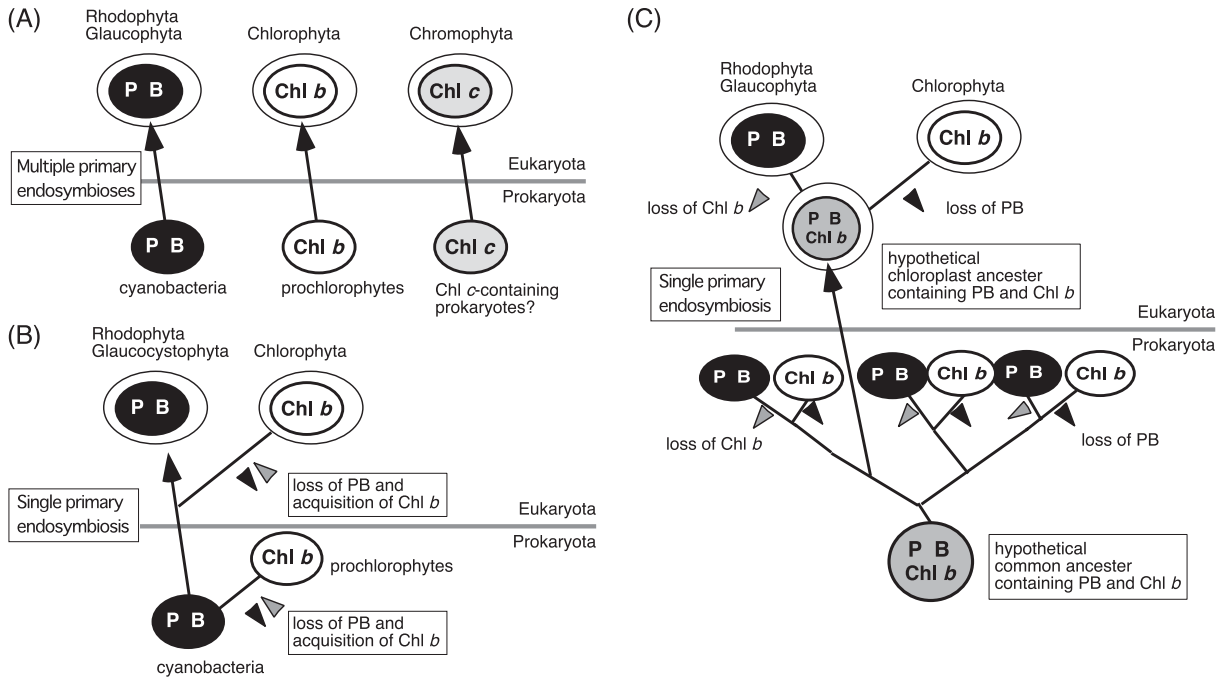

Figure 2. Three evolutionary scenarios on the origin and early evolution of chloroplasts. (A) Multiple origins of chloroplasts. The group of algae containing chlorophyll *c*, such as yellow-green algae and brown algae, has been assigned to the Chromophyta. (B) The acquisitive theory. (C) The reductive theory. Chl: Chlorophyll. PB: phycobilins.

ism containing chlorophyll *b* but not phycobilins (the Prochlorophyta) were discovered, they were suggested to be the progenitors of chlorophyte chloroplasts (Lewin and Withers, 1975; Lewin, 1976). Three species (*Prochloron didemni*, *Prochlorothrix hollandica*, *Prochlorococcus marinus*) have been identified as prochlorophytes. They are distinct from each other morphologically, physiologically and ecologically. *P. didemni* was the first discovered prochlorophyte. It is an exosymbiont of didemnid ascidians in the (sub-) tropical ocean. *P. hollandica* is a filamentous free-living prokaryote inhabiting a freshwater environment. *P. marinus* is a pico-sized planktonic coccoid bacterium. It is found abundantly in marine surface waters of the temperate to tropical ocean.

Despite the chlorophyte-like pigmentation, molecular-phylogenetic analyses based on 16S rRNA (Urbach *et al.*, 1992) and *rpo*C1 (Palenik *et al.*, 1992) have shown that the three prochlorophytes were not the closest relatives of the chlorophyte chloroplasts, but were rather diverged members of the cyanobacteria (Figure 3). In addition, as discussed in the previous section, independent lines of evidence have supported the chloroplast monophyly and the idea of multiple chloroplast origins has been eliminated.

As a more likely scenario, chloroplasts were suggested to have derived from a single cyanobacterium containing phycobilins, with subsequent acquisition of chlorophyll *b* and loss of phycobilins giving rise to the chlorophyte lineage (Figure 2B). In this evolutionary scenario (the acquisitive theory, Delwiche and Palmer, 1997), the ability to synthesize chlorophyll *b* evolved at least four times independently; three times in each of the three known prochlorophyte species and once in the chlorophyte ancestor (Palenik *et al.*, 1992; Urbach *et al.*, 1992).

In contrast, a study of the chlorophyll *b* biosynthesis gene designated as *CAO* (Tanaka *et al.*, 1998) supported another scenario. The analysis of *CAO* from prochlorophytes and chlorophytes suggested that their chlorophyll *b* synthesis genes have a common evolutionary origin (Tomitani *et al.*, 1999). Taking into account the polyphyletic distribution of the prochlorophytes among the cyanobacterial clade, the common ancestor of the prochlorophytes and cyanobacteria, including the progenitor of chloroplasts, could have been derived from a hypothetical ancestor containing both chlorophyll *b* and phycobilins (Figure 2C). In this scenario, subsequent loss of one of the two pigments then gave rise to prochlorophytes and cyanobacteria, as well as the three primary-endosymbiotic algal taxa. This agrees with the reductive theory of pigment evolution (Bryant, 1992; Delwiche and Palmer, 1997), at least for chlorophyll *b* and phycobilins.

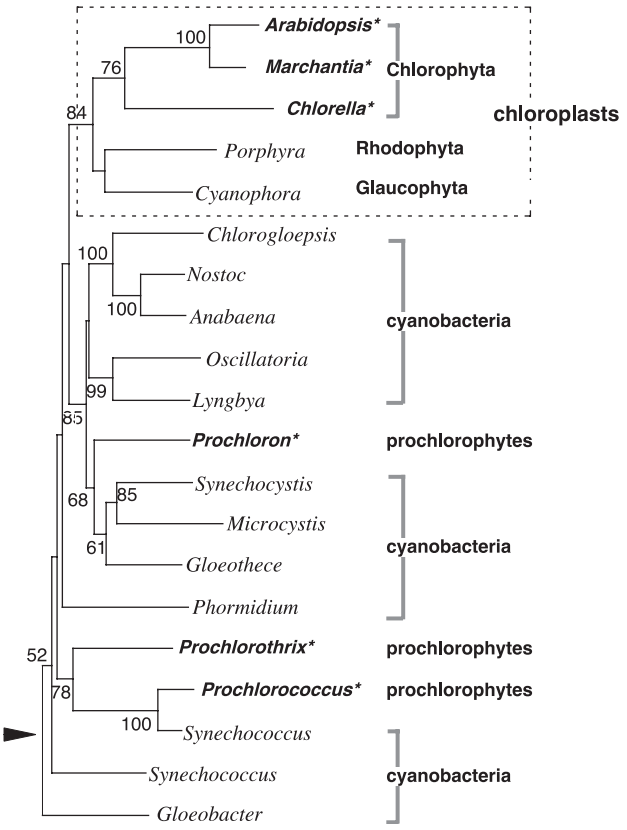

Figure 3. The phylogenetic relationships of cyanobacteria and prochlorophytes inferred from 16S rRNA sequences. The tree was constructed by the neighbor joining method (Saitou and Nei, 1987). Numbers at each branch point are the bootstrap values (Felsenstein, 1985) for percentages of 1000 replicate trees. Only values above 50% are shown. The arrowhead represents the deepest root determined using *Agrobacterium* as outgroup. Asterisks (*) indicate chlorophyll-*b*-containing organisms.

No organisms have been known to have both chlorophyll *b* and phycobilins; however, the *Prochlorococcus marinus* CCMP 1375 contains a type of phycoerythrin, a component of phycobilins (Hess *et al.*, 1996), which has been shown to function as a light-harvesting antenna (Lokstein *et al.*, 1999). The genes for phycoerythrin subunits, *cpe* A and *cpe* B, have also been identified in several other *Prochlorococcus* strains (Ting *et al.*, 2001). *P. marinus* may have derived from the proposed ancestral form without major pigment alterations.

Analysis of prochlorophyte chlorophyll *b* binding proteins presented yet another story about prochlorophyte evolution. Most of the light-harvesting chlorophyll protein complexes (LHCs) analyzed so far belong to members of the LHC superfamily, including the chlorophyll *a/b* complexes (CABs) (Green and Pi-

Table 2. Complete chloroplast genome sequences. Only representative projects are shown here. bp: base pairs.

Taxon	Origins		Sequence length (bp)	Number of Protein-coding genes	References
Chlorophyta	*Arabidopsis thaliana*	(thale cress)	154,478	87	Sato *et al.*, 1999
	Epifagus virginiana	(beechdrops)	70,028	21	Wolfe *et al.*, 1992
	Nicotiana tabacum	(tobacco)	155,943	76	Shinozaki, 1986
	Pinus thunbergii	(pine)	119,707	69	Wakasugi *et al.*, 1994
	Oryza sativa	(rice)	134,525	76	Hiratsuka *et al.*, 1989
	Oryza nivara	(rice)	134,494	76	Shahid Masood *et al.*, 2004
	Zea mays	(maize)	140,387	76	Maier *et al.*, 1995
	Marchantia polymorpha	(liverwort)	121,024	84	Ohyama *et al.*, 1986
	Physcomitrella patens	(moss)	122,890	83	Sugiura *et al.*, 2003
	Chlamydomonas reinhardtii	Chlorophyceae	203,828	72	Maul *et al.*, 2002
	Chlorella vulgaris	Trebouxiophyceae	150,613	78	Wakasugi *et al.*, 1997
	Mesostigma viride	Prasinophyceae	118,360	135	Lemiueux *et al.*, 2000
	Nephroselmis olovacea	Prasinophyceae	200,799	127	Turnel *et al.*, 1999
Rhodophyta	*Porphyra purpurea*		191,028	200	Reith and Munholland, 1995
	Cyanidium caldarium		164,921	199	Glöckner *et al.*, 2000
	Cyanidioschyzon merolae		149,987	207	Ohta *et al.*, 2003
Glaucophyta	*Cyanophora paradoxa*		135,599	136	Stirewalt *et al.*, 1995
Euglenophyta	*Euglena gracilis*		143,170	58	Hallick *et al.*, 1993
Heterokontphyta	*Odontella sinensis*		119,704	124	Kowallik *et al.*, 1995
Cryptophyta	*Guillardia theta*		121,524	183	Douglas & Penny, 1999
Haptophyta	*Emiliania huxleyi*		105,309	150	Sanchez Puerta *et al.*, 2005

chersky, 1994). On the other hand, prochlorophyte chlorophyll *b* binding proteins (Pcb) isolated from the three prochlorophytes were all closely related to IsiA (a cyanobacterial chlorophyll *a*-binding protein induced by iron starvation) and to CP43 (constitutively expressed chlorophyll *a* antenna protein in photosystem II) (La Roche *et al.*, 1996), but not to the LHC superfamily. All the three prochlorophytes, *Prochlorothrix*, *Prochlorococcus*, and *Prochloron*, as well as the chlorophyll *d*-containing oxygenic photosynthetic prokaryote *Acaryochloris marina*, have been shown to contain multiple *pcb* genes (van der Staay *et al.*, 1998; Garczarek *et al.*, 2001; Chen *et al.*, 2005). Phylogenetic analysis of the Pcb, IsiA and CP43 protein sequences indicated a single origin for *pcb*A/B genes and *pcb*C genes. Considering the paraphyletic distribution of the three prochlorophytes and *Acaryochloris*, Chen *et al.* (2005) suggested that *pcb* genes might have spread by multiple lateral gene transfers within the cyanobacterial lineage.

Prochlorophytes are also known to contain other photosynthetic pigments. It has been reported that they contain a chlorophyll *c*-like pigment (Mg 3,8-divinyl-pheoporphyrin a_5) (Larkum *et al.*, 1994). Furthermore, *Prochlorococcus* is known to have a unique pigment composition: it mainly uses divinyl chlorophyll, instead of normal (monovinyl) chlorophyll (Chisholm *et al.*, 1988; Goericke and Repeta, 1992). Recently, the gene for 3,8-divinyl protochlorophyllide *a* 8-vinyl reductase (DVR), which is essential for monovinyl chlorophyll biosynthesis, was identified (Nagata *et al.*, 2005). The *DVR* homologue is present in the cyanobacterium *Synechococcus* WH8102 but not in *Prochlorococcus*, indicating that the divinyl chlorophyll of *Prochlorococcus* may have evolved by losing the *DVR* gene in their progenitor.

The photosynthetic-pigment composition is one of the key characters in algal taxonomy and continued study of the pigment biosynthesis will shed light on chloroplast evolution.

Chloroplast evolution from a genomic point of view

Since chloroplast genomes were first wholly sequenced in 1986, dozens of chloroplast genome sequences have been completely determined from various algae and plants (Table 2). Their comparison with cyanobacterial genomes as well as with host nuclear ones has opened the door to understanding how the chloroplast genome contents have changed (Martin & Herrmann, 1998; Timmis *et al.*, 2004).

Considering that functional chloroplasts contain

Table 3. The complete cyanobacterial genome sequences. Only representative projects are shown here. Mbp: million base pairs. ORF (Open reading frame): a predicted protein-coding region.

Origin	Subsection	Size (Mbp)	Number of ORF	References
Synechocystis sp. PCC 6803	I	3.57	3,215	Kaneko *et al.*, 1996
Gloeobacter violaceus PCC 7421	I	4.66	4,430	Nakamura *et al.*, 2003
Thermosynechococcus elongatus BP-1	I	2.59	2,475	Nakamura *et al.*, 2002
Prochlorococcus marinus SS120	I	1.75	1,884	Dufresne *et al.*, 2003
Prochlorococcus marinus MED 4	I	1.66	1,716	Rocap *et al.*, 2003
Prochlorococcus marinus MIT 9313	I	2.41	2,275	Rocap *et al.*, 2003
Synechococcus sp. WH 8102	I	2.43	2,526	Palenik *et al.*, 2003
Anabaena (*Nostoc*) sp. PCC 7120	IV	6.41	5,368	Kaneko *et al.*, 2001
Nostoc punctiforme ATCC 29133	IV	7.54	7,432	Meeks *et al.*, 2001

about 50 to 200 genes whereas modern cyanobacterial genomes have about 1,700 to 7,500 (Table 2, 3), it appears that the algal chloroplasts have lost most of their genes, probably 90% or more, since departure from the chloroplast progenitor. Where have the chloroplast genes gone? Comparative genomic analyses have shown that most of the chloroplast genes have been transferred to the nucleus (Martin *et al.*, 2002).

One may thus expect that the genes in the ancestral chloroplasts gradually transferred to the host nucleus through algal evolution. Consistent with this idea, the chloroplasts of the unicellar red and green algae appear to retain more genes inherited from the cyanobacteria than do other chloroplasts (Table 2). For example, the chloroplasts of the red algae *Cyanidium caldarium* (Glöckner *et al.*, 2000) and *Cyanidioschyzon merolae* (Ohta *et al.*, 2003) contain five genes involved in the formation of bacterial cell envelopes, namely, four genes (*lipB*, *lpxA*, *lpxC*, *and* ycf82) for lipopolysaccharid synthesis and *glmS* for formation of cell envelopes. Those of the prasinophytes *Nephroselmis olovacea* and *Mesostigma viride* do not contain these five genes, but do contain *ftsI*, a gene involved in peptidoglycan synthesis (Turmel *et al.*, 1999; Lemieux *et al.*, 2000).

However, genome comparison of nine chloroplasts and a cyanobacterium has indicated that chloroplast genome contents may not reflect their evolutionary history (Martin *et al.*, 1998). A phylogenetic tree was constructed using 45 protein-coding genes shared by all the analyzed chloroplast genomes. Martin *et al.* (1998) mapped all 205 protein-coding chloroplast genes on the phylogeny, showing that many gene losses occurred parallelly in the algal lineages. This multiple independent gene loss from the chloroplast genomes may be partly explained by laboratory experiments suggesting that gene transfer from chloroplasts to the nucleus probably occurs at a very high frequency (Huang *et al.*, 2003; Stegemann *et al.*, 2003).

To estimate the number of genes transferred to the nucleus, genomes of three cyanobacteria and *Arabidopsis thaliana* were compared. The analysis has shown that about 18% ($\sim$4500 genes) of the protein-coding genes in the nuclear genome of *A. thaliana* (about 24900 genes in total) were derived from the endosymbiotic cyanobacterium of the chloroplast ancestor (Martin *et al.*, 2002). Comparison of the genome content among 16 algal chloroplasts indicated that 44 of the 274 protein-coding genes are still retained in all the 16 chloroplasts. The 230 left have been lost from chloroplast genomes at least in one algal lineage, and 117 have homologues in the nuclear genome and hence might have been transferred to the host nucleus.

Invention of a mechanism to transport proteins of nuclear-encoded genes back to chloroplasts is a key in conversion of an endosymbiont to a permanent organelle (Cavalier-Smith and Lee, 1985; Cavalier-Smith, 2000). Such a transport system includes N-terminal amino-acid extensions, referred to as transit peptides, which target the chloroplast envelope membranes (for review, see McFadden, 1999). Products of nuclear genes of the secondary endosymbiotic algae additionally contain signal peptides to target the outer chloroplast membrane derived from a primary host organism. Similarity in the mechanism for the nuclear-gene products to retarget the organelles can be regarded as another point of evidence supporting chloroplast monophyly (McFadden and van Dooren, 2004). A computational analysis of the complete *Arabidopsis* genome succeeded in detecting previously unknown chloroplast gene precursors (Emanuelsson *et al.*, 2000). Despite their functional conservatism, however, transit peptides show great diversity in length, composition and organization at the primary structure level (Bruce, 2001).

Successful chloroplast-to-nucleus gene transfer also requires a machinery to transport proteins into the

organelles. It consists of two apparatuses, the Tic and Toc (translocon at the inner/outer envelope membrane of chloroplasts) complexes (for a review, see Soll and Schleiff, 2004). Comparison of algal- and parasite-plastid genomes together with cyanobacterial and nucleomorph ones has shown not only that red and green algal chloroplasts contain remarkably similar important machineries, but also that Tic110 and Toc34, components of the Tic and the Toc respectively, are present in red and green algae (Tic110 is present also in cryptophytes and diatoms), but absent from cyanobacteria, providing strong support for the common ancestry of the chloroplasts (McFadden and van Dooren, 2004). Genome-wide search for proteins of the Tic and Toc subunits suggested that some proteins might have evolved by recruiting cyanobacterial proteins, while others, including Tic110 and Toc34, may have derived from proteins of a host eukaryote (Reumann *et al.*, 2005).

The timing of the chloroplast evolution

Whereas molecular analyses of living photosynthetic organisms reveal the pattern and mechanism of the chloroplast evolution, paleontological and geochemical records are direct evidence of their ancestors, providing time calibration for molecular-evolutionary study.

Grypania spiralis fossils are preserved as spirally coiled ribbonlike films and have been found abundantly in Mesoproterozoic sediments from Montana (Walter *et al.*, 1976), China (Walter *et al.*, 1990) and India (Kumar, 1995). Based on its coiling form and large size (0.7–1.5 mm in width), *G. spiralis* was interpreted as a multicellular alga (Walter, 1990; Runnegar, 1991). Han and Runnegar (1992) identified fossils resembling *G. spiralis* in the 1.87 Ga Negaunee Iron-Formation of Michigan, USA (Schneider *et al.*, 2002). However, the Negaunee coiled fossils lack fine structure such as transverse septa and their eukaryotic interpretation is questioned (Samuelsson and Butterfield, 2001).

Acritarchs of probable eukaryotic origin appear in 1900–1600 Ma rocks, and become abundant in the Mesoproterozoic and morphologically diversified and complex in the Neoproterozoic era (Knoll, 1996). Acritarchs have been generally regarded as remnants of unicellular protists, mostly of phytoplankton (Vidal & Moczydlowska-Vidal, 1997). For instance, *Trachyhystrichosphaera* acritarchs from the ca 700–800 Ma Draken Conglomerate Formation, northeastern Spitsbergen were interpreted as possible prasinophycean green algae (Tappan, 1980; Knoll *et al.*, 1991). However, some Proterozoic acritarchs have been recently reinterpreted as fossils of fungi, which are neither unicellular nor photoautotrophic. Acanthomorphic acritarchs of *Tappania* (the former genera *Tappania* and *Germinosphaera*) from the 900–800 Ma Wynniatt Formation, northwestern Canada have branching septate filamentous processes capable of secondary fusion, which is a synapomorphy of the higher fungi (Butterfield, 2005). Fungus-like characteristics are also seen in other Proterozoic acritarchs such as *Trachyhystrichosphaera*, *Shuiyoushaeridium*, *Dictyoshaera* and *Foliomorpha*, indicating that the diverse Proterozoic acritarchs cannot be ascribed *a priori* to phytoplanktons (Butterfield, 2005).

What fossils can be more confidently linked to the modern algal taxa? The oldest probable algal fossils yet known are *Bangiomorpha* preserved in the 1200 Ma Hunting Formation, arctic Canada, which has been related to the modern bangiophyte red algae (Butterfield *et al.*, 2000). They have complex multicellular filaments characterized by cell differentiation. They also present the earliest record of sexual reproduction. What may be the oldest green algal fossils are uniseriate filaments of *Proterocladus* from the ca. 750 Ma Svanbergfjellet Formation, Spitsbergen (Butterfield *et al.*, 1994). Their branching septate filaments are diagnostic of the modern green alga *Cladophoropsis.*

Paleontological records also provide the upper time limit of secondary-endosymbiotic events. Fossil records of xanthophyte algae were found in the ca. 1000 Ma Lakhanda Group, eastern Siberia (Woods *et al.*, 1998). Fossil xanthophytes have been also documented from the ca. 750 Ma Svanbergfjellet Formation, Spitsbergen (Butterfield, 2004).

Besides paleontological evidence, geochemical signals indicate the presence of early algae. Some organic compounds have been recognized as taxon-specific indicators called biomarkers. Dinosterane, a biomarker of dinoflagellates, becomes abundant between the Permian and Triassic. Although they have been also detected in the Early Cambrian (ca. 520 Ma) and back to Proterozoic sediments, the compounds may not be common in the Precambrian (Summons and Walter, 1990; Summons *et al.*, 1992; Moldowan *et al.*, 1996; Moldowan and Talyzina, 1998).

Geological records are our only direct evidence of ancient life and provide time constraints for molecular-clock analyses. Several calculations have been performed to estimate timing of algal evolution. Yoon *et al.* (2004) used 7 fossil records as time constraints to calibrate a phylogeny inferred from 6 chloroplast genes. Estimated divergence dates were 1474/

1452 Ma for the split of red- and green-algal lineages and 1274/1255 Ma for the chromist divergence, with/without the oldest known algal fossils of the ca. 1200 Ma *Bangiomorpha* (Butterfield *et al.*, 2000), respectively. Hedges *et al.* (2004) estimated the evolutionary timing of major eukaryotic groups, using 20–188 proteins per node by global and local clock methods. Instead of using fossils as direct time constraints, they first used the younger bird-mammal split time (310 Ma) and calculated three divergence dates, which were then used as calibration for deep nodes. They suggested that different methods yielded similar time estimates (within 5% variation) and that the primary endosymbiosis may have occurred 1600 to 1500 Ma. Douzery *et al.* (2004) performed a relaxed molecular clock analysis based on 129 protein sequences from 36 taxonomically diverse eukaryotes, using six fossil records without the *Bangiomorpha* fossils as calibration. They dated the divergence time of chlorophytes and rhodophytes between 825 and 1061 Ma and the split of major eukaryotic kingdoms between 950 and 1259 Ma. The estimates by Douzery *et al.* are in contrast to the other estimates providing deeper dates than the known paleontological records. The discrepancy between the estimated dates indicates that the molecular clock may be subject to the choice of data set, analytical methods and paleontological calibrations.

Concluding remarks

It is now almost certain that chloroplasts derived from a single cyanobacterial endosymbiont and spread widely in different eukaryotic taxa via multiple secondary endosymbiotic events. Extensive phylogenetic analysis will resolve the evolutionary relationships among the algae containing secondary and tertiary endosymbiotic chloroplasts.

The nuclear genome sequences of the rhodophyte *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004) and the diatom *Thalassiosira pseudonana* (Armbrust *et al.*, 2004) have been recently determined. They contain chloroplasts of primary and secondary endosymbiotic origins, respectively, and fill the gap between cyanobacteria and higher plants in the genome-scale investigation of chloroplast evolution. Notably, chloroplast and mitochondria genomes have been completely sequenced for *C. merolae* and *A. thaliana* and we now have truly complete genetic information for these organisms. In addition, the complete nucleomorph genome of the cryptophyte *Guillardia theta* is also available (Douglas *et al.*, 2001). Comparative genomics will further unveil the processes and mechanisms of chloroplast evolution.

Although the chloroplast cluster appear to have branched relatively early in the cyanobacterial radiation, no specific cyanobacterial relative is yet known. A multigene-base phylogeny of taxonomically diverse cyanobacteria and algae may identify the modern cyanobacterium closest to the chloroplast ancestor. On the other hand, studies of the uniquely pigmented prochlorophytes have presented another story about the chloroplast progenitor. Even in the genomic era, extant diversified organisms whose genomes have been mostly uninvestigated hold importance in the reconstruction of complex biological history.

It has been often advocated that lateral gene transfer is universal among prokaryotes (Doolittle, 1999). For example, genome-scale analyses of the five major photosynthetic prokaryote groups indicated that horizontal transfer might have been so common that even core genes for photosynthesis have been transferred (Raymond *et al.*, 2002). Gene transfer is also known in eukaryotic algal evolution. An example is *rbc*L, which codes for the large subunit of Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase). *rbc*L genes of rhodophytes and their derivative secondary chloroplasts of heterokonts, haptophytes and cryptophytes are known to be close to proteobacterial *rbc*L (red-type form I Rubisco), whereas most other algal groups contain a cyanobacterial-type *rbc*L (green-type form I Rubisco) (Delwiche and Palmer, 1996). Furthermore, dinoflagellates contain another type of *rbc*L, similar to some proteobacterial ones (form II Rubisco) (Morse *et al.*, 1995; Delwiche and Palmer, 1996). Careful comparison of genome data will explicate how gene transfer has affected eukaryotic algal evolution.

Last but not least, fossil and geochemical records provide the timing of chloroplast evolution. The available evidence suggests that the primary endosymbiosis may have occurred prior to 1200 Ma, and the first secondary endosymbiosis no later than 1000 Ma. Continuing search for Proterozoic microfossils and biomarkers will place more precise time constraints on early algal evolution. By combining paleontological data with molecular-phylogenetic analyses, the divergence time of red- and green-algal lineages has been estimated: however, the results vary depending on selection of data and methods used for the analyses. Comparison of paleontological records and molecular clock analyses would refine the method for evolutionary time estimates.

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