J Mol Evol (1995) 41:203–210

JOURNAL OF MOLECULAR EVOLUTION

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Analyses of Ribosomal RNA Sequences from Glaucocystophyte Cyanelles Provide New Insights into the Evolutionary Relationships of Plastids

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Received: 30 June 1994 / Accepted: 14 October 1994

Abstract. Glaucocystophyte algae (sensu Kies, *Berl. Deutsch. Bot. Ges.* 92, 1979) contain plastids (cyanelles) that retain the peptidoglycan wall of the putative cyanobacterial endosymbiont; this and other ultrastructural characters (e.g., unstacked thylakoids, phycobilisomes) have suggested that cyanelles are "primitive" plastids that may represent undeveloped associations between heterotrophic "host" cells (i.e., glaucocystophytes) and cyanobacteria. To test the monophyly of glaucocystophyte cyanelles and to determine their evolutionary relationship to other plastids, complete 16S ribosomal RNA sequences were determined for *Cyanophora paradoxa, Glaucocystis nostochinearum, Glaucosphaera vacuolata,* and *Gloeochaete wittrockiana.* Plastid rRNAs were analyzed with the maximum-likelihood, maximum-parsimony, and neighbor-joining methods. The phylogenetic analyses show that the cyanelles of *C. paradoxa, G. nostochinearum,* and *G. wittrockiana* form a distinct evolutionary lineage; these cyanelles presumably share a monophyletic origin. The rDNA sequence of *G. vacuolata* was positioned within the nongreen plastid lineage. This result is consistent with analyses of nuclear-encoded rRNAs that identify *G. vacuolata* as a rhodophyte and support its removal from the Glaucocystophyta. Results of a global search with the maximum-likelihood method suggest that cyanelles are the first divergence among all plastids; this result is consistent with a single loss of the peptidoglycan wall in plastids after the divergence of the cyanelles. User-defined tree analyses with the maximum-likelihood method indicate, however, that the position of the cyanelles is not stable within the rRNA phylogenies. Both maximum-parsimony and neighbor-joining analyses showed a close evolutionary relationship between cyanelles and nongreen plastids; these phylogenetic methods were sensitive to inclusion/exclusion of the *G. wittrockiana* cyanelle sequence. Base compositional bias within the *G. wittrockiana* 16S rRNA may explain this result. Taken together the phylogenetic analyses are interpreted as supporting a near-simultaneous radiation of cyanelles and green and nongreen plastids; these organelles are all rooted within the cyanobacteria.

Key words: Cyanelles — *Cyanophora paradoxa* — Endosymbiosis — Evolution — Glaucocystophyta — Glaucophyta — Phylogeny — Plastid — 16S ribosomal RNA

Introduction

The number and timing of endosymbiotic events comprise two of the most interesting and controversial problems in plastid evolution. The theory of endosymbiosis as an explanation for the origin of plastids from one or more cyanobacterial ancestors is now widely accepted (Mereschkowsky 1905, 1910; Margulis 1981; Gray 1989). The Glaucocystophyta (Kies 1979; Kies and Kremer 1986; synonym Glaucophyta, Skuja 1954), a group of algae with a cyanobacterial cell inclusion ("cyanelle," Pascher 1929), is particularly interesting in the context of plastid evolution. Because cyanelles resemble cyanobacteria in ultrastructure (e.g., unstacked thyla-

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koids, phycobilisomes, carboxysomes) and have retained a peptidoglycan wall (excluding *Glaucosphaera vacuolata,* Hall and Claus 1963; Schnepf 1966; Kies 1974), it has been suggested that cyanelles represent endosymbiotic prokaryotes that have not yet developed into "true" plastids (Geitler 1923; Pascher 1929). The cyanelles of *Cyanophora paradoxa* and *Glaucocystis nostochinearum* were classified as cyanobacterial taxa (Hall and Claus 1963, 1967) to reflect the recent and likely polyphyletic origin of these organelles. The former view is no longer accepted since analyses of the cyanelle genome size in *Cyanophora paradoxa* show that it is comparable to other plastids (Herdman and Stanier 1977). Analyses of gene content, gene order, and phylogeny (e.g., 16S rRNA, *rpo*C1) of the *C. paradoxa* cyanelle also demonstrate that this organelle is more closely related to extant plastids than to cyanobacteria (Bohnert et al. 1982; Giovannoni et al. 1988; Douglas and Turner 1991; Palenik and Haselkorn 1992).

To probe the origins and evolutionary relationships of glaucocystophyte cyanelles, we sequenced the small-subunit ribosomal RNA (SSU rRNA) from *Cyanophora paradoxa, Glaucocystis nostochinearum, Glaucosphaera vacuolata,* and *Gloeochaete wittrockiana* (Glaucocystophyta, sensu Kies and Kremer 1986, 1990; Cavalier-Smith 1987) and compared these to homologous sequences from diverse green (i.e., green algal/land plant) and nongreen (i.e., cryptophyte, euglenophyte, haptophyte, heterokont, and red algal) plastids. Our phylogenetic analyses show that glaucocystophytes that contain peptidoglycan-bound cyanelles (i.e., *C. paradoxa, G. nostochinearum, G. wittrockiana*) form a monophyletic lineage that diverges nearly simultaneously with green and nongreen plastids. The plastid of *G. vacuolata* is positioned within the nongreen plastid lineage and is distinct from glaucocystophyte cyanelles.

Materials and Methods

Preparation of Nucleic Acids. Cyanophora paradoxa Korsch. (Kies strain, Sammlung von Algenkulturen Göttingen [Schlösser 1984], SAG B 45.84) and *Glaucocystis nostochinearum* Itzigs. (SAG 45.88) were grown in unialgal, axenic cultures and on 1% agar plates, respectively, in light at 15°C using a modified Waris solution (McFadden and Melkonian 1986). *Glaucosphaera vacuolata* Korsch. (SAG B 13.82) cells were used directly from stock cultures (SAG Medium 7, Schlösser 1982). Unialgal cultures of *Gloeochaete wittrockiana* Lagerheim (SAG B 46.84) were grown in light at 15°C in a modified Waris solution (McFadden and Melkonian 1986). Cells were collected by centrifugation (2,000*g*) and disrupted by vortexing in the presence of glass beads (2 min) followed by five freeze/thaw cycles (in liquid nitrogen and in a 65°C waterbath, 10 min). Total nucleic acids were isolated using a modified CTAB protocol (Doyle and Doyle 1990).

PCR Experiments. Plastid SSU rRNAs from *C. paradoxa, G. nostochinearum,* and *G. vacuolata* were amplified using the polymerase chain reaction protocols (PCR, Saiki et al. 1988) with primers complementary to the 5′ and 3′ termini of 16S SSU rRNAs (Huss and Giovannoni 1989). Several attempts to obtain an axenic culture of *G. wittrockiana* failed resulting in the PCR amplification of contaminating eubacterial 16S rRNAs (verified by sequence analysis) from the total nucleic acid extracts. Four cyanelle-specific primers were constructed to amplify this coding region in *G. wittrockiana.* The 16S rRNA sequences from *C. paradoxa, G. nostochinearum,* green and nongreen plastids, cyanobacteria, and eubacteria were used in an alignment to identify sequences unique to the cyanelles that were proximal to the 5′ and 3′ termini of these coding regions. Two primers, complementary to the coding and noncoding strands of cyanelle 16S rRNAs at position 200 (respective to the *E. coli* coding region: e.g., 200 FORWARD: $^{5'}$TAATCTGCCTGG/AA/GGAAGAGC$^{3'}$) and two primers complementary to the coding and noncoding strands at position 838 (respective to the *E. coli* coding region; e.g., 838 FORWARD: $^{5'}$CGTATC-GACCCGTACAGTAC$^{3'}$) were used in combination with both 5′ and 3′ "general" primers to amplify complete coding and noncoding strands of the *G. wittrockiana* cyanelle 16S rRNA. Single-stranded template DNA was prepared for sequencing with biotinylated amplification primers and the Dynabeads M-280 system (Dynal, Hultman et al. 1991). PCR products were sequenced directly (solid-phase sequencing) using the dideoxy sequencing method (Sanger et al. 1977) with either the Sequenase 2.0 sequencing kit (USB) or with a PCR sequencing kit (SequiTherm, Epicentre) with a collection of oligonucleotide primers complementary to conserved regions of 16S rRNAs (Huss and Giovannoni 1989). Complete sequences were determined over both strands except for the 5′ and 3′ regions corresponding to the PCR amplification primers in the 16S rRNA for *C. paradoxa, G. nostochinearum,* and *G. vacuolata.* Only the single-strand sequence was determined for the 5′ 200 nucleotides of the *G. wittrockiana* rRNA sequence. Comparison of the existing *Cyanophora paradoxa* cyanelle 16S rRNA sequence (Pringsheim strain, Giovannoni et al. 1988, Turner personal communication) with the 16S rRNA of the Kies strain of *C. paradoxa* analyzed in this study showed at least seven differences due to substitutions and one indel. The insertion, located between positions 173–187 in the 16S rRNA of the Kies strain, allows the unambiguous differentiation of these two closely related taxa.

Phylogenetic Methods. The plastid SSU rRNA coding regions of *C. paradoxa, G. nostochinearum, G. vacuolata,* and *G. wittrockiana* were manually aligned with rRNA sequences from 18 diverse plastids, four cyanobacteria, one α-purple, one γ-purple, and two β-purple bacteria. From this sequence set, 1,403 unambiguously aligned nucleotide positions were used as input for maximum-likelihood, maximum-parsimony, and distance analyses. For the maximum-likelihood method (fastDNAml, V.1.0, Olsen et al. 1994), the global search option was used with rearrangements of partial trees crossing one branch and rearrangements of the full tree crossing 24 branches; this phylogeny was midpoint rooted between the cyanobacterial and the other eubacterial sequences. From the "best" maximum-likelihood phylogeny, topologies that address alternative hypotheses for the evolutionary relationships of cyanelle 16S rRNAs were created with the RETREE program (Phylip V.3.5c, Felsenstein 1993). The log-likelihoods of all trees were compared using the test of Kishino and Hasegawa (1989) with the DNAML program (Phylip V.3.5c, Felsenstein 1993). Phylogenetic trees were also inferred with the maximum-parsimony method (PAUP V3.1.1, Swofford 1993) using weighted sequence positions (maximum consistency index, over a range from 1 to 1,000, Swofford 1993) and a heuristic search procedure with a branch-swapping algorithm (tree bisection-reconnection) and the neighbor-joining method (Phylip V.3.5c, Felsenstein 1993) using evolutionary distances calculated according to Kimura (1980). The maximum-parsimony and neighbor-joining phylogenies were midpoint rooted between the divergence of the cyanobacteria and the other eubacterial rRNA sequences. Bootstrap analyses (100 replications, Felsenstein 1985) were used to assess the stability of monophyletic groups in the maximum-parsimony and neighbor-joining phylogenies. Taxon addition was jumbled and a tran-

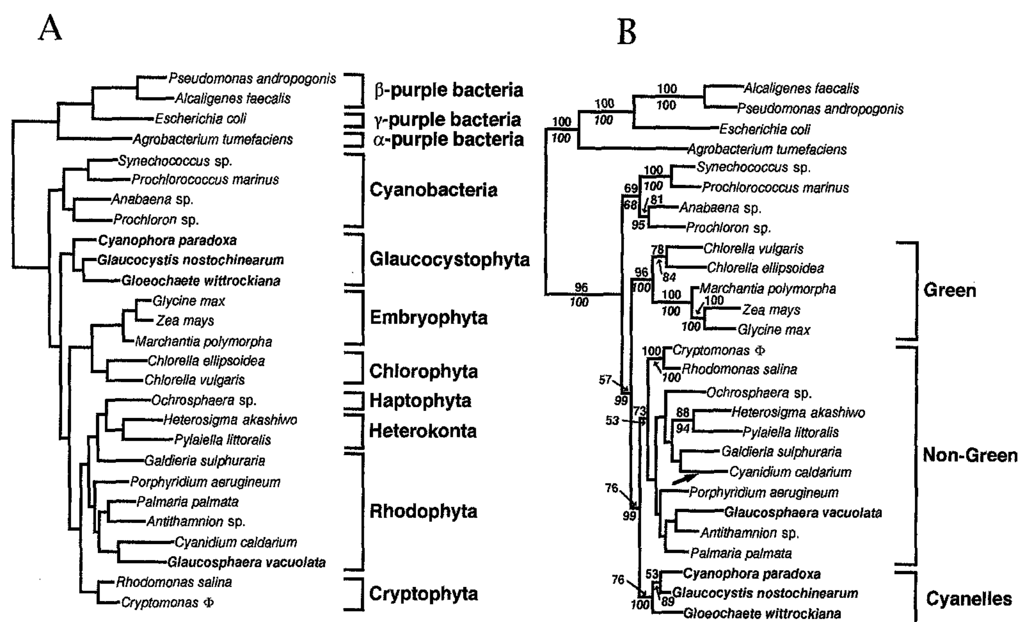

Fig. 1. Phylogenies of 16S rRNA sequences based on the comparison of 1,403 unambiguously aligned nucleotide positions. **A** This phylogeny was inferred with the maximum-likelihood method (fastDNAml, Olsen et al. 1994) using empirically determined base frequencies. The global search option was used with rearrangements of partial trees crossing one branch and rearrangements of the full tree crossing 24 branches. **B** Bootstrap consensus phylogram inferred with the maximum-parsimony method (PAUP V3.1.1, Swofford 1993) using weighted sequence positions and a heuristic search method with a branch-swapping algorithm (TBR, tree bisection-reconnection); this tree has a consistency index = 0.56. Bootstrap values (100 replications, Felsenstein 1985) are shown above the internal nodes in Fig. 1B; only values greater than 50% have been recorded. The bootstrap values shown below the internal nodes in italic text were inferred from a neighbor-joining analysis (Saitou and Nei 1987) using evolutionary distances calculated according to Kimura (1980). The maximum-likelihood and neighbor-joining phylogenetic methods used a jumbled species input and a transition/transversion ratio = 2 and were midpoint rooted on the branch joining the cyanobacteria with the other eubacteria. The *arrow* on the *Cyanidium caldarium* branch marks the divergence point of the euglenophyte 16S rRNAs when these are included in the maximum parsimony analysis. The neighbor-joining analysis (not shown) positioned these rRNAs in the identical position.

sition/transversion ratio = 2 was used in the maximum-likelihood and neighbor-joining methods.

Plastid SSU rRNA sequences used in this study (with EMBL/Genbank numbers where available) are as follows: *Agrobacterium tumefaciens* (M11223), *Alcaligenes faecalis* (M22508), *Anabaena* sp. (X59559), *Antithamnion* sp. (X54299), *Astasia longa* (X14386), *Chlorella ellipsoidea* (X12742), *Chlorella vulgaris* (X16579), *Cryptomonas* ϕ (X56806), *Cyanidium caldarium* (Giovannoni unpubl.), *Epifagus virginiana* (X62099), *Escherichia coli* (M24836), *Euglena gracilis* (X12890), *Galdieria sulphuraria* (X52985), *Glycine max* (X06428), *Marchantia polymorpha* (X04465), *Ochrosphaera* sp. (X65101), *Olisthodiscus luteus* (=*Heterosigma akashiwo*, M82860), *Palmaria palmata* (Z18289), *Porphyridium aerugineum* (Maid and Zetsche unpubl.), *Prochlorococcus marinus* (X63140), *Prochloron* sp. (X63141), *Pseudomonas andropogonis* (X67037), *Pylaiella littoralis* (X14873), *Pyrenomonas salina* (=*Rhodomonas salina*, X55015), *Synechococcus* sp. (=*Anacystis nidulans*, X03538) and *Zea mays* (X01365).

Results

Plastid Phylogeny

A phylogeny deduced from the maximum-likelihood analysis of 16S rRNA coding regions from 27 species is shown in Fig. 1A. The cyanelles of the Glaucocystophyta *C. paradoxa, G. nostochinearum,* and *G. wittrockiana* form a monophyletic group that is positioned at the base of all plastid lineages. In the maximum-parsimony and neighbor-joining analyses (Fig. 1B), the cyanelles form a monophyletic group with high bootstrap support (76%, 100%, respectively) that diverges at the base of the non-green plastids (also with strong bootstrap support, 76%, 99%, respectively). The *G. vacuolata* sequence is positioned within the non-green plastid lineage as a sister group to *Cyanidium caldarium* in the maximum-likelihood analysis and as a sister group to *Antithamnion* sp. in the maximum-parsimony analysis (Fig. 1A,B); in the neighbor-joining tree, *G. vacuolata* is positioned as a sister group to a red algal cluster defined by *Antithamnion* sp., *Palmaria palmata,* and *Porphyridium aerugineum* (not shown).

User-Defined Tree Analyses

To test the phylogeny shown in Fig. 1A (tree 1), user-defined trees were created that position the cyanelle sequences of *C. paradoxa, G. nostochinearum,* and *G. wittrockiana* in two alternative branching patterns: (1) at the base of the green plastid lineage (tree 2) and (2) at the base of the nongreen plastid lineage (tree 3). To test the position of *G. vacuolata,* this sequence was positioned as an early divergence from the cyanelle lineage (tree 4).

Table 1. Comparison of log-likelihoods (Kishino and Hasegawa 1989) of "best" tree with user-defined trees in maximum-likelihood analyses (Phylip V.3.5c, Felsenstein 1993)

	Log-likelihood	Difference in log-likelihoods	Standard deviation	Significantly worse?
Tree 1				
Best	−14,571.58	—	—	—
Tree 2				
Base of greens	−14,582.52	−10.94	8.01	No
Tree 3				
Base of Nongreens	−14,572.40	−0.082	10.48	No
Tree 4				
Glaucosphaera with cyanelles	−14,658.07	−86.49	24.61	Yes

These user-defined trees were used to "constrain" the maximum-likelihood method. Results of these analyses demonstrate that there are no significant differences between the "best" tree shown in Fig. 1A (tree 1) and the two alternative topologies that address the divergence point of the cyanelles (Table 1). With the maximum-likelihood method, the divergence of the cyanelles and green and nongreen plastids may be interpreted as a near-simultaneous radiation of these lineages with an early divergence of the cyanelles as the favored topology. Creation of a monophyletic group of the cyanelle and the *G. vacuolata* plastid sequences produced a significantly "worse" topology in the maximum-likelihood analysis (Table 1).

Effects of Species Exclusion in the Maximum Parsimony and Neighbor-Joining Analyses

The robustness of the maximum-parsimony and neighbor-joining phylogenies (Fig. 1B), which favored a close evolutionary relationship between cyanelles and nongreen plastids, was tested with exclusion of particular cyanelle 16S rRNA coding regions in bootstrap analyses. Species exclusion had profound effects on the maximum-parsimony and neighbor-joining methods (Table 2). *G. wittrockiana* alone diverged with high bootstrap support within the nongreen plastid lineage. In contrast, *C. paradoxa* or *G. nostochinearum* alone was positioned at the base of all plastids in the maximum-parsimony analysis and at the base of the nongreen plastids in the neighbor-joining analysis. These and other results, summarized in Table 2, show that the maximum-parsimony method was most affected by the inclusion/exclusion of the *G. wittrockiana* sequence in the analyses, whereas the neighbor-joining method consistently positioned the cyanelles at the base of the nongreen plastid lineage (except when *G. wittrockiana* alone was used in the analysis). The effect of the *G. wittrockiana* rRNA sequence in both maximum-parsimony and neighbor-joining analyses was to "pull" the cyanelles within the nongreen plastid lineage.

Discussion

Comparison of Nuclear and Plastid SSU rRNA Phylogenies and the Origin of Cyanelles

The evolutionary relationships of the glaucocystophytes (sensu Kies 1979; Kies and Kremer 1986) and their cyanelles have long been in question. *Cyanophora paradoxa,* for example, has sometimes been included in the Cryptophyta (Bourrelly 1970; Gillott 1989) and *G. vacuolata* in the Rhodophyta (McCracken et al. 1980). Analyses of nuclear-encoded SSU rRNA show that *C. paradoxa, G. nostochinearum,* and *G. wittrockiana* are a closely related monophyletic group that putatively forms a sister group to cryptophyte algae (Bhattacharya et al. 1995b). This former result corroborates the taxonomy of Kies (1979) and is supported by the presence of several shared characters in these taxa (e.g., presence of flattened cortical vesicles [lacunae] under the plasma membrane, cruciate flagellar roots with associated multilayered structures [MLS], and a cyanelle bound by a peptidoglycan wall). In support of McCracken et al. (1980), the nuclear rRNA phylogenies position *G. vacuolata* with the red algae. The plastid of *G. vacuolata* does not have a bounding peptidoglycan wall and contains R-phycocyanin whereas cyanelles contain C-phycocyanin (McCracken et al. 1980). Further, McCracken et al. (1980) described only one deeply lobed rhodoplast without a pyrenoid instead of several lens-shaped cyanelles (Korshikov 1930) in *G. vacuolata.*

The plastid SSU rRNA phylogenies are consistent with a monophyletic origin of the cyanelle in the common ancestor of the glaucocystophytes, *C. paradoxa, G. nostochinearum,* and *G. wittrockiana.* Variation in cyanelle size and shape (Kies 1992) and ultrastructure (e.g., carboxysome shape, Hall and Claus 1967) within these taxa is not an indication of a polyphyletic origin. The positioning of the *G. vacuolata* 16S rRNA sequence within the nongreen plastid lineage as a sister group to the red algae is also consistent with the "host" cell phylogenies (Bhattacharya et al. 1995b).

Table 2. Bootstrap support for the positioning of glaucosystophyte cyanelle(s) sequences within the 16S rRNA phylogeny using the maximum-parsimony and neighbor-joining methods[a]

	Maximum-parsimony							Neighbor-joining						
	Cp	Gn	Gw	Cp Gn	Cp Gw	Gn Gw	Cp Gn Gw	Cp	Gn	Gw	Cp Gn	Cp Gw	Gn Gw	Cp Gn Gw
Base of all plastids	72	47			79_{Cp}									
Base of greens														
Base of non-greens				67		77	76	76	84		85	98	99	99
Within non-greens			94		75_{Gw}					100				

[a] The maximum-parsimony method was inferred from a heuristic search and a branch-swapping algorithm (TBR, tree bisection-reconnection, PAUP, V3.1.1, Swofford 1993), and the neighbor-joining method (Saitou and Nei 1987) was implemented with evolutionary distances calculated according to Kimura (1980). Species shown are Cp (*Cyanophora paradoxa*), Gn (*Glaucocystis nostochinearum*), and Gw (*Gloeochaete wittrockiana*)

The Evolution of Plastids

Phylogeny of Euglenophyte Plastids

Phylogenetic analyses of the 16S rRNAs of the Euglenophyta have generally positioned this group within the nongreen plastid lineage (Fig. 1B, Douglas and Turner 1991; Giovannoni et al. 1993) whereas protein phylogenies position the chlorophyll-*a*+*b*-containing euglenophytes with green plastids (Morden et al. 1992). Lockhart et al. (1992, 1994) and Turner (pers. comm.) have suggested that euglenophyte plastids are positioned within the nongreen plastid lineage in 16S rRNA phylogenies due to base compositional bias (i.e., the position of the euglenophyte rRNAs reflects their base composition and not their evolutionary history). To address this issue we calculated the frequencies of each base in the rRNA sequences used in our phylogenetic analyses (Table 3). These data clearly demonstrate that the euglenophyte 16S rRNAs have an elevated adenosine (A) composition (similar to that in nongreen plastids) and a lower G+C content relative to that found in green plastid rRNAs. Their positioning within the nongreen plastid lineage may, therefore, reflect shared sequence biases. Reanalysis of our data with the LogDet transformation method (Lockhart et al. 1994), which is a more robust analysis for finding the correct tree under cases of differing nucleotide composition, supports this hypothesis. Usage of only parsimony sites (627) of the data set shown in Fig. 1, plus the sequences of *Euglena gracilis* and *Astasia longa,* and the LogDet matrix analysis (SplitsTree V1.0, Huson and Wetzel 1994) with the neighbor-joining phylogenetic reconstruction method, shows that the euglenophyte 16S rRNA sequences form an early divergence within the green plastid lineage (tree not shown but available from authors). Other evolutionary relationships within the LogDet distance phylogeny remained essentially unchanged from that shown in Fig. 1B except for minor rearrangements in the evolutionary positions of the cryptophyte and haptophyte sequences.

Application of the LogDet method, using only parsimony sites, to address the position of the cyanelle sequences also provided some insights into their evolutionary relationships. With the full data set (including the Euglenophyta) using a LogDet matrix as input for a neighbor-joining analysis, the cyanelles were positioned as a weak sister group to the nongreen plastids. Exclusion of the *G. wittrockiana* 16S rRNA sequence, which has a high A content and low G+C content (similar to nongreen plastids) relative to the other cyanelle sequences (Table 3), resulted in the positioning of the *C. paradoxa* and *G. nostochinearum* cyanelle sequences at the base of all plastids. These results point out the strong effect that base composition can have on the evolutionary relationships of rRNA sequences (Lockhart et al. 1992, 1994) and underline the need to address these problems with either species exclusion or more refined analyses. In this regard, we have confidence that the evolutionary position of the *Glaucosphaera vacuolata* 16S rRNA sequence, as a sister group to the red algae, is not a reflection of the shared elevated A content and low G+C content of these coding regions, relative to the cyanelle sequences of *C. paradoxa* and *G. nostochinearum;* the consistent positioning of the *G. vacuolata* sequence with the red algae in all analyses, including the LogDet method, plus the results of the user-defined maximum-likelihood analysis, supports this result.

Plastid Origins

Our phylogenetic analyses suggest that there are, minimally, three plastid lineages: (1) the green plastids that contain chlorophylls-*a*+*b* (including euglenophytes), (2) the nongreen plastids with the red algae containing chlorophyll-*a* and phycobilisomes, the heterokont and haptophyte algae containing chlorophylls-*a*+*c,* and the cryptophyte algae containing chlorophylls-*a*+*c* and phycobilisomes, and (3) the glaucocystophytes containing chlorophyll-*a* and phycobilisomes (Douglas and Turner 1991; Gibbs 1993).

The positioning of the cyanelles from glaucocystophytes at the base of all plastids or within a near-simultaneous radiation of cyanelle and green and non-green plastids is generally consistent with results of other

Table 3. Base compositions of 16S rRNAs

	Sequence	f(A)	f(C)	f(G)	f(U)	f(AG)	f(GC)	f(AC)
Eugl.	*Euglena gracilis*	0.281	0.191	0.286	0.242	0.57	0.48	0.47
	Astasia longa	0.310	0.161	0.254	0.275	0.56	0.42	0.47
Green	*Chlorella vulgaris*	0.266	0.225	0.309	0.200	0.57	0.53	0.49
	Chlorella ellipsoidea	0.267	0.217	0.305	0.212	0.57	0.52	0.48
	Marchantia polymorpha	0.256	0.237	0.319	0.189	0.57	0.56	0.49
	Zea mays	0.253	0.239	0.323	0.185	0.58	0.56	0.49
	Glycine max	0.253	0.238	0.323	0.185	0.58	0.56	0.49
Non-Green	*Cryptomonas* ϕ	0.279	0.208	0.292	0.220	0.57	0.50	0.49
	Rhodomonas salina	0.274	0.218	0.298	0.210	0.57	0.52	0.49
	Heterosigma akashiwo	0.283	0.202	0.284	0.230	0.57	0.49	0.49
	Pylaiella littoralis	0.289	0.192	0.281	0.238	0.57	0.47	0.48
	Porphyridium aerugineum	0.272	0.212	0.298	0.217	0.57	0.51	0.48
	Ochrosphaera sp.	0.271	0.212	0.296	0.221	0.57	0.51	0.48
	Galdieria sulphuraria	0.281	0.206	0.288	0.224	0.57	0.49	0.49
	Cyanidium caldarium	0.290	0.204	0.275	0.230	0.57	0.48	0.49
	Glaucosphaera vacuolata	0.289	0.200	0.282	0.229	0.57	0.48	0.49
	Palmaria palmata	0.272	0.226	0.299	0.203	0.57	0.52	0.50
	Antithamnion sp.	0.286	0.203	0.281	0.230	0.57	0.48	0.49
Cyanel.	*Cyanophora paradoxa*	0.262	0.221	0.310	0.207	0.57	0.53	0.48
	Glaucocysis nostochinearum	0.265	0.215	0.313	0.207	0.58	0.53	0.48
	Gloeochaete wittrockiana	0.278	0.208	0.297	0.218	0.57	0.50	0.49
	Synechococcus sp.	0.250	0.236	0.323	0.192	0.57	0.56	0.49
	Anabaena sp.	0.263	0.226	0.317	0.194	0.58	0.54	0.49
	Prochlorochoccus marinus	0.254	0.233	0.314	0.199	0.57	0.55	0.49
	Prochloron sp.	0.272	0.225	0.315	0.189	0.59	0.54	0.50
	Agrobacterium tumefaciens	0.244	0.233	0.323	0.200	0.57	0.56	0.48
	Escherichia coli	0.256	0.229	0.318	0.197	0.57	0.55	0.48
	Pseudomonas andropogonis	0.255	0.232	0.318	0.195	0.57	0.55	0.49
	Alcaligenes faecalis	0.255	0.236	0.308	0.201	0.56	0.54	0.49

16S rRNA and plastid protein phylogenetic analyses. Usage of partial sequences, different outgroups and species sets, and different phylogenetic methods renders impossible, however, a direct comparison of these data to our phylogenies. A partial (1,118 nt) 16S rRNA sequence analysis using a least-squares method with the Jukes-Cantor (1969) correction positioned *C. paradoxa,* with moderate bootstrap support (75.5%), as a sister group to nongreen plastids with eubacteria as the outgroup (Douglas and Turner 1991). Giovannoni et al. (1993), using a similar data set (1,184 nt) and the maximum-parsimony method with *Anabaena* sp. as the outgroup, positioned the *C. paradoxa* 16S rRNA sequence at the base of all plastids. Bootstrapped maximum-parsimony analyses of *atp*B amino acid sequences positioned *C. paradoxa,* with weak bootstrap support (45%), as a sister group to cryptophytes (Douglas and Murphy 1994). Maximum-parsimony analysis of *psb*A amino acid sequences positioned *C. paradoxa* at the base of all plastids whereas parsimony analysis of *tuf*A sequences positioned *C. paradoxa* at the base of the green plastid lineage; this latter result has, however, no bootstrap support (9%, Morden et al. 1992).

In contrast to the above data that are consistent with a monophyletic origin of all plastids from a cyanobacterial ancestor, analyses of large- and small-subunit ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L and *rbc*S) coding regions support a polyphyletic origin of these organelles (Douglas et al. 1990; Valentin and Zetsche 1990; Morden et al. 1992). In the *rbc*L phylogenies, euglenophyte, green algal/land plant, and the *C. paradoxa* cyanelle sequences share a monophyletic origin that is rooted within the cyanobacteria whereas the cryptophyte, heterokont, and red algal plastids are rooted within proteobacteria (Martin et al. 1992; Morden et al. 1992). Though initially interpreted as proof for a polyphyletic origin of these plastids (Douglas et al. 1990; Morden and Golden 1991), these data are now more commonly believed to demonstrate a lateral transfer of the RUBISCO operon from a proteobacterial source into the common ancestor of the red algae that may have given rise, via secondary endosymbiosis(es), to cryptophyte and heterokont plastids (Gibbs 1981, 1993; Leipe et al. 1994; McFadden et al. 1994), or, more likely, to the "differential retention of two RUBISCO operons present in the cyanobacterial ancestor of plastids" (Palmer 1993).

Assuming that plastids with two envelope membranes

(cyanelles, chloroplasts, rhodoplasts) originated from the single primary endosymbiosis of a cyanobacterium (Cavalier-Smith 1987), the close evolutionary relationship between red algal and all other nongreen plastids may be explained by secondary endosymbiosis(es) of the ancestor of the red algae into the "host" cells of taxa now containing nongreen plastids (Gibbs 1981, 1993; Whatley 1993). The existence of additional envelope membranes in cryptophytes, haptophytes, and heterokonts (four) supports this scenario (Gibbs 1993; Whatley 1993) as does the positioning of the cryptophyte nucleomorph (vestigial nucleus of the endosymbiont, Greenwood et al. 1977) as a sister group to red algae in nuclear-encoded SSU rRNA phylogenies (Douglas et al. 1991; Bhattacharya et al. 1995b). The number of secondary endosymbioses in plastid evolution is not known though it is likely greater than one due (in addition to the origin of the cryptophyte plastid) to the positioning of the euglenophyte host cell as a relatively early divergence in the nuclear-encoded SSU rRNA phylogenies (Sogin et al. 1986) independent of the green algae, whereas its plastid is positioned as a sister group to chloroplasts. The origin of the plastids in cryptophytes, heterokonts, and chlorarachniophytes may also be independent of each other since these host cells do not share a monophyletic origin in the SSU rRNA phylogenies (Bhattacharya et al. 1995a,b). For the opposing view that plastids with four-envelope membranes share a monophyletic origin, see Cavalier-Smith (1993).

In conclusion, our 16S rRNA evolutionary analyses demonstrate that rather than being cyanobacteriallike cell inclusions, cyanelles are "mature" plastids that share a monophyletic origin and are part of a near-simultaneous radiation with green and nongreen plastids. If the maximum-likelihood phylogeny most accurately reflects the interrelationships of 16S rRNAs, then the peptidoglycan wall surrounding the cyanelle was lost once in plastid evolution (Palmer 1993). Though this hypothesis is most parsimonious, the rRNA analyses do not exclude the possibility that the peptidoglycan wall may have been lost twice in the common ancestor of the green and nongreen plastids as suggested by the maximum-parsimony and neighbor-joining analyses.

Acknowledgments. This research was supported by a grant from the Deutsche Forschungsgemeinschaft (ME 658/11-2) to M.M. and a research award from the Alexander von Humboldt Foundation to D.B. The authors wish to thank U. Powalowski (Köln) for expert cultivation of the glaucocystophytes, Drs. Giovannoni, V.A.R. Huss, U. Maid, and Prof. K. Zetsche for kindly providing access to unpublished sequences, and G. Wahl (Köln) for computational help.

Note Added in Proof

The 16S rRNA nucleotide sequences of *Cyanophora paradoxa, Glaucocystis nostochinearum, Glaucosphaera vacuolata,* and *Gloeochaete wittrockiana* will appear in the EMBL, Genbank, and DDBJ Nucleotide Sequence Databases under the following accession numbers—X81840, X82496, X81903 and X82495, respectively.

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