

# Phylogeny of Nuclear-Encoded Plastid-Targeted Proteins Supports an Early Divergence of Glaucophytes within Plantae

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The phylogenetic position of the glaucophyte algae within the eukaryotic supergroup Plantae remains to be unambiguously established. Here, we assembled a multigene data set of conserved nuclear-encoded plastid-targeted proteins of cyanobacterial origin (i.e., through primary endosymbiotic gene transfer) from glaucophyte, red, and green (including land plants) algae to infer the branching order within this supergroup. We find strong support for the early divergence of glaucophytes within the Plantae, corroborating 2 important putatively ancestral characters shared by glaucophyte plastids and the cyanobacterial endosymbiont that gave rise to this organelle: the presence of a peptidoglycan deposition between the 2 organelle membranes and carboxysomes. Both these traits were apparently lost in the common ancestor of red and green algae after the divergence of glaucophytes.

## Introduction

Eukaryotic photosynthesis traces its origin to an ancient (e.g., Yoon et al. 2004), putative single primary endosymbiotic event in which a phagotrophic protist (the host), engulfed and retained permanently an oxygenic photosynthetic cyanobacterium (the endosymbiont; Cavalier-Smith and Lee 1985; Bhattacharya et al. 2004). The captured cyanobacterium evolved into a 2-membrane-bound photosynthetic organelle. The result of the endosymbiosis was the emergence of the first algae that ultimately gave rise to the green (including land plants), red, and glaucophyte algae. These lineages form the putative supergroup Plantae (or Archaeplastida; Cavalier-Smith 1998; Adl et al. 2005) and are considered to be monophyletic based on nuclear and plastid phylogenies (e.g., Rodriguez-Ezpeleta et al. 2005; Hackett et al. 2007 [but see Nozaki et al. 2007; Stiller 2007 and discussion in Rodriguez-Ezpeleta et al. 2007]), shared components of the plastid protein import system (McFadden and van Dooren 2004), common gene replacements for plastid pathways (e.g., Fast et al. 2001; Reyes-Prieto and Bhattacharya 2007), and the presence of a family of genes involved in plastid solute transport that putatively originated in the Plantae ancestor (Weber et al. 2006; Tyra HM, Linka M, Weber APM, Bhattacharya D, unpublished data).

The biflagellate *Cyanophora paradoxa* is the best-studied glaucophyte, particularly with respect to the plastid import machinery (Steiner and Löffelhardt 2005), the cyanelle peptidoglycan layer (CPL) (Panzagl et al. 1996), and other aspects of plastid function (e.g., Gross et al. 1994; Nickol et al. 2000). To establish branching order within Plantae, we generated a 19-protein tree of nuclear-encoded plastid-targeted proteins using complete genome and expressed sequence tag data (see table 1 and Methods in Supplementary Material online) that included *C. paradoxa* and another glaucophyte *Glaucocystis nostochinearum*. Based on the abundant evidence cited above, we assumed that the Plantae form a monophyletic group that shares not only the plastid of cyanobacterial derivation but also the set of plastid-targeted proteins that reside in their nucleus and

have originated through endosymbiotic gene transfer from the prokaryote (see Martin et al. 2002; Reyes-Prieto et al. 2006). Under this scenario, the phylogeny of the ancestrally shared nuclear-encoded plastid-targeted proteins recapitulates the host tree.

The maximum likelihood (RAxML) 19-protein phylogeny (fig. 1A) supports the expected separation of the Plantae (RAxML bootstrap support, RBS = 100%; PHYML bootstrap support, PBS = 100%; Bayesian posterior probability, BPP = 1.0) from the cyanobacteria in our data set as well as the monophyly of each of its constituent groups. The red and green algae are united with high bootstrap and Bayesian support (RBS = 97%, PBS = 100%, BPP = 1.0) identifying the glaucophytes as the earliest diverging Plantae. This branching order is consistent with some analyses of plastid (e.g., Martin et al. 1998; Yoon et al. 2004) and nuclear (Hackett et al. 2007) genes in Plantae but conflicts with the basal position of red algae found with a recent analysis of nuclear genes (Rodriguez-Ezpeleta et al. 2005; albeit with low maximum likelihood bootstrap support = 64%, see also Rodriguez-Ezpeleta et al. 2007). To test our result, we used the approximately unbiased (AU) test to assess the likelihoods of all alternative positions of glaucophytes in the tree shown in figure 1A. This analysis shows that the early divergence of glaucophytes has the highest probability among these set of alternative rearrangements ( $P = 0.99$ ). The other 2 key alternative positions as sister to red or green algae were rejected by the AU test ( $P = 0.012$  and  $P = 0.0002$ , respectively; see table 2). All other alternative topologies (see Methods, Supplementary Material online) were rejected at  $P < 0.05$ . To further test these results, we removed the class of fastest evolving amino acid sites from the multiprotein alignment and reran the phylogenetic analyses and the AU test. This operation resulted in a RAxML tree that was identical to figure 1A (see fig. 1B) and now more strongly supported the early divergence of glaucophytes within Plantae (RBS, PBS = 100%; BPP = 1.0). Consistent with this result, the AU test now provided significant support for the “glaucophytes first” hypothesis ( $P = 0.997$ ) and rejected the alternative positions of this clade as sister to red and green algae at a higher significance level ( $P = 0.005$  and  $P = 0.006$ , respectively) as well as all other alternative positions in the tree (see table 2).

Why then do other analyses with larger nuclear protein data sets have difficulties in resolving the branching order within Plantae (Rodriguez-Ezpeleta et al. 2005, 2007)?

Key words: *Cyanophora paradoxa*, glaucophytes, endosymbiosis, Plantae, plastid-targeted proteins.

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**Table 1**  
**The 16 Taxa and the 19 Nuclear-Encoded Plastid-Targeted Proteins of Cyanobacterial Origin that Were Analyzed in This Study**

Taxa	Proteins																			Amino acids	% Missing	
	TL	pO	AD	pH	FH	pC	ID	GH	PG	RP	PK	AC	PS	UD	RF	uG	uA	nL	pP			
<i>Cyanophora</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4557	11.3
<i>Glaucocystis</i>	●	●	○	●	○	●	○	○	●	●	●	●	○	○	○	○	○	○	○	○	1396	72.8
<i>Arabidopsis</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	5100	0.8
<i>Chlamydomonas</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4795	6.7
<i>Ostreococcus</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4674	9
<i>Oryza</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	5083	1.1
<i>Cyanidioschyzon</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	5037	1.9
<i>Galdieria</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4996	2.8
<i>Porphyra</i>	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	2469	51.9
<i>Chondrus</i>	●	●	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	1640	68.1
<i>Anabaena</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	5050	1.7
<i>Crocospaera</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4953	3.6
<i>Lyngbya</i>	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	5051	1.7
<i>Synechocystis</i>	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	4737	7.8
<i>Thermosynechococcus</i>	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	4600	10.5
<i>Trichodesmium</i>	●	●	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	5051	1.7
Recent gene duplication		L	L	G		G, C	L	G	L	R	L	L	G	G					G			

NOTE.—TL, transketolase; pO, oxygen-evolving enhancer protein 1; AD, delta-aminolevulinic acid dehydratase; pH, ferredoxin–NADP reductase; FH, coenzyme F420 hydrogenase/dehydrogenase, beta subunit; pC, cytochrome b6-f complex iron-sulfur (Rieske) protein; ID, imidazoleglycerol-phosphate dehydratase; GH, glyceraldehyde 3-phosphate dehydrogenase; PG, phosphoglycerate kinase; RP, ribulose-phosphate 3-epimerase; PK, phosphoribulokinase; AC, ATP synthase gamma subunit; PS, phytoene desaturase; UD, uroporphyrinogen decarboxylase; RF, peptide chain release factor 1; uG, magnesium protoporphyrin IX methyltransferase; uA, chlorophyll *a* synthase; nL, nitrogen-fixing NifU-like protein; pP, photosystem II oxygen-evolving complex protein. Filled circles denote complete or partial protein sequence. Open circles indicate missing data. L, land plants. G, land plants and *Ostreococcus*. C, *Cyanophora*. R, *Cyanidioschyzon*. The amount, presence/absence, and proportion of missing amino acid data are indicated.

A limited taxon sampling is likely a major issue here. A recent in-depth analysis suggests that the position of *Cyanidioschyzon merolae* (Cyanidiales), which is often used to represent the red algae in eukaryotic host trees based on genome data is strongly affected by long-branch attraction (LBA) artifacts (Rodriguez-Ezpeleta et al. 2007). The attraction of the red algae to other long branches in the eukaryotic tree results in their position either at the base of the Plantae or outside of this supergroup altogether (see Nozaki et al. 2007; Rodriguez-Ezpeleta et al. 2007). This is not sur-

prising because Cyanidiales are highly specialized extremophilic algae that live in hot acidic springs and fumaroles and have undergone extensive genome reduction (Barbier et al. 2005). Although not formally tested, our data set of 19 nuclear-encoded plastid-targeted proteins appears to not suffer from LBA (i.e., compare the relative branch lengths among Plantae in fig. 1B) and provides robust bootstrap and AU test support for the glaucophytes first hypothesis (table 2).

Phylogenetic analyses in which we excluded *C. merolae* (supplementary figs. S1A and B, Supplementary

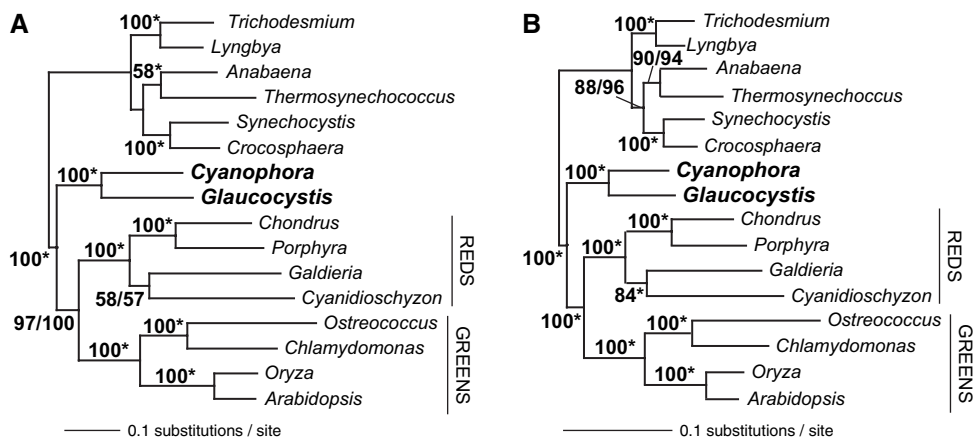


FIG. 1.—Maximum likelihood (RAxML) tree of a concatenated data set of 19 nuclear-encoded plastid-targeted proteins in Plantae. The results of bootstrap analyses using RAxML are shown at the branches with the PHYML bootstrap values to the right only when they differ in value. The results of a Bayesian inference are shown as asterisks associated with the bootstrap values only when the node has a posterior probability of 1.0. The branch lengths in this tree are proportional to the number of substitutions per site (see scale in figure). (A) Tree inferred from the entire amino acid data set. (B) Tree inferred after removal of the class of fastest evolving amino acid sites. The branch leading to the cyanobacteria was used to root these trees. The glaucophytes are shown in larger bold text and the red and green algae are indicated as such.

**Table 2**  
**Results of the AU Test Using Different Subsets of the Complete 19-protein Alignment to Assess Alternative Hypotheses for within-Plantae Phylogeny**

Earliest Diverging Group	Rank	ΔlnL	AU Test Probability	Tree Topology
Complete data				
Glaucophytes	1	−42.9	0.99	(Cp,Gn,(((At,Os),(Cr,Ot)),(Cm,Gs),(Pp,Cc))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Red algae	2	42.9	0.012	(Cp,Gn,(((At,Os),(Cr,Ot)),(Cm,Gs),(Pp,Cc))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Green algae	3	54.3	2.00E-04	(Cp,Gn,(((At,Os),(Cr,Ot)),(Av,Te),(Cw,Ss)),(Ls,Tr))),((Cm,Gs),(Pp,Cc)))
"Fast" sites excluded				
Glaucophytes	1	−38.7	0.997	(Cp,Gn,(((At,Os),(Cr,Ot)),(Cm,Gs),(Pp,Cc))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Red algae	2	38.7	0.005	(Cp,Gn,(((At,Os),(Cr,Ot)),(Cm,Gs),(Pp,Cc))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Green algae	3	40.3	0.006	(Cp,Gn,(((At,Os),(Cr,Ot)),(Av,Te),(Cw,Ss)),(Ls,Tr))),((Cm,Gs),(Pp,Cc)))
Only <i>Cyanidioschyzon</i>				
Glaucophytes	1	−31.2	0.98	((Cp,Gn),((At,Os),(Cr,Ot)),Cm),((Av,Te),(Cw,Ss)),(Ls,Tr)))
<i>Cyanidioschyzon</i>	2	31.2	0.032	((Cp,Gn),((At,Os),(Cr,Ot)),Cm),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Green algae	3	35.1	0.02	((Cp,Gn),Cm),((At,Os),(Cr,Ot)),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Cyanidiales excluded				
Glaucophytes	1	−30.3	0.992	((Cp,Gn),((At,Os),(Cr,Ot)),(Pp,Cc)),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Red algae	2	30.3	0.016	((Cp,Gn),((At,Os),(Cr,Ot)),(Pp,Cc)),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Green algae	3	32.3	0.009	((Cp,Gn),(Pp,Cc)),((At,Os),(Cr,Ot)),((Av,Te),(Cw,Ss)),(Ls,Tr)))
>50% data cutoff				
<i>Cyanophora</i>	1	−40.1	0.989	((Cp,((At,Os),(Cr,Ot)),(Cm,Gs))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Cyanidiales	2	40.1	0.012	((Cp,((At,Os),(Cr,Ot)),(Cm,Gs))),((Av,Te),(Cw,Ss)),(Ls,Tr)))
Green algae	3	55.8	6.00E-05	((Cp,(Cm,Gs)),((At,Os),(Cr,Ot))),((Av,Te),(Cw,Ss)),(Ls,Tr)))

NOTE.—Cp, *Cyanophora paradoxa*; Gn, *Glaucozystis nostochinearum*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Cr, *Chlamydomonas reinhardtii*; Ot, *Ostreococcus tauri*; Cm, *Cyanidioschyzon merolae*; Pp, *Porphyra purpurea*; Cc, *Chondrus crispus*; Gs, *Galdieria sulphuraria*; Av, *Anabaena variabilis*; Cw, *Crocospheera watsonii*; Ls, *Lyngbya* sp.; Ss, *Synechocystis* sp.; Te, *Thermosynechococcus elongatus*; Tr, *Trichodesmium erythraeum*.

Material online) or both Cyanidiales (supplementary figs. S1C and D, Supplementary Material online) from the data set also produced trees with high support for the early branching of glaucophytes (RBS, PBS > 95%; BPP = 1.0). Other alternative positions for glaucophytes using these data sets were rejected by the AU test at  $P < 0.05$  (table 2). Furthermore, phylogenetic analyses that included only those taxa with >50% sequence data available (see table 1) supported the basal position of glaucophytes (i.e., *Cyanophora*) within the Plantae (RBS, PBS > 95%; BPP = 1.0; table 2 and supplementary figs. S1E and F [Supplementary Material online]). These results suggest that our analyses are not significantly misled by missing data or by the impact of the long-branched Cyanidiales red algae. And finally, because our work made the key assumption of Plantae monophyly, the multigene data analyzed here provided only a few reasonable alternative positions (i.e., table 2) for the glaucophytes with the distantly related cyanobacteria as the outgroup. In contrast, multigene nuclear trees often include many more closely related eukaryotic outgroup taxa and therefore many more nodes where the glaucophyte algae could potentially diverge. This simplifying feature (given our hypothesis of Plantae monophyly holds) may make our data set less prone to stochastic error that is typical of anciently diverged sequences.

The topology shown in figure 1 is consistent with the presence of 2 glaucophyte characters that have long been postulated as ancestral for Plantae plastids (e.g., see Helmchen et al. 1995). The most important of these so-called "primitive" traits is the CPL (Pfnzagl et al. 1996) that is located between the 2 plastid membranes. Biochemical analyses of the CPL in *C. paradoxa* support a comparable role in this alga to its involvement in cyanobacterial fission (Berenguer et al. 1987). It has been suggested that the CPL

may be involved in osmolarity and volume regulation of the cyanelle, similar to the cell wall in cyanobacteria (Raven 2003). The second primitive feature of cyanelles is the presence of carboxysomes ( $\beta$ -carboxysomes). Carboxysomes are present in cyanobacteria (and other bacteria) and are accumulations of RuBisCO and carbonic anhydrase that play a role in carbon concentration. Given our results, presumably both of these key traits were lost from the ancestor of red and green algae after the glaucophyte divergence. Plastid fructose-1,6-bisphosphate aldolase (FBA) in Plantae provides another important piece of evidence in this puzzle. Glaucophytes possess the presumed ancestral (cyanobacterial) FBA type II (Nickol et al. 2000), whereas the red and green algae contain the of type I isozyme (Gross et al. 1999). Plastid FBA type I may be of host (Gross et al. 1999; Rogers and Keeling 2004) or cyanobacterial (endosymbiotic) origin (Reyes-Prieto and Bhattacharya 2007). Whichever the case for the latter gene, the presence of FBA type I in green and red algae suggests the replacement of the plastid FBA type II in the common ancestor of green and red algae after the divergence of glaucophytes (Rogers and Keeling 2004; Reyes-Prieto and Bhattacharya 2007). Other ancestral glaucophyte characters that fail to provide decisive insights into the branching order within Plantae include the presence of phycobilins (phycobilisomes) and nonstacked thylakoidal membranes (also present in some cyanobacteria) that is shared with red algae (Kies and Kremer 1990). The unique presence of chlorophyll *b* in green algal and land plant plastids suggests that the cyanobacterial plastid ancestor likely contained both phycobilins and chlorophyll *b* for light harvesting (Tomitani et al. 1999) and the latter were lost independently from glaucophytes and red algae. In this case, the more parsimonious scenario of a single loss of chlorophyll *b* in a putative glaucophyte–rhodophyte ancestor is inconsistent with our results.

## Supplementary Material

The Methods section and supplementary figures are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org>).

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