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# Molecular Evidence for the Origin of Plastids from a Cyanobacterium-like Ancestor

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**Summary.** The origin of plastids by either a single or multiple endosymbiotic event(s) and the nature of the progenitor(s) of plastids have been the subjects of much controversy. The sequence of the small subunit rRNA (Ssu rRNA) from the plastid of the chlorophyll *c*-containing alga *Cryptomonas* Φ is presented, allowing for the first time a comparison of this molecule from all of the major land plant and algal lineages. Using a distance matrix method, the phylogenetic relationships among representatives of these lineages have been inferred and the results indicate a common origin of plastids from a cyanobacterium-like ancestor. Within the plastid line of descent, there is a deep dichotomy between the chlorophyte/land plant lineage and the rhodophyte/chromophyte lineage, with the cyanelle of *Cyanophora paradoxa* forming the deepest branch in the latter group. Interestingly, *Euglena gracilis* and its colorless relative *Astasia longa* are more related to the chromophytes than to the chlorophytes, raising once again the question of the origin of the euglenoid plastids.

**Key words:** Molecular phylogeny — Plastid — Algae — Plant — Small subunit RNA

## Introduction

Although the endosymbiotic origin of plastids is now widely accepted (Gray 1989), the number of endosymbiotic events necessary to explain the diversity of modern plastid types remains unresolved. Based on differences in accessory light-harvesting pigments exhibited by the three major lineages of photosynthetic eukaryotes (Chlorophyta, Rhodophyta, and Chromophyta), multiple events involving hypothetical prokaryotes containing those distinct pigment complements (chlorophyll *b*, phycobiliproteins, and chlorophyll *c*, respectively) were proposed to give rise to extant plastids (Raven 1970). Subsequent events involving eukaryotic endosymbionts were proposed to give rise to those plastids surrounded by more than two membranes (Gibbs 1978; Whatley et al. 1979). An alternative hypothesis, extended by Cavalier-Smith (1982), suggested that only two endosymbiotic events were necessary—the first between a cyanobacterium and a nonphotosynthetic phagotrophic eukaryote, and the second between the resulting photosynthetic eukaryote and a different nonphotosynthetic phagotroph. In this latter hypothesis, the diversity of plastid types is proposed to have arisen by modification of the original endosymbiont as it was converted into the different types of plastids found in the three main lineages. All plastids surrounded by only two membranes arose from the first association and those surrounded by three or four membranes from the second association.

The discovery of the prochlorophyte *Prochloron didemni*, a prokaryote containing both chlorophylls *a* and *b* (Lewin 1975), led to the proposal that it could be related to the putative ancestor of the plastids of the chlorophyll *a*/*b*-containing plants. Similarly, it was postulated that the brownish photoheterotroph *Heliobacterium chlorum* could be a representative of the lineage that gave rise to the putative ancestor of the chlorophyll *a*/*c*-containing algal plastids (Margulis and Obar 1985). However, these hypotheses are gainsayed by the results of molecular phylogenetic analyses. Inspection of small

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subunit rRNAs (Ssu rRNAs) showed *H. chlorum* to be a member of the Firmicutes (Gram-positive bacteria sensu Woese) (Woese et al. 1985; Woese 1987) and to bear no close relationship with chrysophyte plastids (Witt and Stackebrandt 1988).

Similar studies indicated that euglenoid and chlorophyte/land plant plastids as well as the cyanelle of *Cyanophora paradoxa* fell within the cyanobacterial line of descent (Giovannoni et al. 1988; Turner et al. 1989). Although phylogenetic studies using *psbA* gene-encoded protein sequences yielded conflicting results between themselves concerning the relationship of prochlorophytes to the plastids of chlorophyll *a/b*-containing eukaryotes (Morden and Golden 1989a,b; Kishino et al. 1990), Ssu rRNA comparisons have shown that prochlorophytes, while falling within the cyanobacterial phylum, are unlikely to be closely related to these organelles (Seewaldt and Stackebrandt 1982; Turner et al. 1989). However, phylogenetic reconstruction using *rbcL* (Douglas et al. 1990; Morden and Golden 1991) and *rbcS* (Morden and Golden 1991) gene-encoded protein sequences indicate a polyphyletic origin for some plastids from the beta and gamma groups of Proteobacteria (purple bacteria sensu Woese), which bear no close relation to the cyanobacteria, based on Ssu rRNA analysis (Woese 1987).

Analyses of Ssu rRNA sequences of plastids from representatives of the various photosynthetic eukaryotic lineages may allow resolution of this controversy. Small subunit rRNA sequences are good molecules for phylogenetic inference as they are present in all organisms, a large database exists, they contain a large number of nucleotide positions for comparison, and sequence alignment is relatively straightforward (Gray et al. 1984). Here we report the sequence of the Ssu rRNA of the *Cryptomonas* Φ plastid, infer phylogenetic trees using this sequence and those of other algal groups and cyanobacteria, and discuss the results in relation to the monophyletic/polyphyletic origin of modern plastids and the possible number of endosymbiotic events giving rise to them.

## Materials and Methods

*Sequence Determination.* Plastid DNA preparation, construction of a clone bank from *Cryptomonas* Φ, and localization of the rRNA genes on a small (5.5-kb) inverted repeat have been described (Douglas 1988). The location of one of the rRNA cistrons on a 15.5-kb *Bam*H I/*Sac* I fragment and the sequence 3′ to the *Sma* I site have been published separately (Douglas and Durnford 1990b). The remaining sequence was obtained from a 1.2-kb *Sac* I/*Sma* I fragment containing most of the 16S rRNA gene, which was subcloned from the 15.5-kb *Bam*H I/*Sac* I fragment, and the adjoining *Sac* I fragment. Both strands were sequenced completely by the dideoxynucleotide chain termination method (Sanger et al. 1977). The 5′ and 3′ termini of the Ssu rRNA gene were tentatively identified by alignment with corresponding regions from published Ssu rRNA sequences.

*Phylogenetic Analysis.* Small ribosomal subunit sequences were aligned by eye and the alignment verified by secondary structure analysis (Gutell et al. 1985). Only positions that are clearly homologous and for which sequence data are available for all organisms (indicated in Fig. 1) were used in the phylogenetic analyses. Accounting for gaps introduced in the alignment process, a total of 862 positions were used in the analysis for the tree presented in Fig. 2 and 1118 for that in Fig. 3.

The least squares, distance matrix program of Olsen was used to infer phylogenetic trees (Olsen 1988). Briefly, similarity values for each pair of sequences were calculated by the formula $S = M/[M + U + (G/2)]$, where S is similarity, M is the number of matching nucleotides, U is the number of nonmatching nucleotides, and G is the number of nonmatching gaps introduced in the alignment process. In the analysis presented here, gaps greater than a single nucleotide were treated as a single gap. Similarity values were converted to estimated evolutionary distances using the formula of Jukes and Cantor (1969), and the resulting distances were used to infer a phylogenetic tree by means of a least squares, distance matrix method (Fitch and Margoliash 1967; Olsen 1988). Confidence values for individual branches of the tree were determined by a bootstrap analysis in which 100 or 200 bootstrap trees were generated from resampled data (Felsenstein 1985).

The possibility of an artifactual tree topology due to variable mutation rates between sequences was investigated by the method of Olsen (Olsen 1987). In brief, a site-to-site variability is assumed whereby 95% of the sequence positions vary in mutation rate around a median value in a manner described by a log-normal distribution. The assumed rate variation ranges from a value of alpha to the reciprocal of alpha (1/alpha), the particular value of alpha being an arbitrary positive number. In this analysis, values of alpha employed were 2, 4, 8, 16, 32, 64, and 99 (the last being the limit of the program).

All analyses were run on a Digital Equipment Company MicroVax II computer. The use of computer programs and hardware was provided by N.R. Pace of Indiana University.

## Results and Discussion

The coding sequence of the 16S rRNA gene from *Cryptomonas* Φ is 1492 nucleotides in length (Fig. 1). These data have been deposited with the EMBL Data Library under accession number X56806. The 3′ terminal sequence contains a classical Shine–Dalgarno sequence (5′-CCTCCTT-3′) that is complementary to upstream sequences of several protein coding genes from *Cryptomonas* Φ (Douglas and Durnford 1989, 1990a; Douglas et al. 1990; Reith and Douglas 1990). Two putative promoter elements, both sharing extensive sequence similarity with the canonical −35 (TTGACA) and −10 (TATAAT) sequences (Pribnow 1975), are found upstream of the 5′ end of the 16S rRNA coding sequence (P1, P2; Fig. 1). There is no evidence of a gene for $tRNA^{Val}$, such as is found in the regions upstream of land plant plastid rRNA cistrons. The 16S rRNA molecule can be folded according to the general secondary structure of Gutell et al. (1985)

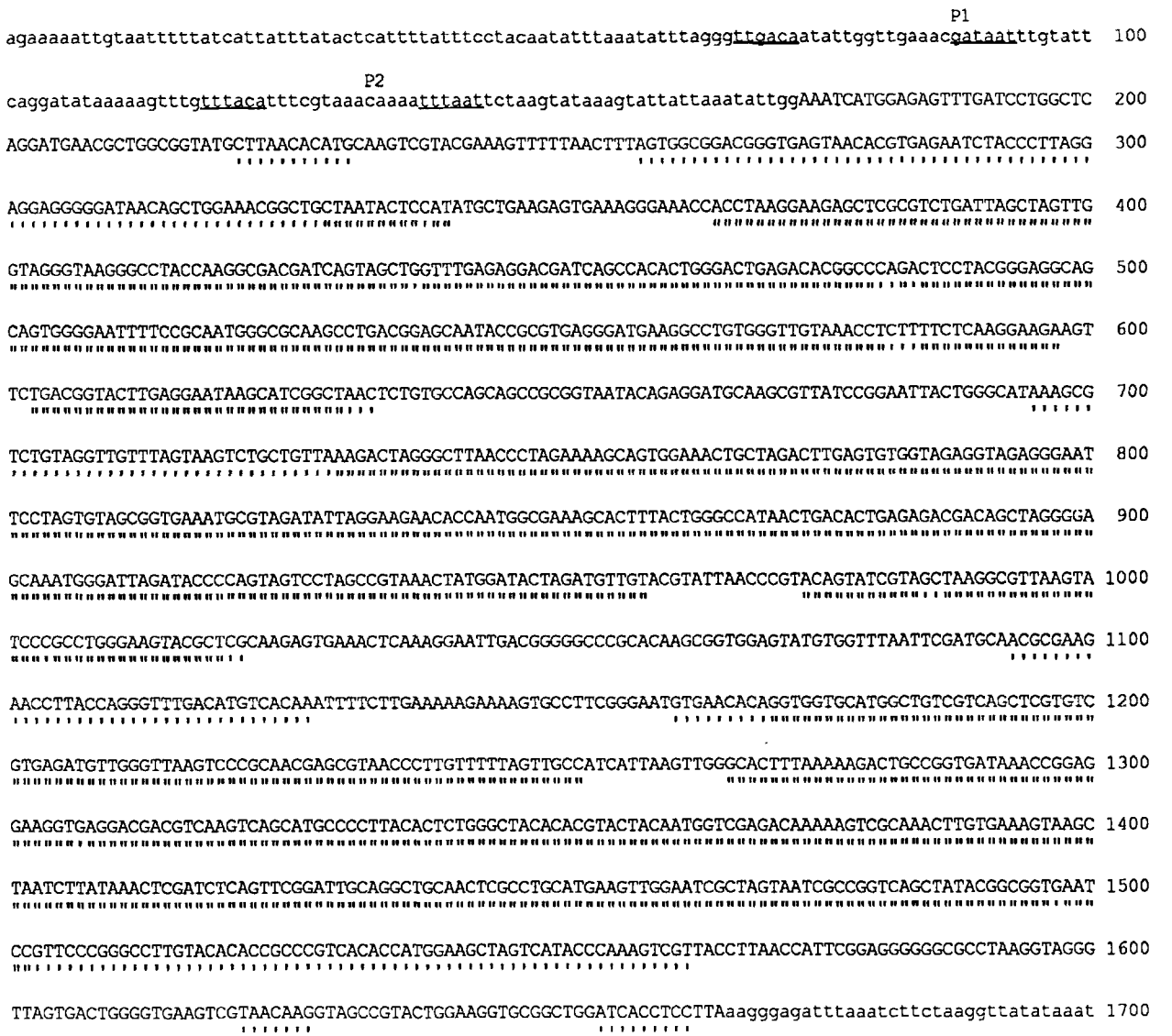


**Fig. 1.** Nucleotide sequence of the noncoding (RNA-like) strand of the 16S rRNA gene (uppercase letters) and flanking regions (lowercase letters) from the *Cryptomonas* Φ plastid. Putative promoter regions, P1 and P2, are underlined. Positions used in the phylogenetic analysis shown in Fig. 2 are indicated by (″); those additional positions used in the phylogenetic analysis shown in Fig. 3 are indicated by (′).

with conserved stems maintained by compensatory base changes (data not shown).

Phylogenetic analysis of Ssu rRNAs from cyanobacteria and the plastids of the chlorophyll *c*-containing alga *Cryptomonas* Φ and other algal and land plant lineages shows that plastids arose from a cyanobacterium-like ancestor (Fig. 2). In a bootstrap analysis the plastids formed a monophyletic group in 99% of the bootstrap trees (Fig. 2). Furthermore, this group fell within the cyanobacterial line of descent in 92% of the cases. Figure 3 represents an augmented analysis of the plastid cluster in Fig. 2. Additional plastid rRNA sequences have been included and the number of sequence positions used in the analysis has been increased.

Following the branching of the plastid cluster within the phylogenetic trees, there is a dichotomy between the chlorophyte/land plant and rhodophyte/chromophyte plastid lineages (Figs. 2 and 3). The deeper branchings in the latter group are comprised of the phycobilin-containing cyanelle of the glaucophyte *C. paradoxa* and plastids of the macrophyte red alga *Palmaria palmata,* the cryptomonad *Cryptomonas* Φ, and the unicellular rhodophyte *Cyanidium caldarium.* The close branching order among this set supports the proposal that the taxonomic separation between glaucophytes and rhodophytes may be artificial, particularly if *C. caldarium* is considered to be a member of the Glaucophyceae (Cavalier-Smith 1987).

The placement of the *Cryptomonas* Φ plastid is indicative of its cyanobacterial ancestry (Figs. 2 and

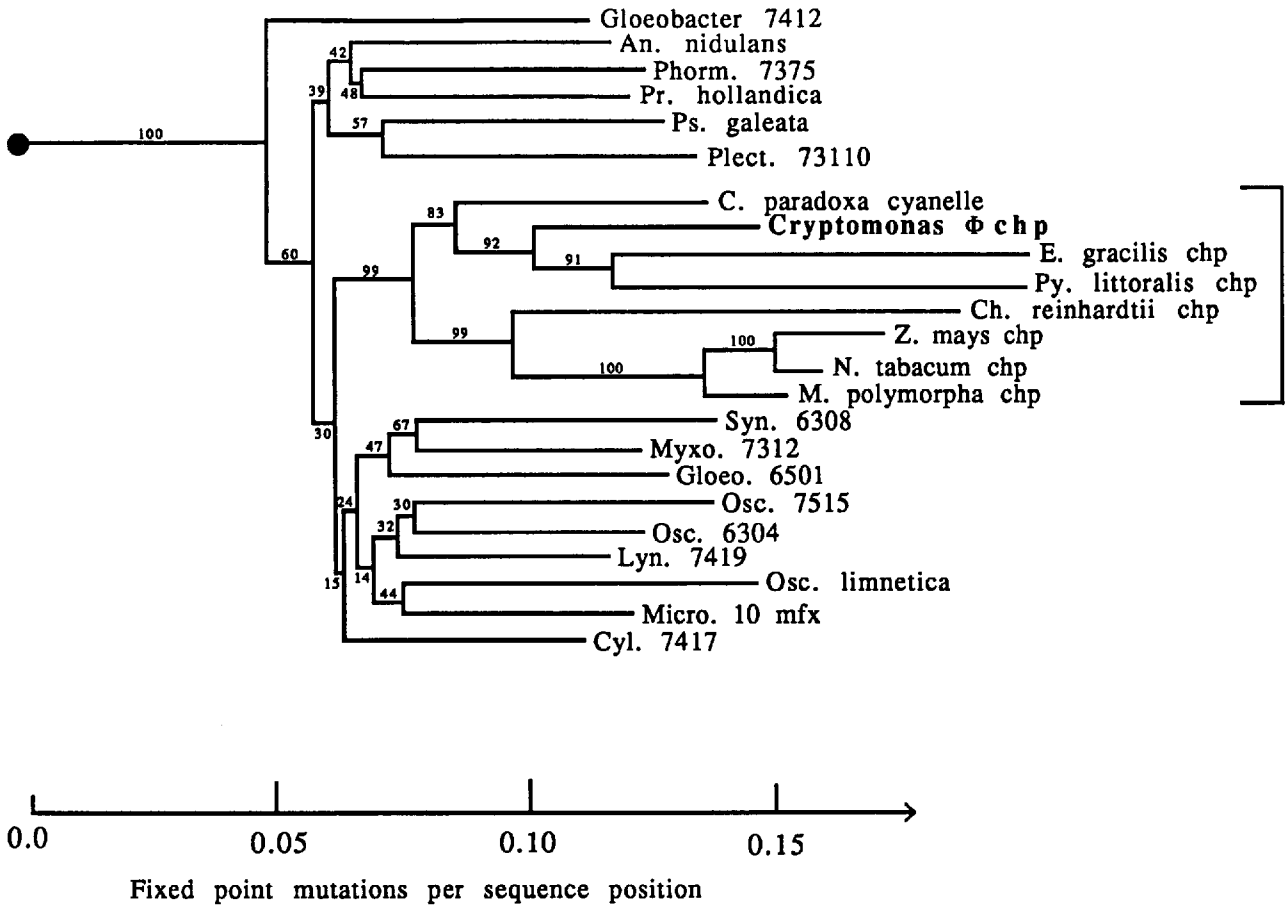


**Fig. 2.** An inferred phylogenetic tree depicting the evolutionary relationships among various cyanobacteria, the prochlorophyte *Procholorothrix hollandica,* and plastids. The tree was produced by a least squares, distance matrix analysis of 862 aligned 16S rRNA sequence positions. The bracket to the right of the tree denotes the cyanelle/plastid cluster. Distances between terminal nodes are equal to the sum of the horizontal segment lengths joining them. Numerical values associated with each internal branch represent the percentage of instances in which that branch occurred in a set of 100 trees generated from a bootstrap resampling of the original data. The 16S rRNA sequences of *Bacillus subtilis* and *Agrobacterium tumefaciens,* whose own relative branch point is represented by ●, were used as outgroups to determine the root of the tree. *Cyanophora paradoxa, P. hollandica,* and cyanobacterial sequence data are from the cyanobacterial 16S rRNA database of N.R. Pace, Indiana Univesity. The references for the other sequences are given in Neefs et al. (1990) with the exception of *Pylaiella littoralis* (Markowicz et al. 1988). Abbreviations are as follows: *An., Anacystis; C., Cyanophora; Ch., Chlamydomonas;* chp., plastid; *Cyl., Cylindrospermum; E., Euglena; Gloeo., Gloeothece; Lyn., Lyngbya; M., Marchantia; Micro., Microcoleus; Myxo., Myxosarcina; N., Nicotiana; Osc., Oscillatoria; Phorm., Phormidium; Pr., Prochlorothrix; Ps., Pseudanabaena; Py., Pylaiella; Syn., Synechocystis; Z., Zea.* Except for *Microcoleus* 10 mfx, strain designation numbers refer to the Pasteur Culture Collection.

3). This contrasts with the results from an analysis of *rbcL* gene-encoded protein sequences in which there was an apparent affiliation between this plastid and the $\beta$ group of Proteobacteria (Douglas et al. 1990). There are several possible explanations for this discrepancy including physical phenomena, such as lateral transfer of the *rbcL* gene, and methodological reasons, such as the relatively limited number of *rbcL* data and the inherent limitations of maximum parsimony methods used to analyze them (Sourdis and Nei 1988; Kishino et al. 1990).

The plastids of both *Euglena gracilis* and its colorless relative *Astasia longa* cluster with those of the chromophyte algae *Ochromonas danica* and *Pylaiella littoralis,* and not with the other chlorophyll *a/b*-containing plastids. A similar association between chromophyte and euglenoid plastids was found by others (Markowicz et al. 1988; Witt and Stackebrandt 1988) and is supported by the bootstrap analyses. These plastids form a monophyletic goup in 87.5% of the bootstrap trees (Fig. 3). Moreover, an analysis that directly addresses the possibility of topological artifacts being produced by differences in evolutionary clock speeds (Olsen 1987) also supports this result. All phylogenetic trees so inferred had topologies identical to that in Fig. 3, even for assumed variations ranging over nearly four orders of magnitude (data not shown). Both euglenoids and chromophyte algae store reserve carbohydrate as $\beta$-1,3-glucans in the cytoplasm, unlike land plants and chlorophyte algae, which store $\alpha$-1,4-glucans in the plastid (Whatley and Whatley 1981). Cavalier-

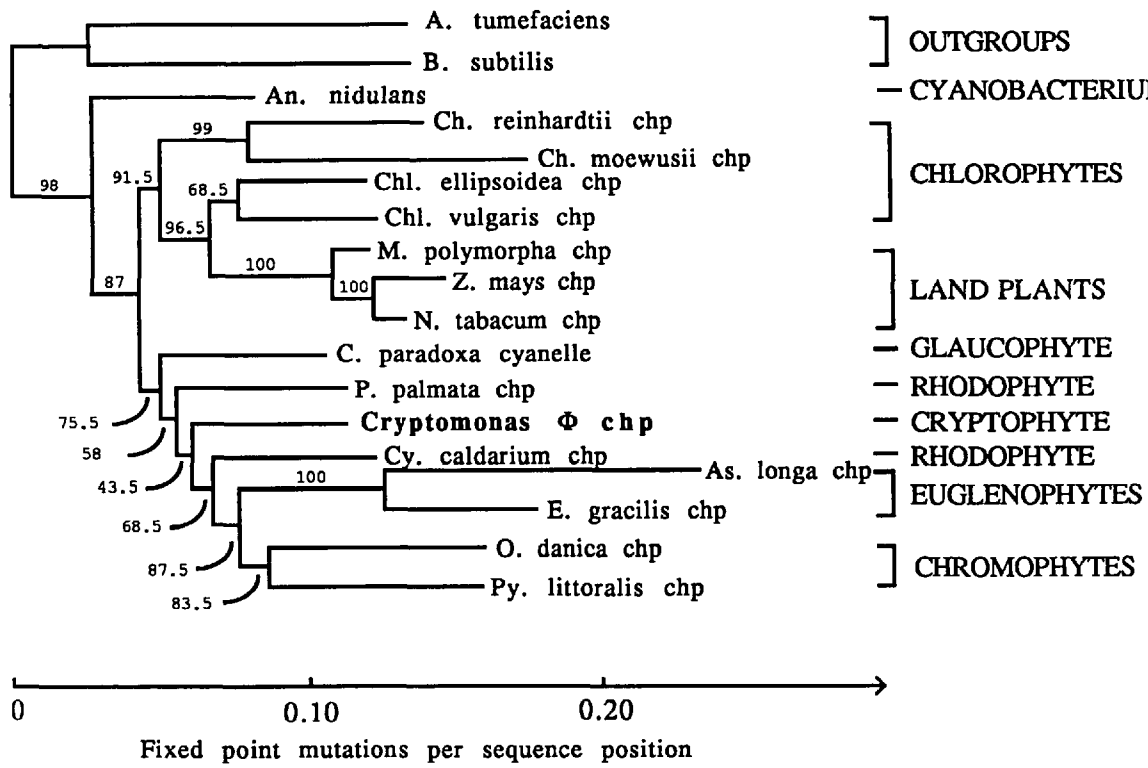


**Fig. 3.** An inferred phylogenetic tree depicting the evolutionary relationships among various plastids. The tree was produced from an analysis of 1118 aligned 16S rRNA sequence positions as described in Fig. 2. Numerical values associated with each internal branch represent the percentage of instances in which that branch occurred in a set of 200 trees generated from a bootstrap resampling of the original data. The references for the sequences used are given in Fig. 2 with the exception of *Astasia longa* (Siemeister and Hachtel 1990), *P. palmata* (Singh, personal communication), and *Cyanidium caldarium* (Maid and Zetsche 1990). Abbreviations are as in Fig. 2 with the inclusion of *A., Agrobacterium; As., Astasia; B., Bacillus; Chl., Chlorella; Cy., Cyanidium; O., Ochromonas; P., Palmaria.*

Smith (1987) has argued that the euglenoid plastid is not related to those of the chlorophyte lineage, based on lipid composition and plastid DNA organization, particularly the tandemly repeated rRNA genes that resemble prokaryotic rRNA cistrons.

The phylogenetic trees depicting plastid relationships (Figs. 2 and 3) do not by themselves address the issue of the number of endosymbiotic events that gave rise to modern plastids. This can be done only by comparing plastid phylogeny with that of the eukaryotic hosts. A number of phylogenetic trees containing members of the major photosynthetic eukaryotic groups have been inferred from analyses of Ssu rRNAs (Bhattacharya et al. 1990; Douglas et al. 1991; Eschbach et al. 1991; Hendriks et al. 1991), large subunit rRNAs (Lsu rRNAs) (Perasso et al. 1989; Lenaers et al. 1991) and 5S rRNAs (Hori and Osawa 1987; van den Eyende et al. 1988). Among these trees, the relative branching orders of the algal groups differ. This is perhaps not surprising because they are derived from analyses of different gene products from different sets of organisms and are inferred by different methods. However, those derived from Ssu and Lsu rRNA sequence analyses have several features in common that can be used as a framework for comparison with the results reported here. Specifically, (1) the rhodophyte, chromophyte, and chlorophyte/land plant groups arise as three distinct lineages within a general eukaryotic radiation, and (2) the euglenoids comprise a very deep branching group with no direct relation to any of the other algal groups.

In Fig. 3, the chlorophyte/land plant plastids branch as a monophyletic group as do the host organisms in the eukaryotic trees. This is consistent with the hypothesis that green algae and land plants arose from a single chloroplast-bearing ancestor. The *Cryptomonas* Φ plastid arises from within the phycobiliprotein-containing algae (Fig. 3), supporting the hypothesis that cryptophytes arose by a secondary endosymbiosis of a primitive red alga that contained chlorophyll *c* (Whatley et al. 1979). Recent studies of the nucleomorph and nuclear Ssu rRNA genes from *Cryptomonas* Φ has confirmed that two separate endosymbiotic events indeed contributed to the formation of cryptomonad algae (Douglas et al. 1991).

The chromophyte plastids appear to have a red algal ancestry whereas in eukaryotic trees, the chromophytes and rhodophytes form distinct lines

of descent. This implies that these plastids are the result of one or more endosymbioses of a red alga or its plastid with a eukaryotic chromophyte ancestor(s). The multiple membrane system surrounding modern chromophyte plastids is consistent with this model. The proposal that rhodophyte and chlorophyte/land plant lineages are sister groups of the Kingdom Plantae and separate from the Kingdom Chromista, which includes chromophytes and cryptophytes (Cavalier-Smith 1986) is not supported by our results or by the eukaryotic Ssu and Lsu rRNA studies cited above.

Furthermore, the relatively shallow placement of the euglenoid plastids coupled with the deep placement of *E. gracilis* in the eukaryotic trees does not support the hypothesis that the common ancestor shared by euglenoids and other photosynthetic eukaryotes contained a plastid (Cavalier-Smith 1982, 1987). It does support the model wherein the euglenoid plastids arose from an endosymbiosis of an alga or algal plastid with an ancestral euglenoid host, albeit the algal symbiont does not appear to have been a chlorophyte as others have proposed (Gibbs 1978; Whatley et al. 1979). However, Gibbs (1970) has noted that the plastids of euglenoids resemble those of both the Chlorophyceae and the more primitive Prasinophyceae (Micromonadophyceae). The discovery of a prasinophyte that contains both chlorophylls *b* and *c* (Wilhelm 1987) raises the possibility that euglenoid plastids arose by uptake of a prasinophyte alga or its plastid by a euglenoid ancestor.

These comparisons are necessarily cursory. A more complete analysis must await the acquisition of more data and a resolution to the problem of differing topologies among both plastid and eukaryotic phylogenetic trees.

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