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The 5S Ribosomal RNA Sequences of a Red Algal Rhodoplast and a Gymnosperm Chloroplast. Implications for the Evolution of Plastids and Cyanobacteria

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Summary. The 5S ribosomal RNA sequences have been determined for the rhodoplast of the red alga *Porphyra umbilicalis* and the chloroplast of the conifer *Juniperus media.* The 5S RNA sequence of the *Vicia faba* chloroplast is corrected with respect to a previous report. A survey of the known sequences and secondary structures of 5S RNAs from plastids and cyanobacteria shows a close structural similarity between all 5S RNAs from land plant chloroplasts. The algal plastid 5S RNAs on the other hand show much more structural diversity and have certain structural features in common with bacterial 5S RNAs. A dendrogram constructed from the aligned sequences by a clustering algorithm points to a common ancestor for the present-living cyanobacteria and the land plant plastids. However, the algal plastids branch off at an early stage within the plastid–cyanobacteria cluster, before the divergence between cyanobacteria and land plant chloroplasts. This evolutionary picture points to the occurrence of multiple endosymbiotic events, with the ancestors of the present algal plastids already established as photosynthetic endosymbionts at a time when the ancestors of the present land plant chloroplasts were still free-living cells.

Key words: Rhodoplast — Chloroplast — Plastid — Endosymbiosis — 5S ribosomal RNA — Nucleotide sequence — Secondary structure

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Introduction

The endosymbiotic theory for the origin of plastids (Margulis 1981) states that they evolved from ancestors of the present-living cyanobacteria. It has also been proposed (Stanier and Cohen-Bazire 1977; Whatley et al. 1979) that *Prochloron* and the chloroplasts, which possess chlorophyll a and b, share a common ancestor different from that of the remaining cyanobacteria and the rhodoplasts, which possess chlorophyll a and phycobiliproteins.

The affiliation of plastids with cyanobacteria, and hence their endosymbiotic origin, has been corroborated by evolutionary trees constructed from 16S RNA oligonucleotide catalogs (Fox et al. 1980), from 5S RNA sequences (e.g., Hori and Osawa 1986; Huysmans and De Wachter 1986), and from complete 16S RNA sequences (Woese 1987). The similarity between cyanobacterial and plastid 5S RNAs has also been stressed by secondary structure studies (Wolters and Erdmann 1984; Delihas et al. 1985). However, a more detailed knowledge of the branching order within the cyanobacteria–plastid cluster could help to resolve the question of whether all plastids descend from a single cyanobacterial ancestor that engaged in endosymbiosis or whether different endosymbiotic events have given rise to different groups of plastids.

5S RNA sequences have been hitherto reported for four species of cyanobacteria, for the cyanelle of *Cyanophora paradoxa,* and for the chloroplasts of 13 species. The latter group includes seven angiosperms, a fern, two mosses, two green algae, and *Euglena gracilis.* In order to permit a more com-

prehensive reconstruction of the evolutionary history of plastids, we have determined the 5S RNA sequences of the chloroplast of the gymnosperm *Juniperus media* and of the rhodoplast of the red alga *Porphyra umbilicalis*. Also, we have corrected the 5S RNA sequence of the chloroplast of the angiosperm *Vicia faba*, for which the sequence reported by Dyer and Bowman (1979) contained errors.

Materials and Methods

Isolation and Sequencing of Plastid 5S RNAs. The red alga *Porphyra umbilicalis* was collected in the intertidal zone at the seashore near Audresselles, France. Young shoots of the conifer *Juniperus* × *media* cv. Hetzii were collected in the garden of one of the authors. Beans of *Vicia faba* were allowed to germinate on a bed of wet vermiculite and shoots were collected after 3 weeks.

In the case of *Vicia faba*, chloroplast ribosomes were isolated according to Bartsch et al. (1982). Ribosomal RNA was prepared by phenol extraction and ethanol precipitation. 5S RNA was obtained by preparative polyacrylamide gel electrophoresis (Fang et al. 1982) with a yield of about 1 A_{260} unit of chloroplast 5S RNA per 100 g of shoots. The extracted RNA was labeled at the 3'-terminus with [5'-^{32}P]pCp (Peattie 1979). After electrophoresis on a high-resolution gel as used for sequencing, autoradiography revealed a single band.

In the case of the red alga *Porphyra umbilicalis* and the conifer *Juniperus media*, 5S RNA was isolated from whole tissue and separated into a cytoplasmic and a plastid fraction by polyacrylamide gel electrophoresis. This obviates the need for plastid purification, a relatively tedious procedure that results in lower yields. Seventy-five grams of plant tissue were homogenized in a Waring Blendor in the presence of approximately 70 ml of phenol equilibrated with an equal volume of 0.01 M Tris-HCl, pH 7.7, 0.001 M $MgCl_2$, 1% SDS. The homogenate was centrifuged and the nucleic acid fraction was recovered from the aqueous layer by precipitation in the presence of an equal volume of isopropanol at −15°C. The redissolved precipitate was separated by preparative polyacrylamide gel electrophoresis. In the case of *Juniperus*, this yielded about 1 A_{260} unit of 5S RNA distributed over two bands with slightly different mobility. RNA extracted from each band was 3'-terminally labeled and fractionated on a high-resolution gel as described above. Autoradiography revealed four bands of different mobility. RNA from each band was subjected to a separate sequence analysis, which showed the three slower-moving bands to consist of plastid 5S RNA possessing length heterogeneity, and the fastest-moving band of cytoplasmic 5S RNA. These two types of 5S RNA are easily distinguishable on the basis of their primary and secondary structure characteristics, as discussed below. Rhodoplast 5S RNA from *Porphyra umbilicalis* was similarly isolated, but in this case the plastid 5S RNA showed a higher mobility than the cytoplasmic 5S RNA. The sequences of the cytoplasmic 5S RNAs will be published elsewhere.

The 3'-terminally labeled plastid 5S RNAs were sequenced essentially by the partial chemical degradation method (Peattie 1979). Where necessary, the sequence in the 5'-terminal area was examined or confirmed by dephosphorylation, 5'-terminal labeling, and partial nuclease degradation (Ursi et al. 1982; Dams et al. 1983).

Dendrogram Construction. Dendrograms were constructed by the clustering algorithm described in detail by Huysmans and De Wachter (1986) and by Dams et al. (1987). This algorithm includes the computation of a dissimilarity matrix for all the sequences involved, correction for multiple substitution, factorial correction for unequal evolutionary rates, and weighted pairwise grouping. The standard deviation on the substitution term of the computed dissimilarities is indicated on each branching point in one of the resulting dendrograms.

Results and Discussion

Structure of the Examined Plastidial 5S RNAs

In Fig. 1, the newly determined sequences of the 5S RNAs of the plastids from *Vicia faba*, *Juniperus media*, and *Porphyra umbilicalis* are shown in alignment with all hitherto published 5S RNA sequences from cyanobacteria and photosynthetic organelles. The alignment is an excerpt from a comprehensive alignment of 457 5S RNA sequences of eukaryotic, eubacterial, and archaebacterial origin, obtainable from the authors. The alignment comprises 154 positions, as compared to 152 positions in other recently published partial alignments from our laboratory (Willekens et al. 1986b; Dams et al. 1987). This is because two new positions, bearing numbers 29 and 77, had to be inserted in order to obtain a satisfactory alignment of the *Chlamydomonas reinhardii* chloroplast 5S RNA.

The *Vicia faba* chloroplast 5S RNA sequence shows six nucleotide changes and one deletion with respect to the structure derived by Dyer and Bowman (1979) from an oligonucleotide catalog rather than a complete sequence. The correction of the errors allows a base pairing scheme (Fig. 1) entirely compatible with that of other 5S RNAs of higher plant plastids.

Secondary structure models for the 5S RNAs of *Juniperus media* chloroplast and of *Porphyra umbilicalis* rhodoplast are shown in Fig. 2. In the case of the gymnosperm *Juniperus*, the model is identical to those applicable to plastid 5S RNAs from other embryophytes (seed plants, ferns, and mosses). These models are defined by the boxes enclosing putative double-stranded areas, superimposed on the alignment (Fig. 1). Both plastid 5S RNA secondary structure models show the possibility of a secondary structure switch in area I_1-C (De Wachter et al. 1982, 1984). The switch structure in *Juniperus* chloroplast has the same shape as in other embryophyte chloroplast 5S RNAs, but in the rhodoplast 5S RNA it is different, with a single nucleotide bulge that can switch between opposite strands of helix C. In comparison to other plastid 5S RNAs, the rhodoplast 5S RNA has an extra nucleotide in hairpin loop H_1, and helix D is lengthened by one base pair at the expense of internal loop I_2. The latter two properties contribute to make the rhodoplast secondary structure the most divergent among the plastid 5S RNAs, but also makes it more similar to 5S RNAs from other eubacterial phyla.

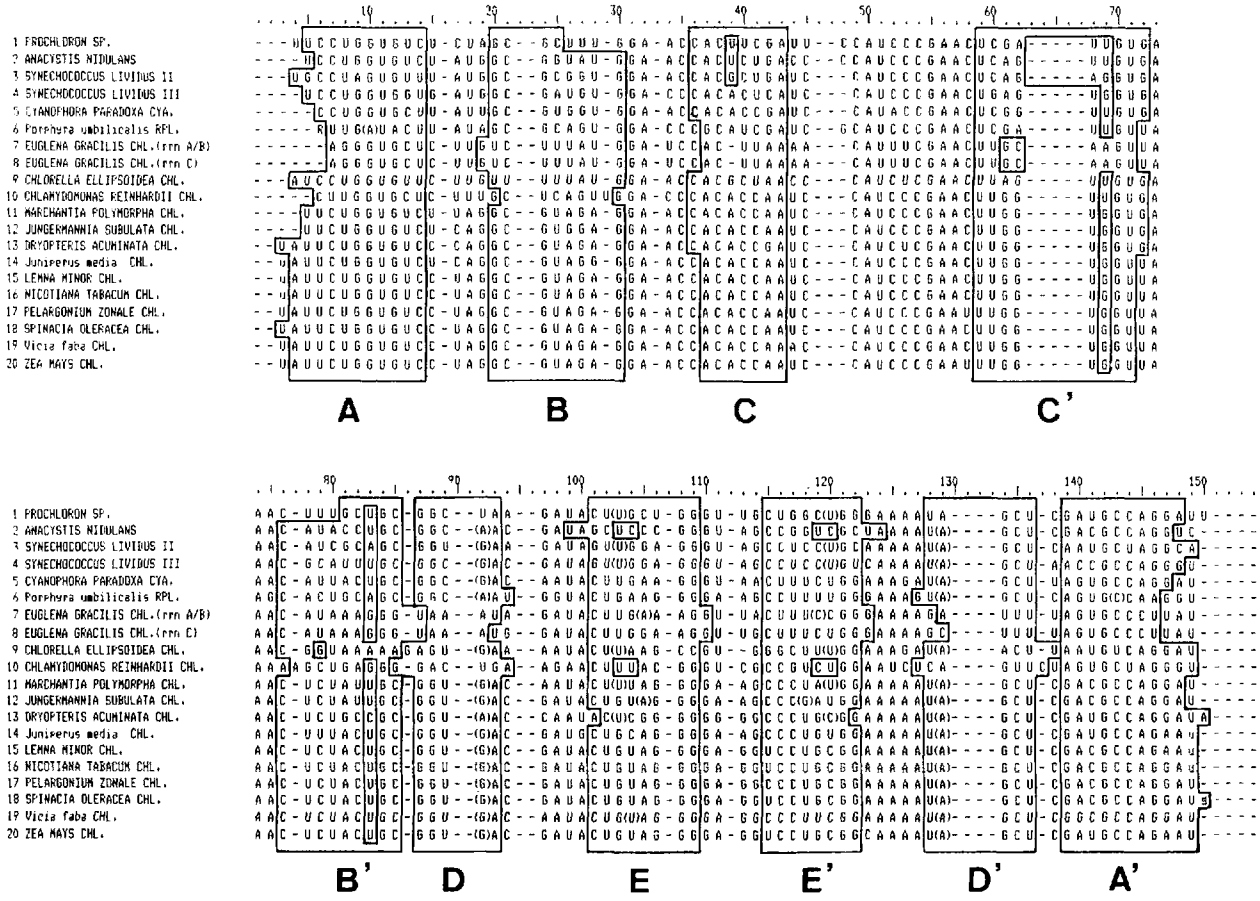

Fig. 1. Alignment of cyanobacterial and plastidial 5S RNA sequences. Species names printed in capitals correspond to previously published sequences. References can be found in Erdmann and Wolters (1986) except for *Chlorella ellipsoidea* chloroplast (Yamada and Shimaji 1986), *Pelargonium zonale* chloroplast (Eck et al. 1987), and *Cyanophora paradoxa* cyanelle (Maxwell et al. 1986). The sequences of *Spirodela oligorhiza* and *Lemna minor* chloroplast 5S RNAs are identical. Abbreviations following the species names are: CHL, chloroplast; CYA, cyanelle; RPL, rhodoplast. Alignment positions that are empty in all listed sequences are needed to accommodate nucleotides in 5S RNAs from other taxonomic groups (see text). Length heterogeneity is indicated by terminal residues printed in lower case characters. Boxes labeled A and A', B and B', etc. enclose complementary strands of helices A, B, etc. in the proposed secondary structure models, two of which are drawn in Fig. 2. Bulges and small interior loops within the helices are enclosed in nested boxes; single odd base pairs (pairs other than G·C, A·U, and G·U) intercalated between standard base pairs (Watson-Crick or G·U pairs) are put in parentheses.

Structural Features Specific for Cyanobacterial and Plastidial 5S RNAs

The alignment and superimposed secondary structure scheme (Fig. 1) shows that there is much more structural variability among the algal plastid 5S RNAs than among the embryophyte plastid 5S RNAs. This variability resides not only in the primary structure, but also in shifted helix boundaries and different helix lengths. There are three structural characteristics that are shared by most or all of the 5S RNAs from cyanobacteria, the cyanelle, and the plastids, and that distinguish them from other eubacterial 5S RNAs. They are listed in Table 1. The first is the presence of an insertion relative to eubacterial 5S RNAs at alignment position 39. This insertion is responsible for the fact that helix C carries a one-base bulge (Fig. 2) as opposed to a two-base bulge in eubacteria (actually in nearly all other 5S RNAs). An exception is found in *Euglena* chloroplast 5S RNA which carries a two-base bulge, though not at the usual position in helix C. A second characteristic is the length of hairpin loop H_1, which contains 13 nucleotides in eubacteria, but 12 nucleotides in most cyanobacteria and plastids. Exceptions are *Prochloron, Anacystis nidulans,* and the rhodoplast. A third noteworthy feature is the nucleotide forming the single-base bulge on helix B. In the great majority of eubacterial 5S RNAs this is A. In most cyanobacteria and most embryophyte chloroplasts it is U. In the algal plastids, however, it is a purine.

The above survey, though based on relatively few data, shows a clear tendency for a closer structural resemblance between the 5S RNAs of cyanobacteria and embryophyte plastids than between each of these

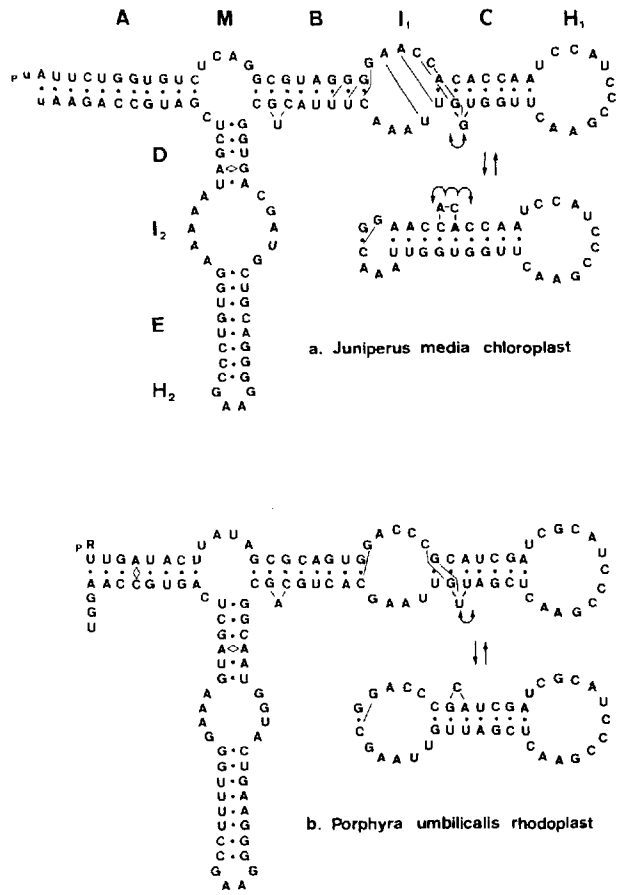

Fig. 2. Secondary structure models for **a** *Juniperus media* chloroplast 5S RNA and **b** *Porphyra umbilicalis* rhodoplast 5S RNA. Helices are labeled A–E, loops are labeled M (multibranched), I (internal), or H (hairpin). Hypothetical odd base pairs intercalated between standard base pairs are indicated by losenges. Two-headed curled arrows indicate alternative positions of migrating bulges (De Wachter et al. 1984). Bases connected by lines at the boundary of loop I_1 and helix C may pair to form the equilibrium structure (De Wachter et al. 1982, 1984) shown in the figures. Base pairing indicated by the lines at the boundary of helix B and loop I_1 would result in an extra bulge on the 5'-strand of helix B, as is possible in all prokaryotic 5S RNAs (Willekens et al. 1986a; Van den Eynde and De Wachter 1987). The rhodoplast 5S RNA shows sequence heterogeneity, with both A and G (symbolized as R) present at the 5'-terminus.

two groups and the 5S RNAs of algal plastids. The latter group also shows the most structural diversity among its members. This is to be expected since the algae dealt with are evolutionarily further apart than are the land plants. The trends sketched above are confirmed in the evolutionary picture reconstructed by clustering analysis and discussed below.

Evolutionary Picture

A dendrogram was constructed (Fig. 3a) from an alignment that comprised the 182 eubacterial 5S RNA sequences known to us at present. This number includes the 20 plastidial and cyanobacterial 5S

Table 1. Structural features distinctive for cyanobacterial and plastid 5S RNAs

Type of 5S RNA	Insertion at position 39[a]	Nucleotides in hairpin loop H_1	Bulge on helix B (position 83)
Eubacteria	—	13	mostly A
Cyanobacteria	A, G, or U	12 or 13	U or A
Cyanelle	A	12	U
Plastids			
Red alga	A	13	A
Euglena	—	12	G
Chlorella	G	12	A
Chlamydomonas	A	12	G
Embryophytes	A	12	U or C

Positions mentioned in the table refer to alignment positions (Fig. 1)

[a] The absence of a nucleotide at this position results in a two-base bulge on helix C, as found in most 5S RNAs; its presence results in a one-base bulge (as in Fig. 2) or in an asymmetric interior loop in helix C

RNA sequences shown in Fig. 1 plus the sequences of 158 other eubacteria belonging to seven different phyla and those of four plant mitochondria. In addition, the alignment included 10 sequences of cytoplasmic 5S RNAs from red algae (Takaiwa et al. 1982; Lim et al. 1983, 1986; our unpublished results) which served as an external reference group allowing a correction for unequal mutation rates among the eubacterial 5S RNAs. The dendrogram shows a number of clusters, one of which comprises all the cyanobacteria and chloroplasts and whose detailed structure is represented in Fig. 3b. The composition and topology of the other eubacterial clusters composing the phenogram has been shown in previous papers (Huysmans and De Wachter 1986; Dams et al. 1987). However the overall topology of the present dendrogram differs from those previously published in the sense that the position of the clusters with respect to each other has undergone a few changes, which are due to the addition of extra sequences to the alignment. One such change is that the cyanobacteria–plastid cluster is at present the deepest diverging branch after the plant mitochondria. A similar view is held, e.g., by Hori and Osawa (1986), who present a 5S RNA tree in which cyanobacteria and chloroplasts emerge first from the eubacterial cluster. Mitochondrial sequences were not included in their analysis, nor in any other comprehensive tree based on 5S RNA data that we know of. On the other hand, the eubacterial phylogenetic tree based on 16S ribosomal RNA sequence analysis, as it is presently known (Woese 1987), shows a substantial amount of difference with respect to the picture based on 5S RNA data, especially with regard to branching order and specific relationships among the major eubacterial phyla. Here cyano-

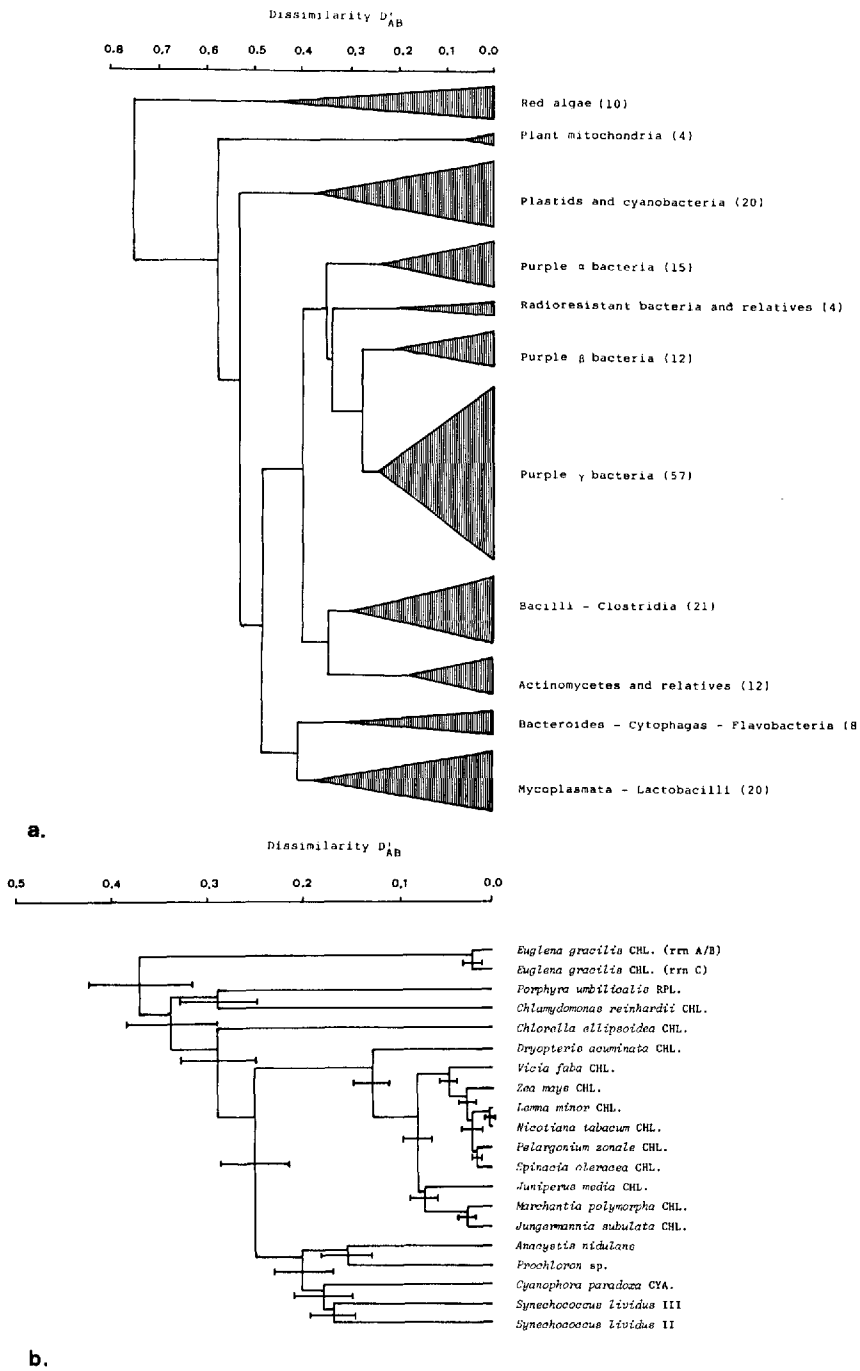

Fig. 3. Dendrogram constructed by cluster analysis of 5S RNA sequences from eubacteria and plant organelles. The complete dendrogram **a** was constructed as described in the Materials and Methods section from an alignment of 182 5S RNA sequences available to us at present plus 10 cytoplasmic 5S RNA sequences from red algae (see text). The latter are used as an external reference group and allow a correction for different evolutionary rates among the eubacterial and organelle sequences. D'_{AB} is the dissimilarity value corrected for unequal evolutionary rates. Each major cluster is represented as an equilateral triangle with its top situated at the D'_{AB} value corresponding to the first divergence within the cluster, and with a base proportional to the number of species in the cluster, mentioned in parentheses after the cluster name. The complete structure of the cluster comprising cyanobacteria and plastids is shown in **b**, with the standard deviation on the substitution term of the computed dissimilarities indicated by a bar.

bacteria and plastids tend to branch off relatively late during eubacterial diversification. When making comparisons between the presently available trees based on 5S RNA and 16S RNA sequences, one has to bear in mind that these are constructed from data for different sets of species. The 16S RNA data set, especially the oligonucleotide catalogs, presently covers more eubacterial species than the 5S RNA data set. It is difficult to predict whether the differences in branching order at the phylum level, observed on comparison of 16S RNA trees and 5S RNA trees, will be emphasized or will fade when more equivalent data sets become available.

Within the cyanobacteria–plastid cluster (Fig. 3b), the algal plastids are situated on three separate branches that diverge at large dissimilarity values. The lowest branch leads to the *Euglena* chloroplast, the second one to the rhodoplast of *Porphyra umbilicalis* and to the chloroplast of *Chlamydomonas reinhardii,* and the third one to the *Chlorella* chloroplast. Next there is a divergence between two clusters, one containing the plastids of the Embryophy-

ta, the other containing all the cyanobacteria including *Prochloron,* and the cyanelle of *Cyanophora paradoxa.* The dichotomy based on pigment composition, with cyanobacteria, cyanelle, and rhodoplasts on the one hand, and *Prochloron* and chloroplasts on the other hand, is not reflected in the divergence pattern. In a study based on 16S RNA oligonucleotide catalogs (Seewaldt and Stackebrandt 1982), *Prochloron* also showed a closer relationship to cyanobacteria than to chloroplasts. On the other hand, among the plastids the rhodoplast of *Porphyridium cruentum* was found to be closer to cyanobacteria than the chloroplasts of embryophytes and green algae according to the same study.

If the picture obtained by 5S RNA sequence clustering approaches the true order of evolutionary events, then our findings mean that the plastids are polyphyletic. Only the chloroplasts of the Embryophyta appear to have a common ancestor. The algal plastids, on the contrary, branch off independently before the divergence between the present-living cyanobacteria and the chloroplasts of the Embryophyta. This points to the occurrence of a series of independent endosymbiotic events. Even taking into account the uncertainties in branching order indicated on the dendrogram of Fig. 3b, it is difficult to reconcile this picture with a single endosymbiotic event—in other words a common cyanobacterial ancestor for all the plastids of the present-day algae and plants. Since a correction for unequal evolutionary rates is built into our tree construction algorithm, it can hardly be argued that the branching topology of Fig. 3b is compatible with a single endosymbiotic event, followed by evolution of the plastids at different rates in independently evolving lines.

The direct filiation hypothesis for the origin of eukaryotes (Cavalier-Smith 1975; Taylor 1976) states that the latter descend from a "Protoalga" or "Uralga," which itself had a cyanobacterial ancestor, and rejects the endosymbiotic origin of plastids. The resemblance between the present-day cyanobacteria and the plastids is explained by a slow evolution of the fraction of the eukaryotic genome secluded in the plastids since the time of the Uralga emergence. Evolutionary pictures such as the one obtained in Fig. 3, which clearly show the plastids to be part of the prokaryotic realm, would then be the consequence of distortions due to gross differences in evolutionary rate. However, as explained by Doolittle (1982), the Uralga hypothesis implies that the eukaryotes, and hence the plastids, arose only once. This hypothesis is therefore contradicted by our finding that the plastids are polyphyletic.

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