Origin and Evolution of Organisms as Deduced from 5S Ribosomal RNA Sequences[1]

Hiroshi Hori and Syozo Osawa†*

*Department of Genetics, GEN-IKEN, Hiroshima University; and †Laboratory of Molecular Genetics, Department of Biology, Nagoya University

A phylogenetic tree of most of the major groups of organisms has been constructed from the 352 5S ribosomal RNA sequences now available. The tree suggests that there are several major groups of eubacteria that diverged during the early stages of their evolution. Metabacteria (=archaebacteria) and eukaryotes separated after the emergence of eubacteria. Among eukaryotes, red algae emerged first; and, later, thraustochytrids (a Proctista group), ascomycetes (yeast), green plants (green algae and land plants), "yellow algae" (brown algae, diatoms, and chrysophyte algae), basidiomycetes (mushrooms and rusts), slime- and water molds, various protozoans, and animals emerged, approximately in that order. Three major types of photosynthetic eukaryotes—i.e., red algae (=Chlorophyll *a* group), green plants (Chl. *a*+*b* group) and yellow algae (Chl. *a*+*c*)—are remotely related to one another. Other photosynthetic unicellular protozoans—such as Cyanophora (Chl. *a*), Euglenophyta (Chl. *a*+*b*), Cryptophyta (Chl. *a*+*c*), and Dinophyta (Chl. *a*+*c*)—seem to have separated shortly after the emergence of the yellow algae.

Introduction

At present, the evolutionary relationships of the major groups of organisms are quite obscure, and the present systems of classification are mainly based on physiological and morphological characters. Since the evolutionary changes of such characters are very complicated and the rate of change is variable in different groups of organisms or in different evolutionary periods, not much confidence can be given to the systems. A more useful approach to this problem is to use DNA or RNA sequences, because the evolutionary change of these molecules is roughly proportional to evolutionary time. The 5S ribosomal RNA (5S rRNA) sequence is particularly useful for establishing the phylogenetic relationship of distantly related organisms (Kimura and Ohta 1973; Hori 1975) because of its low substitution rate (mean ± SE 0.18 ± 0.05 substitution/nucleotide site/10^9 years; Hori et al. [1977]) and because of its basic similarity of structure among all organisms, which makes it possible to align the sequences for the construction of a comprehensive phylogenetic tree.

The 5S rRNA phylogenetic trees for many groups of organisms or organelles have been reported, e.g., for eubacteria (Dekio et al. 1984; Vandenberghe et al. 1985), "the purple eubacterial group" (Lane et al. 1985), the eubacterial family Vibrionaceae (MacDonell and Colwell 1985; MacDonell et al. 1986), Mycoplasmas (Rogers et al. 1985), metabacteria (Fox et al. 1982; Hori et al. 1982), green plants (Hori et al. 1985*a*), Ascomycota (Chen et al. 1984), Basidiomycota (Walker and Doolittle 1982; Huysmans et al. 1983; Gottschalk and Blanz 1984; Walker 1984), protozoans (Kumazaki et al.

1. Key words: 5S rRNA, simplified UPGMA trees, molecular phylogeny.

Address for correspondence and reprints: Dr. Hiroshi Hori, Department of Genetics, GEN-IKEN, Hiroshima University, Kasumi, Hiroshima, 734, Japan.

Mol. Biol. Evol. 4(5):445–472. 1987.

0737-4038/87/0405-0001$02.00

1983*a*), Meso- and Metazoa (Ohama et al. 1984), and organelles (Hori et al. 1982; Wolters and Erdmann 1984). However, a 5S rRNA tree for all groups of organisms has not been constructed. In the present paper, we have employed the 352 sequences of 5S rRNAs now available to construct a phylogenetic tree of a wide spectrum of extant organisms, including organelles, by means of a simplified unweighted-pair-group (UPG) method.

Material and Methods

Sequence Alignment of 5S rRNA

The 352 5S rRNA sequences from various organisms available as of January 1986 have been used in the present study. Representative organisms examined herein are taxonomically summarized in table 1. The alignment of these sequences was obtained mainly by juxtaposing the 5S rRNA secondary structures as described elsewhere (Hori et al. 1985*b*).

Construction of Phylogenetic Trees

The evolutionary distance, *Knuc,* between two sequences was calculated by means of the equation described by Kimura (1980). *Knuc* estimates the number of base substitutions per nucleotide site that have occurred since the separation of the two sequences.

$$Knuc = -(1/2)\log_e[(1-2P-Q)(1-2Q)^{1/2}], \quad (1)$$

where P and Q are the fractions of nucleotide sites between two sequences showing transition- and transversion-type differences, respectively. The SE of the *Knuc,* SE_K, was calculated by using Kimura's (1980) equation. When a gap of length one was paired with one nucleotide, it was counted as equal to one transversion-type substitution. Large deletions in 5S rRNA sequence—e.g., those found in the sequences of *Mycoplasma* species—are likely to be due to single rare events rather than to the compound effect of several separate events. Therefore, a gap of two or more nucleotides was counted as two differences in determining Q.

The G+C content of genomic DNA in eubacteria is diversified to a considerable extent, ranging from 25% to 75%. Since the G+C content of 5S rRNA more or less reflects the genomic G+C content in eubacteria, we introduced a parameter to cancel such an effect that might influence the rate of nucleotide substitution in 5S rRNA molecules. (In eukaryotes and metabacteria, the genomic G+C content does not correlate significantly with the G+C content of 5S rRNA.) To estimate the evolutionary distance between sequences i and j, the following equation was adopted from Hori and Osawa (1986).

$$Dnuc = (c_i/c_j)Knuc, \quad (2)$$

where *Knuc* is the value from equation (1) and c_i and c_j ($c_i \lesssim c_j$) are the G+C contents of sequences i and j, respectively.

With use of the *Knuc* or *Dnuc* values, a phylogenetic tree was constructed by means of a "simplified" method of the UPG method by using arithmetic averages (Sneath and Sokal 1973). For the estimation of the SE of each branching point in the tree, the variance of each branching point was calculated by means of the equation described by Nei et al. (1985). This is given by

$$V(d_{AB}) = [V(d_{kl}) + Cov(d_{kl}, d_{mn})]/(rs)^2, \quad (3)$$

where d_{kl} is the *intercluster* distance between the kth species in cluster A and the lth species in cluster B and r and s are the numbers of species in clusters A and B, respectively; V and Cov are the variance and covariance, respectively. In the actual computation, however, to avoid excessive computational time owing to the large number of 5S rRNA sequences (352 in this case), $(rs)^2$ was conventionally kept ≤ 16 by using representative sequences in each cluster and was used for tree construction by means of the UPG method (="simplified" UPG method).

Results and Discussion

Validity of Phylogenetic Trees Deduced from 5S rRNA Sequences

As mentioned in the Introduction, the 5S rRNA sequences are useful for the construction of phylogenetic trees. However, the following limitations should be kept in mind:

1. The primary sequences of 5S rRNA are more or less specific to each group of organisms, as can be seen from the partial discontinuity of their alignment (fig. 1). The secondary-structure model of 5S rRNAs is fundamentally the same for all organisms, but there exists partial specificity in each group of organisms. In fact, the secondary-structure models of 5S rRNAs may be classified into four types, i.e., eukaryotic type, metabacterial type, eubacterial type (including chloroplasts), and mitochondrial type (see fig. 2; for details, see also Delihas et al. 1984; Wolters and Erdmann 1984; Hori and Osawa 1986). Also, the ribosomal proteins that interact with 5S rRNA differ between prokaryotes and eukaryotes (Wrede and Erdmann 1973). Thus, the rate of nucleotide substitution in 5S rRNA molecules may vary among different groups of organisms to some extent.

2. The genes for 5S rRNA are members of a multigene family, so that it is not improbable that 5S rRNA from the different organisms compared are derived from paralogous genes.

3. The G+C content of genomic DNA has diversified, ranging from 25% to 75% among bacteria and certain eukaryotic groups. Quite recently, we found that the G+C content of 5S rRNA more or less reflects the genomic G+C content in eubacteria, whereas in eukaryotes the mutation pressure operating to alter the genomic G+C content does not seem to affect significantly the G+C content of 5S rRNA (Hori and Osawa 1986). We then introduced equation (2) as a parameter to cancel such an effect, since it might influence the rate of nucleotide substitution in eubacterial 5S rRNAs. The phylogenetic tree constructed with *Dnuc* values is essentially the same as that constructed with *Knuc* values but is more reasonable in some details. We do not know, however, how such a pressure actually affects the rate of nucleotide substitution in the 5S rRNA molecules.

4. The uncertainties discussed in limitations 1–3 may also apply to 16S(18S) and 23S(28S) rRNA. These molecules are much longer than 5S rRNA, and in this sense 16S(18S) rRNA is better than 5S rRNA for reducing the SEs in constructing phylogenetic trees. The phylogenetic position determined from the 5S rRNA sequences of metabacterial (=archaebacterial) members examined in the present paper is different from that determined from 16S(18S) rRNA sequences. (In the present paper, we use the word "metabacteria" instead of "archaebacteria" because, in our view, eubacteria are more ancient than metabacteria.) 5S rRNA sequences as well as a number of molecular properties suggest that metabacteria are more closely related to eukaryotes than to eubacteria. According to 16S rRNA sequences, however, *Halobacterium*

Table 1
Organisms and Their Phylogenetic Subgroups, as Based on 5S rRNA Data

Superkingdom	"Kingdom" or Major Group[a]		Subgroup or Super "Phylum"	Representative Organism(s)
Eukaryotes	Metazoa (80)	Coelomates	Chordata	Vertebrates
			Prochordata	Ascidian
			Hemichordata	Acorn worm
			Echinodermata	Sea urchin, sea cucumber
			Annelida	Sea worm
			Arthropoda	Silkworm
		Noncoelomates	Nemertina	Tapeworm
			Nematoda	*Caenorhabditis* species
			Rotifera	*Brachionus* species
			Porifera	Sponge
			Coelenterata (Cnidaria)	Hydrozoa (jellyfish), Scyphozoa (jellyfish), Anthozoa (see anemone)
			Platyhelminthes (flatworm)	*Planocera* (marine flatworm), *Dugesia* (planaria)
	Mesozoa (1)			*Dicyema*
	Proctista (protozoans, etc.) (22)		Ciliata	*Tetrahymena*
			Zoomastigina	*Crithidia* (trypanosoma)
			Dinophyta	*Crypthecodinium* (dinoflagellate)
			Euglenophyta	*Euglena*
			Cyanophora	*Cyanophora*
			Cryptophyta	*Chilomonas*
			Rhizopoda	*Acanthamoeba* (amoeba)
			Acrasiomycota	*Dictyostelium* (cellular slime mold)
			Oomycota	*Saprolegnia*
			Myxomycota	*Physarum* (plasmodial slime mold)
			Zygomycota	*Phycomyces*
	Basidiomycota (mushroom, smut) (37)			(see table 2)
	"Chromophyta" (yellow algae) (7)[b]		Phaeophyta	*Eisenia* (brown algae)
			Bacillariophyta	*Diatoma* (diatom)
			Chrysophyta	*Hydrurus* (golden-yellow algae)

	Green plants (29)	Angiosperms	*Triticum* (wheat)
		Gymnosperms	*Metasequoia, Cycas* (cycad), *Ginkgo* (maidenhair tree)
		Pteridophyta	*Psilotum* (whisk fern), *Lycopodium* (clubmoss), *Equisetum* (horsetail), *Dryopteris* (fern)
		Bryophyta	*Marchantia* (liverwort)
		Charophyta	*Nitella* (stonewort)
		Gamophyta	*Spirogyra* (conjugating green algae)
		Chlorophyta	*Chlamydomonas, Ulva* (green seaweed), *Chlorella, Scenedesmus*
	Ascomycota (yeast) (25)		(see table 2)
	Thraustochytrids (2)		(see table 2)
	Rhodophyta (red algae) (9)	Florideophyceae	*Batrachospermum*
		Bangiophyceae	*Porphyra*
Metabacteria (=Archaebacteria) (12)		Thermoacidophiles	*Sulfolobus, Thermoplasma*
		Methanogens	*Methanococcus*
		Halophiles	*Halobacterium*
Eubacteria	Gram positive (43)	Actinobacteria (positive C)	*Micrococcus, Streptomyces, Arthrobacter*
		Positive A	*Bacillus, Staphylococcus, Streptococcus*
		Positive B	*Mycoplasma, Clostridium*
	Gram negative (61)	Negative A	*Escherichia, Vibrio, Listonella, Shewanella, Pseudomonas*
		Negative B	*Alcaligenes* species, *Achromobacter*
		Negative C	*Paracoccus, Agrobacterium, Rhodopseudomonas, Rhodospirillum*
	"Cyanobacteria" (3)	Cyanobacteria	*Anacystis*
		Chloroxybacteria	*Prochloron*
	Organelle (21)	Chloroplast	
		Cyanelle	
		Plant mitochondrion	

[a] Group names are mainly those of Margulis and Schwartz (1982). Numbers in parentheses are number of sequences used.

[b] Dinophyta and Chryptophyta (Proctista) are excluded. Source of the sequences: compilation by Erdmann and Wolters (1986) and papers by Lim et al. (1986), MacDonell and Colwell (1985), and Ohkubo et al. (1986). In addition to the published 5S rRNA sequences, the following unpublished sequences from our laboratory were used for the construction of the trees: *Ectocarpus* sp. (brown alga), *Cladophora* sp. (green alga), cytoplasm and cyanelle of *Cyanophora paradoxa,* and chloroplast of *Dryopteris acuminata* (fern).

(metabacteria) and eubacteria diverged after the prokaryote-eukaryote separation (McCarroll et al. 1983; Elwood et al. 1985; Pace et al. 1986; Sogin et al. 1986).

The discrepancy might be due to the influence of the addition of a large number of nucleotides to 16S rRNA, an addition that seems to have occurred after the emergence of eukaryotes. The length of prokaryotic 16S rRNAs is 1,542 nucleotides (nt) in *Escherichia* (eubacteria), 1,567 nt in *Anacystis* (cyanobacteria), and 1,473 nt in *Halococcus* (metabacteria), whereas that of eukaryotic rRNAs is 2,305 nt in *Euglena* (protozoa), 2,251 nt in *Trypanosoma*, 1,771 nt in *Tetrahymena* (ciliata), and 1,875 nt in *Xenopus* (toad). To avoid the length heterogeneity, McCarroll et al. (1983), Pace et al. (1986), and Sogin et al. (1986) have compared "conserved regions" of ~930, ~1,130, and ~950 nucleotides long, respectively. However, an addition of such a large number of nucleotides to an rRNA molecule might have influenced the rate of nucleotide substitution, even when only "conserved regions" are considered. By contrast, the length of the 5S rRNA molecule is ~120 nt and virtually the same for all organisms. Therefore, the 5S rRNA tree would be free from the effect of a drastic change in a molecule.

Keeping the above limitations in mind, we have constructed a tree by assuming that the rate of nucleotide substitution in 5S rRNAs is constant from bacteria to man. Thus, the phylogenetic tree presented in the present paper is a possible tree and is by no means final. However, we believe that it is valuable to have such a phylogenetic tree that covers practically all the major groups of organisms.

Sequence Alignment and Tree Construction of 5S rRNA

Alignment of 5S rRNA sequences (fig. 1, p. 452) clearly reveals that all the sequences were basically uniform, with frequent but nonrandom nucleotide substitutions along the sequences. This strongly suggests that all of these 5S rRNAs are of single origin. Partial discontinuity between different groups of organisms was also noted, suggesting that the same changes took place in the 5S rRNA on emergence of each group (e.g., between eubacteria and metabacteria, between metabacteria and eukaryotes, and between eubacteria and organelles). Especially, the secondary structure of mitochondrial 5S rRNA is quite different from that of other rRNAs (fig. 2), and it is not possible to estimate the exact divergence point.

Outline of the Phylogenetic Tree

A phylogenetic tree of representative groups of organisms (fig. 3) reveals that eubacteria first separated from the metabacteria/eukaryotes branch. *Sulfolobus, Thermoplasma,* and *Halobacterium*, which collectively we call metabacteria (=archaebacteria, according to Woese and Fox [1977]), form a unique group that is phylogenetically closer to eukaryotes than to eubacteria.

The tree in figure 3 reveals that, in early eukaryotic evolution, red algae (Rhodophyta) evolved first and that Thraustochytrids emerged a little later. Then Ascomycota (yeasts, etc.), green plants (green algae including *Chlamydomonas, Chlorella,* multicellular green algae, and land plants), Basidiomycota (mushrooms, etc.) and yellow algae (chrysophytes, diatoms, and brown algae) emerged within a short period, probably in that order. Thus, the three types of algae (red, green, and yellow algae) are remotely related to one another. A little later, a radiation of the molds (Oomycota, Myxomycota/Amoeba, and Zygomycota), cryptomonads (Cryptophyta), animal flagellates (*Euglena* and Zoomastigina), Dinophyta, and ciliates occurred. Note that amoeba and plasmodial slime molds share a common ancestor. Also, *Euglena* and

animal flagellates are relatively close, as are Dinophyta and ciliates. The branching order of the above-mentioned groups is difficult to estimate precisely, because a relatively large SE is associated with each branching point. Note, however, that the order of the lower eukaryotes as deduced on the basis of 5S rRNA sequences agrees well with the classical view (Taylor 1978).

As will be discussed later, many textbooks classify the slime and water molds as fungi along with Ascomycota and Basidiomycota. However, Ascomycota and Basidiomycota apparently emerged much earlier than these molds; the time of appearance of the molds is approximately the same as that of various groups of so-called protozoans. In any case, "molds" and "protozoans" are very heterogeneous. The Mesozoa and Metazoa arose after the emergence of the above-mentioned molds and protozoans.

We will give below the phylogenetic relationships of most of the major groups of organisms. As for the evolution of various eukaryote groups, the results derived from the 5S rRNA sequences will be compared with classifications adopted in systematic biology that are based mainly on phenotypic characteristics. The phylogeny of "protozoans" is discussed in connection with that of other groups of organisms.

Phylogeny of Eubacteria

Figure 4 shows the existence of at least three major groups of eubacteria, i.e., "cyanobacteria", gram-negative bacteria, and gram-positive bacteria. Differentiation of these three major groups occurred during the early stage of eubacterial evolution. The tree also shows that the differentiation of various bacteria began to occur on each branch shortly after separation of the major groups.

A. Gram-negative Bacteria

This group roughly corresponds to the "purple" bacteria studied by Lane et al. (1985). The gram-negative bacteria can be further separated into three subgroups with respect to their relatedness on the tree. Subgroup A includes most of the enterobacteria (such as *Escherichia, Salmonella,* and *Serratia, Vibrio, Pseudomonas, Azotobacter,* etc.) The G+C content of their genomic DNA is between 45% and 63%. A 5S rRNA tree, mainly focused on the Vibrionaceae, recently was reported (MacDonell et al. 1986). Subgroup B contains some *Thiobacillus* species and the typical denitrifying bacteria such as *Alcaligenes* and *Achromobacter* species (Ohkubo et al. 1986). All the bacteria belonging to subgroups A and B have the 120 N–type 5S rRNA (120 nt in the standard length) as discussed elsewhere (Dekio et al 1984). Subgroup C includes *Rhodopseudomonas, Rhodospirillum, Paracoccus,* etc., which have a relatively high (65%) genomic G+C content.

B. "Cyanobacteria"

This group, consisting of Cyanobacteria and Chloroxybacteria (plus chloroplasts and cyanelle of *Cyanophora*), seems to have emerged shortly before other eubacterial groups (fig. 3). Cyanobacteria are gram-negative bacteria and resemble other gram-negative groups in their 5S rRNA structure and genomic G+C content, and some phylogenetic connection may exist between them.

C. Gram-positive Bacteria

The gram-positive group contains three subgroups, i.e., subgroups A, B, and C (=actinobacterial group). The typical gram-positive bacteria, such as *Bacillus* and *Staphylococcus,* belong to subgroup A, all of them having the characteristic 116 N–

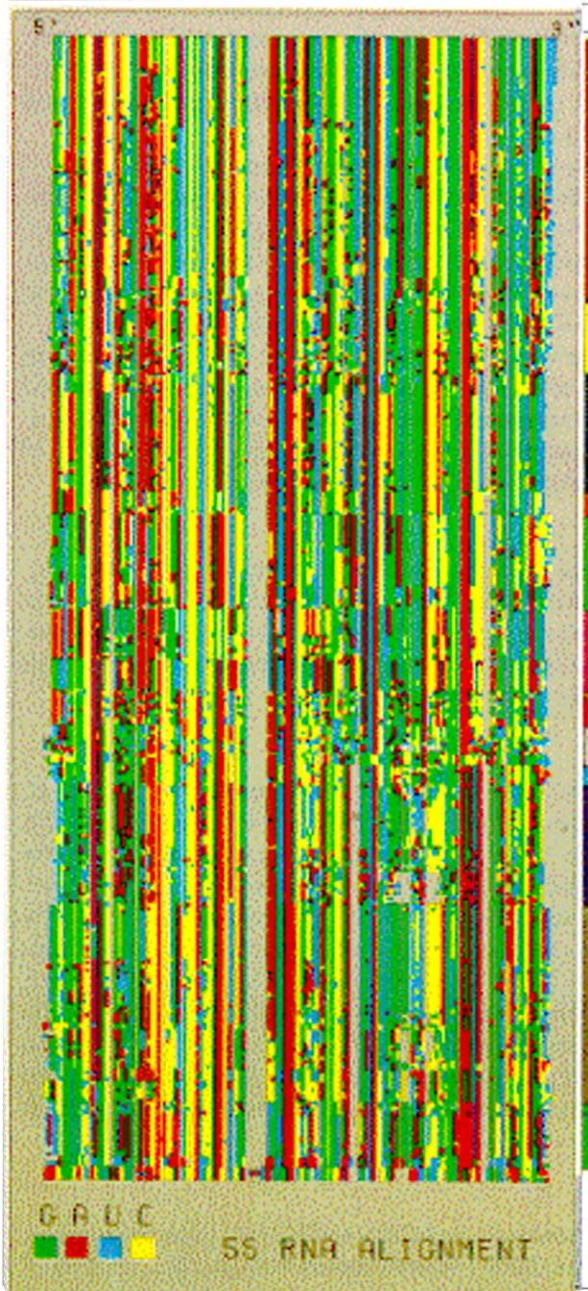

FIG. 1.—Alignment of 5S rRNA sequences. Nucleotide sequences of 352 5S rRNAs—from man (top) to bacteria to organelles (bottom)—were aligned. Red = adenine; blue = guanine; yellow = cytosine; green = uracil; and gray = gaps introduced to improve the alignment. Approximately 60% of these sequences were from eukaryotes (nos. 1–212), 30% from eubacteria (nos. 225–331), and the remainder from metabacteria (nos. 213–224) and organelles (nos. 332–352). Exact number of sequences in each group is shown in parentheses.

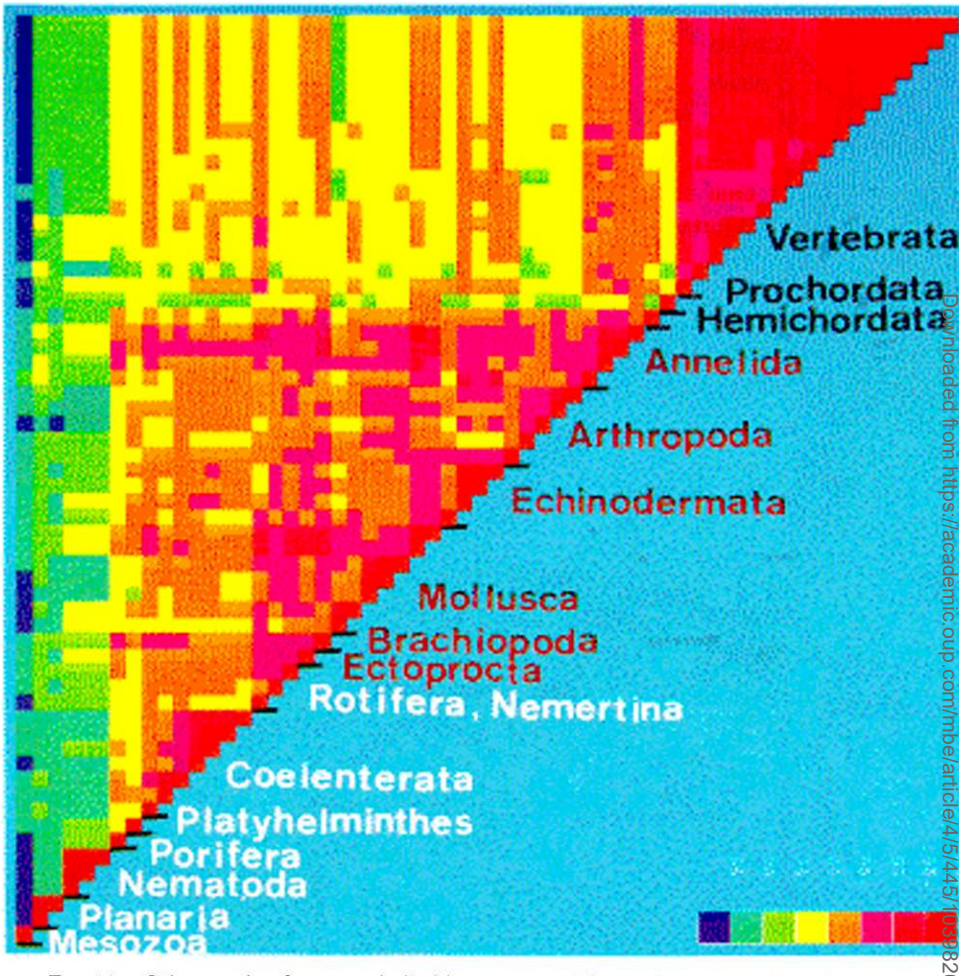

FIG. 11.—Color matrix of percent similarities constructed from 50 representative animal 5S rRNA sequences. Percent similarity of all possible pairs of 5S rRNAs (50-×-50 matrix) was calculated and plotted as color values using a program for the Sony-Techtronix T4116 color graphics system (upper left half of figure = homology triangle). Black = Chordata and relatives; red = phyla of true coleomates; and white = phyla of noncoleomates. "Planaria" is represented only by *Dugesia japonica* (a freshwater planarian; Trichaldia; Platyhelminthes).

type 5S rRNA (116 nt in the standard length; Dekio et al. 1984). *Mycoplasma* and *Clostridium* belong to subgroup B. The *Mycoplasma* 5S rRNA sequences (108 nt) are the shortest in length in all the known 5S rRNAs, with a long deletion in one region (Hori et al. 1981; Walker et al. 1982; Rogers et al. 1985). Subgroups A and B are characterized by a low (35%–45% in subgroup A; 20%–30% in subgroup B) genomic G+C content. The third subgroup includes distinctive gram-positive bacteria, such as *Micrococcus, Streptomyces, Arthrobacter,* and *Mycobacterium,* all of which have a very high (65%–75%) genomic G+C content. Following Margulis and Schwartz (1982), we call these bacteria the actinobacteria. The secondary structure of their 5S rRNA is the "intermediate"-type 5S rRNA between the 116-N type and the 120-N type (118 nt in the standard length; see also Dekio et al. [1984]). Most of the actinobacterial 5S

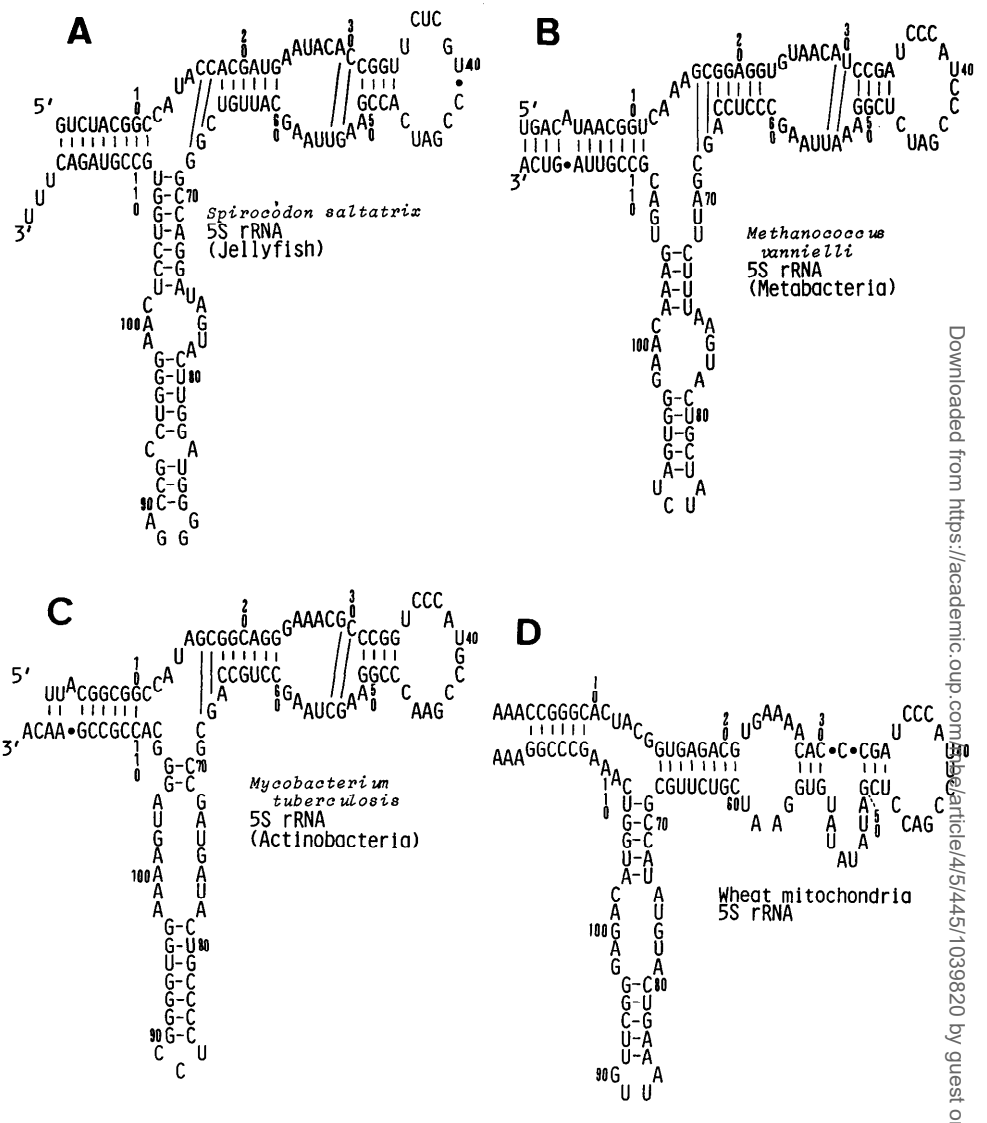

FIG. 2.—Secondary structure of 5S rRNA: *A*, eukaryotic; *B*, metabacterial; *C*, eubacterial; and *D*, mitochondrial. Numbering is based on the *Escherichia coli* 5S rRNA sequence in the alignment (fig. 1). Models *A–C* show fundamentally the same structure, whereas model *D* (mitochondria) has unusual deletions and insertions in certain regions of 5S rRNA. Sequences and secondary-structure models of *B*, *C*, and *D* are according to Vandenberghe et al. (1985), Ohkubo et al. (1986), and Spencer et al. (1981), respectively.

rRNA have a unique bulge in the terminal helix (A-A′ helix; fig. 2*C*), like that in the metabacterial 5S rRNA (fig. 2*B*), suggesting some relationship between actinobacteria and metabacteria (Hori and Osawa 1986).

D. Chloroplast and Mitochondria

Chloroplasts have a typical eubacterial type 5S rRNA (fig. 2*C*), and their phylogenetic position in the tree can be estimated (fig. 3). On the other hand, plant-mitochondrial 5S rRNA is very different from other 5S rRNAs, having unusual in-

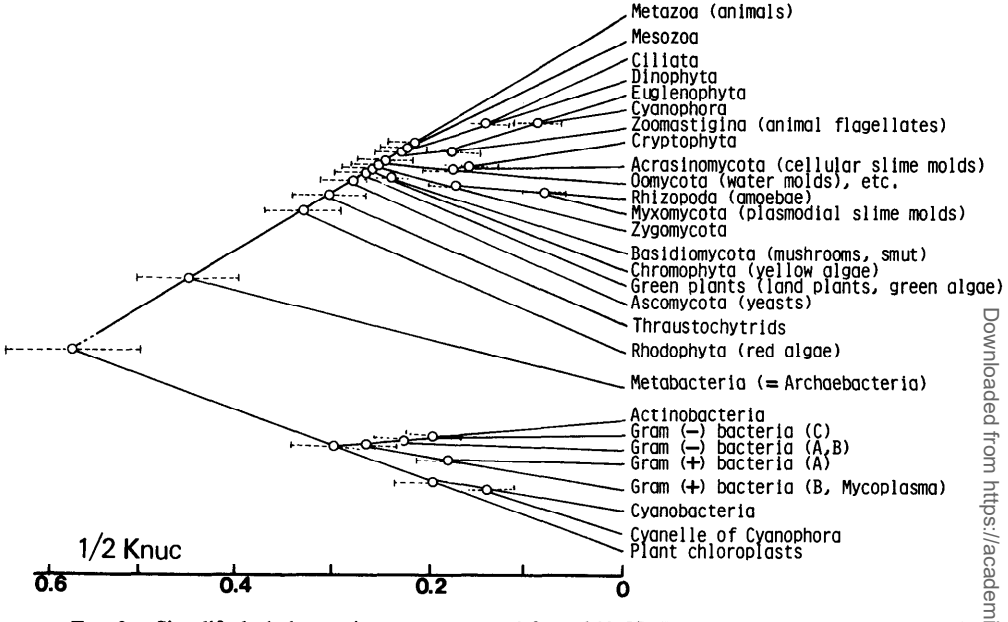

FIG. 3.—Simplified phylogenetic tree constructed from 353 5S rRNA sequences. ½ *Knuc* = relative evolutionary distance deduced from equation (1). |--- O ---| = Range of SE of ½ *Knuc*. SE_K is according to equation (3).

sertions and deletions in certain regions (Spencer et al. 1981; fig. 2*D*). Comparing some conserved regions of 5S rRNAs (52 nucleotide positions), Villanueva et al. (1985) claimed a close relationship between the plant mitochondria and the purple photo-

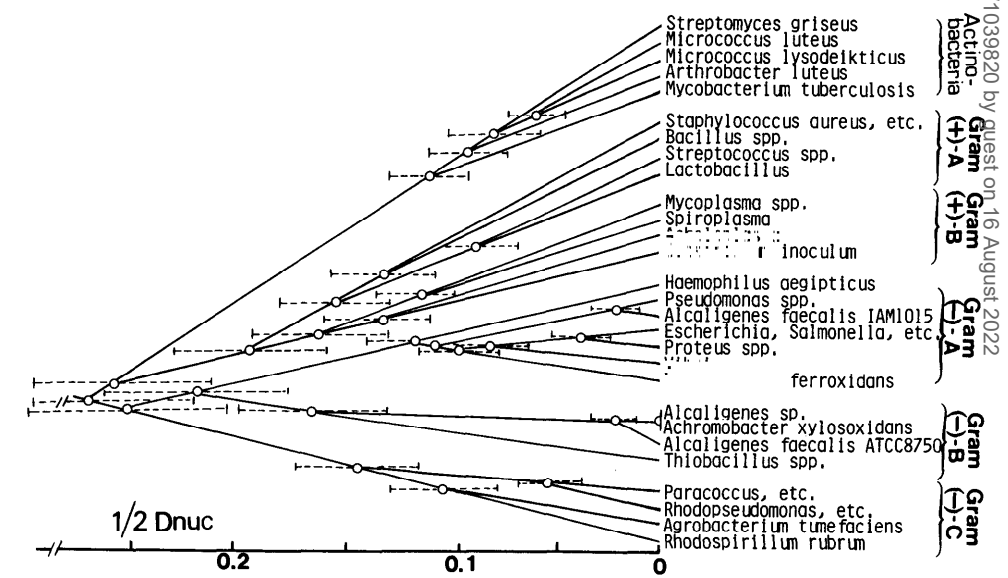

FIG. 4.—Phylogenetic tree of 5S rRNAs from representative eubacteria. ½ *Dnuc* corresponds to ½ *Knuc* but was calculated with consideration of G+C content of 5S rRNAs (see eq. [2] in the text).

synthetic bacteria. However, we believe that it is unreasonable to include the mitochondrial 5S rRNA in the phylogenetic tree, because its structure is drastically different.

Phylogeny of Metabacteria

Sulfolobus, Thermoplasma, halophiles (*Halobacterium* and *Halococcus*), and methanogens form a unique group in bacteria (Woese and Fox 1977). Using their RNase T_1 digest catalog of 16S rRNAs ($=S_{AB}$ method), Woese and his co-workers (see Woese 1981) concluded that these bacteria ("archaebacteria," according to Woese) are the most ancient bacterial group. However, as we have already pointed out (Hori and Osawa 1979; Hori et al. 1982) and as shown in the tree in figure 3, what we call metabacteria (=archaebacteria) are, on the basis of the 5S rRNA sequence comparisons, phylogenetically closer to eukaryotes than to eubacteria. This is consistent with the similarity, in terms of the secondary-structure model of 5S rRNA, between eukaryotes and metabacteria (fig. 2).

A 5S rRNA tree (fig. 5) clearly shows that all metabacterial species examined herein belong to one branch and are not polyphyletic. Emergence of *Sulfolobus* occurred at a very early time, followed by emergence of *Thermoplasma,* various species of methanogens, and, much later, by halophiles. Note that the secondary structure of 5S rRNA from *Thermoplasma* and *Sulfolobus* is somewhat different from that of 5S rRNA from methanogens and halophiles.

Phylogeny of Photosynthetic Eukaryotes

A. Red Algae (Rhodophyta)

The Rhodophyta, consisting of ~4,100 species in 675 genera, is a highly distinctive group, having chlorophyll *a* pigment and phycobilins as accessory photosynthetic pigments.

The 5S rRNA data indicate that all the red algae examined herein belong to one branch (fig. 3) and diverged as shown in figure 6. The emergence point is estimated to be ~1.3–1.4 billion years ago, if the yeast-animal divergence time is taken to be 1.2 billion years ago (Kimura and Ohta 1973). Thus, the emergence of red algae is

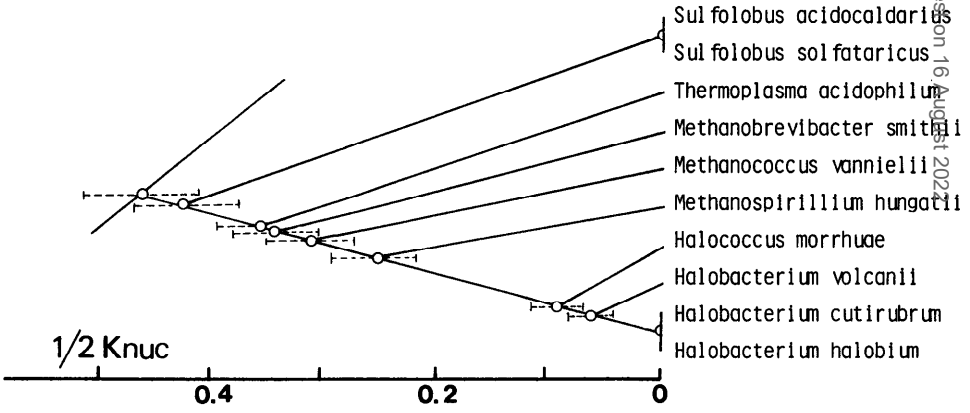

FIG. 5.—Phylogenetic tree of 5S rRNAs from Metabacteria (=Archaebacteria). *Knuc* and its SE are as given in the legend to fig. 3.

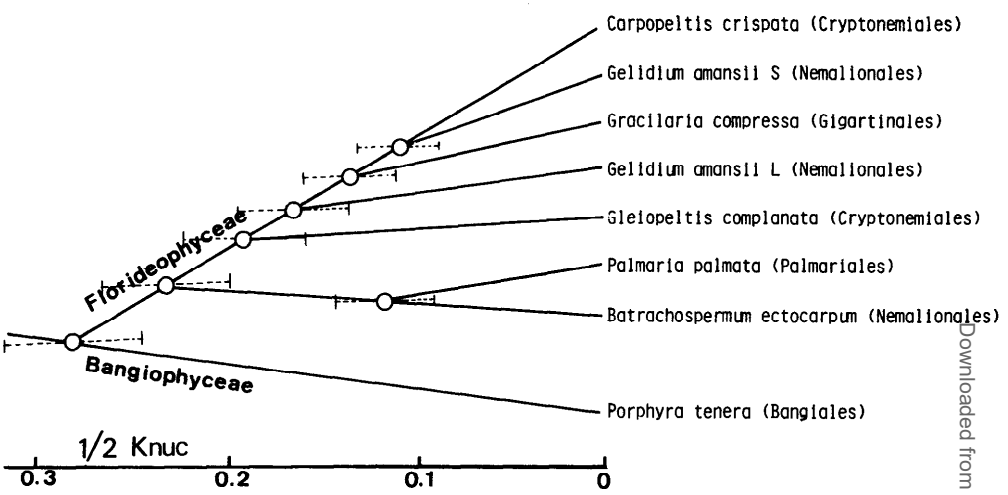

FIG. 6.—Phylogenetic tree of 5S rRNAs from Rhodophyta. Order names in parentheses are according to Kraft (1981). *Knuc* and its SE are as given in the legend to fig. 3.

the most ancient event so far detected in the evolution of eukaryotes, and the separation of the Rhodophyta species examined herein took place at very early times.

Traditionally, the Rhodophyta have been divided into two classes, the "primitive" Bangiophyceae and the "more advanced" Florideophyceae (Dixon 1973). However, there is an opinion that Bangiophyceae species such as *Porphyra* derived from the Nemalionales of Florideophycean algae by means of degeneration (see Kraft 1981). All the Florideophyceae species examined herein—including the species of the Nemalionales, such as *Batrachospermum*—belong to the same branch, whereas *Porphyra* (Bangiophyceae) emerged from the common ancestor of the Florideophyceae species in an early stage of eukaryotic evolution. Thus, the 5S rRNA data support the classical view that the Rhodophyta is divided into two classes, the more primitive Bangiophyceae and the more advanced Florideophyceae (Dixon 1973).

B. Green Plants

All green plants examined herein, such as vascular plants (Pteridophyta and Spermatophyta [=seed plants]), Bryophyta, and green algae, belong to the same green-plant branch (fig. 7). In this branch, emergence of *Chlamydomonas* occurred very early. Various green algae and stonewort (*Nitella*)/land plants then separated from each other. Thus, it is possible that green plants originated from some type of a green, flagellated organism such as *Chlamydomonas* (see Darley 1982; Hori et al. 1985*a*). Among the green algae, *Ulva* separated from *Spirogyra/Chlorella/Scenedesmus* first, and differentiation of these latter three then followed.

Recently, on the basis of comparative ultrastructure work on cell division and zoospore anatomy, Stewart and Mattox (1975) emphasized a close relationship of *Spirogyra* and *Nitella* to land plants and placed these two algae in Charophyta. As mentioned above, however, the 5S rRNA sequence of *Spirogyra* is closely related to the sequences of both the unicellular freshwater green algae (*Chlorella* and *Scenedesmus*) and the multicellular *Ulva* species but not to that of *Nitella*. Thus, the 5S rRNA data do not support their view.

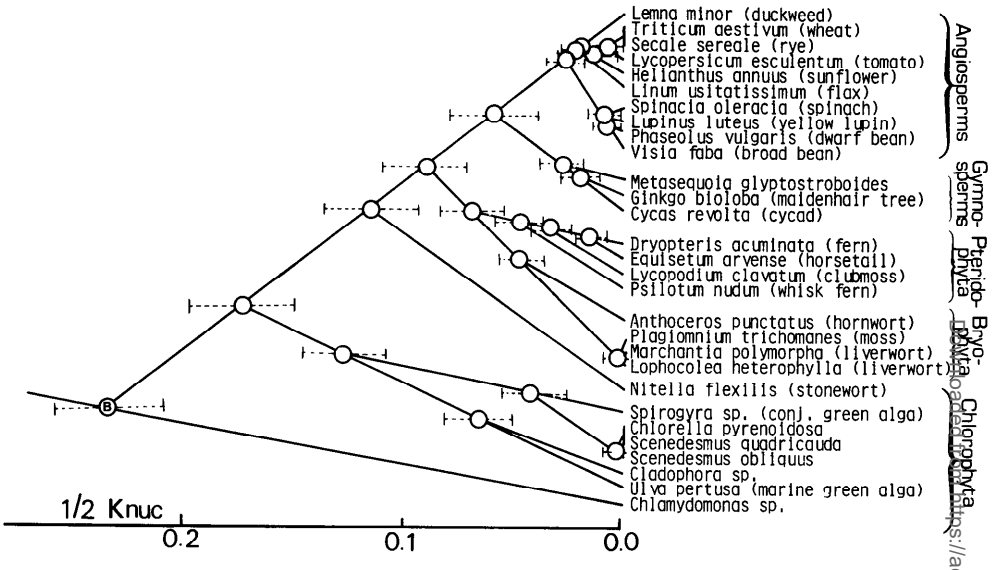

FIG. 7.—Phylogenetic tree of 5S rRNAs from green plants. *Knuc* and its SE are as given in the legend to fig. 3.

It is generally accepted that land plants and green algae have a common ancestor and that land plants were probably derived from some form of Charophyta such as *Nitella* (see Darley 1982). The 5S rRNA comparison between *Nitella* and land plants suggests that Charophyta emerged just before seed plants and Pteridophyta/Bryophyta separated. Thus, this result is consistent with the view that the ancestor of the present-day *Nitella* would be the precursor to land plants.

Seed plants and Pteridophyta are often grouped as vascular plants (see Bold 1970). The general agreement is that these vascular plants evolved from Bryophyta-like organisms lacking a vascular system. However, the 5S rRNA tree shows that Pteridophyta and Bryophyta are sister groups, separate from seed plants (fig. 7). Thus, the tree does not agree with this view and is consistent with the opinion that Bryophyta evolved from ferns by means of degeneration (Schuster 1966; Inoue 1978). This conclusion is also consistent with the fact that Bryophyta fossils have never been found in geological strata earlier than those containing fern fossils.

Within the Bryophyta, the 5S rRNA tree shows that hornworts separated first and that this separation was followed by differentiation of liverworts and mosses (Hori et al. 1985*a*). This picture is in agreement with the classical view that hornworts are evolutionally distinct from liverworts and mosses (see Bold 1970).

From primitive to advanced, the Pteridophyta species examined herein may be arranged, on the basis of anatomical evidence, in the order *Psilotum* (whisk fern), *Lycopodium* (club moss or ground pine), *Equisetum* (horsetail), and *Dryopteris* (fern) (see Bold 1970). The 5S rRNA tree shows that *Psilotum* separated first and that a little later *Lycopodium* separated from the ancestor common to *Equisetum* and *Dryopteris*. The latter two separated more recently. Thus, the branching order deduced from the 5S rRNA sequences agrees perfectly with the classical view.

There are two major hypotheses regarding the evolutionary process within seed plants. The first one is that, after separation from Pteridophyta, the ancestor of seed

plants evolved into two groups—one containing Ginkgophyta (maidenhair tree), Coniferophyta (coniferous trees), and Gnetophyta (e.g., joint fir) and another containing Cycadophyta (cycads) and angiosperms (flowering plants). The latter two share the common ancestor called pteridosperms ("seed-ferns"; see Margulis and Schwartz 1982). The second hypothesis supposes that gymnosperms (including cycads, maidenhair tree, and coniferous trees) and angiosperms (including flowering plants) separated sometime during seed-plant evolution (see Bold 1970). The first hypothesis assumes that cycads are more closely related to flowering plants than to maidenhair tree and coniferous trees, whereas in the second hypothesis cycads, maidenhair tree, and coniferous trees are more closely related to one another than to flowering plants. The 5S rRNA phylogenetic tree clearly shows that *Metasequoia* (a coniferous tree), *Cycas* (a cycad), and *Ginkgo* (maidenhair tree) are closely related. The separation of these three species occurred after their separation from the ancestor of flowering plants, a circumstance supporting the second hypothesis.

C. *"Chromophyta"*

Brown algae (Phaeophyta), diatoms (Bacillariophyta), golden-yellow algae (Chrysophyta), Dinophyta, and Cryptophyta are sometimes grouped together in the superdivision Chromophyta because of their having chlorophylls *a* and *c* and unique storage substances, i.e., laminanin or chrysolaminarin (Taylor 1978; Corliss 1984). The comparison of 5S rRNA sequences from various Chromophyta species indicates that five brown algae, a diatom, and a golden-yellow alga are more closely related to one another (mean identity 74%, range 68%–81%) than to other photosynthetic eukaryote groups (mean identity 63%, range 52%–68%). It would thus appear that three major Chromophyta species examined here are closely related, as shown in the tree of figure 8, a circumstance supporting Taylor's view (1978). On the other hand, the other two Chromophyta groups—i.e., Dinophyta and Cryptophyta—form independent groups (see below). This suggests that "Chromophyta" species examined here (excluding Dinophyta and Cryptophyta) should be grouped together in the superdivision of the Heterokontae or heterokont algae. This view is consistent with the fact that they all have heterokont flagella (see Corliss 1984).

In classical taxonomy, diatoms (Bacillariophyta) are sometimes included in the golden-yellow algal group (Chrysophyta) (see Alexopoulos and Bold 1967). Both have

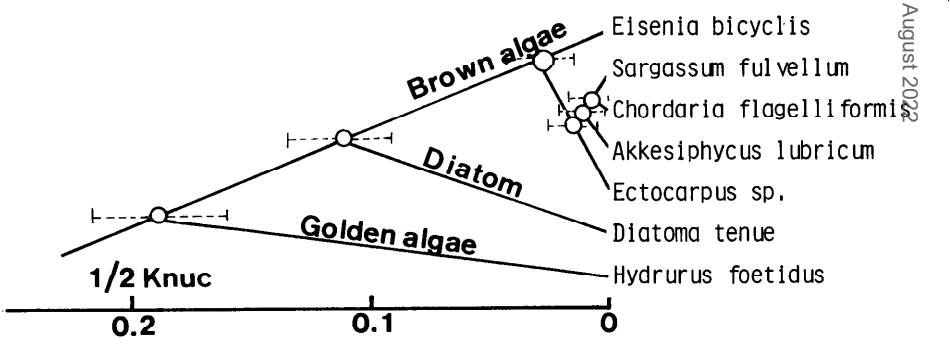

FIG. 8.—Phylogenetic tree of 5S rRNAs from Chromophyta. *Knuc* and its SE are as given in the legend to fig. 3.

chrysolaminarin as a photosynthetic food reserve, although diatoms differ considerably from golden-yellow algae in many important respects (e.g., life-cycle, cell structure, and cell division). The percent similarity between diatoms and golden-yellow algae indicates that the diatom 5S rRNA sequence is more related to sequences from seven species of brown algae (Phaeophyta) (mean identity 81%, range 80%–81%) than to that from the golden-yellow alga *Hydrurus* (Chrysophyta) (mean identity 68%). The 5S rRNA from *Hydrurus* is less similar to those from brown algae and the diatom (mean identity 73%, range 68%–75%). The "Chromophyta" branch of the 5S rRNA phylogenetic tree clearly shows the golden-yellow alga *Hydrurus* separating first, then the diatoms (*Diatoma*) and brown algae (Phaeophyta) separating from each other (fig. 8). Thus, this picture suggests that diatoms and golden-yellow algae are not too closely related, a circumstance supporting the view that these two groups of organisms should be placed in separate taxonomical groups.

Phaeophyta, which consists of 270 genera and 1,500 species, is one of the most morphologically diversified eukaryotic groups. Wynne (1981) suggested that the Phaeophyta should be classified into 14 orders according to the difference in forms and life histories. The sequences of 5S rRNA from five typical brown-algal species—i.e., *Eisenia bicyclis* (order Laminariales), *Sargassum fulvelum* (Fucales), *Ectocarpus* species (Ectocarpales), *Chordaria flagelliformis forma chordaeformis* (Chordariales), and *Akkesiphycus lubricum* (Dictyosiphonales)—which cover the representative major orders of this phylum and have very different morphology and life history, clearly indicate that the percent similarity among them is very high (97%–99%). Thus, all the brown algae examined here separated from one another within a very short time (fig. 8), long after the separation from diatoms. This divergence point was ~0.2 billion years ago.

D. Euglena and Cyanophora

The phylogenetic position of *Euglena* remains unclear; in some cases it has been classified among plants, in other cases among protozoans (Corliss 1984). It has been pointed out that *Euglena* has many biochemical characteristics of animal nature, even though *Euglena* cells normally contain chloroplasts equipped with chlorophyll *a* and *b* (Ragan and Chapman 1978). On the other hand, *Cyanophora* has been considered as one of the Glaucophyta having a chloroplast-like cyanelle. According to the 5S rRNA data, *Cyanophora* is phylogenetically closer to *Euglena* than to other eukaryotes, including *Chilomonas* (a cryptomonad) and green plants. It is interesting that *Cyanophora* contains only chlorophyll *a* (see the following section). The tree in figure 3 also indicates that *Euglena* and *Cyanophora* are phylogenetically more related to other protozoans and animals than to plants, a circumstance supporting the biochemical evidence cited above.

E. Photosystem Evolution

In classical botany (Ragan and Chapman 1978), photosynthetic pigments found in plastids constitute one of the most important characters for classifying each phylum. A recent plant phylogeny based on these pigment characters postulates three major evolutionary lines (Ragan and Chapman 1978; Taylor 1978). Cyanobacteria and Rhodophyta, having only chlorophyll *a*, are supposed to form one ancient line of descent. The second line, having chlorophyll *a* and *c*, includes Chrysophyta, Dinophyta, Cryptophyta, Bacillariophyta, Xanthophyta, and Phaeophyta. The third line, having chlo-

rophyll *a* and *b,* includes Euglenophyta, Chlorophyta, and land plants. Some plant physiologists believe that chlorophyll *a* in these eukaryotes is a result of symbiosis of a prokaryote having chlorophyll *a* (e.g., a prokaryote of a Cyanobacteria species) at an early stage of eukaryotic evolution; chlorophyll *b* and *c*, according to them, then developed independently in the respective lines (see Doolittle 1982). However, this hypothesis demands that all the present-day eukaryotes that once—or even until recently—lacked chloroplasts (e.g., eukaryotes such as fungi, many protozoans, and animals) had had them and then lost them. This is not impossible, but it is more plausible that the symbiosis of photosynthetic prokaryotes to different lines of photosynthetic eukaryotes was due to mutually independent multiple occurrences—i.e., that the symbiosis took place *after* the branching of each respective line. For example, the direct ancestor of the present-day Rhodophyta (all of which have chlorophyll *a*) received a Cyanobacteria carrying chlorophyll *a* sometime after the emergence of the line. Similarly, the direct ancestor of green plants (all of which have both chlorophyll *a* and chlorophyll *b*) received a symbiotic prokaryote after the branching. In this case, chlorophyll *b* either developed after the symbiosis or was brought about by a symbiosis of a prokaryote having both chlorophyll *a* and chlorophyll *b*. The latter possibility is not unlikely, since Chloroxybacteria are said to contain both chlorophyll *a* and chlorophyll *b*. No bacteria having both chlorophyll *a* and chlorophyll *c* have ever been found, although their existence is not improbable.

Dinophyta and Cryptophyta contain chlorophyll *a* and chlorophyll *c* but do not belong to the "Chromophyta" branch in the tree (fig. 3). *Cyanophora* contains only chlorophyll *a* but surely does not belong to the Rhodophyta branch. Also, *Euglena* (having both chlorophyll *a* and chlorophyll *b*) does not belong to the green-plant line. These groups diverged successively at approximately the same time that "Chromophyta" emerged. Thus, the three-lines hypothesis of plant phylogeny, which is based only on pigment characters, is in disagreement with the 5S rRNA data. These discrepancies may easily be explained if we accept as true the multiple symbiotic events discussed above. In the above discussion, the symbiotic organisms are considered to be Cyanobacteria-like prokaryotes. This is probably true for Rhodophyta, green plants, and *Cyanophora* since their chloroplast 5S rRNA (cyanelle 5S rRNA in *Cyanophora*) is very close to that from Cyanobacteria. However, chloroplasts of Dinophyta, Cryptophyta, and *Euglena* are structurally different from those of green plants and red algae in that they are enclosed in three or four membranes (Ludwig and Gibbs 1982). Furthermore, the secondary structure of *Euglena* chloroplast 5S rRNA is quite different from that of the typical cyanobacterial 5S rRNA (Karabin et al. 1983). Gibbs (1978) and Ludwig and Gibbs (1985) have suggested that these chloroplasts have evolved through two sequential symbioses, a prokaryote-eukaryote symbiosis and a eukaryote-eukaryote symbiosis. The *Euglena* chloroplast 5S rRNA might have undergone drastic structural changes during such a complicated symbiotic process; even the rate of nucleotide substitution might have increased, as has been shown to have occurred in the case of mitochondria (Miyata et al. 1982).

Phylogeny of Fungi

A. Outline

Fungi traditionally are classified as being within at least 11 "phyla," as shown in table 2. However, putting all of these groups into one category—i.e., "fungi"—has no logical basis, because a number of fundamental differences exist among them. In fact,

slime molds are often treated as members of Protozoa. Margulis and Schwartz (1982) divided fungi into two "kingdoms", the more "advanced kingdom" of fungi, which includes four groups (Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota), and a more "primitive kingdom" of Proctista, which contains mainly various slime and water molds (table 2). A fine reclassification of each kingdom is complicated and will only be partially given in each pertinent section below. The 5S rRNA sequences available are limited to those shown in table 2. These organisms by no means represent all the fungal groups, so only limited discussions of their phylogeny can be made at present.

The 5S rRNA tree in figure 3 indicates that the thraustochytrid Proctista diverged very early, a little after the Rhodophyta emergence. Ascomycota evolved next, then

Table 2
Classification of Fungi

"Kingdom"	"Phylum" (Common Name[s] of Phylum or of Representative Organisms)	Species Whose 5S rRNA Sequences Have Been Reported
Proctista	Acrasiomycota (cellular slime molds)	*Dictyostelium discoideum*
	Myxomycota (plasmodial slime molds)	*Physarum polycepharum*
	Labyrinthulamycota (net slime molds)	NR
	Plasmodiophoromycota (endoparasitic slime molds)	NR
	Hyphochytridiomycota (anteriorly unflagellated fungi)	NR
	Chytridiomycota (posteriorly unflagellated fungi)	NR
	Oomycota (water molds, white rusts, downy mildews)	*Blastocladiella simplex, Phlyctochytrium irregulare, Phytium hydnosporon, Saprolegnia ferax*
	Thraustochytrids fungi[a]	*Thraustochytrium visurgense, Schizochytrium aggregatum*
Fungi	Zygomycota (bread molds, fly fungi, animal traps)	*Phycomyces blakesleenus*
	Ascomycota (sac fungi, bread yeast)	*Saccharomyces cerevisiae* and eight other species
	Basidiomycota (rusts, smuts, jelly fungi, mushrooms)	*Auricularia auricula-judae* and 21 other species
	Deuteromycota (imperfect fungi)	*Aspergillus niger, A. flavus, A. nidulans, Penicillium chrysogenum, P. patulum, Thermomyces lanuginosus, Acremonium persinum, Rhizoctonia crocorum, R. hiemalis*

NOTE.—Classification is mainly that of Margulis and Schwartz (1982). NR = not reported.
[a] Usually classified in Oomycota but sometimes in Hyphochytridomycota or Labyrinthulamycota.

Basidiomycota, and finally Proctista fungi such as slime and water molds. Thus, along with red algae, thraustochytrids are among the most primitive eukaryotes and are only remotely related to other fungi groups. Furthermore, although the above-mentioned four groups are treated collectively as fungi, they do not belong to one phylogenetic branch in the 5S rRNA tree. Even in the Proctista, the Acrasinomycota (cellular slime molds), Myxomycota (plasmodial slime molds), and Oomycota (water molds) are not phylogenetically close, having emerged independently at approximately the same time as other protozoans (amoeba, flagellates, ciliates, etc.). Although the Zygomycota were classified by Margulis and Schwartz (1982) as belonging to the true fungi, the 5S rRNA data suggest that they emerged at approximately the same time as did the slime and water molds. The protozoan groups mentioned above are also remote phylogenetically, as is the case for the Proctista. Thus, both the Proctista and protozoans comprise very heterogenous entities, and most of their members appear to have diverged fairly early (~0.9–1.0 billion years ago). As already mentioned (see "Outline of the Phylogenetic Tree and *Euglena* and *Cyanophora*" above; also see Kumazaki et al. 1983*a*), plasmodial slime molds and amoeba are more closely related.

B. Ascomycota

Ascomycota is usually divided into the following two subgroups: Hemiascomycetes, in which the asci are produced "singly," and Euascomycetes, in which the asci are formed on ascogenous hyphae, usually within a fluid body. Euascomycetes is subdivided into three groups—Plectomycetes, Pyrenomycetes, and Discomycetes—on the basis of the morphology of the fluid bodies.

The 5S rRNA tree (fig. 9) generally agrees with the above classification: first, Hemiascomycetes (*Saccharomyces, Pichia,* and *Tolulopsis*) and Euascomycetes separated from one another; then, in the Euascomycetes branch, *Plectomycetes* (*Aspergillus* and *Penicillium*) and Pyrenomycetes (*Neurospora*) separated (see Huysmans et al. 1983). *Aspergillus, Penicillium, Thermomyces,* and *Acremonium* are sometimes classified as being within Deuteromycota (imperfect fungi), because of the lack of a sexual stage in the Ascomycota members (table 2). However, the 5S rRNA tree clearly shows that the first three of these four species are included in Plectomycetes (Euascomycetes), whereas *Acremonium* belongs to Pyrenomycetes (Euascomycetes). These four species probably lost the sexual stage during evolution. A fission yeast, *Schizosaccharomyces pombe,* although classified as being within Ascomycota, is related to the Proctista group rather than to Ascomycota.

C. Basidiomycota

The classification of Basidiomycota has been based primarily on the anatomy of basidium. The first group, Heterobasidiomycetes, includes rusts, smuts, and jelly fungi that have either longitudinally or transversely separated basidia, whereas the second group, Homobasidiomycetes, includes mushrooms that have a single-cell basidium (see Alexopoulos and Bold 1967).

The 5S rRNA sequences from Basidiomycota species were phylogenically analyzed by Walker and Doolittle (1982), Huysmans et al. (1983), Gottschalk and Blanz (1984), and Walker (1984). Considering their 5S rRNA tree, Huysmans et al. (1983) claimed that a group of Basidiomycota (Teliomycetes; see below) may be a common ancestor of other Basidiomycota and Ascomycota and suggested a polyphyletic origin of Basidiomycota. However, in the 5S rRNA tree, Ascomycota emerged first, followed by green plants and finally by Basidiomycota including Teliomycetes, a circumstance

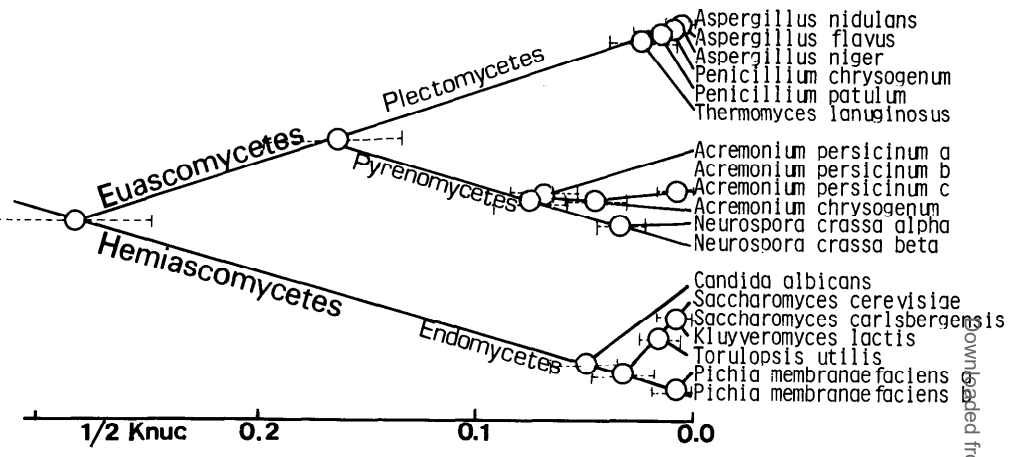

FIG. 9.—Phylogenetic tree of 5S rRNAs from Ascomycota. *Knuc* and its SE are as given in the legend to fig. 3.

suggesting a monophyletic origin of Basidiomycota; all the Basidiomycota species examined herein belong to one branch, with the exception of *Agaricostilbum palmicolum* (Walker 1984). A possible ancestor of Basidiomycota would be some Chromophyta species, since percent homology of 5S rRNA (mean 83%, range 80%–85%) reveals a relatively close relationship between them.

The 5S rRNA tree of Basidiomycota (fig. 10) shows that some smuts (*Ustilago, Rhodosporidium,* and *Aessosporon* [Heterobasidiomycota, Teliomycetes]) separated first, followed by the emergences of several groups of Heterobasidiomycota. Relatively recently, a group of Heterobasidiomycota including other smuts (*Filobasidium* spp.), rusts (*Puccinia* and *Gymnosporangium*), etc. emerged at about the same time as most of the Homobasidiomycota members (mushrooms). Thus certain groups of Heterobasidiomycota mentioned above are more closely related to Homobasidiomycota than to other Heterobasidiomycota. It is also noteworthy that *Tremella* and *Auricularia* (jelly fungi), which in this tree are placed in Heterobasidiomycota, are closely related to the Homobasidiomycota mushrooms. Percent homology between jelly fungi and all the Homobasidiomycota members is 90%–95% (mean 92%), whereas that between jelly fungi and other Heterobasidiomycota species is only 80%–85% (mean 80%). From these data, it is interesting to postulate that early in Basidiomycota evolution various groups of Heterobasidiomycota sequentially emerged. Long after, a group of Heterobasidiomycota such as some smuts (*Filobasidium*, etc.) and rusts began to develop. The ancestor of this group might have served as the precursor of mushrooms. The existence in the 5S rRNA tree of jelly fungi (Heterobasidiomycota) among other mushrooms (Homobasidiomycota) might represent such a process.

D. Deuteromycota (=Fungi Imperfecti)

Since Deuteromycota species (imperfect fungi) lack sexual stages, they are conventionally placed in the "kingdom" Fungi along with Ascomycota and Basidiomycota (perfect fungi) (see table 2). As already has been pointed out (in the Ascomycota section), four species have been classified as being within this group—i.e., *Aspergillus, Penicillium, Thermomyces,* and *Acremonium* belong to Ascomycota—whereas *Rhizoctonia crocorum* and *R. hiemalis* have been found to belong to Basidiomycota.

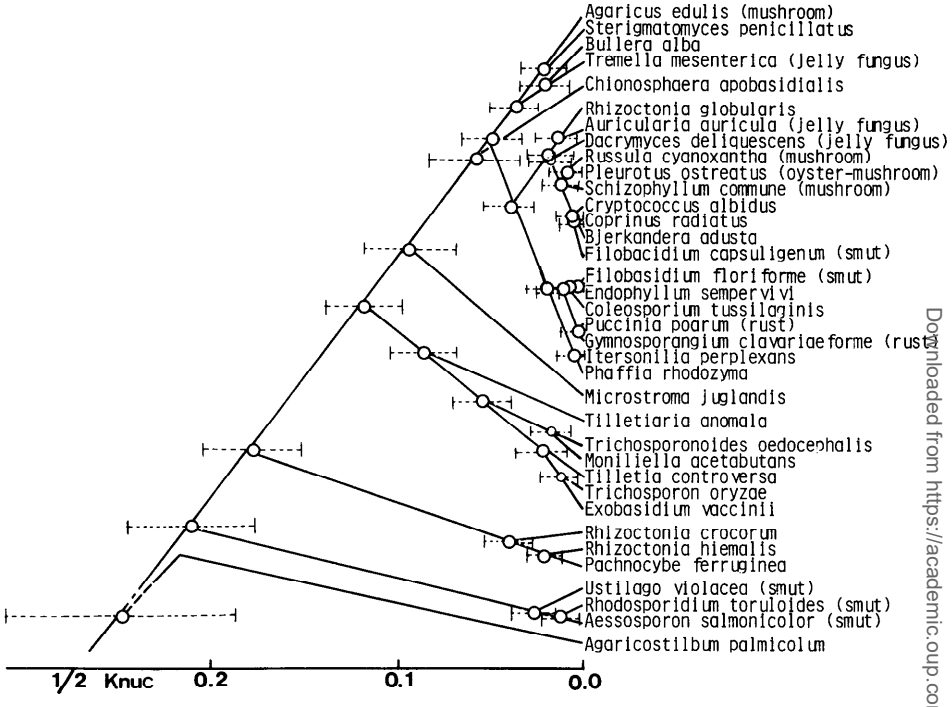

FIG. 10.—Phylogenetic tree of 5S rRNAs from Basidoimycota. *Knuc* and its SE are as given in the legend to fig. 3.

Phylogeny of Animals

A. Origin of Metazoa

Because of animals' enormous phenotypic diversification, phylogenetic relationships among them, especially the origin of metazoan animals, are obscure. Many different phylogenetic trees have been constructed by zoologists (see Hanson 1977). There are at least four hypotheses. (1) The main line of opinion follows Haeckel's gastrea theory, which stipulates that embryogenesis repeats phylogenetic history. Thus, a blastula-like organism, such as ball-shaped flagellates (=*Volvox*-like chlorophyta), would have been the ancestor of animals, from which gastrula-like organisms of radial symmetry, such as Coelenterata, would have emerged next. The animals of bilateral symmetry, such as flatworms (planarians), then would have differentiated from the Coelenterata-like animals, followed by the differentiation of various metazoans. (2) Hadzi (1963) proposed that the most ancient type of metazoan is a flatworm that originated from some ciliated protozoan and that this flatworm then evolved into a nematode-like organism and served as the common ancestor to various metazoan groups, including Coelenterata. (3) The third opinion is that the metazoans are of polyphyletic origin—i.e., that the sponges, Coelenterata, and flatworms emerged independently. The sponges and Coelenterata probably evolved independently from colonial flagellates and the flatworms probably evolved from ciliates—whereas both Haeckel's and Hadzi's schools consider that sponges were derived directly from some protozoan before the development of other metazoans. (4) In addition to the above "key" animal groups, there exists one other group called Mesozoa, which is sometimes considered as an ancestor of metazoans (Lapan and Morowitz 1972), though the recent

majority opinion is that mesozoans evolved from flatworms by means of degeneration (see Margulis and Schwartz 1982).

On the basis of the 5S rRNA alignment (fig. 1), percent similarities of all possible pairs of animal sequences were calculated and summarized in a similarity triangle of 50 representative species (fig. 11, p. 453). This schema does not show as much detail as a dendrogram, but groups with high similarities can be distinctly recognized. For example, all vertebrates consist of a clear red triangle, and each of almost all invertebrate groups forms a cluster. A similar triangle of 352 × 352 dimension that uses all the known 352 sequences (Hori and Osawa 1986) indicates that all metazoan species—including sponges, Coelenterata, and flatworms—form a distinct cluster. This suggests a single origin of all metazoans, in accordance with the 5S rRNA tree (fig. 12) that shows all metazoans to have derived from a common ancestor.

The 5S rRNA phylogenetic tree (fig. 3) shows that ciliated protozoans, Euglenophyta, and mesozoans diverged at approximately the same time during the early stage of metazoan evolution. The separation took place at approximately the point of a ½ *Knuc* value of 0.22 ± 0.04. The *Knuc* values of these three groups are so close to each other that their exact sequence of emergence is difficult to estimate. Thus, there is a possibility that some mesozoan-like organism is the ancestor common to all metazoans, including flatworms (planarians) and nematodes. Another possibility is that the mesozoa are a specialized branch of the protozoans and did not give rise to metazoans. At present, we do not have any conclusive evidence to decide which alternative is correct. It seems clear, however, that the mesozoa are not a degenerate line derived from planarians, since planarians apparently emerged much later (see below).

After the emergence of protozoans and mesozoans, freshwater planarians (*Dugesia*) (but not marine planarians [*Planocera*]) and then nematodes (*Caenorhabditis*) separated from the ancestors of the majority of the metazoan phyla (fig. 12). Their branching points are located at mean ± SE ½ *Knuc* values of 0.16 ± 0.03 and 0.15 ± 0.03, respectively. Other principal metazoan phyla emerged between ½ *Knuc* values of 0.13 and 0.05. The most plausible phylogenetic explanation for these results would be that freshwater planarians and nematodes are of relatively ancient origin in animal evolution. This picture is consistent with Hadzi's (1963) view that planarians and nematodes are ancestors of various metazoans.

B. Invertebrate Evolution

For many years (and even today), biologists divided invertebrates into two groups: (1) those lacking a true coelum (acoelomates and pseudocoelomates [=noncoelomates]) and (2) those that develop a true coelum (coelomates). The color matrix in figure 11 shows the relationship between the invertebrate phyla of coelomates (names shown in red) and those of noncoelomates (names shown in white). The coelomated phyla (Annelida, Arthropoda, Echinodermata, Mollusca, Brachoipoda, and Ectoprocta in fig. 11) and two pseudocoelomated phyla (Rotifera and Nemertinea) may be recognized as a cluster, whereas other noncoelomated phyla—such as Coelenterata, Platyhelminthes, Porifera, and Nematoda—are dissimilar from each other and do not form a cluster. Moreover, the 5S rRNA tree (fig. 12) shows earlier emergences for the noncoelomated phyla than for the coelomated phyla (except for Chordata; see below), suggesting that the emergence of the former preceded the latter, in accordance with the view in the classical textbooks.

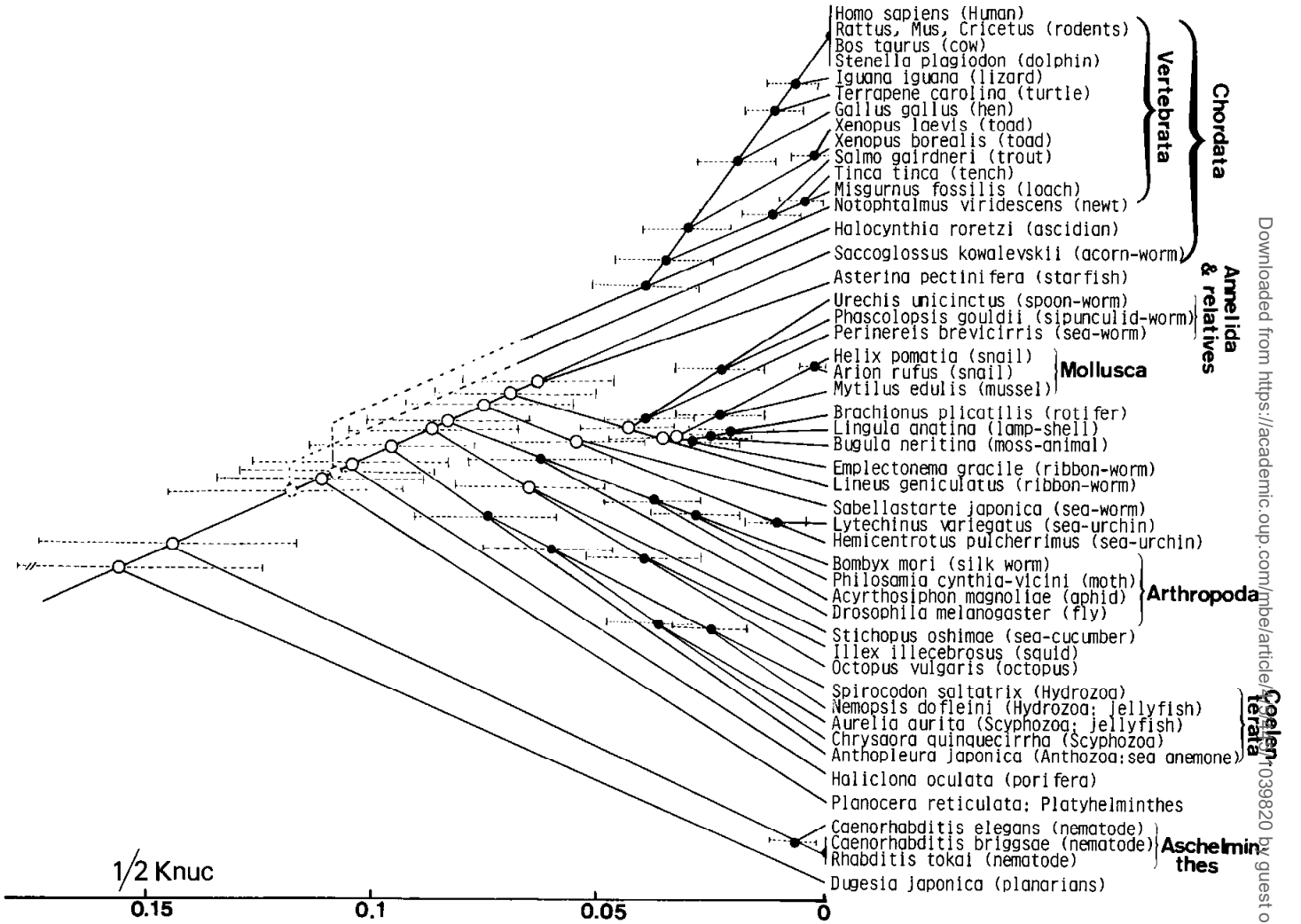

FIG. 12.—Phylogenetic tree of 5S rRNAs from 60 animals. *Knuc* and its SE are as given in the legend to fig. 3. Open circles represent emergence point of major phyla.

In the coelomated invertebrates, however, the branching points of several animals are against expectation (Ohama et al. 1984). For example, (1) a squid and an octopus do not cluster with other molluscans, (2) a sea cucumber and a starfish occupy a peculiar position in the tree, (3) two sea worms are situated separately, etc. Some of such "anomalous" branchings may be due to the large SEs, as shown in the tree (fig. 12). Thus, the branching order of invertebrates, especially of coelomated invertebrates, should be regarded as tentative.

C. Vertebrate Evolution and the Vertebrate-Invertebrate Relationship

In vertebrate evolution, the branching order of major taxa such as fishes, amphibians, reptiles, birds, and mammals is in good accordance with the classical view (fig. 12). However, problematic is the conclusion that chordates (vertebrates and an ascidian) separated from most of the invertebrate groups at a fairly ancient time (fig. 12). At present, the following two possibilities may be considered: (1) The picture is correct, in contrast to the general view postulating a more recent origin of vertebrates. In fact, a picture more or less similar to ours can be seen in Margulis and Schwartz (1982). (2) The 5S rRNA genes are not single copy in any organism. Although their sequences are generally similar, showing only a few base substitutions, the presence in one organism of a heterogeneous 5S rRNA population has been shown for certain animal species (Ford and Southern 1973; Kumazaki et al. 1982, 1983*b*), a finding that implies that sometimes the 5S rRNA genes in one organism can diversify considerably during evolution. Thus, between vertebrates and invertebrates, we might be comparing here 5S rRNA species derived from different genes that separated from one another within their common ancestor and have evolved independently.

Summary

The conclusions of this study may be summarized in outline form as follows:

I. Eubacteria evolution
   A. Eubacteria may be classified into gram-negative bacteria, cyanobacteria, and gram-positive bacteria.
   B. Chloroplasts and cyanelles share a common ancestor with cyanobacteria.

II. Metabacteria evolution
   A. Metabacteria share a common ancestor with eukaryotes.
   B. The emergence of *Sulfolobus* occurred at early stages of metabacterial evolution, followed by the sequencial development of *Thermoplasma,* methanogens, and halophile metabacteria.

III. Plant evolution
   A. Rhodophyta is a group that emerged at the earliest time of eukaryotic evolution.
   B. Three major groups of plants—i.e., Rhodophyta, green plants (Chlorophyta and land plants), and "Chromophyta"—are remotely related to one another.
   C. The emergence of Chlorophyta occurred at early stages of green-plant evolution.
   D. *Nitella*-like green algae would be the direct ancestor of land plants.
   E. Mosses evolved from a fernlike plant by means of degeneration.
   F. Chrysophyta, Bacillariophyta, and Phaeophyta belong to the same group, "Chromophyta" (or the Heterokonta). The Dinophyta and Cryptophyta consist of independent groups.
   G. Various brown algae species (Phaeophyta) diversified quite recently.

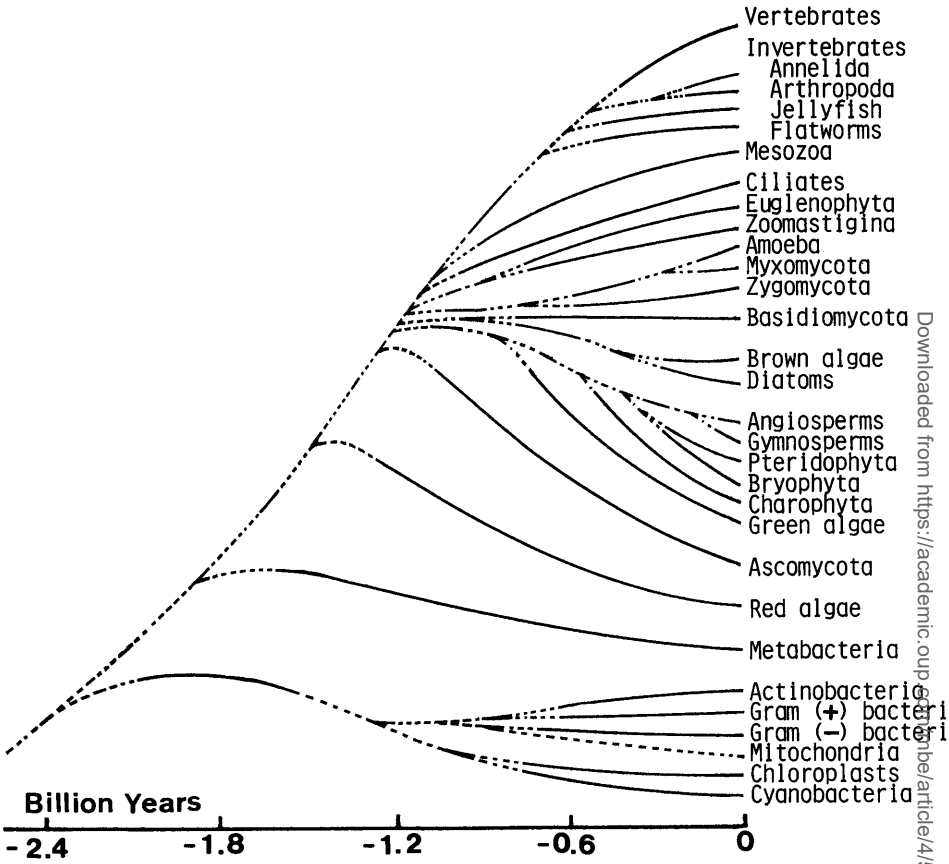

FIG. 13.—Simplified phylogenetic tree as deduced from 352 5S rRNA sequences. Time scale (abscissa) was estimated on the basis of yeast-animal divergence time (1.2 billion years ago; see text) by assuming that the rate of nucleotide substitution in 5S rRNA is constant from eubacteria (½ *Dnuc* scale in fig. 3) to man (½ *Knuc* scale in fig. 2) throughout. Dashed lines indicate SEs.

IV. Fungi evolution
  A. Ascomycota and Basidiomycota are phylogenically remote.
  B. Myxomycota, Zygomycota, Acrasinomycota, and Oomycota emerged independently, much later than Ascomycota and Basidiomycota.

V. Proctista evolution
  A. Amoeba, ciliates, Dinophyta, and animal flagellates/*Euglena* emerged independently, before the emergence of animals.
  B. *Euglena* and *Cyanophora* share a common ancestor.
  C. Myxomycota and amoeba share a common ancestor.

VI. Animal evolution
  A. The metazoans are of a monophyletic origin.
  B. The mesozoa might be the most ancient group of multicellular animals.
  C. The emergence time of the mesozoa is almost the same as that of ciliated or flagellated protozoans.
  D. The branching points of planarians and nematodes are a little earlier than those

of other metazoans, including sponges and jellyfishes, and are followed by the emergence of various metazoan phyla.

This outline is graphically represented in figure 13.

Acknowledgments

We thank those of our colleagues who participated in various phases in the present work and Dr. S. P. Gibbs of McGill University for advice and helpful discussion. This work was supported by a grant-in-aid (projects 5940007, 59107004, 5904131, 60043032, 60540406, and 60224004) from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

ALEXOPOULOS, C. J., and H. C. BOLD. 1967. Algae and fungi. Macmillan, New York.

BOLD, H. C. 1970. The plant kingdom. Prentice-Hall, Englewood Cliffs, N.J.

CHEN, M. W., J. ANNE, G. VOLCKAERT, E. HUYSMANS, A. VANDENBERGHE, and R. DE WACHTER. 1984. The nucleotide sequences of the 5S rRNAs of seven molds and a yeast and their use in studying ascomycete phylogeny. Nucleic Acids Res. **12**:4881–4892.

CORLISS, J. O. 1984. The kingdom protista and its 45 phyla. BioSystems **17**:87–126.

DARLEY, W. M. 1982. Algal biology: a physiological approach. Blackwell, Oxford.

DEKIO, S., R. YAMASAKI, J. JIDOI, H. HORI, and S. OSAWA. 1984. Secondary structure and phylogeny of *Staphylococcus* and *Micrococcus* 5S rRNA. J. Bacteriol. **159**:233–237.

DELIHAS, N., J. ANDERSEN, and R. P. SINGHAL. 1984. Structure, function and evolution of 5S ribosomal RNAs. Prog. Nucleic Acids Res. Mol. Biol. **31**:161–190.

DIXON, P. S. 1973. Biology of the Rhodophyta. Oliver & Boyd, Edinburgh.

DOOLITTLE, W. F. 1982. Molecular evolution. Pp. 307–331 *in* N. G. CARR and B. A. WHITTON, eds. The biology of cyanobacteria. Blackwell, Oxford.

ELWOOD, H. J., G. J. OLSEN, and M. L. SOGIN. 1985. The small-subunit ribosomal RNA gene sequences from the Hypotrichous ciliates *Oxytricha nova* and *Stylonychia pustulata.* Mol. Biol. Evol. **2**:399–410.

ERDMANN, V. A., and J. WOLTERS. 1986. Collection of published 5S, 5.8S and 4.5S ribosomal RNA sequences. Nucleic Acids Res. **14**[Suppl.]: r1–r59.

FORD, P. J., and E. M. SOUTHERN. 1973. Different sequences for 5S RNA in kidney cells and ovaries of *Xenopus laevis.* Nature New Biol. **241**:7–12.

FOX, G. E., K. R. LUEHRSEN, and C. R. WOESE. 1982. Archaebacterial 5S ribosomal RNA. System. Appl. Microbiol. **3**:330–345.

GIBBS, S. P. 1978. The chloroplasts of *Euglena* may have evolved from symbiotic green algae. Can. J. Bot. **56**:2883–2889.

GOTTSCHALK, M., and P. A. BLANZ. 1984. Highly conserved 5S ribosomal RNA sequences in four rust fungi and atypical 5S rRNA secondary structure in *Microstroma juglandis.* Nucleic Acids Res. **12**:3951–3958.

HADZI, J. 1963. The evolution of the metazoa. Pergamon, New York.

HANSON, E. D. 1977. The origin and early evolution of animals. Wesleyan Univ. Press, Middletown, Conn.

HORI, H. 1975. Evolution of 5S RNA. J. Mol. Evol. **7**:75–86.

HORI, H., K. HIGO, and S. OSAWA. 1977. The rates of evolution in some ribosomal components. J. Mol. Evol. **9**:191–201.

HORI, H., T. ITOH, and S. OSAWA. 1982. The phylogenic structure of the metabacteria. System. Appl. Microbiol. **3**:18–30.

HORI, H., B.-L. LIM, and S. OSAWA. 1985*a*. Evolution of green plants as deduced from 5S rRNA sequences. Proc. Natl. Acad. Sci. USA **82**:820–823.

HORI, H., B.-L. LIM, T. OHAMA, T. KUMAZAKI, and S. OSAWA. 1985*b*. Evolution of organisms deduced from 5S rRNA sequences. Pp. 369–384 *in* T. OHTA and K. AOKI, eds. Population genetics and molecular evolution. Springer, Tokyo and Berlin.

HORI, H., and S. OSAWA. 1979. Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species. Proc. Natl. Acad. Sci. USA **76**:381–385, 4157.

———. 1986. Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 352 5S RNA species. BioSystems **19**:163–172.

HORI, H., M. SAWADA, S. OSAWA, K. MURAO, and H. ISHIKURA. 1981. The nucleotide sequence of 5S rRNA from Mycoplasma capricolum. Nucleic Acids Res. **9**:5407–5410.

HUYSMANS, E., E DAMS, A. VANDENBERGHE, and R. DE WACHTER. 1983. The nucleotide sequences of the 5S rRNAs of four mushrooms and their use in studying the phylogenic position of basidiomycetes among the eukaryotes. Nucleic Acids Res. **11**:2871–2880.

INOUE, H. 1978. Life and history of Bryophytes. Idemitsu, Tokyo.

KARABIN, G. D., J. O. NARITA, J. R. DODD, and R. B. HALLICK. 1983. *Euglena gracilis* chloroplast ribosomal RNA transcription units. J. Biol. Chem. **258**:14790–14796.

KIMURA, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**:111–120.

KIMURA, M., and T. OHTA. 1973. Eukaryotes-prokaryotes divergence estimated by 5S ribosomal RNA sequences. Nature New Biol. **243**:199–200.

KRAFT, G. T. 1981. Rhodophyta: morphology and classification. Pp. 6–51 *in* C. S. LOBBAN, and M. J. WYNNE, eds. The biology of seaweeds, Blackwell, Oxford.

KUMAZAKI, T., H. HORI, and S. OSAWA. 1983*a*. Phylogeny of Protozoa deduced from 5S rRNA sequences. J. Mol. Evol. **19**:411–419.

———. 1983*b*. The nucleotide sequences of 5S rRNAs from two ribbon worms: *Emplectonema gracile* contains two 5S rRNA species differing considerably in their sequences. Nucleic Acids Res. **11**:7141–7144.

KUMAZAKI, T., H. HORI, S. OSAWA, N. ISHII, and K. SUZUKI. 1982. The nucleotide sequences of 5S rRNAs from a rotifer, *Brachionus plicatilis,* and two nematodes, *Rhabiditis tokai* and *Caenorhabditis elegans.* Nucleic Acids Res. **10**:7001–7004.

LANE, D. J., D. A. STAHL, G. J. OLSEN, D. J. HELLER, and N. R. PACE. 1985. Phylogenetic analysis of the genera *Thiobacillus* and *Thiomicrospira* by 5SrRNA sequences. J. Bacteriol. **163**:75–81.

LIM, B.-L., H. KAWAI, H. HORI, and S. OSAWA. 1986. Molecular evolution of 5S ribosomal RNA from red and brown algae. Jpn. J. Genet. **61**:169–176.

LUDWIG, M., and S. P. GIBBS. 1985. DNA is present in the nucleomorph of cryptomonads: further evidence that the chloroplast evolved from a eukaryotic endosymbiont. Protoplasma **127**:9–20.

MCCARROLL, R., G. J. OLSEN, Y. D. STAHL, C. R. WOESE, and M. L. SOGIN. 1983. Nucleotide sequence of the *Dictyostelium discoideum* small-subunit ribosomal ribonucleic acid inferred from the gene sequence: evolutionary implications. Biochemistry **22**:5858–5868.

MACDONELL, M. T., and R. R. COLWELL. 1985. Phylogeny of Vibrionaceae, and recommendation for two new genera, Listonella and Shewanella. Syst. Appl. Microbiol. **6**:171–182.

MACDONNELL, M. T., D. G. SWARTZ, B. A. ORTIZ-CONDE, G. A. LAST, and R. R. COLWELL. 1986. Ribosomal RNA phylogenies for the Vibrio-enteric group of eubacteria. Microbiol. Sci. **3**:172–178.

MARGULIS, L., and K. V. SCHWARTZ. 1982. Five kingdoms. W. H. Freeman, San Francisco.

MIYATA, T., H. HAYASHIDA, R. KIKUNO, M. HASEGAWA, M. KOBAYASHI, and K. KOIKE. 1982. Molecular clock of silent substitution: at least six-fold preponderance of silent changes in mitochondrial genes over those in nuclear genes. J. Mol. Evol. **19**:28–35.

NEI, M., J. C. STEPHENS, and N. SAITOU. 1985. Methods for computing the standard errors of

branching points in an evolutionary tree and their application to molecular data from humans and apes. Mol. Biol. Evol. **2**:66–85.

OHAMA, T., T. KUMAZAKI, H. HORI, and S. OSAWA. 1984. Evolution of multicellular animals as deduced from 5S rRNA sequences: a possible early emergence of the Mesozoa. Nucleic Acids Res. **12**:5101–5108.

OHKUBO, S., H. IWASAKI, H. HORI, and S. OSAWA. 1986. Evolutionary relationship of denitrifying bacteria as deduced from 5S rRNA sequences. J. Biochem. **100**:1261–1267.

PACE, N. R., G. J. OLSEN, and C. R. WOESE. 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell **45**:325–326.

RAGAN, M. A., and D. J. CHAPMAN. 1978. A biochemical phylogeny of the protists. Academic Press, New York.

ROGERS, M. J., J. SIMMONS, R. T. WALKER, W. G. WEISBURG, C. R. WOESE, R. S. TANNER, I. M. ROBINSON, D. A. STAHL, G. OLSEN, R. H. LEACH, and J. MANILOFF. 1985. Construction of the mycoplasma evolutionary tree from 5S rRNA sequence data. Proc. Natl. Acad. Sci. USA **82**:1160–1164.

SCHUSTER, H. 1966. Hepaticae and anthocerotae of North America. Columbia University Press, New York.

SNEATH, P. H. A., and R. R. SOKAL. 1973. Numerical taxonomy. W. H. Freeman, San Francisco.

SOGIN, M. L., H. J. ELWOOD, and J. H. GUNDERSON. 1986. Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proc. Natl. Acad. Sci. USA **83**:1383–1387.

SPENCER, D. F., L. BONEN, and M. W. GRAY. 1981. Primary sequence of wheat mitochondrial 5S rRNA: functional and evolutionary implications. Biochemistry **20**:4022–4029.

STEWART, K. D., and K. R. MATTOX. 1975. Comparative cytology, evolution and classification of the green algae with some consideration of the origin of other organisms with chlorophylls *a* and *b*. Bot. Rev. **41**:104–135.

TAYLOR, F. J. R. 1978. Problems in the development of an explicit hypothetical phylogeny of the lower eukaryotes. BioSystems **10**:67–89.

VANDENBERGHE, A., A. WASSINK, P. RAEYMAEKERS, R. DE BAERE, E. HUYSMANS, and R. DE WACHTER. 1985. Nucleotide sequence, secondary structure and evolution of the 5S ribosomal RNA from five bacterial species. Eur. J. Biochem. **149**:537–542.

VILLANUEVA, E., K. R. LUEHRSEN, J. GIBSON, N. DELIHAS, and G. E. FOX. 1985. Phylogenetic origins of the plant mitochondrion based on a comparative analysis of 5S ribosomal RNA sequences. J. Mol. Evol. **22**:46–52.

WALKER, R. T., E. T. J. CHELTON, M. W. KILPATRICK, M. J. ROGERS, and J. SIMMONS. 1982. The nucleotide sequence of the 5S rRNA from *Spiroplasma* species BC3 and *Mycoplasma mycoides* sp. capri. PG3. Nucleic Acids Res. **10**:6363–6367.

WALKER, W. F. 1984. 5S Ribosomal RNA sequences from Atractiellales, and Basidiomycetous yeasts and Fungi Imperfecti. Syst. Appl. Microbiol. **5**:352–359.

WALKER, W. F., and W. F. DOOLITTLE. 1982. Redividing the basidiomycetes on the basis of 5S rRNA sequences. Nature **299**:723–724.

WOESE, C. R. 1981. Archaebacteria. Sci. Am. **244**:94–107.

WOESE, C. R., and G. E. FOX. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc. Natl. Acad. Sci. USA **74**:5088–5090.

WOLTERS, J., and V. A. ERDMANN. 1984. Comparative analysis of small ribosomal RNAs with respect to the evolution of plastids and mitochondria. Int. Soc. Endocytobiol. News Lett. **1**: 1–23.

WREDE, P., and V. A. ERDMANN, 1973. Activities of *B. stearothermophilus* 50S ribosomes reconstituted with prokaryotic and eukaryotic 5S rRNA. FEBS Lett. **33**:315–319.

WYNNE, M. J. 1981. Phaeophyta: morphology and classification. Pp. 52–85 *in* C. S. LOBBAN and M. J. WYNNE, eds. The biology of seaweeds. Blackwell, Oxford.

MASATOSHI NEI, reviewing editor

Received March 18, 1986; revision received February 23, 1987