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Phylogenetic Relationships of the Green Alga *Volvox carteri* Deduced from Small-Subunit Ribosomal RNA Comparisons

Helmut Rausch,[1] Niels Larsen,[2] and Rüdiger Schmitt[1]

[1] Lehrstuhl für Genetik, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany
[2] Biostructural Chemistry, Institute of Chemistry, Aarhus University, DK-8000 Aarhus, Denmark

Summary. The 1788-nucleotide sequence of the small-subunit ribosomal RNA (srRNA) coding region from the chlorophyte *Volvox carteri* was determined. The secondary structure bears features typical of the universal model of srRNA, including about 40 helices and a division into four domains. Phylogenetic relationships to 17 other eukaryotes, including two other chlorophytes, were explored by comparing srRNA sequences. Similarity values and the inspection of phylogenetic trees derived by distance matrix methods revealed a close relationship between *V. carteri* and *Chlamydomonas reinhardtii*. The results are consistent with the view that these Volvocales, and the third green alga, *Nanochlorum eucaryotum*, are more closely related to higher plants than to any other major eukaryotic group, but constitute a distinct lineage that has long been separated from the line leading to the higher plants.

Key words: Green algae — *Volvox carteri* — *Chlamydomonas reinhardtii* — Small-subunit rRNA sequence — Eukaryotic phylogeny

Introduction

Volvox, a multicellular spheroidal green alga, is normally grouped with *Chlamydomonas*, and all of the other normally motile unicellular and colonial green algae, in the order Volvocales (Bold and Wynne 1985). Most Volvocales, whether of the unicellular (*Chlamydomonas*) or colonial type (*Gonium, Pandorina, Eudorina*), have only one type of cell capable of performing all the vegetative and reproductive functions of the organism (Kochert 1973). *Volvox* is the only member of the order that shows a complete division of labor between two fully differentiated cell types, namely, somatic and reproductive cells (Kirk and Harper 1986). It is this range from unicellular to multicellular that recommends the Volvocalean order as a model for examining the genetic origins of multicellularity. To this end, we have initiated a number of phylogenetic studies of *Volvox carteri*, which is the member of the order with the earliest and most complete differentiation of germ and somatic cells.

Prior studies that compared the tubulin gene sequences and codon usage patterns of *V. carteri* and *Chlamydomonas reinhardtii* have reinforced the presumption (based originally on morphological and physiological similarities) that these two genera are closely related, and perhaps even more closely related than might have been anticipated on the basis of organizational differences between the species (Harper and Mages 1988; Mages et al. 1988). At the same time, structural differences of the Volvocalean tubulin genes—and, even more distinctly, of the *Volvox* histone H3 and H4 genes (Müller and Schmitt 1988)—from the corresponding genes of higher plants and animals have suggested a rather unique position of the order Volvocales in the evolutionary scheme. Therefore, we have undertaken comparisons of small-subunit ribosomal RNA (srRNA) sequences, as an independent route to assessing both the relationship between *V. carteri* and *C. reinhardtii*, and the phylogenetic position of the Volvocales relative to other major eukaryotic taxa.

Ribosomal RNAs (rRNAs) have proved to be particularly useful for assessing evolutionary rela-

Offprint requests to: R. Schmitt

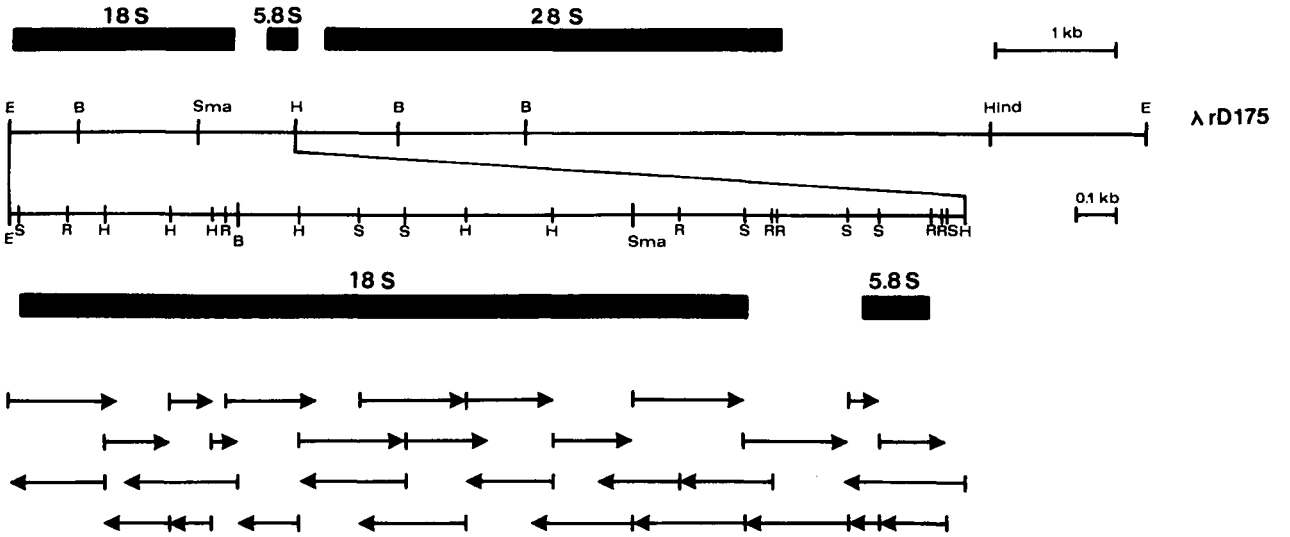

Fig. 1. Restriction maps and sequencing strategy for *Volvox carteri* 18S and 5.8S rRNA genes. One rRNA repeat unit is represented by a 9.4-kb *Eco*RI fragment from the recombinant phage λrD175. The locations of the three rRNA genes within λrD175 are shown above, and an expanded map of the 2334-bp portion that contains the 18S and 5.8S rRNA—with relevant restriction sites used for sequencing—is shown below. B, *Bam*HI; E, *Eco*RI; H, *Hpa*II; Hind, *Hind*III; R, *Rsa*I; S, *Sau*3A; Sma, *Sma*I. Arrows (pointing from 5′ to 3′) indicate single strands that were sequenced from subcloned fragments.

tionships within and between groups of organisms for several reasons. The rRNAs are universal among pro- and eukaryotic organisms, their sequences have changed very slowly during evolution, and their functional role in protein synthesis essentially has remained unchanged (Hori and Osawa 1979; Fox et al. 1980). In particular, the srRNAs contain regions differing so widely in the degree of sequence conservation that they potentially provide measures of divergence over a wide range of phylogenetic distances (Woese 1987).

In this study we have determined the *V. carteri* srRNA sequence and have compared it to that of 17 other eukaryotic taxa, including *C. reinhardtii* (Gunderson et al. 1987) and *Nanochlorum eucaryotum* (Sargent et al. 1988). These data confirm that *V. carteri* and *C. reinhardtii* are very close relatives; most sequence differences between these species reside in variable regions that become apparent in a secondary structure model of the srRNA. Phylogenetically, the order Volvocales appears to be a lineage that diverged early from the line to higher plants.

Materials and Methods

Strains and Chemicals. Volvox carteri f. *nagariensis*, female strain HK10 (UTEX 1885), kindly was supplied by R. Starr (University of Texas Culture Collection of Algae). Cultures were grown in standard *Volvox* medium (Starr 1969) at 28°C under an 8-h dark/16-h light regime, as previously described (Mages et al. 1988). Restriction enzymes, alkaline phosphatase, kinase, DNA polymerase (Klenow fragment), and T4-DNA ligase were purchased from Boehringer (Mannheim, FRG). [γ-^{32}P]ATP was prepared according to Johnson and Walseth (1979). $^{32}P_i$ and [α-^{35}S]dATP were purchased from Amersham Buchler (Braunschweig, FRG). Oligonucleotide primers synthesized by the phosphoamidite method (Matteucci and Caruthers 1981) kindly were supplied by M. Sumper (University of Regensburg, FRG).

Library Screening. The isolation of high molecular weight *Volvox* DNA and construction of a *Volvox* genomic library with phage vector λEMBL3 is described elsewhere (Mages et al. 1988). Approximately 35,000 recombinant phages were screened for rDNA clones by in situ plaque hybridization (Benton and Davis 1977) using Schleicher and Schüll BA85 nitrocellulose membranes. Yeast 18S-like and 28S-like rRNAs were 5′-labeled with [γ-^{32}P]ATP (Maxam and Gilbert 1980) and then used as probes. Southern transfers (Southern 1975) were performed according to Maniatis et al. (1982), with two subsequent washes at 42°C and 65°C.

DNA Sequence Analysis. Fragments of recombinant phage DNA were subcloned into pUC8 (Vieira and Messing 1982) and both strands were sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977) using synthetic primers and a gradient acrylamide gel as described by Heinrich (1986). The ends of the derived srRNA sequence were defined by comparison with yeast srRNA (Rubtsov et al. 1980).

Prediction of Secondary Structure. The secondary structure model of *Zea mays* srRNA (Gutell et al. 1985) served as a pattern for the *Volvox* sequence. All secondary structure predictions are based on evidence for compensating base changes, as described by Leffers et al. (1987), using the graphics editor EDSTRUC (N. Larsen, unpublished). The resulting model of *Volvox* srRNA was plotted using the PLSTRUC program (N. Larsen, unpublished).

Sequence Alignments. A total of 17 eukaryotic srRNA sequences (Dams et al. 1988) were aligned with *Volvox* srRNA by using the sequence editor ALMA (S. Thirup and N. Larsen, unpublished) that first aligns sequences according to common primary structure, then uses well-established secondary structure elements for aligning regions that share little homology, and finally introduces gaps into each individual sequence, as required to achieve optimum alignment.

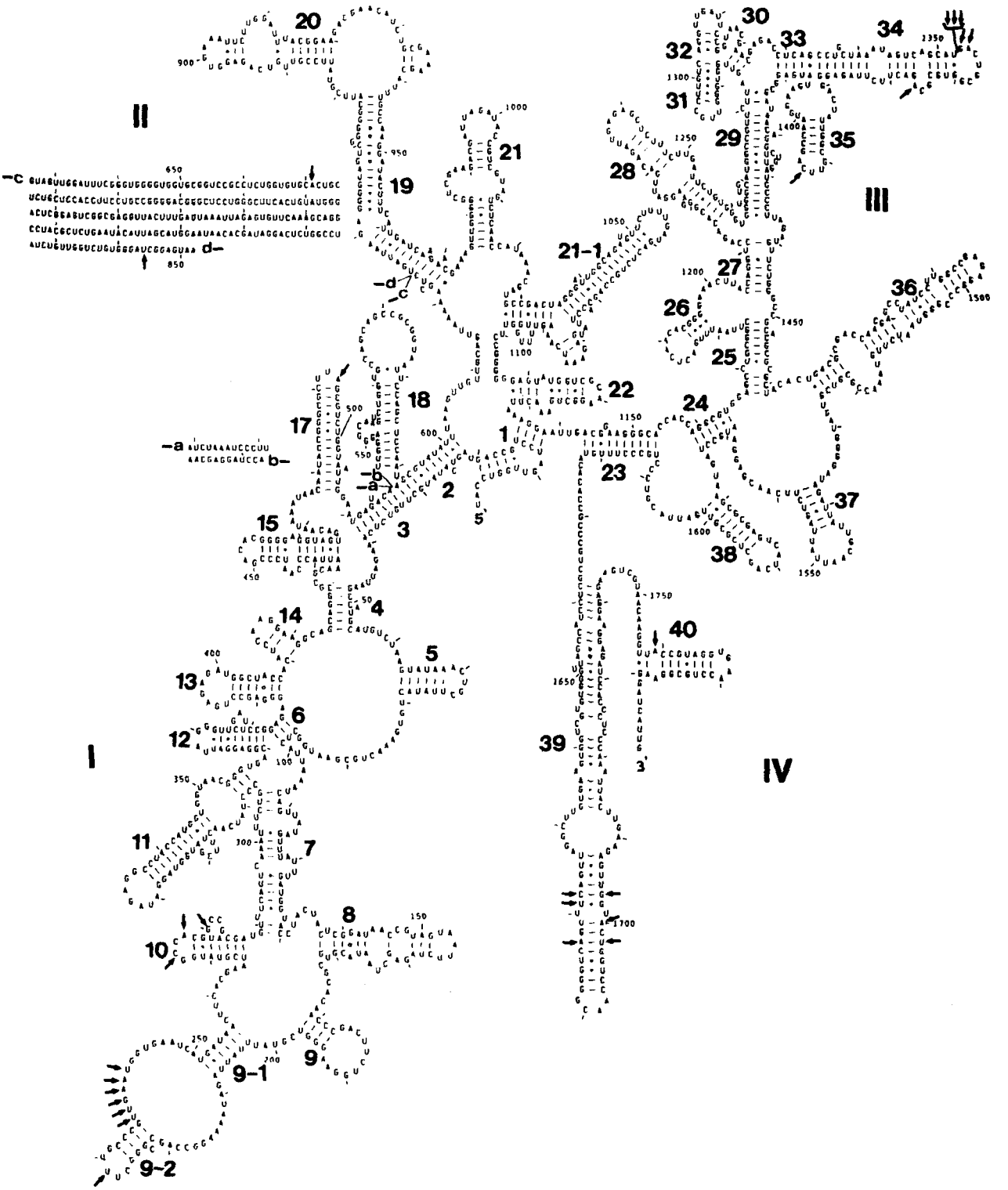

Fig. 2. Secondary structure model for the *Volvox* srRNA. Double-helical segments are numbered (Arabic numerals) according to Huysmans and DeWachter (1986). The division into four domains (Roman numerals, ca. residues 1–605, 606–1140, 1141–1622, and 1623–1788) follows the proposal of Gutell et al. (1985). Twenty-seven positions differing between *Volvox carteri* and *Chlamydomonas reinhardtii* srRNAs are marked with arrows.

Phylogenetic Analysis. Structural similarity values were calculated by pairwise comparisons of aligned srRNA sequences, using the formula: structural similarity = match positions/match positions + mismatch positions. The analysis was restricted to those nucleotide positions that could be aligned unambiguously and exhibited no gaps in all srRNA sequences being compared (Leffers et al. 1987). In a second approach, total srRNA sequences were compared, giving each gap (regardless of size), transition, or transversion a value of one. Structural distance values (Knuc values = the average number of mutational events per sequence

Fig. 3. Sequence of the srRNA-coding region of *Volvox carteri* (V.c.), aligned with those of *Chlamydomonas reinhardtii* (C.r.), *Nanochlorum eucaryotum* (N.e.), *Zea mays* (Z.m.), *Glycine max* (G.m.), *Mus musculus* (M.m.), and *Saccharomyces cerevisiae* (S.c.). Sequences were aligned on the basis of primary structure similarities and phylogenetically conserved secondary structure (Fig. 2). Helix and nonhelix positions are indicated by lower-case and upper-case lettering, respectively, in the *Volvox* sequence. Alignments were refined by introducing gaps, where length variations occurred. These alignments were influenced by consideration of 11 other eukaryotic srRNA sequences (see text).

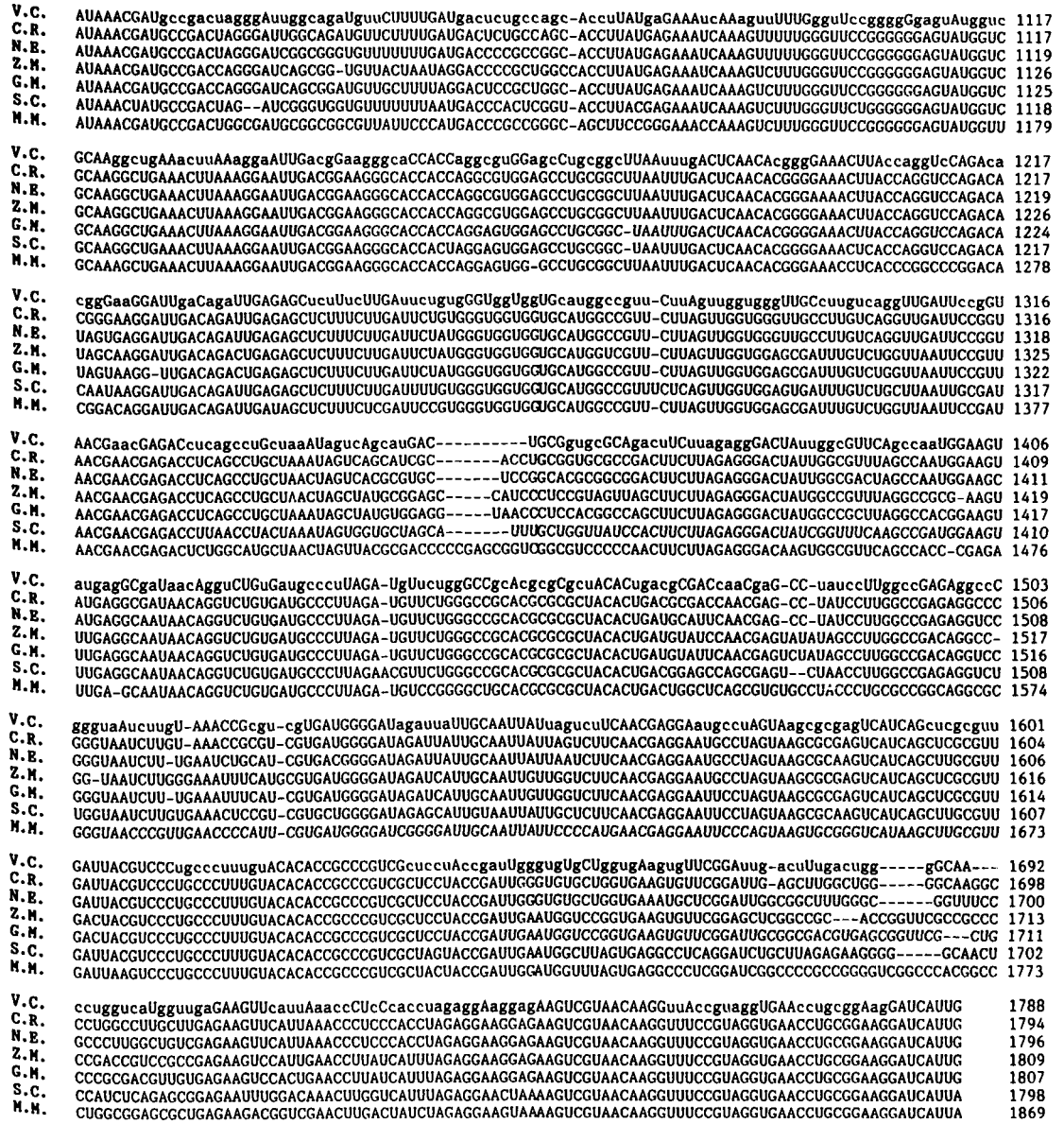

Fig. 3. Continued

position) were calculated according to Hori and Osawa (1979) using the program NUCTREE. Phylogenetic trees were examined by using distance matrix methods (Fitch and Margoliash 1967; Felsenstein 1981), and the best fit was determined by independent application of the FITCH, DNAPARS, and DNAML programs (Felsenstein 1985). All calculations were performed on a DEC VAX 11/780 computer. The programs ALMA, EDSTRUC, NUCTREE, PLSTRUC, and complete listings of the 18 aligned srRNA sequences are available from N. Larsen on request.

Results

Among 19 positive clones from a λEMBL3-based library of *V. carteri* screened with an srRNA probe from yeast, λrD175 was chosen for physical mapping and DNA sequence analysis. As shown in Fig. 1, λrD175 contains one rDNA repeat unit on an approximately 9.4-kb *Eco*RI fragment. Southern hybridization (not shown) of various clones digested with *Bam*HI, *Eco*RI, and *Hind*III with yeast small-subunit and large-subunit rRNA probes indicated that the rRNA genes are arranged in tandemly repeated units, with each unit containing an 18S and 28S rRNA gene; subsequently, sequencing data indicated the presence of a 5.8S rRNA gene between the 18S and 28S genes. The pattern of Southern blots from *Eco*RI- and *Bam*HI-digested genomic DNA probed with yeast rDNA (not shown) was consistent with that observed with λrD175 DNA, suggesting that this clone was representative of the *Volvox* multigene family. The haploid *Volvox* genome contains approximately 1200 rDNA repeats, as estimated by titration with a yeast 18S rRNA probe (data not shown).

The sequencing strategy for the region of λrD175 encoding the 18S and 5.8S rRNAs is illustrated in Fig. 1. As a guide for designing the *Volvox* srRNA

Table 1. Structural similarity[a] and distance[b] (Knuc) values calculated from the aligned nongap regions of srRNA sequences

	V.c.	C.r.	N.e.	O.s.	G.m.	Z.m.	P.m.	S.c.	O.n.	S.p.	P.t.	X.l.
V.c.		99.21	94.88	91.40	91.01	90.94	86.94	85.89	84.06	83.92	83.14	82.68
C.r.	0.008		94.95	91.27	90.94	90.88	86.94	85.89	84.19	83.99	83.14	82.74
N.e.	0.053	0.052		91.99	91.40	91.86	87.53	86.81	85.43	85.56	83.40	82.68
O.s.	0.091	0.093	0.085		97.24	99.08	87.53	85.89	85.56	85.63	83.66	83.40
G.m.	0.096	0.097	0.091	0.028		96.85	87.60	86.22	85.83	85.83	83.40	82.61
Z.m.	0.097	0.097	0.086	0.009	0.032		87.20	85.89	85.24	85.43	83.46	83.33
P.m.	0.144	0.144	0.136	0.136	0.136	0.140		86.15	88.06	87.86	84.97	82.02
S.c.	0.156	0.156	0.145	0.156	0.152	0.156	0.153		86.22	85.89	84.12	82.94
O.n.	0.179	0.178	0.162	0.160	0.157	0.164	0.130	0.152		98.95	87.73	80.31
S.p.	0.181	0.180	0.160	0.160	0.157	0.162	0.132	0.156	0.011		87.60	80.05
P.t.	0.191	0.191	0.188	0.184	0.188	0.187	0.168	0.178	0.134	0.136		79.72
X.l.	0.197	0.196	0.197	0.188	0.198	0.186	0.206	0.194	0.228	0.232	0.236	
M.m.	0.200	0.199	0.200	0.189	0.200	0.188	0.212	0.195	0.231	0.235	0.238	0.036
E.a.	0.242	0.240	0.219	0.225	0.267	0.230	0.187	0.226	0.153	0.155	0.201	0.266
T.t.	0.244	0.245	0.233	0.226	0.223	0.229	0.211	0.196	0.175	0.178	0.160	0.277
D.d.	0.274	0.277	0.273	0.265	0.261	0.269	0.267	0.267	0.261	0.268	0.274	0.306
T.b.	0.411	0.412	0.423	0.411	0.411	0.413	0.405	0.426	0.429	0.433	0.420	0.425
E.g.	0.423	0.420	0.425	0.416	0.425	0.418	0.413	0.415	0.435	0.436	0.410	0.442

Abbreviations used: Chlamydomonas reinhardtii (C.r.), *Dictyostelium discoideum* (D.d.), *Euglena gracilis* (E.g.), *Euplotes aediculatus* (E.a.), *Glycine max* (G.m.), *Mus musculus* (M.m.), *Nanochlorum eucaryotum* (N.e.), *Oxytricha nova* (O.n.), *Oryza sativa* (O.s.), *Paramecium tetraurelia* (P.t.), *Prorocentrum micans* (P.m.), *Saccharomyces cerevisiae* (S.c.), *Stylonychia pustulata* (S.p.), *Tetrahymena thermophila* (T.t.), *Trypanosoma brucei* (T.b.), *Volvox carteri* (V.c.), *Xenopus laevis* (X.l.), *Zea mays* (Z.m.)

[a] Upper right

[b] Lower left

secondary structure model shown in Fig. 2, we used the pattern proposed for *Zea mays* srRNA, because the two srRNA sequences bear over 90% identity. The *Zea mays* model has been established in a phylogenetic framework using a large multi-kingdom set of srRNA sequences for the comparison (Gutell et al. 1985). By reexamining the base pairs at the helical termini in the *Volvox* srRNA secondary structure model using the sequence compilations of Huysmans and DeWachter (1986), we did not find evidence for additional base pairing that would expand the helices. For the 227-nucleotide sequence between positions 626 and 852 and the 25-nucleotide sequence between positions 519 and 543 we do not propose any secondary structure. Satisfactory base-pairing schemes for these two sections that are compatible with the available evidence have not yet been established (Gutell et al. 1985).

The secondary structure bears typical features of the universal model of srRNA including about 40 helices and a division into four domains (Gutell et al. 1985). In addition to the unstructured portion of domain II, there are three other variable regions located in domain I (between nucleotides 127 and 292), in domain III (helix 34), and in domain IV (helix 39). As indicated in Fig. 2 (arrows), most of the positions differing between the closely related *V. carteri* and *C. reinhardtii* are concentrated in domains I and III.

The secondary structure for *Volvox* srRNA proposed here provided us with a basis for accurate sequence alignments of conserved regions. As shown in Fig. 3, the 1788-nucleotide sequence of *Volvox* srRNA has been aligned with those of *C. reinhardtii* (Gunderson et al. 1987), *N. eucaryotum* (Sargent et al. 1988), *Z. mays* (Messing et al. 1984), *Glycine max* (Eckenrode et al. 1985), *Mus musculus* (Raynal et al. 1984), and *Saccharomyces cerevisiae* (Mankin et al. 1986). These alignments were influenced by sequences from 11 other srRNAs representative of major eukaryotic phyla, namely, those of *Dictyostelium discoideum* (McCarrol et al. 1983), *Euglena gracilis* (Sogin et al. 1986a), *Euplotes aediculatus* (Sogin et al. 1986b), *Oryza sativa* (Takaiwa et al. 1984), *Oxytricha nova* (Elwood et al. 1985), *Paramecium tetraurelia* (Sogin and Elwood 1986), *Prorocentrum micans* (Herzog and Maroteaux 1986), *Stylonychia pustulata* (Elwood et al. 1985), *Tetrahymena thermophila* (Spangler and Blackburn 1985), *Trypanosoma brucei* (Sogin et al. 1986a), and *Xenopus laevis* (Salim and Maden 1981). The boundaries of the mature srRNA of *Volvox* were operationally defined by consensus with the experimentally defined termini of yeast srRNA (Rubtsov et al. 1980).

For the initial calculation of structural similarity and structural distance values, shortened versions of the aligned sequences, i.e., 1524 unambiguously aligned, nongap positions (covering 85% of the *Volvox* sequence) were compared as described under Materials and Methods. Structural similarity and structural distance data from all 18 eukaryotic srRNA sequences considered here are compiled in Table 1 in order of decreasing structural similarity values, relative to *V. carteri*.

Table 1. Extended

M.m.	E.a.	T.t.	D.d.	T.b.	E.g.
82.41	79.33	79.20	77.03	68.37	67.65
82.48	79.46	79.19	76.84	68.31	67.85
82.48	81.04	79.99	77.10	67.65	67.59
83.27	80.58	80.51	77.69	68.37	68.04
82.41	80.45	80.71	77.95	68.37	67.59
83.40	80.18	80.25	77.43	68.24	67.98
81.56	83.46	81.63	77.56	68.70	68.24
82.81	80.51	82.74	77.56	67.52	68.11
80.12	86.15	84.38	77.95	67.32	66.99
79.86	86.02	84.12	77.49	67.13	66.93
79.59	82.35	85.63	77.03	67.85	68.44
96.52	77.62	76.84	74.87	67.59	66.60
	77.17	76.31	74.54	68.11	66.80
0.272		79.92	74.34	65.16	65.62
0.285	0.234		74.67	66.08	65.68
0.311	0.314	0.309		67.26	65.16
0.415	0.469	0.452	0.430		66.60
0.439	0.460	0.459	0.406	0.442	

Immediately obvious from these data is the very close relationship (99.2% identity) between the srRNA sequences of *V. carteri* and *C. reinhardtii.* The microalga *N. eucaryotum* (94.9% identity) is next closest, but the structural similarity values for the *O. sativa, G. max,* and *Z. mays* sequences (91.4 to 90.9%) indicate that among the nonchlorophyte taxa considered in this study, Anthophyta are the closest relatives of the Volvocales. Ascomycota, represented by *S. cerevisiae* (similarity value of 85.9%) come next, with Chordata (about 82.5%) and Ciliata (86.9 to 79.2%) not far behind. The other species that were compared had similarity values of 77% or less. The structural distance, or Knuc, values that were obtained from the similarity data (Table 1) were converted into an unrooted phylogenetic tree by using the method of Fitch and Margoliash (1967). This approach treats the branch lengths (derived from Knuc values) as adjustable parameters; the tree that best fits the data (by a least squares analysis) is considered as the correct one (Woese 1987). The root of the resulting distance matrix tree subsequently was established by using as a reference outgroup the archaebacterial srRNA consensus sequence (Woese et al. 1983). The result is shown in Fig. 4A.

In a second set of comparisons, similarity and distance values were calculated for nine complete, aligned srRNA sequences, including that of *Volvox* and the eight species with the most closely related srRNAs. For this purpose, gaps (regardless of size), transitions, and transversions were all weighted equally (given a value of one). This increases distance values (Table 2) and potentially provides improved resolution within closely related groups, as demonstrated by the corresponding phylogenetic tree shown in Fig. 4B.

In accord with other studies (Sogin et al. 1986a; Woese 1987), the overall phylogenetic tree (Fig. 4A) indicates a rather tight clustering of the branches leading to Ascomycota, Anthophyta, and Chordata. The Volvocalean branch is shown here to be part of this cluster, emerging from the main trunk between branches leading to the higher plants and animals, but closer to the former.

It was somewhat surprising to find that the data in Table 1 show *Volvox* and *Chlamydomonas* separated by an even smaller distance (Knuc = 0.008) than the two grasses, *Zea* and *Oryza* (Knuc = 0.009), as *Volvox* and *Chlamydomonas* are thought to represent the multicellular and unicellular extremes among algae of the order Volvocales. Calculations from total srRNA sequences increased the distance values (Table 2) and the corresponding branch lengths (Fig. 4B), but did not alter dramatically either the distance relationships of *Volvox/Chlamydomonas* (0.020) vs *Zea/Oryza* (0.029) or the overall tree topology. These trees indicate that the chlorophyte branch represents the earliest divergence from the plant lineage to be inferred to date from srRNA sequences. But they also indicate that the diversification of the Volvocacean branch of the Volvocales (the branch leading to *Volvox*) may have occurred much more recently.

Discussion

We have determined the 1788-nucleotide sequence of *V. carteri* srRNA and have aligned it with 17 known srRNAs as part of our ongoing effort to deduce the phylogenetic relationship of green algae in the order Volvocales to one another and to members of other major eukaryotic groups.

Southern blots of genomic restriction fragments hybridized to large and small subunit probes, together with DNA sequence analysis indicated that *Volvox* rRNA genes, like those of most eukaryotes, are organized in a tandem array of repeats that contain 18S, 5.8S, and 28S genes, in that order. It has been estimated that the haploid *Volvox* genome contains approximately 1200 such rDNA repeats. This rDNA copy number ranges above that of most animals (100–1000), but is an order of magnitude lower than that of wheat or barley (about 10,000 copies; Gerlach and Bedbrook 1979). It may reflect the need for a highly efficient translation machinery during the maturation of *Volvox* juveniles (Yates and Kochert 1976).

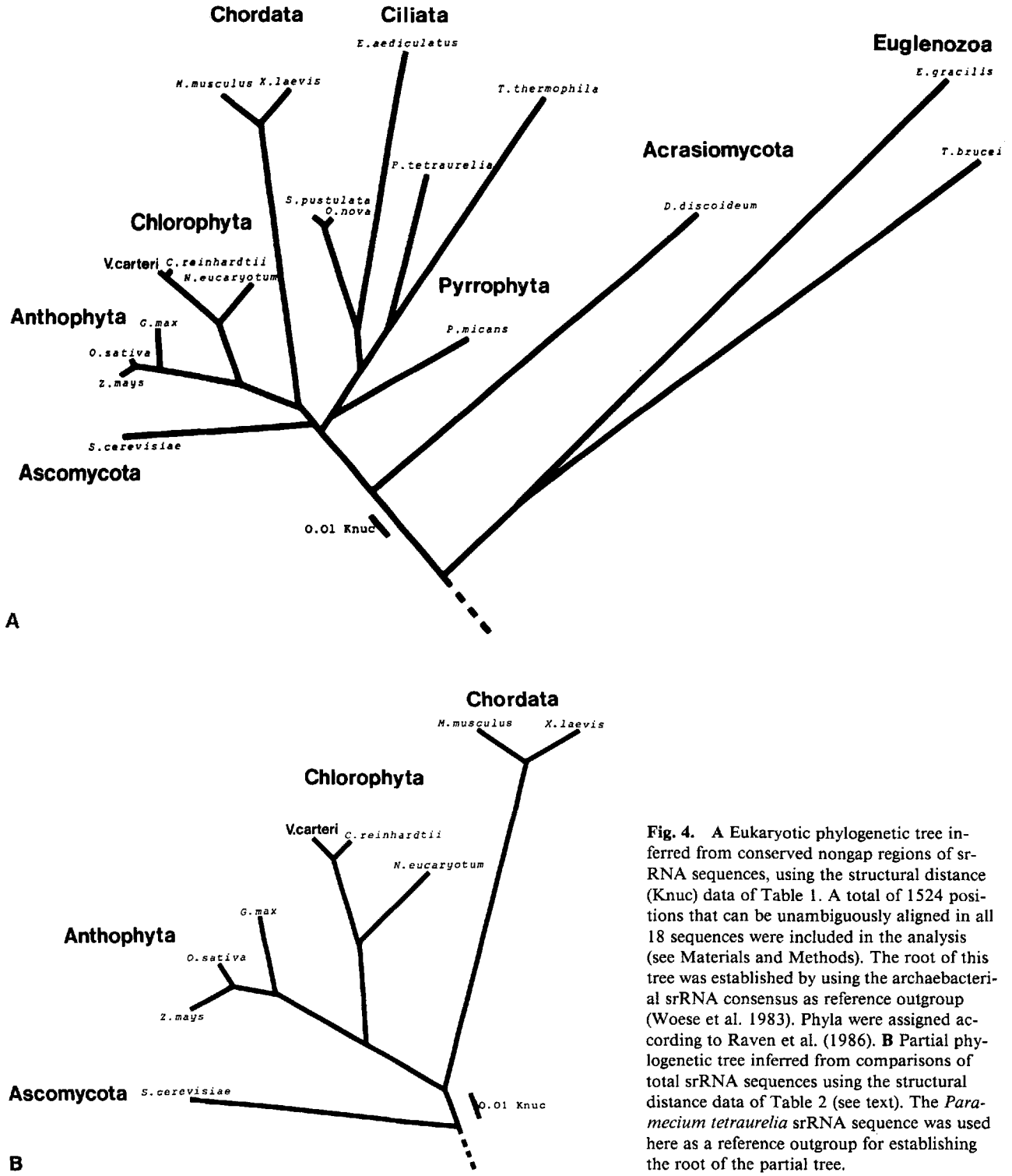

Fig. 4. **A** Eukaryotic phylogenetic tree inferred from conserved nongap regions of srRNA sequences, using the structural distance (Knuc) data of Table 1. A total of 1524 positions that can be unambiguously aligned in all 18 sequences were included in the analysis (see Materials and Methods). The root of this tree was established by using the archaebacterial srRNA consensus as reference outgroup (Woese et al. 1983). Phyla were assigned according to Raven et al. (1986). **B** Partial phylogenetic tree inferred from comparisons of total srRNA sequences using the structural distance data of Table 2 (see text). The *Paramecium tetraurelia* srRNA sequence was used here as a reference outgroup for establishing the root of the partial tree.

The phylogenetic tree in Fig. 4A was deduced from the distance matrix given in Table 1. It was derived initially as an unrooted tree, and the root was then established by use of a consensus sequence for archaebacterial srRNA (Woese et al. 1983) as an outgroup. It is consistent with the eukaryotic tree proposed by Woese (1987), but omits the earliest microsporidian branch (Vossbrinck et al. 1987).

The connections between the eukaryotic, eubacterial, and archaebacterial kingdoms, and the radiations within these kingdoms that are indicated by rRNA sequence data have been discussed extensively in the past (Woese 1987; Sogin et al. 1986a). In this context, it has been pointed out that four major eukaryotic lineages—animals, plants, fungi, and ciliates—form a cluster within which there has

Table 2. Structural similarity[a] and distance[b] (Knuc) values from total srRNA sequences

	V.c.	C.r.	N.e.	O.s.	G.m.	Z.m.	S.c.	X.l.	M.m.	P.t.
V.c.		97.99	91.71	86.31	86.19	85.21	79.52	76.74	76.54	75.55
C.r.	0.020		91.99	86.53	86.19	85.60	79.61	76.83	76.32	75.50
N.e.	0.086	0.083		86.81	86.40	86.15	80.73	76.85	76.74	75.43
O.s.	0.151	0.148	0.145		94.33	97.19	79.64	78.83	78.64	75.59
G.m.	0.152	0.152	0.149	0.059		93.49	80.04	77.63	77.37	75.47
Z.m.	0.164	0.159	0.153	0.029	0.068		79.44	78.47	78.38	75.30
S.c.	0.238	0.236	0.221	0.237	0.231	0.239		77.69	77.15	76.61
X.l.	0.278	0.276	0.276	0.251	0.265	0.253	0.262		95.18	71.78
M.m.	0.281	0.284	0.278	0.251	0.269	0.254	0.271	0.049		71.50
P.t.	0.292	0.291	0.294	0.294	0.295	0.298	0.275	0.351	0.356	

Abbreviations as in Table 1
[a] Upper right
[b] Lower left

been considerably less rRNA sequence divergence than has occurred since these major lineages all diverged from a number of other protistan groups, such as the Acrasiomycota and Euglenozoa. The present analysis places *Volvox,* along with its unicellular relative *Chlamydomonas,* right in the midst of this major eukaryotic cluster, but nearly as divergent from each of these major groups as they are from one another. The location of *Volvox* on a branch lying between the branches leading to plants and animals finds reflection in the mixture of plant-like features (e.g., chloroplasts and photoautotrophy) and animal-like features (e.g., motility and a germ–soma dichotomy) that characterize this genus.

At a finer scale (Fig. 4B and Table 2), the present data place *Volvox* and *Chlamydomonas* near one another on a branch that diverges from the eukaryotic trunk closest to the branch leading to higher plants. These data indicate that the Volvocales shared a common ancestor with higher plants more recently than they did with any other major group, but they also indicate that the Volvocalean lineage must have separated from the plant lineage relatively early in the history of the group.

The srRNA sequences of *V. carteri* and *C. reinhardtii* have diverged somewhat less than those of the two cereal grasses, *Zea* and *Oryza,* that were included in our comparisons (Table 2). Recognizing that it is not possible to extrapolate with any precision from the extent of nucleotide sequence divergence to the time of separation, these data lead us to the working hypothesis that the evolution of *Volvox,* a multicellular organism with division of labor between germ and soma, from a *Chlamydomonas*-like unicellular ancestor may have occurred in as brief an interval as that, during which the radiation of cereal grasses has occurred [perhaps 50 million years (Myr) or less]. This estimate, although somewhat surprising perhaps, is compatible with the close relationship between *V. carteri* and *C. reinhardtii* that was deduced, when it was found that the tubulin genes of these species have diverged by less than 1% in the amino acid sequence that they encode, and by less than 15% in their nucleotide sequences (Harper and Mages 1988; Mages et al. 1988).

When the aforementioned tubulin sequence data are used to calculate the extent of silent nucleotide changes that distinguish the two species (corrected for unobserved secondary exchanges by established methods: Ochman and Wilson 1987), values of 46% and 59% are obtained for the α- and β-tubulin genes, respectively. Using a value of 0.7–1% silent substitution rate per Myr (Ochman and Wilson 1987), this leads to an estimated divergence time for *Chlamydomonas* and *Volvox* of 50–70 Myr. This estimate corresponds rather well to that derived from the 1.5% difference in nucleotide sequence of the srRNAs observed here: assuming a mutation rate of 1% per 50 Myr for srRNA (Ochman and Wilson 1987), these data indicate that *V. carteri* and *C. reinhardtii* diverged some 75 Myr ago.

These data suggest that the close relationship among Volvocalean algae that has long been inferred on the basis of similarities in cellular morphology and physiology is reflected at the DNA sequence level, and reinforce the potential utility of this group as a model for exploring the genetic basis for the evolution of multicellular organisms from unicellular ancestors.

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