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Evolution

Phylogenetic relationships between chlorophytes, chrysophytes, and oomycetes

(evolution/ribosomal RNAs/*Ochromonas danica*/*Chlamydomonas reinhardtii*/*Achlya bisexualis*)

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ABSTRACT **The phylogenetic relationships among the chlorophyte *Chlamydomonas reinhardtii*, the chrysophyte *Ochromonas danica*, and the oomycete *Achlya bisexualis* were explored by comparing the sequences of their small-subunit ribosomal RNA coding regions. Comparisons of similarity values or inspection of phylogenetic trees constructed by distance matrix methods reveal a very close relationship between oomycetes and chrysophytes. The separation of chrysophytes from chlorophytes is comparable to that of plants from animals, and both separations are far antedated by the divergence of a number of other protist groups.**

Traditional analyses based on phenotypic criteria frequently depict chlorophytes (green algae) and chrysophytes (golden-brown algae) as representatives of lineages that diverged soon after the appearance of the earliest protists (1, 2). Comparisons of numerous characteristics such as features of nuclear division, chloroplast structure and pigment types, kinetid ultrastructure, the nature of the cell wall, and mitochondrial crista structure suggest a long and separate evolutionary history for these algal lineages. The presence of tubular mitochondrial cristae in chrysophytes and lamellar mitochondria cristae in chlorophytes (3) may be particularly significant. Since no recognizably monophyletic protistan groups are split with respect to crista type, and related groups seem to be connected by this characteristic, fundamental differences in mitochondrial structure are thought to reflect ancient divergences and to be of significance at a high taxonomic level.

Certain colorless protistan lineages may be more closely related to the chrysophytes than to the chlorophytes. Among these groups are the "lower" fungi. These forms are widely regarded as a polyphyletic assemblage of uncertain relationships to "higher" fungi (4). For example, the oomycetes have traditionally been grouped with the "true" fungi but comparisons of several biochemical and morphological characteristics suggest a close relationship to xanthophytes and chrysophytes (4–7). Taxonomic placement of the oomycetes is still controversial; in some schemes they are grouped with the higher fungi (8), and in others they are grouped with the chrysophytes and their relatives (9, 10).

Since there is little agreement about which phenotypic characteristics are most reliable for inferring evolutionary relationships, a consensus phylogeny for protists has never emerged. A phylogenetic tree, which reflects true genotypic similarity, can be inferred from comparisons of macromolecular sequences. Ribosomal RNAs have been used extensively for measuring evolutionary distances (11, 12) and these can be converted into phylogenetic trees by parsimony or distance matrix analyses. Because of their large size (relative to that of 5S and 5.8S rRNAs) and the existence of highly conserved and partially conserved sequence elements, the 16S-like or small-subunit rRNAs have been particularly useful for measuring both close and distant phylogenetic relationships (13, 14). As part of a program to investigate protistan evolution through comparisons of small subunit rRNA gene sequences, those of the chrysophyte *Ochromonas danica*, the chlorophyte *Chlamydomonas reinhardtii*, and the oomycete *Achlya bisexualis* were determined.¶

EXPERIMENTAL PROCEDURES

Cloning of Small-Subunit rRNA Genes. DNA and RNA from *O. danica* and *A. bisexualis* were prepared by phenol extraction of cells disrupted by homogenization in the presence of sodium dodecyl sulfate. DNA was purified by equilibrium centrifugation in cesium chloride (15). A genomic library for *O. danica* was prepared by inserting *Pst* I restriction fragments into the plasmid pBR325. Partial libraries for *A. bisexualis* were prepared by inserting restriction fragments into the *Eco*RI or *Hin*dIII sites of pBR325. *Escherichia coli* HB101 was transformed with recombinant plasmids. The *C. reinhardtii* rDNA gene was identified in a genomic library constructed in 1 EMBL4 (16). Radioactive probes for detecting recombinant colonies or plaques containing rDNA genes were prepared by using bulk RNA populations from the cognate organisms as templates in primer extension syntheses. Reverse transcriptase reactions containing dATP[^{35}S] were "primed" with synthetic oligonucleotides (15–18 nucleotides long) that are complementary to evolutionarily conserved regions in eukaryotic small-subunit rRNAs (12, 17). Since the primers only hybridize to specific sites on the cytoplasmic small-subunit rRNA templates, the synthesis of DNA probes that are specific for rRNAs encoded by the nucleus is assured.

DNA Sequence Analyses. Recombinant plasmids containing the *O. danica* and *A. bisexualis* small-subunit rRNA coding regions were grown in *E. coli* HB101 and amplified in the presence of spectinomycin (300 μg/ml). A 6.0-kilobase (kb) *Pst* I fragment defining the *O. danica* small-subunit rRNA was subcloned into the single-stranded phage M13mp19 as described (18). The *A. bisexualis* coding region resides on overlapping 5.8-kb *Eco*RI and 4.4-kb *Hin*dIII fragments, which were subcloned into the single-stranded phage M13mp18. The *C. reinhardtii* small-subunit rRNA coding region resides on a 2.4-kb *Sal* I fragment (19), which was also subcloned into M13mp18. The recombinant M13mp18 and M13mp19 phages propagated in *E. coli* strain JM 109 and single-stranded templates for directing DNA synthesis in the

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¶The sequences reported in this paper are being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos: J02949, *C. reinhardtii*; J02950, *O. danica*; and J02951, *A. bisexualis*).

dideoxynucleotide chain-termination sequencing protocols were prepared as described (18). DNA synthesis in the dideoxynucleotide chain-termination sequencing protocols was initiated using synthetic oligonucleotide primers that are well conserved in eukaryotic small-subunit rRNA genes (12). Complete sequences were determined for both strands of the small-subunit ribosomal RNA genes, except for the 20 positions at the 5′ and 3′ termini, which were sequenced in only one direction. Homologous regions in the small-subunit rRNA coding regions were aligned by procedures that consider the phylogenetic conservation of both primary and secondary structural features (12). Homologies (referred to here as "structural similarities") between all the unambiguously aligned sequence positions in the small-subunit rRNA coding region were used to infer phylogenetic trees as described elsewhere (12).

RESULTS

Aligned small-subunit rRNA coding regions from *C. reinhardtii, O. danica*, and *A. bisexualis* are shown in Fig. 1. The alignments were based on the juxtaposition of primary and secondary structures that are conserved in eukaryotic small-subunit rRNAs and were influenced by sequences not shown from rabbit (20), rat (21), *Xenopus laevis* (22), *Artemia salina* (23), *Zea mays* (24), rice (25), soybean (26), *Acanthamoeba castellanii* (27), *Saccharomyces cerevisiae* (28), *Neurospora crassa* (29), *Podospora anserina* (M.L.S., unpublished data), *Blastocladiella emersonii* (M.L.S. and H.E., unpublished data), *Tetrahymena hegewischi* (13), *Paramecium tetraurelia* (30), *Stylonychia pustulata* (12), *Oxytricha nova* (12), *Euplotes aediculatus* (31), *Prorocentrum micans* (32), *Plasmodium berghei* (33), *Dictyostelium discoideum* (34), *Trypanosoma brucei* (14), *Crithidia fasciculata* (35), and *Euglena gracilis* (14). The lengths of the *C. reinhardtii, O. danica*, and *A. bisexualis* coding regions are 1791, 1789, and 1809 nucleotides, respectively, and they define transcripts that contain all of the primary and secondary structure features found in other eukaryotic small-subunit rRNAs. Primer-extension analyses of cytoplasmic RNA preparations from the cognate organism (27) demonstrated that transcripts of the coding regions were represented in mature small-subunit rRNA populations (data not shown).

Pairwise comparisons of ≈1530 nucleotide positions that can be unambiguously aligned in all eukaryotic small subunit rRNA sequences were used to calculate structural similarity and structural distance values as described (12). Representative comparisons of the small-subunit rRNA sequences of *C. reinhardtii, O. danica*, and *A. bisexualis*, as well as those of other major eukaryotic groups are shown in Table 1. These data show that the chlorophyte–chrysophyte schism appeared after the divergence of several other protist lineages. From inspection of the similarity values or structural distance data it is apparent that the small subunit rRNAs of *C. reinhardtii* or *O. danica* are more closely related to *Rattus norvegicus* (similarity values of 0.830 and 0.813, respectively) than the small-subunit rRNAs of *D. discoideum* or *E. gracilis* are to *R. norvegicus* (similarity values of 0.749 and 0.650, respectively). We have previously shown that *E. gracilis* represents an early eukaryotic divergence that was followed by the successive branching of lineages leading to *D. discoideum, P. berghei*, and the more recent and nearly simultaneous divergence of animals, plants, fungi, ciliates, and several other protist groups. Since the structural distance value between *O. danica* and *C. reinhardtii* is similar to the distance values between plants, fungi, and ciliates, the divergence of chrysophytes from chlorophytes could not have been an early event in protistan evolution. The structural distance data in Table 1 also reveal an unexpectedly close relationship between oomycetes and chrysophytes. The similarity value of 0.916 between *O. danica* and *A. bisexualis* is similar to values of 0.916 from a comparison of *S. cerevisiae* to *N. crassa* or 0.913 from a comparison of *C. reinhardtii* to *Z. mays*. On the basis of structural distance data, it appears that chrysophytes and their relatives are closely related to the oomycete *A. bisexualis*.

The structural similarity data shown in Table 1 were expanded to include rabbit, rice, soybean, *A. castellanii, Podospora anserina, O. nova, Plasmodium berghei*, and *T. brucei*. The resulting structural distance data were converted into the phylogenetic tree shown in Fig. 2 using a modification (12) of the distance matrix methods (36). As observed through direct comparisons of the similarity data, the separation of *O. danica* and *C. reinhardtii* corresponds to a relatively recent period of radiative evolution that gave rise to plants, fungi, animals, and a number of protist groups. The *O. danica* lineage appears to have shared a common evolutionary history with that of *A. bisexualis*. Finally, the separation of the dinoflagellate *Prorocentrum micans* does not appear to have been an early event in the evolutionary history of the Eukaryota.

DISCUSSION

The usefulness of several characteristics that are commonly viewed as indicators of primitiveness or early evolutionary branchings among protistan lineages is brought into question by the phylogeny inferred from comparisons of small-subunit rRNA coding region sequences. As shown in Fig. 2, the split between the chrysophytes and chlorophytes does not extend to the base of the protistan phylogenetic tree. The chlorophyte *C. reinhardtii* is closely related to the Metaphyta, and the chrysophyte *O. danica* appears to represent a lineage that diverged from other protists shortly before the nearly simultaneous separation of lineages leading to plants, fungi, and animals. Therefore, the split between organisms with nondiscoidal lamellar cristae and organisms with tubular cristae does not appear to be very ancient. The tree does segregate those protists with lamellar and tubular mitochondrial cristae (only *A. castellanii* is misplaced), but in an unexpected way. Organisms with lamellar cristae appear to be direct descendants of those with tubular cristae. It remains to be determined whether the separate types arose from different purple bacteria (37) or if lamellar cristae arose from tubular cristae. The positions of *Chlamydomonas* and *Ochromonas* indicates that neither the difference in crista structure nor the difference in chlorophyll types should be used as indicators of early diverging lineages in eukaryotic evolutionary trees.

The peculiar nucleus of the dinoflagellates has suggested to many that they are a very ancient group, ancestral to other eukaryotes (38). Their chromosomes are permanently condensed and lack histones. The nuclear membrane remains intact during mitosis and the spindle is entirely extranuclear in most forms. It initially appeared as though chromosomes are separated from each other during mitosis through the expansion of the nuclear membrane to which they are attached. The rRNA-based phylogeny indicates that dinoflagellates (as represented by *Prorocentrum micans*) are not a group that appeared early in protistan evolution. They are specifically related to ciliates. This association was not recognized in an earlier phylogenetic analysis that failed to include ciliates or other protist groups known to represent earlier evolutionary branches (32). From comparisons of a limited number of secondary structure features, Herzog and Marteaux (32) suggested that the *Prorocentrum micans* small-subunit rRNA closely resembles the archaebacterial homologue and precedes the divergence of *D. discoideum*. However, on the basis of simple comparisons of structural similarity values or phylogenetic trees constructed by using

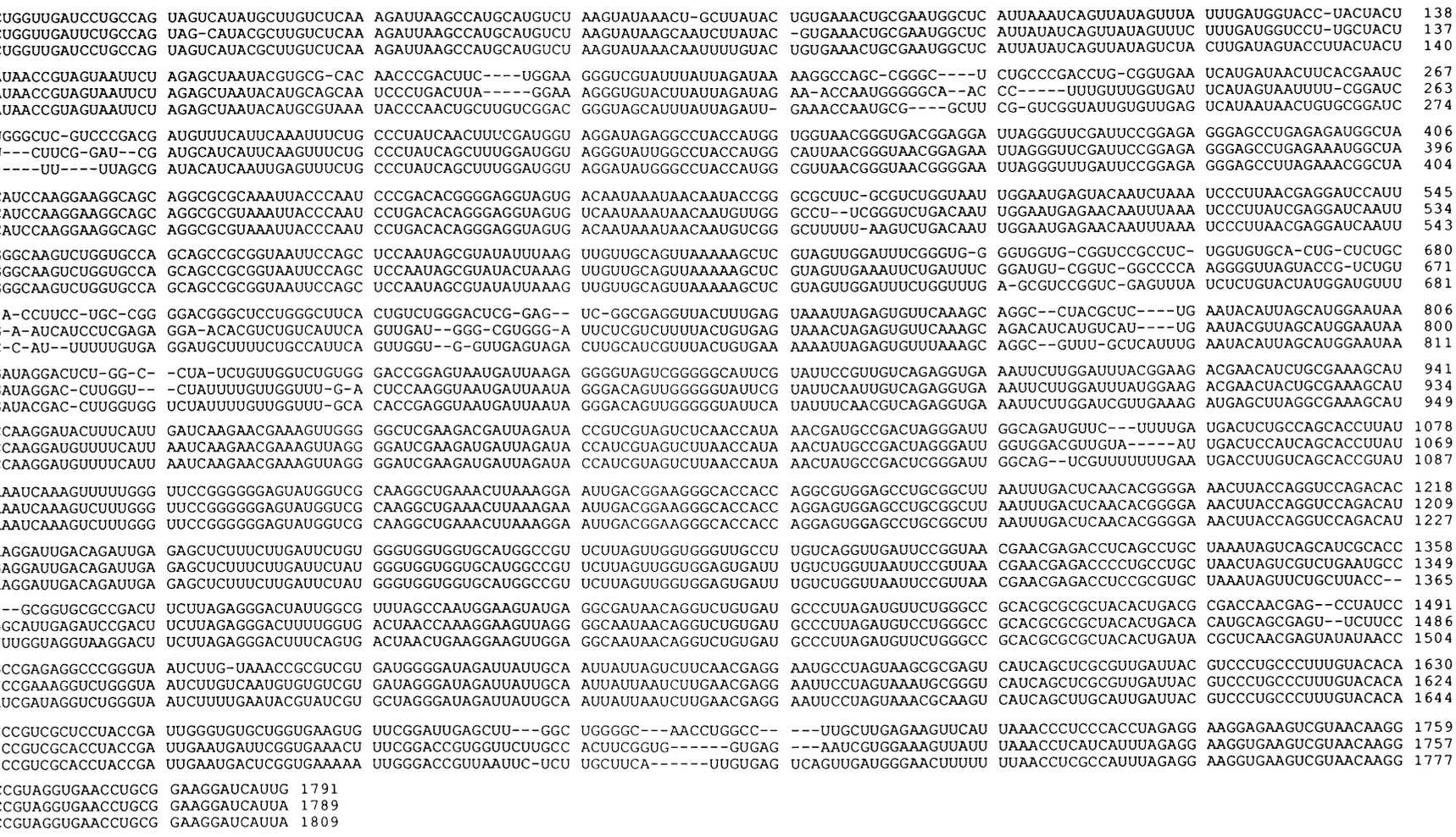

FIG. 1. The sequences of the small-subunit rRNA coding regions from *C. reinhardtii* (a), *O. danica* (b), and *A. bisexualis* (c). Homologous sequence positions were initially aligned on the basis of obvious primary structure similarity. The locations of phylogenetically conserved secondary structures were then juxtaposed to refine the alignments where length variation occurred. The alignments were influenced by sequences from 23 other eukaryotic small-subunit rRNA coding regions (see text).

Table 1. Structural similarity and distance data between eukaryotic small-subunit rRNA gene sequences

Organism	*R.n.*	*X.l.*	*A.s.*	*Z.m.*	*C.r.*	*S.c.*	*N.c.*	*O.d.*	*A.b.*	*S.p.*	*P.m.*	*D.d.*	*E.g.*
R. norvegicus		0.966	0.884	0.835	0.830	0.832	0.821	0.813	0.820	0.806	0.812	0.749	0.650
X. laevis	0.034		0.882	0.837	0.831	0.833	0.820	0.818	0.826	0.809	0.817	0.758	0.653
A. salina	0.125	0.127		0.833	0.826	0.836	0.832	0.821	0.824	0.807	0.818	0.765	0.652
Z. mays	0.186	0.184	0.189		0.913	0.871	0.863	0.866	0.878	0.859	0.877	0.780	0.676
C. reinhardtii	0.193	0.191	0.197	0.092		0.875	0.871	0.870	0.884	0.857	0.873	0.772	0.674
S. cerevisiae	0.190	0.188	0.185	0.141	0.137		0.916	0.867	0.878	0.876	0.872	0.781	0.667
N. crassa	0.204	0.205	0.189	0.150	0.141	0.088		0.852	0.870	0.858	0.866	0.764	0.657
O. danica	0.215	0.209	0.203	0.148	0.143	0.146	0.164		0.916	0.865	0.879	0.797	0.679
A. bisexualis	0.206	0.198	0.199	0.132	0.126	0.132	0.142	0.089		0.884	0.889	0.800	0.680
S. pustulata	0.224	0.219	0.223	0.156	0.158	0.135	0.157	0.149	0.126		0.897	0.784	0.666
P. micans	0.216	0.210	0.207	0.134	0.138	0.139	0.147	0.132	0.119	0.110		0.782	0.674
D. discoideum	0.305	0.291	0.281	0.260	0.272	0.258	0.283	0.236	0.232	0.254	0.257		0.652
E. gracilis	0.472	0.465	0.467	0.423	0.427	0.440	0.459	0.419	0.417	0.441	0.427	0.468	

The upper right half of the table gives structural similarity values (12) for regions that can be unambiguously aligned in all of the considered small-subunit rRNA sequences. A total of 1530 positions were considered. The structural distances (average number of base changes per sequence position) are shown in the lower left half of the table.

distance matrix methods, we suggest that *Prorocentrum micans* shared a common ancestry with the Ciliophora. The affiliation of *Prorocentrum micans* with the Ciliophora is observed in similar tree constructions that include a larger number of ciliates, other eukaryotes, or representatives from the two prokaryotic kingdoms (data not shown). The peculiar nucleus and type of mitosis found in dinoflagellates probably arose secondarily from a type more typical of eukaryotes. It is now known that at least some dinoflagellates have chromosomes connected to microtubules of the spindle by kinetochores during mitosis. The possible affiliation of dinoflagellates with ciliates has been previously suggested on the basis of kinetid ultrastructure and the unusual arrangement of membranes at the cell surface (39).

On the basis of great differences in life cycle, mode of nutrition, and nuclear division, a relationship between chrysophytes (as represented by *O. danica*) and oomycetes (as represented by *A. bisexualis*) is unexpected. However, the comparisons of small-subunit rRNAs convincingly demonstrate that oomycetes and chrysophytes are separated by only a short evolutionary distance. By the criterion of rRNA homology, the extent of genetic relatedness between oomycetes and chrysophytes is similar to the genetic distances between *N. crassa* and *S. cerevisiae* or *C. reinhardtii*

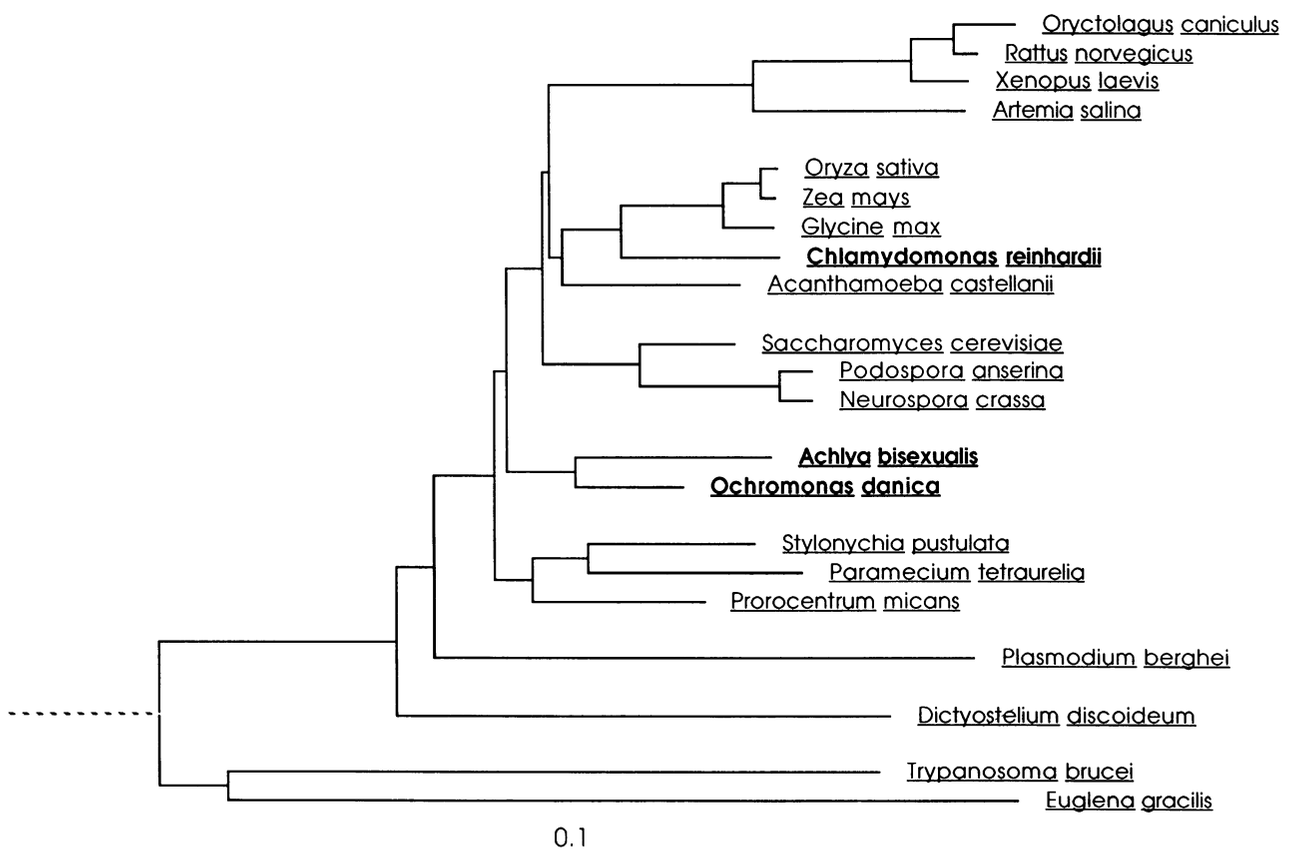

FIG. 2. Eukaryotic phylogeny inferred from small-subunit rRNA sequence similarities. A phylogenetic tree was inferred by using the structural distance data in Table 1. The analysis is limited to ≈1530 positions. The evolutionary distance between nodes of the tree is represented in the horizontal component of their separation in the figure.

and *Z. mays*. Furthermore, the oomycete/chrysophyte lineage is separate and distinct from the fungal line of descent. A close evolutionary relationship between chrysophytes and oomycetes can hardly be denied and the taxonomic separation of the lower fungi from the higher fungi seems reasonable. The oomycetes apparently evolved a mode of life similar to that of the higher fungi but independently from them. The oomycetes are unlike the true fungi in having tubular cristae, cellulose cell walls (although some have chitin, as the true fungi usually do), and in synthesizing lysine by the diaminopimelic acid pathway (5, 7). They are specifically like chrysophytes (and their relatives) in having heterokont flagellated stages and very similar kinetids (4, 6).

The results described in this paper illustrate the difficulty of determining which morphological features reliably indicate fundamental schisms in protistan evolution, or which organisms are primitive. Like the dinoflagellates, the red algae are postulated to be very primitive eukaryotes, and as in the case of dinoflagellates, this is contradicted by rRNA sequence information (J.H.G. and M.L.S., unpublished data). It remains to be determined which extant protistan groups diverged from other forms near the base of the protistan evolutionary tree and which phenotypic characteristics can be used to identify such divergences.

If trees produced from small-subunit rRNA sequences are even approximately correct, it indicates that nuclear and mitotic characteristics evolve too rapidly and contain too many convergent characteristics (at least as currently analyzed) to estimate similarities between intermediate or distantly related forms. On the other hand, kinetid ultrastructural characteristics may provide quite reliable evidence, at least over the evolutionary distances between which the structures are still comparable.

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