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Novel Eukaryotes from the Permanently Anoxic Cariaco Basin (Caribbean Sea)†

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Present knowledge of microbial diversity is decidedly incomplete (S. J. Giovannoni and M. S. Rappé, p. 47-84, *in* D. Kirchman, ed., *Microbial Ecology of the Oceans*, 2000; E. Stackebrandt and T. M. Embley, p. 57-75, *in* R. R. Colwell and D. J. Grimes, ed., *Nonculturable Microorganisms in the Environment*, 2000). Protistan phylogenies are particularly deficient and undoubtedly exclude clades of principal ecological and evolutionary importance (S. L. Baldauf, Science 300:1703-1706, 2003). The rRNA approach has been extraordinarily successful in expanding the global prokaryotic record (S. J. Giovannoni and M. S. Rappé, p. 47-84, *in* D. Kirchman, ed., *Microbial Ecology of the Oceans*, 2000; E. Stackebrandt and T. M. Embley, p. 57-75, *in* R. R. Colwell and D. J. Grimes, ed., *Nonculturable Microorganisms in the Environment*, 2000) but has rarely been used in protistan discovery. Here we report the first application of the 18S rRNA approach to a permanently anoxic environment, the Cariaco Basin off the Venezuelan coast. On the basis of rRNA sequences, we uncovered a substantial number of novel protistan lineages. These included new clades of the highest taxonomic level unrelated to any known eukaryote as well as deep branches within established protistan groups. Three novel lineages branch at the base of the eukaryotic evolutionary tree preceding, contemporary with, or immediately following the earliest eukaryotic branches. These newly discovered protists may retain traits reminiscent of an early eukaryotic ancestor(s).

Until recently, biodiversity studies of unicellular eukaryotes focused on gross morphology and ultrastructure. These approaches led to erection of dozens of clades of the highest taxonomic order (28). However vast this picture of protistan diversity may appear, it probably represents just the tip of the iceberg (3, 27, 28). Several recent rRNA-based surveys (2, 5, 7, 21, 26, 31, 36) have left little doubt that eukaryotic lineages of principal evolutionary and ecological importance still await discovery.

Anoxic environments occupy a special place in the search for novel forms of eukaryotic life (34). Eukaryotes may have evolved before the planet's biosphere became oxygenated (9). Oxygenation notwithstanding, anoxic systems have existed continuously throughout Earth's history. It follows that such environments may still contain organisms that branched early in the history of eukaryotes and may retain ancestral eukaryotic lineages. Until now this hypothesis was rarely tested because modern biodiversity research has barely examined Earth's largest permanently anoxic ecosystems (5). Here we report on the use of 18S rRNA approach in protistan discovery in one such ecosystem, the Cariaco Basin in the Caribbean Sea on the northern continental margin of Venezuela.

The Cariaco Basin is the world's largest, truly marine permanently anoxic basin. It exhibits pronounced vertical chemical gradients controlled by physical transport of oxygen downward and reduced compounds upward countered by biological demands. Typically, oxygen concentrations decrease from saturation at the surface to 0 μM between 250 and 350 m; deeper waters have remained anoxic and sulfidic down to the basin's floor (≈1,400 m) over timescales of centuries to millennia (32). This water column is characterized by pronounced and predictable vertical layering of microbial communities and productivity (38, 39). Significant enrichments in abundances of prokaryotes, protozoans and viruses and prokaryotic productivity are routinely observed in the sulfidic waters immediately underlying the oxic/anoxic interface (38, 39). Prokaryotic diversity in these anoxic waters has been addressed to a limited extent by using 16S ribosomal DNA libraries (23), and until now microeukaryotic diversity was ignored entirely.

MATERIALS AND METHODS

Sampling site. The sampling site was located in the eastern subbasin of the Cariaco Basin at 10.50°N, 64.66°W. This is the location of the time series station of the cooperative U.S.-Venezuelan Carbon Retention in a Colored Ocean (CARIACO) program (39).

Microbial abundance and production. Sampling was conducted aboard the B/O *Hermano Gines*, operated by Estación de Investigaciones Marinas (EDIMAR), Fundación la Salle de Ciencias Naturales, located on Margarita Island, Venezuela. Water samples were collected at 18 depths with a SeaBird rosette equipped with 12 TFE-lined, 8-liter Niskin bottles. Dissolved O_2 concentrations were provided by a YSI oxygen probe mounted on the rosette. Sampling protocols are described in references 38 and 39.

At each depth, whole-water samples (200 ml) were preserved with 2% (final concentration) borate-buffered formaldehyde and were stored at 5°C. In the laboratory, standard 4′,6′-diamidino-2-phenylindole-stained slides were prepared on dark 0.2- or 0.8-μm Poretics polycarbonate membranes for enumeration of prokaryotes or flagellated protozoans by epifluorescence microscopy, respectively (29). Heterotrophic and chemoautotrophic bacterial production was estimated from incorporation of [^{3}H]leucine into protein and incorporation of [^{14}C]bicarbonate into particles, respectively, as previously described in reference

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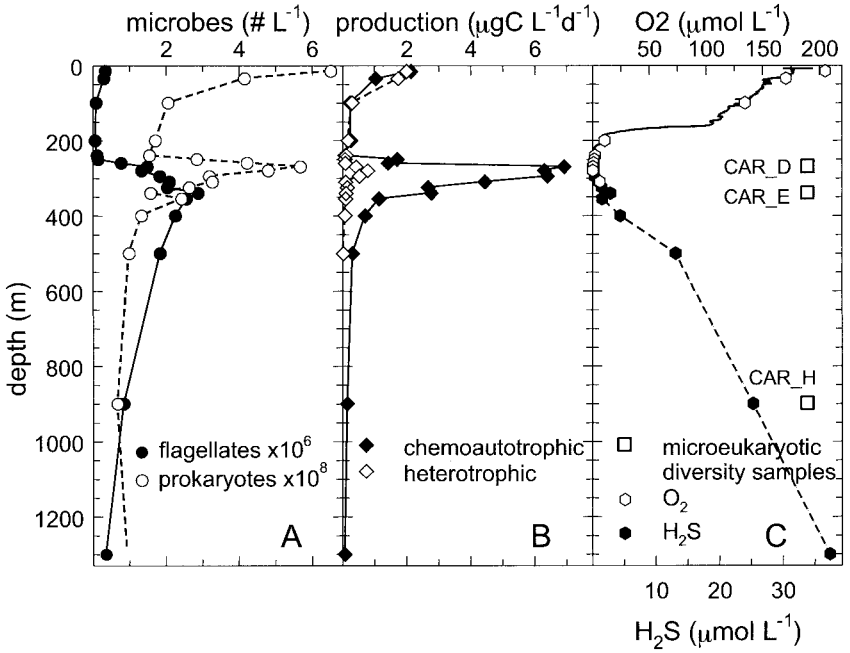

FIG. 1. Vertical distributions of microorganisms (A), bacterial production (B), and oxygen, hydrogen sulfide, and samples for phylogenetic analysis (C) at Station CARIACO on 8 May 2002. Chemoautotrophic production was determined by assimilation of $^{14}CO_2$ into particles in the dark, and heterotrophic production was estimated from [^{3}H]leucine incorporation into protein.

39. Protocols used to measure dissolved O_2 and H_2S in discrete samples are also described in reference 39.

Sampling for phylogenetic studies. Samples were collected at 270-m (CAR_D), 340-m (CAR_E), and 900-m (CAR_H) water depths and were withdrawn from Niskin bottles on deck under N_2 atmosphere to sterile, evacuated 2-liter hospital intravenous bags (non-DEHP vinyl; Secure Medical Inc., Whitewater, Wis.) immediately after retrieval. The depths corresponded to oxic/anoxic interface and anoxic layers. Duplicate bags from each depth ($n = 6$) were stored immersed in seawater maintained at ≤10°C in total darkness and were processed upon return to the shore lab (EDIMAR), within 40 h of collection. Cells from each bag were collected separately on 47-mm Durapore membranes (0.65-μm pore size) by using an in-line filtration system connected directly to the intravenous bag under gentle vacuum (<25 cm Hg) provided by a hand pump. Sample volumes processed varied between 2.3 and 2.8 liters and readily passed through a single membrane. Sampling and processing protocols prevented exposure to the atmosphere and minimized alterations in the water's redox potential. Immediately after filtration, membranes were individually placed in 2.0-ml cryovials containing 1 ml of DNA extraction buffer (41). Then 5 μl of proteinase K was added before freezing of the sample at −20°C. Samples were returned to the United States frozen in a block of ice.

DNA isolation, PCR amplification, cloning, and sequencing. Nucleic acids were obtained from one of the two samples obtained at each depth. High-molecular-weight DNA was extracted as described in reference 25. In short, the samples were heated to 65°C for 2 h in a high-salt extraction buffer (100 mM Tris-HCl [pH 8], 100 mM Na_2EDTA [pH 8], 100 mM $NaPO_4$ [pH 8], 1.5 M NaCl, and 1% cetyltrimethylammonium bromide [CTAB]) with proteinase K (final concentration, 100 μg ml^{-1}), sodium dodecyl sulfate (20%), and CTAB (1%). The lysates were purified twice by extraction with an equal volume of chloroform-isoamyl alcohol (24:1) and were precipitated with 0.6 volume of isopropanol. Potential inhibitors of downstream applications were removed by DNA purification with the resin-based Wizard DNA cleanup system (Promega, Madison, Wis.). The integrity of the total DNA was checked by agarose gel electrophoresis (0.8%).

We amplified an ≈1,200-bp fragment of the 18S rRNA gene by using a nested PCR. In the first reaction, nearly full-length 18S rRNA genes were PCR amplified by using a universal eukaryotic primer set (EukA 1 to 21 and EukB 1795 to 1772 [25]) and HotStar *Taq* DNA polymerase (Qiagen, Valencia, Calif.). The PCR protocol consisted of an initial hot-start incubation (15 min at 95°C) followed by 30 identical amplification cycles (denaturing at 95°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2.5 min) and final extension at 72°C for 7 min. Negative control reactions included bacterial (*Escherichia coli*) or archaeal (*Halobacterium salinarium* ATCC 19700) DNA as templates. In the second reaction, the eukaryotic forward primer 360FE and the universal reverse primer 1492R (25) together with 1 to 3 μl of the first PCR mixture were used to amplify the target fragments by using the same PCR protocol as described above.

The PCR products were used to construct one clone library for each of the three depths sampled by using the TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Plasmids were isolated from overnight cultures by using the Qiaprep Spin Miniprep kit (Qiagen). The presence of the target insert was confirmed by PCR reamplification by using the primer set and PCR protocol described above. Between 200 and 400 ng of positive amplification products of the target size was digested with 7.5 U of the restriction endonuclease *Hae*III (New England Biolabs, Beverly, Mass.) for 60 min at 37°C, followed by an inactivation step for 20 min at 80°C. The resulting bands were separated by electrophoresis in a 2.5% low-melting-point agarose gel at 80 V for 2 to 3 h. Clones with identical fragment (ARDRA) patterns were considered members of the same operational taxonomic unit (OTU). At least one clone of each OTU was sequenced at the University of Maine DNA Sequencing Facility by using an Applied Biosystems (ABI) 373 DNA Stretch sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.0 Cycle Sequencing Ready Reaction kit. In total, 130 clones with unique ARDRA patterns were sequenced bidirectionally. Low-quality sequence reads and nontarget metazoan sequences were excluded from the phylogenetic analyses.

Phylogenetic analyses. Environmental 18S rRNA gene sequences obtained were compared to those in GenBank by using gapped BLAST analysis (1) and to >5,000 prealigned eukaryotic small-subunit rRNAs in the Ribosomal Database Project (24). Multiple alignments were obtained by using ClustalX (40) and were manually refined by using phylogenetically conserved secondary structures. The RNA secondary-structure prediction and comparison tools of the Vienna RNA Package (18) were used to construct secondary-structure models, which were verified against known conserved secondary-structure regions (4). Environmental gene sequences were tested by using the Ribosomal Database Project CHECK_CHIMERA program (24) to detect potential chimeric gene artifacts (10, 33, 37). Further phylogenetic analyses included only conserved nucleotides at unambiguously aligned positions (their number is indicated in Fig. 2 to 5). Minimum-evolutionary-distance trees were constructed by using the PAUP* software package 4.0b10 (37). All heuristic searches were performed by using random, stepwise addition of taxa with the TBR branch-swapping algorithm. We used the program Modeltest (30) to choose the model of DNA substitution that best fit our data sets from among 56 possible models. Modeltest was run for each

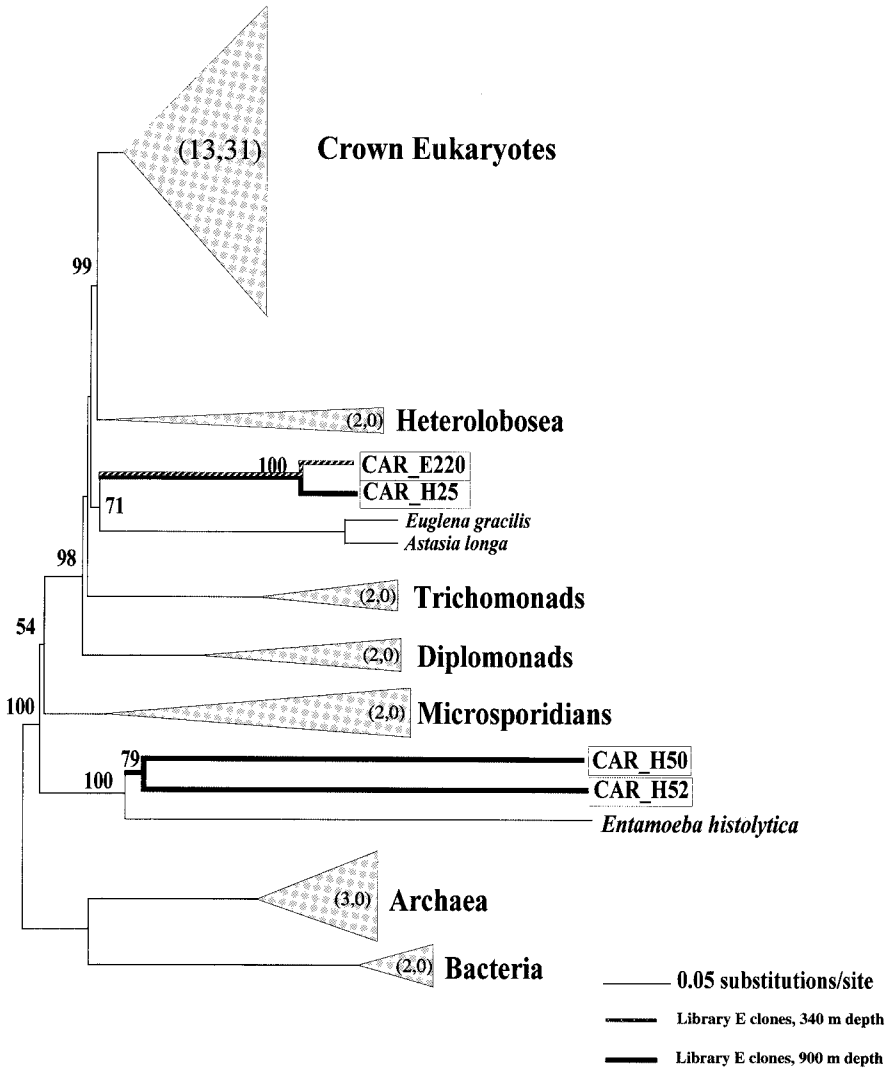

FIG. 2. Minimum-evolution phylogenetic tree of eukaryotic small-subunit rRNA showing the positions of clones E220, H25, H50, and H52. The tree was constructed under maximum-likelihood criteria by using a general-time-reversible DNA substitution model with the variable-site gamma distribution shape parameter (G) at 0.9831, based on 657 aligned positions. Distance bootstrap values over 50% are given at the respective nodes. The size of each shaded triangle corresponds to the total number of sequences in the respective group used in the analysis. The first and second numbers in parentheses correspond to the numbers of GenBank sequences and Cariaco sequences, respectively, included in the group.

individual data set. The DNA substitution models as well as the parameter settings for each tree constructed are described in detail in the legend of the respective figure. We assessed the relative stability of tree topologies by using 1,000 bootstrap replicates. Heuristic searches for bootstrap analyses employed stepwise addition starting trees with simple addition of sequences and TBR branch swapping. Relative similarities between two different clones are based on ClustalX alignments of homologous regions of these two clones. Relative rate tests were conducted by using MEGA version 2.1 (20).

Nucleotide sequence accession numbers. The gene sequences from this study have been deposited in the GenBank database (accession numbers AY256203 through AY256336).

RESULTS AND DISCUSSION

At the time of sampling, the oxic/anoxic interface appeared to be at approximately 270 m, where peaks in prokaryotic abundance and chemolithotrophic production were evident (Fig. 1). Our shallowest sample was obtained from the interface (CAR_D, 270 m), which is probably best categorized as a microxic environment. Immediately below the oxic/anoxic interface, we detected a broad maximum in protistan abundance and a narrower peak in heterotrophic bacterial production (Fig. 1). The diversity within this zone was captured in sample CAR_E (depth, 340 m). We consider the environments of this sample and a deeper sample (CAR_H; 900 m) anoxic because both depths were sulfidic with undetectable oxygen (Fig. 1).

Clone libraries. We constructed three clone libraries designated D, E, and H corresponding to the three depths sampled (270, 340, and 900 m, respectively). These libraries totaling over 500 clones contained 43 (D), 46 (E), and 42 (H) unique ARDRA patterns. A random check of 10 pairs of clones with identical patterns showed low (<0.01%) to no sequence divergence within a single pattern. Therefore, each ARDRA pattern was considered a single and unique OTU. The libraries contained 7 (D), 5 (E), and 10 (H) unique metazoan nontarget OTUs, which, together with low-quality and chimeric sequences, were excluded from further phylogenetic analyses. The phylogenetic analyses included 36 (D), 41 (E), and 32 (H) unique protistan OTUs.

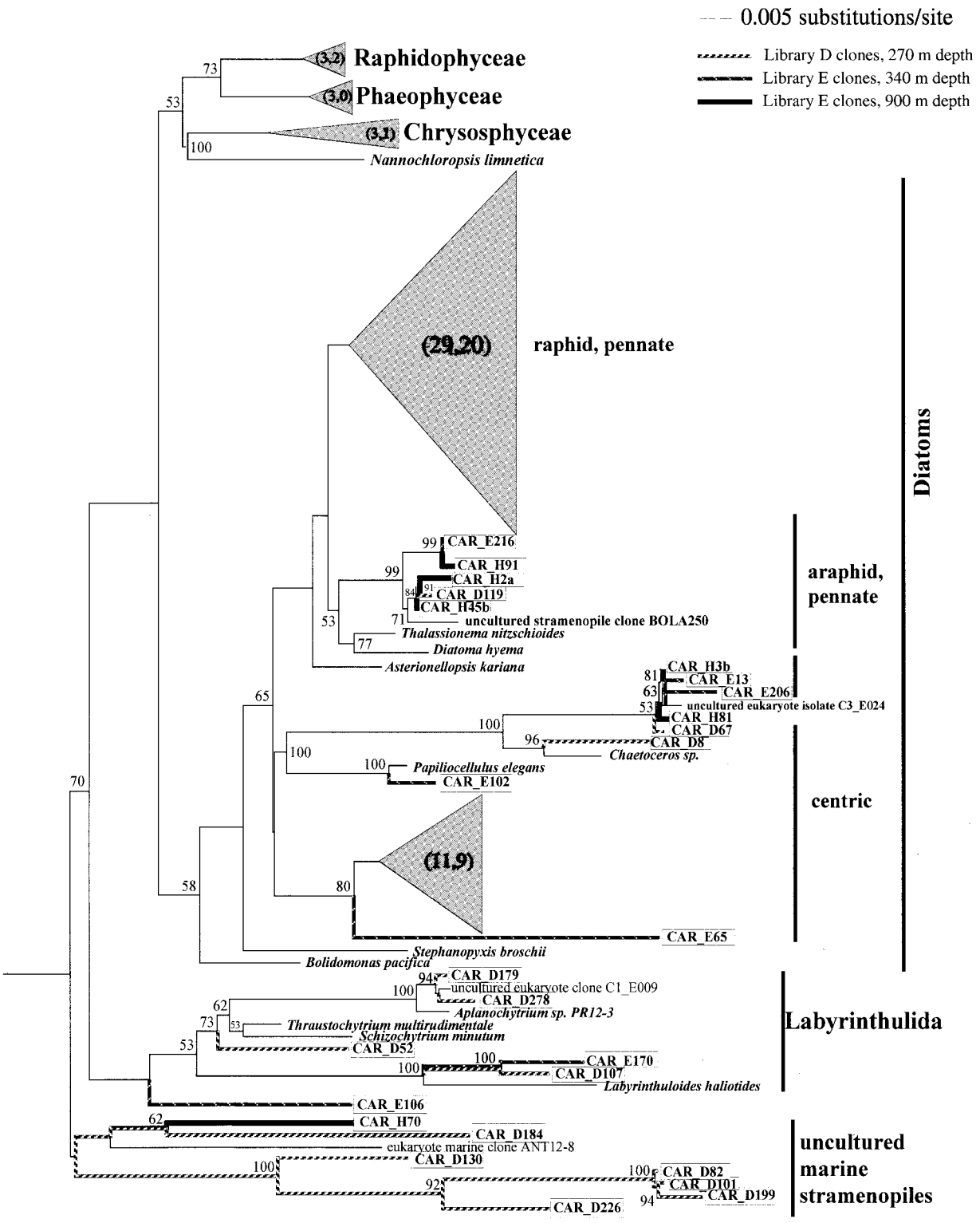

FIG. 3. Minimum-evolution phylogenetic tree of stramenopiles. The tree topology was obtained under a Kimura–two-parameter DNA substitution model with the variable-site gamma distribution shape parameter (G) at 0.4830 and the among-site rate variation proportion of invariable sites (I) at 0.2945 based on 648 aligned positions. Distance bootstrap values over 50% are given at respective nodes. The size of each shaded triangle corresponds to the total number of sequences in the respective group used in the analysis. The first and second numbers in parentheses correspond to the numbers of GenBank sequences and Cariaco sequences, respectively, included in the group.

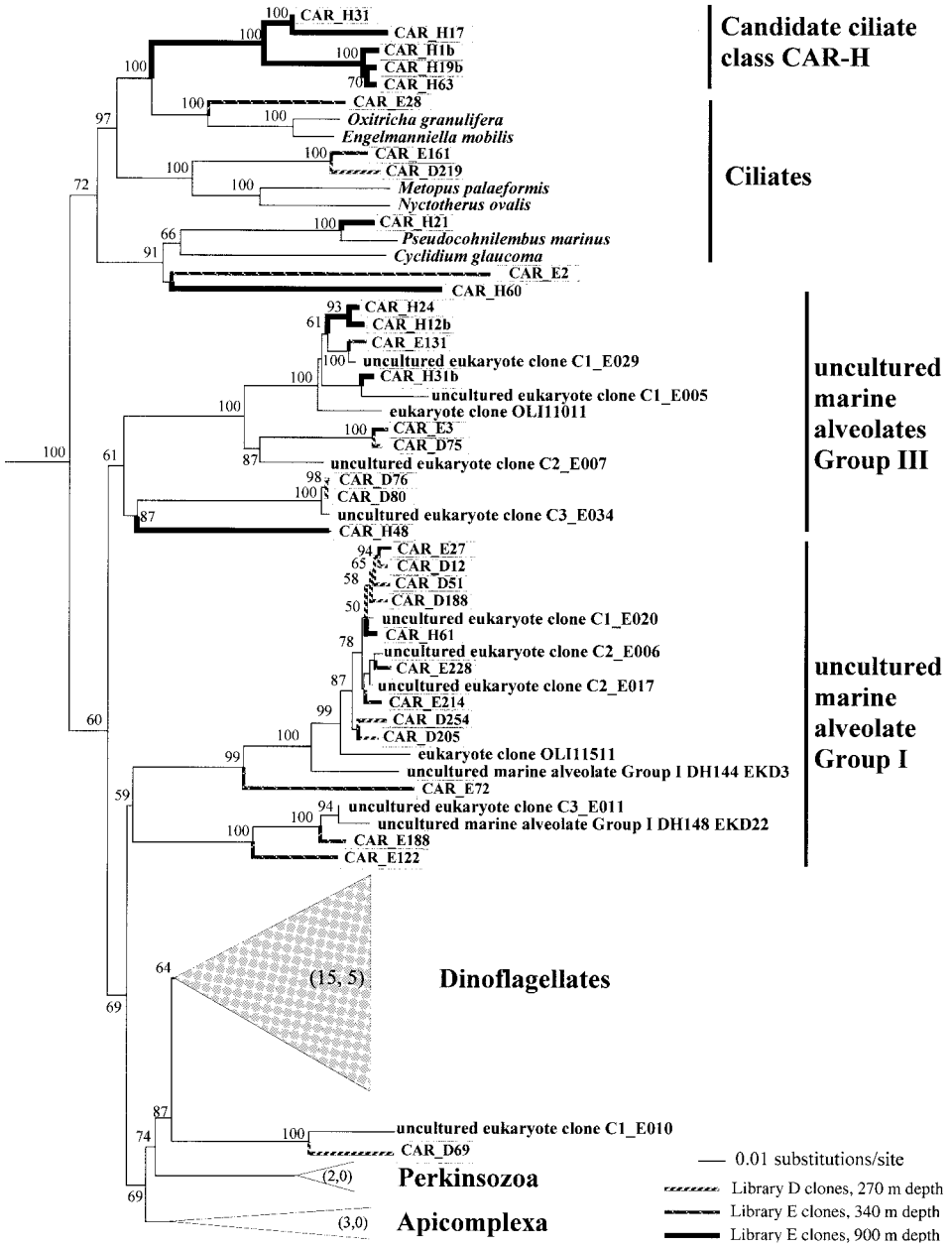

FIG. 4. Minimum-evolution phylogenetic tree of alveolates. The tree topology was obtained under a Tamura-Nei DNA substitution model with equal base frequencies and variable-site gamma distribution shape parameter (G) at 0.5042, based on 529 aligned positions. Distance bootstrap values over 50% are given at respective nodes. The size of each shaded triangle corresponds to the total number of sequences in the respective group used in the analysis. The first and second numbers in parentheses correspond to the numbers of GenBank sequences and Cariaco sequences, respectively, included in the group.

The 18S rRNA gene sequences obtained are consistent with the hypothesis of a vast diversity of early branching novel protists in this oxygen-depleted environment. Clones H50 and H52 recovered from 900 m represent two especially deeply rooted lineages on the 18S rRNA eukaryotic tree (Fig. 2). Although their basal position is reasonably bootstrap supported, this position may also be due to these sequences' divergent nature and/or lack of sufficient taxon sampling, since they are not specifically related to any recognized eukaryotic clade or to each other (43.7% similarity between the two). Similarly, the exact phylogenetic position of another early branching clade consisting of two clones, E220 (also present in library H) and H25, is difficult to determine. In spite of low bootstrap support for the branching order of these four clones, it is likely that they form three novel eukaryotic lineages of the highest taxonomic order branching very early in the course of eukaryotic evolution. Organisms in these lineages may retain traits reminiscent of an early eukaryotic ancestor(s).

We detected rRNA signatures that may be important for the phylogeny of stramenopiles, one of the most diverse groups of

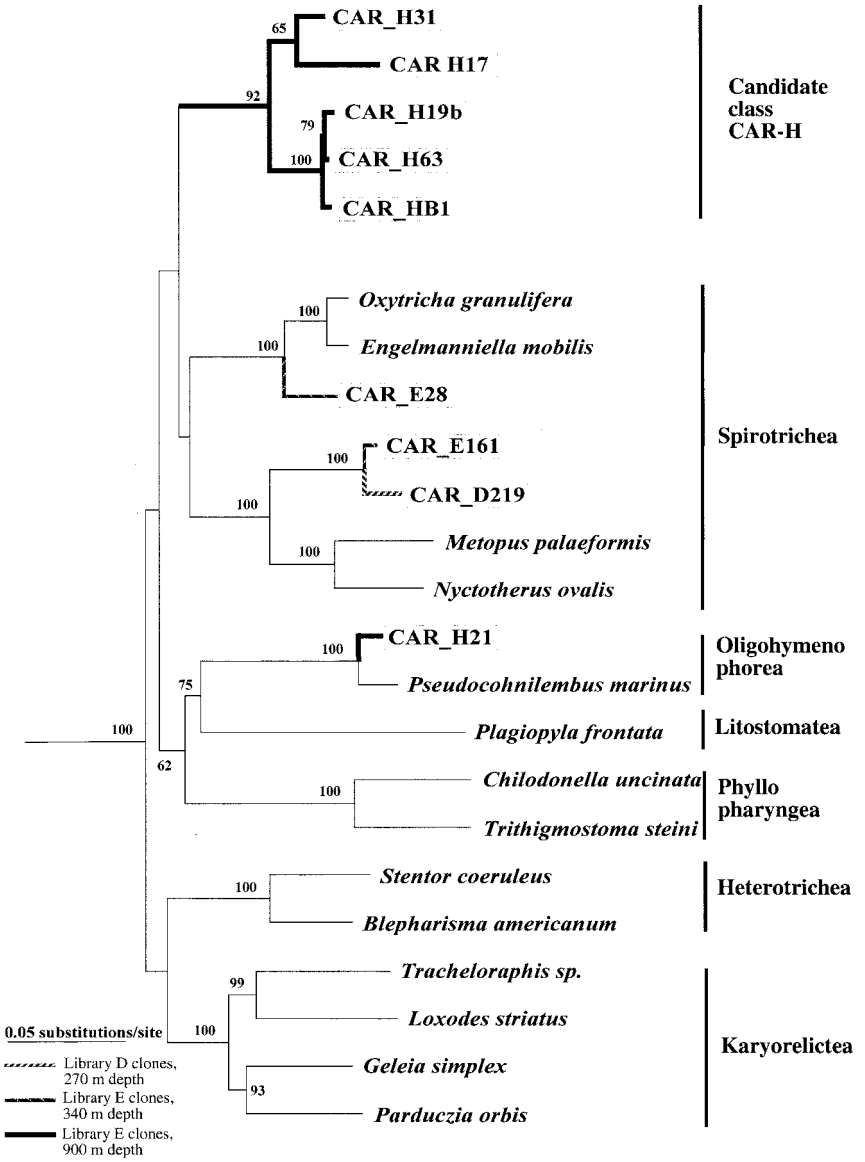

FIG. 5. Minimum-evolutionary-distance phylogenetic tree of ciliated protozoans. The tree was constructed under maximum-likelihood criteria by using a general-time-reversible DNA substitution model with the variable-site gamma distribution shape parameter (G) being 0.4671, based on 506 aligned positions. Distance bootstrap values over 50% are given at respective nodes. Class-level classification is done according to the information in reference 22 and is well supported by the rRNA data. Candidate class CAR_H is a sister group of classical ciliate classes.

eukaryotes (28). Clones D82, D101, D130, D199, and D226 form a novel, well-supported protistan clade that appears to be either an ancestral or sister group to all the other stramenopiles (Fig. 3). A second clade of stramenopiles is formed by clones H70 and D184, together with one rRNA sequence detected previously in Antarctic waters (21) (Fig. 3). Collectively, these clones make it abundantly clear that a significant level of undetected diversity exists within the stramenopiles, possibly representing basal stramenopile lineages. More information on these novel groups will help to elucidate early events in the evolution of stramenopiles.

We obtained a substantial number of clones that form deeply rooted lineages within known protistan clades. Several findings are particularly noteworthy. Clones E106 and identical D79 form a lineage that appears ancestral to Labyrinthulida, a unique heterotrophic phylum branching early within the stramenopiles (Fig. 3). Clones D67, E13, E206, H3b, and H81 support a clade of novel uncultivated centric diatoms; the clade also includes ribosomal sequences uncovered earlier in anoxic sediments at a hydrothermal site (7) (Fig. 3). Clones D119, E216, H2a, H45b, and H91 increase diversity in a clade of uncultivated pennate diatoms also retrieved from anoxic intertidal marine sediments (5) (Fig. 3).

We identified a large undescribed diversity within a major eukaryotic group, the alveolates. Some of the sequences obtained fell into a previously described group, group I, of uncultivated alveolates (21) and included an ancestral signature to this ubiquitous group (clone E72; Fig. 4). Others formed a

TABLE 1. Distribution of clones detected at more than one depth

Clone	Taxon	Occurrence	Closest relative (% similarity)
CAR_D3	Stramenopile	270 m, 340 m, 900 m	*Skeletonema pseudocostatum* (98.4)
CAR_E142	Stramenopile	270 m, 340 m, 900 m	*Amphora montana* (95.6)
CAR_H11a	Stramenopile	270 m, 340 m, 900 m	*Thalassiosira rotunda* (98.6)
CAR_D94	Stramenopile	270 m, 340 m, 900 m	*Navicula cryptocephala* (97.5)
CAR_D102	Stramenopile	270 m, 340 m, 900 m	*Peridinium balticum* endosymbiont (96.2)
CAR_D66	Stramenopile	270 m, 340 m, 900 m	*Eolimna minima* (94.8)
CAR_H3b	Stramenopile (uncultured)	270 m, 340 m, 900 m	Uncultured eukaryote clone C3_E024 (99.6)
CAR_H45b	Stramenopile (uncultured)	270 m, 340 m, 900 m	Uncultured stramenopile clone BOLA250 (98.0)
CAR_E214	Alveolate (uncultured)	270 m, 340 m, 900 m	Uncultured eukaryote isolate C2_E017 (99.0)
CAR_E230	Alveolate	270 m, 340 m, 900 m	*Gymnodinium* sp. strain MUCC284 (97.7)
CAR_H48b	Fungi	270 m, 340 m, 900 m	*Penicillium namyslowski* (98.8)
CAR_E77	Stramenopile	270 m, 340 m	*Pseudonitzschia multiseries* (97.4)
CAR_D52	Stramenopile	270 m, 340 m	*Thraustochytrium multirudimentale* (93.6)
CAR_E112	Stramenopile	270 m, 340 m	*Cylindrotheca closteriva* (95.7)
CAR_E108	Stramenopile (uncultured)	270 m, 340 m	Uncultured eukaryote clone C2_E024 (92.2)
CAR_D12	Alveolate (uncultured)	270 m, 340 m	Uncultured eukaryote clone C1_E020 (99.3)
CAR_D75	Alveolate (uncultured)	270 m, 340 m	Uncultured eukaryote clone C2_E007 (94.3)
CAR_E161	Alveolate	270 m, 340 m	*Metopus palaeformis* (89.5)
CAR_H70	Stramenopile (uncultured)	270 m, 900 m	Eukaryote marine clone ANT12-8 (89.9)
CAR_E63	Stramenopile	340 m, 900 m	*Phaeodactylum tricornutum* (93.3)
CAR_E131	Alveolate (uncultured)	340 m, 900 m	Uncultured eukaryote isolate C1_E029 (99.4)

novel deeply rooted alveolate lineage, termed here group III, of uncultivated alveolates. This alveolate group contains no named species and shows high species richness. Similar to the uncultured marine alveolate group I, it appears to be ubiquitously distributed because related sequences have been recovered from anaerobic sediments at a hydrothermal site (7) as well as in surface waters of the equatorial Pacific (26).

Within the ciliated protozoans, we discovered a diverse group of organisms that formed a well-supported, potentially deep branching clade (clones H1b, H17, H19b, H31, and H63 [Fig. 3 and 5]). This clade is represented in the deepest anoxic sample and does not include sequences of any known anaerobic ciliates. The clade appears to be a sister group to the established ciliate classes and may thus deserve a designation at the same taxonomic level (Fig. 5). If so, this would signify discovery of the first new class of ciliates in decades. This is remarkable because the ciliates are among the best-described protistan groups, and after 2 centuries of research on their systematic attributes (6, 8, 13–16, 19), some investigators contend that most, if not all, ciliate species had already been described (11, 12). Whatever the correct taxonomic status of the discovered clade, its very existence suggests that it is too early to establish limits for protistan diversity even for the best-studied taxa.

On the basis of the data obtained, it is difficult to reconstruct the exact pattern of protists' vertical distribution. However, the existence of a vertical pattern is strongly suggested by a high degree of differential recovery of the majority of clones. Most of the groups were observed exclusively or predominantly at one or two depths (e.g., uncultured marine stramenopiles, candidate ciliate class CAR_H, Labyrinthulida, and uncultured marine alveolate group I [Fig. 3 to 5]). However, several taxa seem to have been present throughout the sampled water column (e.g., uncultured marine alveolate group III, traditional ciliate classes, and diatoms [Fig. 3 and 4]). The same applies to individual clones, some of which were detected at all three depths (Table 1). However, for some organisms, e.g., diatoms, such spatial homogeneity may be an artifact caused by sinking of dead cells, while in others (e.g., ciliates), it is probably a reflection of their colonization of the entire water column. Therefore, the pattern of clone recovery suggests that some uncultured protists have depth-specific distribution, whereas the distribution of others may be depth independent. The protistan communities at different depths are therefore distinct but interconnected.

To conclude, this analysis represents the first 18S rRNA survey of a major reservoir of permanently anoxic waters and reveals a significant number of novel 18S rRNA gene sequences. Some of these sequences branch very early in analyses of eukaryotic rRNA, while others represent novel lineages that form sister groups to described taxa. Considering their evolutionary and potential ecological importance, visualization of these organisms and their quantification by using fluorescence in situ hybridization, their retrieval for scanning electron microscopy-transmission electron microscopy ultrastructural studies, and cultivation to resolve their physiology and biochemistry are all very promising avenues for future research.

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