Large-scale patterns in biodiversity of microbial eukaryotes from the abyssal sea floor

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Eukaryotic microbial life at abyssal depths remains "uncharted territory" in eukaryotic microbiology. No phylogenetic surveys have focused on the largest benthic environment on this planet, the abyssal plains. Moreover, knowledge of the spatial patterns of deep-sea community structure is scanty, and what little is known originates primarily from morphology-based studies of foraminiferans. Here we report on the great phylogenetic diversity of microbial eukaryotic communities of all 3 abyssal plains of the southeastern Atlantic Ocean—the Angola, Cape, and Guinea Abyssal Plains—from depths of 5,000 m. A high percentage of retrieved clones had no close representatives in genetic databases. Many clones were affiliated with parasitic species. Furthermore, differences between the communities of the Cape Abyssal Plain and the other 2 abyssal plains point to environmental gradients apparently shaping community structure at the landscape level. On a regional scale, local species diversity showed much less variation. Our study provides insight into the community composition of microbial eukaryotes on larger scales from the wide abyssal sea floor realm and marks a direction for more detailed future studies aimed at improving our understanding of deep-sea microbes at the community and ecosystem levels, as well as the ecological principles at play.

benthic | clone library | deep sea | protists | protozoa

The abyssal sea floor (3,000–6,000 m depth) represents the most common benthic environment on this planet, covering 54% of the Earth's surface (1). Studies of the abyss have been impeded by the considerable technical difficulties involved in accessing this remote environment. Almost all studies to date have focused on prokaryotes and metazoans. Whereas diversity estimations of metazoans of meiofaunal size have a long and rich history (2), microbial eukaryotes (protists) have received much less attention, and studies have concentrated mainly on a single taxonomic group, the foraminiferans (3). There remains a significant lack of information on the community structure of other deep-sea microbial eukaryotes (4, 5). Environmental molecular surveys have revolutionized our understanding of microbial systems (6). Phylogenetic surveys also have been applied successfully to study the community composition of eukaryotic microbes at several deep-sea sites (7–16); however, to date, no study has addressed the phylogenetic diversity of eukaryotic microbial life of one of the largest habitats, covering over half of the Earth's surface: the abyssal plains.

Abyssal plains are among the Earth's flattest and smoothest regions and are covered with muddy soft sediments. At one time, these plains were assumed to be vast, desert-like, contiguous habitats with relatively constant physical and chemical parameters. Recent studies of prokaryotes have shown that even the deepest parts of the Earth teem with a wide variety of life (17); however, information on the diversity and distribution of microbial eukaryotes at abyssal depths and beyond remains scarce, despite these eukarocytes' important role in the material flux in other aquatic ecosystems of the biosphere. Knowledge of the role of microbial eukaryotic communities is essential to understanding global biogeochemical cycles (18).

The perceived homogeneity of abyssal environments, with little environmental variation, has led to the assumption that species have broad distribution ranges. This is in fact supported by studies of foraminiferans, which in some cases have ranges encompassing entire abyssal plains (19). But environmental gradients do shape the deep-sea community structure, especially in benthic environments (20). Except for the studies by Countway et al. (13), molecular studies of microbial eukaryote communities in the deep sea have all been carried out on a local scale. Local-scale studies of benthic deep-sea microbial communities have reported differences in the community structures between adjacent sampling sites (11, 21). Although currently scant, knowledge of large-scale patterns of deep-sea community structures is necessary to allow the assessment of the forces driving biodiversity and biogeography in the deep.

In the present study, we used environmental cloning and sequencing techniques to investigate microbial eukaryotes in the three abyssal plains of the southern Atlantic Ocean: the Angola, Cape, and Guinea Abyssal Plains (Fig. 1). We addressed and compared the phylogenetic diversity and community structure of these abyssal plains using a multiple PCR primer approach, including general eukaryotic and group-specific (Heterokonta, Cercozoa, and Kinetoplastea) primers. The data reveal a high phylogenetic diversity and demonstrate that large-scale patterns of eukaryotic microbial biodiversity exist at the abyssal sea floor.

Results

Sampling Efficiency and Estimation. We were able to retrieve a total of 763 protistan clones with an average length of 594 bp, 442 of them with general eukaryotic primers and 331 of them with group-specific primers (Heterokonta, $n = 205$; Cercozoa, $n = 59$; Kinetoplastea, $n = 57$). In addition, 26 clones were identified by RDP Chimera Check, Pintail, or our manual procedure as potential chimeric sequences and consequently were excluded from the analysis. All clones were grouped into operational taxonomic units (OTUs), following mean (5.21%; $OTU^{5.21}$) and median (0.80%; $OTU^{0.80}$) values for OTU delineation estimated with the SILVA data set (22) (Fig. S1), resulting in 180 (for $OTU^{5.21}$) and 387 (for $OTU^{0.80}$) different OTUs. Rank-abundance curves of the entire data set (deducible from the bar charts of Figs. 2 and 3; rank-abundance curves for each region are shown in Fig. S2) showed high percentages of singletons (59% for $OTU^{5.21}$ and 73% for $OTU^{0.80}$). Rarefaction curves calculated with both the general

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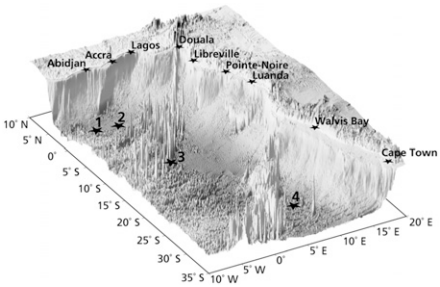

Fig. 1. Map of the southeastern Atlantic with sampling locations: western Guinea Abyssal Plain (1), eastern Guinea Abyssal Plain (2), Angola Abyssal Plain (3), and Cape Abyssal Plain (4).

eukaryotic and the group-specific primers always leveled off, but did not reach saturation (Fig. S3). The total number of OTUs was estimated at 408 for $S^{5.21}_{ACE}$ [95% confidence interval (CI), 328–535] and 1,240 for $S^{0.80}_{ACE}$ (95% CI, 1006–1562).

Large Amount of Novel Phylotypes. Comparisons of all clones with published sequences resulted in a very high percentage of clones having neither a nearest named neighbor (73%) nor any nearest neighbor in GenBANK (63%). We defined the nearest neighbor as the Basic Local Alignment Search Tool (BLAST) hit with the highest score and no more than 5.21% *p*-distance to the respective clone. The nearest named neighbor was accordingly defined as the highest scoring BLAST hit with no more than 5.21% *p*-distance to the respective clone and representing a sequence with full species name annotated. The mean genetic *p*-distances between clones and their first BLAST hit (i.e., the BLAST hit with the highest score), as well as first named BLAST hit (i.e., the BLAST hit with the highest score representing a sequence with full species name annotated), were accordingly high, with mean values of 11% and 13%, respectively (Fig. S4). This was mainly the result of novel euglenozoan clones. The first BLAST hit within this group was nearly always a highly divergent named sequence and no environmental sequence, indicating that these clones rarely appeared in other clone libraries. Removing euglenozoans from the analysis resulted in mean genetic *p*-distances between clones and published sequences of 6.5% (first BLAST hit) and 12.4% (first named BLAST hit). Calculating mean genetic *p*-distances between clones and published sequences without clones from group-specific clone libraries raised the mean values to 15.9%–16.0% (Fig. S4), due to the fact that most euglenozoan clones (all diplonemids and 78% of all kinetoplastids) were present in clone libraries retrieved with general eukaryotic primers.

High Phylogenetic Diversity with Many Parasites. All retrieved clones belonged to well-established high-rank taxonomic groups. All phylogenetic groups generally found in deep-sea molecular surveys were present in our clone libraries (Figs. 2 and 3). The most abundant phylogenetic groups in clone libraries constructed with general eukaryotic primers were Alveolata ($n = 168$), Euglenozoa ($n = 161$), Heterokonta ($n = 39$) and Rhizaria ($n = 26$). This resembles the phylogenetic composition of other deep-sea clone libraries. Well-known major phylogenetic clades, such as the uncultured marine alveolates (UMA/MALV/NA) or marine stramenopiles (MAST), were abundant. Lineages and clades with no close representatives in GenBANK (5.21% threshold) were retrieved for all major phylogenetic groups, even within well-studied groups, such as the Ciliophora (red long-dashed lines in Figs. 2 and 3). Furthermore, numerous clones had parasites as nearest neighbors, including *Amoebophrya*, *Cryptocaryon*, *Cytauxzoon*, *Duboscquella*, *Haramonas*, *Ichthyobodo*, *Miamiensis*, *Paradinium*, *Perkinsus*, *Pirsonia*, and *Rhizidomyces*.

Community Comparisons of Deep-Sea Abyssal Plains. Clone libraries constructed with general eukaryotic primers were statistically compared. Rank-abundance curves for each sampling region (deducible from Figs. 2 and 3 and Fig. S2) show that the numerically dominant OTUs generally were present in all 4 sampling regions, highlighting the similarity of the underlying communities based on relative clone abundances. However, OTU-based similarity indices showed significant dissimilarity between the communities of the Cape Abyssal Plain and those of the Angola Abyssal Plain and the eastern and western Guinea Abyssal Plain (0.20–0.26 $\theta^{5.21}_{YC}$), but considerably greater similarity of the communities of the Angola Abyssal Plain and the Guinea Abyssal Plain (0.72–0.87 $\theta^{5.21}_{YC}$). F_{ST} (Table S1) and Unifrac (Table S2) tests revealed significant differences among the communities of the Cape Abyssal Plain and the other abyssal plains (Fig. 4). Differences in the community structures of the Angola and the Guinea Abyssal Plain turned out to be statistically nonsignificant; however, the P-test (Table S2) was nonsignificant only between the communities of the Angola and the western Guinea Abyssal Plains. According to SIMPROF-tests ($OTU^{5.21}$- and $OTU^{0.80}$-based Bray–Curtis similarity indices), communities of the Cape Abyssal Plain differed from those of the Angola and the 2 regions of the Guinea Abyssal Plain ($P > .01$). The communities of the Angola and the Guinea Abyssal Plains could not be distinguished with the present data set ($P < .01$). ∫-LIBSHUFF tests (Table S3) also could not distinguish between communities of the Angola Abyssal Plain and the 2 regions of the Guinea Abyssal Plain ($P > .05$); communities of both of these abyssal plains were mostly significantly different from communities of the Cape Abyssal Plain ($P < .01$).

Tests with group-specific clone libraries showed that communities from the same abyssal plain (the eastern and western Guinea Abyssal Plain) were very similar (Table S4). This highlights the remarkable similarity of communities retrieved from the same abyssal plain. Sampling regions from different abyssal plains were generally significantly different from each other.

All clone libraries were significantly different ($P < .01$; Fig. 4) from other published deep-sea clone libraries.

Discussion

High Diversity and High Numbers of Novel Phylotypes. The most obvious feature of microbial eukaryotes from abyssal plains is the substantial species richness of their communities (408 $S^{5.21}_{ACE}$; 1,240 $S^{0.80}_{ACE}$), which is as high as that at proclaimed diversity hot spots, such as hydrothermal vents, anoxic and hypersaline environments, and methane seeps (8–10, 12, 14–16) with mean estimates ranging from 410 ($S^{5.21}_{ACE}$) to 489 ($S^{0.80}_{ACE}$). Furthermore, the mean genetic *p*-distances between all clones and both their first BLAST hits (11.0%) and first named BLAST hits (13.0%) are higher than those reported from other deep-sea environments (2.5% and 10.6%, respectively; Fig. S4). These values point to a specific assemblage of microbial eukaryotes at benthic abyssal sites and are supported by high F_{ST} values between our clone libraries and other published clone libraries (Fig. 4). To some extent this is the result of a single taxonomic group: the Euglenozoa (i.e., Diplonemea and Kinetoplastea). Although group-specific primers have proven useful in assessing the phylogenetic diversity of some groups, such as the Cercozoa (23) and Diplonemea (24), only one-third of the kinetoplastid clones were retrieved from group-specific clone libraries, whereas two-thirds were retrieved with general eukaryotic primers. Thus, the high representation of euglenozoans in our clone libraries is the result of their high occurrence in these environments, rather than due to a bias resulting from group-specific primers able to detect lineages that otherwise may not appear in general eukaryotic clone libraries.

The first BLAST hit within the euglenozoans was mostly a highly genetically divergent sequence, indicating that these deep-sea euglenozoans rarely appear in published clone libraries and

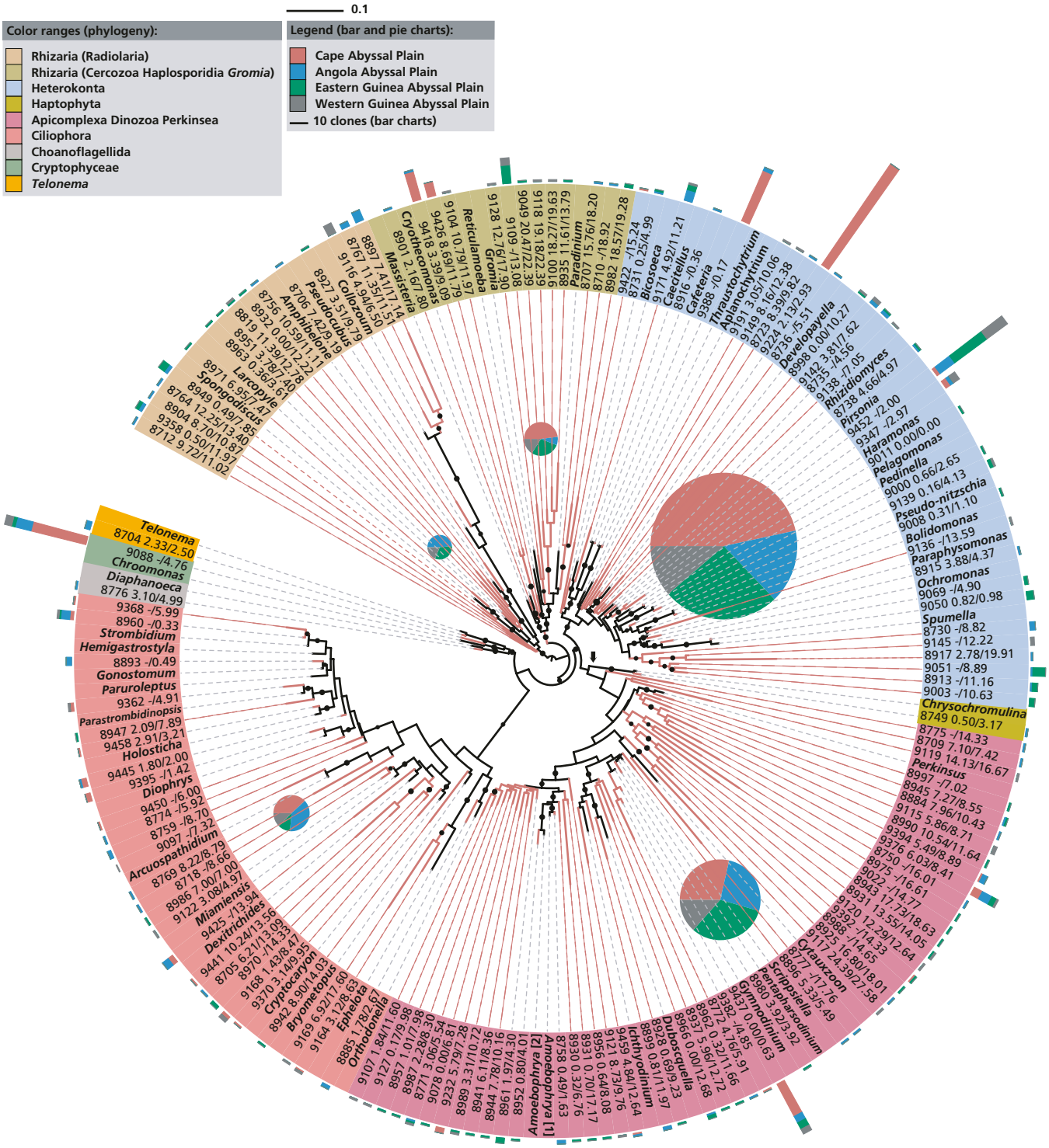

Fig. 2. Midpoint rooted maximum likelihood tree of all clones (general eukaryotic and group-specific primers). Because of their very long branches, the euglenozoans were removed and are displayed separately (Fig. 3). Their branching position is marked by an arrow. Bootstrap support values ≥ 70 are highlighted by black circles. Branches in red are clones; branches in black are named sequences retrieved from GenBANK. For clarity, clones were grouped into OTUs (5.21% threshold; Fig. S1), and only 1 representative clone from each OTU was included in the phylogenetic tree. These clones are identified by the last 4 numbers of their GenBANK accession numbers (GU218701-GU219463) followed by the lowest *p*-distance to the first BLAST hit and, after the slash, the lowest *p*-distance to the first named BLAST hit within each OTU. When the first BLAST hit was already a named sequence, the first value was omitted. Bar charts represent the number of clones per abyssal plain obtained for each OTU; pie charts represent the relative number of clones obtained from each abyssal plain for the major phylogenetic groups. Sequences in bold represent the nearest named neighbor for each OTU. Phylogenetic branches are extended toward their labels by gray dotted lines and by red dotted lines if the lowest *p*-distance to the first named BLAST hit within the respective OTU was > 5.21%. These branches are also highlighted by long dashes if the lowest *p*-distance to the first BLAST hit was already > 5.21%. Species names and GenBANK accession numbers of the reference sequences are listed in Table S6.

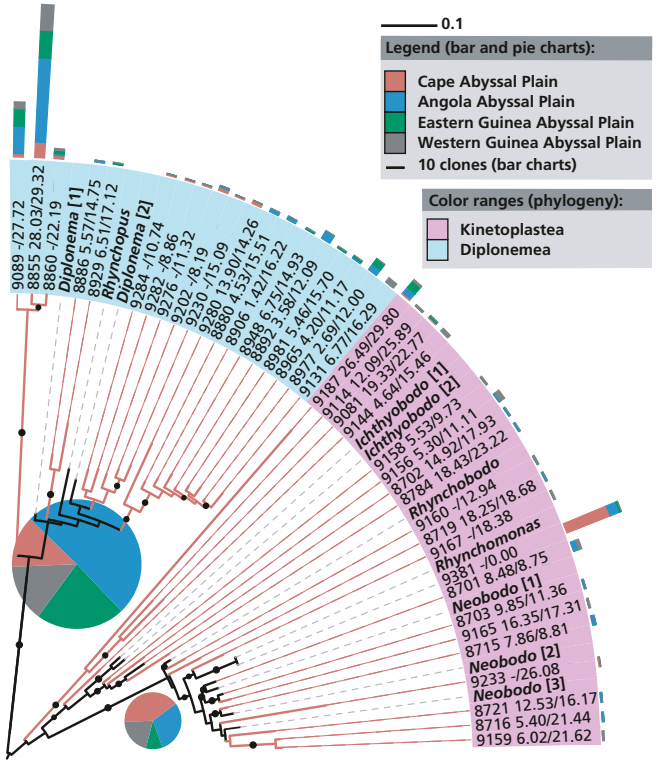

Fig. 3. Maximum likelihood tree of Euglenozoa. For more details, see Fig. 2.

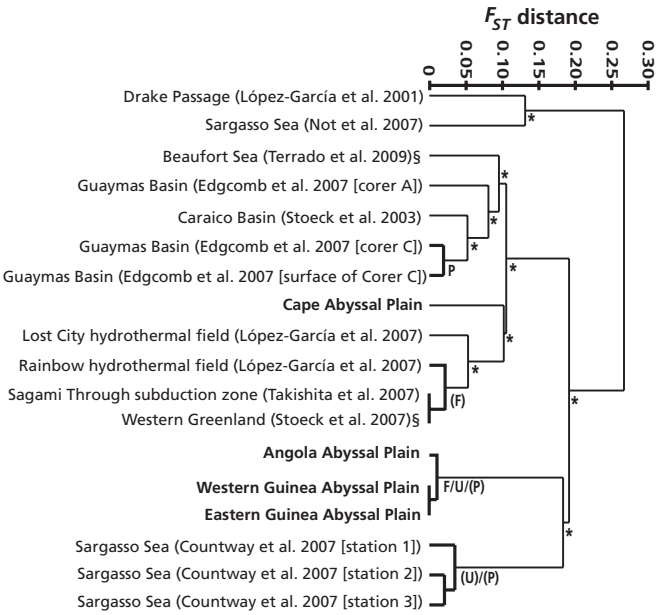

Fig. 4. Dendrogram of pairwise F_{ST} values. Clone libraries of microbial eukaryotes from depths ≥ 750 m constructed using general eukaryotic primers were compared. Clone libraries from this study are highlighted in bold. For comparision, two clone libraries from non–deep-sea environments were included in the analysis (denoted by §). Significant P values for F_{ST}, UniFrac, and P-tests are indicated by an asterisk ($P < .01$). Nonsignificant P values are indicated by bold lines, and the respective tests are denoted by F (F_{ST} test), U (UniFrac test), and P (P-test). When only some pairwise tests within a cluster were insignificant, the respective tests are given in parentheses.

have not yet been taxonomically studied. This was an unexpected finding for the euglenozoan group Kinetoplastea, because this group has been studied in detail. This supports the idea of a specific benthic deep-sea community of euglenozoans and is in agreement with the fact that most nonparasitic diplonemids and kinetoplastids have been described as typical benthic species, and some diplonemid lineages have been hypothesized to preferentially inhabit the deep oceans (24).

Removing euglenozoans from the analysis did lower the mean p-distance between all clones and their first BLAST hits, although the mean values (Fig. S4) were still higher than those reported from most other deep-sea habitats. This contradicts the assumption that the abyss is simply a "sink habitat" (25), as has been suggested for some metazoans, and indicates that the diversity of benthic, deep-sea microbial eukaryotic communities can be attributed only partially to the sedimentation of organisms from the pelagial.

"Rare Biosphere." Most of the OTUs obtained in this study occurred at low abundance, giving the distribution of clone relative abundances a very long right-hand tail, with rare species present as singletons. This "rare biosphere" (26, 27) is a common phenomenon in microbiology and has traditionally been thought to indicate the presence of a seed bank of potential new colonizers, according to the "everything-is-everywhere" hypothesis (28). Interestingly, this is consistent with observations of rare species of metazoans present in the deep sea that led to the idea of a high deep-sea diversity (29). These rare species challenge the traditional concept of diversity and are likewise hypothesized to form a pool of transient immigrants (30). Several other explanations have been given to explain this rare biosphere in eukaryotic microbiology (27).

The diversity of microbial eukaryotes in the deep sea seems to be undersampled, as indicated by rarefaction curves. Thus, future studies may reveal many additional new phylotypes. Group-specific clone libraries seem to be especially useful for evaluating this missed diversity. This applies particularly to phylogenetic groups that have been underreported in environmental sequencing surveys (e.g., amoebozoans and foraminiferans). At present, we cannot provide any reliable estimates of this missing diversity, however (31).

High Diversity of Parasites. The presence of many lineages and clades associated with parasitic species or groups, such as the Apicomplexa, emphasizes the importance of parasitism in aquatic (in particular, marine and deep-sea) environments reported by many recent molecular surveys (e.g., 32). Nevertheless, the putative parasitic diversity (in both phylogenetic diversity and relative clone abundance) reported herein is noteworthy. Although inferring the trophic role of an organism by its sequence is difficult, parasitism appears to be a major force shaping microbial community structure at abyssal depths, a force that has been underestimated in current conceptualizations of the marine carbon cycle. Lafferty et al. (33) have concluded that up to 75% of all food web links include parasites.

Studies of deep-sea metazoan parasites have reported declines in parasitic abundance and diversity with depth but increases close to the sea floor (34, 35). This phenomeon has been linked to the increased biomass at benthic sites and thus to an increased availability of potential intermediate hosts, resulting in higher parasite abundance and diversity. This also may explain the high occurrence of microbial eukaryotic parasites at the abyssal sea floor. One possible explanation for the paradox of a species-rich deep-sea floor, according to Snelgrove et al. (36), was accorded to the underestimation of positive species interactions in the deep sea due to parasites (37).

Large-Scale Patterns in Biodiversity. Communities of the Angola and the Guinea Abyssal Plains were relatively similar and differed from the community of the Cape Abyssal Plain. The

community structure of microbial eukaryotes showed little geographic variation on large spatial scales encompassing different abyssal plains located thousands of kilometers apart. Although protistan biogeography is controversial (e.g., refs. 28 and 38) and no consensus has been reached, it is well known that at least some deep-sea microbial eukaryotes, including several foraminiferan species (19), exist with large distribution ranges at benthic abyssal sites, similar to some deep-sea metazoans. Allen and Sanders (39) noted that 50% of North Atlantic bivalves have geographic ranges encompassing entire abyssal plains, a finding echoed by Rex et al. (25) for gastropods. A recent study (13) reported similar community compositions of microbial eukaryotes on a regional scale in the abyssopelagial zone of the northwestern Atlantic Ocean. At abyssal depths, the perceived homogeneity of sea floor habitats thus appears to be paralleled by the homogeneity of their communities. However, by pooling the DNA from each region, we were not able to capture the local heterogeneity possibly present at each sampling region as reported by other deep-sea studies (10, 21).

In the present study, the numerically dominant OTUs were generally present at all 4 sampling regions, in contrast to many rare OTUs (deducible from bar charts of Figs. 2 and 3 and Fig. S2). It is generally assumed that the dominant taxa contribute the most to an ecosystem's function and consequently best describe that ecosystem. According to this assumption, different environments with a high overlap of the dominant species should contribute similarly to ecosystem processes. There is an ongoing debate regarding the role of rare species in ecosystem functioning, however (e.g., ref. 27). If this rare biosphere turns out to play an important role, then abundance-based statistics (e.g., Bray–Curtis similarity indices) might not adequately describe the underlying communities. Nevertheless, all statistics showed high similarity between the Angola and Guinea Abyssal Plains. Consequently, although our sampling was far from exhaustive, and many more especially rare taxa might be present at the sampling sites, virtually identical communities over large spatial scales, as reported here, indicate the prevailing homogeneous environmental conditions on the abyssal sea floor. Thus, the differences in community structure between the Cape Abyssal Plain and the other 2 abyssal plains studied underscore the importance of ecological parameters in shaping community composition. This finding is in accordance with, for example, studies of eukaryotic picoplankton from marine surface waters of the Southern Ocean showing similar community structure over long geographic distances and changes between different hydrographic areas (40). This might also be supported by the significant differences between several deep-sea clone libraries from different habitats, as shown in Fig. 4. Keep in mind, however, that we cannot state with confidence to what extent these differences are the result of PCR biases or represent true differences between the communities (41).

Studies of metazoans and foraminiferans suggest that 2 of the most important factors controlling deep-sea species distribution are water quality and food supply (42, 43). At the water surface, the Angola and Guinea Abyssal Plains lie within the influence of the South Equatorial Current, whereas the sampling stations at the Cape Abyssal Plain are under the influence of the cold, less-productive Benguela Oceanic Current (44). At the sea floor, the Angola and Guinea Abyssal Plains are filled with North Atlantic Deep Water, modified by injections of Antarctic Bottom Water through the Mid-Atlantic Ridge System, whereas the Cape Abyssal Plain is filled with Lower Circumpolar Deep Water (also referred to as Antarctic Bottom Water), which has lower temperature, salinity, and dissolved oxygen (44). All 3 abyssal plains are furthermore separated by geographic barriers, namely the Guinea Fracture Zone in the North and the Walvis Ridge in the South (Fig. 1).

The high similarity of communities from the Guinea and the Angola Abyssal Plains assume that some abyssal plains form a vast interconnected environment. Thus, communities of microbial eukaryotes might not be shaped primarily by geographical distance. The differences in the ecological conditions between our sampling regions suggest that ecological parameters might be the decisive factors in shaping deep-sea communities of microbial eukaryotes on larger scales. It must be noted, however, that we sampled only 4 regions, and further sampling might challenge our findings.

Conclusion

Our findings challenge the concept of lower biodiversity on the abyssal sea floor than in other marine environments. The high percentage of lineages with no close representatives in genetic databases points to rich, and to some extent specific, communities of microbial eukaryotes at the abyssal sea floor with a potentially high percentage of parasites.

The abyssal sea floor appears to be a contiguous habitat for microbial eukaryotes on regional scales. Nevertheless, the significant differences between the Cape Abyssal Plain and the other 2 abyssal plains studied suggest that ecological parameters might be the decisive factors in shaping microbial eukaryote distribution patterns and zonation on large spatial scales at abyssal depths. The abyssal sea floor seems to be a mosaic of semi-isolated habitats, shaped and maintained on larger scales by diverse environmental gradients.

Investigating the diversity and distribution of natural microbial communities in Earth's largest habitat is critical to our understanding of global biogeochemical cycles. Unique techniques (27) and large-scale studies (45), as well as long-term surveys/time series (46), may further elucidate the diverse composition of deep-sea communities over both space and time (43).

Materials and Methods

Sampling Site. Sampling regions, depths, and sample volumes were the western Guinea [Fig. 1 (1); 0° 50′ N 5° 35′ W; 5,136–5,142 m], eastern Guinea [Fig. 1 (2); 0° 0′ S 2° 25′ W; 5,060–5,066 m), Angola [Fig. 1 (3); 9° 56′ S 0° 54′ E; 5,646–5,655 m], and Cape Abyssal Plains (Fig. 1 (4); 28° 7′ S 7° 21′ E; 5,033–5,038 m]. Samples were taken with a multicorer. Each region was sampled by 3 multicorers; thus, each sampling region consisted of several cores located several kilometers apart. From each multicorer, water from several corers was obtained and directly filtered over polycarbonate filters with a pore size of 0.2 μm (GTPB; Millipore) and then stored in lysis buffer (47) at -20°C until further treatment. All materials were autoclaved before use.

DNA Extraction, Purification, Cloning and Sequencing of PCR-Amplified SSU rDNA. Genomic DNA was extracted from the filters using a general phenol/chloroform/CTAB extraction protocol (47) and further purified by Sepharose 4B/PVPP columns (48). Genomic DNA from the same sampling region was pooled together. SSU rDNA was amplified with FastStart Taq DNA polymerase (Roche Applied Science) under standard conditions as specified by the manufacturer. Amplified gene fragments were cloned (StrataClone PCR Cloning Kit; Stratagene) and sequenced (positions 500–1,300) in both directions (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems) following the manufacturers' protocols. The primers used are listed in Table S5 From each sampling region, clone libraries were constructed with general eukaryotic primers, kinetoplastid-specific primers, cercozoan-specific primers, and heterokont-specific primers.

Phylogenetic Analysis. The obtained sequences were corrected and assembled manually. Chimeric sequences were detected using the Check Chimera (49) program and by comparing each clone with its first BLAST hit, both manually and with the Pintail program (50). Multiple alignments for phylogeny were calculated using SINA Webaligner (22) and phylogenetic analysis with RAxML v7.0.4 (51) under the GTRCAT model with 1,000 bootstrap replicates. Phylogenetic trees were drawn using iTOL (52). Representative clones for Figs. 2 and 3 were chosen as described in *SI Materials and Methods.*

Statistical Analysis. *P*-distance matrixes were built from pairwise *p*-distances inferred from pairwise alignments for each possible combination using

ClustalW2 v2.0.10 (53) and distmat (54). Based on these matrixes, pairwise statistics were calculated with Arlequin v3.11 (F_{ST} tests) (55), UniFrac (unweighted UniFrac and P-tests) (56), DOTUR v1.53 (OTU assignment, rarefaction curves, and richness estimations) (57), SONS v1.0 (OTU-based statistics) (58), ∫-LIBSHUFF v1.22 (59), and PRIMER v6 (PRIMER-E Ltd; OTU-based SIMPROF tests).

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