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Diversity of the Microeukaryotic Community in Sulfide-Rich Zodletone Spring (Oklahoma)†

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The microeukaryotic community in Zodletone Spring, a predominantly anaerobic sulfide and sulfur-rich spring, was examined using an 18S rRNA gene cloning and sequencing approach. The majority of the 288 clones sequenced from three different locations at Zodletone Spring belonged to the *Stramenopiles*, *Alveolata*, and *Fungi*, with members of the phylum *Cercozoa*, order *Diplomonadida*, and family *Jakobidae* representing a minor fraction of the clone library. No sequences suggesting the presence of novel kingdom level diversity were detected in any of the three libraries. A large fraction of stramenopile clones encountered were monophyletic with either members of the genus *Cafeteria* (order *Bicosoecida*) or members of the order *Labyrinthulida* (slime nets), both of which have so far been encountered mainly in marine habitats. The majority of the observed fungal clone sequences belonged to the ascomycetous yeasts (order *Saccharomycetales*), were closely related to yeast genera within the *Hymenobasidiomycetes* (phylum *Basidiomycetes*), or formed a novel fungal lineage with several previously published or database-deposited clones. To determine whether the unexpected abundance of fungal sequences in Zodletone Spring clone libraries represents a general pattern in anaerobic habitats, we generated three clone libraries from three different anaerobic settings (anaerobic sewage digester, pond sediment, and hydrocarbon-exposed aquifer sediments) and partially sequenced 210 of these clones. Phylogenetic analysis indicated that clone sequences belonging to the kingdom *Fungi* represent a significant fraction of all three clone libraries, an observation confirmed by phospholipid fatty acid and ergosterol analysis. Overall, this work reveals an unexpected abundance of *Fungi* in anaerobic habitats, describes a novel, yet-uncultured group of *Fungi* that appears to be widespread in anaerobic habitats, and indicates that several of the previously considered marine protists could also occur in nonmarine habitats.

The utilization of culture-independent approaches for describing microbial assemblages in various ecosystems has altered our view on the breadth of prokaryotic diversity. For example, by extensive 16S rRNA gene-based analysis of various marine and terrestrial ecosystems, the number of bacterial divisions and candidate divisions has increased from 12 in 1987 (68) to at least 52 (53). Similarly, numerous novel archaeal lineages, many of which have a global distribution, have been detected in both marine (11) and terrestrial (7) ecosystems.

Recently, a similar approach utilizing small subunit (SSU) rRNA gene amplification, cloning, and sequencing has been applied to study eukaryotic diversity. The few available studies primarily examined the microeukaryotic community in different marine environments, including the photic and aphotic zones of pelagic oceans (12, 13, 25, 39, 43, 61), anoxic marine salt marsh sediments (60), and hydrothermal vents (17, 38). Collectively, these studies suggest an unexpected level of diversity with the detection of sequences representing potential novel lineages within known eukaryotic groups, as well as sequences suggesting the presence of novel kingdom level diversity within the microeukaryotic community. The use of 18S rRNA gene-based analysis to study freshwater anaerobic communities has received relatively less attention than marine surveys. However, the work of Dawson and Pace (10) suggests the presence of a similar broad diversity at the kingdom level within freshwater lake sediments.

An argument has recently been presented stating that SSU rRNA surveys overestimate the level of eukaryotic diversity at the megaevolution level (6, 9). The reasoning is that there is an inadequate number of available 18S rRNA gene sequences representing some of these novel fast-evolving groups, chimeric sequences have been incorporated into phylogenetic analysis, long branch attraction artifacts may have occurred, and there are a relatively high number of described eukaryotic taxa with no available sequence data (6).

The present work examines the microeukaryotic communities in Zodletone Spring in southwestern Oklahoma. Zodletone Spring has a high dissolved sulfide concentration in the emergent water (8 to 10 mM) and maintains hypoxic conditions in the water and anoxia in the underlying sediments throughout the course of the spring. Another important characteristic of the spring is the continuous bubbling of short-chain alkanes (methane, ethane, and propane) from the source of the spring, resulting in high hydrocarbon levels, especially in the source area. Recent surveys of the bacterial and archaeal communities in Zodletone Spring revealed an extremely diverse microbial community with several novel bacterial and

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archaeal lineages (18, 19). The study of the microeukaryotic community in the spring will add to our knowledge regarding microeukaryotes that can thrive under anaerobic conditions in general and under extreme conditions of high sulfide concentrations and hydrocarbon exposure in particular. The resulting increase in the 18S rRNA gene sequences in databases will lead to better phylogenetic resolution for both known and novel eukaryotic lineages that suffer from low sampling. Also, extensive 18S rRNA gene sampling in Zodletone Spring will allow us to practically test the two previously outlined hypotheses regarding the level of eukaryotic diversity at the kingdom level. Finally, comparing the overall patterns of eukaryotic diversity in Zodletone Spring with patterns observed in other anaerobic environments examined in this and other studies will contribute to our understanding of the global patterns of microeukaryotic diversity within anaerobic habitats. Our results support the view that the number of novel high-level eukaryotic lineages is much lower than previously inferred from other SSU rRNA surveys, and we document the presence of several eukaryotic groups previously thought to be restricted to marine environments. We also show an unexpected abundance of fungi in all studied ecosystems and suggest a wide distribution of a novel fungal lineage in anaerobic environments.

MATERIALS AND METHODS

Site description. Zodletone Spring emerges near Zodletone Mountain in the Anadarko basin in southwestern Oklahoma. Water emerges at the spring source at a rate of 8 liters/s and flows for about 20 m before discharging at a neighboring creek (Stinking Creek). The source is mainly an anaerobic area of sulfide-saturated sediments which approximately 50 cm of water overlies. As a result of light exposure and constant high sulfide concentrations, microbial mats of differing structures are visible throughout the spring. It has been shown that due to phototrophically driven sulfide oxidation mediated by the spring microbial community, barite and calcite are formed throughout the spring and as mineral crusts located on the bank of an adjacent creek (55). These crusts appear to precipitate from sulfide-laden groundwater that percolates out of the creek banks.

Sampling. Eukaryotic diversity was examined in three locations in Zodletone Spring: the sulfide-saturated, hydrocarbon-exposed spring source; the microbial mat community; and the bacterial crust formations on the banks of the creek. Samples were collected using a sterile spatula and frozen immediately on dry ice. They were transferred to the laboratory within 3 h of sampling, where they where stored at −20°C. In addition to samples obtained in Zodletone Spring, samples were also obtained from three different anaerobic locations: an anaerobic sewage digester from a sewage treatment plant in Norman, OK (clone library ww), anaerobic sediments from a gas-condensate-contaminated site near Ft. Lupton, CO (clone library ftlp), and anoxic sediment (5 cm deep) from a freshwater pond (duck pond) in Norman, OK (clone library dp). All samples were collected in August 2003, frozen immediately, and stored at −20°C for a maximum of 1 week prior to DNA extraction. Subsurface sediment used for constructing the fl library was collected in June 2001 and stored frozen for 2 years.

DNA extraction, PCR amplification, cloning, and sequencing. DNA isolation was carried out using a lysis-bead-beating protocol (15). The custom primers (Invitrogen Corp., Carlsbad, CA) used for amplifying the 18S rRNA gene were 82f and 1520r (10). We checked the primers for specificity by confirming the length of the amplification product using agarose gel electrophoresis. As well, all sequences obtained from the cloning procedure were 18S rRNA gene sequences. 18S rRNA genes were amplified from the bulk community DNA in a 50-μl reaction mixture containing (final concentration): 2 μl of 1:100 dilution of extracted DNA, 1× PCR buffer (Invitrogen), 1.5 mM $MgSO_4$, 0.2 mM (each) deoxynucleoside triphosphate mixture, 2.5 U of platinum *Taq* DNA polymerase (Invitrogen), and 10 μM (each) forward and reverse primers. PCR amplification was carried out on a Gene Amp PCR system 9700 thermocycler according to the following protocol: denaturation for 5 min at 94°C, 30 cycles of 94°C for 30 s, 55°C for 15 s, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. PCR products were cloned into a TOPO-TA cloning vector and sequenced as previously described (19).

Phylogenetic analysis. Shannon-Weiner Index, species evenness, average nucleotide diversity (θ), and percentage of coverage were determined according to previously described procedures (32, 42). For phylogenetic placement, sequences initially were compared to the GenBank nr database and checked using BLAST (1). Sequences with more than 98% similarity were considered to be of the same operational taxonomic unit (OTU). The generation of chimeric sequences during PCR-based diversity studies has been frequently observed (36), and several studies have demonstrated the accumulation of chimeric bacterial, archaeal (31), and eukaryotic (6) rRNA gene sequences in the databases. The presence of chimeric sequences in our data set was checked by screening all sequences with the CHECK-CHIMERA program, available through the ribosomal database website (40). Also, sequences with low (<92%) database similarity were manually inspected to detect the presence of universally conserved regions. In addition, we used a partial treeing analysis approach (31), in which trees were generated using various segments of the 18S rRNA gene sequence and the resulting tree topologies were compared to each other and to the full-length tree. An example of partial treeing analysis for the fungal data set is provided as supplementary material (see Fig. S1a-c in the supplemental material). Overall, 12 chimeric sequences (10 from *Zeuk* and 1 each from the dp and ftlp clone libraries) were detected and removed from the data set. Zodletone sequences and GenBank-downloaded sequences were aligned using the ClustalX program (63). The program ModelTest (52) was used to choose the optimum model of DNA substitution for each data set. Phylogenetic trees were constructed using representatives of closely related reference sequences to highlight the phyogenetic affiliation of clones obtained in this study. Evolutionary distance (neighbor-joining algorithm) and maximum-parsimony trees were constructed using PAUP (version 4.01b10; Sinauer Associates, Sunderland, Mass.). Bayesian analysis for the fungal data set were performed with MrBayes version 3.0b4 (30), using the GTR+G model of evolution with 500,000 generations run and 5,000 trees sampled. The first 100 trees were discarded, and the consensus tree was computed from the remaining trees using PAUP 4.01b. All phylogenetic trees show the frequencies of occurrence of specific OTUs in the source, mat, and crust clone libraries in parentheses from Zodletone samples (designated *Zeuk*), as well as in ww, dp, or ftlp libraries.

Phospholipid fatty acid (PLFA) and ergosterol analysis. Sediment or solids were extracted with the single-phase chloroform-methanol buffer system of Bligh and Dyer (8) as modified by White et al. (67). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (28). The neutral lipids (sterols) were saponified and then derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (46), while the polar lipids were transesterified to the fatty acid methyl esters by a mild alkaline methanolysis (28).

Ergosterol and PLFA methyl esters were analyzed by gas chromatography/mass spectroscopy using a Hewlett-Packard 6890 series gas chromatograph interfaced to a Hewlett-Packard 5973 mass selective detector using a 50-m *DB-1* column (0.1-mm inner diameter, 0.1-μm film thickness). The injector and detector were maintained at 290°C and 300°C, respectively. The column temperature was programmed at 60°C for 1 min, then ramped at 20°C min^{-1} to 150°C, held for 4 min, then ramped at 7°C min^{-1} to 230°C, held for 2 min, and finally ramped at 10°C min^{-1} to 300°C and held for 3 min. Mass spectra were determined by electron impact at 70 eV. Methyl nonadecanonate was used as the internal standard, and the PLFA and ergosterol were expressed as the equivalent peak response to the internal standard.

Nucleotide sequence accession numbers. Sequences obtained in this study were deposited in GenBank under accession numbers AY916560 through AY916665.

RESULTS

Composition and comparative diversity of Zodletone source, mat, and crust clone libraries. A library of 108, 95, and 85 clones was constructed and sequenced from the source, mat, and crust clone libraries, respectively. Figure 1 summarizes the group level affiliation of clone sequences. Clone sequences belonging to the *Stramenopiles*, *Fungi*, and *Alveolata* collectively represented 97, 94, and 93% of the source, mat, and crust clone libraries, respectively, while those belonging to the *Cercozoa*, *Diplomonadida*, and *Jakobidae* represented a minor fraction of the clone libraries. In spite of sequencing a total of 288 clones from Zodletone Spring, no novel kingdom level diversity was detected. Selection for a few dominant species was evident, as five OTUs belonging to the orders *Bicosoecida*,

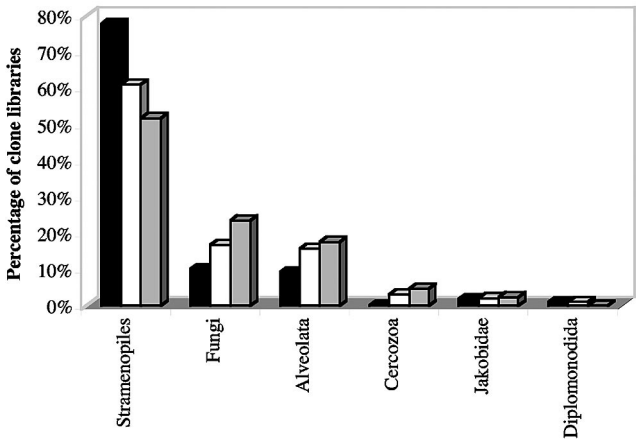

FIG. 1. Distribution of Zodletone source (black columns), mat (white columns), and crust (gray columns) clones among various eukaryotic groups.

Labyrinthulida, and *Bacillariophyceae* made up 54% of the total number of clones sequenced. Various diversity indices (see Table S1 in the supplemental material) suggested a lower level of diversity within the source clone library, compared to the mat and crust clone libraries. This lower diversity is probably a reflection of the harsher conditions (higher sulfide and hydrocarbon concentration) at the spring source.

Phylogenetic placement. (i) Stramenopiles. Within the three Zodletone clone libraries, *Stramenopiles* clone sequences belonged to four different groups: the pinnate diatoms (class *Bacillariophyceae*), golden algae (class *Chrysophyceae*), order *Bicosoecida*, and slime nets (order *Labyrinthulida*) (Fig. 2). *Bicosoecida* clones (2 OTUs, 81 clones) were monophyletic, with strong bootstrap support, to members of the genus *Cafeteria*. All currently known *Cafeteria* spp. had been obtained from marine sources such as marine plankton and hydrothermal vents (2, 5, 23, 34). *Labyrinthulida* clone sequences (2 OTUs, 11 clones) were only 93% similar to their closest relatives (*Aplanochytrium kerguelense*) (Fig. 2). Similar to *Cafeteria*, all members of the *Labyrinthulida* have so far been isolated from marine and estuarine environments (51), and culture-independent studies have often encountered this group in anaerobic, marine habitats (17, 39, 43, 61). Therefore, this work shows that this group of microeukaryotes could also occur in other aquatic habitats.

(ii) Fungi. Fungal clones represented a significant fraction of the clone libraries in Zodletone Spring (16% of the total number of clones) (Fig. 1). Fungal sequences are often observed (10) in clone libraries during surveys of eukaryotes from anaerobic environments (10, 17, 60, 61) but are rarely discussed. In Zodletone clone libraries, most fungal clones belonged to four main groups (Fig. 3). (i) Several clone sequences (3 OTUs, 16 clones) had no affiliation with any of the known fungal phyla and, together with several published or database-deposited clones (10, 66), form a novel lineage, which we named novel fungal lineage LKM, after clone LKM11 (AJ130849), the first published sequence belonging to this group (66). (ii) Members of the tremallales clade within the *Hymenomycetes* (phylum *Basidiomycetes*), with their closest relatives being members of known yeast genera, e.g., *Bullera*, *Fellomyces*, and *Kockovaella*. (iii) Members of the plant pathogenic class *Ustilaginomycetes* within the *Basidiomycetes* with close (99%) similarity to the smut fungi *Ustilago hordei* and *Ustilago maydis*. (iv) Members of the class *Saccharomycetes* within the *Ascomycota*. The source clone library had a high percentage (55% of the total fungal clones) of *Hymenobasidiomycetes*, clone sequences related to yeast genera. In contrast, the majority of the fungal clone sequences in the mat and crust clone libraries belonged to the novel LKM group.

(iii) Alveolates. All alveolates from Zodletone belonged to the class *Ciliophora* (ciliates), the presence of which has long been established in anaerobic environmments (22). Zodletone ciliates either belonged to the classes *Spirotrichea* or *Colpodea* or formed a novel lineage within the ciliates that is designated candidate ciliate class *Zeuk* (Fig. 4A)

***Jakoba*, *Diplomonadida*, and *Cercozoa*.** Zodletone *Jakoba* clones were closely related to *Jakoba incarcerata*, isolated from intertidal sediments in Quibray Bay, Australia (Fig. 4B). Two *Diplomonadida* clones were detected from the Zodletone source, and mat samples and were only 91 to 93% similar to their closest relative, the free-living amitochondrial flagellate *Hexamita inflata* (Fig. 4B). These *Diplomonadida* clones were the only sequences encountered in this study that belonged to one of the early branching amitochondrial eukaryotic groups. *Cercozoa* clones belonged to the order *Cercomonadida* that has been often encountered in anoxic habitats (5, 10).

Comparison of the Zodletone Spring microeukaryotic community to other anaerobic environments. Samples from an anaerobic sewage digester (ww sequences), a gas-condensate-impacted aquifer (ftlp sequences), and freshwater pond sediments (dp sequences) were used to construct clone libraries, and a total of 210 clones were partially sequenced. The composition of all three libraries is given in Table 1. Interestingly, fungus-affiliated clone sequences represented a significant fraction in all three clone libraries (Fig. 3) (63% in the ww library, 35% of the fl library, and 34% of the dp library). Moreover, clone sequences belonging to the novel fungal LKM group were present in all three environments, suggesting a widespread distribution of this group in anaerobic ecosystems. This group represented 82% of the fungal clones ($n = 27$) and 28% of the total number of clones ($n = 79$) in the dp clone library. The remaining fungal clone sequences in the dp clone libraries were members of the phyla *Glomeromycota* and *Chytridiomycota* and the class *Ustilaginomycetes*. Fungal clone sequences from the anaerobic digestor were either saccharomycetous yeasts or members of the fungal LKM group (Fig. 3). Anaerobic aquifer fungal clone sequences were ascomycetous or basidiomycetous yeasts and belonged to the LKM group as well as the orders *Pezizales* and *Ustilaginomycetes* (Fig. 3).

PLFA and sterol analysis. To estimate the relative abundance of eukaryotes in general and fungi in particular in anaerobic environments, we quantified the total, prokaryotic, and eukaryotic PLFA, two fungus-specific PLFAs (18:2w6 and 18:3ω3), and ergosterol in Zodletone Spring as well as in ww, dp, and ftlp samples. Ergosterol, a fungus-specific sterol used as an indicator of living fungal biomass (27), was detected in all environments except Zodletone crust samples and ftlp samples (Table 2). However, fungus-specific PLFAs were detected in all samples analyzed, confirming the presence of fungi in all of the anaerobic ecosystems examined. These fungus-specific

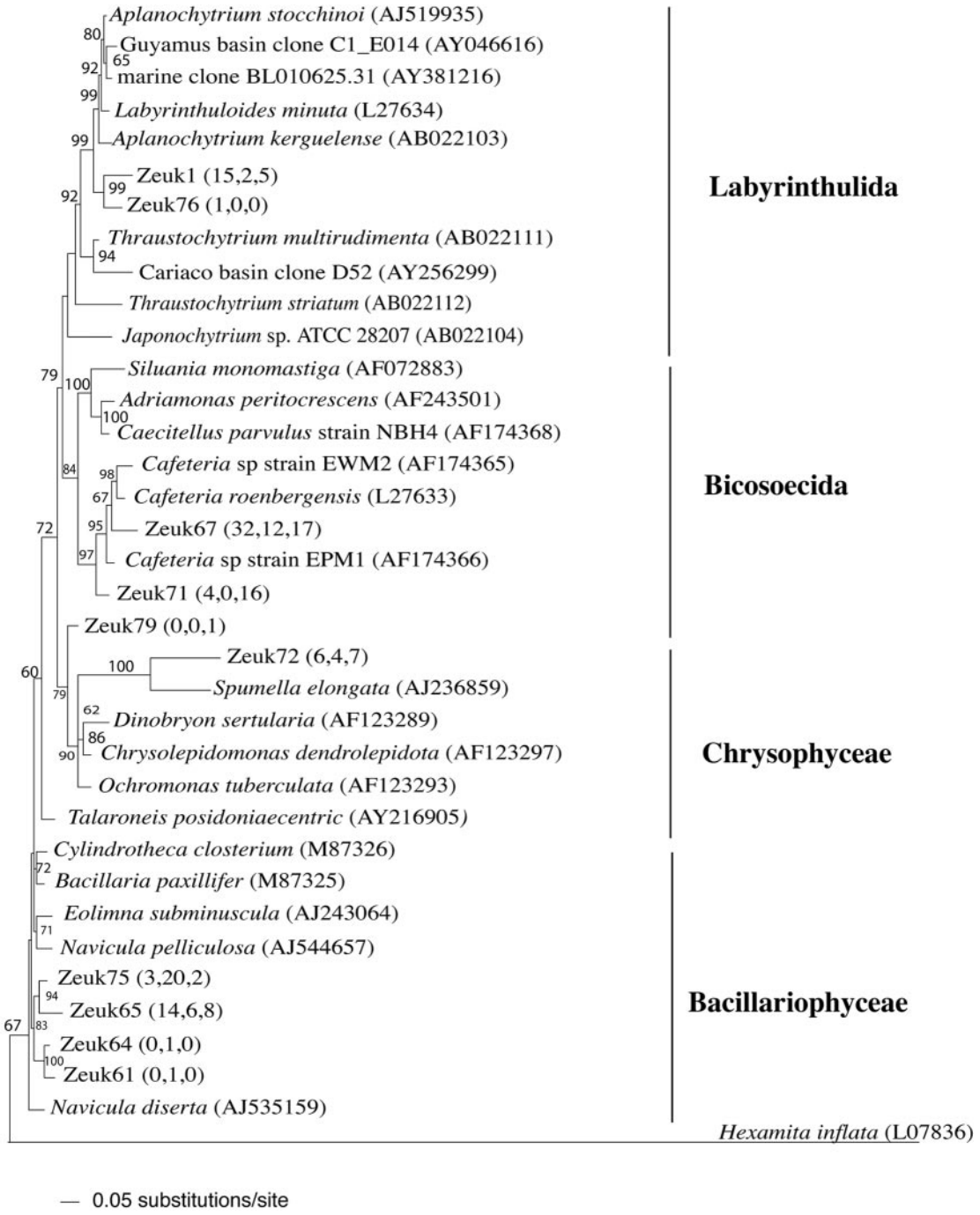

FIG. 2. Distance dendrogram based on the 18S sequences of stramenopile OTUs encountered in Zodletone source, mat, and crust clone libraries. The tree was constructed using a general time reversible substitution model, with a proportion of invariable site value of 0.13128 and a variable site gamma distribution shape parameter at 0.3493. *Hexamita inflata* (L07836) was used as the outgroup. Bootstrap values (in percentages) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. Numbers in parentheses represent the frequencies of occurrence of a specific OTU in the source, mat, and crust clone libraries, respectively.

PLFAs (18:2ω6 and 18:3ω3) represented 4 to 6% of the total PLFAs in the dp, ww, and ftlp samples and represented a significant fraction (between 35% in Zodletone crust samples and 79% in Zodletone source samples) of the total eukaryotic PLFAs in all samples (Table 2). It is interesting to note that among all three Zodletone Spring samples, the highest total eukaryotic PLFA and lowest prokaryotic/eukaryotic ratios were observed in the source sample. Also, the source samples contained more fungus-specific PLFAs (226 pmol/g [dry weight]) than the mat and crust samples (Table 2). The fact that the source has a higher sulfide concentration and lower exposure to light and atmospheric oxygen compared to the mat

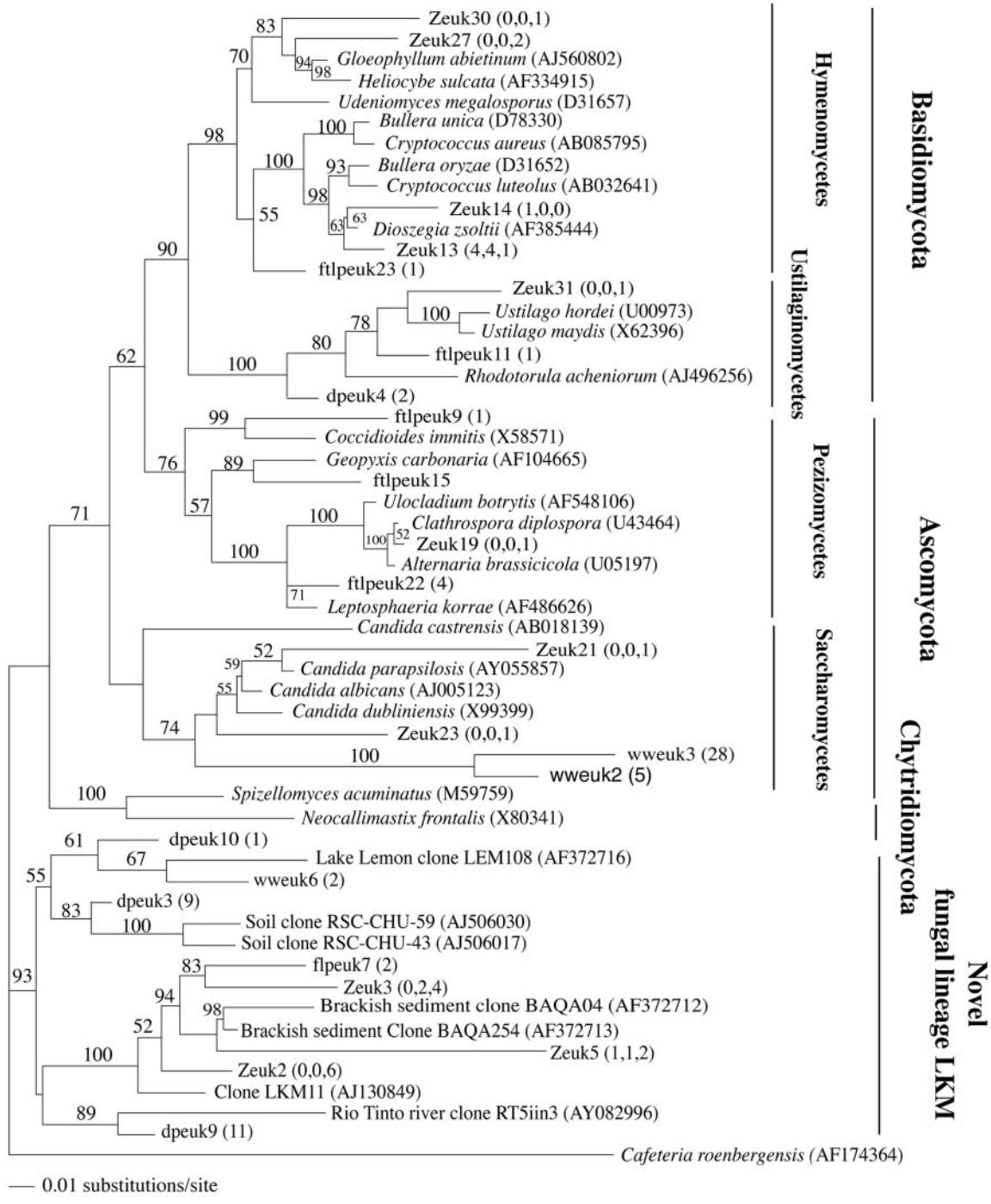

FIG. 3. Distance dendrogram based on the 18S sequences of fungal OTUs encountered in Zodletone source, mat, and crust clone libraries as well as in ww, ftlp, and dp clone libraries. The tree was constructed using a Tamura-Nei substitution model, with a proportion of invariable site value of 0.1218 and a variable site gamma distribution shape parameter at 0.4114. *Cafeteria roenbergensis* was used as the outgroup. Bootstrap values (in percentages) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. Numbers in parentheses represent the frequencies of occurrence of a specific OTU in the source, mat, and crust clone libraries, respectively.

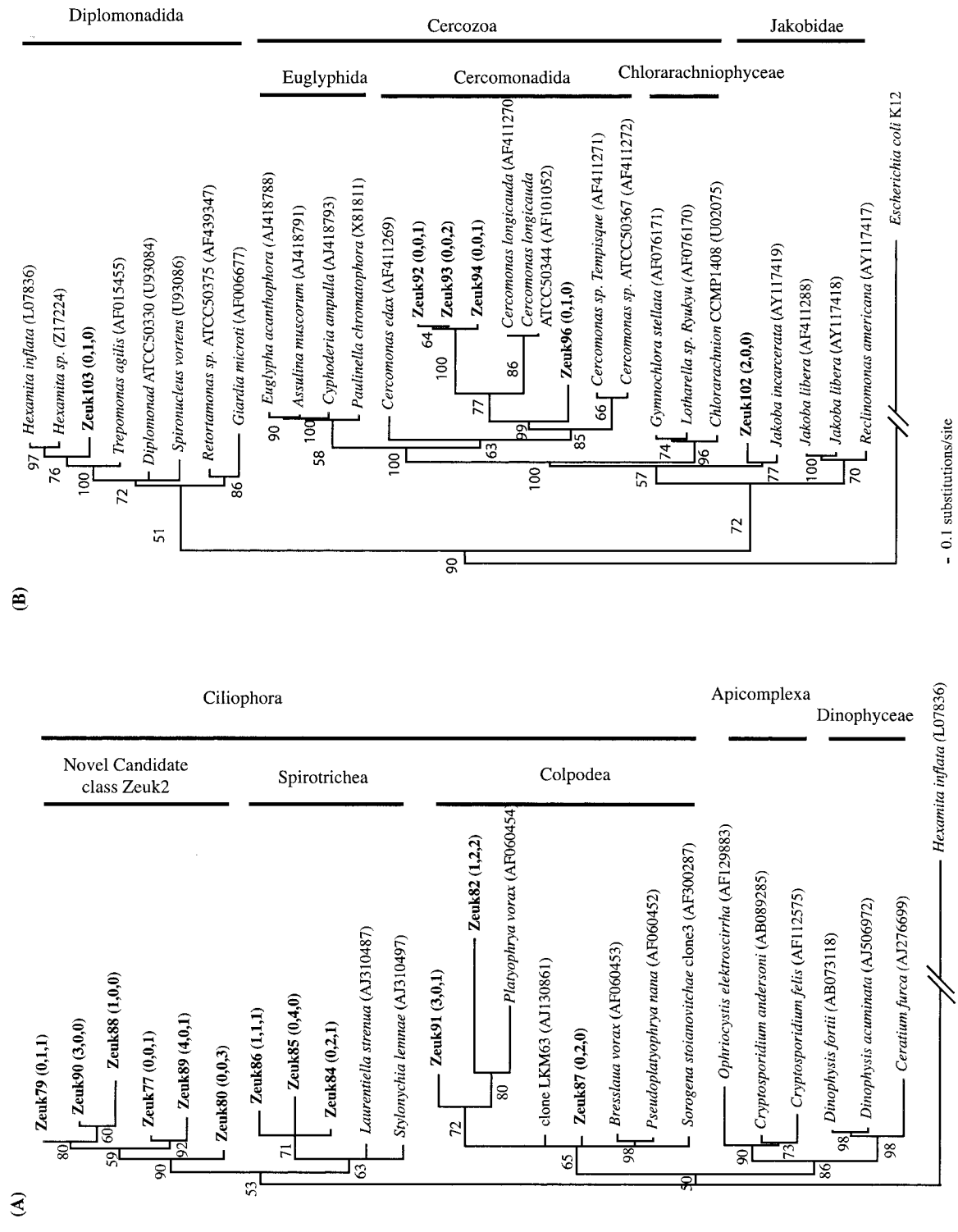

FIG. 4. (A) Distance dendrogram based on the 18S sequences of *Alveolata* OTUs encountered in Zodletone source, mat, and crust clone libraries. The tree was obtained under a Tamura-Nei substitution model, with a variable-site gamma distribution parameter at 0.4022. *Hexamita inflata* (L07836) was used as the outgroup. (B) Distance dendrogram based on the 18S sequences of *Cercozoa*, *Diplomonadida*, and *Jakoba* encountered in Zodletone source, mat, and crust clone libraries. The tree was obtained under a Tamura-Nei substitution model, with a variable-site gamma distribution parameter at 0.5402. *Escherichia coli* strain K-12 16S rRNA gene was used as an outgroup. Bootstrap values (in percentages) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. Numbers in parentheses represent the frequencies of occurrence of a specific OTU in the source, mat, and crust clone libraries, respectively.

and crust attests to the ability of eukaryotic groups detected in Zodletone Spring, including *Fungi*, to survive and grow under strict anaerobic, highly reduced conditions.

DISCUSSION

In this study, we surveyed the microeukaryotic community in Zodletone Spring to determine the identity of eukaryotes in this anaerobic, sulfide and sulfur-rich, hydrocarbon-impacted environment. Although this study did not reveal the presence of any novel kingdom level diversity, it suggests the presence of novel lineages within known groups (e.g., novel fungal lineage LKM within the fungi or novel *Zeuk* lineage within the ciliates) and expands the geographical presence of some groups (genus *Cafeteria*, order *Labyrinthulida*, and genus *Jakoba*) previously thought to be restricted to marine habitats.

The presence of ciliates within the anoxic community in Zodletone Spring is not surprising since ciliates have long been known to be inhabitants of anaerobic environments (22). However, several Zodletone clone sequences have low similarity to all GenBank-deposited ciliate 18S rRNA genes and potentially represent a novel uncultured candidate class within the ciliates.

Cafeteria species have been shown to be abundant in a variety of marine habitats. They have been isolated from anaerobic tidal waves (2), pelagic ocean, and hydrothermal vents (2). Here, we show that members of *Cafeteria* spp. are not restricted to marine settings but could also be encountered in other aquatic ecosystems. This conclusion is also true for *Jakoba* spp. and the order *Labyrinthulida*, both of which have previously been detected using culture-dependent and -independent analyses solely in marine environments (17, 39, 43, 51, 61). It is interesting to note that all three previously mentioned groups have been shown to be an integral component of hydrothermal vent ecosystems. Similarly, many clone groups within the prokaryotic community in Zodletone Spring have been observed in hydrothermal vent prokaryotic communities (18, 19, 62). The resemblance between a deep-sea hydrothermal vent and a mesophilic spring is quite unexpected. However, both environments have several geochemical characteristics in common, including the low redox potential, high sulfide content, and high levels of short-chain gaseous alkanes. Therefore, it appears that these previously mentioned factors could play an important role in shaping the microbial communities in both ecosystems.

In Zodletone Spring, samples with sequences suggesting the presence of two different phototrophic groups (within the *Stramenopiles*) were observed: the classes *Chrysophyceae* (golden algae) and *Bacillariophyceae* (diatoms). The fact that almost all *Chrysophyceae* species described so far are oxygenic phototrophs suggests these organisms are introduced into this anaerobic environment with the input of exogenous organic matter (e.g., wood, leaves) to the stream. Alternatively, in the case of the mat and crust, the *Chrysophyceae* might be localized on the uppermost, air-exposed layers and hence directly exposed to atmospheric air and sunlight. These assumptions could also be true with members of the *Bacillariophyceae*, especially since they represent a larger fraction of the clone libraries of the light-exposed microbial mats compared with the less light-exposed source. It should be noted, however, that some of the closest relatives of Zodletone *Chrysophyceae* clone sequences are closely related to the genus *Spumela* (Fig. 2), all of which are obligately phagocytic (33, 37).

Perhaps the most interesting finding of this study is the unexpected abundance and the novel fungal diversity in Zodletone Spring as well as in all other surveyed anaerobic environments (Fig. 1 and 3; Tables 1 and 2). Fungal clones were previously detected and often represented a significant fraction of the 18S rRNA sequences in clone libraries from anaerobic environments (10, 17, 39, 60). Apart from members of the class *Neocallimasticaceae* within the *Chytridiomycota*, no strict anaerobic fungal species has yet been described and fungi have always been thought to play a minor role in ecosystem processes in anaerobic ecosystems (14, 41). Within the various groups encountered in all six clone libraries examined, fermentative metabolism and anaerobic growth abilities are known to exist among yeast species of the order *Saccharomycetales*. It had been shown that *Candida albicans* and *Saccharomyces cerevisiae* could be grown and maintained under strict anaerobic conditions (16, 58). Also, basidiomycetous yeasts are known to be saprophytes, thriving in high-organic-content environments (20). Although fermentation occurs in only very few of the basidiomycetous yeasts (20), some species had been isolated from seemingly anaerobic environments like subseafloor habitats (45). These fermentative capabilities could explain the presence of these two groups of fungi in Zodletone Spring as well as in other clone libraries. A third group of fungal clones in the libraries is characterized by their close similarity to well known aerobic, wood- and plant-associated fungi e.g., *Ustilago* sp. and *Pezizales*, and their presence is probably a reflection of plant input to the system.

The final group of fungal sequences encountered in Zodletone Spring as well as other environments is the novel fungal lineage LKM (Fig. 3). Since no pure culture representative of this group is yet available, we can only speculate on their metabolic capabilities based on the environments where they have been encountered. A database search revealed the presence of clones belonging to this group in anaerobic brackish sediments and anaerobic freshwater lake sediments (10). This group has also been encountered in soil and lake sediments where no definite information regarding the redox potential is available (66) (GenBank accession numbers AJ506017 and AJ506030), as well as in extremely acidic river sediments (69). However, the fact that several studies using the 18S rRNA gene sequence to study the fungal communities in highly oxic systems, including soil (65), the rhizosphere (57, 64), and freshwater habitats (47), did not detect this group, coupled with their presence in all clone libraries tested in this study as well as several other anaerobic habitats, provides strong evidence for the indigenous nature of this group and hence the ability to thrive under anaerobic conditions. However, it is important to note that some of the previous studies examining fungal diversity utilizing the large ribosomal subunit (rRNA) as a biomarker have detected several unique yet uncultured fungal lineages (54). Comparing the phylogeny of the novel fungal lineage obtained in this study to that of novel lineages detected in large-subunit rRNA-based studies is not feasible.

PLFA and ergosterol determinations confirmed the presence of fungi in all ecosystems studied. The fact that ergosterol and fungal PLFA levels were highest in anaerobic digester

TABLE 1. Phylogenetic affiliation of the microeukaryotic community encountered in wastewater, hydrocarbon-contaminated, and pond sediment clone libraries[a]

Sample	Fungi		Stramenopiles		Alveolates		Green algae		Metazoa (%)	Higher plants (%)
	%	Components[b]	%	Components[b]	%	Components[b]	%	Components[b]		
Wastewater (ww)	63	*Saccharomycetales* (34) LKM group (3)	0	NA	0	NA	0	NA	37	0
Gas condensate-contaminated aquifer (ftlp)	35	*Saccharomycetales* (7) LKM group (1) *Pezizomycetes* (14) *Hymenobasidiomycetes* (2) *Ustilaginomycetes* (1)	13	*Labyrinthulida* (1) *Oomycetes* (6) *Bacillariophyceae* (2)	7	*Suessiales* (1) *Stichotrichia* (2) *Cyrtophorida* (1) *Cyrtolophosidida* (1)	0	NA	24	22
Freshwater pond (dp)	34	LKM group (22) *Chytridomycota* (2) *Glomeromycetes* (1) *Ustilaginomycetes* (2)	24	*Labyrinthulida* (1) *Oomycetes* (1) *Bacillariophycease* (16) *Raphidophyceae* (1)	25	*Armophorida* (4) *Eimeriida* (1) *Halteriidae* (3) *Peniculida* (3) *Gymnodiniales* (1) *Euplotida* (5) *Pleuronematida* (3)	1 4	*Volvocales* (7) *Chlorococcales* (2) *Chlorosarcinales* (1) *Prasiolales* (1)	4	0

[a] Detailed phylogenetic placement of fungal clones is given in Fig. 2.
[b] Number of clones encountered belonging to each phylogenetic group is given in parentheses. NA, not applicable.

samples is in agreement with the observation that *Fungi* constituted the majority of clone sequences (63%) in ww clone library. Assuming a conversion factor of 5.4 mg ergosterol g^{-1} fungal biomass (35), fungal concentrations were 0.06 to 0.07 mg fungal biomass per gram of sediment in the Zodletone source and mat samples, respectively, 0.24 mg per gram in the pond, and 2.02 mg per gram in sewage digester samples. The lower fungal biomass in Zodletone Spring is in agreement with previous studies showing that ergosterol content decreases considerably with depth-associated change from aerobic to anaerobic redox potential in salt marsh sediments (41). However, fungal biomass values in Zodletone Spring are on average only 2 orders of magnitude less than values obtained in studies examining fungal biomass in aerobic, fungus-rich habitats such as compost (35), fungus-infested sludge (24), leaf litter (26, 59), and biocontaminated building materials (50). Moreover, it has been suggested that ergosterol measurements might underestimate the fungal biomass of yeast cells (50). The fact that several Zodletone clones are closely related to yeast lineages suggests that ergosterol analysis might have underestimated the fungal biomass in Zodletone Spring.

The fact that many of the clones in dp, ftlp, and ww clone libraries are derived from the metazoa and *Viridiplantae* (Table 1), coupled with the unexpected abundance of fungi in these settings and relatively small number of clones sequenced renders this study far from a comprehensive survey of the microeukaryotic community in common anaerobic freshwater settings. Studies examining similar environments using culturing techniques targeting a single group, e.g., ciliates or flagellates, have isolated several microeukaryotes, many of which appear to be numerous but that we failed to detect (5, 21, 22, 29, 48, 49, 56). Nevertheless, the 18S rRNA gene-based study and PLFA analysis collectively suggest that culture-based studies generally underestimate fungal levels in anaerobic ecosystems.

Finally, the lack of novel kingdom level diversity or even clones affiliated with recently described novel kingdoms in all six clone libraries analyzed (a total of 496 clones) is surprising. Previous studies collectively suggest the presence of a large number of yet-undescribed eukaryotic kingdoms (10, 17, 44, 60). However, a recent reanalysis of sequences proposed to represent these kingdoms led the authors to propose only five

TABLE 2. Biomass, ergosterol, and PLFA analysis of sediments from samples examined using 18S rRNA gene analysis in this study[a]

	Total				Ergosterol biomass[b]	Fungus-specific PLFA (18:2ω6 and 3ω3)[b]	
	PLFA biomass[b]	Prokaryotic[b]	Eukaryotic[b]	Prokaryotic/eukaryotic ratio		Biomass	mol%
Zodletone source	14,755 (3,222)	14,469 (3,135)	286 (86)	52 (4)	0.8 (0.2)	226	<1
Zodletone mat	9,246 (3,116)	9,194 (3,095)	52 (20)	177 (11)	0.9 (0.8)	19	<1
Zodletone crust	11,557 (415)	11,392 (359)	165 (55)	74 (22)	0	58	<1
Freshwater pond (dp)	22,756 (597)	20,722 (718)	2,034 (121)	11 (1)	3.3 (1.5)	1,315	6
Wastewater (ww)	1,198,626 (299,952)	1,137,662 (279,713)	60,964 (20,239)	20 (2)	27.6 (1.5)	44,726	4
Gas condensate-contaminated sediment (ftlp)[c]	3,053	2,882	171	18	0	143	5

[a] Standard deviation values are given in parentheses.
[b] Values are expressed as pmol/g (dry weight).
[c] Only one sample was analyzed; for all other samples, $n = 2$.

novel lineages, three of which were detected in more than one study (6). These five lineages may represent new eukaryotic kingdoms; however, additional studies are likely needed to validate them. The view that we may be overestimating the level of eukaryotic diversity at the kingdom level based upon 18S rRNA gene analysis is supported by recent studies which used multiple gene analysis to show that most of the eukaryotic diversity could be grouped into eight supergroups and that many of the lineages previously thought to be early diverging branches of the eukaryotic tree based on the 18S sequence are related to groups belonging to the crown radiation eukaryotes (3, 4).

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