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ORIGINAL ARTICLE

Diversity and Distributional Patterns of Ciliates in Guaymas Basin Hydrothermal Vent Sediments

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ABSTRACT

Little is known about protists at deep-sea hydrothermal vents. The vent sites at Guaymas Basin in the Gulf of California are characterized by dense mats of filamentous pigmented or nonpigmented *Beggiatoa* that serve as markers of subsurface thermochemical gradients. We constructed 18S rRNA libraries to investigate ciliate assemblages in *Beggiatoa* mats and from bare sediments at the Guaymas vent site. Results indicated a high diversity of ciliates, with 156 operational taxonomic units identified in 548 sequences. Comparison between mat environments demonstrated that ciliate and bacterial assemblages from pigmented mats, nonpigmented mats, and bare sediments were significantly different and highly correlated with bacterial assemblages. Neither bacterial nor ciliate assemblages were correlated with environmental factors. The most abundant ciliates at Guaymas were more likely to be represented in clone libraries from other hydrothermal, deep-sea, and/or anoxic or microaerophilic environments, supporting the hypothesis that these ciliate species are broadly distributed. The orange mat environment included a higher proportion of ciliate sequences that were more similar to those from other environmental studies than to cultured ciliate species, whereas clone libraries from bare sediments included sequences that were the most highly divergent from all other sequences and may represent species that are endemic to Guaymas.

CILIATED protists are a diverse group that includes free-living species as well as commensal, parasitic, and mutualistic species (Lynn 2008). They are an integral part of freshwater and marine ecosystems and have adapted to hypoxic and anoxic environments (Fenchel and Finlay 1992; Park et al. 2008). Ciliates feed on bacteria, other protists, and fungi, and provide an important avenue for the transfer of carbon from microbial populations to higher trophic levels (Calbet and Saiz 2005; Hlaili et al. 2008; Sherr and Sherr 1987; Šimek et al. 1995; SimeNgando et al. 1995). Global species richness among ciliates has been a controversial topic in recent years (Fenchel et al. 1997; Finlay 2002; Finlay and Esteban 1998; Foissner et al. 2008; Katz et al. 2005). Foissner et al. (2008) calculated that the approximately 4,500 extant free-living ciliate species that have been described represent less than 20% of the true diversity of ciliates. This speculation is supported by culture-independent studies of ciliate diversity based on phylogenetic analysis of 18S rRNA gene libraries (Doherty et al. 2007, 2010; Dopheide et al. 2009; Lara et al. 2007). Phylogenetically diverse ciliates and novel lineages of ciliates have been identified in clone libraries of microeukaryotes from a wide range of extreme environments, including suboxic and anoxic waters of the Baltic Sea (Stock et al. 2009), deep-sea hydrothermal vent sites (Edgcomb et al. 2002; Lopez-Garcia et al. 2003, 2007; Sauvadet et al. 2010), sulfide-rich anoxic waters of the Framvaren Fjord (Behnke et al. 2006, 2010) and Zodletone Spring (Luo et al. 2005), hypersaline anoxic waters of the Mediterranean Sea (Alexander et al. 2009; Edgcomb et al. 2009; Stock et al. 2012), the permanently anoxic Cariaco Basin in the Caribbean Sea (Edgcomb et al. 2011; Orsi et al. 2011, 2012a,b; Stoeck et al. 2003), the abyssal sea floor (Scheckenbach et al. 2010), suboxic and sulfidic waters of the Black Sea (Wylezich and Jurgens 2011), and deep-sea methane cold seeps at Sagami Bay (Takishita

et al. 2010). Within extreme environments, however, few studies have specifically focused on ciliate assemblages, or examined the diversity of ciliates over a range of habitats in conjunction with physicochemical characterizations of the environment (Lara et al. 2007; Orsi et al. 2012a). Therefore, the extent of ciliate species richness in these ecosystems and the environmental factors governing their distributions are poorly understood.

The Guaymas Basin hydrothermal vent site in the Gulf of California (hereafter referred to as Guaymas) is characterized by layers of organic sediments that can reach up to 400 m in thickness (Simoneit 1985). The sediments promote diffuse venting of highly reduced hydrothermal fluids that are enriched to varying degrees in hydrogen sulfide, low molecular weight organic acids, petroleum-like aliphatic and aromatic hydrocarbons, ammonia, and methane (Kawka and Simoneit 1987, 1990; Leif and Simoneit 1995; Simoneit et al. 1996). Temperatures also vary, with surface temperatures near ambient seawater and subsurface temperatures approaching 300 °C at some sites (Einsele et al. 1980). The hydrothermal sediment environment at Guaymas supports dense bacterial mats of filamentous, sulfide-oxidizing *Beggiatoa* spp., and other bacterial and archaeal species that specialize in sulfate reduction, degradation of hydrocarbons, or methane oxidation (Biddle et al. 2012; Dhillon et al. 2003; Pearson et al. 2005; Teske et al. 2002). *Beggiatoa* mats can be several meters in diameter and are spatially distinct, separated by areas of bare sediments. Mats can be nonpigmented (white) or highly pigmented (yellow or orange), and are composed of phylogenetically distinct species of *Beggiatoa* (McKay et al. 2012). The pigmentation is due to the accumulation of sulfur and/or *c*-type cytochromes (Prince et al. 1988) and is thought to be related to distinct trophic modes of *Beggiatoa* spp. (Hagen and Nelson 1996; Nelson et al. 1989; Nikolaus et al. 2003). Studies of *Beggiatoa* from cold seep environments, for example, demonstrated that nonpigmented strains are chemoautotrophic, using CO_2 as their primary carbon source, whereas pigmented *Beggiatoa* are heterotrophic (Nikolaus et al. 2003). Pigmentation may also be indicative of the environment. Recent analysis of the underlying geochemistry of *Beggiatoa* mats at Guaymas demonstrated that carbon sources were more concentrated near the sediment surface beneath orange mats due to greater hydrothermal temperature gradients (McKay et al. 2012). In addition, the considerable biomass associated with *Beggiatoa* mats at Guaymas suggests that the bacterial community may have a significant and highly localized effect on biogeochemistry of the mat environment and the microbial communities associated with the mats.

The bacterial population in *Beggiatoa* mats likely provides a significant food source sustaining ciliates and other protistan bacterivores (Fenchel 1968). Indeed, a molecular survey of microeukaryotic gene sequences described in Edgcomb et al. (2002) demonstrated the presence of a broad diversity of protistan species, including ciliates, in surface sediments of cores extracted from Guaymas. Nothing is known, however, regarding the distribution of ciliate species across mat environments. The objectives of this study were to investigate and compare the diversity and distributional patterns of metabolically active ciliates in mat environments and bare sediments at the Guaymas vent site. 18S rRNA cDNA libraries were constructed from duplicate cores collected at *Beggiatoa* mats and from bare sediments at the Guaymas hydrothermal vent site. These assemblages were then correlated with bacterial populations and with geochemistry associated with each site to evaluate factors affecting the diversity and distributional patterns of metabolically active ciliates in Guaymas sediments.

MATERIALS AND METHODS

Sample collection

Sediment cores were collected from the *Alvin* submersible using piston corers. Duplicate cores were collected from bare sediments and from the center of three microbial mats consisting of orange, yellow, and nonpigmented (white) *Beggiatoa* at the Guaymas hydrothermal vent field during dives 4479, 4480, and 4481 (Table 1).

Temperature and chemical analysis

Temperature at the sediment–water interface was recorded prior to core collection with the submersible's high-temperature thermocouple probe (± 0.1 °C accuracy). Vertical temperature gradients in the sediments below *Beggiatoa* mats were measured with a five-sensor Heatflow probe (± 0.2 °C accuracy; Woods Hole Oceanographic Institution) that recorded temperatures at 5- to 10-cm intervals down to a depth of 30–40 cm. Temperature gradients below the bare sediment surface (the "no mat" environment) were not measured due to instrument failure. The heat flow probe was inserted into the sediment and temperature readings were allowed to stabilize (~2 min) before recording for at least a further 3 min at 1 Hz. A small volume (10 ml) discrete water sampler, the Sipper, consisting of 12 gas tight syringes (Di Meo-Savoie et al. 2004) was used to collect samples at the sediment surface prior to core collection. Duplicate samples were collected at each location; one syringe was preload with zinc acetate and was used for estimating total sulfide, the other analyzed for pH, total iron, and trace elements. The syringes were returned to the ship and immediately transferred to ice (4 °C) and processed within 2 h. pH values were obtained using a 1-ml aliquot of sample and a standard pH meter calibrated daily using a three-point calibration (accuracy ± 0.05 pH units). Standard spectrophotometric methods (Grassoff et al. 1999) were used to determine total sulfides (methylene blue) and total iron (ferrozine) on the ship. Trace element samples (3 ml) were filtered through 0.2-μm cellulose nitrate membrane filters into acid-washed glass vials, acidified with 50 μl of concentrated ultra-pure HNO_3 and stored at 4 °C until analysis. These samples were prepared for analysis by 50-fold dilution with ultra-pure 2% HNO_3 and analyzed on

Table 1. Location, depth and physical and chemical characteristics of mat environments

Dive	Mat color	Location (Lat/Long)	Depth (m)	Surface temperature (°C)	pH	Sulfide (mg/L)[b]	Fe (mg/L)
4479	No Mat	27°25.0619′N 110°58.9956′W	2002	2.6[a]	7.07	ND[c], 0.40	0.04
4479	White	27°25.0609′N 110°58.9756′W	2003	13.29	6.8	7.17, 2.83	ND
4480	Yellow	27°7.5042′N 111°22.6829′W	2002	5.5	7.45	ND	ND
4481	Orange	27°1.16678′N 111°23.0244′W	1998	19.00	7.09	ND, ND	0.04

[a]Temperature taken with Sipper.
[b]Duplicate values given for discrete samples.
[c]ND, not detected.

shore using a Perkin Elmer Elan SCIEX DRC II (PerkinElmer, Waltham, MA) inductively coupled plasma mass spectrometer. A 2% HNO_3 blank solution and Marek standards were run periodically to correct for mass bias and instrument drift.

RNA extraction and cDNA synthesis

Sediment cores were sealed during transport to the ocean surface and stored undisturbed at 4 °C until processing, within 8 h after collection. Overlying water was removed from the tops of sample cores with a sterile pipette and the top 1 cm of sediments from each core, including the microbial mat, was collected into individual sterile Petri dishes and briefly homogenized by mixing. Approximately 2 g of homogenized sediments from cores designated 4479-1 and -3 (white mat), 4479-11 and -12 (no mat), 4480-1 and -5 (yellow mat), and 4481-2 and -3 (orange mat) was transferred to sterile 15-ml polystyrene tubes and RNA was extracted on board using the MO BIO PowerSoil RNA Extraction Kit (MO BIO, Carlsbad, CA). The quantity of RNA extracted from each core was determined spectrophotometrically and its quality was evaluated by gel electrophoresis.

Approximately, 1.0 μg of RNA was DNase-treated with DNase I (Life Technologies, Grand Island, NY) as described in Coyne and Cary (2005) and reverse transcribed with random hexamer primers using SuperScript III First Strand Synthesis Supermix (Life Technologies).

Amplification, cloning, and sequencing

cDNA and control reactions in which no reverse transcriptase was added were diluted 1:20 and amplified by PCR in 20 μl reaction volumes. Each reaction contained 1 μl of diluted cDNA, 1X Jump-Start Taq Polymerase buffer (Sigma Chem. Co., St. Louis, MO), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 μM Ciliophora 18S rRNA primers 384F and 1147R (Dopheide et al. 2008), and 0.5 units of Jump-Start Taq Polymerase (Sigma Chem. Co.). Reactions proceeded for 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min.

PCR products were cloned into pCR™4-TOPO® plasmid vector (Life Technologies) following the manufacturer's protocols. A total of 96 clones from each library were sequenced by the Sanger method at the Genome Sequencing Center at Washington University, St. Louis, MO.

Phylogenetic analysis

Sequences within each ciliate 18S rRNA library were evaluated using the program Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and chimeric sequences were removed from the libraries. Remaining sequences were aligned using ClustalW (Thompson et al. 1994) and visually inspected for errors. Sequences were then binned at the 95% and 99% similarity levels into operational taxonomic units (OTUs) using the Microbial Eukaryote Species Assignment program (Caron et al. 2009).

Phylogenetic analysis was conducted for the most commonly occurring OTUs (containing sequences present three times or more in the combined libraries). Sequences were aligned with ciliate sequences from other environmental studies and closely related ciliate taxa (Table S1) using Geneious v.4.8.5 (Biomatters Ltd, Auckland, NZ). Four alveolate sequences (AF022153, DQ028763, FJ541187, and U97523) were used as outgroups. Highly divergent regions in the alignment were then removed using GBlocks (Castresana 2000; Talavera and Castresana 2007) to yield 492 informative sites for phylogenetic analysis. Resulting sequences were analyzed using Bayesian inference implemented on MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Phylogenetic analysis was conducted by running 2,000,000 generations in four chains, using default temperatures and saving every 100th tree. Trees were constructed using a GTR+I+G DNA substitution model, taking into account a gamma-shaped variation in substitution rates across sites. The log-likelihood score of each tree was plotted against the number of generations to determine the point where log-likelihood scores became stationary (burn-in). The first 5,000 sampled trees (the burn-in) were discarded and a consensus tree was constructed from the remaining trees.

A second tree of ciliate sequences forming a deep-sea clade (Takishita et al. 2010) was constructed using 14 OTUs from this investigation (Gy-08-3, -28, -55, -67, -84, -90, -93, -102, -122, -123, -129, -139, and -143) along with sequences obtained from GenBank for deep-sea cold seep ciliate clones RM2-SGM09 (AB505517), RM2-SGM10 (AB505518), RM2-SGM11 (AB505519), RM1-SGM12 (AB505469), and RM1-SGM13 (AB505470). Sequences were aligned along with 22 representative sequences from the Intramacronucleata (Prostomatea, Plagiopylea, Oligohymenophorea, Spirotrichea, Armophorea, Litostomatea, and the recently described Cariacotrichea; Table S1) using Geneious v.4.8.5 (Biomatters Ltd., Auckland, NZ). Three alveolate sequences (AF022153, DQ028763, and FJ541187) were used as outgroups. Highly divergent regions in the alignment were then removed using GBlocks (Castresana 2000; Talavera and Castresana 2007) to yield 302 informative sites for phylogenetic analysis. Resulting sequences were analyzed using Bayesian inference as described above.

Bacterial Community DNA Fingerprinting Analysis

Bacterial communities were profiled using terminal-restriction fragment length polymorphism (T-RFLP). T-RFLP employs a restriction enzyme to digest partial PCR amplicons of the 16S rRNA gene, creating a profile of length polymorphisms in the restriction fragments that reflect the composition of bacterial communities and the relative abundances of their constituents (Schutte et al. 2008).

PCR amplifications for T-RFLP were performed in triplicate and pooled to reduce stochastic interreaction variability (Polz and Cavanaugh 1998). Each 30 μl reaction contained 10 ng diluted cDNA, 1.5 U Platinum Taq DNA polymerase (Life Technologies), 1x Platinum Taq PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 μM each of primers 787F (5′-Fam-ATTAGAWACCCBGGTAGTC-3′) and 1391R (Ashby et al. 2007), and PCR-grade water. PCR master mixes were treated with ethidium monoazide before template DNA was added to inhibit potential contaminating DNA present in PCR components (Rueckert and Morgan 2007). Thermal cycling conditions were 94 °C for 3 min, 30 cycles of 94 °C for 20 s, 53 °C for 20 s, and 72 °C for 45 s, followed by 72 °C for 3 min. Reactions were pooled and size-selected using a QuickClean DNA Gel Extraction Kit (GenScript USA Inc., Piscataway, NJ). For restriction digestion, 400 ng of PCR amplicon was digested with 2 U *Msp* I (Roche Applied Science, Indianapolis, IN) and 1x Roche SuRE/Cut Buffer L (in 20 μl) at 37 °C for 5 h and terminated at 65 °C for 20 min.

Length profiles of fluorescent-labeled T-RFLP fragments were determined by capillary electrophoresis using an ABI 3130 Genetic Analyzer (Life Technologies) at 10 kV and a separation temperature of 44 °C for 2 h. The resulting fragment profiles were exported in PeakScanner v.1.0 (Life Technologies) using the minimal 5 RFU cutoff and analyzed to identify signal peaks and perform size-binning (Abdo et al. 2006), with the following modifications: peak noise was modeled using a log-normal distribution, and only signal peaks with $p < 0.01$ were included (i.e., occurrence of peak not due to random noise). In addition, all peak heights exceeding 100 RFU were assigned as signals. The resulting signal peaks were binned at a width of 1 nt and normalized by peak height to the total of all peak heights within each sample.

Statistical Analysis

Ciliate OTU abundances (binned at the 95% similarity level) and bacterial T-RFLP profiles were imported into PRIMER 6 v. 6.1.6 (PRIMER-E, Plymouth, UK, 2006) and used to calculate Sørensen similarities (one minus Bray–Curtis dissimilarity) for each group across samples. Agglomerative, hierarchical clustering of similarities was performed using the CLUSTER method, and the resulting clusters were tested using the SIMPROF method at a cutoff of $p = 0.05$ to identify nonrandom grouping patterns. Relationships between ciliate and bacterial communities were evaluated using the RELATE method in PRIMER 6 (PRIMER-E). The BEST (Biota-Environment STepwise matching) function in PRIMER 6 was used to determine whether any subset of environmental parameters might explain differences in ciliate OTU distributions and bacterial T-RFLP profiles. BEST calculates the value of Spearman's rank correlation coefficient (ρ) using every possible combination of variables until it finds the subset whose Euclidean distance matrix gives the highest value ρ ("best" match) for a given resemblance matrix (Clarke et al. 2008).

RESULTS

Temperature and chemistry

Temperature and water chemistry immediately above the four sampling locations varied considerably. Surface temperature ranged from 2.6 °C in bare sediment to 19.0 °C in the orange mat (Table 1). Differences in surface temperature reflected subsurface gradients, with temperatures at 30-cm depth reaching 40 °C, 69 °C, and 103 °C in the yellow, white, and orange mat, respectively (Fig. S1). The lowest pH (6.8) and highest sulfide concentration (224 μM) were recorded above the white mat (Table 1). At the other locations, pH was always above 7 and sulfide concentrations were very low ("no mat") or not detected (yellow and orange mats). It should be noted, however, that pH may have increased due to out-gassing of CO_2 during transit to the surface. For this reason, pH values reported here are not absolute, but should be considered as relative pH for comparison between samples. The majority of trace metal concentrations did not vary substantially (i.e., by less than a factor of 2–3) among locations (Table S2). For those elements that did vary, Al and P concentrations were much higher in the orange mat compared with the other three sites. The white mat had an order of magnitude lower P concentration and the yellow mat a similar reduction in Zn concentration. Both the white and orange mat had lower Cu concentrations

compared with the yellow mat and bare sediment. However, one-way repeated measures ANOVA of trace metal concentrations revealed no statistically significant differences between any two samples ($p > 0.05$).

cDNA library analysis

A total of 550 quality sequences of approximately 680 bases each were obtained, yielding 158 OTUs when binned at the 99% similarity level. These were designated Gy-08-1 through Gy-08-158 in order of overall abundance and deposited in the GenBank database under accession numbers JX268805 through JX268960. Two sequences of 550 were more similar to other alveolate groups. These were removed from the library, yielding 548 ciliate sequences binned into 156 OTUs (Table 2).

Correlation with bacterial community and environmental characteristics

cDNA-based T-RFLP analysis of bacterial communities revealed distinctly and consistently localized communities according to the presence and pigmentation of the bacterial mat, whereas the ciliate communities displayed similar clustering patterns, but with less well-defined distinction between yellow and orange mats (Fig. S2). Sørensen similarity analysis of sequence abundance data revealed that ciliate assemblages in duplicate cores from the same environment were not significantly different (Fig. S2). Ciliate assemblages in pigmented (yellow and orange) mats were also not significantly different. However, there were significant differences between ciliate assemblages in the bare sediments and mat environments, and between the assemblages in the white mat and pigmented mats ($p = 0.01$). Comparison of bacterial T-RFLP results and ciliate OTUs suggested that these assemblages were highly correlated ($p = 0.003$). However, ciliate and bacterial assemblages were not significantly correlated with environmental variables as determined by BEST analysis ($p > 0.05$). As ciliate assemblages between duplicate cores collected from each environment were not significantly different from each other, libraries from duplicate cores were combined for further analysis.

Diversity

Rarefaction curves were calculated for OTUs from each mat environment using Analytic Rarefaction v.1.3 (Holland; http://strata.uga.edu/software/index.html). None of the libraries reached saturation (Fig. 1). Observed species richness was evaluated using Menhinick's richness index (Whittaker 1977) based on the number of OTUs in combined libraries from each environment (Table 2). This diversity index was chosen because it is only dependent on the number of taxa and the sample size, and is appropriate for sequence libraries constructed from RNA, where numbers of individuals of each species are not known. Diversity indices based on small data sets such as those presented here should be viewed with caution, however, as they do not account for unseen diversity. Estimated ciliate species richness was also calculated for duplicate samples collected at each mat environment using Chao2 richness estimators (Table 2; Colwell and Coddington 1994; Chao 1984) as implemented in EstimateS v.8.2.0 (http://viceroy.eeb.uconn.edu/estimates; Colwell et al. 2012). Chao2 is an incidence-based richness estimator and is also appropriate for RNA-based diversity studies, where the number of individuals within each taxon is not known. Based on these indices, the "no mat" environment was the most species-rich, with an estimated 273 species. Based on this estimate, the sequences obtained from the "no mat" environment represented less than 30% of the species present. Samples with the lowest species richness were from the orange mat environment, with approximately half (22 of an estimated 43) of the species richness represented in our libraries. The white and yellow mat environments had intermediate species richness compared with the orange and "no mat" environments, but the number of sequences from the white mat environment represented only about 23% of the estimated number of ciliate species present.

Similarity to cultured and uncultured ciliate sequences

The similarity of each OTU to other sequences available in GenBank was evaluated through BLAST analysis (Altschul et al. 1990), first by excluding all environmental or uncul-

Table 2. Analysis of ciliate library data for each environment

Dive	Mat color	Quality sequences[a]	Number of OTUs (95%)	Number of OTUs (99%)	Menhinick's richness index (D)[b]	Estimated Richness Chao2 (SD)[c]
4479	No Mat	155	66	80	6.42	273 (74)
4479	White	88	30	42	4.48	179 (77)
4480	Yellow	156	27	29	2.32	87 (36)
4481	Orange	149	22	22	1.88	43 (15)
	Total	548	109	156		

Data from duplicate cores were combined. Richness index and estimated richness were calculated based on OTUs binned at the 99% level. SD = standard deviation.

[a]Quality sequences used for alignment after chimeric sequences and two nonciliate sequences were removed.

[b]Menhinick's richness index is the ratio of the number of taxa to the square root of sample size.

[c]Estimated richness based on uncorrected Chao2.

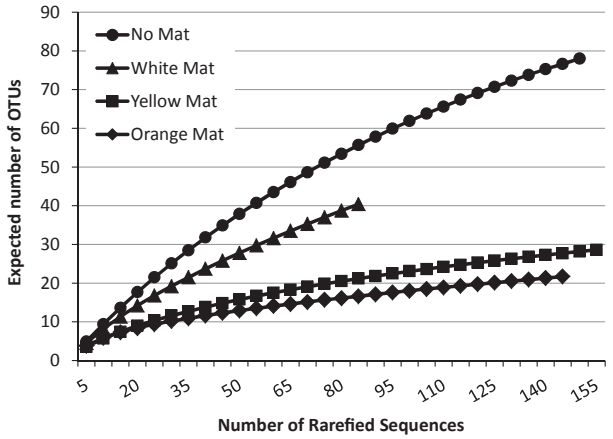


Figure 1 Species accumulation (rarefaction) curves for OTUs generated from each mat environment. Libraries from duplicate cores were combined before analysis and OTUs were binned at 99% similarity. OTU, operational taxonomic units.

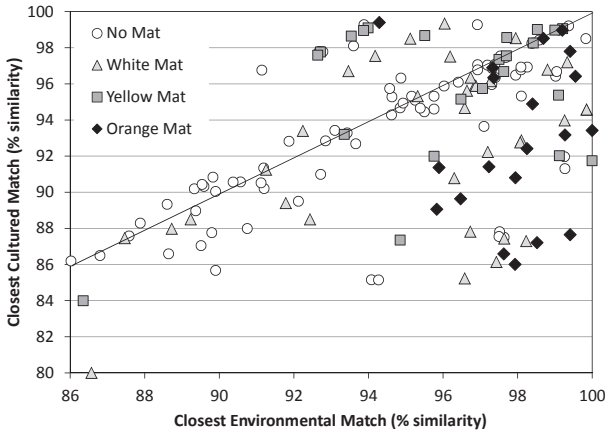


Figure 2 Analysis of sequence similarity to closest cultured match vs. closest environmental match for OTUs from each environment.

tured ciliate sequences and again by excluding all cultured ciliate sequences. The percent similarity to environmental sequences was then plotted against percent similarity to cultured ciliate sequences (Fig. 2). All of the OTUs from the orange mat environment were > 94% similar to environmental clone sequences in GenBank, but only one OTU was more similar to a sequence from culture than to environmental sequences. In addition, six of the OTUs from the orange mat and five from the white mat environment were > 96% similar to uncultured ciliates, but < 90% similar to sequences from cultured ciliates. In contrast, sequences from the "no mat" environment were about equal in similarity to uncultured vs. cultured ciliates and fell near the 1:1 line on the plot. OTUs from this environment included 10 representatives that were < 90% similar to any sequence in GenBank.

Two OTUs (Gy-08-4 and -6) from this investigation perfectly matched (i.e., 100% identity over the full length of the sequence) existing environmental clone sequences in GenBank. Gy-08-4, found exclusively in the yellow mat environment, was a match to environmental clone NA1_2D3 (GenBank accession no. EF526720) from the lower redox transition zone along the O_2/H_2S gradient in the Framvaren Fjord, Norway (Behnke et al. 2006). Gy-08-6, represented in both the yellow and orange mat libraries, matched environmental sequence RM1-SGM18 (GenBank accession no. AB505475), a ciliate sequence from the deep-sea cold seep sediments of Sagami Bay (Takishita et al. 2010). The highly abundant sequence Gy-08-2, found exclusively in libraries from the orange mat, partially matched clone A1_E015 (AY046682), isolated from the surface of a bacterial mat in a previous study of protists at Guaymas (Edgcomb et al. 2002). Due to differences in PCR primers used for this study, the sequences overlapped by only 418 bp of the 667 bp in the Gy-08-2 sequence.

Phylogenetic analysis

Operational taxonomic units were further classified according to ciliate class, based on BLAST similarities. Sequences were identified from eight described ciliate classes and a novel class of deep-sea ciliates previously identified by Takishita et al. (2010). Representative sequences corresponding to the Colpodea, Nassophorea, and Phyllaphorangea classes were not found in our libraries. As the ciliate primers have been used successfully to amplify sequences from these classes in other environments (Dopheide et al. 2008), it seems likely that ciliates from these classes were not present or were in very low abundance at the Guaymas vent sites. In addition, there were no representative sequences for the newly described class, Cariacotrichea (Orsi et al. 2011, 2012b) in the Guaymas library. Although the reverse primer used here (1187R; Dopheide et al. 2008) matches sequences obtained for the Cariacotrichea, it is possible that the forward primer used in this study (384F; Dopheide et al. 2008) was not specific to the newly described class, as all available sequences from the Cariacotrichea were truncated at the 5′-end compared to those obtained here.

The distributions of the nine classes that were obtained in the total library are shown in Fig. 3. Sequences from the "no mat" environment represented ciliates from eight classes, and included ciliates in classes that were common to all other environments sampled except for the Armophorea, which were only detected in libraries from the orange mat. OTUs from the yellow and orange mats were grouped into seven different classes, with no representatives from the Karyorelictea and Plagiopylea in the orange mat, and from the Prostomatea and Armophorea in the yellow mat. Ciliates in the white mat environment had the lowest class-level diversity, with representatives from only six classes of ciliates, lacking species from the Litostomatea, Armophorea, and Heterotrichea classes. This may be due to the low number of sequences obtained from this environment, representing less than a quarter of the estimated species richness (Table 2).

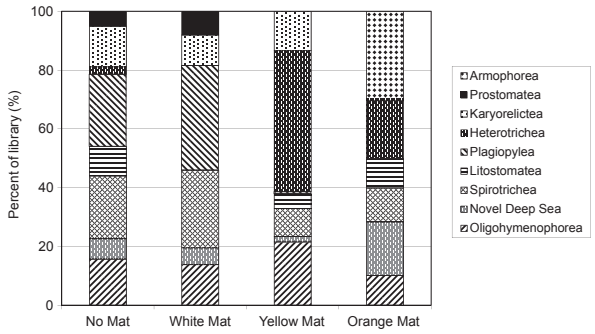


Figure 3 Bar graph depicting relative proportions of ciliate classes represented in libraries from each mat environment.

Phylogenetic analysis of the most abundant ciliates in Guaymas sediments (Table S3) demonstrated the presence of nine well-supported clades (Fig. 4). Closely related sequences from other environmental studies and from cultured ciliates were included for comparison (Table S1). Phylogenetic analysis was also conducted to evaluate sequences that grouped with a novel deep-sea clade of ciliates identified in Takishita et al. (2010). This analysis included 14 sequences from this study (Table S4) and five sequences from Sagami Bay deep-sea cold seep environments (Takishita et al. 2010). Results of this analysis indicated that the ciliates sequences within this group are phylogenetically distinct from other ciliates within the subphylum Intramacronucleata, and can be further divided into two, well-supported clades (Fig. 5).

DISCUSSION

The diversity and numerical dominance of ciliates at vent sites were first noted by Small and Gross (1985) who examined preserved specimens collected at the 21°N hydrothermal vent site in the Pacific Ocean. Many of these specimens also included bacterial remains in food vacuoles, indicating that they were viable and metabolically active members of the microbial community in this environment. In this study, we utilized 18S rRNA libraries to investigate the diversity and distribution of metabolically active members of ciliate assemblages in bacterial mats and bare sediments at Guaymas hydrothermal vents. Analysis of ciliate sequences from Guaymas sediments indicated the presence of a diverse assemblage of ciliates, with 157 unique sequences out of 550 total sequences. The samples obtained from the bare sediments (the "no mat" environment) were the most diverse, followed by samples from the white and yellow mat environments, whereas samples from the orange mat harbored the fewest species. A comparison of the number of OTUs at the 95% and 99% similarity level also indicates greater microdiversity among ciliates in the "no mat" and white mat environments compared with the yellow and orange mats, and may indicate a greater level of functional redundancy (Naeem 1998) within these assemblages. It should be noted, however, that the ciliate sequences obtained likely represented the most abundant species at each site, and true extent of the diversity within each community was not measured.

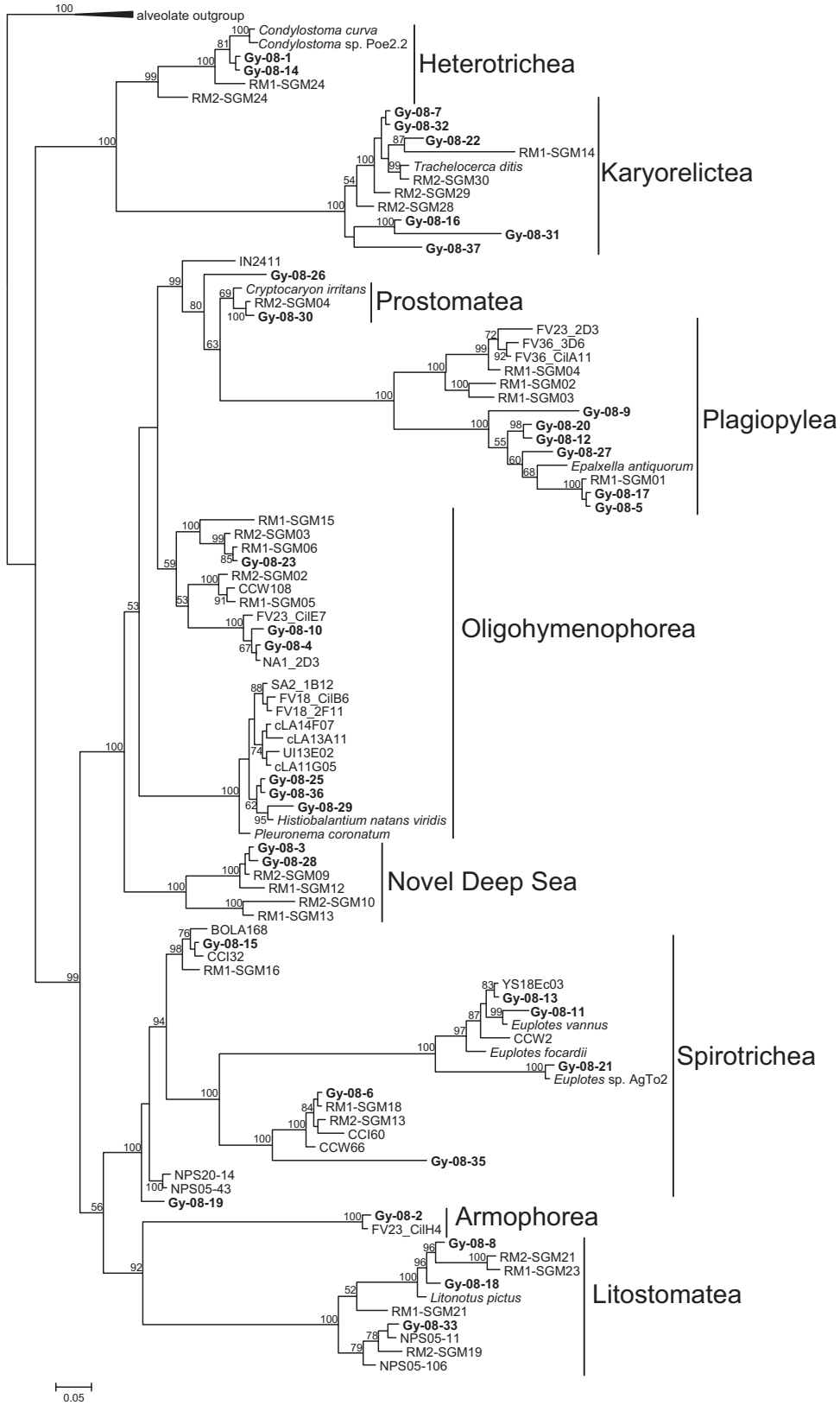
The analysis presented here is based on molecular sequence data, so that predictions about ciliate physiology or their evolutionary adaptation to deep-sea hydrothermal vent environments can only be made in the context of what is known about ciliates and other microbes in similar environments. Structural changes to membrane lipids or the amino acid composition of structural proteins, for example, have been noted in barophilic bacterial species (Campanaro et al. 2008), whereas tolerance to elevated temperatures has been suggested as a factor in structuring ciliate communities (Andrushchyshyn et al. 2009; Li et al. 2011). Genetic adaptation may not be solely responsible for the success of these species, however. Previous studies of barotolerant microbial species have also demonstrated that combined high pressure and elevated temperature can act synergistically to enhance growth rates above those achieved with only one variable (Kaye and Baross 2004).

Environmental factors

In this study, the ciliate and bacterial assemblages within each mat environment were significantly correlated, but did not appear to be correlated with surface temperature. Metal concentrations may also have an effect on community structure (Ancion et al. 2013; Díaz et al. 2006; Gutiérrez et al. 2003). Water overlying the yellow mat had elevated Ni and Cu—both of which can be toxic to ciliates (Díaz et al. 2006; Madoni 2000)—and may have contributed to lower class-level diversity in this mat environment. Zinc can also be toxic to ciliates at the concentrations recorded in this study (Fernandez-Leborans and Antonio-Garcia 1986; Gallego et al. 2007), with highest concentrations of 98 and 86 μM in the "no mat" and white mat environments, respectively. Sulfide detected in the white mat (224 μM) and "no mat" environments (12 μM) may have protected ciliates from heavy metal toxicity, however, by formation of metal sulfides (Edgcomb et al. 2004). Al was also elevated in the orange mat, but the circumneutral pH measured here suggests that Al was not present in soluble form required for toxicity (reviewed by Gensemer and Playle 1999). In addition, statistical analysis did not identify any significant correlations between ciliate or bacterial assemblages with any of the environmental parameters collected, suggesting that other physical or chemical factors, such as subsurface thermochemical gradients (McKay et al. 2012), may have played a larger role in shaping these microbial communities.

Distributional patterns

In spite of the lack of correlation with environmental parameters, analysis of ciliate OTU distributions in the four sampled environments revealed some interesting patterns. Phylogenetic analysis indicated that ciliates in Guaymas sediments fell within nine recognized classes and one novel class of ciliates. The most diverse class of



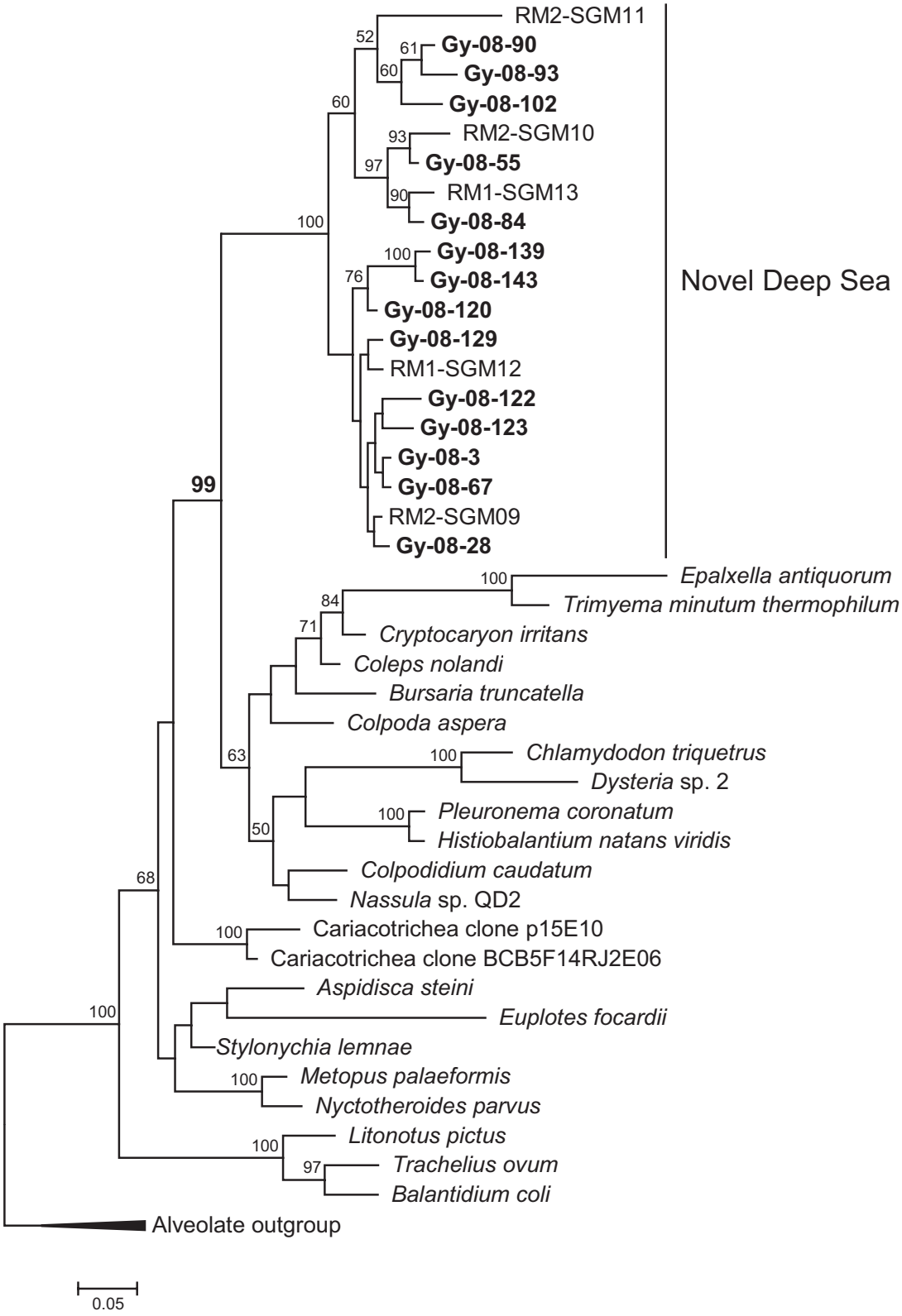
ciliates in our libraries was the Oligohymenophorea, with 86 sequences (15% of the total library) representing 30 OTUs distributed among all four environments sampled. This is not surprising as this class has a broad distribution and is the most diverse class within the Ciliophora (Lynn 2008). It is possible that ciliates within this class played a role in shaping the bacterial community as most free-living oligohymenophorean ciliates are bacterivorous, with some species capable of consuming > 4,000 cells/h (e.g. Šimek et al. 1996). In addition to high grazing rates, selective grazing by these species has been shown to impact bacterial size distributions and community composition (Posch et al. 2001). The most abundant OTUs within this class (Table S3) were divided between two clades (Fig. 2). Notably, one clade consisting of OTUs Gy-08-4, -10, and -23, from white, yellow, and orange mats, respectively, was populated only by environmental sequences from anoxic or microaerobic environments (Table S1). Sequences within this clade were only distantly (< 93% similarity) related to cultured ciliate species within this class. The second clade was populated by Gy-08-25, -29, and -36, with OTUs that were most closely related to *Histiobalantium* and *Pleuronema* spp. within the Order Pleuronematida. In contrast to the first clade, two of the OTUs in this clade were found in the "no mat" environment only.

The Spirotrichea were also very diverse in Guaymas libraries, with 26 sequences represented in all four libraries. Like the oligohymenophorean ciliates, spirotrichs play an important role in microbial food webs, consuming bacteria in addition to other flagellates and ciliates. Phylogenetic analysis of the most abundant ciliates within this class shows that they are distributed among four clades. One clade consisted of Gy-08-19 was found exclusively in the "no mat" environment and is basal to the other three clades in this class. Three OTUs (Gy-08-11, -13, and -21), isolated from yellow and white mat environments, formed a second well-supported clade with several *Euplotes* species along with sequences obtained from other microaerobic and anoxic environments. The *Euplotes* are a cosmopolitan and species-rich genus that is highly divergent from other spirotrichs (Petroni et al. 2002), suggesting that sequences from Guaymas within this clade likely represent novel species within the genus *Euplotes*. A third clade within this class comprises two OTUs from the Guaymas library (Gy-08-06 and -35) along with sequences from deep-sea cold seeps of Sagami Bay and samples from anoxic and/or microaerophilic environments of Great Sippewisset salt marsh, Cape Cod, MA (Table S1). These two OTUs share only 93% and 87% similarity to cultured ciliate species sequences in GenBank, respectively, suggesting that ciliates within this clade are novel. The fourth clade of ciliate sequences within this class includes Gy-08-15 along with environmental sequences from other similar environments. Gy-08-15 was the only OTU shared between all four environments sampled at Guaymas. This OTU was 99% similar to sequences from the cultured ciliate *Trachelostyla pediculiformis*, a stichotrichous ciliate in an isolated lineage that may be ancestral to other oxytrichids (Shao et al. 2007).

There was also some evidence for class-specific preferences or tolerances with respect to mat environment. Ciliates belonging to the class Karyorelictea, for example, were not found in the orange mat environment. The most abundant Guaymas sequences within this class of ciliates (Gy-08-7, -16, -22, -32, and -37) fall within the Trachelocercidae, a family characterized by large vermiform cells, some > 5 mm in length, so that their contribution to the rRNA pool would be conspicuous in communities of low diversity. Their absence in 18S rRNA libraries from the orange mat environment suggests that members of this class may be sensitive to environmental conditions at this site, and are either absent or in very low abundance compared with other taxa. Sequences within this clade were closely related to several environmental ciliate sequences from Sagami Bay (Takishita et al. 2010), however, which is characterized by high concentrations of methane and sulfides. The absence of Karyorelictea species in the orange mat environment at Guaymas suggests that other geochemical factors such as elevated surface temperature and/or the large temperature gradient in the orange mat (Fig. S1) may be a factor preventing colonization by these species.

Ciliates within the Plagiopylea also appear to be selected within certain environments in Guaymas. The most abundant OTUs in this class, representing 10% of the total library, were only found in libraries from the white mat and "no mat" environments. Their presence in sediments with elevated sulfide is consistent with previous investigations of ciliate communities in high sulfide, anoxic sediments (Dyer 1989; Esteban et al. 1993). In addition to those described here, several ciliate sequences previously obtained from Guaymas in an earlier investigation (Edgcomb et al. 2002) also grouped within Plagiopylea (analyzed by Stoeck et al. 2007), supporting the hypothesis that this class of ciliates is abundant in Guaymas sediments. Four sequences within this class (Gy-08-9, -12, -20, and -27) from the "no mat" environment, however, were < 93% similar to other sequences currently in GenBank and included the two most abundant ciliate OTUs in the "no mat" libraries. Two other sequences within the Plagiopylea included the most abundant OTU from the white mat environment (Gy-08-05) and were only 94% similar to the closest cultured ciliate, *Epalxella antiquorum*. We also compared sequences in the Guaymas libraries

Figure 4 Phylogenetic tree of sequences represented three times or more in Guaymas ciliate libraries. Sequences from this study are represented by Gy-08-01 (most abundant OTU) through Gy-08-37. GenBank accession numbers and environmental characteristics for other sequences used in the alignment are provided in Table S1. The tree was constructed using Bayesian inference. The consensus tree is shown for trees generated based on 2,000,000 generations and saving every 100th tree (after discarding the first 5,000 trees). Posterior support values > 50% are indicated. The tree was rooted using the 18S rRNA sequences from four alveolate species. Scale bar, 0.05 substitutions per site.



with several sequences representative of a potentially novel genus within the Plagiopylea as identified by Stoeck et al. (2007). These included environmental sequences (FV23_2D3 and FV36_3D6, shown in Fig. 2) from anoxic and sulfidic water samples from Framvaren Fjord, Norway (Behnke et al. 2006). None of the Guaymas sequences included in the phylogenetic analysis presented here or those from a prior study (Edgcomb et al. 2002; evaluated by Stoeck et al. 2007), however, grouped with this novel clade.

Similar to the Plagiopylea, the most abundant OTUs within the class Prostomatea were found only in white and "no mat" environments. One OTU within this class, Gy-08-30, grouped with *Cryptocaryon irritans*, the fish parasite responsible for white spot disease (Burgess and Matthews 1995). The life cycle of *C. irritans* and other species within the Prostomatea includes a protomont stage, which adheres to sediments to form a tomont, or reproductive cyst. This stage may last for several weeks in *C. irritans* (Colorni and Burgess 1997). OTUs within this class may represent tomont stages of parasitic species associated with fish or other metazoans at the vent sites. A second OTU, Gy-08-26, formed a clade by itself and was basal to other sequences in the Prostomatea. This OTU was < 90% similar to other sequences in GenBank, but shared some similarity to ciliate clone IN2411, isolated from hydrothermal sediments at the Mid-Atlantic Ridge vent site (Lopez-Garcia et al. 2003).

Ciliate sequences from Guaymas sediments within the class Litostomatea were from the subclass Haptoria, a group of free-living predators that graze on flagellates and other ciliates as well as small metazoan species. Haptorian ciliates are often the top predator in microbial communities, using toxicysts to immobilize their prey (reviewed by Lynn 2008). The most abundant ciliate sequence from this class (Gy-08-8) made up 8% of the library from the orange mat environment, and may have contributed to low species richness in this environment by grazing on other members of the ciliate population. This OTU shared 98% similarity with sequences from the marine pleurostomatid ciliate *Litonotus pictus*, and groups with other environmental ciliate sequences from the cold seeps of Sagami Bay. A second clade within this class includes Guaymas sequence Gy-08-33, also from the orange mat, that groups with ciliate sequences from Sagami Bay sediments and from nonpolluted soil near an aromatic hydrocarbon-polluted site in Andujar, Spain (Table S1). Both Gy-08-33 and deep-sea ciliate sequence RM2-SGM19 are 97% similar to sequences for *Arcuospathidium* sp., a genus composed of large, cosmopolitan "flagship" species that are typically found in terrestrial environments. Their presence in extreme environments such as deep-sea marine sediments suggests a wider niche for this genus than previously thought, or the possibility of a novel genus of ciliate closely related to the *Arcuospathidium*.

There was some evidence that separate microenvironments existed within sediments from the orange mat, based on the ciliate sequences. The most abundant OTU from Guaymas sediments (Gy-08-1) belonged to the class Heterotrichea. Along with Gy-08-14, these two sequences make up 18% of the library and were found in the yellow and orange mats only. Gy-08-1 and -14 were 99% similar to large benthic marine ciliates within the *Condylostoma* genus. Species within this genus are rarely found in anoxic environments, instead occupying the thin oxidized layer at the surface of the sediments (Simpson et al. 1998), where they graze on other ciliates, flagellates, and small metazoan species (Fenchel 1968; Lei et al. 2010). In contrast, OTU Gy-08-2, found exclusively in the orange mat environment, belongs to the Armorphorea. Species within this class have a restricted distribution and are found exclusively in anoxic environments (Lynn 2008). They lack mitochondria, but have specialized organelles (hydrogenosomes) that reduce hydrogen ions to H_2, using reductant provided by oxidation of pyruvate (Embley et al. 2003). Although we did not measure oxygen concentrations in the mats, the presence of both *Condylostoma* spp. and ciliates belonging to the Aromophorea within the orange mat suggests the presence of both oxic and anoxic microenvironments within the sediment sample.

Novel deep-sea clade of ciliates

Several sequences in our library showed strong similarity to sequences of a novel clade of ciliates identified in clone libraries from the deep-sea methane cold seeps of Sagami Bay (Takishita et al. 2010). These are oxygen-depleted environments with geochemically produced methane- and sulfide-rich fluids seeping out through the sediments. Similar to Guaymas hydrothermal vent sites, filamentous bacterial mats at the Sagami Bay vent site were found to be good markers for active fluid venting (Henry et al. 2002). Phylogenetic analysis indicates that the novel deep-sea class of ciliates at Guaymas vent sites and Sagami Bay cold seeps falls within the Subphylum Intramacronucleata and is most closely related to ciliates from the Phyllopharyngea and Nassophorea (Fig. 5). Putative members of this novel class were detected in all four environments sampled here (Table S4) and accounted for 8.3% of the total library, suggesting that they are a significant component

Figure 5 Phylogenetic tree of sequences representing a novel deep-sea clade of ciliates. Sequences from Guaymas hydrothermal vent sites (designated GY-08-XX) were aligned with those from cold seeps in Sagami Bay, Japan (designated RMX-SGMXX) and representative sequences from Plagiopylea, Nassophorea, Colpodea, Phyllopharyngea, Oligohymenophorea, Spirotrichea, Cariocotrichea, Armophorea, and Litostomatea. GenBank accession numbers for sequences used in the alignment are provided in Table S1. The tree was constructed using Bayesian inference. The consensus tree is shown for trees generated based on 2,000,000 generations and saving every 100th tree (after discarding the first 5,000 trees). Posterior support values > 50% are indicated. The tree was rooted using the 18S rRNA sequences from alveolate species. Scale bar, 0.05 substitutions per site.

of the benthic ciliate community in the Guaymas hydrothermal vent environment. Although the majority of these sequences occurred only once in our libraries, Gy-08-03, found in the orange and yellow mat environments, was the third most abundant OTU in the libraries, accounting for 5% of the total number of sequences. Phylogenetic analysis of ciliates from Guaymas that fell within this novel group further indicate the presence of two well-supported clades. Notably, one clade included five representative sequences (Gy-08-55, -84, -90, -93, and -102) that were found only in the libraries from the "no mat" environment, three of which (Gy-08-90, -93, and -102) were < 90% similar to all other sequences in GenBank.

Abundant ciliates at Guaymas have a broad distribution

Results of this investigation are consistent with the hypothesis that protists are broadly distributed in similar environments and that distributional patterns are similar for these species on a global scale (Finlay 2002; Finlay et al. 2001). That is, the most abundant ciliates sequences in the libraries from Guaymas were more likely to be represented in clone libraries from other, similar environments, whereas rare sequences were less likely to be found elsewhere. The eight most abundant OTUs in our libraries, for example, were 99–100% identical to sequences previously deposited in GenBank, and accounted for 46% of all sequences obtained (Table S3). Conversely, only 15% (18 out 119) of the OTUs represented by only one or two sequences in the total library were ≥ 99% similar to other sequences in GenBank. The majority of the ciliate sequences that shared identity with those from Guaymas (25 of the OTUs with ≥ 99% similarity) were from environmental clone libraries from deep-sea, hydrothermal and/or microaerophilic to anoxic environments (Behnke et al. 2006, 2010; Dawson and Pace 2002; Lara et al. 2007; Takishita et al. 2010). The high level of gene flow among ciliate assemblages from geographically isolated regions suggests that ciliates are dispersed in marine environments at a rate that conceals evolutionary or historical events (Bass et al. 2007), at least at the 18S rRNA level. Recruitment of these species may be facilitated by the ephemeral and patchy nature of the vents at Guaymas, such that frequent formation of new vent sites provides a period of reduced competitive pressure, allowing colonization by nonindigenous species. Closer analysis of these results indicates that the orange mat environment harbored the highest proportion of ciliate species with a high level of similarity to other environmental sequences, but low level of similarity to cultured ciliates (Fig. 2). The "no mat" environment, on the other hand, had the highest proportion of species that shared < 90% identity with any other sequence in GenBank, suggesting a greater degree of genetic divergence and perhaps endemicity among members of the ciliate population in the "no mat" environment. A more detailed investigation of these ciliates, including microscopic analysis and isolation, would enhance our understanding of adaptation to extreme environments and the role ciliates play within these ecosystems.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Sediment temperature profiles below the surface of *Beggiatoa* mats.

Fig. S2. Cluster analysis of Sørensen similarities for ciliate and bacterial communities.

Table S1. Sequence identity and relevant environmental information for ciliate sequence data included in phylogenetic analysis.

Table S2. Trace metal concentrations (μM) of water samples collected above each mat. Numbers below elements refer to atomic weights.

Table S3. Most abundant sequences and their distribution across mat environments. Numbers indicate the number of sequences in duplicate cores from each environment. Closest environmental and cultured ciliate matches are indicated for each OTU along with % similarity and GenBank accession numbers.

Table S4. Distribution of Novel Deep-Sea clade of ciliates across mat environments at Guaymas. Numbers indicate the number of sequences for the OTU in duplicate cores from each environment.