

Distribution and diversity of oligotrich and choreotrich ciliates assessed by morphology and DGGE in temperate coastal waters

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ABSTRACT: We assessed the diversity of coastal planktonic ciliates (Oligotrichia and Choreotrichia, Class: Spirotrichea) in Fishers Island Sound off the coast of Connecticut, USA using a combination of denaturant gradient gel electrophoresis (DGGE) and morphological analyses. To test the hypothesis that ciliate communities followed water masses, we sampled 3 times at a surface drifter (i.e. floating buoy) and twice at intervening times from points 1 km away. By repeating both PCRs and gel electrophoresis under varying conditions, we confirmed that DGGE is an appropriate tool to capture the ciliate community composition, based on the high repeatability of samples. The 2 methods used for assessing ciliate community structure showed similar levels of diversity for tintinnids. However, there was a mismatch in diversity estimates for the aloricate ciliates, especially for the oligotrichs where the estimated diversity was greater by DGGE than by microscopy. This may be attributable to biases in the DGGE (e.g. primer or gene copy number issues) and/or misidentification by morphology. However, the pattern of community structure assessed by cluster analyses is similar for both microscopy and DGGE, suggesting that the mismatch between these methods does not introduce bias in biogeographical analysis. Analyses of both DGGE haplotypes and microscopically observed morphospecies revealed that the ciliate communities were not static within the water mass. Community structure and abundance patterns varied with salinity and currents. These changes could be due to mixing with adjacent estuarine and coastal assemblages, ciliate behavior, and/or other factors that cause assemblages to be dynamic over short (i.e. ultradian) periods.

KEY WORDS: Oligotrichia · Choreotrichia · DGGE · Morphology · Ciliate assemblage

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INTRODUCTION

Assessing ciliate diversity is important for understanding relationships within the planktonic food web (Ducklow 1993, Calbet et al. 2008, Grattepanche et al. 2011b). Indeed, numerous studies have shown that ciliates are the major trophic link from pico- and nanoplankton to higher trophic levels such as copepods and fish (e.g. McManus & Fuhrman 1988, Sherr & Sherr 1994, Calbet & Saiz 2005, Landry & Calbet

2005, Grattepanche et al. 2011a). Morphological studies suggest limited global diversity and cosmopolitan distributions of individual species, but this is controversial as molecular studies reveal patterns of high diversity over short spatial scales (cf. Fenchel et al. 1997, López-García et al. 2001).

Application of molecular techniques has shown considerable numbers of cryptic species underlying some morphospecies (Katz et al. 2005, Foissner 2008) and provided a fine-scale assessment of ciliate diver-

sity (Foissner et al. 2008, Tamura et al. 2011, Caron et al. 2012, Coyne et al. 2013). Katz et al. (2005) assessed the genetic diversity of the ciliate morphospecies *Strombidium oculatum* and *Halteria grandinella*, and found evidence of cryptic species in both. The presence of cryptic species within these ciliates is supported by subsequent studies (Foissner 2006, Simon et al. 2008, McManus et al. 2010). Studies of ciliate diversity and biogeography have reported different assemblages by location (Doherty et al. 2010), depth (Countway et al. 2007) and/or season (Countway et al. 2010, Steele et al. 2011). Tamura et al. (2011) used a combination of clone libraries and denaturant gradient gel electrophoresis (DGGE) to assess ciliate diversity across salinity gradients in Long Island Sound, USA. This study revealed (1) similar results comparing clone libraries and DGGE, (2) high diversity of ciliates, with a few abundant and numerous rare haplotypes, and (3) a species-richness pattern that varied with depths and stations.

In the present study we combined DGGE and microscopy to elucidate the pattern of ciliate diversity in relation to spatial and temporal factors and the possible link to water masses. We tested the hypothesis that the ciliate community follows the water mass by tracking a surface drifter (a buoy following the water mass into which it was placed) over a period of 2.5 h (35 ± 19 min between each sampling). To quantify the ciliate community structure, we sampled 5 stations at the surface: 3 stations at the surface drifter and 2 further stations located 1 km away. We used a serial filtration system to estimate genetic diversity within the nano- (~ 2 to $10 \mu\text{m}$) and micro-size (~ 10 to $80 \mu\text{m}$) fractions. We analyzed the resulting data to assess the patterns in community structure associated with water mass and other environmental variables.

MATERIALS AND METHODS

Station locations and sample collection

On 30 May 2012, we sampled 5 stations in Fishers Island Sound while following a surface drifter made from 2 pairs of crossed plastic pipes connected by a 1 m length of nylon fabric, neutrally buoyant at the surface. Three stations (Stns 1, 3 and 5) were sampled at

the surface drifter as it followed the falling tide, and 2 intervening stations (Stns 2 and 4) were sampled approximately 1 km west and east, respectively, of the surface drifter position (Fig. 1, Table 1). At each station, 2 l of seawater was collected at the surface (in duplicate) in polycarbonate carboys (Table 1). Vertical measurements of temperature, salinity, and chlorophyll fluorescence were made with a SeaBird CTD instrument and a factory-calibrated WetStar fluorometer. Horizontal currents were measured with a ship-mounted acoustic Doppler current profiler (ADCP).

Microscopy

At each station, 500 ml of surface seawater were preserved with acid Lugol's solution (2% final concentration) to classify and quantify the ciliate morphospecies. These samples were settled and concentrated to 50 ml by aspiration of the supernatant. One to 2 subsamples of 3 ml per concentrated sample were examined under an inverted microscope. Some aloricate ciliates (i.e. ciliates without a 'shell' or covering) with easily identifiable morphology and all tintinnids were classified to genus or species according to Maeda & Carey (1985) and Alder (1999), respectively. The remaining aloricate ciliates were classified into morphological groups based on their shape and size, and identified to the level of family or order according to Lynn (2008).

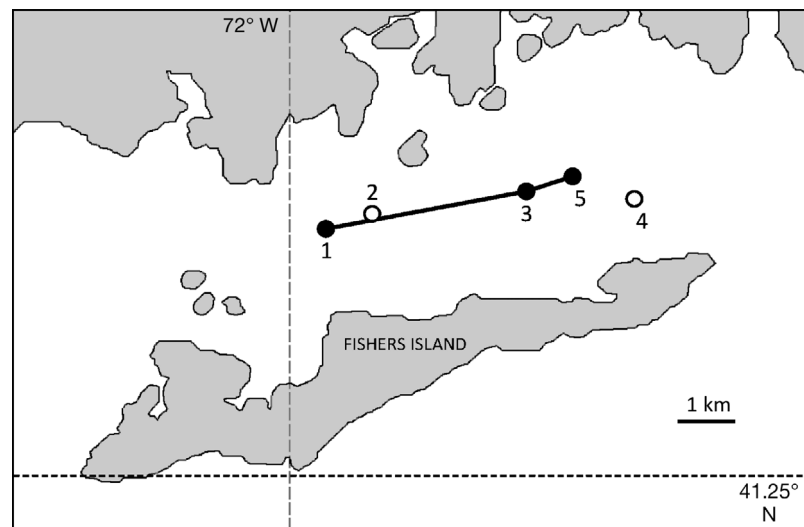


Fig. 1. Sampling stations to assess the diversity of coastal planktonic ciliates in Fishers Island Sound off the coast of Connecticut, USA. The 3 surface drifter stations (1, 3, and 5) were sampled over approximately 2.5 h on a falling tide. In between surface drifter samples, we collected water at 2 further stations (2 and 4) that were ca. 1 km west and east, respectively, from the surface drifter's position at the time of sampling

Table 1. Environmental conditions at sampling stations to assess the diversity of coastal planktonic ciliates in Fishers Island Sound, Connecticut, USA. See Fig. 1 for location of sampling stations (Stns 1 to 5). The ‘away’ Stns 2 and 4 were located 1 km away from the surface drifter position at the time of sampling. Values for salinity, temperature, current and fluorescence are the mean \pm SD for the first meter depth from the CTD data. The direction of current (northeastwards or southwestwards) is shown in parentheses. Fluorescence is used as a proxy for chlorophyll concentration

| Station | Stn 1 Drifter | Stn 2 Away | Stn 3 Drifter | Stn 4 Away | Stn 5 Drifter |
|------------------------------|----------------------|----------------------|----------------------|-----------------------|----------------------|
| Time (EST) | 9:51 | 10:55 | 11:24 | 11:47 | 12:13 |
| Longitude | 41°17.82'N | 41°18.05'N | 41°18.25'N | 41°18.19'N | 41°18.35'N |
| Latitude | 72°0.08'W | 71°58.39'W | 71°57.50'W | 71°56.20'W | 71°56.91'W |
| Depth (m) | 19.8 | 22.3 | 19.4 | 10.8 | 15.9 |
| Salinity | 29.7 \pm 0.04 | 29.4 \pm 0.02 | 29.4 \pm 0.05 | 29.7 \pm 0.03 | 29.9 \pm 0.05 |
| Temperature (°C) | 14.8 \pm 0.01 | 15.0 \pm 0.02 | 15.4 \pm 0.01 | 15.3 \pm 0.01 | 15.2 \pm 0.02 |
| Current (m s ⁻¹) | 0.58 \pm 0.01 (NE) | 0.28 \pm 0.03 (NE) | 0.26 \pm 0.01 (NE) | 0.16 \pm 0.002 (NE) | 0.08 \pm 0.04 (SW) |
| Fluorescence | 3.9 \pm 0.1 | 4.2 \pm 0.1 | 4.3 \pm 0.1 | 5.6 \pm 0.2 | 4.8 \pm 0.09 |

DNA extraction and processing

Between 1 and 2 l of seawater were filtered with a serial filtration on 80 μ m mesh, 10 μ m and 2 μ m polycarbonate filters, using a peristaltic pump with low filtration pressure. We used serial filtration to avoid filter clogging and minimize organism disruption. To compare with the size fractionation, we collected a separate sample of water onto a 3 μ m nitrocellulose filter without prescreening. All filters were immediately placed in DNA preparation buffer (100 mM NaCl, Tris-EDTA at pH 8, and 0.5% SDS) until extraction. DNA was extracted and purified using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989, Ausubel et al. 2002) adapted to filters by Costas et al. (2007). The final DNA pellet was air dried and resuspended in 50 μ l Tris-EDTA and 0.1 μ l RNase.

The DNA from the 10 μ m and 2 μ m filters for each station was amplified with Phusion polymerase (NEB) under conditions aimed at minimizing PCR recombination (i.e. low starting template, minimal cycles; Lahr & Katz 2009). The master mix used in this PCR consisted of 12.9 μ l PCR water, 4 μ l 5 \times GC buffer (NEB), 0.2 μ l MgCl (0.5 mM), 1.0 μ l BSA (50 mM), 0.4 μ l dNTP (50 μ M each), 0.2 μ l of each primer used (0.25 pM), and 0.1 μ l Phusion polymerase. The SSU rDNA primers 152+ and 528-GC were used (Tamura et al. 2011). Target regions were amplified with PCR under the following conditions: initial denaturing temperature 98°C for 3 min, 30 cycles of 98°C for 15 s, 58°C for 15 s, 72°C for 1 min and a 10 min final extension at 72°C.

The DGGE method used in this study is described in Tamura et al. (2011) with the following changes. For each sample, either 3 or 5 PCR products were pooled prior to DGGE. For preliminary gels, 0.05 ng

μ l⁻¹ of genomic DNA was used in each of 3 PCRs (see Fig. S2 in the Supplement at www.int-res.com/articles/suppl/a071p211_supp.pdf) while the remainder of the gels use 5 PCR products amplified from a 1:10 dilution of genomic DNA (0.34 and 1.56 ng μ l⁻¹). To test the robustness of DGGE, we replicated each gel using PCR reactions that had been run on different days. The brightest bands and all common bands were excised from the gels and sequenced by the Sanger method after reamplification (10 cycles with the same cycling conditions) to assess consistency between gels and for comparison with morphology-based identification (sequences are available at the National Center for Biotechnology Information [NCBI] under accession numbers KF385016 to KF385037). In order to compare the DGGE experiments, we created a set of markers representing known haplotypes (see Fig. 3 and Table S2, the latter in the Supplement at www.int-res.com/articles/suppl/a071p211_supp.pdf).

Using morphospecies sequences from NCBI as references, we constructed a tree of the sequenced bands using Randomized Axelerated Maximum Likelihood For High Performance Computing (RAXML-HPC) BlackBox (Stamatakis 2006, Stamatakis et al. 2008) in the CIPRES Science Gateway (Miller et al. 2010). The GTR model of evolution with a model of rate heterogeneity and a proportion of invariable sites was used, as previously identified with jModelTest (Darriba et al. 2012) under the Akaike's information criterion (AIC).

The community structure obtained by morphology and DGGE was analyzed with Fast UniFrac software (Hamady et al. 2010) based on band pattern and intensity. Band intensity was measured using Kodak molecular imaging software (Carestream Health). For Fast UniFrac inputs, we used a star tree for morphology and DGGE, a map of haplotypes or morphospecies

presented at each station, and a map of the environmental conditions at each station. For DGGE we also performed the analysis using a phylogenetic tree with sequenced bands and morphospecies from NCBI. The stations were clustered using the unweighted pair group method with arithmetic mean (UPGMA). The robustness of the UPGMA clusters was tested with jackknife analysis based on 50 permutations.

RESULTS

Environmental conditions

The physical parameters (depth, salinity, temperature, current) varied among the stations (Table 1). Depth to the bottom ranged from 11 to 22 m among stations. The current speed at the surface ranged from 0.59 to 0.03 m s⁻¹ and was generally northeastward with the falling tide until Stn 5, when it began to turn southwest after slack water (Table 1). The temperature and salinity were relatively constant among stations and depths, although the salinity was slightly lower in the upper 1 m and this varied over time as the drifter passed through areas of freshwater influence from coastal watersheds and saline influence from the incoming tide. Chlorophyll *a* concentration at the surface ranged from 3.9 to 5.6 µg l⁻¹ across samples, with the highest concentration at Stn 4 (Table 1).

Oligotrichia and Choreotrichia ciliates assessed by morphology

The number of morphospecies varied from 24 to 36 among stations, with the richest assemblage observed

at Stn 4 (Fig. 2 and Table S1, the latter in the Supplement). Abundance ranged from 2.4 to 7.1 × 10³ cells l⁻¹, showing the maximum and minimum values at Stns 3 and 5, respectively. More oligotrich than choreotrich morphospecies were observed at all stations (in total, 43 Oligotrichia versus 23 Choreotrichia; Table S1 in the Supplement). Oligotrich morphospecies also dominated in terms of abundance, representing between 71.8 and 79.7% of total abundance, except at Stn 3 where choreotrich and oligotrich morphospecies had similar proportions (53.4 and 46.6% of total abundance, respectively).

Repeatability of DGGE

To ensure that the serial filtration and DGGE methods were robust enough to capture the ciliate community, we replicated DGGE gels multiple times using varying PCR cycling conditions (Figs. 3 & S1, the latter in the Supplement). Comparing number, position and brightness of bands between unfractionated samples and the micro- and nanosize samples (Fig. S1), all bands in the unfractionated (whole water) lane were also found in the nano- and/or microsize lanes. This comparison indicates good preservation of cells in our serial filtration system. We repeated the DGGE up to 8 times, pooling 3 or 5 PCR products to (1) confirm that the common haplotypes are observed regardless of the number of PCR products pooled and (2) observe the rarer haplotypes. The DGGE repeated with the 2 replicates and with 3 or 5 PCR products pooled generated the same band pattern for each sample (Figs. 3 & S1, where 5 and 3 PCR products are pooled, respectively).

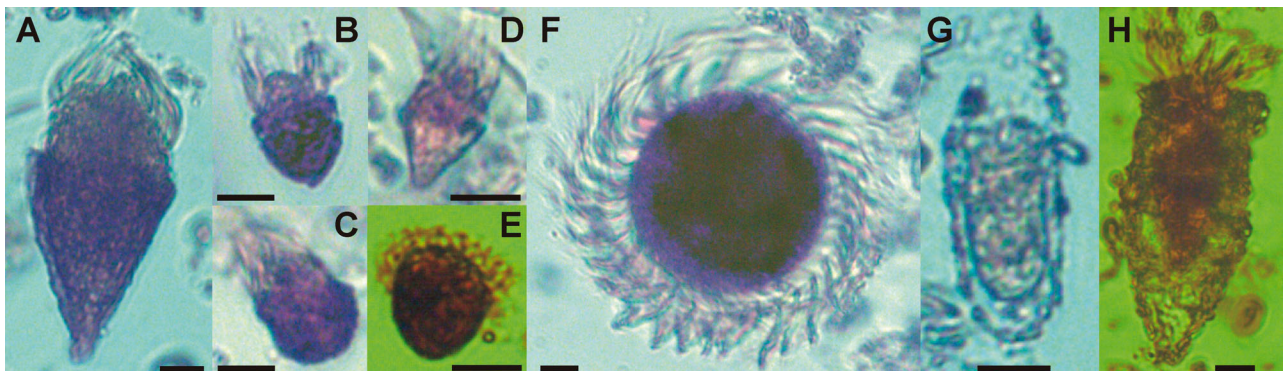


Fig. 2. Examples of morphospecies observed by microscopy: (A) microplanktonic Oligotrichia (*Strombidium* cf. *conicum*); (B to D) nanoplanktonic Oligotrichia (*Strombidiidae* 8, 4, 32, respectively; morphospecies nos. shown in Table S1 in the Supplement at www.int-res.com/articles/suppl/a071p211_supp.pdf); (E & F) nanoplanktonic and microplanktonic aloricate Choreotrichia (*Choreotrichida* 2, 8, respectively); (G & H) loricate Choreotrichia (*Tintinnidium* sp. 1, *Tintinnopsis* sp. 2, respectively). Scale bar = 10 µm

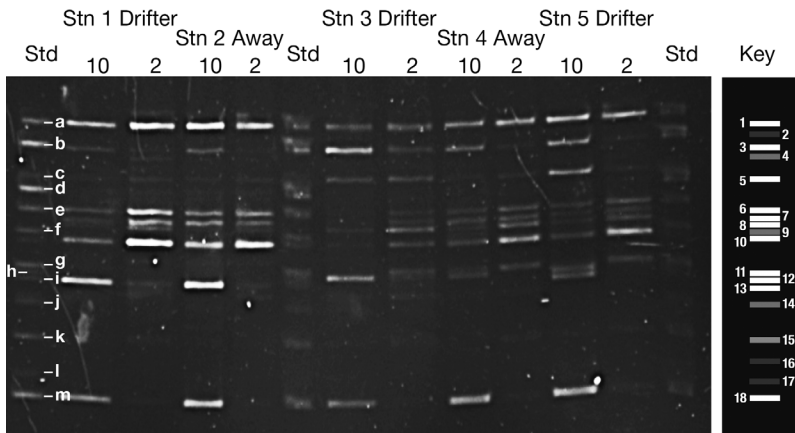


Fig. 3. Structure of the oligotrich and choreotrich ciliate community at surface assessed by DGGE gel. Std: standard based on previously collected DNAs or bands cut from gels in this study; for description of bands ('a' to 'm') see Table S2 in the Supplement at www.int-res.com/articles/suppl/a071p211_supp.pdf. For location of sampling stations see Fig. 1. The 'away' Stns 2 and 4 were located 1 km away from the surface drifter position at the time of sampling. 10 = microsize fraction between 80 and 10 µm; 2 = nanosize fraction between 10 and 2 µm. The key to the right indicates the positions of all haplotypes observed across the various stations

The robustness of DGGE was further confirmed by excising and sequencing bands from multiple gels and across stations. Of the 18 bands excised, 3 sequences had poor quality that may suggest the presence of multiple sequences at the same location in the DGGE gel (Bands 4, 5 and 9; Figs. 3 & 4). Of the 12 bands sequenced successfully, 5 and 7 haplotypes fell within Oligotrichia and Choreotrichia, respectively. Sequences for bands at the same position on different gels corresponded to the same haplotypes e.g. Band 1 of Stns 1 and 2 represented the same sequence (closely related to *Pelagostrobilidium neptuni* AY541683; Fig. 4) as did Band 12 of Stns 1 and 3 (closely related to *Tintinnopsis* sp. JN831850 sampled in an estuary of the SW Atlantic; Santoferrara et al. 2013). Some haplotypes (notably the haplotypes of Bands 10, 11 and 12) showed closest similarity to the same NCBI morphospecies sequence, *Strombidium* cf. *basimorphum* JF791016 (Fig. 4). The varying brightness and distribution in size (nano- or microsize lanes) of these 3 bands indicate that Band 10 could represent a nanosized haplotype, Band 12 a microsized haplotype and Band 11 a rarer haplotype of *S.* cf. *basimorphum* JF791016 without a clear size signature (Figs. 3 & 4).

Only one haplotype (Band 17; Fig. 3) was related to a non-ciliate, the dinoflagellate *Heterocapsa triquetra*, showing the relative specificity of our primers for the Oligotrichia and Choreotrichia subclasses. The bands of this haplotype, as with 2 others, were not bright and were found only by combining 5 PCR products before running the DGGE (Bands 2, 16 and 17; Figs. 3 & 4).

Oligotrichia and Choreotrichia ciliates assessed by DGGE

The total number of unique haplotypes varied from 10 (3 PCR products pooled; Fig. S1 in the Supplement) to 18 (5 PCR products pooled; Fig. 3 in our initial trials). Thereafter, we used the DGGE with 5 PCR products

pooled for capturing the ciliate communities. Haplotype number (number of bands present) ranged from 8 to 12 within the 2 size fractions (Table 2). The structure and size distribution of ciliate communities as indicated by the serial filtration varied across stations. The nanosize and microsize ciliates presented similar overall patterns; haplotype num-

Table 2. Comparison of the number of taxa assessed by morphology and by DGGE at sampling stations (Stns 1 to 5) in Fisher Island Sound. Data for morphospecies show the total number of taxa and the number of taxa of common morphospecies accounting for >2, >5 and >10% of total abundance. For morphospecies accounting for >2% of total abundance and haplotypes obtained by DGGE, numbers of choreotrich and oligotrich taxa are shown; the numbers in parentheses show the percentage of abundance (morphospecies) or band intensity (haplotypes). C/O: Morphospecies not clearly classifiable as choreotrich or oligotrich; nd: bands possibly containing >1 sequence

| Station | Stn 1 Drifter | Stn 2 Away | Stn 3 Drifter | Stn 4 Away | Stn 5 Drifter |
|----------------------------|------------------|---------------|------------------|---------------|------------------|
| Number of morphospecies | | | | | |
| Total | 25 | 24 | 29 | 36 | 25 |
| >2% | 12 | 10 | 9 | 13 | 16 |
| >5% | 6 | 5 | 3 | 6 | 6 |
| >10% | 2 | 4 | 3 | 4 | 4 |
| Common morphospecies (>2%) | | | | | |
| Choreotrich | 3 (24.9) | 4 (18.5) | 2 (58.3) | 3 (20.7) | 5 (27.5) |
| Oligotrich | 9 (75.1) | 6 (81.5) | 7 (41.7) | 8 (71.7) | 11 (72.5) |
| C/O | | | | 2 (7.6) | |
| Haplotypes | | | | | |
| Total | 12 | 8 | 10 | 12 | 12 |
| Choreotrich | 6 (47.3) | 5 (58.1) | 5 (62.4) | 6 (64.8) | 5 (57.1) |
| Oligotrich | 4 (50.2) | 2 (41.3) | 4 (26.8) | 4 (21.1) | 5 (30.5) |
| nd | 2 (2.5) | 1 (0.6) | 1 (10.8) | 2 (14.1) | 2 (12.4) |

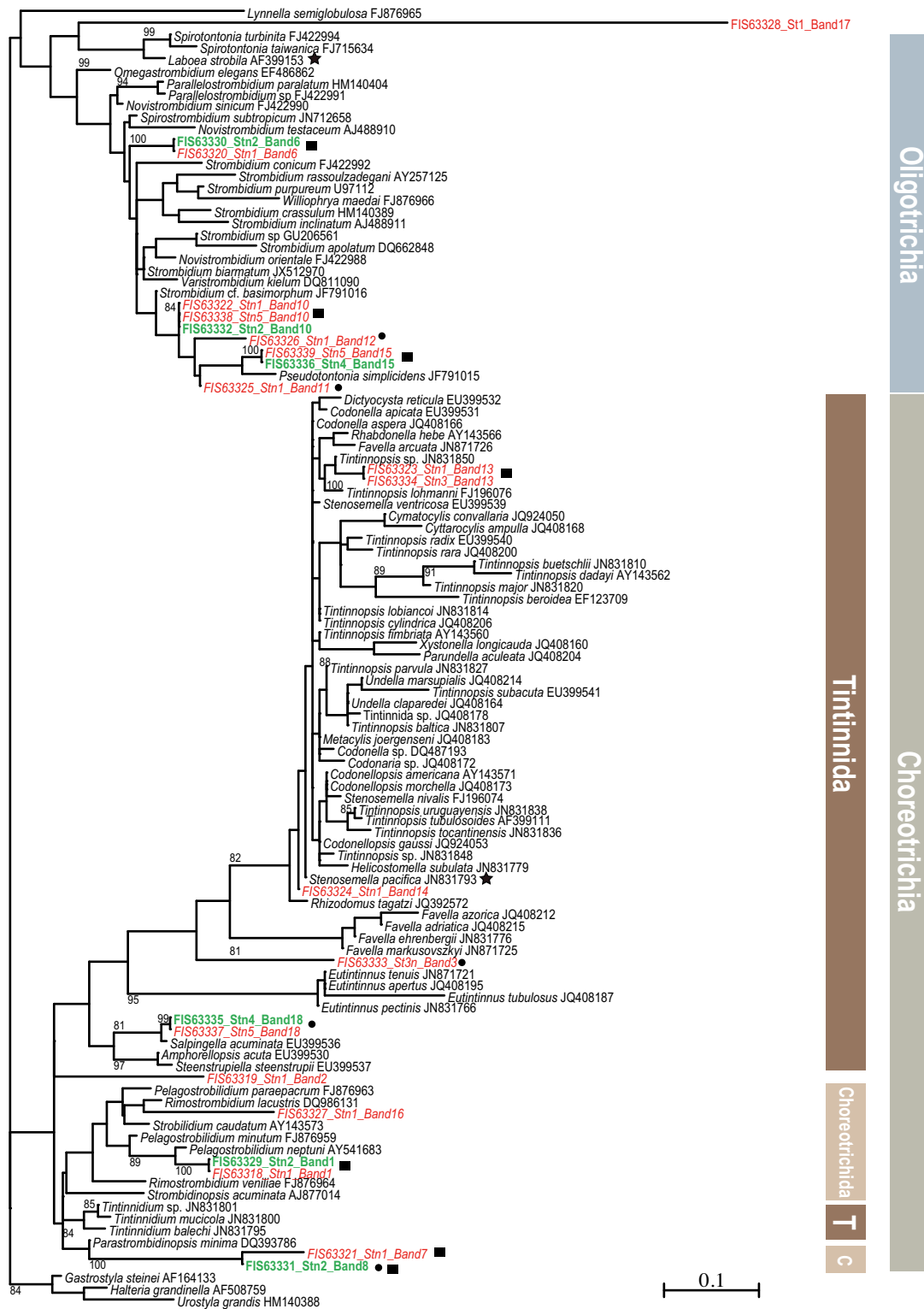


Fig. 4. Phylogenetic tree constructed using Randomized Axelerated Maximum Likelihood (RAXML) software from sequenced bands and morphospecies from the National Center for Biotechnology Information (NCBI) showing similarity of haplotypes across stations and some size-specific haplotypes. Scale bar represents the number of differences per base pair. Sequences from drifter Stns 1, 3 and 5 are shown in italic and red; and from 'away' Stns 2 and 4 in bold and green. (●) Microsize haplotype; (■) nanosize haplotype; (★) morphospecies found by microscopy. Sequences from NCBI (in black) are identified by accession number

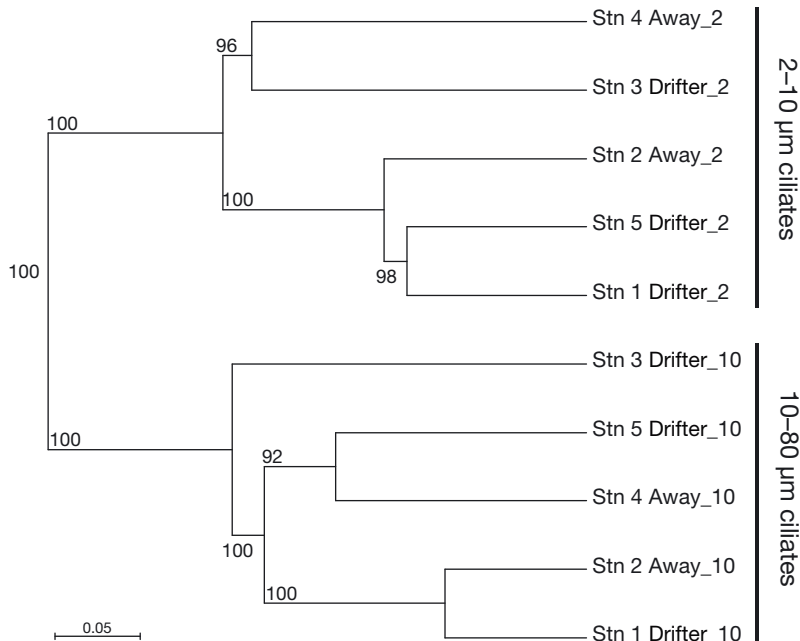


Fig. 5. Clustering of ciliate communities at sampling stations and by size based on the unweighted pair group method with arithmetic mean (UPGMA) and clustering stations software (Fast UniFrac) analysis of community structure obtained by DGGE. See Fig. 3 legend for explanation of codes indicating station number, location and ciliate size fractions. The scale bar shows the distance between clusters in UniFrac units (distance matrix where 0 = identical samples, 0.5 = samples composed by different ciliates)

ber varied between 6 and 9 within the microsize fraction and from 4 to 10 for the nanosize fraction (Fig. 3). The size fractionation did select for different community members as we observed that some bands were specific to one size fraction or the other. For example, Band 18 was only found in the microsize fraction and Band 10 was always brighter in the nanosize fraction (Fig. 3). The UPGMA tree indicating clustering of stations confirms visual observation of the gels as the nanosize and microsize samples cluster separately from one another (Fig. 5).

To assess further the variation in ciliate community structure, we used band intensity as a proxy for the relative abundance of haplotypes in a sample. Qualitatively, the nanosize lane had brighter bands compared to the microsize lane for Stn 1 while the brightness of the 2 lanes was equivalent for the other stations (Fig. 3). After sequence analysis, we compared band intensity within a lane to assess diversity patterns and found that the *Choreotrichia*-related sequences dominated the community among all stations ($60.6 \pm 3.6\%$) except for Stn 1 where band intensity related to choreotrich and oligotrich haplotypes was similar (Table 2).

Comparison of ciliate communities assessed by morphology and DGGE

A higher total number of morphospecies was observed under the microscope compared to the number of DGGE haplotypes, which is consistent with the fact that DGGE captures only the common haplotypes (Table 2). Thus, we used only the morphospecies representing more than 2% of the total abundance to compare the 2 approaches. Comparing just these common morphospecies with the DGGE haplotypes, the total number per station was similar. In addition, we observed the same pattern of variation between haplotypes and morphospecies for the loricate choreotrichs (i.e. the order Tintinnida: $23.3 \pm 16.4\%$ and $20.6 \pm 9.5\%$ of total abundance by microscopy and DGGE, respectively; Fig. S2B in the Supplement). However, the microscope observations found that the aloricate forms were dominated by oligotrich morphospecies ($68.3 \pm 12.8\%$ of total abundance) while the DGGE showed a more even

split between oligotrichs and non-tintinnid choreotrichs (34.0 ± 11.7 and $37.3 \pm 6.1\%$ of total relative abundance based on the sequenced bands, respectively; Table 2, Fig. S2A in the Supplement).

DISCUSSION

Our assessment of ciliate diversity in Fishers Island Sound yielded 3 main observations: (1) DGGE is a reliable and reproducible tool for assessing ciliate community structure; (2) despite some biases, DGGE is comparable to microscopy; (3) variation over time in ciliate assemblages within a water mass was as great as variation on a spatial scale of 1 km.

DGGE is a reproducible tool for assessing ciliate community structure

We found that DGGE is robust to numerous parameters including repeating PCRs and gel electrophoresis. We found the same pattern in up to 8 replicates of DGGE protocols. Replicates, both PCR and electrophoresis, yielded the same patterns in terms of

brightest bands as well as for some lesser bands, such as Bands 2, 16 and 17 (Figs. 3 & S1, the latter in the Supplement). Moreover, sequencing bands at identical positions both within and between gels generated identical sequences, providing further support for the replicability of DGGE. Haplotypes shared between the 2 size fractions (e.g. Bands 1, 2 and 10; Fig. 3) could result from variation within some ciliate morphospecies (e.g. size, flexibility, symmetry) and/or different life history stages (e.g. cyst vs. feeding stages). Thus, DGGE appears to be a good tool for assessing ciliate community structure, as reported in other recent studies (e.g. Sweet & Bythell 2012).

Despite some biases, DGGE is comparable to microscopy

To assess the reliability of DGGE, we compared microscopy and DGGE results. Working in the same system, Tamura et al. (2011) showed that this method is appropriate to study the ciliate community using a clone library for comparison. By comparing our DGGE results with microscopy data for the same samples, we observed a similar proportion of tintinnids and aloricate ciliates but differences within the aloricate forms in the relative abundances of oligotrichs and non-tintinnid choreotrichs (Table 2, Fig. S2 in the Supplement). This mismatch could be explained by several possibilities including (1) DGGE primer bias, (2) variation of gene copy number among the subclasses and/or (3) misidentification of the morphospecies (e.g. naked choreotrichs wrongly identified as oligotrichs; Fig. S2B). Using the same system, primer bias was implicated in explaining overabundance of oligotrichs compared to microscopy (Doherty

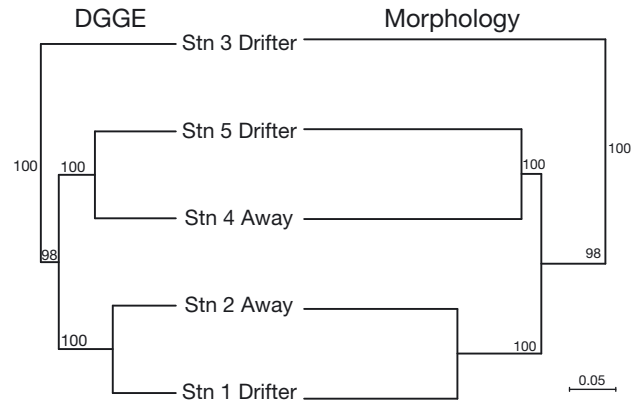


Fig. 6. Clustering of ciliate communities at sampling stations based on the unweighted pair group method with arithmetic mean (UPGMA) and Fast UniFrac analysis of community structure obtained by morphology and DGGE, showing the same pattern of clustering in both cases. For DGGE, the nanosize and the microsize fractions were grouped together. The scale bar shows the distance between clusters in UniFrac units

et al. 2007) and underestimation compared to clone libraries (Tamura et al. 2011). However, if the primers were biased towards choreotrichs, then presumably tintinnids would have been overrepresented in the DGGE results as well, but they were not. Alternatively, differences between diversity estimates by DGGE and microscopy could be due to variation in gene copy number as higher per cell SSU gene copy number has been reported for choreotrichs compared to oligotrichs (Gong et al. 2013). Again, this should have been reflected in the tintinnid results. Finally, some of the differences between morphology and DGGE may be attributed to misidentification as some small choreotrichs (e.g. the genera *Leegardiella* and *Lohmanniella*) can be confused with oligotrichs when

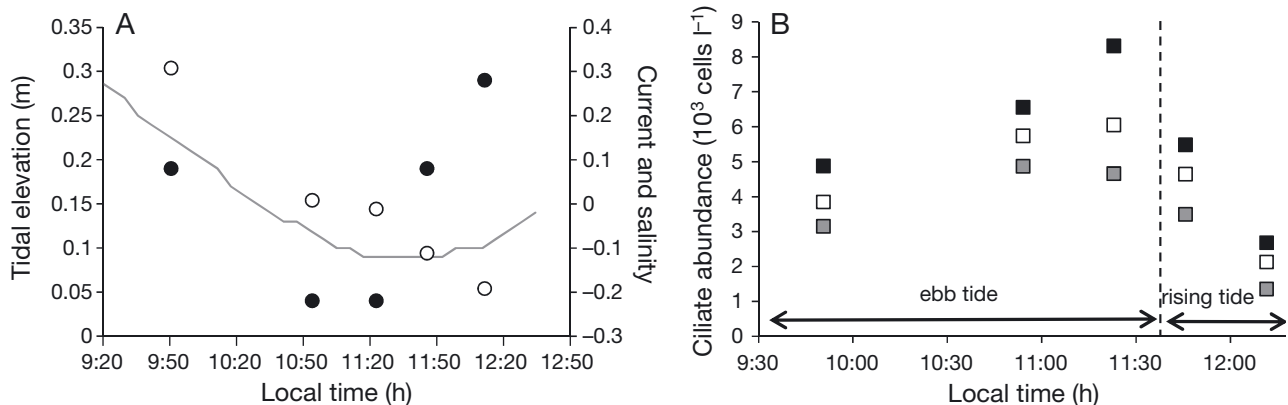


Fig. 7. (A) Tidal elevation (—), current speed (O) and surface salinity (●) over time during the study (tidal elevation from NOAA tide gauge station at New London, CT; speed and salinity expressed as difference from the mean). (B) Ciliate morphospecies abundance (10^3 cells l^{-1}) in relation to tidal stage and time. Total abundance of all morphospecies (■); abundance of species with >2% of total abundance (□); abundance of species with >5% of total abundance (▣)

silver staining methods are not used for identification (Lynn 2008). Despite the differences in diversity estimates, the relationships among the communities are the same when estimated from morphology and DGGE using Fast UniFrac (Fig. 6). This observation suggests that discordance between morphology and DGGE estimates of biodiversity does not affect inferences about biogeography.

**Variation over time in ciliate assemblages
within a water mass was as great as variation
on a spatial scale of 1 km**

In contrast to our expectations that the samples taken at the surface drifter (Stns 1, 3, and 5) would cluster together because they were sampled from the same water mass, we found a more complex biogeographic pattern. Analyses of both DGGE and morphological data reveal that samples taken at the surface drifter clustered more closely to samples taken 1 km away within 30 to 60 min: Stn 1 (surface drifter) clustered with Stn 2 (1 km away), while Stn 5 (surface drifter) clustered with Stn 4 (1 km away). One possible explanation is that the surface drifter did not stay with the water mass into which it was placed. However, the displacement of the surface drifter followed the speed and direction of the currents as measured by ADCP as the ebbing tide moved from west to east in the Sound.

An alternative explanation is that Stns 4 and 5 were more ocean-influenced, while Stns 1 and 2 were more representative of the upstream (Long Island Sound) waters and Stn 3 was a mixture of the 2 environments. The surface salinity declined slightly from surface drifter Stns 1 to 3 and then increased at Stn 5 (Fig. 7), suggesting some mixing with fresher waters from the Mystic or Thames rivers as the surface drifter moved from Stns 1 to 3 and subsequent mixing with higher salinity coastal water (at Stn 4) as the tide turned to flood just before Stn 5. Though salinity varied only slightly, we assume it is a good marker of water mixing and hypothesize that the ciliates we observed represent a combination of 2 ciliate communities (one oceanic and another more estuarine). The variation of haplotype number and common morphospecies supports this to some extent, as there is a decrease of diversity until the low tide and increase during the rising tide (Table 2). Ciliate grazing activity has been related to the salinity across a surface estuarine-coastal interface (Lehrter et al. 1999), so it is also possible that salinity causes changes in grazing behavior and thereby in the community structure, as observed in our study.

Besides physical processes such as horizontal and vertical mixing, which may alter ciliate communities on short time scales, such as tidal or weather cycles, biological processes may result in short-term and small spatial-scale variations. Some ciliates have been shown to perform vertical migrations (Jonsson 1989, Rossberg & Wickham 2008) and migration of even a few species by a few tens of centimeters during our surface sampling could have altered the community composition. Ciliates have been argued to contain their own ultradian rhythms of cell division or locomotion (i.e. repeated cycles throughout a day; for reviews see Wille & Ehret 1968, Lloyd & Kippert 1987, Lloyd 1998) that are independent of environmental variables (Kippert & Hunt 2000). Thus, it may be naïve to expect coherent communities within a given water mass, especially in energetic coastal environments, even over the time scale of a few hours that we examined.

The potential for ultradian behavior should be considered for future studies of community structure. This further emphasizes the usefulness of DGGE and other relatively rapid molecular techniques. These methods allow efficient estimation of common community members and high-resolution sampling of communities on both spatial and temporal scales. Combining these approaches with high throughput sequencing will enable determination of physical and biological processes that structure ciliate communities on time scales commensurate with their life cycles and behaviors.

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LITERATURE CITED

- Alder VA (1999). Tintinninoidea. In: Boltovskoy D (ed) South Atlantic Zooplankton. Backhuys, Leiden, p 321–384
- Ausubel FM, Brent R, Kingston RE, Moore DD and others (2002). Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology, Vol 2. Wiley, New York, NY
- Calbet A, Saiz E (2005) The ciliate-copepod link in marine ecosystems. *Aquat Microb Ecol* 38:157–167
- Calbet A, Trepát I, Almeda R, Saló V and others (2008) Impact of micro- and nanograzers on phytoplankton assessed by standard and size-fractionated dilution grazing experiments. *Aquat Microb Ecol* 50:145–156

- Caron DA, Countway PD, Jones AC, Kim DY, Schnetzer A (2012) Marine protistan diversity. *Annu Rev Mar Sci* 4: 467–493
- Costas BA, McManus G, Doherty M, Katz LA (2007) Use of species-specific primers and PCR to measure the distributions of planktonic ciliates in coastal waters. *Limnol Oceanogr Methods* 5:163–173
- Countway PD, Gast RJ, Dennett MR, Savai P, Rose JM, Caron DA (2007) Distinct protistan assemblages characterize the euphotic zone and deep sea (2500 m) of the western North Atlantic (Sargasso Sea and Gulf Stream). *Environ Microbiol* 9:1219–1232
- Countway PD, Vigil PD, Schnetzer A, Moorthi SD, Caron DA (2010) Seasonal analysis of protistan community structure and diversity at the USC Microbial Observatory (San Pedro Channel, North Pacific Ocean). *Limnol Oceanogr* 55:2381–2396
- Coyne KJ, Countway PD, Pilditch CA, Lee CK, Caron DA, Cary SC (2013) Diversity and distributional patterns of ciliates in guaymas basin hydrothermal vent sediments. *J Eukaryot Microbiol* 60:433–447
- Darriba D, Taboada GL, Doallo R, Posada D (2012). jModel-Test 2: more models, new heuristics and parallel computing. *Nat Methods* 9:772
- Doherty M, Costas BA, McManus GB, Katz LA (2007) Culture-independent assessment of planktonic ciliate diversity in coastal northwest Atlantic waters. *Aquat Microb Ecol* 48:141–154
- Doherty M, Tamura M, Costas BA, Ritchie ME, McManus GB, Katz LA (2010) Ciliate diversity and distribution across an environmental and depth gradient in Long Island Sound, USA. *Environ Microbiol* 12:886–898
- Ducklow HW (1993) Bacterioplankton distributions and production in the northwestern Indian Ocean and Gulf of Oman, September, 1986. *Deep-Sea Res II* 40:753–771
- Fenchel T, Esteban GF, Finlay BJ (1997) Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. *Oikos* 80:220–225
- Foissner W (2006) Biogeography and dispersal of microorganisms: a review emphasizing protists. *Acta Protozool* 45:111–136
- Foissner W (2008) Protist diversity and distribution: some basic considerations. *Biodivers Conserv* 17:235–242
- Foissner W, Chao A, Katz LA (2008) Diversity and geographic distribution of ciliates (Protista: Ciliophora). *Biodivers Conserv* 17:345–363
- Gong J, Dong J, Liu X, Massana R (2013) extremely high copy numbers and polymorphisms of the rDNA operon estimated from single cell analysis of oligotrich and peritrich ciliates. *Protist* 164:369–379
- Gratsepanche JD, Breton E, Brylinski JM, Lecuyer E, Christaki U (2011a) Succession of primary producers and micrograzers in a coastal ecosystem dominated by *Phaeocystis globosa* blooms. *J Plankton Res* 33:37–50
- Gratsepanche JD, Vincent D, Breton E, Christaki U (2011b) Microzooplankton herbivory during the diatom-*Phaeocystis* spring succession in the eastern English Channel. *J Exp Mar Biol Ecol* 404:87–97
- Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *ISME J* 4:17–27
- Jonsson PR (1989) Vertical distribution of planktonic ciliates – an experimental analysis of swimming behaviour. *Mar Ecol Prog Ser* 52:39–53
- Katz LA, McManus GB, Snoeyenbos-West OLO, Griffin A, Pirog K, Costas B, Foissner W (2005) Reframing the ‘Everything is everywhere’ debate: evidence for high gene flow and diversity in ciliate morphospecies. *Aquat Microb Ecol* 41:55–65
- Kippert F, Hunt P (2000) Ultradian clocks in eukaryotic microbes: from behavioural observation to functional genomics. *Bioessays* 22:16–22
- Lahr DJG, Katz LA (2009) Reducing the impact of PCR-mediated recombination in molecular evolution and environmental studies using a new-generation high-fidelity DNA polymerase. *Biotechniques* 47:857–866
- Landry MR, Calbet A (2005) Reality checks on microbial food web interactions in dilution experiments: responses to the comments of Dolan and McKeon. *Ocean Sci* 1: 39–44
- Lehrter JC, Pennock JR, McManus GB (1999) Microzooplankton grazing and nitrogen excretion across a surface estuarine-coastal interface. *Estuaries* 22:113–125
- Lloyd D (1998) Circadian and ultradian clock-controlled rhythms in unicellular microorganisms. *Adv Microb Physiol* 39:291–338
- Lloyd D, Kippert F (1987) A temperature-compensated ultradian clock explains temperature-dependent quantal cell cycle times. *Symp Soc Exp Biol* 41:135–155
- López-García P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. *Nature* 409:603–607
- Lynn DH (2008). The ciliated protozoa: characterization, classification, and guide to the literature. Springer, New York, NY
- Maeda M, Carey PG (1985). An illustrated guide to the species of the family Strombidiidae (Oligotrichida, Ciliophora), free swimming protozoa common in the aquatic environment. *Bull Ocean Res Inst, Univ Tokyo* 19:1–68
- McManus GB, Fuhrman JA (1988) Control of marine bacterioplankton populations: measurement and significance of grazing. *Hydrobiologia* 159:51–62
- McManus GB, Xu DP, Costas BA, Katz LA (2010) Genetic identities of cryptic species in the *Strombidium stylifer/apolatum/oculatum* cluster, including a description of *Strombidium rassoulzadegani* n. sp. *J Eukaryot Microbiol* 57:369–378
- Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proc Gateway Computing Environments Workshop (GCE), New Orleans, 14 Nov 2010. Institute of Electrical and Electronics Engineers, New Orleans, LA, p 1–8
- Rosser M, Wickham SA (2008) Ciliate vertical distribution and diel vertical migration in a eutrophic lake. *Fund Appl Limnol* 171:1–14
- Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning, Vol 2. Cold Spring Harbor Laboratory Press, New York, NY
- Santoferrara LF, McManus GB, Alder VA (2013) Utility of genetic markers and morphology for species discrimination within the order Tintinnida (Ciliophora, Spirotrichea). *Protist* 164:24–36
- Sherr EB, Sherr BF (1994) Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. *Microb Ecol* 28:223–235
- Simon EM, Nanney DL, Doerder FP (2008) The ‘*Tetrahymena pyriformis*’ complex of cryptic species. *Biodivers Conserv* 17:365–380

- Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690
- Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol* 57: 758–771
- Steele JA, Countway PD, Xia L, Vigil PD and others (2011) Marine bacterial, archaeal and protistan association networks reveal ecological linkages. *ISME J* 5: 1414–1425
- Sweet M, Bythell J (2012) Ciliate and bacterial communities associated with white syndrome and brown band disease in reef-building corals. *Environ Microbiol* 14:2184–2199
- Tamura M, Katz LA, McManus GB (2011) Distribution and diversity of oligotrich and choreotrich ciliates across an environmental gradient in a large temperate estuary. *Aquat Microb Ecol* 64:51–67
- Wille JJ, Ehret CF (1968) Light synchronization of an endogenous circadian rhythm of cell division in *Tetrahymena*. *J Protozool* 15:785–789

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