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Molecular diversity and biogeography of benthic ciliates in the Bohai Sea and Yellow Sea

Guihao Li[1, 2], Lei Su[1, 2], Qianqian Zhang[1], Xiaoli Zhang[1], Jun Gong[3]*

[1] Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] School of Marine Sciences, Sun Yat-Sen University, Zhuhai 519082, China

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Abstract

This study explored the molecular diversity and biogeography of benthic ciliates in Chinese marginal seas, the Bohai Sea (BHS), North Yellow Sea (NYS) and South Yellow Sea (SYS). From a previous 18S rRNA gene pyrosequencing dataset of the benthic microeukaryotes, we retrieved the sequences affiliated with phylum Ciliophora and analyzed alpha and beta diversities of ciliate communities. We found that BHS had the highest ciliate operational taxonomic unit (OTU) richness than NYS and SYS, whereas the richness was not significantly different between summer and winter. Among all the measured environmental variables, water depth showed consistently the strongest correlations with alpha diversities. Overall, the class Spirotrichea (mostly Choreotrichia and unassigned lineages within the class) dominated the communities in terms of both relative proportion of sequences (77.0%) and OTU richness (66.5%). OTU-level ciliate community structure was significant different among the three basins, but not between the seasons. Structurally, significant differences in relative proportion among the basins were detected for the class Litostomatea, but not for other classes. Partial Mantel tests demonstrated that water depth difference was more important than geographic and environment distances in shaping the community structure of benthic ciliates in the studied area. About 60% OTUs were not assigned at a class or order level and at least 45% OTUs shared a sequence similarity no more than 97% with the described species, indicating a great potential for ciliate species discovery in the offshore sediments. Compared with previous morphology-based surveys, the spatial pattern of ciliate diversity (decreasing from NYS to SYS) is also identified in the present study. Nevertheless, structurally, the dominant class appeared to be Spirotrichea in the sequencing dataset, which differs from previous morphology-based results (dominance of classes Prostomatea and Karyorelictea in biomass). The potential causes for the discrepancies between molecular and morphological findings are also discussed.

Key words: 18S rDNA, benthic ciliates, diversity, biogeography, depth decay relationship

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1 Introduction

Marine ciliates (phylum Ciliophora) are heterotrophic and mixotrophic protists that are of high biodiversity and playing important ecological roles in coastal and marine ecosystems (Song et al., 2003, 2009; Lynn, 2008). In pelagic food webs, marine ciliates usually act as microzooplankton that transfers carbon and energy to higher trophic levels (e.g., metazoans) by predating on bacteria, pico- and nano-sized protists (Stoecker and Capuzzo, 1990). Many ciliate species are also abundant in marine benthos, especially the water-sediment interface layer (Fenchel, 1969; Gao et al., 2017; Su et al., 2018), due to high adaptation to low oxygen contents, or even hypoxic environments (Park et al., 2008).

Studies of diversity and distribution of marine benthic ciliates were mainly based on microscopical identification and enumeration. The species richness and abundance of benthic ciliates were investigated using cell staining techniques for different sites along coastal line of the Black Sea (Azovsky and Mazei, 2005) and Yellow Sea (Meng et al., 2012; Zhou and Xu, 2016). While the application of these traditional methodologies has contributed greatly in ciliate diversity and ecology, a large part of ciliate diversity might have been missing. For example, fragile and nano-sized ciliates sometimes are abundant in marine habitats (Dong et al., 2014), but could be easily ignored due to loss of morphological integrity, cell disruption, and difficulty in telling from sediment particles (Caron and Countway, 2009). In the past decade, molecular approaches, especially high-throughput sequencing, have circumscribed these methodological difficulties and been widely applied in studying benthic protist diversity and biogeography (Doherty et al., 2010; Massana et al., 2015; Zhao and Xu, 2016). Recently, using molecular method based on high-throughput sequencing, Gong et al. (2015) have revealed that depth shapes the diversity and community composition and

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*Corresponding author, E-mail: gongj27@mail.sysu.edu.cn structure of microbial eukaryotes in the surficial sediments of the Bohai Sea and Yellow Sea of China, and ciliates appear to be important members in the benthic microeukaryotic communities. However, the variations in ciliate genetic diversity and community in these regions have not been analyzed, and it remains to be explored what factors determined the community assembly of coastal benthic ciliates across the Bohai Sea and Yellow Sea.

In our study, we focus on ciliates by extracting their sequences from the existing 454 sequencing dataset for benthic microeukaryotes in the Bohai Sea and Yellow Sea (Gong et al., 2015). We are particularly interested in the following questions: (1) What ciliate phyla or groups exist in these basins, and how the ciliate community was assembled and varied across space and time? (2) Is the biogeographic pattern of benthic ciliates different from the protists or microeukaryotes?

2 Materials and methods

2.1 *Sampling information, environmental variables and sequence analyses*

The sequences and environmental data used in this study were derived from Gong et al. (2015). Briefly, forty sediment samples were collected from the surface layer (top 0–5 cm) at 20 stations in the Bohai Sea (BHS), North Yellow Sea (NYS), South Yellow Sea (SYS, Fig. 1), during the summer and winter cruises of 2011. Sediment cores were put into cryovials and transferred immediately to liquid nitrogen for further DNA extraction, the rest of sediments was preserved at 4°C for physicochemical analyses. A total of 25 variables were recorded. These included: temperature, salinity, chlorophyll *a* (Chl *a*), pH and dissolved oxygen (DO) of bottom waters, and nitrate (NO$_3^-$), nitrite (NO$_2^-$), ammonium (NH$_4^+$), total organic carbon (TOC) and nitrogen (TON), and grain size of sediments. Moreover, metal concentrations in sediment and geographic distances were also measured or calculated. Total environmental DNA was extracted and purified using the FastDNA SPIN Kit (Q-BIOgene) for Soil. The V2 and V3 regions of 18S rDNA was then amplified using the universal eukaryotic primers 82F (5′-GAAACTGCGAATGGCTC-3′) and 516R (5′-ACCAGACTTGCCCTCC-3′). Pyrosequencing was performed on a Roche 454 GS FLX Titanium sequencer by the BGI company (Shenzhen, China). The raw sequence data have been deposited in the GenBank under the accession number SRA157723. Sequence data were processed by quality controlling, denoising, and discarding chimera and singletons. The clean sequences were clustered and aligned, and classified against the Protist Ribosomal Reference Database (PR2 database). Operational taxonomic units (OTU) were clustered at a sequence similarity of 97%. One winter sample from NYS (NY01W) was failed to amplify and was not included in the subsequent analysis. Rarefaction curves were depicted and inspected to confirm that a majority of ciliate diversity had been recovered by the retrieved ciliate sequences (Fig. S1). To minimize the bias associated with sequencing coverage and allow comparing alpha-diversity estimators, we rarefied the datasets to 100 sequences per sample (the lowest number of sequences recovered for the 36 samples) using *multiple_rarefactions_even_depth.py* command. Three samples (SH08W, NH10W and NH24W) were excluded due to low sequence numbers (<100). The alpha-diversity estimators (OTU richness, Simpson, Shannon, and Chao1 indices) were calculated. To explore the distribution pattern of the communities of benthic ciliates, nonmetric multidimensional scaling (NMDS) was conducted based on a Bray-Curtis matrix using the package PRIMER v.6.0 (Plymouth Marine Laboratory, UK).

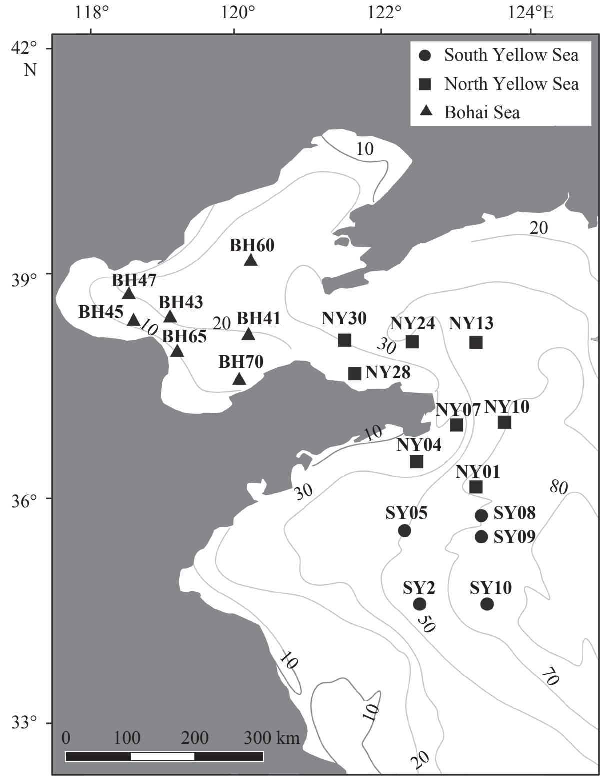

Fig. 1. Sampling sites in the Bohai Sea, North Yellow Sea and South Yellow Sea. Lines indicate isobaths of given depths (m).

2.2 *Statistical analyses*

One-way ANOVA and student's *t*-tests (two-tailed) were performed to examine the differences in alpha-diversity estimators and taxon-specific read proportions of benthic ciliates among BHS, NYS and SYS basins and between two seasons. Spearman's correlation analysis was conducted to examine the associations between alpha-diversity estimators and environmental factors, and betweem relative proportions of ciliate taxa and environmental factors. All these analyses above were executed using SPSS v.13.0 (SPSS, Chicago, IL, USA).

To test the effects of season and basin on the variations in community structure of benthic ciliates, ANOSIM (analysis of similarity) was performed with the Bray-Curtis distance-based matrix using PRIMER. Because the spatial scale of this study was relative large, the horizontal (geographic) distance could limit the dispersal of benthic ciliates thus affect the geographic distribution pattern of these microorganisms. To test this hypothesis, simple and partial Mantel tests were conducted to partitionate the effects of geographic distance, depth, and environmental factors on the community structure of benthic ciliates using the *vegan* package in R statistical software (R 3.1.2, R Development Core Team, www.r-project.org).

3 Results

3.1 *Alpha-diversity of benthic ciliates*

A total of 25 608 reads affiliated with phylum Ciliophora were obtained for the 39 sediment samples, with 12 to 2 343 (median 500) reads per sample (Table S1). Classification of the pyroseqencing reads indicated that a total of six ciliate classes were recovered in this study. These included Spirotrichea, Oligohymenophorea, Phyllopharyngea, Litosomatea, Colpodea and Karyorelictea (Fig. 2a). Overall, a total of 481 OTUs were detected in our non-rarefied dataset. The number of OTUs obtained from each site varied from 4 to 156, with an average of 69 (Table S1). Among the 481 OTUs identified in our dataset, more than 1/3 OTUs were unique to one or two sites. Only four OTUs (about 0.8%) were nearly common to all the sites. Most OTUs detected in all the 39 samples were affiliated with classes Spirotrichea (66.5%), Litostomatea (11.2%) and Oligohymenophorea (3.7%). Within Spirotrichea, the subclass Choreotrichia (17.5%) dominated in terms of OTU richness, followed by Stichortichia (2.3%), Oligotrichia (1.7%) and Hypotrichia (0.8%, Fig. 2a). Of the rarefied dataset (normalized to 100 reads per sample), alpha-diversity estimators of benthic ciliates were high and varied greatly among samples (Table S1, Fig. 3), with OTU richness ranging from 8 to 42, Simpson indices of 0.347–0.954, Shannon indices 1.21–4.90 and Chao1 indices 11–90 (Table S1). Between two seasons, alpha-diversity estimators appeared to be higher in the winter than in the summer, which were, however, not statistically significant (*t*-tests, $P>0.07$, Fig. 3). Among these three basins, the alpha-diversity estimators in the BHS were usually the highest ($P<0.05$), whereas there were no significant differences between NYS and SYS ($P>0.05$).

3.2 *Community composition and structure of benthic ciliates*

In terms of sequence proportions, the class Spirotrichea dominated (77.7%), followed by Litostomatea (4.6%) and Oligohymenophorea (1.5%), whereas the relative proportions of other classes (i.e., Phyllopharyngea, Colpodea, Karyorelictea) were each on average less than 1%. About 15.8% sequences remained unclassified at the class level (Fig. 2a). The proportion of Spirotrichea sequences ranged from 9.8% to 98.4% among individual samples, with many sequences assigned to the subclasses Choreotrichia (0.0% to 46.6%), Stichotrichia (0.0% to 10.0%), Oligotrichia (0.0% to 6.0%) and Hypotrichia (0.0% to 3.4%%), and the unclassified (0.8% to 95.5%) (Fig. 2b).

The NMDS plot showed the OTU-level community distance among the 39 samples (Fig. 4). In the plot, the samples were largely clustered by basin, with NYS and SYS samples clearly separating from each other and the BHS samples nested within a space mainly occupied by SYS samples. Seasonal separation was not distinct. These results were well supported by the ANOSIM, which indicated that the basin-wise differences were significant ($R=0.31$, $P=0.001$, Table 1), but the seasonality was indistinct ($R<0.01$, $P=0.396$, Table 1).

Comparison of relative proportions of rDNA sequences could reveal the source taxa whose proportion changes contributed

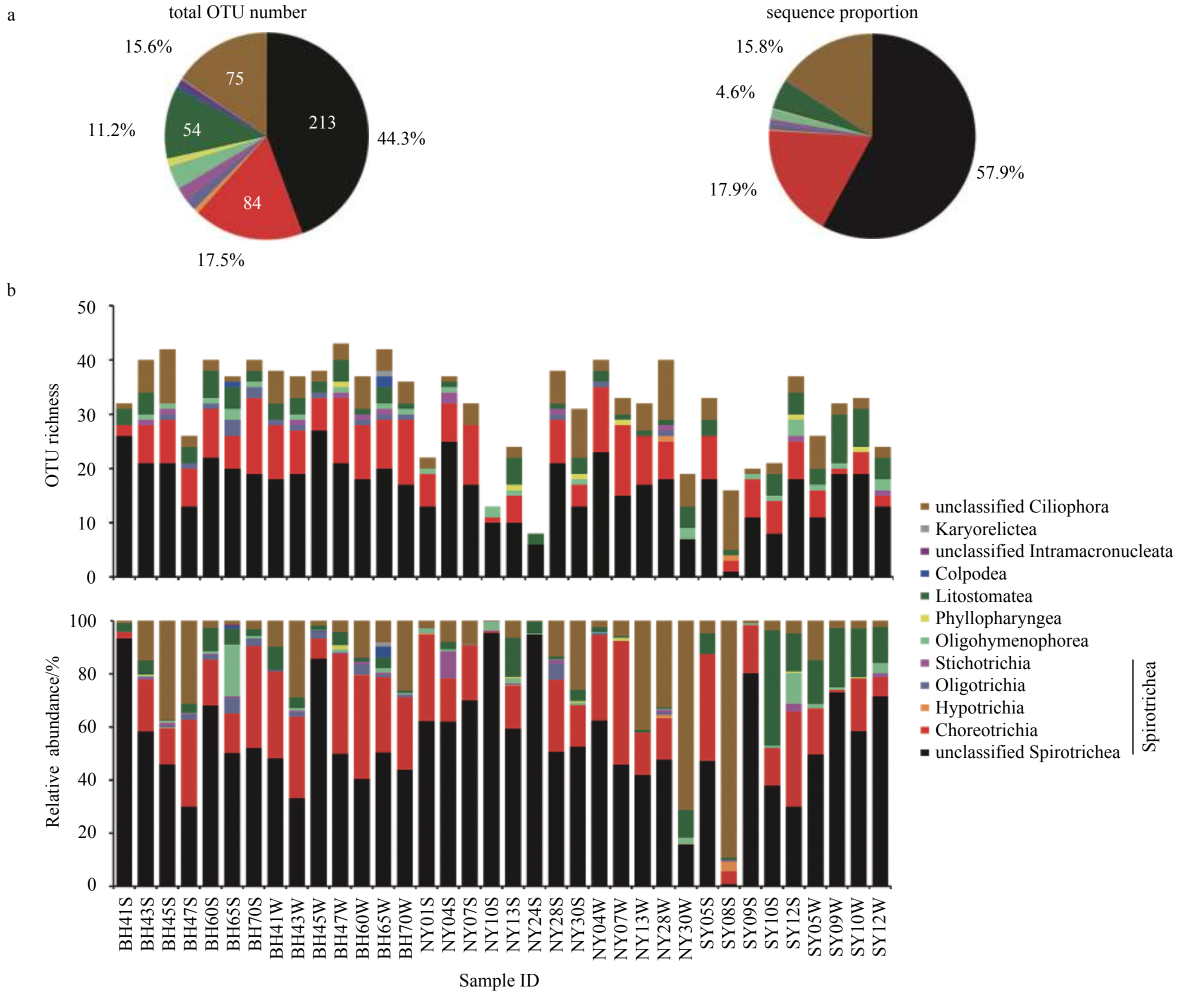

Fig. 2. The proportions of major taxonomic groups (classes/subclasses) in benthic ciliates communities. a. Overall OTU and sequence proportions and b. OTU and sequence proportions in individual samples. Abbreviations in sample IDs indicate the sampling locations and seasons: BH for Bohai Sea, NY North Yellow Sea, SY South Yellow Sea, S summer, and W winter.

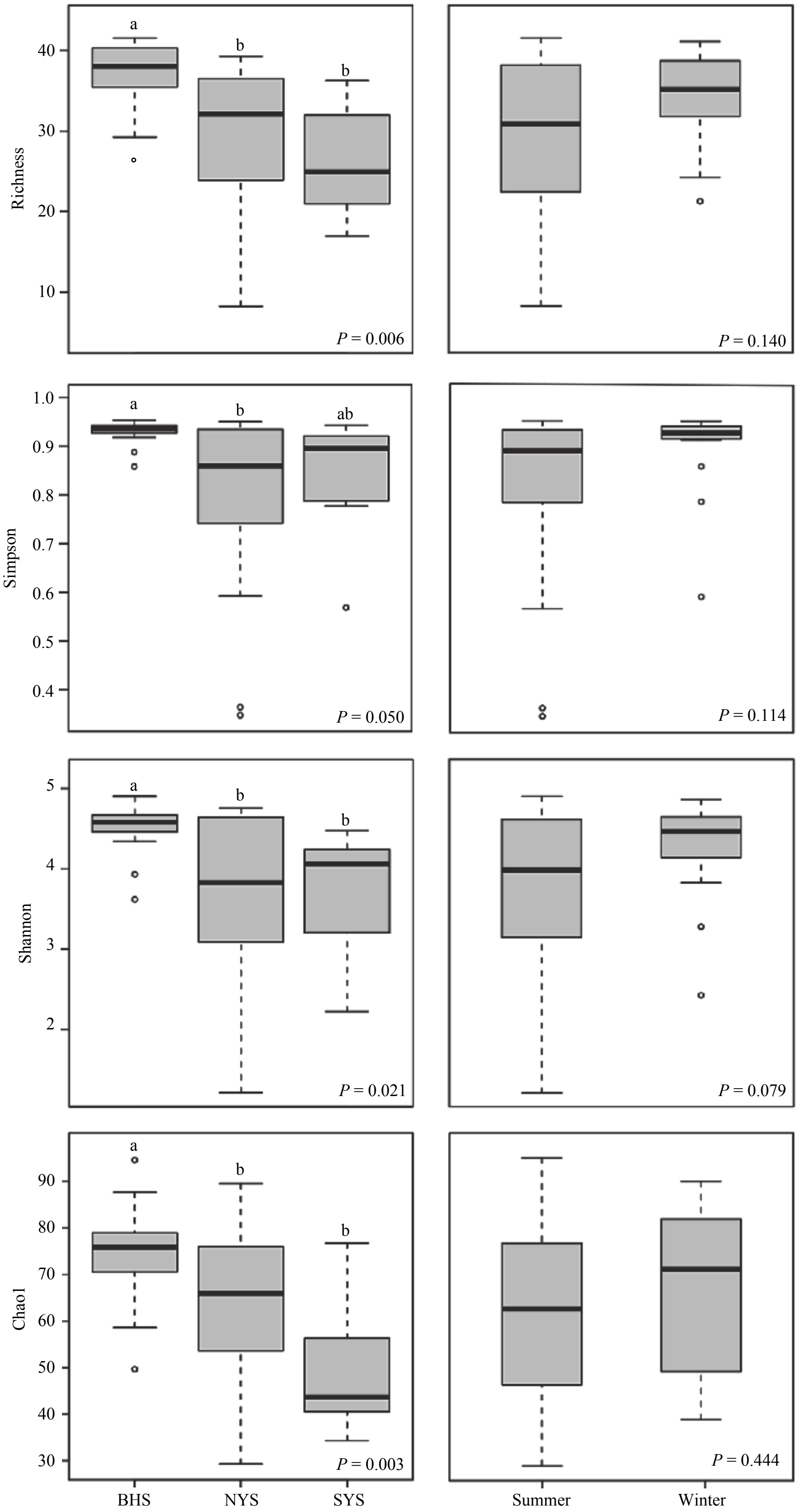

Fig. 3. Comparisons of alpha diversity estimators of benthic ciliates. *P* values are given for the comparisons among basins (BHS, NYS and SYS) using one-way ANOVA and between two seasons (summer and winter) using *t*-tests. Basins sharing no lowercase letters above their boxes indicate significant differences in pairwise comparisons (*t*-tests, $P<0.05$).

mostly to the overall community structure variations (Table 2). For most classes and orders examined, their relative sequence proportions were not significantly different among basins ($P>0.05$), nor between two seasons ($P>0.05$), exhibiting a relative

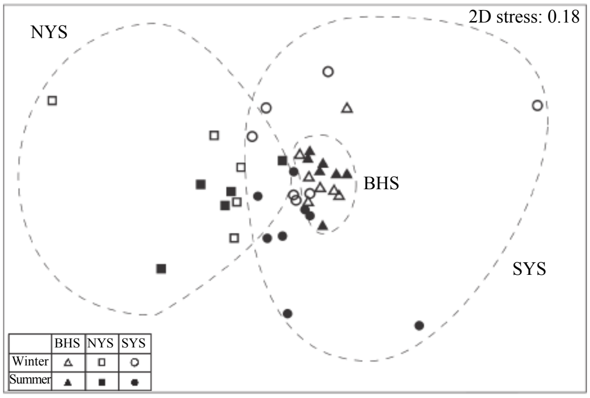

Fig. 4. Nonmetric multidimensional scaling plot showing the differences in the community structure of benthic ciliates collected from different basins and seasons. The stress value of 0.18 indicates a good ordination.

stable community organization at higher taxonomic levels. The only exception was the class Litostomatea, which had substantially higher proportions (mean 16%) in the ciliate communities in SYS than these in the BHS (mean 4.0%) and NYS (mean 3.0%) ($P<0.05$). Apparently, the order Haptoria was one of the major contributors to these differences (Table 2). The relative proportion of Spirotrichea seqeunces progressively decreased from BHS (80%±3.08%) to NYS (81%±5.66%) and SYS (67%±7.84 %), but this pattern was not statistically significant (ANOVA, P=0.64, Table 2).

Table 1. ANOSIM statistics testing the difference of ciliate communities among basins and between seasons

Grouping	R	P
Basin (global test)	0.31	**0.001**
BHS vs. NYS	0.18	**0.001**
NYS vs. SYS	0.22	**0.009**
BHS vs. SYS	0.66	**0.001**
Season (global test)	<0.01	0.396

Note: Significant differences ($P<0.05$) are highlighted in bold.

3.3 *Correlations of alpha-diversity estimators and relative proportions with environmental factors*

Ciliate OTU richness had the strongest correlation with water depth ($\rho=-0.71$, $P<0.001$), followed by temperature ($\rho=0.64$, $P<0.001$), salinity ($\rho=-0.63$, $P<0.001$), longitude ($\rho=-0.57$, $P<0.001$), pH ($\rho=0.55$, $P<0.001$) and Chl *a* ($\rho=0.49$, $P=0.002$, Table 3). Simpson, Shannon and Chao1 exhibited similar patterns. Among the sedimentary properties measured in our study, several trace metals showed significant correlations with richness, including vanadium (V, $\rho=-0.47$, $P=0.003$), zinc (Zn, $\rho=-0.44$, $P=0.008$), arsenic (As, $\rho=0.44$, $P=0.008$) and chromium (Cr, $\rho=-0.43$, $P=0.010$).

The associations between relative proportion of major taxa and environmental factors were also explored (Table 4). The proportion of Litostomatea exhibited a positive relationship with C:N ($\rho=-0.34$, $P=0.032$) and Pb ($\rho=-0.38$, $P=0.021$), whereas the relative abundance of Oligohymenophorea was negatively and significantly correlated with grain size ($\rho=-0.32$, $P=0.042$). No strong correlations were observed for the class Spirotrichea with all environmental factors measured in this study. At the subclass level, Oligotrichia and Haptoria were negatively correlated with water depth and positively with temperature. Furthermore, there were strong associations between Oligotrichia and several other variables, i.e., salinity ($\rho=-0.56$, $P<0.001$), pH ($\rho=0.50$, $P=0.001$), Chl *a* ($\rho=0.49$, $P=0.002$) and concentrations of some metals (V, Cr, Fe,

Table 2. Pairwise comparisons of relative read proportions (mean±SE, %) of ciliate classes and orders in the benthic communities among the Bohai Sea (BHS), North Yellow Sea (NYS) and South Yellow Sea (SYS) or between two seasons, using t-test

Taxonomy	Basin			Season	
	BHS	NYS	SYS	Summer	Winter
Spirotrichea	80.0±3.08	81.0±5.66	67.0±7.84	77.4±4.69	75.6±4.52
Choreotrichia	24.4±3.18	19.8±4.00	15.9±4.33	19.1±2.73	22.0±3.59
Tintinnida	5.2±1.19	6.2±2.93	0.8±0.61	2.4±0.76	6.6±2.34
Choreotrichida	2.8±0.96	1.0±0.41	1.5±1.27	1.0±0.46	2.6±0.87
Unclassified Choreotrichia	16.4±2.83	12.6±2.38	13.5±4.04	15.6±2.47	12.7±2.31
Oligotrichia	1.7±0.44	4.5±3.86	0±0.00	1.0±0.41	3.8±3.04
Stichotrichia	0.5±0.11	0.9±0.66	0.5±0.30	1.0±0.50	0.3±0.10
Hypotrichia	0.1±0.02	0.1±0.09	0.3±0.34	0.2±0.17	0.1±0.06
Unclassified Spirotrichea	53.6±4.81	55.5±4.80	49.9±7.03	57.1±5.24	49.5±3.70
Litostomatea	**4.0±0.71^{b}**	**3.0±1.12^{b}**	**16.0±3.92^{a}**	6.4±2.19	7.0±1.77
Haptoria	**1.0±0.25^{ab}**	**0.9±0.55^{b}**	**3.5±1.82^{a}**	0.9±0.29	2.4±1.04
Unclassified Litostomatea	**2.9±0.68^{b}**	**1.9±1.00^{b}**	**12.4±3.79^{a}**	5.3±2.20	4.6±1.34
Oligohymenophorea	2.1±1.41	1.3±0.49	4.8±2.86	2.4±1.09	2.6±1.49
Peritrichia	1.4±1.37	0±0.00	0±0.00	1.0±0.96	0.0±0.00
Scuticociliatia	0.1±0.11	0.5±0.46	0±0.00	0.0±0.00	0.5±0.37
Apostomatida	0.0±0.00	0.1±0.05	0.0±0.02	0.0±0.03	0.0±0.01
Unclassified Oligohymenophorea	**0.4±0.10^{b}**	**0.7±0.24^{b}**	**4.8±2.71^{a}**	1.2±0.57	2.1±1.48
Colpodea	0.4±0.29	0	0	0.1±0.05	0.2±0.21
Phyllopharyngea	0.1±0.12	0.2±0.09	0.1±0.07	0.1±0.04	0.2±0.10
Cyrtophoria	0.1±0.11	0±0.00	0±0.00	0.0±0.01	0.1±0.08
Suctoria	0.0±0.00	0.1±0.04	0.1±0.04	0.1±0.03	0.0±0.02
Karyorelictea	0.1±0.12	0	0	0.0±0.00	0.1±0.08
Unclassified Ciliophora	13.2±3.38	14.8±5.18	12.6±8.61	13.6±4.66	14.3±4.22

Note: Superscripts sharing no letters indicate significant differences ($P<0.05$).

Table 3. Spearman's correlation coefficients between alpha-diversity estimators and environmental factors

	Richness	Simpson	Shannon	Chao1
Longitude	**–0.57****	**–0.49****	**–0.55****	**–0.49****
Latitude	**0.39***	0.22	0.31	**0.42***
Depth	**–0.71****	**–0.65****	**–0.70****	**–0.59****
Temperature	**0.64****	**0.59****	**0.65****	**0.45****
Salinity	**–0.63****	**–0.56****	**–0.61****	**–0.57****
DO	–0.20	–0.25	–0.23	–0.07
pH	**0.55****	**0.50****	**0.55****	**0.46****
Chl *a*	**0.49****	**0.38***	**0.45****	**0.40***
Grain size	0.29	0.16	0.21	**0.35***
NO_3^-	–0.27	–0.26	–0.25	–0.26
NO_2^-	0.27	0.21	0.26	0.06
NH_4^+	0.21	0.32	0.28	0.12
DIN	0.12	0.22	0.19	0.04
TOC:TON	–0.22	–0.12	–0.18	–0.32
Pb	–0.24	–0.07	–0.13	**–0.34***
V	**–0.47****	**–0.33***	**–0.39***	**–0.47****
Cr	**–0.43****	–0.26	–0.32	**–0.41***
Mn	0.29	0.29	0.31	0.20
Fe	**–0.42***	–0.29	**–0.34***	**–0.37***
Co	–0.21	–0.03	–0.09	–0.31
Ni	–0.20	–0.04	–0.10	–0.30
Cu	–0.26	–0.12	–0.17	–0.36*
Zn	**–0.44****	**–0.34***	**–0.37***	**–0.40***
As	**0.44****	**0.42***	**0.44****	**0.33***
Cd	–0.03	0.09	0.05	–0.11

Note: ** $P < 0.01$; * $P < 0.05$.

Zn and As, $\rho>0.34$, $P<0.05$). The correlations of orders Choreotrichida and Tintinnida generally followed the subclass Oligotrichia (Table 4).

3.4 *Water depth, geographic distance and environmental controls on beta-diversity of benthic ciliates*

We also distinguished the effects of horizontal (geographic distance) and vertical (depth) spatial factors and environmental variables on community composition using Mantel tests (Table 5). The simple Mantel tests showed that the changes in community structure of benthic ciliates were significantly correlated with both geographic distance (r=0.21, P<0.001) and depth (r=0.29, P<0.001), but not with environment distance (r=0.10, P=0.251, Table 5). In the partial Mantel tests, however, when water depth was controlled, the effect of geographic distance became insignificant (r=0.02, P=0.350), whereas the effect of depth remained when geographic distance was controlled (r=0.20, P=0.001), indicating that water depth was the most important factor shaping the ciliate community in the studied areas.

4 Discussion

Previously, the benthic ciliate diversity and community composition and structure in the Yellow Sea (including NYS and SYS) were studied using morphology-based methods (MBM), e.g., Ludox density centrifugation and quantitative protargol stain (Meng et al., 2012; Zhou and Xu, 2016). The present study expands the sampling regions (including the Bohai Sea) and revisits the topic from a molecular perspective, revealing several consistent characteristics in comparison with the previous work. For example, the regional pattern of OTU-based ciliate species richness, much like these described in the MBM studies, exhibits a decreasing tendency from NYS to SYS (Fig. 3); the benthic ciliate richness was positively correlated with bottom water temperature, but negatively with bottom water salinity and water depth (Table 3). The depth-dependent pattern of alpha diversity of ciliates is much like that previously observed for microeukaryotes (Gong et al., 2015), in which limitation of sunlight and primary productivity may be also responsible for the reduction of ciliate species number in the benthos.

Table 4. Coefficients (ρ) of Spearman's correlations between the relative abundance of major groups of benthic ciliates and environmental variables

	Depth	Temp	Sal	DO	pH	Chl *a*	Grain size	NO_2^-	C:N	Pb	V	Cr	Fe	Co	Zn	As
Oligohymenophorea							–0.32*									
Peritrichia		0.35*														
Scuticociliatia																
Pleurostomatida																
Spirotrichea																
Choreotrichia																
Choreotrichida	–0.39*	0.45**	–0.50**		0.49**						–0.43**	–0.32*	–0.40*		–0.32*	
Tintinnida	–0.68**	0.48**	–0.60**		0.49**	0.52**				–0.32*	–0.41*	–0.45**	–0.44**		–0.43**	0.40*
Oligotrichia	–0.62**	0.44**	–0.56**		0.50**	0.49**			–0.37*		–0.49**	–0.45**	–0.45**		–0.58**	0.34*
Stichotrichia	–0.37*			0.33*		0.41*			–0.40*							
Urostylida		0.39*				0.37*		0.33*								
Sporadotrichida																
Hypotrichia																–0.34*
Euplotida																
Phyllopharyngea																
Cyrtophoria																
Dysteriida																
Litostomatea									0.34*	0.38*				0.32*		
Haptoria	–0.37*	0.33*														
Haptorida																
Apostomatida																

Note: * P<0.05; ** P<0.01; *** P<0.001; and insignificant correlations were left blank. Temp represents temperature and Sal salinity.

Table 5. Simple and partial Mantel tests for correlations between geographic distance, depth, environmental factors, and changes in community structure based on Bray-Curtis distance

	Simple Mantel test		Control for	Partial Mantel test	
	r	*P*		*r*	*P*
Geo_distance	0.21	**0.001**	Depth	0.02	0.350
Geo_distance	–	–	Environment	0.19	**0.004**
Depth	0.29	**0.001**	Geo_distance	0.20	**0.001**
Depth	–	–	Environment	0.27	**0.001**
Environment	0.10	0.251	Depth	–0.04	0.682
Environment	–	–	Geo_distance	0.01	0.446

Note: Significant *P*-values (<0.01) are highlighted in bold. Geo_distance represents pairwise geographic distances between samples.

There are also notable discrepant results between these two types of investigations. Our study using MiSeq approach obtained a higher species-level richness (~480 OTUs) than the MBM studies (200–250 morphospecies), indicating the possibility that some benthic ciliate species may escape from the fixation and microscopical observations. Furthermore, Spirotrichean ciliates are known to have highly diverse morphological characters and ecological adaptations, and many new marine ciliate species estimated in recent years come from this class (Gao et al., 2016, 2017). Our molecular survey of the Bohai Sea and Yellow Sea showed that over 40% OTUs were unclassified within the class Spirotrichea (Fig. 2), indicating that the species discovery of spirotricheans (including the highly diverse choreotrichians) in marine habitats, especially from benthos, is far from being ended, and that potentially many new taxa or poorly known morphospecies are needing to be morphologically described and linked to molecular information. In fact, the hidden diversity of ciliates is not just restricted within a few classes, and there were about 16% ciliate OTUs unclassified at the class level (Fig. 2). BLASTing these unclassified OTUs against GenBank showed that 215 OTUs (about 45% of all OTUs detected in this study) shared a similarity not greater than 97% with those of the morphologically identified ciliate species (Fig. S2, Tables S2 and S3), highlighting the potential for discovering new ciliate taxa in the coastal sediments. However, high levels (up to 1% difference) of intragenomic polymorphisms of rRNA genes in ciliates were reported (Gong et al., 2013). Selecting a proper OTU cutoff (e.g., 97% or lower) could reduce this risk of species diversity overestimation. In the future, much effect has yet to be made, for example, to cultivate and isolate the morphospecies in marine benthos, and to obtain longer sequences for resolving their systematic ranks.

It is well known that the proportion of 18S rRNA gene sequences of a taxon does not necessarily correlated to their relative abundance in cells, because there are great copy number variations within and between protist species (Gong et al., 2013). Our recent single-cell analysis indicate that the sequence-based relative proportion of a taxon with protistan communities more reflect their relative biomass rather than cell abundance (Fu and Gong, 2017), providing a reasonable basis for bridging these cell-based and rDNA-based characteristics. In comparison with the morphological surveys of benthic ciliate communities in the Yellow Sea, our molecular investigation showed some marked differences in the ciliate community composition and structure. The MBM studies generally found the classes Prostomatea and Karyorelictea dominated in biomass (Meng et al., 2012; Zhou and Xu, 2016), which are contrast with the present study indicating the dominance of Spirotrichea sequences (biomass) (Fig. 2 and Table 2). Approximately a quarter of sequences in this study were affiliated with the two spirotrichean subclass Choreotrichia and Oligotrichia, which are typical planktonic groups (Santoferrara et al., 2016; Zhang et al., 2017), and were hardly observed in the MBM studies involving fixation and morphological examination (Meng et al., 2012). It is possible that these planktonic cells have been broken during physical mixing with sediment particles and extraction using Ludox density centrifugation, thus lost the integrated morphology for proper identification under microscopes, because these protists generally have a relatively thinner cortile membrane and more fragile than most other ciliates (Song et al., 2003, 2009). Previous studies have also demonstrated microscopic observations may miss a large number of the existent species (e.g., Tamura et al., 2011). Alternatively, these ciliates presented as cyst forms in the surface sediments (e.g., Chao et al., 2013), thus could not be readily identified and enumerated in previous morphology-based surveys. However, every coin has two sides. The rDNA-based surveys could not tell which community members are viable or dormant, thus have to be interpreted as a reflection of recent situation of the benthic community (Zhu et al., 2018). It is also likely that the eukaryotic universal primers used in this study are biasing against prostomatean ciliates, thus fail to amplify their 18S rDNA sequences. Another cause for the absence of prostomatean rDNA sequences may be that they have been PCR amplified but not properly classified due to the lack of enough reference sequences in the publically available databases. By building phylogenetic trees containing reference and sequences yielded in this study (data not shown), and BLASTing against the GenBank (Tables S2 and S3), we did not obtain any evidence for the notion that some prostomatean sequences might be lumped into the category of unclassified ciliates. This possibility can thus be ruled out. To some extents, the MBM may have an advantage over rDNA-based approaches in detecting the most viable members, which are assumed to be directly involved in the ecological and biogeochemical processes in the microbial food webs at the sampling time points.

Our analyses point to a distinct pattern of regional variations in ciliate community structure among the BHS, NYS and SYS (Table 1), which was not previously recognized. In the MBM studies, the benthic ciliate community structure was not significantly different between the NYS and SYS (Meng et al., 2012; Zhou and Xu, 2016), indicating that the inactive members may be quite differently assembled among these regions. This implies that classical taxonomic work has to expand the geographic range of sampling sites from coastlines to off-shore locations for species discovery. In addition, the present study provides new data on benthic ciliate diversity in the BHS, and shows there is much higher species richness of ciliates in the benthos of this basin than in the NYS and SYS, illustrating that the shallow basin could be a hotspot for benthic ciliate diversity.

It is interesting to note that the relative proportion of Litostomatea sequences was significantly higher in the deeper SYS than in the shallow NYS and BHS, a statistically supported characteristic for the community structure changes among the three basins (Table 2). The litostomateans are carnivorous ciliates primarily feeding on other protozoans even small-sized metazoans. The increasing biomass contribution of these carnivorous in deeper sites reflects a decrease of bacteriophages and herbivores, which are sustained by bacterial and microalgal food (microphytobenthos) dwelling in or depositing on the surface sediments. With the increasing water depth, the declining temperature and light will increasingly limit the growth of microphytobenthos and hence bacterial and algal predators, resulting in the depth-dependent pattern of litostomateans observed in this study. However, again this pattern disagrees with the MBM studies in the similar regions, in which the carnivorous biomass seemed to be decreasing in the SYS (Meng et al., 2012; Zhou and Xu, 2016), suggesting that the changes in relative abundance/biomass of a given taxon in the communities does not necessarily follow its biological abundance in the samples, as we previously discussed (Zhu et al., 2018).

The spatial scale of the present study is relatively large (spanning about 500 km), it is thus important to consider geographic distance in addressing the relative importance of spatial factors (horizontal and vertical distances that are related to biological dispersal) and environmental filtering in shaping the benthic ciliate communities in the BHS, NYS and SYS (Martiny et al., 2006; Gong et al., 2015). Our study shows that water depth is the most important factor in benthic ciliate community assembly, a result similar to the biogeographic study of microeukaryotes (Gong et al., 2015), but different from that of fungi, which showed distinct seasonality (hence environmental filtering is the most important driver) in the similar studied area (Wang et al., 2017). This demonstrates that different microeukaryotic groups may have different biogeographies. Life history, trophic style, growth kinetics, and ecological association may be key factors explaining these differences, which is an interesting issue warranted further investigations.

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Supplementary information:

Fig. S1. Rarefaction curves of 39 sediment samples showing the sequences sampled and the numbers of OTUs obtained.

Fig. S2. Distribution of the numbers of the unclassified ciliate OTUs that fall in three identity categories (95%<identity⩽97%, 90%<identity⩽95%, and ⩽90%). The identities were relative to their closest described species which were located by BLASTing the OTUs against the GenBank. a. The OTUs unclassified at the class level and b. the OTUs unclassified in the class Spirotrichea.

Table S1. Summary of the pyrotags and alpha-diversity of benthic ciliates in sediment samples.

Table S2. Results of BLASTing against GenBank with the representative sequences of ciliate OTUs that are unclassified at the class level.

Table S3. Results of BLASTing against GenBank with the representative sequences of spirotrichean ciliate OTUs that are unclassified at lower ranks.

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