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Genetic diversity pattern of microeukaryotic communities and its relationship with the environment based on PCR-DGGE and T-RFLP techniques in Dongshan Bay, southeast China

Wenjing Zhang[a,*], Yuanyuan Mo[a], Jun Yang[b], Jing Zhou[a,c], Yuanshao Lin[a], Alain Isabwe[b], Jian Zhang[a], Xiu Gao[a], Zheng Yu[a,d]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China
[b] Aquatic EcoHealth Group, Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[c] School of Environmental Studies, China University of Geosciences, Wuhan, China
[d] Department of Chemical Engineering, University of Washington, Seattle, WA, USA

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ABSTRACT

Microeukaryotes play important roles in aquatic ecosystems, and could act as drivers of the biological nutrient cycling processes. However, compared with prokaryotic ones, little is known about the genetic diversity pattern of their community, and the environmental factors affecting their ecological pattern, especially in marine ecosystems. In this study, we used denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) to explore the genetic diversity structure of microeukaryotic communities in Dongshan Bay, southeast China. Our results revealed that microeukaryotic diversity ranged from 31 to 48 phylotypes (on average, 42) using the DGGE approach, while from 22 to 38 phylotypes (on average, 30) based on T-RFLP method, and the Shannon-Wiener diversity (DGGE-based) was relatively higher, suggesting that DGGE displayed a slightly higher resolution than T-RFLP. Surprisingly, the DGGE showed significant horizontal difference among microeukaryotic communities, but was similar with T-RFLP analysis that had no significant influence on microeukaryotic diversity at vertical scale. Further, redundancy analysis (RDA) indicated that the DGGE-based microeukaryotic communities distribution was significantly correlated with three environmental factors (Chl-a, TP and salinity), whereas microeukaryotic community revealed by T-RFLP was affected by four environmental factors namely salinity, temperature, depth and NO_X-N. Compared with RDA, BIO-ENV analysis showed that heterotrophic bacteria and NO_X-N were important environmental variable influencing microeukaryotic communities in both methods. These differences may be attributed to the noisy effects caused by the relatively large number of environmental variables. Generally speaking, despite differences in beta-diversity ordination for both DGGE and T-RFLP methods, there exists some consistency in the results of both techniques in terms of microeukaryotes responses to the environmental variables. These results suggested that environmental parameters had a great effect on spatial distribution of microeukaryotic community and contributed to marine ecosystem health to be further evaluated.

1. Introduction

In natural aquatic systems, microeukaryotes are critical components of microbial food webs and play important roles in influencing the biological nutrient cycles processes (Koid et al., 2012). For example, a little change of microeukaryotic community compositions tends to generate significant variations at all trophic states (Liu et al., 2011). However, study on microeukaryotic communities lagged far behind prokaryotic ones (Caron et al., 2009), which have been detected with the small subunit (SSU) 16 S rRNA gene served as a proxy since the late 1980s (Narasingarao et al., 2012). Recently, some studies demonstrated that eukaryotic assemblages are very diverse based on 18 S rRNA genes (Koid et al., 2012; Bochdansky et al., 2017). However, in contrast to prokaryotes, the factors driving the spatial distribution of microeukaryotic diversity along environmental gradients are poorly understood (Liu et al., 2013), especially across water stratification (Yu et al., 2014).

In the past decades, molecular techniques based on small subunit

* Corresponding author.
E-mail address: zhangwenjing@xmu.edu.cn (W. Zhang).

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ribosomal RNA (SSU rRNA) genes such as clone library (Díez et al., 2001a), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997) were widely applied in studying the diversity of microbial community (Not et al., 2008; Vigil et al., 2009; Yu et al., 2015). These molecular techniques provide useful information on microbial community dynamics, whereas any a molecular method, only reflects a portion of the real biodiversity because of their large populations and small size (Aguilera et al., 2006). Therefore, it's relatively difficult to detect the complete microbial assemblage (Gans et al., 2005; Smalla et al., 2007).

To improve some of the shortcomings of traditional approaches, some researchers performed two or even more methods to explore microbial assemblages (Nunan et al., 2005). For example, Not et al. (2008) combined multi-technique approaches to assess microeukaryotic diversity across the Indian Ocean, which allowed them to investigate general patterns and even the fine scale structure of assemblages. Moreover, few studies tried different methods of DNA extraction (Koid et al., 2012) or different restriction enzymes (Zhang et al., 2008) to modify the current techniques. Among these available molecular techniques, both DGGE and T-RFLP are most widely applied and effective methods in evaluating the spatial and temporal dynamics of microbial assemblages (Gao et al., 2012). DGGE takes advantage in high resolution based on the same size fragments that are separated (Muyzer and Smalla, 1998), in contrast to T-RFLP approach that has high throughputs, reproducible and even web-based Ribosomal Database Project (RDP) database (Marsh et al., 2000). In addition, T-RFLP allows a rapid and rather accurate estimation in microbial richness and community composition (Danovaro et al., 2006), and also avoids gel-to-gel comparison bias (Enwall and Hallin, 2009). However, the application of two methods (DGGE and T-RFLP) for assessing microeukaryotic diversity response to environmental changes has not received adequate attention, thus, in this study, the combination of both approaches would provide a more comprehensive understanding into the microeukaryotic community in the marine ecosystem.

The Dongshan Bay is located in the southwest of the Taiwan Strait, south of Fujian Province, China. The bay is surrounded by hills on three sides, and is an irregular pear shape, about 20 km long and 15 km wide. The Zhangjiang River, flowing into the bay from the northwest, is a main river in the south of Fujian Province with a drainage area of 961 km^2. Importantly, in recent years, a variety of environmental problems emerged in Dongshan Bay that were attributed to the social and economic development in the area. Moreover, increasing discharge of aquaculture and domestic wastewater in offshore areas has lead to the increasing water pollution. The coastal forest cover has suffered serious damage from human exploitation (Ni et al., 2015). However, few studies about ecological responses of microeukaryotic community to environmental changes have been reported in Dongshan Bay. Therefore, studying microeukaryotic community diversity and structure is crucial to understand ecosystem function of Dongshan Bay.

In this study, both DGGE and T-RFLP were used to investigate genetic diversity pattern of microeukaryotic community from Dongshan Bay. Specifically, the main aims of our study were (i) to explore the differences in the genetic diversity pattern of microeukaryotic community across spatial scale and (ii) to compare the DGGE and T-RFLP methods in assessing which major factors significantly affect the spatial distribution of the microeukaryotic communities.

2. Materials and methods

2.1. Sample collection

A field cruise was conducted across Dongshan Bay (117°29'–117°36' E, 23°34'–23°54' N) in early August 2011 and 10 stations were covered during the cruise. The surface (0.5 m) and bottom (2.0–18.0 m) water samples were collected in a pollution/environmental gradient along Dongshan Bay (Fig. 1). Then the water samples were sequentially filtered through a 200 μm sieve and a 0.22-μm-pore-size membrane (Millipore, Bedford, MA, USA) to collect microbial community. Filters were frozen at − 80 °C immediately after filtration until DNA extraction.

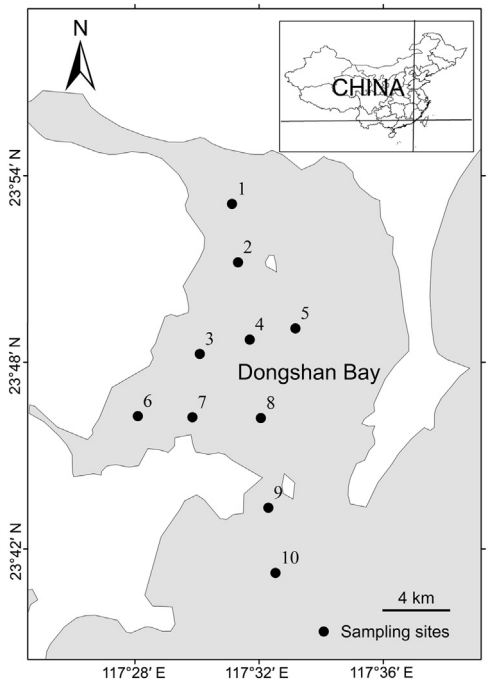

Fig. 1. Map of the Dongshan Bay showing sampling locations.

2.2. Environmental variables

Sampling depth, temperature and salinity were measured by using an onboard conductivity-temperature-depth (CTD, SBE-917) via probes. Chlorophyll-a (Chl-a) concentration was measured using a Turner Fluorometer (10-AU-005). Dissolved oxygen (DO), dissolved organic carbon (DOC), nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N), total nitrogen (TN), reactive phosphorus (RP), total phosphorus (TP), reactive silicate (RSi) and pH were measured according to the standard methods established in the Offshore Marine Chemical Survey Technical Regulations (Office of the State Oceanic Administration, 2006). Seawater samples for the heterotrophic bacterial (HB) counts were filtered onto black 0.2 Am Nuclepore polycarbonate membrane, stained with DAPI (Porter and Feig, 1980; Fuks et al., 2005). Suspended solids (SS) were detected gravimetrically by filtering 350 ml water sample through a pre-weighed filter (pore size of 0.45 μm), finally weighing the filter again after drying at 105 °C (Liu et al., 2011).

2.3. DNA extraction and PCR amplification of the 18S rRNA gene

Total microeukaryotes DNA was extracted using the FastDNA SPIN Kit for Soil (MP Qbiogene, CA, USA) according to the manufacturer's instructions. All DNA extracts were eluted with 50 μl TE buffer and stored at − 20 °C until further analyses.

The eukaryotic 18 S rRNA gene fragments were amplified using primer set Euk 1A and Euk 516r for both DGGE and T-RFLP analysis. The primer Euk 1A was 5′ labeled with hexachlorofluorescein (Operon Technologies) when used in T-RFLP method, while a 40 bp GC-clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) was added to the Euk 516r (5′) to increase the separation efficiency of DNA bands in DGGE gel (Díez et al., 2001b). The PCR amplification mixture contained: 0.3 mM of each primer, 1 × Ex Taq Buffer, 1.5 mM

of $MgCl_2$, 200 μM each of deoxynucleoside triphosphate, 2.5 U of Ex Taq polymerase, approximately 50 ng of template DNA, and double–distilled water in a final reaction volume of 50 μl. The Ex Taq polymerase, Ex dNTP Mix and 10 × Ex Taq Buffer were purchased from TaKaRa (TaKaRa, Japan). The PCR program included an initial denaturation at 94 °C for 10 min and 10 touchdown cycles of denaturation at 94 °C for 1 min, annealing at 65 °C (with the temperature decreasing by 1 °C each cycle) for 1 min, and extension at 72 °C for 1 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

2.4. Denaturing gradient gel electrophoresis (DGGE)

The PCR products were separated using the Dcode mutation detection system (Bio-Rad, Hercules, CA, USA). Approximately equal amounts of PCR products of different stations were loaded onto 0.75-mm-thick 6% polyacrylamide gels (acrylamide: bisacrylamide, 37.5: 1) in 1 × TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4). The electrophoresis was performed at 60 °C with a constant voltage of 100 V for 16 h in a linear 30–45% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide). Gels were stained with SYBR Green nucleic acid stain for 30 min and then visualized with UV radiation using Gel Doc EQ imager and analyzed with the Quantity One software (Bio-Rad, USA) as described previously (Schauer et al., 2000). The peak areas of fingerprint patterns were used to indicate the intensities, the intensity matrix was then constructed, taking into account the relative contribution of the band (as a percentage) to total intensity of the lane.

2.5. Terminal-restriction fragment length polymorphism (T-RFLP)

Fluorescently labeled PCR products were purified by using Eizard PCR purification columns (Promega, Madison, WI, USA). Aliquots of purified PCR products were digested with two restriction enzymes, *Msp*1 (USA Amersham International) and *Hha*1 (Thermo Fisher Scientific/Catalogue number: ER1851, India), individually according to the manufacturer's protocol. PCR products from each sample, peak over a threshold of 50 fluorescence units were used and T-RFs of < 30 bp and > 500 bp were excluded from the analysis to avoid detection of primers and uncertainties of size determination, respectively (Zhang et al., 2008). Subsequently, the remaining terminal restriction fragments (T-RFs) data were imported into T-REX software and profiles were aligned by using a clustering of 0.5% (Culman et al., 2009).

2.6. Statistical analysis

The Shannon-Wiener index (*H*) was calculated as follows:

$$H = -\sum \frac{n_i}{N} \ln \frac{n_i}{N}$$

where n_i is the area of a peak and *N* is the sum of all peak areas in each sample. Principal component analysis (PCA) was used to group the samples according to environmental variables using the Pearson correlation coefficient. Specifically, the PCA procedure basically calculates new synthetic variables (principal components), which are linear combinations of the original variables, and that account for as much of the variance of the original data as possible (Hotelling, 1933). The PCA is implemented using CANOCO 4.5 software package (Ter Braak and Smilauer, 2002). PCA results are generally displayed as a biplot (Jolicoeur and Mosimann, 1960), where the axes correspond to the new system of coordinates, and samples (dots) and environmental factors (arrows) are represented (Fig. 2). On the one hand, each arrow points in the direction of the steepest increase of the values for corresponding sample. The angle between arrows and dots indicates the sign of the correlation between the environmental factors and sample: the approximated correlation is positive when the angle is sharp and negative

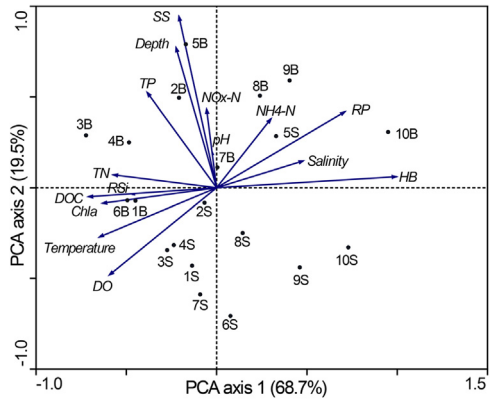

Fig. 2. PCA plots showing the relationship of 15 environmental variables of the sampling sites in the Dongshan Bay. Numbers refer to sampling locations as shown in Fig. 1; S represents for surface samples and B indicates bottom samples, respectively. DO- dissolved oxygen, DOC- dissolved organic carbon, TN- total nitrogen, NH_4-N- ammonium, TP- total phosphorus, RP- reactive phosphorous, Chl-a- Chlorophyll-a, SS- suspended solids, RSi- reactive silicate, HB- Heterotrophic bacteria.

when the angle is larger than 90 degrees; the angle is smaller, which indicates that there is a stronger relationship between the environmental factors and samples, otherwise, meaning there is a weaker relationship. On the other hand, the length of the arrow is a measure of fit for the environmental factors. The correlation is greater between the environmental factors and samples when the length of the arrow is longer (Ter Braak and Smilauer, 2002).

Although a Jaccard index matrix was generally performed from the presence/absence matrix, the result was the same as Bray-Curtis matrix in our study. Therefore, to enhance validity, the obtained fingerprint data were analyzed using presence/absence and relative abundance-based matrices using Bray-Curtis distance measure (Enwall and Hallin, 2009). Cluster analysis (Clarke and Gorley, 2001) and non-metric multidimensional scaling ordination (NMDS) (Clarke and Gorley, 2001) of microeukaryotic communities were performed using the PRIMER v5.0 package. The significant variance ($P < 0.01$) between groups of samples was assessed with the analysis of similarity (ANOSIM). The global R ranges from 0 to 1, and R = 0 represents no separation, whereas complete separation is demonstrated by R = 1.

Detrended correspondence analysis (DCA) for the biological data was applied to determine whether linear or unimodal ordination approaches should be selected. Then, redundancy analysis (RDA) revealed the detailed relationships between microeukaryotic communities and environmental variables using CANOCO 4.5 software package (Ter Braak and Smilauer, 2002). Physicochemical factors were ln(x) transformed to improve their homoscedasticity and normality with exception of the pH. The environmental variables with high variance inflation factor (VIF > 20) were eliminated from RDA analyses to avoid the collinearity among variables. The forward selection was then performed to select the minimum set of environmental factors explaining a significant percentages among the community variations ($P < 0.05$) (Ter Braak and Smilauer, 2002), and the significance of relationships between molecular bands data (DGGE and T-RFLP profiles) and environmental variables was determined by Monte Carlo permutation test (999 permutations) (Ling et al., 2012). RELATE analysis was applied to compare the microeukaryotic communities' genetic pattern as revealed by both DGGE and T-RFLP methods, and both the relativity intensity (%) similarity matrix and the presence/absence (0/1) similarity matrix were involved. Further, the significance of the Biota-Environment (BIO-ENV), correlation was tested using the BIO-ENV procedure, which finds out the best combination based on all environmental factors to explain the spatial patterns in the biological data. Independent-samples *t*-test was applied to assess significant differences between the surface and

bottom waters. All data analyses were performed with the PRIMER 5.0, the CANOCO 4.5 software package and the SPSS 19.0 (IMB Corp., Armonk, NY, USA).

3. Results

3.1. Environmental characteristics

The basic environmental parameters of the surface and bottom water samples were shown in Table S1. The depth, suspended solids and total phosphate showed significant difference (*t*-test, $P = 0.001$, 0.017 and 0.048, respectively) between the surface and bottom waters.

The first PCA axis explained a large proportion of the total variance (68.7%) and the proportion increased to 88.2% when the second axis was added (Fig. 2). The environmental variables contributing most to the first axis were heterotrophic bacteria counts (r = 0.99), reactive phosphorus (r = 0.71), temperature (r = −0.65), Chl-a (r = −0.63), DO (r = −0.59), TN (r = −0.57) and DOC (r = −0.71), while those to the second axis were suspended solids (r = 0.94), depth (r = 0.77), TP (r = 0.52).

3.2. Diversity of marine microeukaryotic communities revealed by DGGE and T-RFLP

A total of 91 distinct bands were detected (the relative intensity exceeded 0.5%) in 20 different samples on the DGGE fingerprint, among which 4 bands were common to all sites and 2 bands appeared only in one site (details not shown). It can be also seen that the banding patterns among all the samples were distinct. The band numbers varied from 31 (4S sample) to 48 (6S sample) with a mean of 42. The highest and lowest Shannon-Wiener diversity were obtained in the bottom water of site 2 and site 3, respectively. And the range of Shannon-Wiener diversity value varied from 3.099 to 3.728, and the mean value was 3.517 (Fig. 3a).

Based on values greater than 0.5% contribution to total peak areas, there were 109 unique T-RFs in all samples. The number of T-RFs varied from 22 (4B, 7B and 10B samples) to 38 (6S sample) with a mean of 30, while the Shannon-Wiener diversity value varied from 1.955 to 3.369, mean 2.956 (Fig. 3b). In one word, the eukaryotic plankton communities revealed by DGGE showed a higher diversity than those revealed by T-RFLP.

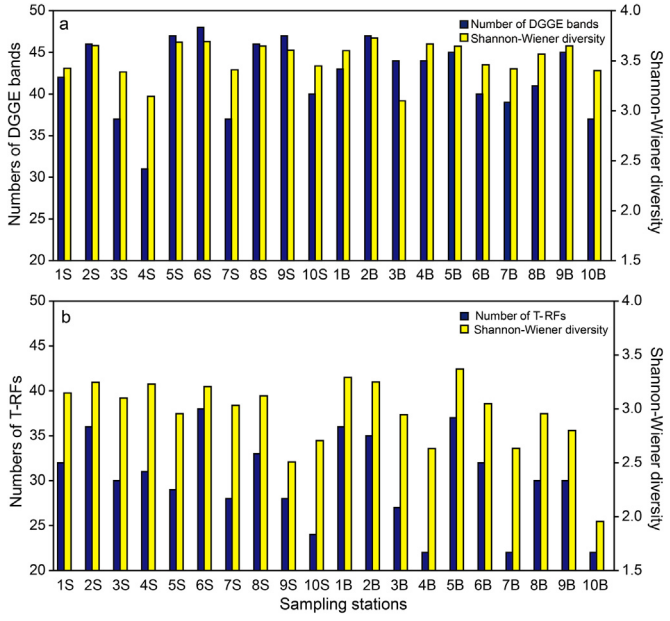

Fig. 3. Variations in richness (Number of DGGE bands and T-RFs) and Shannon-Wiener index of microeukaryotic communities based on DGGE (a) and T-RFLP (b) fingerprints. Numbers on x-axis refer to sampling locations, S and B refer to surface and bottom, respectively.

Table 1
Comparison of the microeukaryotic communities as revealed by DGGE and T-RFLP methods using the Spearman test (RELATE) analysis.

Correlation	DGGE (%)	DGGE (0/1)	T-RFLP (%)	T-RFLP (0/1)
DGGE (%)	—	0.622	0.305	0.291
DGGE (0/1)	0.001**	—	0.364	0.180
T-RFLP (%)	0.007**	0.001**	—	0.719
T-RFLP (0/1)	0.009**	0.056	0.001**	—

The "%"denotes relative intensity data, "0/1" stands for presence/absence data, respectively.
Upper and lower triangular matrices are Spearman Rho coefficients and *P*-value for each pairwise correlation.
** $P < 0.01$.

3.3. Correlation between DGGE and T-RFLP methods

RELATE analysis confirmed that the relativity intensity was closely related to the presence/absence in DGGE method (Rho = 0.622, $P = 0.001$) (Table 1). The two matrices revealed by T-RFLP also showed a significant correlation (Rho = 0.719, $P = 0.001$). When taking the relative abundance of DGGE bands and T-RFLP peaks into consideration, the composition of microeukaryotic communities was significant correlated with each other (Rho = 0.305, $P = 0.007$). As observed for the Bray-Curtis similarity matrices of presence/absence-based were not significant correlated between the DGGE and T-RFLP patterns (Rho = 0.180, $P = 0.056$). Thus, our results showed that the genetic diversity patterns of microeukaryotic communities analyzed by the same molecular method are much similar.

3.4. Spatial patterns in genetic diversity of microeukaryotic community

The 20 samples were greatly distinguished based on the DGGE band profiles (Figs. 4a and 4b). As cluster analysis classified the microeukaryotic communities into three groups at a similarity level of 60.02% (Fig. 4a). Group 1 included the samples from sites 1, 2 and 4 S, Group 2 consisted only of two samples from the bottom waters (4B and 6B), and Group 3 comprised the 13 samples from both surface and bottom. Similarly, non-metric multidimensional scaling ordination (NMDS) produced a similar result that all the samples were divided into three groups (Fig. 4b). In addition, ANOSIM analysis showed the global R value of 0.647 ($P = 0.001$), suggesting that each group from 20 samples was well separated (Fig. 4b).

Compared with DGGE method, there were some different distribution patterns (beta-diversity) by T-RFLP. Based on a similarity level of 53.76%, the microeukaryotic communities were divided into three groups as well as the result of NMDS (Figs. 4c and 4d). Specifically, Group 1 was only composed of 10B sample, Group 2 comprised the samples from the surface and bottom of sites 1, 2, and Group 3 included other sites samples from both surface and bottom. The results of ANOSIM analysis further demonstrated that the microeukaryotic communities were significantly different across the three groups (R = 0.564, $P = 0.001$).

At the vertical scale distribution, NMDS analysis revealed that the microeukaryotic communities from surface and bottom waters were not separated (R = 0.052, $P = 0.207$) in terms of DGGE band profiles (Fig. S1a). Interestingly, there was no significant difference between surface and bottom microeukaryotic communities based on the present/absence of T-RFLP data (R = 0.005, $P = 0.440$) (Fig. S1b).

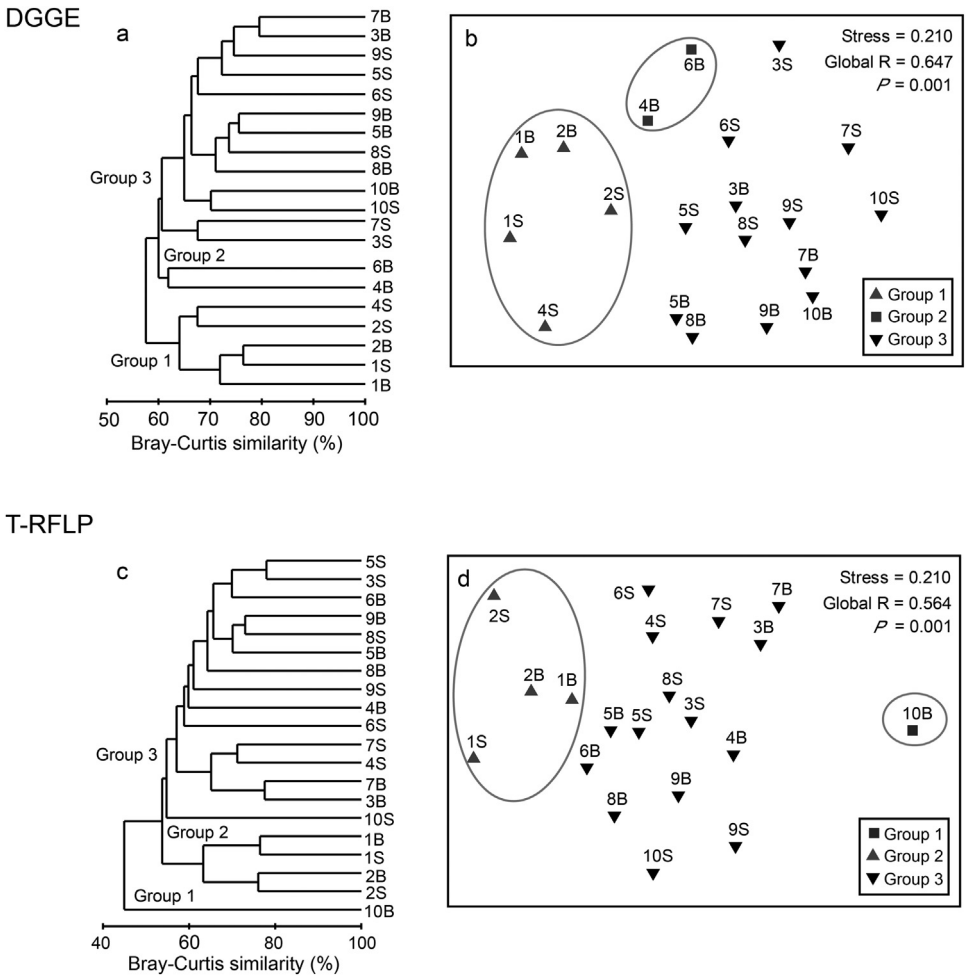

Fig. 4. Cluster analysis and NMDS ordination of microeukaryotic communities based on presence/absence data by DGGE profiles (a and b) and T-RFLP data (c and d). S represents for surface samples and B indicates bottom samples, respectively.

3.5. Comparisons of microeukaryotic community genetic structure based on DGGE and T-RFLP and their relationships with environmental parameters

Both redundancy analysis (RDA) and BIO-ENV procedure were applied to evaluate the relationship between environmental variables and microbial community structure. Here four matrices of microbial community structure were involved in our analysis and each of them showed a different result (Fig. 5 and Table 2).

RDA carried out using abundant DGGE bands (the relatively intensity exceeded 0.5%) together with environmental variables revealed that the Chl-a was related to the changes of the microeukaryotic communities ($P < 0.05$) (Fig. 5a), while the salinity and total phosphorus were the most significant factors associated with the changes of the microeukaryotic communities based on the present/absence of DGGE bands data in Dongshan Bay ($P < 0.05$) (Fig. 5b). The cumulative variances of the microbial community structure and environmental relation were explained by the first two axes, which were 26.5% and 17.9% for the abundant DGGE bands and the present/absence of DGGE bands data, respectively (Figs. 5a and 5b). This mismatch also occurred when the area of peaks (the relatively area of peaks that exceeded 0.5%) and the presence/absence in T-RFLP patterns combined with environmental variables were analyzed by RDA. According to Monte Carlo test, both temperature and depth showed significant correlations with the area of peaks, while temperature, salinity and NO_X-N were significantly related to the presence/absence of T-RFs. The first two axes of RDA explained 25.5% and 21.8% for the area of peaks and the present/absence of T-RFLP pattern data, respectively (Figs. 5c and 5d).

In addition, the BIO-ENV procedure gave us another approach to demonstrate the importance of different environmental variables in determining the structure of microbial community in Dongshan Bay (Table 2). The results of BIO-ENV indicated that heterotrophic bacteria were the most strongly correlated with the abundant DGGE bands (BIO-ENV Spearman rank correlation coefficient = 0.283), while the strongest correlation with the present/absence of DGGE bands was found in the combination of NO_X-N and reactive silicon (BIO-ENV Spearman rank correlation coefficient = 0.552). For the T-RFs, the subset strongly related to the area of peaks included salinity and heterotrophic bacteria (BIO-ENV Spearman rank correlation coefficient = 0.484), whereas the best significantly correlated to the present/absence of T-RFs included temperature, NO_X-N as well as heterotrophic bacteria (BIO-ENV Spearman rank correlation coefficient = 0.501).

4. Discussion

4.1. Diversity of microeukaryotic community

Microeukaryotic communities are ubiquitous and play a crucial role in the function of marine ecosystems (Caron et al., 2009). In our study, the eukaryotic 18 S rRNA gene could be separated by both DGGE and T-RFLP fingerprint methods. The average of the band numbers was 42 based on DGGE method, whereas the average number of T-RFs was 30 (Fig. 3), indicating that DGGE has a quite higher resolution than T-RFLP for 18 S rRNA gene. There was some probable differences in amplification efficiencies between DGGE and T-RFLP methods. Some previous

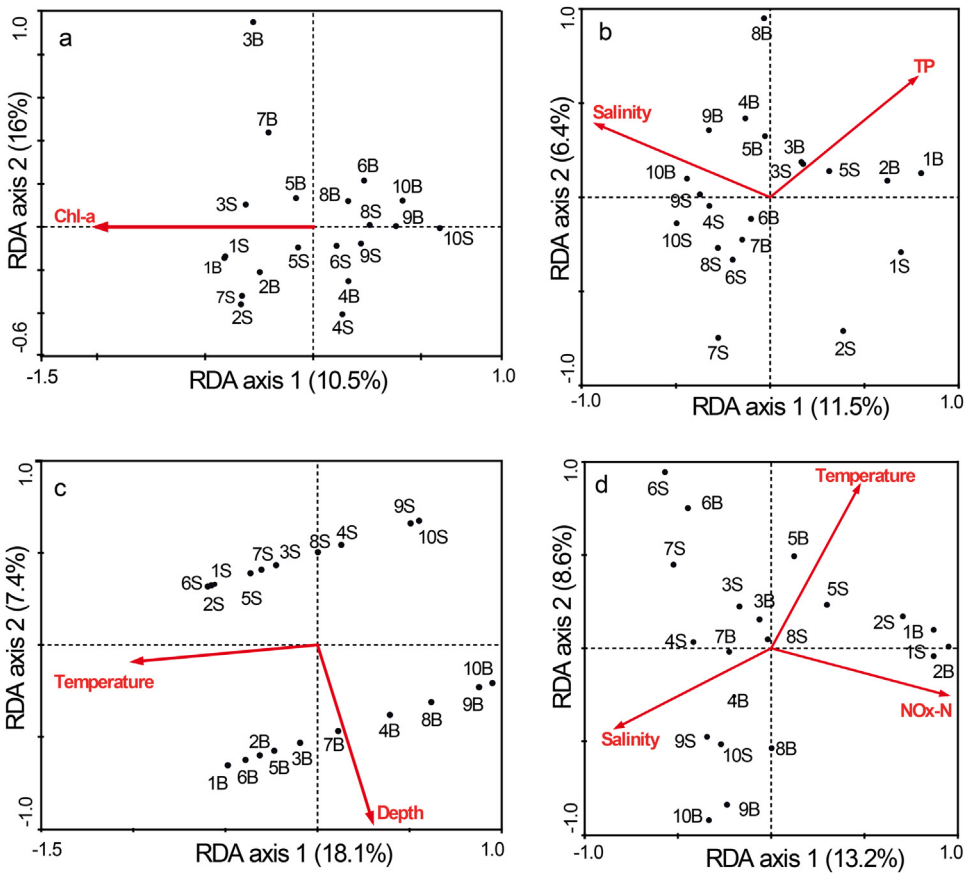

Fig. 5. The RDA ordination of genetic diversity pattern of microeukaryotic community in the Dongshan Bay in relation to the statistically significant environmental factors ($P < 0.05$) as revealed by DGGE (a: relative intensity, b: presence/absence) and T-RFLP methods (c: relative intensity, d: presence/absence). The numbers indicate the sampling sites, S stands for surface and B stands for bottom, respectively.

studies yielded opposing opinions to our results, showing a higher resolution of T-RFLP over DGGE (Horz et al., 2001; Nunan et al., 2005). This indicates that T-RFLP can be a more suitable alternative for community diversity analysis of large numbers of samples compared to DGGE. As the same PCR primer set and programs were used for both DGGE and T-RFLP in this study, confirming that there was almost no influence of the PCR biases. Our results agreed with Enwall and Hallin (2009) who found that DGGE had a higher resolution for *nosZ* gene of denitrifiers compared with T-RFLP in agriculture soils. However, Gao et al. (2012) found that DGGE and T-RFLP exhibited similar results in soil fungal community, regardless of the differences between *Hinf*I and *Taq*I based on T-RFLP analysis. These differences might result from different research subjects and backgrounds, for instance, research object of Gao et al. (2012) was soil fungal community, whereas marine microeukaryotic community was investigated in our study. In fact, different microbial communities (soil fungal community and marine microeukaryotic community) have different characteristics such size, abundance, diversity and evolution, so that there are different results despite using the similar method (DGGE and T-RFLP).

Additionally, Shannon-Wiener diversity index (H′) ranged between 3.099 and 3.728 based on DGGE, and from 1.955 to 3.369 based on T-RFLP data, indicating a wide range of diversity of the microeukaryotic community. Interestingly, the highest Shannon-Wiener diversity was found in the 2B and 5B samples for DGGE and T-RFLP data, respectively. On the contrary, the lowest Shannon-Wiener diversity was detected in the samples of 3B and 10B, respectively. One possible reason is

Table 2
Correlation coefficients associated with the best subset of environmental variables describing biological community structure from the exploratory multivariate analysis (BIO-ENV procedure). The values in bold are the variables that best selected with BIO-ENV.

DGGE						T-RFLP					
Relative intensity			Presence/absence			Relative intensity			Presence/absence		
No. variables	Selections	Correlation	No. variables	Selections	Correlation	No. variables	Selections	Correlation	No. variables	Selections	Correlation
1	**15**	**0.283**	2	**9, 13**	**0.552**	2	**3, 15**	**0.484**	3	**2, 9, 15**	**0.501**
1	13	0.265	2	3, 13	0.503	2	3, 7	0.428	3	3, 9, 15	0.490
1	14	0.250	2	11,13	0.485	2	3, 9	0.420	3	2, 7, 9	0.470
1	6	0.186	2	7, 13	0.446	2	3, 13	0.420	3	2, 9, 12	0.467
1	7	0.173	2	6, 13	0.395	2	2, 3	0.409	3	9, 14, 15	0.461
1	12	0.171	2	7, 9	0.392	2	9, 15	0.405	3	7, 9, 15	0.461
1	8	0.155	2	13, 14	0.391	2	0, 3	0.388	3	9, 12, 15	0.458
1	2	0.150	2	2, 13	0.388	2	3, 5	0.375	3	2, 3, 15	0.450
1	3	0.131	2	9,14	0.385	2	2, 9	0.372	3	6, 9, 15	0.445
1	9	0.080	2	3, 11	0.332	2	0, 2	0.211	3	2, 3, 9	0.435

Selections: 0, Depth; 2, Temperature; 3, Salinity; 4, pH; 5, SS; 6, DO; 7, DOC; 8, TN; 9, NO_X-N; 10, NH_4-N; 11, TP; 12, RP; 13, RSi; 14, Chl-a; 15, HB.

that complex environments have great effects on microeukaryotic diversity. For instance, comparing with 3B and 10B samples, the 2B and 5B samples were primarily affected by depth, suspended solids and total phosphorus (Fig. 2), which suggested the total phosphorus may provide more suitable setting for diverse microeukaryotes as was previously found (Liu et al., 2011). Some studies also found that free-living microbial eukaryotes, such as the planktonic algae are related to the trophic status (Reynolds, 1984; Yang et al., 2012). Further, Shannon-Wiener diversity based on the DGGE bands was higher than that of T-RFLP data except for the surface water sample of site 4 (Fig. 3), suggesting that T-RFLP had a slightly lower resolution. These results were also supported by the fact that highly similar DNA amplicons can be separated by DGGE (Myers et al., 1985), while only the separation of T-RFLP depends on the nucleotides corresponding to the restriction sites of the selected enzymes (Enwall and Hallin, 2009). Some researchers showed an increasing of the resolution of T-RFLP by using several enzymes (Horz et al., 2001), however, increasing information might result in adding noise in the statistical analysis (Enwall and Hallin, 2009).

4.2. Spatial pattern of the microeukaryotes community

The spatial dynamics of the marine microeukaryotic community structure have yet to benefit from the explosion of molecular-based investigations into microbial ecology and biogeography (Thomas et al., 2012). The clone library (Zhang et al., 2008) as well as fingerprinting techniques, such as ARISA (Danovaro et al., 2006), DGGE (Liu et al., 1997; Díez et al., 2001a) and T-RFLP (March, 1999), have recently been widely used in fresh and marine aquatic ecological research, allowing faster and deeper to compare the differences of samples from microbial assemblages. In this study, both DGGE and T-RFLP were used to observe the spatial distribution of microeukaryotes in Dongshan Bay.

Similar findings were obtained using both fingerprinting methods. NMDS analysis of 18 S rRNA gene, based on both DGGE profiles and T-RFLP, revealed that there was a significant difference among microeukaryotic communities (Fig. 4). This suggested that both methods could distinguish between the different microeukaryotic communities at a statistically significant level ($P < 0.01$). Previous study has shown significant variation in sludge plant communities by both fingerprinting methods based on a statistically significant level (Evans et al., 2014). However, no significant difference was observed in microeukaryotic communities between surface and bottom water of the Dongshan Bay with DGGE profiles (Fig. S1a, $R = 0.052$, $P = 0.207$) as well as T-RFLP approach (Fig. S1b, $R = 0.005$, $P = 0.440$). It is implied that there was no significant influence of vertical environmental variation on microeukaryotic diversity, consistent with the patterns observed for large tintinnid ciliates in Shenhu Bay (Wang et al., 2014). The possible explanation is that the water is not deep enough since the maximum distance of surface water from the bottom layer was less than 10 m at vertical scale. Indeed, the seawater is more likely to be well mixed based on vertical level, which might lead to hydrological characteristics of seawater to cause small change in the whole water columns (Wang et al., 2014). In addition, the coastal environmental conditions suffer serious effects of human activities around and in the Dongshan Bay (Ni et al., 2015). Consequently, at the vertical scale, the microeukaryotic communities had a relatively homogeneous distribution pattern in the study area, whereas it varied horizontally among sites at a statistically significant level when using both DGGE and T-RFLP methods.

4.3. Environmental effects on microeukaryotes community distribution

There exist some studies suggesting that the possible changes in the presence/absence or in the variation of intensity of a single band is a useful way to analyze DGGE fingerprinting patterns (Murray et al., 1996; Fromin et al., 2002). Though the presence/absence data was previously considered to better depict the changes taking place in the microeukaryotic community than the relative intensity, because of the large differences of the cell size between the microeukaryotes (Díez et al., 2001a; Muylaert et al., 2002; Yu et al., 2014), both data matrices were involved in our analysis for revealing the microbial diversity and their rank abundance (Ranjard et al., 2000). Thus, when using the same statistical method (RDA and BIO-ENV), different combinations of environmental variables were proved to be significantly related to microeukaryotic communities revealed by presence/absence profile and relative intensity data, respectively. In this paper, it is suggested that presence/absence and relative intensity of DGGE bands as well as the T-RFs may have different ecological significance.

In DGGE analysis, there were significant relationship between spatial patterns of microeukaryotic communities and environmental factors. DGGE revealed that microeukaryotic communities were significantly associated with Chl-a, TP and salinity (Figs. 5a and 5b). Similar result was observed in a previous study of marine system, finding a significant relationship between microeukaryotes and environmental variables such as salinity (Yu et al., 2015). In addition, BIO-ENV analysis showed that NO_X-N and reactive silicon were the best combination of variables that determined the distribution of microeukaryotic communities in the present study, and heterotrophic bacteria was also a key factor in affecting microeukaryotic community structure (Table 2). Compared with bacterial community, salinity was also strongly correlated with spatial variation of bacterioplankton community in aquatic ecosystem (Logares et al., 2013). This indicated that salinity was an important environmental factor affecting the spatial distribution of microbial community (Milione and Zeng, 2008), such as microeukaryotic communities in marine system (Zhang et al., 2017).

In T-RFLP data-based analysis, the spatial change of microeukaryotic communities could also be detected. The results of RDA and BIO-ENV analyses indicated that salinity, NO_X-N, and heterotrophic bacteria had a great effect on communities structure as well as DGGE method (Fig. 5 and Table 2). However, community genetic diversity was significantly influenced by some different environmental factors for both DGGE and T-RFLP analyses. For example, T-RFLP also revealed that temperature and depth were significant environmental factors influencing the distribution of microeukaryotic communities, while reactive silicon, Chl-a and TP were revealed by DGGE analysis (Fig. 5). Our result is inconsistent with Yu et al. (2015) who found that temperature had significant influence on the distribution of microeukaryotic community, but is supported by the fact that salinity was another significant environmental factor affecting community structure (DGGE-based). It is implied that DGGE analyses might be related with T-RFLP method to some extent. In fact, the higher resolution was found in DGGE, while the both methods presented a high correlation ($P < 0.01$) in our study, showing that both fingerprinting techniques were suitable for a snapshot of microeukaryotic communities. For example, the major advantage of DGGE is the visibility of the gel and it can provide a direct impression by different band patterns. T-RFLP approach takes advantage in high throughputs (Marsh et al., 2000) and removes gel-to-gel comparison bias (Enwall and Hallin, 2009). Thus, both DGGE and T-RFLP contribute to a better understanding that environmental parameters (heterotrophic bacteria, Chl-a, TP, reactive silicon, salinity, depth, temperature and NO_X-N) had a strong impact on microeukaryotic distribution at spatial scale. In general, RDA and BIO-ENV analyses provided us different information about the factors that drove the spatial distribution of microeukaryotes. It might be because of the relatively large number of environmental variables, compared to small sample size, which increased the noisy effects of multicollinearity among environmental variables (Saiz-Salinas and Urkiaga-Alberdi, 1999). Nevertheless, the fact that both methods illustrated some similar environmental variables as main drivers of community change (e.g. heterotrophic bacteria, salinity and NO_X-N). Indeed, salinity is perceived as the main driver of microbial dynamics in marine environments (Logares et al., 2013; Yu et al., 2015; Zhang et al., 2017).

5. Conclusion

In this research, the horizontal distribution of microeukaryotic communities' diversity in the Dongshan Bay, South China Sea, showed a significant spatial variation, although their diversity was not significantly different among the vertical direction based on both PCR-DGGE and T-RFLP approaches. In DGGE analysis, the salinity, TP and Chl-a seemed to be responsible for the distribution and abundance of microeukaryotes, as revealed by RDA analysis, whereas heterotrophic bacteria, NO_X-N, and reactive silicon well determined their distribution through BIO-ENV analysis. For T-RFLP data, RDA revealed that temperature, salinity, NO_X-N and depth were significantly influencing microeukaryotes, but BIO-ENV showed that salinity, NO_X-N, temperature and heterotrophic bacteria were best combined factors affecting them. While the same DNA samples were extracted from 20 samples of Dongshan Bay, comparative analysis of each same DNA sample was done by DGGE and T-RFLP in separate experiments, respectively. This means that both fingerprinting techniques did not completely generate the same data set of microeukaryotic communities because of different resolution and amplification efficiencies. This is why DGGE and T-RFLP results from the same samples yielded different correlations between plankton communities and environmental variables despite using similar statistical methods. Generally, the genetic diversity pattern of microeukaryotic communities exhibited a distinct trend with the spatial change in certain environmental variables. Though DGGE showed a slightly higher resolution compared with T-RFLP in this study, the DGGE profile was found to be significantly related to the T-RFLP data, indicating that both methods were useful fingerprinting techniques for assessing genetic diversity of microeukaryotic communities in marine ecosystem.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.csr.2018.05.006.

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