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The diversity and structure of marine protists in the coastal waters of China revealed by morphological observation and 454 pyrosequencing

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ABSTRACT

Pyrosequencing of the 18S rRNA gene has been widely adopted to study the eukaryotic diversity in various types of environments, and has an advantage over traditional morphology methods in exploring unknown microbial communities. To comprehensively assess the diversity and community composition of marine protists in the coastal waters of China, we applied both morphological observations and high-throughput sequencing of the V2 and V3 regions of 18S rDNA simultaneously to analyze samples collected from the surface layer of the Yellow and East China Seas. Dinoflagellates, diatoms and ciliates were the three dominant protistan groups as revealed by the two methods. Diatoms were the first dominant protistan group in the microscopic observations, with *Skeletonema* mainly distributed in the nearshore eutrophic waters and *Chaetoceros* in higher temperature and higher pH waters. The mixotrophic dinoflagellates, *Gymnodinium* and *Gyrodinium*, were more competitive in the oligotrophic waters. The pyrosequencing method revealed an extensive diversity of dinoflagellates. *Chaetoceros* was the only dominant diatom group in the pyrosequencing dataset. *Gyrodinium* represented the most abundant reads and dominated the offshore oligotrophic protistan community as they were in the microscopic observations. The dominance of parasitic dinoflagellates in the pyrosequencing dataset, which were overlooked in the morphological observations, indicates more attention should be paid to explore the potential role of this group. Both methods provide coherent clustering of samples. Nutrient levels, salinity and pH were the main factors influencing the distribution of protists. This study demonstrates that different primer pairs used in the pyrosequencing will indicate different protistan community structures. A suitable marker may reveal more comprehensive composition of protists and provide valuable information on environmental drivers.

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1. Introduction

Protists (single cell eukaryotes) play fundamental ecological roles as producers, consumers and decomposers in marine ecosystem. They dominate several primary trophic links, and mediate the carbon and nutrient flow to higher trophic levels through the marine food webs (Caron et al., 2012). Marine protists are sensitive to environmental variation, for example, global warming threatened the Arctic Ocean and resulted in significant loss of protistan diversity (Lovejoy et al., 2006). The continental shelf of the Yellow and East China Seas is one of the most productive areas of the world oceans and waters here are affected by multiple water currents, such as the Changjiang Diluted waters, Taiwan Warm Current and Kuroshio Current (Su, 2001). These currents influence the water temperature, salinity and particularly the nutrient inputs (Tang et al., 2006), and cause variations in the composition and diversity of the protist community (Wu et al., 2000; Chen et al., 2011).

Previous investigations on eukaryotic composition and distribution in the coastal waters of China mainly used traditional microscopic and pigment extraction-based methods (Wu et al., 2000; Chen et al., 2006). The microscopic method mainly relies on identification of the cell morphology, which makes it difficult to distinguish similar taxa. Pigment analysis of protists only targets the autotrophic species and also various groups of marine protists are sensitive to sampling or handling process. A large proportion of these fragile cells may be missed from those traditional procedures, resulting in significant underestimation of its actual diversity

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(Epstein and López-García, 2008). Recently, molecular approaches have been applied to phytoplankton diversity analysis in the coastal waters of China, such as denaturing gradient gel electrophoresis (DGGE) (Sun et al., 2010). The 18S rRNA gene is universally present in living eukaryotes, containing both conserved and hypervariable regions which are convenient for designing primers to distinguish majority of species (Ki, 2012). In addition, there are significant sequence data for comparison in public databases (e.g., GenBank). Thus, the 18S rRNA gene is widely accepted as a valuable 'bar-code' to assess eukaryotic diversity in environmental samples (Moon-Van Der Staay et al., 2001; Countway et al., 2005; Behnke et al., 2011).

454-pyrosequencing can assess microbial communities with high taxonomic resolution, and has opened a new window in microbial biodiversity surveys (Stoeck et al., 2010). This technique has been successfully applied to investigate the protist communities and revealed a very large diversity (Behnke et al., 2011; Kilias et al., 2013; Lima-Mendez et al., 2015; Massana et al., 2015). However, very limited studies have been carried out in the coastal waters of China (Faria et al., 2014). Thus, to get a comprehensive understanding of protist communities and their correlations with environmental conditions in the coastal waters of China, we carried out a large-scale investigation in the Yellow and East China Seas by simultaneously using microscopic observations and 454-pyrosequencing of 18S rDNA.

2. Methods

2.1. Sample collection and environmental measurements

The sampling was performed during a cruise in July 2012 along the coastal line of China (119.5°–126° E longitude, 25.88°–38.75° N latitude) (Fig. 1). Surface seawaters were collected with Niskin bottles. Temperature and salinity were measured by the equipped CTD (conductivity, temperature and depth) sensor. The pH values were determined within 10 min of sampling using a temperature compensating pH meter (Schott pH meter CG 837). Seawater was transferred directly from Niskin bottles to acid-washed (10% HCl) polythene bottles via gravity filtration through a 200 μm Nitex mesh, to exclude metazoan from the following-up molecular analyses. About 0.25 L of filtrates were fixed with 2% v/v acid Lugol's solution. Another 0.5–1 L of filtrates were then filtered onto 3 μm pore-size polycarbonate filters (47 mm diameter, Millipore) using a vacuum pump at low pressure (<100 mbar). The filters were transferred into cryogenic vials and stored immediately in liquid nitrogen for DNA extraction. Filtrates passing through 0.45 μm pore-size cellulose filters were stored in −20 °C and nutrient concentrations were measured using a continuous flow analyzer (SAN^{plus} system, SKALAR Inc.) by colorimetric methods. The analyzed nutrients included DIN (nitrate, nitrite and ammonium), DRP (dissolved reactive phosphate) and silicate.

2.2. Morphological observation

The samples fixed in Lugol's solution were examined at ×200 magnification using an inverted microscope (Olympus IX71; Olympus Co., Tokyo, Japan) after sedimentation in chambers for 24 h (Utermöhl, 1958). The volume of samples settled were determined on experience, to ensure that cells disperse randomly in the bottom of the chambers without overlapping.

2.3. DNA extraction

The genomic DNA of environmental water samples was extracted using the Powersoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer instructions. DNA extracts were stored at −80 °C until being further processed.

2.4. Pyrosequencing analysis

The DNA samples were amplified using a pair of universal eukaryote primers 18S-82F (5′-GAAACTGCGAATGGCTC-3′) and Ek-516r (5′-ACCAGACTTGCCCTCC-3′) (Monchy et al., 2012). The primer set has been designed to amplify a 480-bp amplicon corresponding to the V2 and V3 regions of eukaryotic 18S rRNA gene. Roche A and B adaptors were added to the primers respectively. DNA concentrations of the specific amplicon were quantified using a QuantiFluor™ -ST fluorometer(Promega) and equivalent amounts of the amplicon from each individual samples were loaded onto half of a plate and processed with the Roche 454 GS-FLX platform. The pyrosequencing project was carried out by the Majorbio Bio Tech Co. Ltd (Shanghai, China). Raw sequence reads were deposited in the NCBI Sequence Read Archive under the accession number SRP034609.

Raw sequences were initially filtered to remove invalid reads in Qiime (version 1.17), including short sequences (<200 bp), wrong primer sequence, or sequences containing ambiguous bases or with homopolymers longer than six nucleotides. Primer sequences, barcode and adaptor fragments were subsequently removed prior to further analysis. Resulting reads were firstly strictly dereplicated. Exactly identical sequences (equal length and identical base composition) were grouped as a unique sequence. Unique sequences were aligned in the Mothur software against the Silva reference alignment (http://www.mothur.org/wiki/Align.seqs) with the kmer = 8 parameter. Chimeric sequences were detected and removed with UCHIME (http://drive5.com/uchime). The remaining reads were clustered into operational taxonomic units (OTUs) at the ≥97% similarity level by means of furthest-neighbor clustering (http://www.mothur.org/wiki/Cluster). All singletons, defined as an OTU composed of one single sequence that only occurs once in the whole analysis, were removed to evade possible errors induced by the sequencing process.

2.5. Taxonomic assignment

Taxonomy was assigned to valid sequences by conducting BLASTN searches (Altschul et al., 1990) in the Silva SSU rRNA database (http://www.arb-silva.de/). Only unique sequences with a best BLAST hit of at least 80% sequence similarity were assigned to a taxonomic category. BLAST results were visualized using the MEGAN software (Huson et al., 2007). Sequences affiliated to protists were picked out for further analyses.

2.6. Diversity and community structure analyses

For comparison of the protistan diversity by 454-pyrosequencing, a sub-sampling of the final processed sequences to the lowest read number (1247) was applied in order to normalize the different sequencing depths. The estimators of protistan community richness (non-parametric ACE and the Chao1), diversity (Shannon and Simpson) and Good's coverage were calculated using the Mothur software. Dominant genera were estimated by means of Mcnaughton index (Y) calculated with the following formula:

$$Y = \frac{n_i}{N} \times f_i$$

where n_i was the number of sequences for genus i in all samples, N was the total number of sequence reads for all genera, f_i was the frequency of occurrence for genus i in all samples.

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