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Genetic diversity of *Ulva prolifera* population in Qingdao coastal water during the green algal blooms revealed by microsatellite

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ABSTRACT

Green tides have occurred in Qingdao coast in China for seven consecutive years from 2007 to 2013. To provide information on the genetic structure of these blooms, 210 free-floating green algae samples isolated from the green tide in Qingdao coast on June 19, 2013 were identified based on the ITS, *rbc*L and 5S sequence, and genetic diversity was investigated by microsatellite markers. According to ITS, *rbc*L and 5S sequence, all the 210 samples belonged to *Ulva prolifera*. Nei's genetic diversity and Shannon index estimated using eight microsatellite markers indicated that the genetic diversity of *U. prolifera* population within Qingdao's green bloom in 2013 was low. Taking into account previous reports about life history and physiology of *U. prolifera*, we proposed that the limited origin area of the free-floating biomass and asexual reproduction of *U. prolifera* might be responsible for the lower diversity of free floating *U. prolifera*.

1. Introduction

Green tide is a harmful ecological phenomenon caused by vast accumulations of floating green algae, such as *Ulva*, *Chaetomorpha* and *Cladophora*. In recent years, green tides have frequently occurred in North America, South America, Europe, Asia and Australia, and have been becoming a thorny problem worldwide (Morand and Briand, 1996; Hernández et al., 1997; Blomster et al., 2002; Hiraoka et al., 2004; Merceron et al., 2007). These blooms have significant negative impacts on marine ecological environment such as damaging ecosystem structure and decreasing biodiversity (Berezina et al., 2007; Mcglathery, 2001). Anoxic conditions caused by vast algal decomposition are shown to influence the survival of invertebrate and fishes (Valiela et al., 1997; Rafaelli et al., 1998; Deacutis et al., 2006; Berezina et al., 2007). Furthermore, the accumulation of green algae in coast area not only generates aesthetical displeasure but also produces noxious smell, thus leading to serious economic losses on the local tourism industries. Therefore, much attention has been paid on the identification and physiological character of dominant species, the deleterious effects of green tide and the origin of the massive drifting green tide (Hiraoka et al., 2004; Merceron et al., 2007; Huo et al., 2013; J. Zhang et al., 2014).

In general, eutrophication is considered to be responsible for the outbreak of green algae blooms (Morand and Briand, 1996; Sun et al., 2008). High growth rate and huge biomass accumulation of the green algae are usually found in the environments with sufficient input of nutrients such as estuarine deltas and muddy intertidal zones of the sea (Leskinen et al., 2004; Conley et al., 2009). The green algae have broad tolerance to temperature, salinity and irradiance, and they can grow well at 5-25 °C (Dan et al., 2002; Deng et al., 2012; Han et al., 2013). Large-scaled green tides are usually dominated by one or several co-occurring opportunistic species, and occur in the area closed or semi-closed bay (Callow et al., 1997; Blomster et al., 2002). The majority species of green tides are reported to consist of *Ulva* and *Enteromorpha* (Blomster et al., 2002). However, *Enteromorpha* was later reduced to synonymy with *Ulva* (Hayden et al., 2003). The growth rate of *Ulva* has been shown to be related to the morphology, the capacity to store nutrients during pulses and the ability to adapt to varied environmental conditions (Merceron et al., 2007).

In China, large masses of free-floating green algae have accumulated in the Yellow Sea successively in summer from 2007 to 2013 (Sun et al., 2008; Liu et al., 2009; D. Liu et al., 2010; Shen et al., 2012; Zhao et al., 2011, 2013; J. Zhang et al., 2014; L. Zhang et al., 2014). Especially in the summer of 2008, the world's largest green algal blooms covering about 2400 km^2 attacked off the coast of Qingdao a few weeks before the 2008 Olympic Games (Hu et al., 2010). In terms of the morphological observation and phylogenetic analyses of the nuclear encoded ribosomal DNA internal transcribed spacer region (ITS nrDNA) and the plastid encoded large subunit of ribulose-1, 5-bisphosphate carboxylase/oxgenase gene (*rbc*L), some reports regarded the dominant species of green algae as *Ulva linza-procera-prolifera* (LPP) complex (Leliaert et al., 2009; F. Liu et al., 2010; Wang et al., 2010). Later, Zhao et al. (2013) demonstrated that the ITS and 5S spacer sequences of the

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algae covering the large area of the Yellow Sea from 2008 to 2011 exhibited high similarities, and the bloom-forming algae was *U. prolifera*. However, study on ITS, *rbc*L and 5S sequence by Duan et al. (2012) showed that the green tide was composed of *U. proliera*, *Ulva compressa* and *Ulva pertusa*. Additionally, Han et al. (2013) pointed out a succession of free-floating *Ulva* species in Rudong coast in Jiangsu province, including *U. compressa*, *Uniola flexuosa*, and *U. prolifera*. It is obvious that the exact species composition of the massive green tides occurring in the Yellow Sea remains controversial (Kang et al., 2014). Furthermore, due to the absence of suitable molecular markers, to date very few papers have focused on the population genetic structure of the green tide (L. Zhang et al., 2014). Therefore, very little is known about the genetic diversity of these *Ulva* blooms, though some markers such as inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) have been used to investigate the genetic diversity of *U. prolifera* populations along the coast of China (Zhao et al., 2011, 2015; Liu et al., 2011). Nevertheless, it is crucial to learn the genetic structure of the blooms for understanding the large-scale green tides and correctly identifying the origin of the blooms.

Microsatellite loci, known as simple sequence repeats (SSR), consists of repeated motifs of 1–6 bases existing in both coding and non-coding regions in all prokaryote and eukaryote genomes (Field and Wills, 1996; Li et al., 2004). As a molecular marker with often highly variable loci, microsatellite loci has been widely used to analyze the gene flow and population structure in macroalgae (Alström-Rapaport and Leskinen, 2002; Shan and Pang, 2009). However, owing to the high mutation rate, the use of a specific set of microsatellites is usually restricted to a genus or closely related genera (Kostamo et al., 2008). Accordingly, the microsatellite loci often need to be isolated *de novo* from each species, which is time-consuming and expensive (Dutech et al., 2007). Due to the limited genetic knowledge of the green algae, especially the *Ulva* genus, to date few attempts have been made to exploit this molecular marker to study genetic diversity of green tide. Alström-Rapaport and Leskinen (2002) for the first time reported five polymorphic microsatellite markers in the green algae *Enteromorpha intestinalis*, opening a window for exploring the genetic structure of green tide. Later, Kostamo et al. (2008) developed four new microsatellite markers for this species, and Kagami et al. (2008) reported nine microsatellite markers for the green alga *U. compressa*. In the case of China Sea, L. Zhang et al. (2014) used some microsatellite markers from expressed sequence tag (EST) to analyze the genetic variation of several *U. prolifera* populations in the South Yellow Sea of China, while Zhao et al. (2011, 2015) investigated the genetic variation using ISSR. However, these studies focus on the genetic diversity of *U. prolifera* from the north of the Yellow Sea to the south of the East China Sea, not just in Qingdao coastal water during the blooms (Zhao et al., 2011, 2015; L. Zhang et al., 2014). Due to the limited numbers of samples examined from Qingdao water in these previous studies, the genetic diversity of *U. prolifera* population in Qingdao coastal water, especially during the green algal blooms, remains largely unclear.

In this current study, our objective is to explore the genetic diversity of *U. prolifera* population in Qingdao coastal water during the green algal bloom. So, 210 samples of free-floating green algae were firstly isolated from the green tide off the Qingdao coast on June 19, 2013, and then identified based on the ITS, *rbc*L and 5S sequence. Secondly, eight microsatellite primers were developed using amplified fragments of ISSR and in terms of the EST from GenBank, and the genetic diversity was investigated using these microsatellite markers.

2. Materials and methods

2.1. Collection of samples

Twenty-one sample collection sites were set off the coast of Qingdao, Shandong province, China in 2013 (Fig. 1 and Table 1). At each station, 10 free-floating samples were collected from different sites including the ship's bow, stem and broadside. Each sample consisted of one individual only. Total 210 free-floating samples were collected on Jun. 19, 2013. After collected, the samples were cleaned simply *in situ* and put into cool boxes immediately, then transported to laboratory within 40 h. In laboratory, the free-floating samples were cleaned once again with sterile seawater to remove potential epiphytic diatoms and tiny sand, then stored at −20 °C.

2.2. DNA extraction

Frozen samples were washed three times with sterilized seawater. A piece of tissue was excised randomly from samples for DNA extraction. Total DNA was extracted with a HiPure Plant DNA Mini Kit (Magen, China) according to the manufacturer's specification. DNA quality was confirmed on a 1% TAE agarose gels strained with Gold View (SBS Genetech, China).

2.3. PCR amplification and sequencing

PCR primers used to amplify ITS, *rbc*L and 5S sequence were synthesized by Shanghai Sangon Biotech Co., Ltd., China. The ITS region was amplified as described by Leskinen and Pamilo (1997) using primers FW and RV. The PCR for *rbc*L was performed as described by Manhart (1994) using primer rbcLF and rbcLR (Shen et al., 2012). To further distinguish *U. prolifera*, *U. linza* and *U. procera* within LPP complex, 5S region sequence was obtained according to the protocol reported by Shimada et al. (2008) and Duan et al. (2012) with the primer pair: forward 5′-GGTTGGGCAGGATTAGTA-3′, reverse 5′-AGGCTTAAGTTGCGAG TT-3′, which generated about 400 bp product.

Total genomic DNA (15-20 ng) was added to 20 μl PCR reaction containing 2 × PCR mix (Genstar, China) and 20 mM of each primer. PCR reaction was carried out in a MJ MiniTM Gradient Thermal Cycler (BIORAD, California, USA). PCR products were separated on 1% agarose gel electrophoresis and stained with GoldView. Purified products by a HiPure PCR Pure Mini Kit (Magen, China) were sent to Sangon Biotech Co., Ltd. (China) for sequencing.

2.4. Phylogenetic analysis

The sequences obtained in the current study and the ITS and *rbc*L sequences of the genera *Ulva* and *Blidingia*, as well as 5S sequences of *U. prolifera* and *U. linza* from GenBank were aligned using program MAFFT v6.843 (Katoh et al., 2002). The phylogenetic tree was constructed by neighbor-joining (NJ) method using program Mega 6 (Tamura et al., 2013) with the Kimura 2-parameter model (Kimura, 1980). The reliability of branches was estimated with non-parametric bootstrapping (1000 replicates) (Felsenstein, 1985). Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed.

2.5. SSR development

In this study, eight microsatellite primers were developed to analyze the genetic diversity of *U. prolifera* population. Among them, seven primers were designed in terms of the ESTs from GenBank, and the remaining one was developed using ISSR amplifications as reported by Lian et al. (2001).

Total 6738 ESTs of *U. prolifera* were obtained from GenBank (http://www.ncbi.nlm.nih.gov/nucest/), and saved in FASTA format. The sequences shorter than 100 bp or longer than 1000 bp, as well as Cap and poly A tail of mRNA were removed by est_timmer.pl. (http://pgrc.ipk-gatersleben.de/misa/). The recognition and location of microsatellite sites were carried out using misa.pl (http://pgrc.ipk-gatersleben.de/misa/misa.html). The parameters for SSR search were as follows: di-, tri-, and tetranucleotide motifs with a minimum of six, five, and four repeats, respectively (Xie et al., 2009). Total 118 microsatellite sites were detected from EST sequence, and only 35 microsatellite primers were designed by Primer 3. Among them, seven primers were