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# Genetic analyses of floating *Ulva prolifera* in the Yellow Sea suggest a unique ecotype

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ABSTRACT

Large-scale green tides caused by the green algae *Ulva prolifera* have occurred in the Yellow Sea every summer since 2007. The genetic variation and relationships at the intra-species level among floating and attached samples of *U. prolifera* collected from 2007 to 2011 were analysed using ISSR (inter-simple sequence repeat) markers. The results showed that all of the floating samples collected from the Yellow Sea during the past five years formed a single genetic entity that was different from the attached samples in intertidal zone. A SCAR (sequence characterized amplified region) marker highly specific to the floating samples of *U. prolifera* was identified and it showed that the same population dominated in the blooms from 2007 to 2013. In combination with the morphology and physiological features, the genetic analysis results suggested that a unique ecotype of *U. prolifera* was responsible for the largest green tides in the world. These findings indicated that attached *U. prolifera* along the coast does not seem to be the originator of the green tide in the Yellow Sea and gene flow between attached and floating populations does not readily take place.



## 1. Introduction

Green tides have become a globally severe problem due to the increased eutrophication of coastal waters (Charlier et al., 2007). In addition to various ecological impacts on indigenous biodiversity (Berger et al., 2003) and biogeochemical cycles (Sandjensen and Borum, 1991), these algal blooms affect aquaculture and tourism (Sun et al., 2008). Most green tides involve *Ulva* (Blomster et al., 2002; Shimada et al., 2003) or *Enteromorpha*, which is a synonym of *Ulva* (Hayden et al., 2003). China experienced a small-scale (82 $km^2$) green tide in the Yellow Sea caused by *Ulva* in 2007 from May to July. A green algal bloom on a much larger scale (3489 $km^2$) occurred again in 2008 (Keesing et al., 2011). In the following years, the Yellow Sea underwent consecutive green tide blooms, which have been documented as the largest green tides ever to occur worldwide (Liu et al., 2013a).

*Ulva prolifera* (=*Enteromorpha prolifera*) has been confirmed as the only dominant species in the Yellow Sea green tides by both morphology and molecular analysis (Jiang et al., 2008; Ye et al., 2011; Zhao et al., 2013). Detailed studies tracing the blooms discovered the coexistence of *Ulva compressa*, *Ulva flexuosa* and *Ulva linza-procera-prolifera* (LPP) complex in the floating algal assemblage, but *U. prolifera* gradually dominated during the drifting period (Tian et al., 2011).

Since quite a few attached *Ulva prolifera* populations distributed widely along the coast of China, and some even have high biomass near the bloom area (Zhang et al., 2011), comparative genetic analysis at the intra-species level between populations has been a key to figure out the origin of the floating algae. Preliminary study of the samples collected from 2007 to 2009 using ISSR (inter-simple sequence repeat) markers revealed that the floating samples exhibited a close genetic relationship and that they were different from all of the *U. prolifera* samples attached to hard substrates in coastal areas (Zhao et al., 2011). Phylogenetic analysis of the 5S rDNA spacer region also indicated that the floating *U. prolifera* in the Yellow Sea from 2008 to 2009 formed a monophyletic clade (Lin et al., 2011; Zhang et al., 2011).

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However, after successive blooms of *Ulva prolifera* in the Yellow Sea, gene exchange between the floating and attached populations may occur to a certain extent and the genetic composition of the dominant population of the Yellow Sea green tides might change. Different genotypes of floating *U. prolifera* have been proposed based on the results of phylogenetic analyses using the 5S rDNA spacer region as a genetic marker (Huo et al., 2013; Liu et al., 2013b). But the difference could result from an inhomogeneity of the 5S rDNA spacer region, which has been previously revealed in other organisms (Sajdak et al., 1998; Baum et al., 2001). Thus, genome wide and specific molecular markers are more appropriate for analysis of genetic variation in floating seaweed.

In this study, we applied ISSR markers to investigate the genetic diversity of the floating and attached population samples of *Ulva prolifera* in the Yellow Sea from 2007 to 2011. Compared to the single molecular marker with potential heterogeneous multicopies at limited locus in the genome, such as the 5S rDNA spacer region, the ISSR markers that generate the fingerprint of the whole genome could provide more sufficient resolution to reveal the genetic variations among populations and have been widely used to analyze the intra-species genetic diversity for seaweeds (Wang et al., 2008; Mostafa et al., 2011). We further developed a SCAR (sequence characterized amplified region) marker that is highly specific to the floating population using the ISSR fingerprints. SCAR markers are a type of genetic marker that is used to identify a specific population from other populations (Zijlstra, 2000). To find out if the genetic makeup of the floating *U. prolifera* in lower case unchanged across several years, we examined samples collected through 2007 to 2013.

## 2. Materials and methods

### 2.1. Sample collection

From 2007 to 2011, nine attached population samples from substrates in littoral zone and 14 floating samples of *Ulva prolifera* were collected at different sites in the Yellow Sea and East China Sea, and in 2012 and 2013, an additional 10 floating algae samples were collected (Fig. 1 and Table 1). The species identification of the samples was based on both morphological characteristics and an analysis of the ribosomal DNA internal transcribed spacers (ITS). The algal thalli were rinsed carefully in sterilized seawater to remove debris and epiphytes and then cultured in Von Stosch's Enriched Medium (VSE medium).

### 2.2. DNA extraction

For the samples collected from 2007 to 2011, four fragments of separated fronds from each collection site were selected randomly for DNA extraction; for the samples collected from 2012 to 2013, one fragment was selected to represent a sample. The genomic DNA from each tubular thallus (approximately 5 cm) was separated using a modified CTAB method (Zhao et al., 2010). The quality and concentration of the obtained DNA were tested by comparison with the λDNA/*Eco*R I + *Hin*d III markers (Fermentas, Shanghai, China) on a 0.8% agarose gel.

### 2.3. ISSR amplification

Six out of the 50 ISSR primers made available by the University of British Columbia Biotechnology Laboratory were selected to amplify the profiles of *Ulva prolifera* fronds on the basis that they generated clear, reproducible bands ranging from 200 bp to 2000 bp. The samples collected from 2007 to 2011 were subjected to ISSR analysis. The ISSR-PCR reactions were performed in a volume of 25 μl containing 10.5 μl of dd$H_2O$, 12.5 μl of 2 × Taq Master Mix (Sinobio, Beijing, China), 1 μl of primer (10 μM, Sangon, Shanghai, China) and 1 μl of template DNA (approximately 30 ng/μl). Amplification was conducted by a thermocycler (Biometra, Germany) using a program described by Zhao et al. (2011). The ISSR primers used in this study and their annealing temperatures are listed in Table 2. The ISSR reactions were replicated twice for each frond.

### 2.4. Visualization and scoring of the ISSR fragments

The PCR products were separated on a 2% agarose gel stained with ethidium bromide in 1× TAE buffer and then visualized and scored under ultraviolet light (Bio-Rad, USA). D2000 plus DNA marker (Transgene, Beijing, China) was loaded onto both sides of the gel as the fragment-sized standard. The gel images were processed for scoring using BandScan version 5.0 (Drets, 1978). The use of digital imaging to identify and score bands helped establish a consistent scoring protocol and eliminate bias during the scoring process (Wolfe and Liston, 1998). Polymorphic fragments of each locus were scored in a binary manner as present (1) or absent (0). Only those bands that were clearly identified in both amplified replicates were recorded.

### 2.5. Data analysis

Assuming Hardy–Weinberg equilibrium, the Nei's unbiased genetic distances (*D*) between samples were determined. A matrix of pairwise distances between all of the individual fronds was calculated using the Jaccard similarity coefficient, which emphasizes shared traits among fronds and ignores the absence of shared traits. Clustering with unweighted pair-group mean analysis (UPGMA) was performed based on the Jaccard similarity coefficient using MAGA 4 (Tamura et al., 2007) and NTSYS-pc, version 2.1 (Rohlf, 2000).

### 2.6. SCAR marker development

Based on the ISSR profiles of the samples collected from 2007 to 2009, those bands occurring solely in the floating samples with high intensity, clear separation and a molecular weight between 200 bp to 2000 bp were selected. The selected bands were purified (Gel Midi Purification Kit, TianGen, Beijing, China), ligated into a pMD18-T vector (Takara, Dalian, China), and transformed into Top-10 *Escherichia coli* competent cells. The positive clones were sent to a business enterprise (Co. Majorbio, Shanghai China) for sequencing. SCAR primers consisting of more than 18 bases were designed from the sequences of the cloned ISSR fragments using Primer 5.0 (Clarke and Gorley, 2001) and were synthesized by Sangon (Shanghai, China).

### 2.7. SCAR amplification

The SCAR amplifications were performed in 10 μl reaction mixtures containing 4.2 μl of water, 5 μl of 2 × Taq Master Mix (SinoBio, Beijing, China), 0.2 μl of each primer (10 μM), and 0.4 μl of template DNA (approximately 30 ng/μl). The PCR conditions were 94 °C for 10 min followed by 35 cycles of 94 °C for 45 s, annealing temperature for 45 s, and 72 °C for 1 min and a final elongation at 72 °C for 10 min. The SCAR amplifications were analysed by the electrophoresis of 3 μl of the PCR products on a 2% (w/v) agarose gel stained with ethidium bromide.