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.

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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 km²) 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 km²) 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).
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/EcoR I + Hind 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 ddH2O, 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 (Biomera, 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 (SinBio, 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.
3. Results

3.1. Species identification

The ITS sequences of the 33 samples used in this study have been submitted to GenBank, and their accession numbers were KP966511 – KP966543. A phylogenetic analysis showed that 31 out of the 33 samples were clustered into the LPP clade. The two remaining samples were two floating samples (QD156 and S792) that clustered into the Ulva compressa clade, which was excluded from the intra-specific genetic analysis (Fig. S1). The morphology of all 31 LPP samples exhibited strong characteristics of branched and tubular thalli with monostromatic walls, as described by Wang et al. (2010). Combining the results of molecular and morphological analyses, the 31 samples collected in this study were identified as Ulva prolifera. Only the 31 samples of U. prolifera were considered in a further study of the intra-specific genetic diversity.

3.2. ISSR amplification

A total of 104 high-intensity and well-separated bands were recorded from the profiles generated by the six ISSR primers (Table 2). All of these ISSR fragments were polymorphic among the 92 Ulva prolifera individual fronds collected from 2007 to 2011 (Figs. S2 and S3). The fingerprints were digitized and subjected to further phylogenetic analysis.

3.3. Phylogenetic analysis

The phylogenetic tree of UPGMA based on the Jaccard distance between Ulva prolifera fronds revealed that all of the floating samples were grouped into one cluster and were significantly separated from the attached samples (Fig. 2). The cophenetic

Table 1
Details of the specimens used in this study.

| Sample | Collection site                  | Collection date | Sample state |
|--------|----------------------------------|-----------------|--------------|
| S096   | Yumingzui, Huangdao, Qingdao     | 29 Jul 2007     | Floating     |
| S196   | Sculpture Park, Qingdao          | 15 Jun 2008     | Floating     |
| S266   | Lianyungang                      | 8 Jul 2008      | Floating     |
| S349   | Sculpture Park, Qingdao          | 16 Jul 2008     | Floating     |
| S380   | Donghai Restaurant, Qingdao      | 25 Jul 2008     | Floating     |
| 09DH   | Donghai Restaurant, Qingdao      | 18 Jul 2009     | Floating     |
| 10YY   | No.1 bathing beach, Qingdao      | 23 Jul 2010     | Floating     |
| U146   | Zhujilin, Lvisi, Qingdao         | 2 Jul 2011      | Floating     |
| U149   | Laoqiang, Nantong                | 2 Jul 2011      | Floating     |
| U152   | Sheyang, Yancheng                | 3 Jul 2011      | Floating     |
| U156   | Haitou, Ganuy, Lianyungang       | 4 Jul 2011      | Floating     |
| U158   | Gaunxi, Lianyungang              | 4 Jul 2011      | Floating     |
| U162   | Rizhao                           | 4 Jul 2011      | Floating     |
| N161   | No.1 bathing beach, Qingdao      | 7 Jul 2011      | Floating     |
| QD075  | Donghai Restaurant, Qingdao      | 17 Jun 2012     | Floating     |
| QD076  | No.1 bathing beach, Qingdao      | 17 Jun 2012     | Floating     |
| QD156  | Luqinghe, Qingdao                | 1 Jul 2012      | Floating     |
| S792   | Rudong, Nantong                  | 29 Apr 2013     | Floating     |
| S791   | Rudong, Nantong                  | 7 May 2013      | Floating     |
| S793   | Xuejiaodao, Qingdao              | 7 Jun 2013      | Floating     |
| S795   | No.3 bathing beach, Rizhao       | 7 Jun 2013      | Floating     |
| S796   | No.1 bathing beach, Qingdao      | 8 Jun 2013      | Floating     |
| S989   | Xuejiaodao, Qingdao              | 6 Jul 2013      | Floating     |
| S986   | Haiyang, Yantai                  | 31 Jul 2013     | Floating     |
| S565   | Gangshen Port, Rizhao            | 26 Mar 2009     | Attached     |
| S605   | D'aao Village, Fuqing            | 7 Apr 2009      | Attached     |
| S703   | Fenghua, Ningbo                  | 27 Mar 2009     | Attached     |
| S744   | Dafeng, Yancheng                 | 1 Apr 2009      | Attached     |
| S746   | Dafeng, Yancheng                 | 1 Apr 2009      | Attached     |
| U148   | Rudong, Nantong                  | 2 Jul 2011      | Attached     |
| U150   | New Beilingzha                   | 2 Jul 2011      | Attached     |
| U159   | Guanxi, Lianyungang              | 4 Jul 2011      | Attached     |
| U161   | Beach, Guanxi, Lianyungang       | 4 Jul 2011      | Attached     |

S096, S196, S266, S349, S380, 09DH, S565, S605, S703, S744 and S746 were used in a preliminary study (Zhao et al., 2011) and were considered together with the newly collected samples in the present study.
Correlation indicated a very good fit of the cluster analysis to the data \( r = 0.97 \).

To better understand the pattern of variation among the samples, a UPGMA dendrogram was also produced using Nei’s unbiased genetic distances \( D \) among the samples (Table S1). Similar to the dendrogram obtained based on the distance between fronds, the *Ulva prolifera* samples were grouped into two distinct clusters (Fig. 3). The floating samples formed one major cluster, whereas the nine attached samples formed another cluster.

### 3.4. Results of the amplification of the SCAR marker

A specific band (928 bp) of the floating samples was selected based on fingerprints amplified by ISSR primer 855. Its sequence has been submitted to GenBank, and the accession number is KP966544. No significant homologous sequence was found in the NCBI database. Based on the complete sequencing data, a pair of SCAR primers was designed. The sequences of the primers were YSF-F (ACACC-TACAAACCCTAACC) and YSF-R (TTGTGCGCAAACCACTTC), and the

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**Table 2**

| Primer name | Sequence \((5’ \rightarrow 3’)\) | Annealing temperature \( (^\circ C) \) | Number of polymorphic bands |
|-------------|----------------------|----------------------|------------------|
| 816         | \((CA)_1T\)                    | 46.9                  | 15                |
| 818         | \((CA)_1G\)                    | 49.5                  | 20                |
| 826         | \((AC)_1C\)                    | 49.6                  | 16                |
| 855         | \((AC)_1YT\)                   | 48.8                  | 16                |
| 857         | \((AC)_1YG\)                   | 51.2                  | 21                |
| 890         | HV1(TG)                        | 47.0                  | 16                |

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![Fig. 2. Dendrogram of the UPGMA cluster analysis constructed using the Jaccard distance calculated for 23 samples (92 individuals) of *U. prolifera* based on ISSR fragments. Samples for the Yellow Sea floating algae are shown surrounded by a red oval. All of the floating individuals were grouped together and formed a clade separating from the littoral attached samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).](image-url)
annealing temperature was 50.5 °C. The expected size of the SCAR product obtained for the Yellow Sea floating Ulva prolifera was 830 bp.

We used the developed SCAR marker to test the 33 algal samples collected from 2007 to 2013. The PCR results showed that the target band was amplified for 21 out of the 22 floating Ulva prolifera samples, with the exception of S793. In contrast, none of the nine samples of attached U. prolifera showed the specific band (Fig. 4).

4. Discussion

The results revealed that the floating Ulva prolifera samples collected between 2007 and 2013 were genetically similar (except for one sample in 2013) and that the attached U. prolifera were genetically different from the floating U. prolifera, corroborating previous results (Zhao et al., 2011).

Besides the genetic traits, the floating population was also different from the attached populations on morphology, physiology and living environments. Most remarkably, the bloom-forming thalli exhibited denser branches, which facilitated the long-time and far-distance drifting of them, than the attached thalli (Kong et al., 2011). And the morphological characteristics displayed continuously through several generations cultured in the laboratory (Hiraoka et al., 2011). What is more, living conditions, such as the temperature range (Huo et al., 2013), irradiance (Kim et al., 2011), desiccation (Gao et al., 2011) and salinity (Wei et al., 2010), are obviously different between the littoral attached and the floating populations in the Yellow Sea. Compared to the attached populations, the floating population was better adapted to the habitat on the sea surface (Hiraoka et al., 2011).

Combining the facts that the dominant floating population in the Yellow Sea was distinct in genetics, morphology, physiology and living conditions, it was reasonable to conclude that the Yellow Sea green tide was dominated by a unique ecotype of Ulva prolifera, a genetically distinct population adapted to the drifting life style on sea surface. Many ecotypes have been reported within several HAB (harmful algae bloom)-caused microalgae species based on genetic analysis (Fredrickson et al., 2011; Piccini et al., 2011). The intra-specific variations, such as morphological, genetic characters, maximum growth, photosynthesis rates, and tolerance of adversity (Thessen et al., 2009), were possibly important factors for their competitive success (Hallegraeff et al., 2012). The definition of this floating U. prolifera as a unique ecotype is critical to the further investigation and monitor of the Yellow Sea green tide.

First, the detection of a different ecotype raises a question as to the origin of the green tide. There are two different meanings involved: the annual seed bank for the floating biomass and the initial biological origin of the floating algae since 2007. Upon the annual seed bank, Liu et al. (2013b) proposed that propagules in intertidal seawater and sediments constituted an over-winter “seed stock”, and proliferated attached to Porphyra rafts in the Subei Shoal as a “nursery” (Liu et al., 2010; Zhang et al., 2015). However, to confirm this hypothesis, it would have been necessary to obtain molecular data, by use of SCAR marker specific to the floating ecotype, on algae attached to the rafts, which we did not do. We are uncertain about the initial origin of the green tide. In the early years of green tide in the Yellow Sea, Ulva prolifera were rarely found on the rafts during the Porphyra aquaculture season (Shen et al., 2012). It is only since 2011 that the quantity of U. prolifera attached to the Porphyra aquaculture rafts began to increase significantly (Han et al., 2013). These results suggest that the Porphyra aquaculture rafts may not be the initial origin of the floating U. prolifera in 2007. Our present results indicate that the attached populations along the coast of the Yellow Sea could not be the source, neither. It may be a unique ecotype of U. prolifera may have invaded into the Subei Shoal and initially caused the green tides in 2007.

Second, after successive blooms, genetic variation kept between the floating and the attached populations in the coastal area suggested the limited gene flow between them. That could result from
the asexual reproduction, which was reported to be the dominant proliferation style for the bloom-forming Ulva in eutrophic conditions (Kamermans et al., 1998; Blomster et al., 2002). And the oceanographic factors, such as eddies (Lin et al., 2011) and current variations, might provide the geographical barrier for the floating ecotype, which need to be taken into account.

Third, aside from some necessary environmental conditions, such as eutrophication, the biological property of this floating ecotype may also offer essential contributions to the Yellow Sea green tide and should thus receive greater attention with respect to understanding the mechanism underlying the formation of the bloom.

In addition, the SCAR marker developed in this study was proven to be highly specific to the floating ecotype, which is an effective tool to examine the genetic similarity of the bloom-forming algae in the coming years, to monitor the settlement of the floating ecotype in the coastal area and to assess the gene exchange between the floating and the attached populations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2015.05.027

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