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Discrimination of the common macroalgae (*Ulva* and *Blidingia*) in coastal waters of Yellow Sea, northern China, based on restriction fragment-length polymorphism (RFLP) analysis

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ABSTRACT

Since 2007, reoccurring large-scale green algae blooms have caused deleterious effects to the estuarine ecosystem of Yellow Sea, northern China and subsequent economical losses. Previous surveys indicated the green tides were initiated in the coastal water of southern Jiangsu province where *Porphyra* farming was intensively conducted; however, the main 'seed source' of floating green algae is still under debate. *Ulva prolifera* was confirmed to be the major causative species of green tides. The multiple sympatric ulvoid species in the natural environment has complicated species identification in both field surveys and laboratory studies due to their morphological plasticity. Thus, we developed a genetic identification key based on restriction fragment length polymorphism (RFLP) analysis of the ITS nuclear marker to discriminate the common *Ulva* and *Blidingia* species in the Yellow Sea. Ten genetic lineages (1 in *Blidingia*, 9 in *Ulva*) were detected along the coast of China through phylogenetic analysis of ITS sequences. They can be separated by virtual restriction digestion using the four selected restriction enzymes (BspT107 I, EcoO109 I, Hin1 I and VpaK11B I). With additional PCR amplification of the 5S spacer region, we were able to discriminate *U. prolifera* from *Ulva linza*. Using this genetic key, we screened macroalgal samples collected from the coast of the Yellow Sea, and the results indicated 6 common lineages (*U. prolifera*, *U. linza*, *Ulva compressa*, *Ulva pertusa*, *Clade 6* and *Blidingia* sp.) in this region, which could be explicitly distinguished by a single enzyme (BspT107 I) coupled with 5S spacer polymorphism. *U. prolifera* was confirmed to be present on the *Porphyra* aquaculture rafts with seasonal variation in the species composition. This genetic key will facilitate our long-term field surveys to investigate the origin of the floating *U. prolifera* and furthermore to explore its bloom dynamics along the coast of the Yellow Sea. It also provided a framework for the future inclusion of more *Ulva* species, which will expand the usage of this key.

1. Introduction

Large-scale 'green tides' reoccurred every year since 2007 along the coast of the Yellow Sea, China. It has been claimed to be the world's largest 'green tide' in terms of affected area and biomass it produced (Liu et al., 2009, 2010a). In 2008, the accumulated biomass of green macroalgae was estimated to be 3 million tons and covered an area of 1.29×10^4 km during its peak (Sun et al., 2008; Keesing et al., 2011; Lin et al., 2011). Such large-scale blooms have caused serious detrimental ecological and social-economic impacts to the adjacent coastal areas (Sun et al., 2008; Ye et al., 2011). The causative green tide genus was identified to be *Ulva*, of which *U. prolifera* was dominant (Leliaert et al., 2009; Liu et al., 2010b; Pang et al., 2010; Duan et al., 2012; Shen et al., 2012). Satellite remote sensing and physical oceanographical modeling indicated that *U. prolifera* floating mats were repeatedly formed in the near-shore water of southern Jiangsu province where *Porphyra yezoensis*[1] aquaculture was most intensive (Sun et al., 2008; Hu, 2009; Keesing et al., 2011; Liu et al., 2010a), and transported northward by seasonal winds and surface currents (Lee et al., 2011).

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[1] *Porphyra yezoensis* was re-classified to *Pyropia yezoensis* based on a recent molecular phylogenetic work (Sutherland et al., 2011).

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However, there is still debate regarding the 'origin' or 'source' of the floating *U. prolifera*.

The taxonomy of the Ulvaceae, especially the genus *Ulva*, has followed a tortuous history (Tan et al., 1999; Woolcott and King, 1999; Hayden and Waaland, 2002; Hayden et al., 2003). The tubular *Enteromorpha* and lettuce-like *Ulva* with distromatic blades were originally recognized as two different genera according to their distinct morphological differences. Recent research, however, indicated that they were not evolutionarily distinct entities based on the molecular phylogenetic analyses, and it was suggested that *Enteromorpha* should be synonymized with *Ulva* (Tan et al., 1999; Hayden et al., 2003. Only *Ulva* is used in this paper). Several *Ulva* species have long been recognized in China and they have co-existed ubiquitously in the Yellow Sea with no evident harm to the local habitats (Tseng, 1984). Discrimination of these co-occurring macroalgae based on gross morphology is difficult due to their large intra-specific morphological variations and relatively few inter-specific differences (Mathieson et al., 1981; Blomster et al., 1998, 1999, 2002).

Molecular analysis has been applied to distinguish species within *Ulva* since large-scale blooms occurred in the Yellow Sea (Shimada et al., 2003, 2008; Hiraoka et al., 2011; Duan et al., 2012). The dominating species of macroalgal blooms was confirmed to be *Ulva prolifera* (Leliaert et al., 2009; Wang et al., 2010; Zhao et al., 2012). Previous molecular studies, however, mainly relied on sequencing and phylogenetic analysis which requires tedious laboratory work and complicated program computation. Those assays were not readily accessed and not economically applicable for large-scale, long-term surveys that usually come along with large number of field samples. Thus, the purpose of this study was to: (1) investigate the number of common macroalgal species (or genetic lineages) in our regional survey in the Yellow Sea, based on the available molecular data; (2) develop a reliable and easy to use genetic identification key based on restriction fragment length polymorphism (RFLP) analysis of the ITS nuclear marker; and (3) analyze the species composition of the attached and floating macroalge from *Porphyra* aquaculture rafts and near-shore habitats in the Yellow Sea.

2. Materials and methods

2.1. Sample collection

Macroalgae samples were collected from a *Porphyra* aquaculture area along the coast of Jiangsu province and intertidal areas along the coast of Qingdao (Fig. 1). Attached macroalgae was sampled monthly from the *Porphyra* rafts at three stations (XA, Xiaoyangkou; GA, Gaoni; NA, Niluosha; Fig. 1) except in November of 2011, January, February and August of 2012, due to adverse weather conditions. Floating samples were collected in June, July, September and October in the waters near the *Porphyra* aquaculture area (Fig. 1). Two additional samples (GS, NS) were collected from macroalge clumps settled on the muddy flat at Gaoni and Niluosha. These macroalge clumps were most likely left by *Porphyra* farmers when they cleaned the rafts after the *Porphyra* harvest season (Liu et al., 2010a; Fan et al., personal communication).

For comparison, we also sampled attached macroalgae at the intertidal rocky and sandy beaches of Qingdao in July. Furthermore, we noticed that floating *Ulva* spp. bloomed frequently at a much smaller scale (usually $<100\ m^2$) along the recreational beaches in Qingdao in summer. These blooms usually occurred locally (i.e. not drifting to open area from offshore large-scale *U. prolifera* floating mats). To investigate and compare the species composition of these small-scale blooms with the large-scale more offshore green tides, we sampled these local floating macroalgae in June as well.

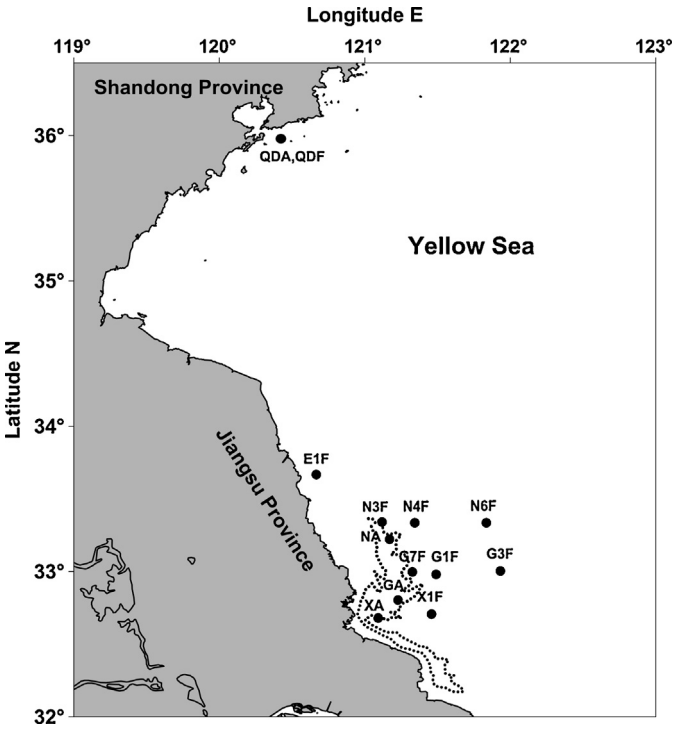

Fig. 1. Map of sample locations along the coast of the Yellow Sea. Dashed line indicated the area of recent expansion of *Porphyra* aquaculture. See Table 2 for site abbreviations.

Fresh macroalgal samples were sealed in the plastic bags and transported to the laboratory in coolers. Individual plants were carefully separated after the macroalge clumps being soaked in distilled (DI) water. The plants were examined carefully and divided into several groups based on their gross morphology (Tseng, 1984; Hayden et al., 2003). Thalli of two to five plants for each morphological group were randomly taken for DNA extraction, and the rest of the sample was weighted, dried at room temperature and stored at 4 °C for further research.

2.2. DNA extraction and PCR amplification

Before DNA extraction, individual thalli were rinsed three times with DI water, frozen and thawed twice and homogenized with pellet pestles. Then genomic DNA was extracted using E.Z.N.A.™ HP plant DNA kits (OMEGA Bio-tek Inc., GA, USA) following the manufacture's protocol.

Polymerase chain reaction (PCR) amplifications of ITS rRNA gene (including ITS1, 5.8S, ITS2 regions) were performed as in Leskinen and Pamilo (1997) and Hayden et al. (2003) using the primer pair: forward 5′-TCGTAACAAGGTTTCCGTAGG-3′, reverse 5′-GCTTATTGATATGCTTAAGTTCAGCGGGT-3′. Amplicons were then electrophoresed on 1% agarose gels and visualized after ethidium bromide staining.

In order to distinguish *Ulva prolifera* and *Ulva linza* which could not be separated by ITS sequence polymorphism (Leliaert et al., 2009), the 5S spacer regions were amplified following the protocol of Yotsukura et al. (2002) and Shimada et al. (2008) using the primer pair: forward 5′-GGTTGGGCAGGATTAGTA-3′ and reverse 5′-AGGCTTAAGTTGCGAGTT-3′. The PCR products were separated on a 2% agarose gel. Based on the sequence analyses on both sequences downloaded from GeneBank database and obtained in this study for *U. prolifera* and *U. linza*, length polymorphism was evident and could be used to differentiate these two taxa (Fig. 2).