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Molecular identification of green algae from the rafts based infrastructure of *Porphyra yezoensis*

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ABSTRACT

To provide more information on the origin of the *Ulva prolifera* bloom in Qingdao sea area in China from 2007 to 2011, the diversity of green algae growing on the rafts of *Porphyra yezoensis* on the coast in Jiangsu Province was investigated based on ITS, *rbc*L and 5S sequences. Eighty-four of green algal samples from various sites and cruises in 2010 and 2011 were collected. According to ITS and *rbc*L sequences, samples from the rafts of *P. yezoensis* fell into four clades: *Ulva linza-procera-prolifera* (LPP) complex, *Ulva flexuosa*, *Blidingia* sp. and *Urospora* spp. However, based on the 5S rDNA, a more resolved DNA marker, only one of the 84 samples belonged to *U. prolifera*. Combined with the previous reports, it is likely that *U. prolifera* bloom in Qingdao sea area might consist of more than one origin, and *Porphyra* cultivation rafts might be one of the causes.

1. Introduction

The excessive growth of green algae species such as *Ulva*, *Enteromorpha*, *Chaetomorpha* and *Cladophora*, has been reported in the formation of macroalgal blooms or green tide events in many parts of the world including Europe, North America, South America, Japan and Australia (Fletcher, 1996; Morand and Briand, 1996; Hiraoka et al., 2004; Morand and Merceron, 2005; Merceron et al., 2007). One of the green tide algae, the filamentous alga *Ulva prolifera*, formerly known as *Enteromorpha prolifera* (Hayden et al., 2003), is broadly distributed along the nearshore coasts of the north-eastern Asia (Shimada et al., 2008). In the Yellow Sea of China, large-scale green algal blooms have occurred for five consecutive years from 2007 to 2011 (Jiang et al., 2008; Sun et al., 2008; Tian et al., 2011). Especially in the summer of 2008, the world's largest green tide occurred along the coast of the Yellow sea near Qingdao, China, which caused severe social problems as well as marine ecological issues. The dominant bloom algal species in 2007–2009 was identified to be the *Ulva linza-procera-prolifera* (LPP) based on ITS and *rbc*L analysis (Hayden et al., 2003; Leliaert et al., 2009; Wang et al., 2010; Liu et al., 2010b,c). Later, Duan et al. (2012) amended it to *U. prolifera* based on 5S phylogenetic analysis.

It is crucial to correctly identify the origin of the bloom for understanding the large-scale green tide and exploring solutions to the problems it can potentially cause. According to satellite images from 2008 to 2009, the drifted biomass initiated offshore of the coast of Jiangsu Province and was transported across the Yellow Sea to Qingdao coast by seasonal winds and surface currents (Sun et al., 2008; Liu et al., 2009). However, the original "seed" source of the drifting bloom remained unclear. Some reports proposed that the accumulation and disposal of waste *U. prolifera* from *Porphyra* cultivation rafts was the most probable cause of the blooms (Keesing et al., 2011; Wang et al., 2007; Liu et al., 2009). *U. prolifera* was the dominant fouling species growing on the rafts based infrastructure of *Porphyra yezoensis* aquaculture. It was estimated that about 91–505 kg/ha *U. prolifera* was attached to *P. yezoensis* in the coast of Jiangsu Province, and a total biomass came up to 4956 tons during the harvesting of *P. yezoensis* (Liu et al., 2010a). This was sufficient to seed a bloom when they were dislodged from the rafts as a result of harvesting practice (Liu et al., 2010a). However, based on the ribotype analysis of the free-floating *U. prolifera* samples in 2008 and 2009 blooms, Duan et al. (2012) proposed that the bloom might be derived from the Sea of Japan. Similarly, Pang et al. (2010) found that the haplotypes of the Yellow Sea free-floating *U. prolifera* were closely related to those from Japanese coast but less to European and American algae. Together with the similarity of samples from *P. yezoensis* farming rafts to *U. linza* in the morphology, they presented that land-based animal aquaculture ponds along the Jiangsu Province coast were the source of the green tide algae.

To provide more information on the origin of the massive drifting green tide, here we investigated the diversity of the green algae growing on the rafts of *P. yezoensis* in the Gaoni, Niluosha, Xiaoyangkou of the Jiangsu Province in China from November

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2010 to April 2011. The growing green algae twined on the rafts, with highly variable morphology at different developmental stages and environmental conditions. So, morphological identification was insufficient to distinguish among the different algae species, especially among the *Ulva* genus. In this study, the diversity of the green algae was investigated based on the phylogenetic analysis with the sequences of 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). In addition, 5S rDNA phylogenetic analysis was conducted to distinguish among the species in the LPP complex (Shimada et al., 2008; Duan et al., 2012).

2. Materials and methods

2.1. Collection of samples

Rudong (RD), is the main coastal city for *P. yezoensis* aquaculture in Jiangsu Province. It is located in the south-western coast of the Yellow Sea. So, three sites near to RD, Xiaoyangkou, Gaoni and Niluosha were chosen for exploring the distribution of green algae attached to the *P. yezoensis*. Green algae samples at the three sites (Fig. 1) were taken monthly from November-2010 to May-2011 (Table 1). To learn the relationship between diversity of the green algae attached to the *P. yezoensis* and dominant bloom algal species in Qingdao, totally 84 samples from *P. yezoensis* and 3 samples (qingdao1, qingdao2 and qingdao 724) from Qingdao sea area in China were collected during the 2011 bloom.

2.2. Treatment of algae

The green algae collected from the rafts of *P. yezoensis* were cleaned *in situ* and brought back to the laboratory in cooled box within 24 h. After cleaning again with sterilized seawater, the algal samples in good growth condition were sorted out and cultured in PES medium (Berges et al., 2001) for one week to remove epiphytic diatoms for further DNA analyses.

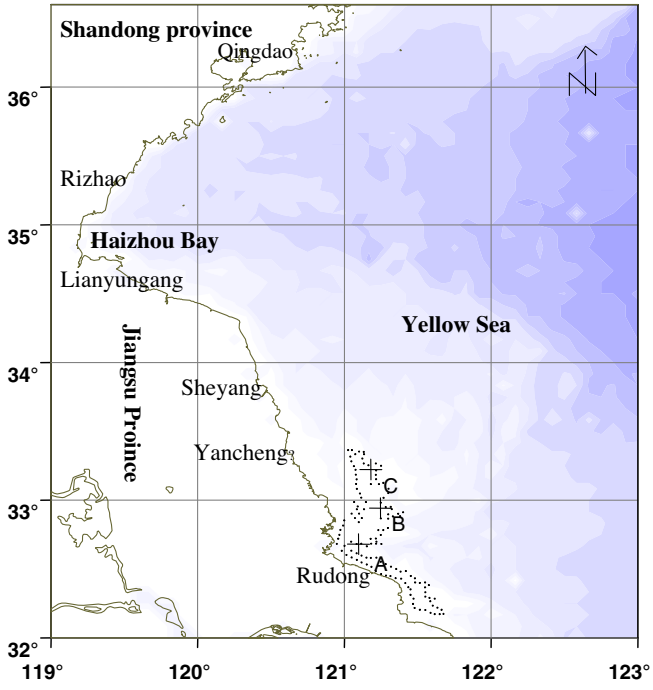

Fig. 1. The sampling sites in the Jiangsu coast. A, B and C indicate Xiangyangkou, Gaoni and Niluosha, respectively.

2.3. DNA extraction

The fresh algal samples were washed three times with sterilized water, and then dried with filter paper. Unialgal material for each sample was detached carefully for DNA extraction. Total DNA was extracted according to the manual of HP plant DNA extract kit (Omega, USA). DNA quality was examined by 1% TAE agarose gels stained with GoldView.

2.4. ITS rDNA, rbcL gene and 5S rDNA spacer amplification and sequencing

PCR primers for ITS, *rbc*L and 5S listed in Table 2 were synthesized by Shanghai Sangon Biologic Engineering Technology and Service Co. Ltd., China. The PCR amplifications of ITS nrDNA and *rbc*L genes were performed as described by Leskinen and Pamilo (1997) and Manhart (1994). As for 5S rDNA, the PCR was conducted as reports by Shimada et al. (2008) and Duan et al. (2012), in which the primer pair 5SF-5SR locates to the 5S rDNA tandem arrays amplified multiple DNA fragments. Total genomic DNA (30–40 ng) was added to 50 μL PCR reactions containing 1× PCR buffer (Takara, Dalian, China), 0.8 mM dNTPs (Takara), 25 mM of each primer and 1.6 U *Taq* Polymerase (Takara). PCR was carried out in a MJ MiniTM Gradient Thermal Cycler (BIO-RAD). PCR profiles for different genes were set as follows: ITS nrDNA amplification included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min 10 s, 54 °C for 50 s and 72 °C for 1 min 30 s; the *rbc*L gene was amplified according to the reaction profile (94 °C for 3 min, followed by 35 cycles of 94 °C for1 min, 45 °C for 2 min, and 65 °C for 3 min) and the final step at 72 °C for 10 min; the 5S amplification reaction profile included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were resolved by 1.0% agarose gel electrophoresis, excised by the gel purification method using the QIAquick DNA Gel Purification Kit (QIAGEN, USA) and sequenced by Invitrogen (Invitrogen, China). When the ITS and 5S rDNA spacer fragments could not be directly sequenced, PCR products were purified using the QIAquick DNA Gel Purification Kit (QIAGEN). PCR products were cloned into pMD19-T Vector (Takara, China) according to the manufacturer's instructions, and sequenced by Invitrogen (Invitrogen, China).

2.5. Phylogenetic analysis

The phylogenetic trees were constructed by neighbor-joining (NJ) method using the program Mega 4.0 (Tamura et al., 2007). The reliability of branches was evaluated with non-parametric bootstrapping (1000 replicates) (Felsenstein, 1985). The evolutionary distances of the NJ tree were computed using the Kimura 2-parameter method (Kimura, 1980). Branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances were used to infer the phylogenetic tree.

3. Results

3.1. Morphology and phylogenetic analysis based on ITS and rbcL sequences

Based on the morphology, we found that green algae were widely distributed in *Porphyra* cultivation rafts. Among the 84 samples, 41 samples belonged to *Blidingia* sp. However, it was difficult to further distinguish among the green algae due to the variability