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Population differentiation in the dominant species (*Ulva prolifera*) of green tide in coastal waters of China

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Abstract

Since 2015, green tides with *Ulva prolifera* as the dominant species in the Qinhuangdao coastal waters have continued to occur. In this study, the relationship between green tides in Qinhuangdao and the Yellow Sea (setting sites in Rudong and Qingdao) was evaluated by genetic analyses of *U. prolifera*. Single nucleotide polymorphism (SNP) markers were used to analyze genetic diversity and genetic relationships among groups. Genetic differentiation was lower among floating *U. prolifera* populations in Rudong and Qingdao than in Qinhuangdao. The floating *U. prolifera* population had higher genetic diversity and polymorphism levels in Qingdao and Rudong than in Qinhuangdao. Physiological experiments showed that the growth rate and net buoyancy of floating *U. prolifera* were highest in Qinhuangdao and Qingdao, respectively, under the same environmental conditions (temperature and light). Overall, these findings showed that *U. prolifera* populations in the Qinhuangdao and Yellow Sea green tides (Rudong and Qingdao) differ significantly at the molecular and physiological levels. Therefore, the Qinhuangdao green tide is not correlated with the Yellow Sea green tide and has a different origin and development mode. This study provides insight into the mechanism underlying green tide blooms in coastal waters of China.

Key words: *Ulva prolifera*, green tide, dominant species, population differentiation, Qinhuangdao

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1 Introduction

Green tide is an ecological anomaly caused by large attached green algae floating away from adhesion substrates and continuously proliferating or aggregating (Taylor et al., 2001; Blomster et al., 2002). It is a common ecological disaster in coastal countries worldwide. A small-scale green tide with *Ulva prolifera* as the dominant species was the first green tide event reported in China in the coastal waters of Qingdao in 2007 (Wang et al., 2015). Large-scale *U. prolifera* blooms have occurred every year since 2008 in the Yellow Sea, seriously impacting the economy and ecological environment along the coasts of Jiangsu and Shandong (Liu et al., 2009; Huo et al., 2015). Moreover, green tides have been reported in coastal areas of Qinhuangdao from April to September every year since 2015 from the Tanghe River Estuary to Jinshanzui Beach (Han et al., 2019; Song et al., 2019a, b). Several algae accumulate on the beach during the green tide bloom, seriously affecting the local ecological environment and tourism. Qinhuangdao is the second offshore area in China and the first area in the Bohai Sea with periodic green tide blooms (Han et al., 2019; Song et al., 2019b).

The Yellow Sea green tide originates from attached green algae on the *Pyropia yezoensis* aquaculture rafts on the Subei Shoal (Han et al., 2013, 2020; Fan et al., 2015; Wang et al., 2018; Cui et al., 2019; Liu et al., 2021). Morphological and molecular analyses have revealed five to seven species of attached algae on the *P. yezoensis* aquaculture rafts, including *Blidingia* sp., *U. prolifera*, *U. linza*, *U. compressa*, *U. intestinalis, U. flexuosa,* and *U. clathrata* (Duan et al., 2012; Fan et al., 2015). *U. prolifera* is the dominant species of the green tide in the Yellow Sea due to its rapid growth and floating ability (Wang et al., 2015; Zhao et al., 2015). Floating *U. prolifera* first appeared in Rudong coastal areas in Jiangsu Province (Fan et al., 2012). The green tide algae then moved northward due to the monsoon current, eventually forming a large-scale green tide in Qingdao offshore areas (Zhang et al., 2013; Huo et al., 2014). Buoyancy promotes *U. prolifera* migration over long distances, since the species relies on the surface current for movement (Collins et al., 2010).

The Qinhuangdao green tide mainly affects Jinmenghaiwan Beach and occurs at the same time as the green tide in the Yellow Sea, from April to September. *Ulva prolifera* is the dominant species of the Qinhuangdao green tide (Han et al., 2019; Song et al., 2019b). It is unclear whether *U. prolifera* in this area originated from large-scale green tides in the Yellow Sea. Furthermore, it is unclear whether there are differences among the floating *U.*

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prolifera populations in the Yellow Sea and coastal waters of Qinhuangdao. The field survey revealed that most *U. prolifera* in the coastal waters of Qinhuangdao were filamentous and suspended in the water column. Therefore, there may be differences in growth and floating ability compared to *U. prolifera* causing green tide in the Yellow Sea.

The 2b-RAD technology is widely used for population genetic and evolutionary analyses, auxiliary genome assembly, whole-genome selection, and other applications (Wang et al., 2016). This technique is widely used owing to the uniform size of the enzymatically cut DNA fragments, the simplicity and speed of library construction, the ease of adjusting the label density, the low cost, and the high accuracy. In this study, differences among populations of floating *U. prolifera* in coastal waters of China were evaluated at the molecular and physiological levels. Floating *U. prolifera* samples collected from the Yellow Sea (setting sites in Rudong and Qingdao) and the coastal waters of Qinhuangdao were evaluated by 2b-RAD simplified genome sequencing and physiological assays. Our results provide a scientific basis and theoretical support for understanding the mechanism underlying green tide blooms in coastal waters of China.

2 Materials and methods

2.1 *Sample collection*

Ten floating *U. prolifera* samples were collected separately in Rudong (32.68°N, 121.09°E), Qingdao (36.05°N, 120.43°E), and Qinhuangdao (39.89°N, 119.54°E) from May to July 2019 based on the time of green tide blooms in the Yellow Sea and Qinhuangdao. Sample information, including sampling locations, are shown in Table 1 and Fig. 1. Samples were cleaned thrice using sterilized seawater on-site, dried with absorbent paper, stored in liquid nitrogen, and transported to the laboratory within 48 h.

2.2 *Species identification*

The macroalgal samples were rinsed with sterile seawater to remove sediment, debris, and epiphytes. The samples were then placed on white enamel discs filled with sterile seawater. The green macroalgal species were identified and separated based on shape, branching, cell arrangement, and locations of pyrenoids and chloroplasts (Ding et al., 2008; Duan et al., 2012).

Total genomic DNA was extracted from samples according to the manual of the Plant Genomic DNA Kit (Tiangen Biotech, China) and stored at −20°C for further analysis. Internal transcribed spacer (ITS) sequences and 5S ribosomal DNA (5S rDNA) spacer sequences were chosen for species identification (Han et al., 2013). The neighbor-joining method was used to construct a phylogenic tree and 1 000 bootstrap replicates were used to evaluate branch support.

2.3 *Sequencing*

The concentration and quality of DNA products were detected by gel electrophoresis and the NanoDrop2000. The Illumina HiSeq Xten platform (Shanghai Oe Biotech Co., Ltd., China) was used for genome determination. A tag sequencing library of 30 samples was constructed using 2b-RAD technology, and paired-end sequencing was performed with HiSeq X-TEN platform. All samples were connected with standard 5′-NNN-3′ joint and enzyme digestion tags to construct a library. Clean reads were obtained by removing (1) reads containing the joint sequence, (2) reads containing >8% N bases, and (3) low-quality reads (more than 15% of bases with quality <Q30).

Table 1. Sample information

Location	Sample number	Collection date
Rudong	RD-1, RD-2, RD-3, RD-4, RD-5, RD-6, RD-7, RD-8, RD-9, RD-10	May 21, 2019
Qingdao	QD-1, QD-2, QD-3, QD-4, QD-5, QD-6, QD-7, QD-8, QD-9, QD-10	June 24, 2019
Qinhuangdao	QHD-1, QHD-2, QHD-3, QHD-4, QHD-5, QHD-6, QHD-7, QHD-8, QHD-9, QHD-10	July 18, 2019

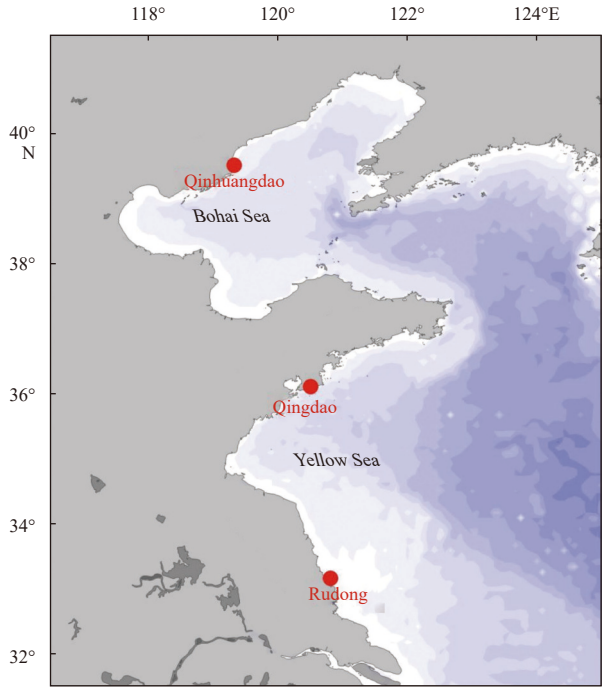

Fig. 1. Sampling stations in the surveyed sea.

2.4 *Whole-genome single nucleotide polymorphism (SNP) screening and genotyping analysis*

Reads containing enzyme recognition loci were extracted from sequencing data. Ustacks (version 1.34) from the Stacks package was used to construct the reference sequence. Simple Object Access Protocol (SOAP) (version 2.21) was used to compare the sequencing data with the reference sequence. The maximum likelihood method was used for genotype calling. Loci with call rates of less than 80%, a minor allele frequency of <0.01, containing only one allele, containing four alleles, containing only one genotype, and containing more than two SNPs were eliminated.

2.5 *Buoyancy and growth rate of U. prolifera in different areas*

Floating algae samples were collected separately in Rudong, Qingdao, and Qinhuangdao during the green tide bloom in 2019, stored at 4–6°C, and taken to the laboratory within 48 h. Morphological and molecular analyses were then used to identify the samples as *U. prolifera*. The algal samples were cultured at 20°C for 3 d; then, buoyancy and growth experiments were conducted.

Ulva prolifera was continuously cultured for 7 d under different environmental conditions to measure buoyancy. The light intensity was set to 50 μmol/(m²·s), 100 μmol/(m²·s), 150 μmol/(m²·s), and 200 μmol/(m²·s) and the temperature was set at 20°C. The buoyancy of *U. prolifera* (F_B) was measured as shown below. A positive F_B represents the vertical upward force, indicating that algae can keep floating in the upper layer of water. Three replicates per sample were evaluated to reduce measurement error.

$$F_B = -gV(\rho_a - \rho_w), \quad (1)$$

The parameters of g, V, ρ_a, and ρ_w indicate the acceleration of gravity (9.81 m/s²), volume of algae, density of algae, and seawater density, respectively. The volume of algae was determined by measuring the volume of water increase in the cylinder after completely immersing the algae. An electronic balance was used to measure the mass of algae (±0.01 g) before the experiment. An absorbent paper was used to dry the algae before measuring weight. The density of algae was estimated by dividing the mass of each alga by its volume. A hydrometer was used to determine the density of the seawater in the field.

Ulva prolifera was continuously cultured for 7 d under different environmental conditions to measure its growth rate. *Ulva prolifera* (0.5 g) was cultured in 1 L sterile seawater with 20 mL of Provasoli-enriched seawater and 1 mL of 5 mg/L GeO_2 under a light intensity of 100 μmol/(m²·s) with a light cycle of 12 h:12 h. The temperatures were set at 10°C, 15°C, 20°C, and 25°C. Each group had three parallel experiments. The growth rate (GR, %/d) of macroalgae was calculated as follows:

$$GR = [\ln(W_t/W_0)]/t \times 100, \quad (2)$$

where W_0 and W_t indicate the wet weight of macroalgae at the beginning and at day t, and t represents the number of days of the experiment.

2.6 *Statistical analysis*

Genepop (version 4.2.2), PowerMarker (version 3.25), Genome-wide Complex Trait Analysis (version 1.25.0), ustacks (version 1.34), and SOAP (version 2.21) were used to analyze the sequencing results. All data measured or calculated are displayed as mean±standard deviation. Statistical analyses were performed using Origin 8.0. The significance level used was p<0.05.

3 Results

3.1 *Species identification*

The green algae collected in Rudong were dark green with filiform branches (Figs 2a, b). Algae were light green and yellow and were mostly filled with gas as they approached the offshore area of Qingdao (Figs 2c, d). The floating *U. prolifera* samples collected in the coastal waters of Qinhuangdao were similar to those in the Rudong area (Figs 2e, f). An ITS sequence analysis showed that the samples clustered in the *Ulva linza–procera–prolifera* complex (LPP) group (Fig. 3). Further 5S rDNA analysis of the LPP group showed that the samples all clustered in *U. prolifera* (Fig. 4).

3.2 *Sequencing quality and population genetic structure*

The average ratio of high-quality reads containing enzyme

Fig. 2. Morphological structure of floating *Ulva prolifera* at different locations. Green algae and its filiform branches of Rudong (a, b); green algae and its filiform branches of Qingdao (c, d); green algae and its filiform branches of Jinmenghaiwan, Qinhuangdao (e, f).

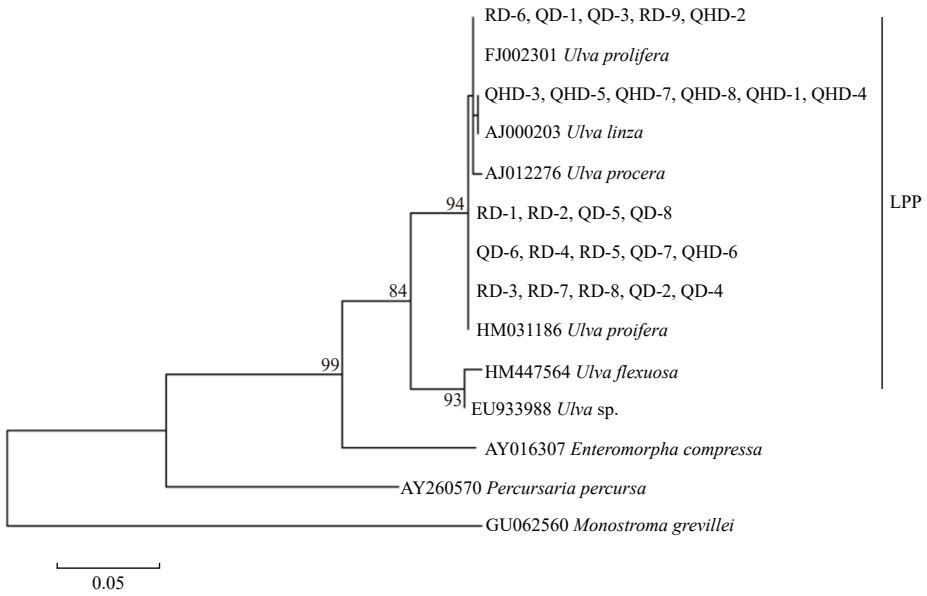

Fig. 3. Phylogenetic tree based on internal transcribed spacer sequences of *Ulva prolifera* at different locations. Bootstrap values (1 000 replicates) larger than 50% are indicated at the nodes. LPP is the abbreviation of *U. linza-procera-prolifera*.

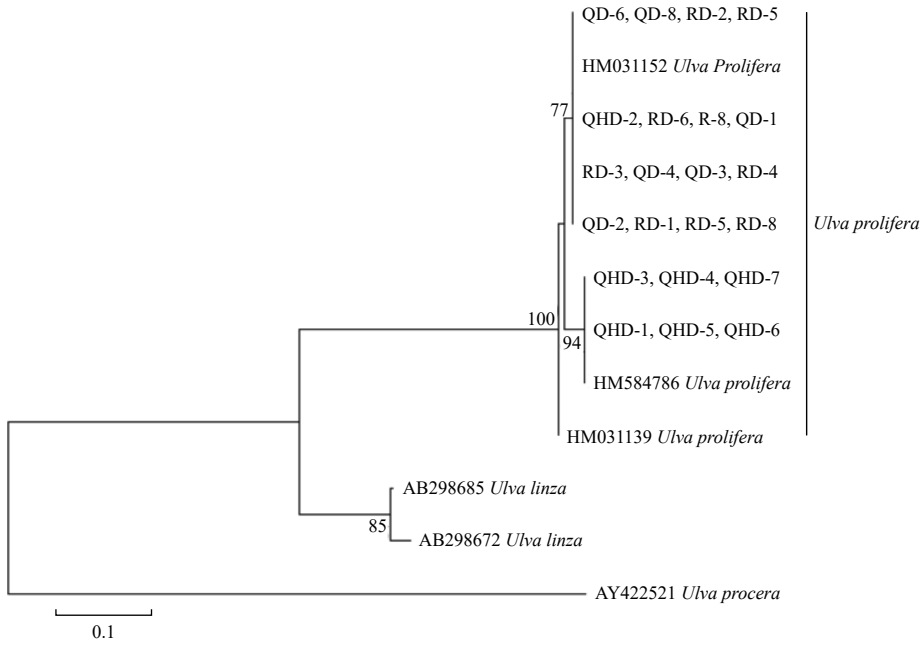

Fig. 4. Phylogenetic tree based on 5S sequences of *Ulva prolifera* from different locations. Bootstrap values (1 000 replicates) larger than 50% are indicated at the nodes.

loci and original reads sequenced in 30 sequencing libraries exceeded 55.14%. The label depth ranged from 13.4 to 81.6 (average value, 42.4), indicating a good sequencing quality of the floating *U. prolifera* library. Genetic differentiation (F_{ST}) was estimated for each SNP locus obtained after screening (1 295 SNP loci). The F_{ST} values for pairwise combinations of floating *U. prolifera* populations from Rudong, Qingdao, and Qinhuangdao ranged from 0.064 1 to 0.862 4. The F_{ST} value between Rudong and Qingdao was 0.064 1, indicating low genetic differentiation between the two populations. The F_{ST} values for Qinhuangdao vs. Rudong and Qinhuangdao vs. Qingdao were 0.862 4 and 0.859 4, respectively, indicating high genetic differentiation.

3.3 *Selective elimination analysis and population genetic diversity*

Tajima's D (a test of neutrality) was highest in the Rudong population (0.816 7) and lowest in the Qinhuangdao population (0.173 4). θ_π (an indicator of the degree of polymorphism within populations) was highest in the Rudong population (0.154), followed by the Qingdao population (0.120) and the Qinhuangdao population (0.083).

Average heterozygosity reflects genetic variation within populations, where higher values indicate higher diversity. The average observed heterozygosity (H_o) and expected heterozygosity (H_e) of floating *U. prolifera* populations were higher in Qingdao and Rudong than in Qinhuangdao. The polymorphism information content (PIC) is used to measure locus polymorphism; higher values indicate higher levels of variation and greater genetic diversity. PIC values for each SNP locus were highest in the Qingdao population (0.17) and lowest in the Qinhuangdao population (0.07) is shown in Table 2. These results indicated that genetic diversity in the floating *U. prolifera* population was higher in the Yellow Sea (Rudong and Qingdao) than in Qinhuangdao.

3.4 *Principal component analysis*

A principal component analysis (PCA) was used to evaluate the floating *U. prolifera* populations in Rudong, Qingdao, and Qinhuangdao. All samples in the three areas were represented in a scatter plot based on the first two principal components (PCs). PC1 and PC2 explained 68.53% and 30.59% of the total variance, respectively (cumulative contribution, 99.21%). Three-dimensional clustering showed that the floating *U. prolifera* populations in Rudong and Qingdao were closely related, with a lack of clear separation in the PCA plot. In contrast, the *U. prolifera* populations in Qingdao were clearly separated from the *U. prolifera*

Table 2. Genetic diversity in three *Ulva prolifera* populations

Population		H_e	H_o	PIC
Qinhuangdao	range	0–0.54	0–0.89	0–0.47
	average value	0.11	0.16	0.07
Qingdao	range	0–0.50	0–1	0–0.38
	average value	0.16	0.26	0.17
Rudong	range	0–0.50	0–1	0–0.38
	average value	0.14	0.21	0.15

Note: PIC is the abbreviation of polymorphism information content.

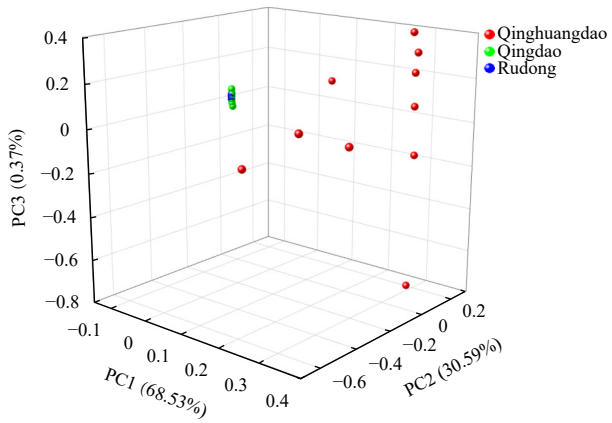

Fig. 5. Principal component analysis of three groups of *Ulva prolifera*.

populations in Qingdao and Rudong, with distinct classification boundaries and a scattered distribution (Fig. 5). The results showed that the Rudong and Qingdao populations had low genetic differentiation, while Rudong and Qinhuangdao populations and Qingdao and Qinhuangdao populations had high genetic differentiation.

3.5 *Phylogenetic analysis of U. prolifera*

SNPs in the 30 samples were used to construct a phylogenetic tree of *U. prolifera* populations. In total, 12 945 SNP markers were obtained for all samples. The *U. prolifera* populations in Rudong and Qingdao were closely clustered, while samples from Qinhuangdao clustered separately (Fig. 6). These results showed that *U. prolifera* populations in Rudong were closely related to those in Qingdao but not to those in Qinhuangdao.

3.6 *Growth rate and buoyancy under different temperatures and light conditions*

The growth rate and net buoyancy of floating *U. prolifera* in the Rudong, Qingdao, and Qinhuangdao areas affected by green tide were measured under different environmental conditions. The growth rate was significantly higher in the Qinhuangdao coastal area (23.6%) than in Rudong and Qingdao coastal areas (p<0.05) under the same temperature. Moreover, the growth rates in Rudong, Qingdao, and Qinhuangdao increased with increasing temperatures (Fig. 7). The net buoyancy was highest in Qingdao (0.052 N) and lowest in Qinhuangdao (p<0.05) under the same light conditions. The net buoyancy in the Rudong, Qingdao, and Qinhuangdao areas increased with increasing light intensity (Fig. 8).

4 Discussion

4.1 *Genetic diversity in U. prolifera populations*

In this study, 2b-RAD simplified genome sequencing technology was used to analyze genetic diversity in *U. prolifera* populations in Rudong, Qingdao, and Qinhuangdao coastal waters to verify the relationship between the green tide in coastal waters of Qinhuangdao and the Yellow Sea. The genetic differentiation between floating *U. prolifera* populations was low in Rudong and Qingdao, and large in Qinhuangdao. The Yellow Sea green tide originates from Subei Shoal coastal waters. The green tide algae cluster in Rudong and Dafeng coastal waters and then move northward under the action of the monsoon and current, finally forming a large-scale green tide in coastal waters of Qingdao after long-distance migration (Zhang et al., 2013; Huo et al., 2014). *Ulva prolifera* is the dominant species of the Yellow Sea green tide in Rudong and Qingdao. Therefore, it is easy to explain the low divergence of floating *U. prolifera* populations in these areas. Moreover, dominant species of the Yellow Sea green tide is a single species, with no gene flow, with other green algae during migration (Zhao et al., 2013, 2015; Zhang et al., 2018). Genetic differentiation among *U. prolifera* populations was high between Rudong and Qinhuangdao, and between Qingdao and Qinhuangdao, indicating substantial differences. Population genetic analysis, principal component analysis, and phylogenetic analysis based on individual shared tag sequences demonstrated that there is a high degree of divergence in the dominant species *U. prolifera* between the Yellow Sea and offshore waters of Qinhuangdao. Therefore, the Qinhuangdao green tide is not correlated with the green tide in the Yellow Sea, with a distinct origin and progression.

The 2b-RAD technology enables whole-genome high-throughput SNP screening and genotyping by a bioinformatics

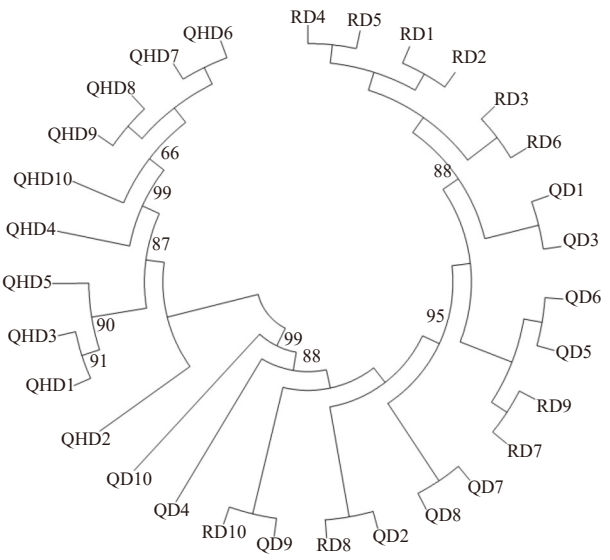

Fig. 6. Evolutionary relationships among *Ulva prolifera* populations based on the neighbor-joining method. RD: Rudong; QD: Qingdao; QHD: Qinhuangdao.

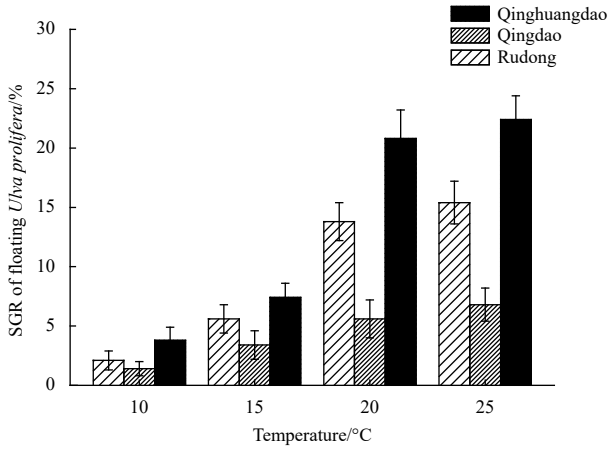

Fig. 7. Specific growth rates (SGR) of *Ulva prolifera* in different regions at various temperatures.

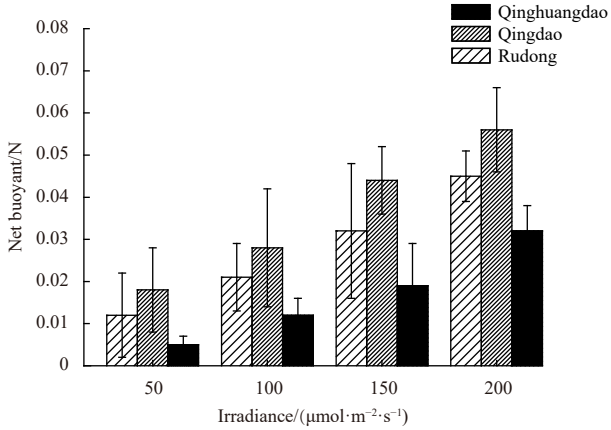

Fig. 8. Net buoyancy of *Ulva prolifera* in different areas under various light intensities.

approach. Batch SNP loci can be used for analyses of population genetic diversity (Fu et al., 2013). SNP genotyping was used in this study to compare the PIC and population genetic diversity of floating *U. prolifera* populations in different coastal areas. Tajima's D takes a value of zero under the standard neutral evolutionary model and deviates from zero under non-neutral evolution and demographic changes (Van Inghelandt et al., 2010). Herein, Tajima's D was highest in the Rudong population (0.816 7), indicating that this population might be affected by population expansion and natural selection. Tajima's D values for the Qingdao and Qinhuangdao populations deviated from zero to different degrees. θ_{π} is an important index of the degree of polymorphism within populations. Herein, θ_{π} was highest in the Rudong population, followed by the Qingdao population and the Qinhuangdao population. Average heterozygosity and the PIC are additional indicators of population genetic diversity. The average heterozygosity and PIC of floating *U. prolifera* populations were higher in Qingdao and Rudong coastal areas than in Qinhuangdao. In conclusion, the floating *U. prolifera* population in the Yellow Sea (Rudong and Qingdao) has higher genetic diversity and higher degree of polymorphism within populations than Qinhuangdao.

4.2 *Differences in physiological characteristics of U. prolifera populations in different areas*

Studies have shown that temperature significantly affects the growth rate, reproduction, and community succession of attached green algae (Fan et al., 2015; Song et al., 2015; Wang et al., 2018). The suitable temperature range for *U. prolifera* growth is 10–20°C. The *U. prolifera* growth rate can reach 23% when the temperature exceeds 15°C (Taylor et al., 2001; Largo et al., 2004). The growth rates of *U. prolifera* differed significantly under different temperatures and states (Cui et al., 2015). *Ulva prolifera* in Qinhuangdao was bright green with filiform branches, different from that in Rudong and Qingdao, which had a good physiological status. The growth rate of *U. prolifera* was significantly higher in Qinhuangdao than in Rudong and Qingdao under the same temperature. Floating *U. prolifera* first appears in the adjacent waters of Rudong in Jiangsu Province from March to May (Fan et al., 2012). *Ulva prolifera* has been declining in the coastal waters of Qingdao. However, our results indicated that *U. prolifera* in Qingdao has a certain growth capacity after a period of culturing under appropriate environmental conditions. Moreover, the growth rate of floating *U. prolifera* was significantly lower in Qingdao than in Qinhuangdao and Rudong in Jiangsu Province waters. Floating *U. prolifera* in the offshore waters of Qinhuangdao had a higher growth capacity than that of the dominant species of the green tide in the Yellow Sea (Qingdao and Rudong).

Ulva prolifera has a hollow tube composed of a single layer of cells. Oxygen produced during photosynthesis fills the inner tube of the algae, thus maintaining buoyancy (Lin et al., 2011). Buoyancy is achieved through the accumulation of air produced by photosynthesis inside the filamentous algae. Previous studies have shown that the morphology of *U. prolifera* is highly plastic and varies with respect to environmental conditions during the green tide bloom (Zhang et al., 2013; Gao et al., 2016). Herein, the morphology of *U. prolifera* varied, from dark green and filamentous in the early stage of the Yellow Sea green tide (Rudong) to yellow and tubular (full of gas) in the late stage of the green tide (Qingdao). The morphological changes are considered a physiological response to various environmental changes, such as changes in nutrient levels and light (Zhang et al., 2013; Gao et al., 2016). The *U. prolifera* samples collected from coastal waters of Rudong and Qinhuangdao had similar morphologies (i.e., mostly filamentous). *Ulva prolifera* samples from Rudong and Qinhuangdao had a significantly lower net buoyancy than that of samples from the coastal waters of Qingdao. These results indicate that the buoyancy of *U. prolifera* is closely related to its morphological and physiological status.

Buoyancy is also highly dependent on light conditions (Fu et al., 2019). The gas in the *U. prolifera* tube is mainly oxygen. Light intensity significantly affects the amount of oxygen produced via photosynthesis. Low light may reduce the amount of oxygen in tubular algae, making them sink (Xu et al., 2014). *Ulva prolifera* also sink after successive cloudy or rainy days (Fu et al., 2019), possibly due to reduced oxygen production caused by insufficient light. Therefore, environmental factors, especially light intensity, affect the buoyancy of macroalgae. Our results also showed that light intensity is positively correlated with the buoyancy of macroalgae. The buoyancy of *U. prolifera* increased in different areas with increasing light intensity within a certain range. Floating capacities were higher for the *U. prolifera* population in the Yellow Sea (Rudong and Qingdao) area than in the Qinhuangdao coastal area. A field investigation also showed that most *U. prolifera* in Qinhuangdao coastal area were suspended on the water surface, unlike *U. prolifera* in Rudong and Qingdao coastal area, floating on the sea surface. However, the molecular mechanisms underlying these physiological phenomena are still unclear. Wang et al. (2019) found that several growth-related genes, including pyruvate kinase (PK) and nitrate transporter (NRT), are enriched in *U. prolifera* compared with three co-occurring *Ulva* species, further explaining the dominance of *U. prolifera* in the green tide in the Yellow Sea. Therefore, the molecular mechanisms underlying the growth and floating capacity of *U. prolifera* in coastal waters of China will be evaluated in subsequent studies.

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