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Article

Distribution and Identification of *Ulva aragoensis* (Ulvaceae, Chlorophyta), a Constituent Species of Green Tides in the Southern Yellow Sea, Based on Molecular Data

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Abstract: Green tides are a serious global ecological disaster; the largest occur in the Southern Yellow Sea (SYS). Early-stage green tides in the SYS are composed of four species (*Ulva prolifera*, *Ulva flexuosa*, *Ulva linza*, and *Ulva compressa*). We found that *U. aragoensis* is a constituent species of green tides in the SYS based on molecular data. Furthermore, this study re-evaluated the proportion of *U. aragoensis* in green tides and found that it was more prevalent in micro-propagules cultured from surface seawater during an early-stage green tide in 2021. The internal transcribed spacers, *tuf* A, 18S, *rbc*L, large subunit, *psb*A, and rps2-*trn*L gene sequences were compared; the *tuf* A and *rbc*L gene sequences were the most suitable DNA barcodes for distinguishing *U. aragoensis*. A haplotype analysis of the sequences of floating *U. aragoensis* and its micro-propagules was performed to study the correlation between green tide macroalgae and micro-propagules; close haplotype similarities occurred between them. This study further clarified the species composition of SYS green tides and provided a reference for assessing the relationship between micro-propagules and green tide macroalgae.

Keywords: green tide; *Ulva aragoensis*; *Ulva flexuosa*; micro-propagule; Southern Yellow Sea; haplotype

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1. Introduction

With the acceleration of urbanization, green tides are occurring frequently globally [1–4]. They are an ecological disaster caused by green macroalgae. The main causative genera are *Ulva*, *Chaetomorpha*, *Cladophora*, *Rhizoclonium*, *Percursaria*, and *Ulvaria* [5]. The green tides in the Southern Yellow Sea (SYS) are the largest in the world. They accumulate in the waters around the Subei Shoal from April to May every year. Driven by wind currents, the green tides constantly propagate and drift northward. From June to August, they land on the shore of Shandong Province, causing huge economic losses and ecological threats [6,7].

Studying the species composition of green tides is a prerequisite for exploring the source of green tides and for making effective management decisions. In the early stage of green tides in the SYS, the macroalgae species include many *Ulva* species such as *Ulva flexuosa*, *U. compressa*, *U. linza*, and *U. prolifera* [8]. *U. prolifera* is the dominant species because of its strong ability to resist stress and its rapid growth and reproduction rates [9]. The initial species composition of the green tides in the SYS is closely related to the species composition of the green macroalgae attached to *Neoporphyra* cultivation rafts in the radial sandbanks of Jiangsu Province, and the internal transcribed spacers (ITS) sequences of floating *U. flexuosa* and *U. compressa* in the early period of the green tides have shown a 100% homology with the green macroalgae attached on the *Neoporphyra* cultivation rafts [10]. This indicates that the floating species of the green tides in the SYS may originate from *Neoporphyra* cultivation rafts. From 2009 to 2012, *U. flexuosa* and *U. compressa* were the main floating species in the early-stage green tides; *U. prolifera* was not present. However, since

2012, *U. prolifera* has become the first floating green tide macroalgae in the early stage, and it occurs throughout the whole process of the early floating green tide. This may be related to the annual increase in sea temperature [10,11].

Due to the morphological similarity among species, *Ulva* exhibits large intraspecies morphological and cytological changes under different growth stages and environmental conditions [12,13], which makes it difficult to distinguish green tide species based on morphology alone. A convenient and effective method for distinguishing morphologically indistinguishable green macroalgae is the use of molecular markers. The *rbc*L gene sequence is widely used for distinguishing *Ulva*; however, it cannot effectively distinguish between *U. linza-prolifera-procera* (LPP) clusters [6,12,14]. Similarly, ITS cannot distinguish between LPP clusters, and the *tuf* A marker gene has a higher sensitivity than *rbc*L and ITS; however, the use of *tuf* A for *U. linza* and *U. prolifera* distinction remains controversial [5,15]. The 5S ribosomal DNA (5S rDNA) spacer sequence is the most widely used method for distinguishing LPP clusters, and ITS+5S rDNA is an effective method for distinguishing green tide macroalgae [16]. Furthermore, rps2-*trn*L can also effectively distinguish *U. prolifera*, *U. flexuosa*, *U. compressa*, and *U. linza*, which are commonly found in SYS green tides [17]. While DNA-based approaches provide a better understanding of species boundaries, they are associated with issues such as conflicts between different methods or markers and species-level differences indicated by the level of uncertain sequence divergency [18,19]. In addition to morphology-based taxonomy, crossing experiments have been used to test biological species concepts and refine species boundaries, with considerable application in *Ulva*. Recent studies have combined DNA and morpho-anatomical data and performed crossing experiments to describe new species such as *Ulva ohnoi* and *Ulva limnetica* [20,21] or to elucidate species boundaries in taxonomically challenging complexes such as that of *U. prolifera* and *U. linza* [22].

Ulva flexuosa is a significant constituent of early green tides and micro-propagules [10,19]. Through hybridization, Hiraoka et al. [22] discovered another species, which was previously mistaken as *U. flexuosa* and *U. californica*, and named it *Ulva mediterranea*, the genetic information of which was updated in GenBank. *Ulva mediterranea* is based on the type specimen of *Enteromorpha aragoensis* Bliding (1960, p. 174, 'Aragoensis') from Banyuls, Mediterranean France; however, the name *Ulva mediterranea* is a superfluous name change [23–25]. It is widely distributed globally and is particularly concentrated along the eastern coast of the United States and the Gulf Coast [26]. *Ulva aragoensis* may be an early floating species and micro-propagule species of green tides in the SYS. Therefore, it is necessary to reassess the species composition of green tides in the SYS. Investigating the role of *U. aragoensis* in green tide outbreaks in the SYS is of great significance for assessing its potential to cause such ecological disasters.

At present, it is widely accepted that the green tides in the SYS originate from *Neoporphyra* cultivation rafts in the north of the Subei Shoal [7,27]; however, the stage from macroalgae detachment to floating is still unclear. A large number of micro-propagules have been found across a wide area of the SYS. Micro-propagules including gametes, spores, zygotes, micro-germlings, and vegetative fragments of macroalgae are part of the life history of opportunistic macroalgae [28,29]. The number of micro-propagules is related to the biomass of green tide macroalgae [28,30]. However, it remains difficult to determine the contribution of micro-propagules to green tides in the SYS. The micro-propagules germinate and grow on the *Neoporphyra* cultivation rafts and form green tide after detachment. The haplotypes of the micro-propagule *U. aragoensis* and the floating green tide *U. aragoensis* should be closely related in gene sequences.

Therefore, this study investigated the proportion of *U. aragoensis* in an area of a green tide outbreak in the SYS in 2021 and conducted multiple primer (ITS, *tuf* A, 18S, large subunit [LSU], *psb*A, *rbc*L, and rps2-*trn*L) sequencing for *U. aragoensis* in order to improve understanding of the species composition of the green tides and micro-propagules. The sequences of *U. aragoensis* and other *Ulva* species in the National Center for Biotechnology Information (NCBI) database were compared and analyzed based on the molecular markers.

The findings of this study can help clarify the classification of *U. aragoensis* in the *Ulva* genus and determine its role in green tides in the SYS, which is of great importance for the management and control of green tides.

2. Materials and Methods

2.1. Sampling Point Setting and Sampling Process

A total of three sample collection voyages (6–9 May 2021, 14–16 May 2021, and 15–18 July 2021) were conducted during a green tide outbreak in the SYS. The sampling site was located in the green tide outbreak area of the SYS (between 33°–37° N and 119°–122° E) (Figure 1). A WP2 zooplankton net was used to randomly collect large floating green macroalgae at the sampling site, and 1.5 L of surface seawater was collected using a water-sampler (HQM-1, Jiangsu, China). The surface seawater was filtered through a sieve (200 μm) and transported to the laboratory where it was stored at 4 °C away from light for further micro-propagule cultivation.

2.2. Micro-Propagule Cultivation and Pretreatment of Green Macroalgae Samples

The surface seawater samples from each site were divided into three 500 mL beakers, used as parallel samples (n = 3). The concentrations of dissolved inorganic N and PO_4-P in the water were 500 μmol/L and 30 μmol/L, respectively, after being enriched with von Stosch's enriched medium. At the same time, 500 μL of saturated GeO_2 was added to the water to inhibit microalgal growth. The enriched surface seawater samples were then incubated in a light incubator with a temperature of 18 ± 2 °C, a light intensity of 100 μmol/(m^2·s), and a light cycle of 12 h light (L):12 h dark (D). After 3–4 weeks of incubation, green macroalgae seedlings approximately 5–10 cm long on the walls and bottom of the beaker were visible to the naked eye. The seedlings were carefully scraped out of the beaker, and the number of individual seedlings was counted and regarded as the number of micro-propagules in the water. The seedlings were washed with sterilized seawater, dried with absorbent paper, and stored in a refrigerator (Haier, Qingdao, China) at −20 °C [11,31–33]. The abundance of micro-propagules (A, inds·L^{-1}) was calculated as follows:

$$A = N/V \text{ (N: total number of germlings, V: volume of water sample cultured).} \quad (1)$$

In addition, floating green macroalgae were collected at each sampling point (Figure 1) and transported back to the laboratory at 4 °C. After removing miscellaneous algae from the surface of the macroalgae with a brush in the laboratory, the samples were cleaned with sterilized seawater, dried with absorbent paper (Vinda, Jiangmen, China), and stored in a refrigerator at −80 °C.

2.3. Identification of U. aragoensis

The collected green tide floating macroalgae and surface seawater micro-propagules were roughly classified according to their morphological characteristics, and the morphology of the macroalgae was recorded under a microscope (Nikon, Tokyo, Japan) in the laboratory. Three single algae (n = 3) of each form from the green tide floating macroalgae and surface seawater micro-propagules at each site were then selected and cleaned with sterile seawater for DNA extraction.

The DNA of macroalgae samples was extracted using Dzup (Plant) Genomic DNA Isolation Reagent produced by Sangon Biotechnology (Shanghai) Co., Ltd. (Shanghai, China). The *rbc*L sequence primers used are shown in Table 1, and the amplification procedure is detailed in Table 2. The amplification products of the *rbc*L sequence were sent to Sangon Biotechnology Co., Ltd. for Sanger generation sequencing [32].

The *rbc*L gene sequences obtained by Sanger sequencing were manually proofread, and the Basic Local Alignment Search Tool of the NCBI was used to confirm the species of the macroalgae samples. The proportion of *U. aragoensis* in the floating green macroalgae

and micro-propagule samples was calculated, and Origin 2022 (OriginLab, Northampton, MA, USA) was used to plot the histogram and for data analysis.

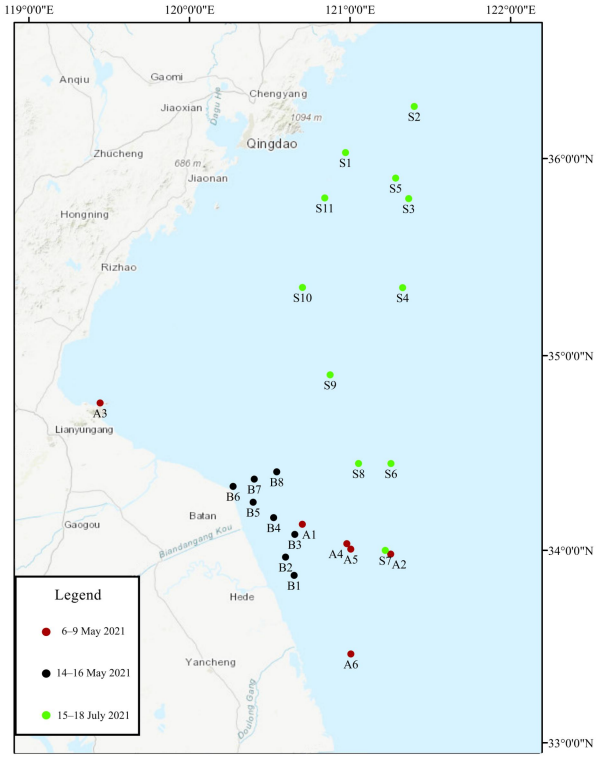

Figure 1. Distribution of green tide outbreak sampling sites in the Southern Yellow Sea in 2021. Different colors represent different sample collection times. Red represents samples collected from 6 May 2021 to 9 May 2021, black represents samples collected from 14 May 2021 to 16 May 2021, and green represents samples collected from 15 July 2021 to 18 July 2021.

2.4. Sequencing of Multiple DNA Barcodes and Phylogenetic Tree Construction of U. aragoensis

Twenty-five *U. aragoensis* samples were identified by *rbc*L gene sequences, and then multiple pairs of DNA-barcoding polymerase chain reaction amplification of the *tuf* A, 18S, LSU, *psb*A, and rps2-*trn*L gene sequences were performed on the detected *U. aragoensis*. At the same time, three *U. flexuosa* subsp. *flexuosa* (Uf1, Uf2, and Uf3) collected at 121°40′5.746″ E, 32°3′20.664″ N and *U. californica* (Uc1, Uc2, and Uc3) collected at 120°16′6.326″ E, 34°18′13.421″ N were identified by ITS sequences in the laboratory [33]. Their ITS, *rbc*L, *tuf* A, rps2-*trn*L, 18S, and LSU sequences were amplified for further analysis. The primer sequences are shown in Table 1, and the polymerase chain reaction procedures are shown in Table 2. The sequences of the relevant species were downloaded from the NCBI. Clustal X [34] software was used for multiple sequence alignment analysis of the proofread *U. aragoensis* sequences. A Kimura 2-Parameter model with MEGA v.7 software was used to construct the phylogenetic tree with the adjacency method, and the reliability of each branch was verified with bootstrap values for 1000 replicates [35].

Table 1. Polymerase chain reaction primers used in the study.

Primers	Sequence	Direction	Reference
ITS-F	5′-TCGTAACAAGGTTTCCGTAGG-3′	Forward	[36]
ITS-R	5′-TTCCTTCCGCTTATTGATATGC-3′	Reverse	[36]
tuf A-F	5′-GGNGCNGCNCAAATGGAYGG-3′	Forward	[37]
tuf A-R	5′-CCTTCNCGAATMGCRAAWCGC-3′	Reverse	[37]
18S-F	5′-GGAGGATTAGGGTCCGATTCC-3′	Forward	[38]
18S-R	5′-CTTCCGTCAATTCCTTTAAG-3′	Reverse	[38]

Table 1. *Cont.*

Primers	Sequence	Direction	Reference
LSU-F	5′-AMAAGTACCRYGAGGGAAAG-3′	Forward	[15]
LSU-R	5′-GCACTAATCATTCGCTTTACC-3′	Reverse	[15]
*psb*A-F	5′-YTHTAYCCWATHTGGGAAGC-3′	Forward	[39]
*psb*A-R	5′-GGGAAGTTRTGNGCRTTRCG-3′	Reverse	[39]
*rbc*L-F	5′-ATGTCACCACAAACAGAAACTAAAGC-3′	Forward	[40]
*rbc*L-R	5′-AATTCAAATTTAATTTCTTTCC-3′	Reverse	[40]
rps2-*trn*L-F	5′-AAAATCAAAATCTAGTAAACCAGGC-3′	Forward	[41]
rps2-*trn*L-R	5′-GCTAGCGATTCTTAACGCGATTGGG-3′	Reverse	[41]

Table 2. Polymerase chain reaction profiles for different gene.

Target	PCR Reaction Profile
ITS	94 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 55 °C for 40 s, and 65 °C for 70 s, with the final step at 65 °C for 10 min
tuf A	94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 1 min, with the final step at 72 °C for 10 min
18S	95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 4 min, with the final step at 72 °C for 6 min
LSU	94 °C for 5 min, followed by 38 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with the final step at 72 °C for 7 min
*psb*A	94 °C for 10 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 50 s, with the final step at 72 °C for 10 min
*rbc*L	94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 45 °C for 2 min, and 65 °C for 3 min, with the final step at 72 °C for 10 min
rps2-*trn*L	94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 40 s, with the final step at 72 °C for 5 min

2.5. Haplotype Analysis of U. aragoensis

The sequences amplified by the various primers (*rbc*L, *tuf* A, *psb*A, 18S, ITS, LSU, and rps2-*trn*L) were manually corrected. The DNASP5 software [42] was then used to calculate and analyze the haplotypes of the samples that were identified as *U. aragoensis*. The number of haplotypes, haplotype diversity, and nucleotide diversity were calculated. The PopART software [43] was next used to construct a median joining network for the amplified and spliced sequences to assess the genetic relationship between *U. aragoensis* samples (Table 3) from the SYS.

Table 3. Description of the different macroalgae and micro-propagules.

Sample No.	Station	Type
FUm1-1	A1	Macroalgae
FUm1-2	A1	Macroalgae
FUm1-3	A2	Macroalgae
FUm1-4	A3	Macroalgae
FUm2-1	B1	Macroalgae
FUm2-2	B2	Macroalgae
FUm2-3	B3	Macroalgae
FUm3-1	S9	Macroalgae
MUm1-1	A1	Micro-propagules
MUm1-2	A1	Micro-propagules
MUm1-3	A1	Micro-propagules
MUm1-4	A3	Micro-propagules
MUm1-5	A5	Micro-propagules
MUm1-6	A5	Micro-propagules
MUm1-7	A5	Micro-propagules
MUm1-8	A6	Micro-propagules

Table 3. *Cont.*

Sample No.	Station	Type
MUm2-1	B4	Micro-propagules
MUm2-2	B5	Micro-propagules
MUm2-3	B5	Micro-propagules
MUm2-4	B7	Micro-propagules
MUm2-5	B7	Micro-propagules
MUm3-1	S7	Micro-propagules
MUm3-2	S8	Micro-propagules
MUm3-3	S9	Micro-propagules
MUm3-4	S9	Micro-propagules

3. Results

3.1. Morphological Characteristics of U. aragoensis

Ulva aragoensis in the floating green tide macroalgae mostly comprised broken algae without pseudorhizes (Figure 2a). It was dark green and singular, or with a few branches at the base. The macroalgae thickened from the base to top, the lower part was tubular or approximately cylindrical, and the upper part was sometimes slightly flattened, irregularly rotated and twisted, and contained folds. The morphology of the micro-propagules was quite complete, with pseudorhizes, obviously long main branches, and small branches at the base (Figure 2b). The cells were arranged irregularly and were nearly rectangular, square, or polygonal, with two to six pyrenoids (Figure 2d,e). *Ulva aragoensis* is morphologically similar to *U. flexuosa* (Figure 2c).

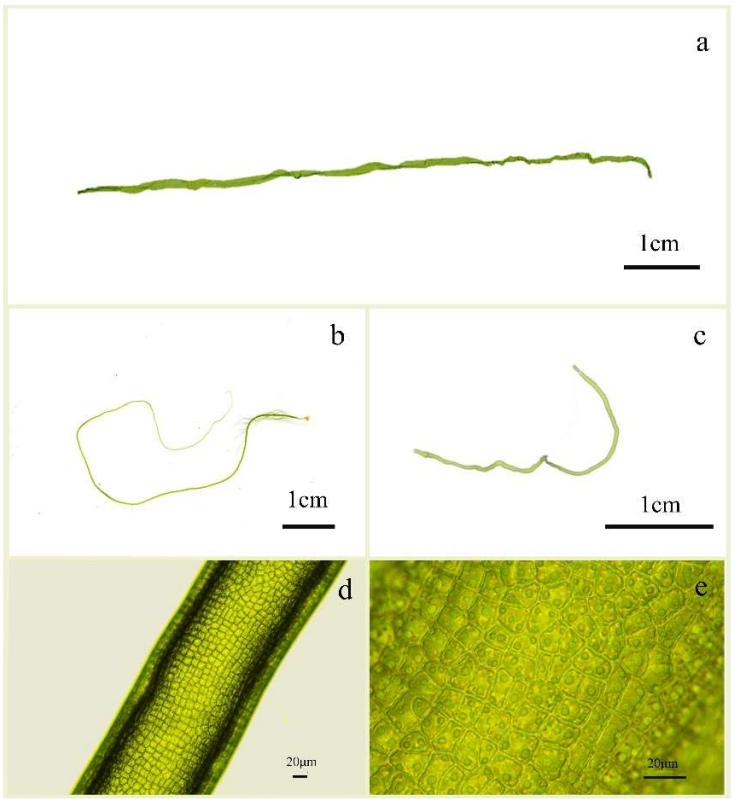

Figure 2. Morphology and cell diagram of *U. aragoensis* and *U. flexuosa* subp. *flexuosa*; (**a**) Morphology of *U. aragoensis* floating in the early-stage green tide of the Southern Yellow Sea. (**b**) Micro-propagules of *U. aragoensis* cultured from the surface seawater in the area of green tide outbreak in the Southern Yellow Sea. (**c**) Morphology of *U. flexuosa* subp. *flexuosa*. cultured from the surface seawater in the Southern Yellow Sea. (**d**) The middle part of *U. aragoensis* under a microscope at 40 × 10 magnification. (**e**) The middle part of *U. aragoensis* under a microscope at 10 × 10 magnification.

3.2. Proportion of U. aragoensis at Each Site

During the three surveys, a total of 25 sites were investigated, among which *U. aragoensis* was found in the floating green macroalgae at six sites (Figure 3) and in the seawater micro-propagules at 16 sites (Figure 4). The proportion of *U. aragoensis* in the micro-propagules at each site was greater than that in the floating green macroalgae, and the maximum proportion of *U. aragoensis* in the floating macroalgae was only 9% (Figure 3). However, *U. aragoensis* accounted for 33.3% of the micro-propagule samples (Figure 4). In two early-stage green tide surveys conducted from 6 May 2021 to 9 May 2021, and 14 May 2021 to 16 May 2021, the average proportion of *U. aragoensis* was much larger than that in the third survey conducted from 15 July 2021 to 18 July 2021, which was during the dissipation period of the green tide (Figure 5). This indicated that the proportion of *U. aragoensis* decreased gradually as the green tide moved northward.

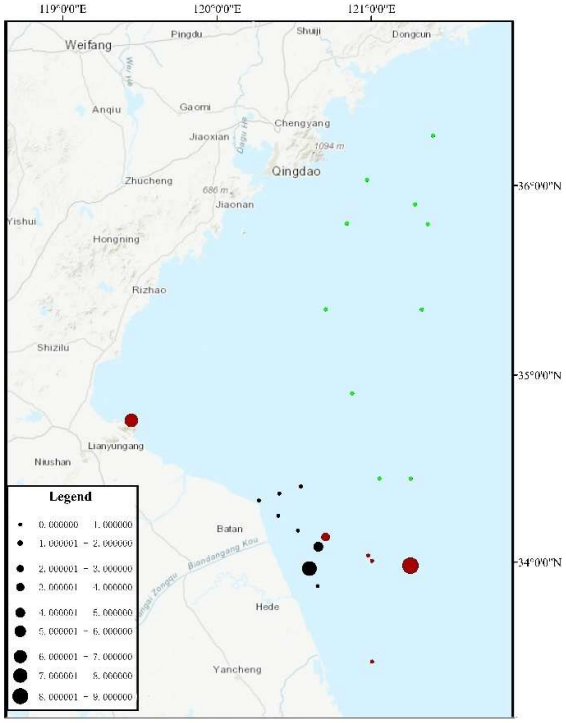

Figure 3. Percrntage of *U. aragoensis* in the floating green macroalgae. Circle size represents the proportion of *U. aragoensis*. Different colors represent different sample collection times. Red represents samples collected from 6 May 2021 to 9 May 2021, black represents samples collected from 14 May 2021 to 16 May 2021, and green represents samples collected from 15 July 2021 to 18 July 2021.

3.3. Phylogenetic Tree Results of Each DNA Barcode

The *rbc*L sequences of all samples were clustered with MZ582407, indicating that all of the samples were *U. aragoensis* (Figure 6). All samples of *U. aragoensis* had a high degree of consistency in the *tuf* A, *rbc*L, and *psb*A sequences (Figures 6–8). In addition, there were differences in the ITS, 18S, LSU, and rps2-*trn*L sequences (Figures 9–12), indicating that *U. aragoensis* had a faster evolution rate in the ITS, 18S, LSU, and rps2-*trn*L sequences. The *tuf* A sequences of *U. aragoensis* were clustered with MF614789 *U. flexuosa*, HE600177 *U. flexuosa*, and MT 859810 *U. aragoensis* (Figure 7), and were different from the standard *U. flexuosa* samples (Uf1, Uf2, and Uf3) and *U. californica* samples (Uc1, Uc2, and Uc3). The *psb*A sequences were clustered as one large branch, and there was no significant difference (Figure 8). The 18S sequences and ITS sequences of *U. aragoensis* were different from those of *U. flexuosa* and *U. califonica* (Figures 9 and 10). The *rbc*L sequences of *U. aragoensis* were divided into two clades with HM447574 *U. flexuosa* and HM447566 *U. flexuosa* (standard sequence) and clustered with *U. flexuosa* annotated in KP233758 and KC411905 in the NCBI database. This indicated that KP233758 and KC411905 were *U. aragoensis* and not *U. flexuosa*

(Figure 6). The LSU sequences of the FUm1-2, FUm1-3, and MUm3-1 samples were not completely clustered with other *U. aragoensis* samples (Figure 11), and the sequences of the other samples of *U. aragoensis* were clustered with MZ596067 in the NCBI database. In addition to the *psb*A sequences, the other sequences of *U. aragoensis*, *U. flexuosa*, and *U. californica* were obviously different. Furthermore, after comparing with the standard *U. flexuosa* sequences and *U. californica* sequences, there were a large number of sequences of *U. aragoensis* that were misidentified as *U. flexuosa* in the NCBI database (Figure 12).

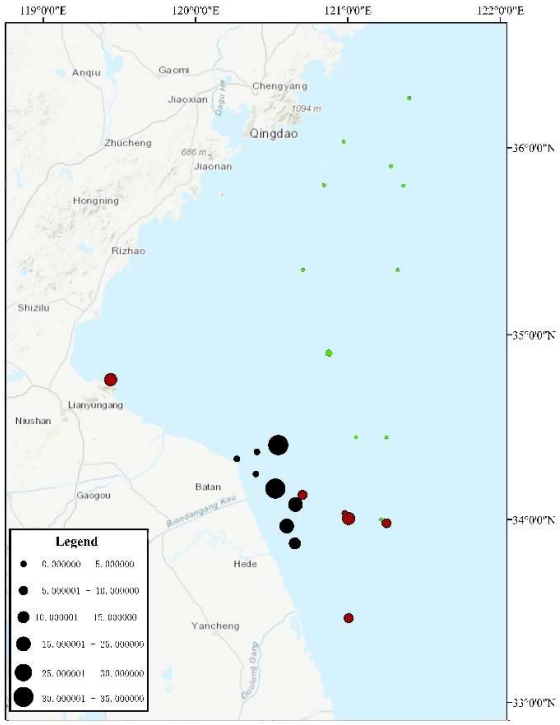

Figure 4. Percrntage of *U. aragoensis* in the micro-propagules. Circle size represents the proportion of *U. aragoensis*. Different colors represent different sample collection times. Red represents samples collected from 6 May 2021 to 9 May 2021, black represents samples collected from 14 May 2021 to 16 May 2021, and green represents samples collected from 15 July 2021 to 18 July 2021.

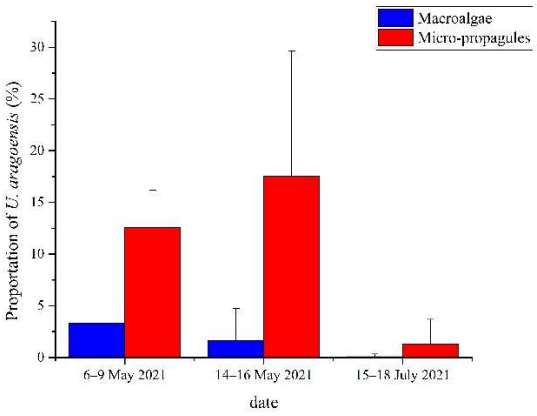

Figure 5. Average proportion of *U. aragoensis* in the floating green macroalgae and micro-propagules in the three surveys (6–9 May 2021, 14–16 May 2021, and 15–18 July 2021).

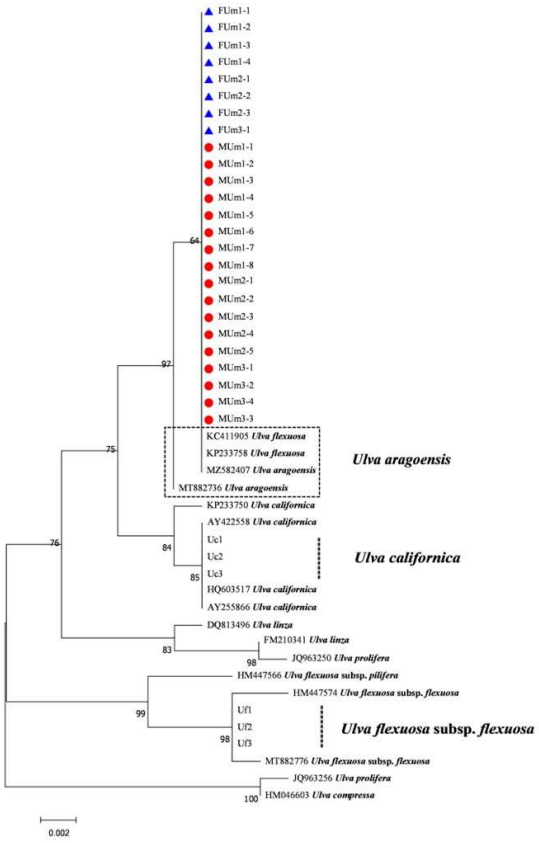

Figure 6. Maximum likelihood tree constructed from the analysis of *rbc*L gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

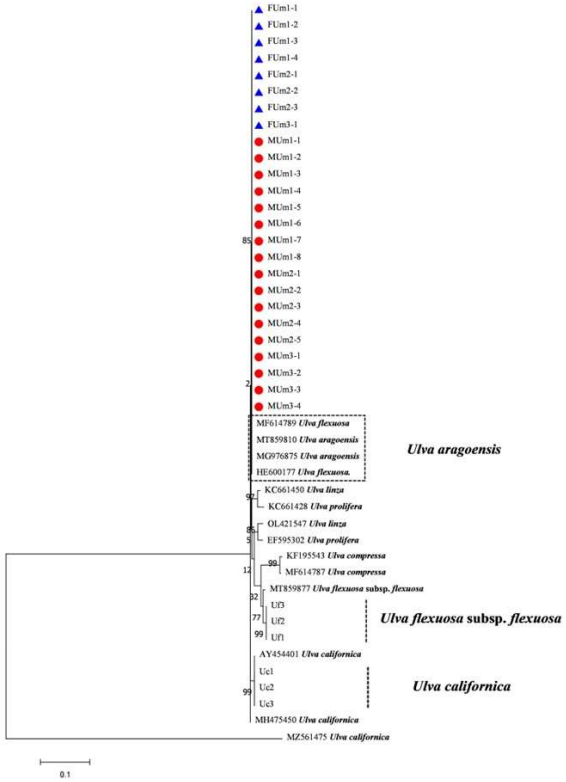

Figure 7. Maximum likelihood tree constructed from the analysis of *tuf*A gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

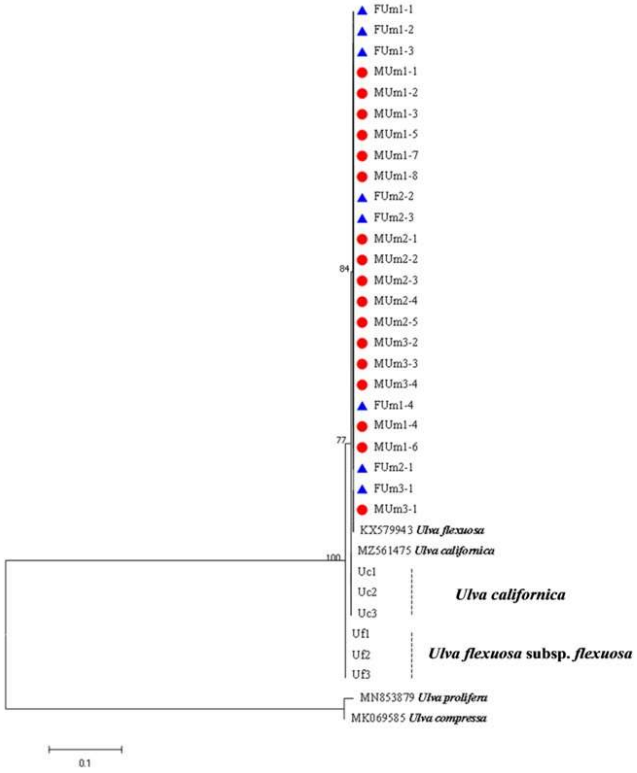

Figure 8. Maximum likelihood tree constructed from the analysis of *psb*A gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

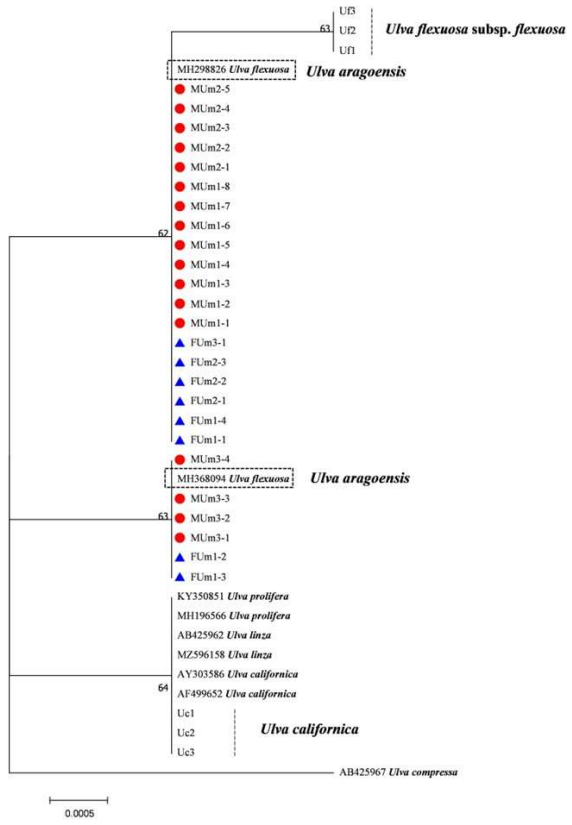

Figure 9. Maximum likelihood tree constructed from the analysis of 18S gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

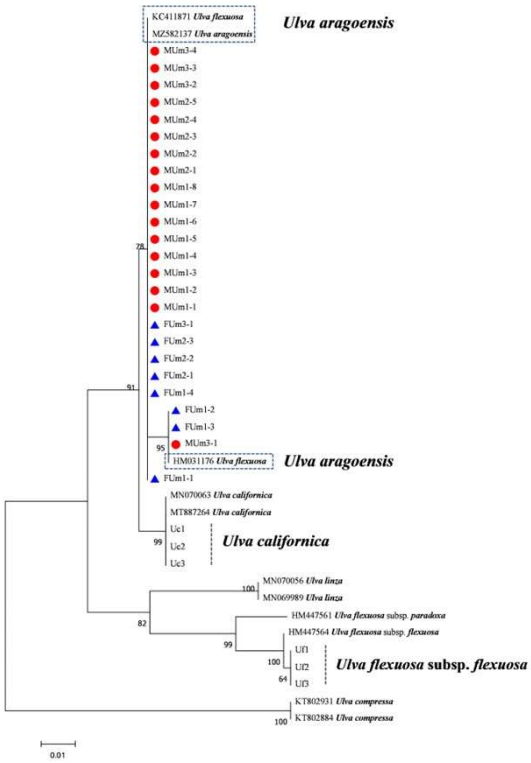

Figure 10. Maximum likelihood tree constructed from the analysis of internal transcribed spacer gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

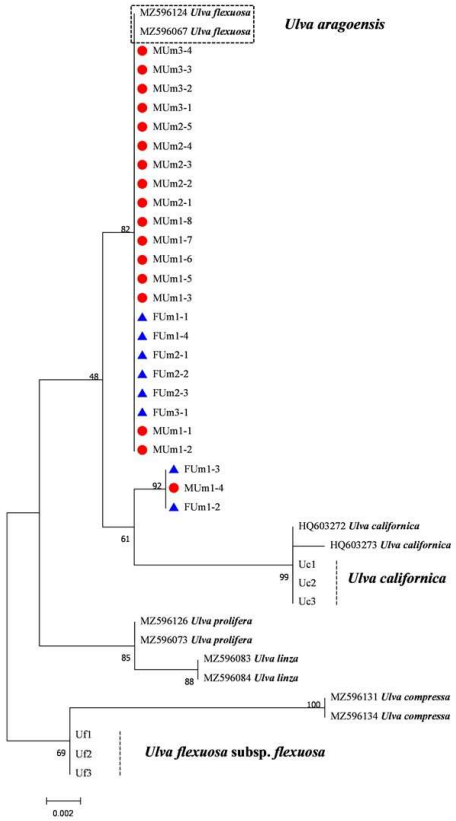

Figure 11. Maximum likelihood tree constructed from the analysis of large subunit gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

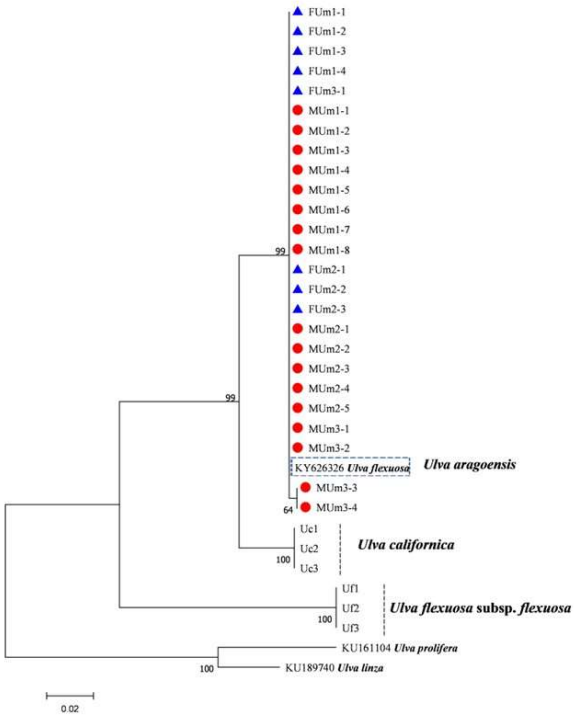

Figure 12. Maximum likelihood tree constructed from the analysis of rps2-*trn*L gene sequences of *U. aragoensis* samples and those downloaded from GenBank.

3.4. Analysis of Haplotype Relationship between Macroalgae and Micro-Propagules

According to the sample source, the sequences were divided into floating macroalgae species or micro-propagule populations. Because the sequences of *rbc*L, *tuf* A, and *psb*A of the 25 samples were completely consistent, haplotype analysis could not be carried out. Therefore, a haplotype analysis was performed on the sequences of the four different primers (18S, ITS, LSU, and rps2-*trn*L). The sequences of the four primers were divided into two haplotypes, and the differences between the two haplotypes were small (Figure 13a–d). The rps2-*trn*L sequences had the smallest difference and the LSU sequences had the largest. The two haplotypes of the 18S, ITS, and LSU sequences contained both macroalgae samples and micro-propagule samples, while the rps2-*trn*L sequences contained only two different sources of samples in Hap_1 and only micro-propagule samples in Hap_2, which may be the reason for the small number of samples of floating *U. aragoensis*.

The sequences of all four primers were spliced according to the sequence of 18S-ITS-LSU-rps2-*trn*L for haplotype analysis. There were four haplotypes, namely Hap_1, Hap_3, Hap_4, and Hap_6, in the micro-propagule samples, and three haplotypes in the floating macroalgae samples, namely Hap_1, Hap_2, and Hap_5. Hap_1 had the largest number of samples, including not only six floating samples but also twelve micro-propagule samples. Hap_2 and Hap_5 in only the macroalgae samples, and Hap_3, Hap_4, and Hap_6 in only the micro-propagule samples showed little difference (Figure 13e). Therefore, there was a close relationship between the micro-propagules and macroalgae samples.

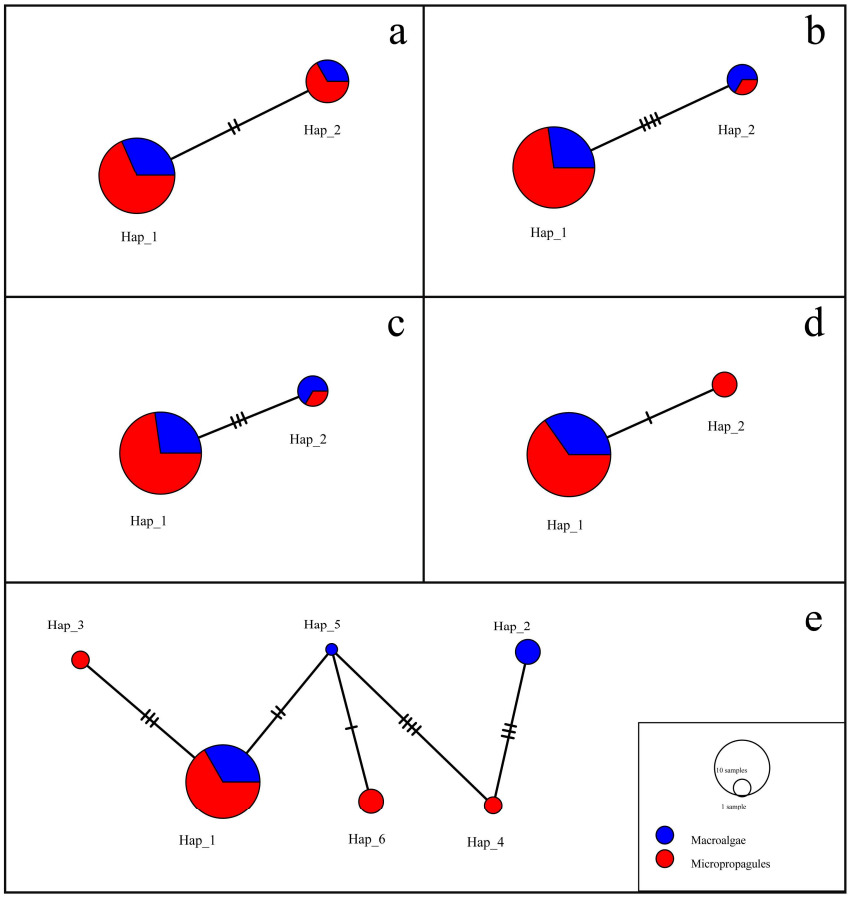

Figure 13. Network diagram for the *U. aragoensis* haplotypes derived from the 18S (**a**), internal transcribed spacers (**b**), large subunit (**c**), rps2-*trn*L (**d**), and 18S-internal transcribed spacers-large subunit-rps2-*trn*L (**e**) gene sequences. The circle symbol represents one haplotype, and its size reflects the number of different haplotypes. Different colors represent different sources, from which the haplotypes were derived, and blank circles indicate undetected haplotypes.

4. Discussion

4.1. Ulva Aragoensis Is a Constituent Species of the Early Green Tide in the SYS

Ulva aragoensis is morphologically highly similar to *U. flexuosa* (Figure 2), with wide blades that are unbranched or present at the base. Previously, *U. flexuosa* comprised 68.70% of the green macroalgae on *Neoporphyra* cultivation rafts in December in the SYS [44] and also represented a certain proportion in the micro-propagules [31]. *Ulva flexuosa* was an important component of the early-stage green tide in a previous study [9], while in the present study, *U. aragoensis* comprised a large proportion in the early-stage green tide. It comprised up to 33.3% in the micro-propagules. The *Ulva* genus shows a high degree of morphological plasticity in different environments, and the combination of ITS sequences and 5S rDNA gene sequences is the most commonly used method for species identification of green tide macroalgae in the SYS [16]. Some *U. aragoensis* in the NCBI database have been mistakenly considered as *U. flexuosa* and *U. californica*. In the phylogenetic tree constructed from the ITS and *rbc*L sequences of *U. aragoensis*, the sample sequences were also clustered with *U. flexuosa* in the NCBI database. This indicated that a large number of *U. aragoensis* were also present in the early-stage floating green macroalgae and micro-propagules and were previously identified as *U. flexuosa*. Hiraoka et al. [22] corrected the ITS sequences of *U. aragoensis* in the NCBI database, and the present authors entered the sequences of *rbc*L, *tuf* A, and rps2-*trn*L, three commonly used molecular markers of green tide macroalgae previously mistakenly considered *U. flexuosa* (Table 4) in order to provide a basis for species identification of green tide macroalgae in the SYS.

Table 4. Sequences of *U. aragoensis* mistakenly identified as *U. flexuosa* in the NCBI database.

DNA Makers	*rbc*L	*tuf* A	rps2-*trn*L
Accession in NCBI	KX579943 *Ulva flexuosa* KP233758 *Ulva flexuosa* KC411905 *Ulva flexuosa* KC411904 *Ulva flexuosa* KC411892 *Ulva flexuosa* KP233759 *Ulva flexuosa*	HE600177 *Ulva flexuosa* MH475451 *Ulva flexuosa* OL604529 *Ulva flexuosa* MH475452 *Ulva flexuosa*	KY626326 *Ulva flexuosa* KX455878 *Ulva flexuosa* MH013470 *Ulva flexuosa*

4.2. The Proportion of U. aragoensis Decreased as the Green Tide Moved Northward

As the green tide gradually moved northward, the temperature and light intensity increased, and the proportion of *U. aragoensis* in the floating green algae decreased. This may be because *U. aragoensis* is not suited to floating for a long time and cannot adapt to the high temperature and strong light in the coastal waters of Shandong Province [9,45,46]. Therefore, *U. aragoensis* did not contribute significantly to the green tide in the SYS. In addition, the proportion of *U. aragoensis* in the micro-propagules was much larger than that in the floating green macroalgae (Figure 5), and the large number of *U. aragoensis* attached on *Neoporphyra* cultivation rafts may be the main source of micro-propagules (unpublished data). In the process of green tide drift, a large number of pieces of bamboo used in the cultivation of *Neoporphyra* also drifted with the green tide. These floating pieces of bamboo provided a suitable attachment substrate for *U. aragoensis*, which is not suited for floating life. The *U. aragoensis* attached to the floating pieces of bamboo also contributed to the micro-propagules in the sea.

4.3. Appropriate Molecular Markers for Green Tide Macroalgae Identification

The ITS, *rbc*L, and *tuf* A sequences are molecular markers commonly used to identify macroalgae. The ITS sequence is a moderately conserved region from the process of evolution, with an interspecific difference of more than 14%. It is often used as a molecular marker to study the interspecific level of algae, but LPP clusters cannot be distinguished. In addition, the difference between the ITS sequences of *U. aragoensis* and *U. flexuosa* is small, only 2.4% [19]. Furthermore, the intraspecific difference in the ITS sequences of *U. aragoensis* is large, which increases the difficulty of its identification. Therefore, ITS is not suitable for distinguishing *U. flexuosa* from *U. aragoensis* [19]. The 5S rDNA spacer sequence is a highly variable non-transcribed (100–900 bp) conserved coding region with two to ten times more phylogenetic information than the ITS region, and it is a suitable fragment for identifying LPP clusters [16]. However, it does not seem to be able to amplify the 5S gene sequence of *U. flexuosa*. Three samples in the LSU sequence were isolated into a clade, which may be related to the higher evolutionary rate in the LSU fragment. Therefore, it was not suitable for the identification of *U. aragoensis*. The *psb*A sequences of *U. aragoensis* are difficult to distinguish due to the high similarity with *U. flexuosa* and *U. californica*. The rps2-*trn*L marker has played an effective role in the identification of four species of green tide macroalgae (*U. prolifera*, *U. flexuosa*, *U. linza*, and *U. compressa*) [17]. The rps2-*trn*L sequences of *U. aragoensis* were consistent with KY626326 *U. flexuosa* in the NCBI database, but not with the real *U. flexuosa*. This indicated that KY626326 *U. flexuosa* in the database was *U. aragoensis*, and there were significant differences between *U. aragoensis* and real *U. flexuosa* and *U. californica*. However, there are few reference rps2-*trn*L sequences in the NCBI database, and the identification of green tide macroalgae by rps2-*trn*L sequences still needs to be further improved.

Compared with LSU, *rbc*L, and UPA, the *tuf* A gene sequences data of green macroalgae contained almost no introns, had a high amplification success rate, a higher evolutionary rate, an obvious discrimination effect, and a lower pollution level. It is a standard molecular marker for the classification of green algae. The *rbc*L enzyme (Rubis-Co) promotes the fixation of primary CO_2 in photosynthesis and widely exists in algae. The

sequence information of *rbc*L in Genbank is abundant and conserved. As a molecular marker, *rbc*L is universal, easy to amplify, and easy to contrast. The *rbc*L sequences of *U. aragoensis* in the 25 samples were consistent, thereby making it a relatively conserved molecular marker for distinguishing *U. aragoensis* from *U. flexuosa*. The *tuf* A sequences of *U. aragoensis* in the present study were stable and specific and clearly distinguishable from *U. californica* and *U. flexuosa*; however, it is not clear whether *tuf* A can distinguish *U. linza* from *U. prolifera* [5,15]. Therefore, it is proposed that *tuf* A+5S rDNA sequences are a suitable approach for identifying green tide macroalgae. Furthermore, *rbc*L can also be used to identify green tide macroalgae outside LPP clusters. For the determination of *U. aragoensis* population geographic relationships, 18S, ITS, LSU, and rps2-*trn*L, with higher evolutionary rates, are more appropriate approaches.

4.4. The Micro-Propagule U. aragoensis and the Floating Green Tide U. aragoensis Are Related

Micro-propagules exist widely in the SYS in spring and summer, but the source of green tide outbreaks is particularly concentrated. Furthermore, green tide micro-propagules seem to have no ability to germinate directly in seawater [47] and must be attached to a certain substrate for germination; therefore, the micro-propagules cannot directly provide an initial biomass for green tide outbreaks. However, a large number of *Neoporphyra* cultivation rafts in the Subei Shoal provide suitable attachment substrates for micro-propagules [48]. The micro-propagules in the SYS attach to *Neoporphyra* cultivation rafts and then germinate in spring and summer every year. A large number of green macroalgae detach and fall into the water, providing the initial biomass for a green tide [49,50]. In autumn and winter, the micro-propagules are released into the seawater and sediments to survive the adverse environment to germinate again next year. This indicates that there should be close species overlap between floating green tide macroalgae, macroalgae attached to *Neoporphyra* cultivation rafts, and micro-propagules. This study revealed the existence of *U. aragoensis* in both green tide macroalgae and micro-propagules, in addition to the *U. aragoensis* from *Neoporphyra* cultivation rafts [51]. Furthermore, the haplotypes of the micro-propagule *U. aragoensis* and the floating green tide *U. aragoensis* were closely related in gene sequences, and the haplotypes were highly coincident in Hap_1, indicating that the micro-propagules indirectly contributed to the green tide.

5. Conclusions

In this study, we found that *U. aragoensis* was a constituent species of the early green tide in the SYS. The proportion of *U. aragoensis* decreased gradually as the green tide moved northward, and the proportion of *U. aragoensis* in the micro-propagules at each site was greater than that in the floating green macroalgae. This suggests that *U. aragoensis* is not suited to floating for a long time. Through amplification of the ITS, *rbc*L, rps2-*trn*L, *tuf* A, 18S, LSU, and *psb*A gene sequences of the three confused species (*U. aragoensis*, *U. flexuosa*, and *U. californica*), there were obvious gene sequence differences between these three species except *psb*A. The sequences of *tuf* A and *rbc*L were stable and suitable for the identification of *U. aragoensis*. Furthermore, through the analysis of the haplotypes of the floating *U. aragoensis* and the micro-propagules *U. aragoensis*, they were highly consistent, which also provided evidence for the indirect contribution of micro-propagules to the green tides.

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