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Development of chloroplast marker for identification of *Ulva* species*

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Abstract Large-scale *Ulva*-caused green tides has posed various environmental and ecological problems as well as economic consequences. It is important to identify them accurately and quickly. The current universal markers based on ITS, *rbc*L, and 5S spacer sequences were defective to distinct closely related species in the genus of *Ulva*. In this study, by investigating the intergenic regions of chloroplast (cp) genome, a novel marker of c15 (based on *ycf*3-*rps*7) were reliable for discrimination of *Ulva* species. Notably, the resolution of c15 was suitable for resolve the closely related species of *U. linza* and *U. prolifera*, which may provide tools for deciphering the blooming mechanisms of green tides.

Keyword: *Ulva*; linza-procera-prolifera (LPP) complex; molecular makers; *ycf*3-*rps*7 region

1 INTRODUCTION

As a type of harmful algal boom, green tide caused by *Ulva* spp. is a source of marine pollution (Blomster et al., 2002). Recently, large-scale green tides occur frequently in coastal areas worldwide (Smetacek and Zingone, 2013). In China, *Ulva* green tides occurred in the Bohai Sea, Yellow Sea, and South China Sea in last decade, which damaged local marine environments and ecological systems (Zhang et al., 2019; Xie et al., 2020b). Specifically, the world's largest green tide in the Yellow Sea have lasted for 15 consecutive years since 2007, whose causal species were identified as *U. prolifera* (Pang et al., 2010; Zhang et al., 2011, 2018, 2019; Zhao et al., 2012; Qi et al., 2016). Based on laboratory culturing analysis, it showed that the multiple life cycle modes were exhibited for *U. prolifera*, involving sexual reproduction with biflagellate gametes and quadriflagellate meiospores, and asexual reproduction from biflagellate spores, quadriflagellate spores, and unfertilized gametes (Liu et al., 2015; Zhang et al., 2017). Although *U. prolifera* can be recognized from its related taxa in adult stage, it is hard to morphologically discriminate each another in the young stage even for the experts. Phylogenetically, *U. prolifera*, *U. procera*, and *U. linza* could not be distinct and formed a clade called linza-procera-prolifera (LPP) clade based on solo ITS or *rbc*L markers (Shimada et al., 2008, 2016). And the hybridization studies indicated that *U. prolifera* and *U. linza* was the closely related *Ulva* species with complex speciation history, as the marine strains of *U. prolifera* were complete reproductive isolated with *U. linza* exhibiting by gamete incompatibility (Hiraoka et al., 2011), whereas some brackish strains of *U. prolifera* showed partial gamete compatibility with *U. linza* (Xie et al., 2020a).

To solve the relationships among closely related

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species in *Ulva*, several molecular approaches were reported. Besides the combination of ITS or *rbc*L markers, Shimada et al. (2008) firstly introduced the 5S rDNA spacer sequences to analyze the genetic diversity within LPP complex, and this marker was widely used for studying origin of floating *U. prolifera* under intra-specific level (Zhao et al., 2019). However, the using of 5S rDNA marker has some deficiencies as the PCR products of 5S rDNA marker were not specific as several bands were observed during electrophoretic analysis (Liu et al., 2022). On the other hand, as 5S rDNA marker was from nuclear genome, it will confuse the analysis if specimens were heterozygous. In another study, a PCR-based marker of sequence characterized amplified region (SCAR) was designed for identifying the *U. prolifera* populations (Zhao et al., 2015), but the application of SCAR marker is limited as only floating *U. prolifera* could be specifically amplified. In addition, the ribosomal large subunit (LSU) and intergenic spacer (IGS) sequences in *U. prolifera* were investigated, but their ability to delimitate closely related species remains uncertain (Shen et al., 2019). Therefore, specific markers should be exploited to identify the closely related species in a stable manner.

In land plants, the molecular markers have developed from chloroplast genome, including gene-coding genes of *rbc*L and *mat*K, as well as intergenic regions of *trn*H-*psb*A and *trn*L-F, which were widely used for species delimitation and phylogenetic analysis (Mishra et al., 2016). However, in algae, few studies were involved in the application of molecular markers from chloroplast (cp) genome. In this study, by investigating the intergenic regions of cp genome, we tried to develop novel marker with purpose to discrimination of *Ulva* species.

2 MATERIAL AND METHOD

2.1 Sampling and species identification

The specimens of *Ulva* spp. used in this study were collected from the coast and offshore areas of China, which distributed in ecologically diverse locations (Fig.1). For morphologically identification, the healthy adult individuals were selected and washed several times with sterile seawater to remove surface attachments, and then herbarium comparation were carried out according to the taxonomic features of *Ulva* taxa. Voucher herbarium specimens were deposited in the Marine Biological Museum of the Chinese Academy of Sciences (MBMCAS) (Fig.2). For molecular identification, the genome DNA of each specimen were extracted and ITS barcoding were characterized via PCR amplification, sequencing, and Blast analysis.

2.2 DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from fresh specimens with a DNeasy Plant kit (Tiangen Biotech. Co. Ltd., Beijing, China) following manufacturer's instructions. The ITS region was amplified by primers designed for *Ulva* taxa by Hayden et al. (2003) (F: 5′-TCTTTGAAACCGTATCGTGA-3′; R: 5′-GCTT-ATTGATATGCTTAAGTTCAGCGGGT-3′), and the c15 region of cp genomes were designed in this study (F: 5′-CTGACATACCATCACGATAGT-3′; R: 5′-CGTATTATTTCACCAGACCCTT-3′). The PCR reaction were carried out by TaKaRa Ex Taq enzyme in 25-L reaction column (TaKaRa, Shiga, Japan), with condition of an initial denaturation at 94 °C for 5 min, 36 cycles at 94 °C for 50 s, 54 °C for 50 s, and 72 °C for 1 min, and a final elongation step of 10 min at 72 °C. All PCR products were examined by electrophoresis on a 1% agarose gel and then sequenced using autosequencer (ABI, 3730) according to manufacturer's instructions (ABI, BigDye® Terminator v3.1 Cycle Sequencing Kit).

2.3 Data analysis

To develop high-resolution markers, the cp genomes of *U. prolifera* (KX342867), *U. linza* (KX058323), and *U. flexuosa* (KX579943) were recruited for comparative genomic analysis. The sequences alignment of cp genomes was analyzed and the conserved regions were identified by mVISTA online software (https://genome.lbl.gov/vista/mvista/submit.shtml). Thus, several less conserved regions were selected for primers design and evaluated via routine PCR and sequencing analysis. For phylogenetic analysis, the sequences were aligned using Clustal module in MEGA software v.11 (Tamura et al., 2021) and then manually adjusted. The phylogenetic trees based on ITS and *ycf*3-*rps*7 sequences were constructed with Neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) methods, respectively. NJ analysis was carried out by MEGA software v.11 with 1 000 bootstrap replicates, whereas ML and BI analysis were carried out by PhyloSuite software (Zhang et al., 2020) following corresponding instructions. Trees were visualized using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Fig.1 Sampling of *Ulva* species

a. floating on the sea; b. floating on aquaculture ponds; c. attaching on rocks in intertidal zone; d. attaching on aquaculture ropes; e and f. attaching on rocks in subtidal zone.

3 RESULT

3.1 Chloroplast genomes comparison and marker development

Based on the cp genomes alignment of *Ulva prolifera*, *U. linza*, and *U. flexuosa*, a high degree of conservation of synteny was exhibited, indicting the evolutionary relationships of these species was closely related (Fig.3). It is shown that most coding regions were highly conserved, reflecting the evolutionary conservation of these genes to the function of chloroplast. In addition, there were conserved no-coding sequences (CNS) scattered in the alignment, suggesting these sequences might

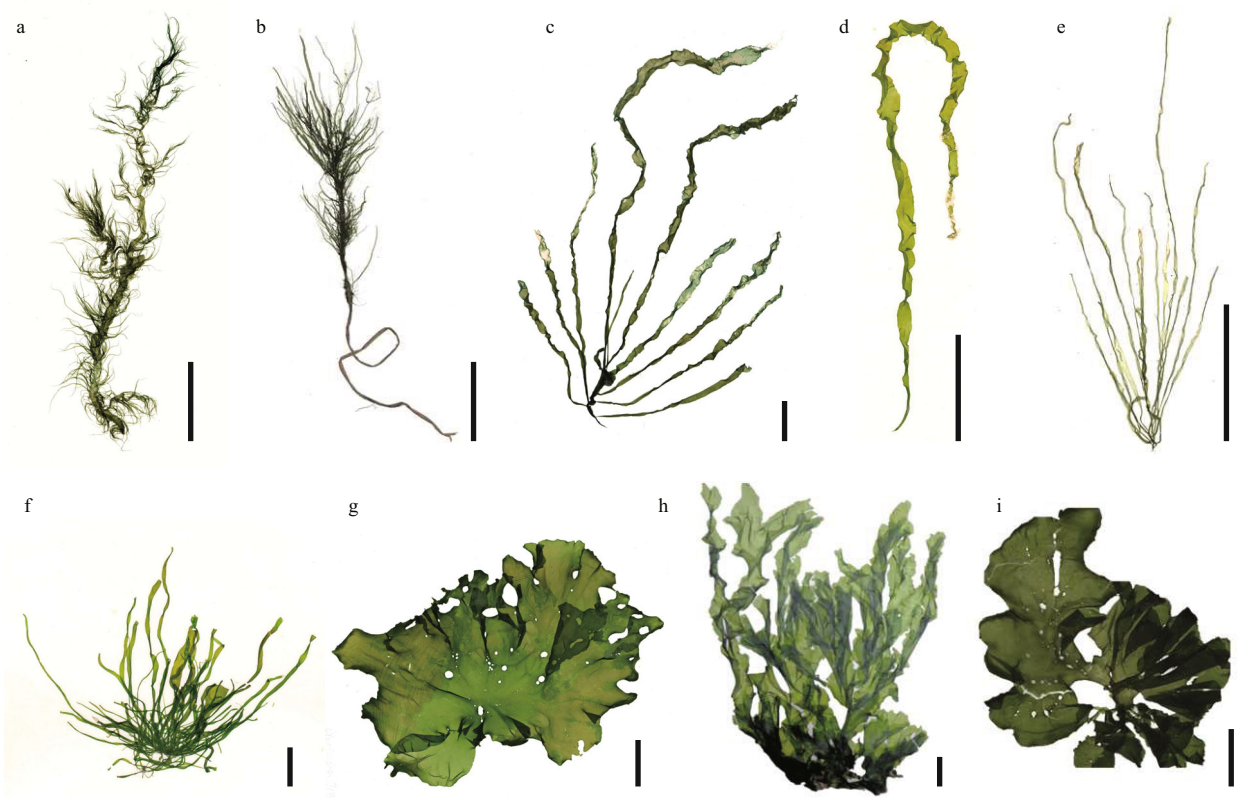

Fig.2 The specimens of representative species in the genus of *Ulva*

a. *U. prolifera* (floating); b. *U. prolifera* (attaching); c. *U. compressa*; d. *U. linza*; e. *U. intestinalis*; f. *U. flexuosa*; g. *U. australis*; h. *U. fasciata*; i. *U. rigida*. Scale bars: 2 cm.

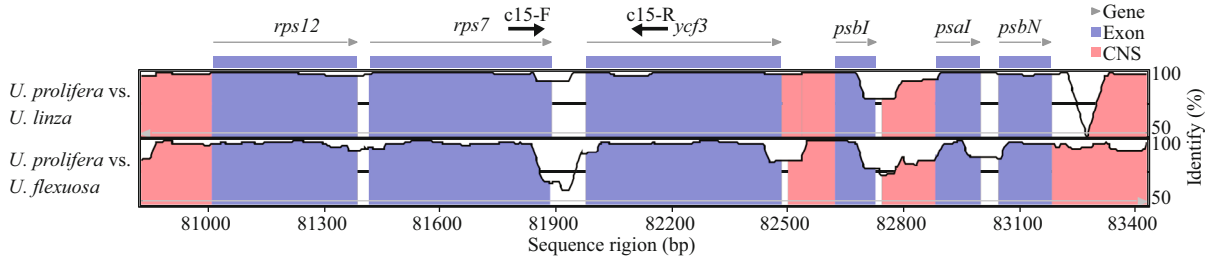

Fig.3 Comparative analysis of the chloroplast genome of three *Ulva* species

The arrows show the position of designed primers.

have conserved regulatory roles to the genes. Therefore, the less conserved sequences located in intergenic region could be theoretically used to develop molecular markers. Accordingly, to PCR-amplify these intergenic regions, dozens of primers were designed based on *U. prolifera* genomic sequences. After evaluation by gel electrophoresis and sequencing, it is showed that majority of primer pairs were failed to obtain a specific and unique PCR product. Nevertheless, a specific PCR product corresponding to the intergenic region of *rps*7 and *ycf*3 genes could be stabilized amplified by one primer pair labelled c15, exhibiting a potential role as novel molecular marker.

3.2 Sequence analysis of *ycf*3-*rps*7 region in *Ulva* taxa

To evaluate the usability of *rps*7-*ycf*3 region, the sequence divergence between species was analyzed. The corresponding amplification regions of primer pair c15, which include intergenic sequences of *rps*7-*ycf*3, were retrieved from 9 published *Ulva* cp genomes. According to the sequence alignment results, the coding region were highly conserved (Fig.4). Specifically, there were two highly variable regions (HVR1 and HVR2) located in the intergenic regions, suggesting these sequences divergence could be used for markers to distinguish species.

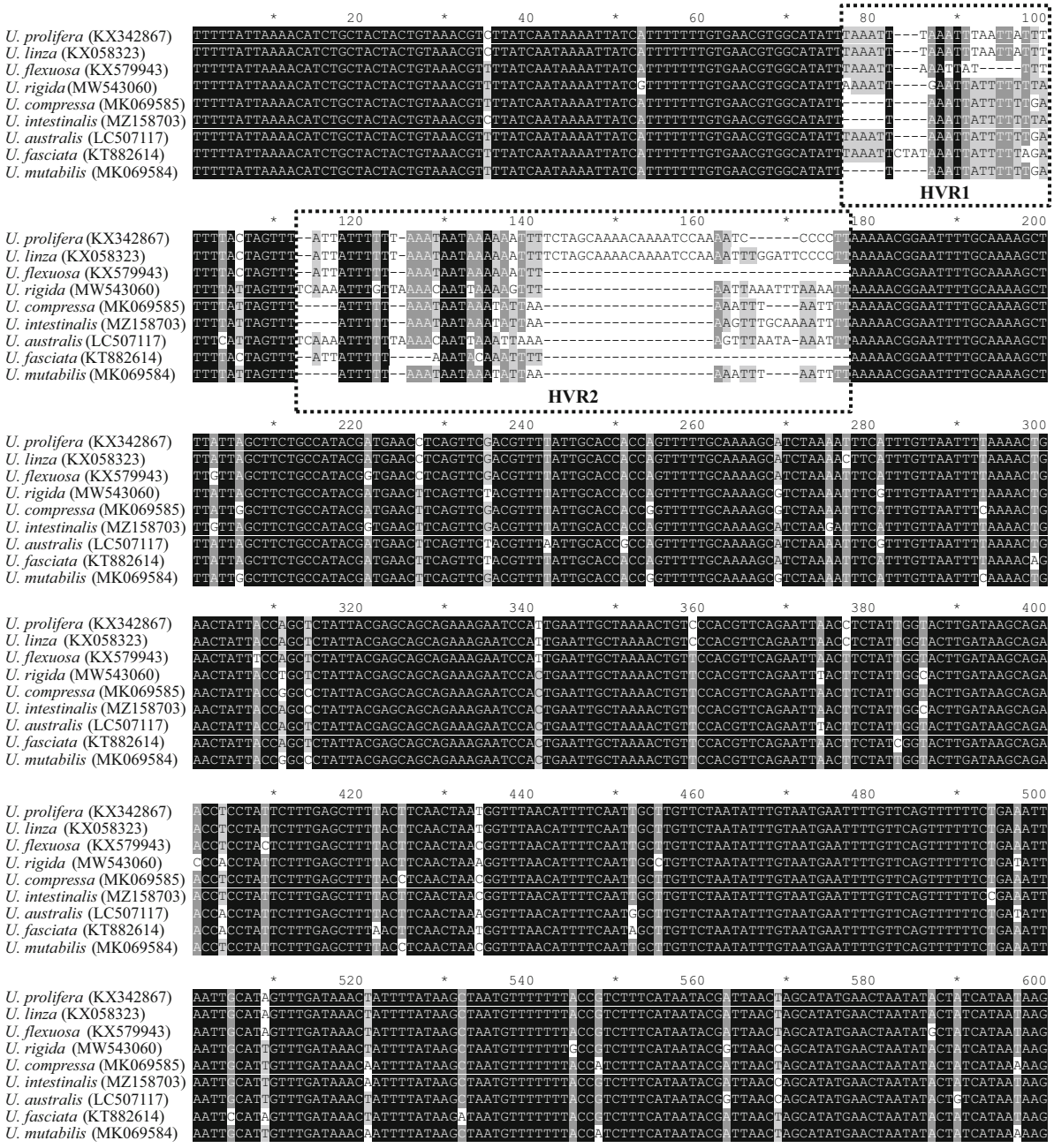

Fig.4 Sequence alignment of *rps*7-*ycf*3 regions of representative *Ulva* cp genomes

The dotted rectangle is two highly variable regions. HVR: highly variable region.

3.3 Discrimination of *U. linza* and *U. prolifera* through phylogenetic analysis

To validate the resolution of the c15 marker, the specimens collected from the coastal area of China were used for phylogenetic analysis (Table 1). These specimens could be identified as eight *Ulva* species based on their distinguished morphological characteristics (Fig.2). *U. linza* and *U. prolifera* could not separate as monophyly in the ITS tree, which clustered into LPP complex as previous studies (Fig.5a). However, in the c15 tree, *U. linza* and *U. prolifera* split into two separated clades clearly, suggesting the high resolution of this marker (Fig.5b). Furthermore, the monophyly of other six *Ulva* species were unaffected, which was consistent with ITS or *rbc*L markers.

4 DISCUSSION

The key to control the *Ulva* green tides is to identify the causal species accurately, which is important to prevent the occurrence of bloom. Several studies have demonstrated that the *U. prolifera* on bamboos,

Table 1 Samples information used in the study

Species	Sample ID	Sampling location	Latitude and longitude	Sampling habitat
U. compressa	MBMD-02402	Qingdao, Shandong	36.051 0°N, 120.366 4°E	Floating in coastal waters
U. compressa	MBMD-02403	Weihai, Shandong	37.526 5°N, 122.016 4°E	Attached on rocks in seashore
U. compressa	MBMD-02404	Lianyungang, Jiangsu	34.779 7°N, 119.292 6°E	Attached on rocks in seashore
U. flexuosa	MBMD-02504	Qingdao, Shandong	36.051 0°N, 120.366 4°E	Attached on bamboos of seawaters
U. flexuosa	MBMD-02505	Dalian, Liaoning	38.815 8°N, 121.400 6°E	Attached on rocks in seashore
U. flexuosa	MBMD-02506	Tianjin	39.150 0°N, 117.822 0°E	Attached on rocks in seashore
U. intestinalis	MBMD-02605	Weihai, Shandong	37.450 4°N, 122.481 3°E	Attached on rocks in seashore
U. intestinalis	MBMD-02606	Zhanjiang, Guangdong	20.224 0°N, 109.920 5°E	Attached on sands in seashore
U. intestinalis	MBMD-02607	Qingdao, Shandong	36.053 8°N, 120.345 2°E	Attached on rocks in seashore
U. linza	MBMD-02702	Qingdao, Shandong	36.053 8°N, 120.345 2°E	Attached on rocks in seashore
U. linza	MBMD-02703	Weihai, Shandong	37.450 4°N, 122.481 3°E	Attached on rocks in seashore
U. linza	MBMD-02704	Changdao Is., Shandong	37.965 3°N, 120.733 8°E	Attached on rocks in seashore
U. linza	MBMD-02705	Rongcheng, Shandong	37.034 8°N, 122.534 3°E	Attached on rocks in seashore
U. prolifera	MBMD-02801	Yancheng, Jiangsu	33.427 1°N, 120.705 6°E	Attached on rocks in estuary
U. prolifera	MBMD-02802	Haiyang, Shandong	36.655 3°N, 121.136 4°E	Floating in coastal waters
U. prolifera	MBMD-02803	Lianyungang, Jiangsu	34.665 7°N, 119.478 0°E	Attached on rocks in seashore
U. prolifera	MBMD-02805	Rushan, Shandong	36.823 5°N, 121.681 6°E	Attached on rocks in aquacultural pond
U. prolifera	MBMD-02806	Dalian, Liaoning	38.815 8°N, 121.400 6°E	Attached on aquacultural raft in coastal waters
U. prolifera	MBMD-02807	Nanji Is., Zhejaing	27.487 0°N, 121.066 5°E	Attached on rocks in seashore
U. prolifera	MBMD-02808	Tianjin	39.150 0°N, 117.822 0°E	Attached on rocks in seashore
U. prolifera	MBMD-02809	Dandong, Liaoning	39.972 2°N, 124.299 3°E	Attached on rocks in seashore
U. prolifera	MBMD-02901	Qingdao, Shandong	36.050 1°N, 120.349 1°E	Floating in coastal waters
U. rigida	MBMD-03603	Dalian, Liaoning	39.024 4°N, 122.718 1°E	Attached on rocks in seashore
U. rigida	MBMD-03604	Dalian, Liaoning	39.024 4°N, 122.718 1°E	Attached on rocks in seashore
U. rigida	MBMD-03605	Sanya, Hainan	18.536 2°N, 110.107 0°E	Attached on rocks in seashore
U. fasciata	MBMD-03801	Ningbo, Zhejiang	29.584 4°N, 121.787 4°E	Attached on mudbank
U. fasciata	MBMD-03802	Wenchang, Hainan	19.373 9°N, 108.691 6°E	Attached on rocks in seashore
U. fasciata	MBMD-03805	Sanya, Hainan	18.536 2°N, 110.107 0°E	Attached on rocks in seashore
U. australis	MBMD-04102	Weihai, Shandong	37.450 4°N, 122.481 3°E	Attached on rocks in seashore
U. australis	MBMD-04103	Zhanjiang, Guangdong	20.224 0°N, 109.920 5°E	Attached on rocks in seashore
U. australis	MBMD-04108	Qingdao, Shandong	35.922 9°N, 120.211 7°E	Attached on rocks in seashore

nets and ropes of aquaculture infrastructure were the seed source to green tides in the Yellow Sea (Liu et al., 2013, Zhang et al., 2017). As there were four major species of *U. linza*, *U. compressa*, *U. flexuosa* and *U. prolifera* been identified in *Neopyropia* cultivation area (Zhang et al., 2011), it is difficult to morphologically discriminate each another in the young germlings. Thus, the early interference by recognizing the *U. prolifera* specifically will increase the efficiency of controlling green tides blooms.

In this study, a novel marker was developed from cp genome for phylogenetically discriminating of *Ulva* species in a high-resolution manner. Specifically, comparing with tradition markers, c15 could precisely resolve the species relationships of *U. linza* and *U. prolifera* in LPP complex (Fig.5b). Although the nuclear marker 5S rDNA were applied to distinct the species in LPP complex (Shimada et al., 2008, 2016), the specificity of this marker was doubted as incomplete concerted evolution (Liu et al., 2020). Moreover, the new c15 marker could be used for other frequently distributed *Ulva* species, indicating its potential to work as universal barcode to investigate the cryptic biodiversity of this taxa.

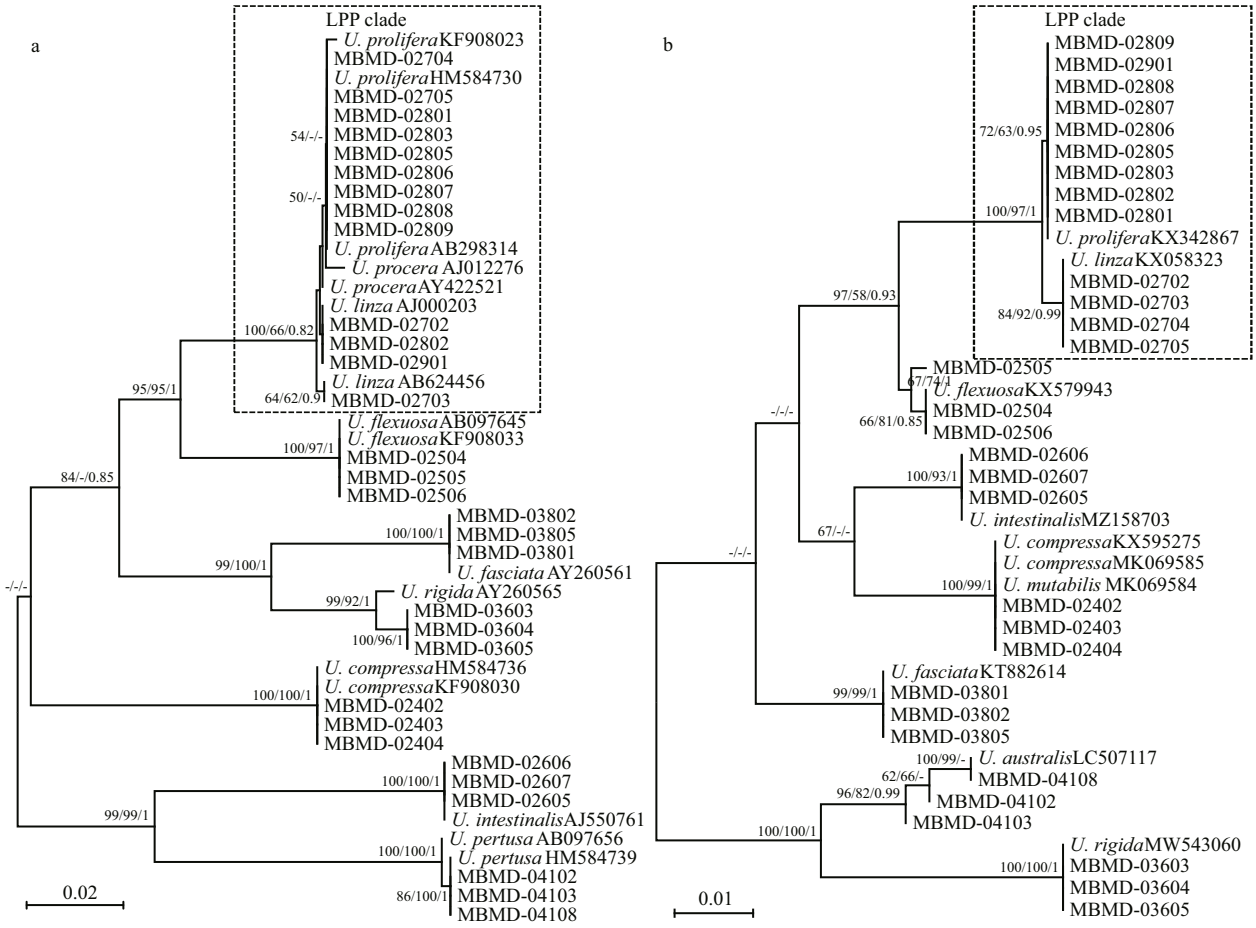

Fig.5 Phylogenetic trees based on ITS (a) and *rps*7-*ycf*3 (b) sequences

Bootstrap values and posterior probabilities are indicated in the branches (NJ/ML/BI). A bootstrap value or posterior probabilities below 50% or 0.50 is indicated by "-". The LPP clade is highlighted with dotted rectangle for each tree.

The identification of closely related species by molecular method was challengeable as the events of hybridization and gene flow between species occur frequently. As a result, different strategies are selected to solve problematic genera when resolution is desired. In most cases, the molecular markers developed from organelle genomes are uniparental inherited and single copy, providing resolution up to inter or intra-species levels (Lahaye et al., 2008; Matiz-Ceron et al., 2022). For example, the markers of *atp*F-*atp*H and *trn*H-*psb*A can simultaneously discriminate all the 46 representative medicinal plant species of 28 families (Thakur et al., 2019). However, given the different evolutionary pattern and reproductive mode between land plant and *Ulva* species, whether the cp genome of *Ulva* was inherited uniparentally need to be investigated.

In our analysis, the interspecific relationships were not completely identical between ITS and c15 phylogenies. The reasons for this inconsistent could be the different evolutionary rate and patterns of distinct markers (Wang et al., 2017). As both ITS and c15 are from no-coding intergenic region, it is suitable to identify taxa but not analyze phylogenetic relationships. Therefore, emerging phylogenomic analysis combining protein-coding markers could expected to better understanding the phylogenetic relationships for *Ulva* species. Besides, the intraspecific diversification in *Ulva* species were not well-solved, which need novel markers been developed.

5 CONCLUSION

In this study, by investigating the intergenic regions of chloroplast (cp) genome, a novel marker of c15 (based on *ycf*3-*rps*7 region) were reliable for discrimination of *Ulva* species. Notably, the resolution of c15 was suitable for resolve the closely related species of *U. linza* and *U. prolifera*, which may provide tools for deciphering the blooming mechanisms of green tides.

6 DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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