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Research Article

Sequences Analysis of ITS Region and 18S rDNA of *Ulva*

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Ulva, as the main genera involved in green tides in the Yellow Sea, has attracted serious concern in China. Especially, *Ulva prolifera* is one of the causative species of the occurring. This paper focused on the complete sequences analyses of ITS, 18S, and the combined data to determine phylogenetic relationships among taxa currently attributed to *Ulva*, *Monostroma*, and some other green algal. The samples are all concluded in the area of Yellow Sea, China. The results showed the content of G+C in 18S was approximately concentrated upon 49% in average of 19 subjects while the ITS region content of base G and C is obviously higher than A and T. Comparing the ITS and 18S rDNA sequences obtained in this paper to other species retrieved from GenBank, the genetic distance and the ratio of sequence divergence reflect that *U. pertusa* and *U. prolifera* had closer genetic relationship with an 18S rDNA, which had genetic distance of 0.007 while ITS had further genetic distance. According to further comparison, *Ulva prolifera* has closest genetic distance with *Chloropelta caespitosa* (0.057) and *Ulva californica* (0.057), which is a reverification coincided *Chloropelta*, *Enteromorpha*, and *Ulva* are not distinct genera.

1. Introduction

Green tide is an ecological phenomenon that occurs globally in which fixed growth alga break away from the shallow beaches of calm bays, resulting in accumulation of extensive biomass of free-floating green alga [1, 2]. Eutrophication is the main reason for green tide [3, 4]. The main species involved in green tides are *Ulva* sp. and *Enteromorpha* sp. Large scale green tides have occurred continuously four times in the Yellow Sea, from 2007 to 2010 in China, [5, 6], which had serious influences on the local environment and the life of coastal residents. As well known, these green tides consist of *Enteromorpha* sp. [7, 8]; however, their sources are still subject to debate. It has been suggested that the southern coast of the Yellow Sea is the ultimate source; accordingly, this region has become the focus of many investigations into the subject [9]. *Ulva* sp. has been considered the main causative species of green tides, which have bloomed continuously in recent years in the central and southern Yellow Sea in China. *Enteromorpha* [10] belongs to *Ulvaceae*, *Ulvales*, *Chlorophyceae*, *Chlorophyta*. More than 100 species of this organism have been recorded worldwide; however, only about 30 species can be recognized based on their morphology [11]. In addition, 23 species of *Enteromorpha* have been recorded in China [12–16]. Most of them are marine species, which are widespread along the coast of China.

Due to the divergence of *Enteromorpha* or *Ulva*, the *Ulva* sp. is named according to Tan et al. [20] and Hayden et al. [21] in this study. *Ulva* sp. has wide acclimatization and can grow well in a broad range of temperatures and salinities, but the morphological characteristics are changing easily in response to the environment [16]. Various morphological changes in the intraspecies and less differences that are difficult to identify, even among interspecies. Therefore, making an identification of *Ulva* sp. by using classical taxonomic methods is very difficult [22, 23]. Molecular biology and cross-hybridization methods [24, 25] have been introduced into this field to clarify the species. By this method, some reports have suggested that green tides are formed by an *Ulva linza-procera-prolifera* (LPP) complex instead of individual species [5, 26]. Analyses of nuclear ribosomal internal transcribed spacer DNA (ITS nrDNA; 29 ingroup taxa including the type species of *Ulva* and *Enteromorpha*), the chloroplast-encoded rbcL gene (for a subset of taxa), and a combined dataset were carried out. Combined with

Table 1: Primers used for PCR amplification and sequencing.

Gene	Region and direction	Primer name	Sequence (5′-3′)
ITS region	5′ end (F)	F	TCT TTG AAA CCG TAT CGT GA
	3′ end (R)	R	GCT TAT TGA TAT GCT TAA GTT CAG CGG GT
18S rRNA	5′ end (F)	NS1[1]	GTA GTC ATA TGC TTG TCT C
	~1150 (R)	NS4[1]	CTT CCG TCA ATT CCT TTA AG
	~1150 (F)	NS5[1]	AAC TTA AAG GAA TTG ACG GAA G
	3′ end (R)	NS8[1]	TCC GCA GGT TCA CCT ACG GA
	5′ end (F)	CRN5[2]	TGG TTG ATC CTG CCA GTA G
	~1137 (R)	1137[2]	GTG CCC TTC CGT CAA T
	~337 (F)	AB1[3]	GGAGGATTAGGGTCCGATTCC
	~1131 (R)	TW4[3]	CTTCCGTCAATTCCTTTAAG
	~1056 (F)	MonS	GCGGGTGTTTGTTTGA
	~1328 (R)	MonA	CTATTTAGCAGGCTGAGGT

[1] From White et al. [17], [2] from Booton et al. [18],[3] from Van Oppen [19].

earlier molecular and culture data, these data provide strong evidence that *Ulva*, *Enteromorpha*, and *Chloropelta* are not distinct evolutionary entities and should not be recognized as separate genera [21]. Partial sequences of the genes coding for rbcL and the 18S rRNA were used to determine the phylogenetic position of the order Prasiolales among other members of the Chlorophyta. Sequence divergence values within the Prasiolales for the rbcL gene (0–6.1%) and the 18S rRNA gene (0.4–3.8%) are both low compared to values among the other green algal sequences. Parsimony and distance analyses of the two subject genes sequences indicate that the Prasiolales is a well-delineated order of green algae containing both Prasiola and Rosenvingiella [27].

In this study, complete sequences of ITS and 18S are used to analyze the phylogenetic relationship among three common species (*Ulva prolifera*, *Ulva pertusa*, and *Monostroma grevillei*) in the Yellow Sea, China and some other green algal around the world. Especially, *Ulva prolifera* is the main causative species of green tides in China. Assessing the phylogenetic position of *Ulva* contributes to the investigation of the process of phylogenetic.

2. Material and Methods

2.1. Plant Material. *Ulva prolifera* were collected from Lianyungang coast of Jiangsu Province. And *Monostroma grevillei* and *Ulva lactuca* were collected from Qingdao coast of Shandong Province. Thallus were cleaned up, dried, and stored at −20°C for further analysis. The samples were reanimated several hours to unfold entirely and then soaked in 0.7% KI solution for ten minutes, followed by scouring with ddH_2O.

2.2. DNA Extraction. The cleaned-up samples were stored at −70°C overnight and then were triturated thoroughly with a chilled mortar and pestle. They were transferred into microcentrifuge tubes, which 2% sea snail enzyme with 2 M glucose was added into. The samples were digested for three hours at 25°C in swing bed and then were filtered and collected. Total genomic DNA was extracted by CTAB method, which was modified according to the samples. In brief, each sample was suspended with DNA extraction solution (3% CTAB, 0.1 mol L^{-1} Tris-HCl, pH 8.0, 0.01 mol L^{-1} EDTA, 1.4 mol L^{-1} NaCl, 0.5% β-mercaptoethanol, 1% PVP) and digested with protease-K with a final concentration of 300 μg mL^{-1}. The mixture was incubated at 55°C for half an hour with shaking every ten minutes, and then it was cooled down to room temperature. One-third volume of 5 M KAc was mixed with the solution before extracting the protein with phenol : trichloromethane : isoamyl-alcohol (25 : 24 : 1 v/v/v). The extract was precipitated by isopropyl-alcohol at −20°C for two hours. The solution was centrifuged and then the sediment was collected and cleaned with 70% ethyl alcohol. Finally, the sediments were dissolved in TE buffer (10 mmol L^{-1} Tris-HCl, pH 8.0, 1 mmol L^{-1} EDTA) and could be used as template for the following PCR amplification.

2.3. PCR Amplification and Purification. The primers F&R were designed according to ITS region sequence and MonS & MonA were designed according to 18S rDNA sequence retrieved from software Primer 5.0. And the primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Table 1).

The reactions for PCR amplification were performed with a final volume of 50 μL, containing 27.5 μL ddH_2O, 10 μL 5×ExTaq Buffer (Mg^{2+}), 6 μL dNTPMix (2.5 mM), 5 μL of the DNA template, 2 μL of each PCR primer (50 pmol μL^{-1}), and 0.5 μL ExTaq DNA polymerase (5 U μL^{-1}).

The amplification of ITS region was performed with an initial denaturation at 94°C for 5 min, 35 cycles at 94°C for 1 min, 45°C for 2 min, and 65°C for 3 min. And the products of amplification were preserved in 4°C.

The amplification of 18S rDNA was performed with an initial denaturation at 95°C for 2 min, 35 cycles at 95°C

Table 2: List of species used in this study and GenBank accession numbers for ITS and 18S.

Taxon	ITS accession number	18S rRNA accession number
Ulva lactuca	AY422499	AF499666
Klebsormidium flaccidum	EU434019	M95613
Chlorella vulgaris	FM205855	X13688
Mantoniella squamata	FN562451	X73999
Ulva prolifera	**HQ902007**	**HQ850569**
Monostroma grevillei	**HQ902006**	**HQ850570**
Paulschulzia pseudovolvox	AF182428	U83120
Trebouxia asymmetrica	AF344177	Z21553
Kornmannia leptoderma	AF415168	AF499661
Monostroma nitidum	AF415170	AF499665
Blidingia minima	AJ000206	AF499659
Enteromorpha intestinalis	AJ000210	AF189077
Ulva fenestrata	AJ234316	AF499653
Chloropelta caespitosa	AY016309	AF499656
Ulvaria obscura var. blyttii	AY260571	AF499657
Ulothrix zonata	Z47999	AY278217
Ulva pertusa	**HQ902008**	**HQ850571**
Ulva californica	AY422518	AF499652
Percursaria percursa	AY016305	AF499658

for 1 min, 55°C for 1 min (while the AB1&TW4 was 50°C and the MonS&MonA is 44°C), 72°C for 4 min, and a final extension step at 72°C for 6 min. And the products of amplification are preservation in 4°C.

The PCR products were confirmed by electrophoresis in 1% agarose gel. The gels were stained with ethidium bromide and photographed by Bio-IMAGING System. The PCR products were purified by TaKaRa Agarose Gel DNA Purification Kit Ver.2.0.

2.4. Sequencing and Phylogenetic Tree Construction. The products were sequenced by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The sequences were aligned using Clustal X, and further manually adjusted using BioEdit. The gained sequences should be ITS region and 18S rDNA by homology investigation with BLAST on the website of NCBI (http://www.ncbi.nih.gov/) by defining the boundary and length [28]. The sequences were aligned in order to observe the resemblance and to analyze the differences using the software DNAMAN with default parameters. With the multialignment analysis, we applied the program MEGA 3.1 with Kimura's two-parameter model [29] to calculate the base composition, Kimura two-parameter distance and the ratio of sequence divergence. For phylogenetic analysis, we used MEGA3.1 (neighbor-joining method) to study the relationship of different species to construct the phylogenetic tree, and detect the degree of bootstrap confidence from 1000 replicates. For comparative analysis, sequences were taken from the GenBank, of which entrance numbers were shown in Table 4.

3. Results

3.1. Splicing of ITS Region and 18S rDNA Sequences Fragments. With the primers mentioned above, the genomic of the ITS region and 18S rDNA fragments were successfully amplified (Figure 1). We got the overall length of ITS region and 18S rDNA sequences about *U. prolifera*, *U. pertusa*, and *M. grevillei*.

3.2. ITS Region and 18S rDNA Sequences of the Three Species. The base size of *U. prolifera* was 536 bp in ITS and 1718 bp in 18S. And the base size of *M. grevillei* was 541 bp in ITS and 1755 bp in 18S. And the base size of *U. pertusa* was 567 bp in ITS and 1761 bp in 18S. The base sizes of the three species were almost the same in ITS region and 18S rDNA. After sequencing the ITS region and 18S rDNA, we got the accession numbers from GenBank. For comparative analysis, sequences of other sixteen species were also taken from the GenBank. All these data were shown in Table 2.

3.3. Base Composition of ITS Region and 18S rDNA Sequences. The base composition of ITS region and the content of G+C are shown in Table 3. And the base composition of 18S rDNA and the content of G+C are shown in Table 4. The ITS region content of G+C was about 58.45% in average in nineteen species. However, the content of G+C varied obviously from 51.65% to 65.78% in ITS region while 18S rDNA content of G+C was about 48.98% in average in nineteen species. Comparing with the content of G+C in ITS region, the content of G+C in 18S was approximately concentrated upon 49%. The ITS region content of base G and C in average in nineteen species is obviously higher than A and T.

3.4. Genetic Distance in Interspecies. Comparing the ITS region and 18S rDNA sequence obtained in this study and other species retrieved from GenBank, the results of the genetic distance and the ratio of sequence divergence are shown in Tables 5 and 6. *U. pertusa* and *U. prolifera* had closer genetic relationship with genetic distance of 0.007 in 18S rDNA. While, its had further genetic distance in ITS.

A comparison of the sequences of the ITS of the three strains evaluated in this study to those of other species of green algal retrieved from GenBank is shown in Table 5. Obviously, CC, UC, HT, UL, EI, SC, UF, UO, and PPE are subordinate to *Ulvaceae*. The region distance (ranged form 0.189 to 0.035) and the ratio of sequence divergence (ranged from 0.026 to 0.010) are lower than those comparisons within other 10 species from different families. Particularly prominent, CC, HT, and UC had the closest relationship

Table 3: Base composition of ITS region.

Taxon	A (%)	T (%)	G (%)	C (%)	G+C (%)
Ulva lactuca	21.37	15.88	28.24	34.51	62.75
Klebsormidium flaccidum	21.78	19.42	28.71	30.10	58.81
Chlorella vulgaris	20.00	23.45	25.32	31.22	56.55
Mantoniella squamata	22.76	21.27	28.17	27.80	55.97
Ulva prolifera	20.15	17.16	28.92	33.77	62.69
Monostroma grevillei	22.92	21.26	24.95	30.87	55.82
Paulschulzia pseudovolvox	24.26	24.09	24.75	26.90	51.65
Trebouxia asymmetrica	20.27	26.93	26.48	26.32	52.80
Kornmannia leptoderma	22.78	22.06	26.16	29.00	55.16
Monostroma nitidum	24.18	23.26	25.27	27.29	52.56
Blidingia minima	22.45	22.08	25.55	29.93	55.47
Enteromorpha intestinalis	18.85	18.85	28.84	33.46	62.29
Ulva fenestrata	20.04	17.12	28.99	38.85	62.84
Chloropelta caespitosa	20.51	19.15	29.06	31.28	60.34
Ulvaria obscura var. blyttii	20.42	18.13	28.44	33.02	61.45
Ulothrix zonata	23.98	24.18	24.56	27.27	51.84
Ulva pertusa	18.87	15.34	30.34	35.45	65.78
Ulva californica	20.41	19.11	28.39	32.10	60.48
Percursaria percursa	19.86	14.81	29.62	35.71	65.33
Average	21.36	20.19	27.41	31.31	58.45

Table 4: Base composition of 18S rDNA.

Taxon	A (%)	T (%)	G (%)	C (%)	G+C (%)
Ulva lactuca	26.37	24.50	28.78	20.35	49.13
Klebsormidium flaccidum	26.08	26.80	26.53	20.59	47.11
Chlorella vulgaris	24.97	25.42	27.64	21.97	49.61
Mantoniella squamata	26.01	26.80	26.74	20.45	47.19
Ulva prolifera	24.80	25.67	27.76	21.77	49.53
Monostroma grevillei	25.13	26.55	27.35	20.97	48.32
Paulschulzia pseudovolvox	24.91	25.49	28.02	21.58	49.60
Trebouxia asymmetrica	25.06	26.00	27.34	21.60	48.94
Kornmannia leptoderma	26.51	24.10	28.92	20.48	49.40
Monostroma nitidum	26.17	24.83	28.84	20.16	49.00
Blidingia minima	26.34	23.66	29.14	20.86	50.00
Enteromorpha intestinalis	24.87	25.74	27.78	21.61	49.39
Ulva fenestrata	26.30	24.43	28.84	20.43	49.27
Chloropelta caespitosa	26.44	24.43	28.84	20.29	49.13
Ulvaria obscura var. blyttii	26.44	24.30	28.97	20.29	49.27
Ulothrix zonata	25.09	27.01	27.07	20.83	47.90
Ulva pertusa	24.70	25.95	27.77	21.58	49.35
Ulva californica	26.30	24.57	28.84	20.29	49.13
Percursaria percursa	26.17	24.43	28.97	20.43	49.40
Average	25.72	25.30	28.11	20.87	48.98

among each other because of the lower region distance (HT/CC 0.057; HT/UC 0.057; CC/UC 0.035) and the ratio of sequence divergence (HT/CC 0.012; HT/UC 0.012; CC/UC 0.010). These data illustrate HT/UC/CC has extremely related species. According to other *Ulva* species, the sequence homology of these three species is closer which verified *U. prolifera* and *C. caespitosa* belonged to *Ulva* genera. On the other hand, JM, UZ, BM, MN, and KL had higher homology relationship within each other, the region distance ranged from 0.335 to 0.133, and the ratio of sequence divergence ranged from 0.033 to 0.021. The data are relatively stable, and in these five species, JM/UZ is the closest pair because of

Table 5: Kimura 2-parameter ITS region distance (below) and the ratio of sequence divergence (above).

Species	UL	JM	HT	SC	UZ	UO	CC	UF	EI	BM	MN	KL	TA	PP	MS	UC	PPE	CV	KF
UL		0.039	0.017	0.019	0.038	0.025	0.016	0.019	0.015	0.039	0.041	0.038	0.053	0.048	0.057	0.014	0.024	0.049	0.049
JM	0.377		0.037	0.040	0.021	0.037	0.037	0.038	0.037	0.025	0.032	0.033	0.051	0.048	0.048	0.039	0.035	0.045	0.044
HT	0.105	0.338		0.018	0.037	0.023	0.012	0.016	0.016	0.039	0.040	0.039	0.049	0.045	0.054	0.012	0.023	0.046	0.047
SC	0.118	0.369	0.102		0.041	0.026	0.019	0.015	0.018	0.042	0.044	0.042	0.053	0.049	0.059	0.017	0.023	0.046	0.051
UZ	0.364	0.133	0.355	0.398		0.036	0.037	0.037	0.039	0.022	0.028	0.033	0.046	0.049	0.052	0.038	0.037	0.043	0.045
UO	0.196	0.335	0.163	0.181	0.333		0.025	0.024	0.024	0.035	0.038	0.040	0.053	0.049	0.059	0.025	0.016	0.044	0.047
CC	0.085	0.341	0.057	0.116	0.344	0.189		0.018	0.016	0.039	0.039	0.038	0.054	0.048	0.057	0.010	0.024	0.048	0.048
UF	0.118	0.346	0.089	0.076	0.360	0.178	0.099		0.018	0.039	0.040	0.040	0.051	0.048	0.054	0.017	0.023	0.045	0.048
EI	0.070	0.348	0.085	0.112	0.360	0.185	0.079	0.109		0.039	0.040	0.038	0.050	0.045	0.056	0.015	0.022	0.048	0.048
BM	0.368	0.189	0.360	0.398	0.146	0.329	0.354	0.369	0.363		0.029	0.030	0.045	0.049	0.053	0.039	0.038	0.046	0.049
MN	0.399	0.257	0.370	0.416	0.219	0.349	0.378	0.374	0.374	0.243		0.036	0.048	0.046	0.053	0.040	0.039	0.039	0.046
KL	0.379	0.285	0.370	0.406	0.297	0.381	0.369	0.389	0.374	0.256	0.335		0.050	0.046	0.055	0.040	0.041	0.050	0.048
TA	0.510	0.486	0.464	0.508	0.438	0.476	0.510	0.485	0.474	0.442	0.449	0.503		0.036	0.053	0.051	0.051	0.048	0.049
PP	0.477	0.479	0.440	0.489	0.475	0.480	0.477	0.465	0.444	0.478	0.465	0.459	0.315		0.055	0.048	0.048	0.048	0.047
MS	0.549	0.474	0.527	0.571	0.520	0.558	0.551	0.537	0.548	0.534	0.529	0.565	0.519	0.554		0.055	0.056	0.059	0.042
UC	0.072	0.365	0.057	0.099	0.363	0.181	0.035	0.092	0.073	0.368	0.393	0.394	0.480	0.476	0.538		0.023	0.048	0.048
PPE	0.178	0.329	0.164	0.167	0.351	0.095	0.174	0.163	0.174	0.370	0.377	0.391	0.476	0.450	0.539	0.174		0.046	0.045
CV	0.476	0.423	0.441	0.448	0.397	0.434	0.460	0.443	0.456	0.429	0.383	0.483	0.480	0.458	0.611	0.470	0.435		0.049
KF	0.492	0.432	0.485	0.508	0.446	0.460	0.502	0.478	0.479	0.490	0.452	0.473	0.501	0.468	0.389	0.496	0.443	0.506	

Note: UL, *U. lactuca*; KF, *K. flaccidum*; CV, *C. vulgaris*; MS, *M. squamata*; HT, *U. prolifera*; JM, *M. grevillei*; PP, *P. pseudovolvox*; TA, *T. asymmetrica*; KL, *K. leptoderma*; MN, *M. nitidum*; BM, *B. minima*; EI, *E. intestinalis*; UF, *U. fenestrate*; CC, *C. caespitosa*; UO, *U. obscura var. blyttii*; UZ, *U. zonata*; SC, *U. pertusa*; UC, *U. californica*; PPE, *P. percursa*.

Table 6: Kimura 2-parameter 18S rDNA distance (below) and the ratio of sequence divergence (above).

Species	EI	CC	BM	UC	JM	SC	HT	CV	UL	PPE	UO	UF	TA	MS	PP	KF	UZ	MN	KL
EI		0.002	0.010	0.000	0.009	0.003	0.003	0.011	0.002	0.004	0.005	0.002	0.013	0.014	0.011	0.013	0.009	0.009	0.008
CC	0.003		0.010	0.002	0.010	0.002	0.002	0.011	0.002	0.004	0.005	0.002	0.013	0.014	0.011	0.014	0.009	0.009	0.008
BM	0.070	0.073		0.010	0.010	0.010	0.010	0.012	0.010	0.010	0.010	0.010	0.013	0.015	0..012	0.015	0.010	0.010	0.006
UC	0.000	0.003	0.070		0.009	0.003	0.003	0.011	0.002	0.004	0.005	0.002	0.013	0.014	0.011	0.013	0.009	0.009	0.008
JM	0.064	0.067	0.069	0.064		0.010	0.010	0.010	0.010	0.010	0.009	0.010	0.010	0.013	0.010	0.013	0.003	0.005	0.008
SC	0.007	0.004	0.078	0.007	0.072		0.003	0.011	0.003	0.005	0.005	0.003	0.013	0.015	0.011	0.014	0.009	0.009	0.009
HT	0.005	0.003	0.076	0.005	0.070	0.007		0.011	0.003	0.004	0.005	0.003	0.013	0.015	0.011	0.014	0.009	0.009	0.009
CV	0.086	0.089	0.108	0.086	0.067	0.093	0.092		0.011	0.011	0.011	0.011	0.009	0.011	0.009	0.010	0.009	0.009	0.011
UL	0.004	0.003	0.075	0.004	0.069	0.007	0.005	0.090		0.004	0.004	0.000	0.013	0.015	0.011	0.014	0.009	0.009	0.008
PPE	0.011	0.014	0.072	0.011	0.066	0.018	0.017	0.083	0.015		0.004	0.004	0.013	0.014	0.011	0.013	0.009	0.009	0.008
UO	0.018	0.017	0.073	0.018	0.064	0.021	0.019	0.091	0.017	0.012		0.004	0.012	0.014	0.012	0.014	0.009	0.009	0.008
UF	0.004	0.003	0.075	0.004	0.069	0.007	0.005	0.090	0.000	0.015	0.017		0.013	0.015	0.011	0.014	0.009	0.009	0.008
TA	0.113	0.116	0.115	0.113	0.075	0.121	0.119	0.054	0.114	0.108	0.105	0.114		0.011	0.011	0.011	0.010	0.011	0.012
MS	0.141	0.145	0.150	0.141	0.114	0.150	0.148	0.079	0.146	0.137	0.141	0.146	0.087		0.012	0.011	0.013	0.013	0.014
PP	0.086	0.089	0.104	0.086	0.078	0.093	0.092	0.064	0.090	0.086	0.094	0.090	0.081	0.102		0.012	0.010	0.010	0.011
KF	0.124	0.127	0.139	0.124	0.111	0.132	0.130	0.078	0.129	0.122	0.129	0.129	0.086	0.084	0.098		0.012	0.012	0.014
UZ	0.058	0.061	0.067	0.058	0.008	0.066	0.064	0.063	0.063	0.060	0.061	0.063	0.074	0.109	0.072	0.104		0.004	0.008
MN	0.060	0.063	0.069	0.060	0.018	0.067	0.066	0.063	0.064	0.061	0.066	0.064	0.078	0.114	0.073	0.104	0.011		0.008
KL	0.054	0.057	0.034	0.054	0.046	0.061	0.060	0.084	0.058	0.054	0.054	0.058	0.092	0.130	0.085	0.123	0.046	0.049	

Note: UL, *U. lactuca*; KF, *K. flaccidum*; CV, *C. vulgaris*; MS, *M. squamata*; HT, *U. prolifera*; JM, *M. grevillei*; PP, *P. pseudovolvox*; TA, *T. asymmetrica*; KL, *K. leptoderma*; MN, *M. nitidum*; BM, *B. minima*; EI, *E. intestinalis*; UF, *U. fenestrate*; CC, *C. caespitosa*; UO, *U. obscura var. blyttii*; UZ, *U. zonata*; SC, *U. pertusa*; UC, *U. californica*; PPE, *P. percursa*.

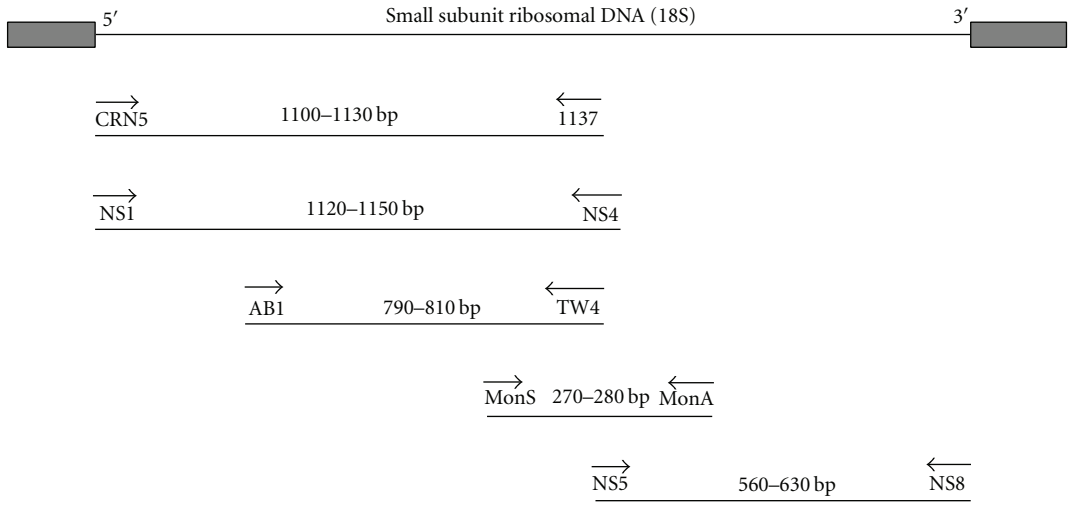

Figure 1: Splicing of 18S rDNA sequences fragments with the primers mentioned in Table 1.

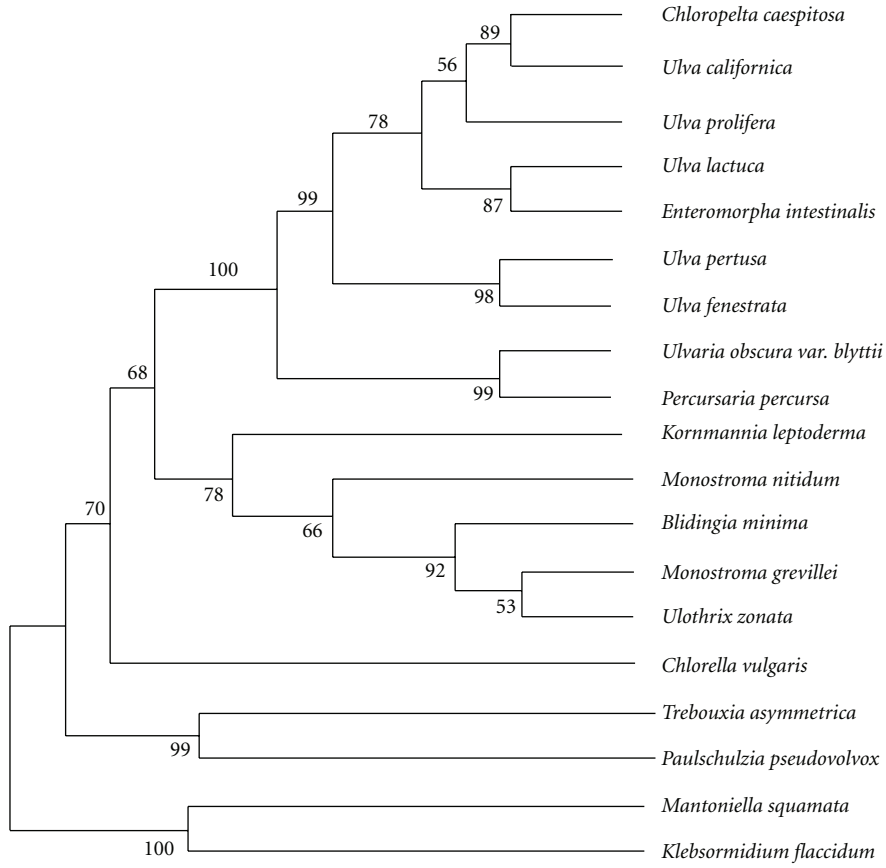

Figure 2: NJ phylogenetic tree constructed from ITS sequences.

the lowest region distance (0.133) and the ratio of sequence divergence (0.021).

A comparison of the sequences of the ITS of the three strains evaluated in this study to those of other species of green algal retrieved from GenBank is shown in Table 6. The same result with ITS, IE, CC, UC, SC, HT, UL, PPE, UO, and UF are subordinate to *Ulvaceae*. The region distance (ranged form 0.021 to 0.000) and the ratio of sequence divergence (ranged from 0.005 to 0.000) are lower than those comparisons within other 10 species from different families. Meanwhile, JM, UZ, BM, MN, and KL had higher homology relationship within each other, the region distance ranged

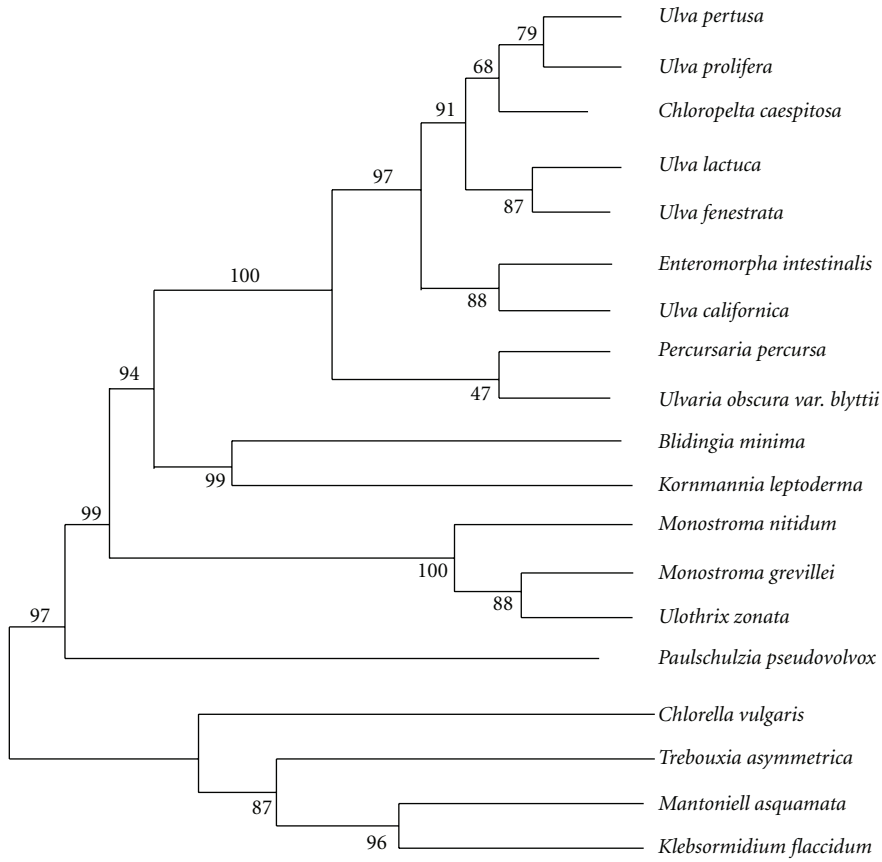

Figure 3: NJ phylogenetic tree constructed from 18S sequences.

from 0.069 to 0.008, and the ratio of sequence divergence ranged from 0.010 to 0.004.

3.5. Phylogenetic Tree Analysis. From the results of NJ phylogenetic tree constructed from ITS and 18S sequences (Figures 2 and 3), *U. pertusa* and *U. prolifera* belong to one branch. The homology of *U. pertusa*, and *U. prolifera* was obviously higher than *M. grevillei*.

The tree resulting from NJ analysis of the combined data of ITS is shown in Figure 2. As in previous analyses, the *Ulvaceae* and JM/UZ/BM/MN/KL were well supported. *C. caespitosa* and *U. californica* formed a tiny branch and *U. prolifera* joined them. *U. lactuca* and *E. intestinalis* composed another branch as *U. pertusa* and *U. fenestrata* did. There is strong support for the grouping of *C. caespitosa* with *U. californica* and *U. prolifera* in this phylogenetic tree, which coincided with the result from Hayden and Waaland [30]. *M. grevillei* and *U. zonata* formed one branch and then joined with *B. minima*, *M. nitidum*, and *K. leptoderma* forming one lager clade. The NJ tree and distance data both supported that *M. grevillei* has closer phylogenetic relation with *U. zonata* than *M. nitidum*.

The tree resulting from NJ analysis of 18S sequence is shown in Figure 3. In this tree, there is still strong support for the grouping of *C. caespitosa* with *U. californica* and *U. prolifera* while *U. pertusa* and *U. prolifera* formed a branch and *C. caespitosa* joined with them, which is different from the result of ITS in Figure 2. Additionally, *E. intestinalis* came to the same branch with *U. californica* which confirmed well with the data of region distance and the ratio of sequence divergence in Table 6. *B. minima* and *K. leptoderma* became one clade. *M. grevillei* and *U. zonata* formed one branch again and *M. nitidum* joined them firstly. In the NJ tree of 18S, the smaller branch of *B. minima* and *K. leptoderma* is further away from the bigger one of *M. nitidum*, *M. grevillei,* and *U. zonata*, which is a more significant diversity compared with the NJ tree of ITS.

4. Discussions

During the past three decades, green tide has been gaining in scale and frequency in both marine and estuary environment all over the world. And in recent years, green tide massively occurred in China Yellow Sea. The main species involved in green tides are *Ulva* sp. and *Enteromorpha* sp.

The traditional taxonomy uses the morphology characters as the criterion. The thallus that consists of two layer cells is the genus *Ulva* while the monolayer cell form hollow tubular is *Enteromorpha*. But the *Enteromorpha* internal tubular thallus contained villiform protuberances and meshwork structures which were composed of glycoprotein. When the number of cells in cross-section of tubular thallus reached

30–50 h, the villiform protuberances disappeared and the meshwork structures became tight, then the mural cells of tubular thallus adhered and the foliolose thallus formed [31]. In culture studies of European *Ulva* species, Gayral [32] observed the development of both tubular and blade thalli from single populations of zoospores and parthenogenetic gametes.

Ulva sp. has wide acclimatization and can grow well in a broad range of temperatures and salinities, but change morphological characteristics easily in response to the environment [16]. Various morphological changes in the intraspecies and less differences among interspecies make identification of *Ulva* sp. using classical taxonomic methods very difficult [22, 23].

Molecular biology methods such as chloroplast *rbc*L, nuclear ITS, and 18S rDNA sequence analysis [5, 26, 27], alone or in combination with morphological methods, have been applied to species identification since green tides have occurred in the Yellow Sea. Despite multidisciplinary study, classification of the main causative species of green tides is still difficult. Several researchers have suggested that green tides are formed by an *Ulva linza-procera-prolifera* (LPP) complex instead of individual species [5, 17].

Although the thallus of *U. pertusa* is thick with many holes, it is different from *U. prolifera* in morphology. From the phylogenetic tree constructed from 18S sequences, it can be found that *U. pertusa* and *U. prolifera* group in one branch while *U. lactuca*, *U. fenestrate*, and *U. californica* are in another branch. From the phylogenetic tree constructed from ITS sequences, *U. prolifera* and *U. pertusa* are not in one branch. But *U. pertusa* and *U. fenestrate* grouped in one branch. No matter if the phylogenetic tree is constructed from ITS sequences or 18S sequences, it is clear that the clade of *Ulvaceae* is comprised of *Chloropelta*, *Enteromorpha*, *Percursaria*, *Ulva* and *Ulvaria*. These results are consistent with that of Hayden and Waaland [30] which proved that in different areas it had the same conclusion.

Hayden et al. [21] preferred that *Enteromorpha* should be transferred to *Ulva* and *Chloropelta, caespitosa* should be named *Ulva tanneri* according to the molecular phyletic evolution research. From the phylogenetic tree constructed from ITS and 18S sequences in our study, it is obviously showed that *Enteromorpha* and *Ulva* have closer phyletic evolution relationship.

Trees inferred from phylogenetic analysis of ITS and 18S both showed that *Monostroma nitidum* and *Monostroma grevillei* were not in one branch while *Monostroma grevillei* and *Ulothrix zonata* are in one branch. *Monostroma grevillei* has a closer 18S genetic distance with *Ulothrix zonata* (0.008) than with *Monostroma nitidum* (0.018). The same result is in ITS genetic distance with *Ulothrix zonata* (0.133), other than that with *Monostroma nitidum* (0.257). These results are also consistent with that of Hayden and Waaland [30].

So, we can make use of sequences not only in 18S rDNA to analyse genera, but also in ITS region and other sequences, such as 28S rDNA and chloroplast *rbc*L, all these together are the better way to analyze the whole phyletic evolution relationship.

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