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The Amplification and Application of Ribosomal RNA (rDNA) Gene Sequences of *Blidingia minima* (Chlorophyta, Blidingia)

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Abstract

The sequence of the ribosomal RNA gene (rDNA) plays an important role in species identification and phylogenetic analysis. However, the only published full-length sequence of a ribosomal gene of green algae is that of *Ulva mutabilis*. In this study, we amplified the full-length sequence of each ribosomal gene unit of the ribosomal gene of *Blidingia minima*. The full-length sequence of the ribosomal gene in *Blidingia minima* is 8676 bp, including the 1759 bp 18S rDNA, 576 bp internal transcribed spacer (ITS) + 5.8S rDNA, 3282 bp 28S rDNA, and 3059 bp intergenic spacer (IGS) region. We then carried out a series of genetic analyses based on the ITS and IGS sequences, to verify whether IGS sequences are useful for studying the genetic diversity of green algae from different locations. We amplified the IGS sequences of *Blidingia minima* from 10 different locations in the Yellow Sea. Multiple alignments of the IGS sequences of samples from these 10 different sites revealed varying degrees of base differences, and comparative analysis of the ITS sequences revealed that our amplified species was classified as *Blidingia minima* and distinct from other green algae. In conclusion, our full-length amplified ribosomal gene provides useful information to enrich the data on green algae ribosomal genes and provides an effective molecular marker for the analysis of the interspecies and intraspecies relationships of *Blidingia minima*.

Keywords

Blidingia minima, rDNA, IGS (Intergenic Spacer), Intraspecies Relationships

1. Introduction

From 2007 to 2018, large-scale green tide events occurred every year in the Yellow Sea of China. The event was caused by the excessive deposition of fertilizers

combined with increased temperatures [1] [2], and such events have a negative impact on the local economy and the environment. In addition, green tides can be dispersed by wind and currents to other coastal areas [3]. The world's largest green tide events were mainly caused by *Ulva prolifera* O. F. Müller (Chlorophyta, Ulvophyceae), which erupted in 2008 in Qingdao on the Yellow Sea coast in north-eastern China. Based on satellite remote sensing images, it has been postulated that the origin of the green tide was closely related to the expansion of Pyropia aquaculture regions along the coastline of Jiangsu Province [4] [5]. There was molecular evidence that the species attached on the raft in Jiangsu Province and the species that caused the green tide were the same [3]. In addition to *U. prolifera*, a variety of other green algae were also identified on the *Pyropia* farming raft. Another of the fixed green algae species growing on the *Pyropia* farming rafts throughout the year is a *Blidingia* species with extensive biomass (**Figure 1**). *Blidingia* has similar habits to *U. prolifera* on the *Pyropia* farming rafts. The growth of large quantities of green algae on the *Pyropia* farming raft, especially *Ulva prolifera* and *Blidingia minima*, causes large economic losses to the aquaculture industries in China. During the outbreak of green tide, the biomass of *Ulva prolifera* is predominant. The biomass of other green algae, such as *Ulva flexuosa* and *Ulva compressa*, gradually decreased, but the amount of *Blidingia minima* remained almost unchanged. Few studies have shown why *Blidingia minima* can grow on *Pyropia* farming rafts throughout the year. Therefore, our amplified ribosomal gene cluster can provide some molecular information for subsequent research.

In eukaryotes, nuclear ribosomal DNA (nrDNA) is a cluster structure composed of multiple tandem transcription units, and each rDNA transcriptional unit consists of three rRNA coding sequences (the 18S rDNA gene, 5.8S rDNA gene and 28S rDNA gene), two internal transcribed spacers (ITSs) and one intergenic spacer (IGS) [6] [7] [8]. Due to different evolutionary rates, different regions of ribosomal transcription units can be regarded as molecular markers for species identification and phylogenetic studies at different levels in marine algal research. Reference [9] combined 18S rDNA and rbcL to analyse the phylogeny of *Chloromonas* and *Chlamydomonas* (Chlorophyceae, Volvocales), with an emphasis on snow and other cold-temperature habitats. Reference [10] demonstrated that *Phytophthora de Bary* and *Peronosporales* were a common natural group in phylogenetic studies based on nuclear large subunit ribosomal DNA sequences.

Figure 1. A large amount of *Blidingia minima* is fixed on a *Pyropia* farming raft.

Reference [11] combined ITS data, including the 5.8S rDNA sequence, with morphological data and found that the bloom-forming algae in the Yellow Sea in 2009 and 2010 were the same species. Reference [12] used the intergenic spacer (IGS) region to analyse the genetic variability of *Pyropia haitanensis* in different areas. Similarity analysis and multisequencing alignment of the sequences indicated that the IGS sequences of different regions of *P. haitanensis* had notable variability. Thus, the sequences of ribosomal transcription units are of great significance in phylogenetic research on marine algae.

This article provides additional information on the classification and identification of green algae. In addition, the methods outlined in this article can be used to obtain the entire ribosomal RNA gene (rDNA) sequence in other green algae taxa.

2. Materials and Methods

2.1. Sample Collection

Samples of *Blidingia minima* were collected from ten different sites in the Yellow Sea of China (Table 1). Once the samples were obtained at each site, they were immediately washed with seawater and dried in the shade to a moisture content of 30% - 40%. Finally, they were transported to the laboratory in an insulated specimen box at 4˚C. All samples used in this experiment were stored in the Algae Laboratory of the School of Basic Medical and Biological Sciences, Soochow University, and when fresh algae were obtained, the primary axis, diverged branches, colours and basal part (holdfasts) were recorded and photographed. The samples were sectioned into thin slices with a knife for observation under a microscope. Then, the cell shapes, sizes and arrangements were observed horizontally and vertically; the cell interior structures, such as chromatophores and pyrenoids, were also measured. A microscope (Nikon N90i) and a stereomicroscope (NiKon SMZ 1500) with an image collection system were applied in this research to obtain the main characteristics of the samples (Figure 2).

Table 1. Details of the specimens used in this study.

Sample ID	Collection site	Collection date	Latitude and longitude
A	Rudong, Nantong	18 Apr 2018	121˚20'E, 32˚48'N
B	Rudong, Nantong	21 Apr 2017	121˚18'E, 32˚55'N
C	Rudong, Nantong	20 Apr 2018	121˚21'E, 33˚24'N
D	Rudong, Nantong	15 Apr 2017	121˚08'E, 32˚37'N
E	Rudong, Nantong	16 Apr 2017	121˚25'E, 32˚26'N
JG1	Jianggang, yancheng	24 July 2018	121˚03'E, 32˚73'N
QG	Qionggang, yancheng	17 Apr 2017	121˚01'E, 32˚71'N
LYG	Lianyungang	25 Nov 2018	119˚30'E, 34˚87'N
ZQ1	zhaiqiancun, Haiyang	24 May 2018	121˚13'E, 36˚41'N
ZQ2	zhaiqiancun, Haiyang	24 May 2018	121˚21'E, 36˚36'N

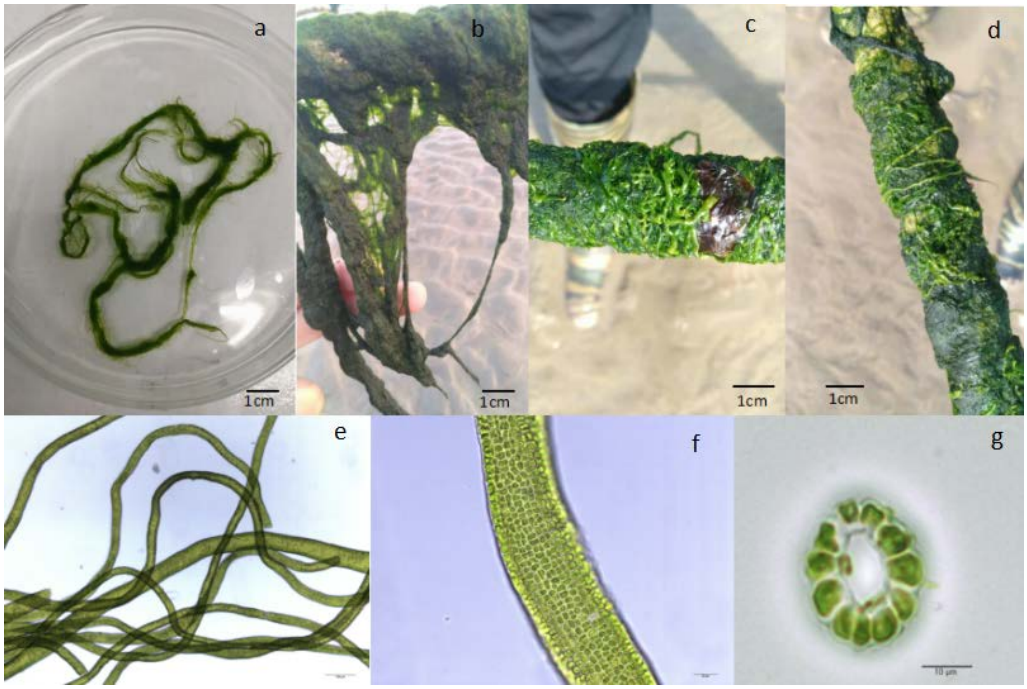

Figure 2. Morphological characteristicsof *Blidingia minima*. Thallus light-green, simple or branching, Lithophyte. Littoral, in exposed and sheltered localities (a)-(d). Microscopic details of *Blidingia minima*. The cells located on the lateral side of the body wall and are square or vertical, 5 - 10 μm long and 7 - 10 μm wide. 1 starch core and chloroplast (e)-(g).

2.2. Total RNA Extraction

Fresh algae samples were ground to a powder using an RNase-free mortar and pestle with liquid nitrogen, followed by the addition of 1 ml of Total RNA Extractor (Sangon Biotech, Shanghai) to completely cover the powder, according to the manufacturer's instructions. The concentration and quality of the extracted RNA were preliminarily tested by electrophoresis on 1% agarose gels. RNA samples were stored at −80˚C until use.

2.3. DNA Extraction

The algae were removed from the low-temperature refrigerator and placed in sterile double-distilled water for several hours. The algal surface was constantly brushed to remove the debris on the surface of the leaf thallus. The algae were then soaked in 0.7% KI for 10 min and rinsed several times with sterile seawater. Total genomic DNA was extracted by using a Plant Genomic DNA Kit (TIANGEN, BIOTECH (BEIJING) CO., Ltd) according to the instruction manual.

2.4. 3' RACE cDNA Preparation

First, a poly(A) tail was added to the 3' end of the RNA using poly(A) polymerase (Takara Biotechnology (Dalian) Co., Ltd). Then, 11 μL of RNA was combined with 1 μL of 3'-CDS primer A, heated to 72˚C for 3 min and cooled to 42˚C for 2 min. Next, 4.0 μL of 5× First-Strand Buffer, 0.5 μL of 100 mM DTT, 1.0 μL of 20 mM dNTPs, 0.5 μL of 40 U/μL RNase inhibitor and 2.0 μL of

SMARTScribe Reverse Transcriptase were added to each reaction tube according to the instructions of the SMART RACE Kit (Clontech). The 20 μL reaction was incubated at 42˚C for 90 min and then cooled to 70˚C for 10 min. The cDNA products were diluted with 90 μL of EDTA buffer.

2.5. PCR Amplification and Sequencing

An overview of the sequence cloning method of the ribosomal RNA gene in *Blidingia minima* is presented in Figure 3. The primers (Table 2) used in this study were designed with Primer 5.0 software. PCR amplification was carried out in a 50 μL volume containing 32.7 μL of ddH_2O, 5 μL of dNTP Mix (2.5 mM), 5 μL of the template DNA, 5 μL of 10× LA Taq Buffer (Mg^{2+}), 1 μL of each primer (20 nmol/L), and 0.3 μL of LA Taq DNA polymerase (Takara Biotechnology (Dalian) Co., Ltd.). The 3' RACE amplification was performed in a 50 μL volume containing 15.5 μL of PCR-Grade H_2O, 25.0 μL of 2× SeqAmp Buffer, 1.0 μL of SeqAmp DNA Polymerase (Clontech), 2.5 μL of cDNA, 5 μL of 10× UPM, and 1 μL of 10 μM Gsp. Colony PCR was performed in a final volume of 20 μL containing 13.8 μL of ddH_2O, 2 μL of dNTP Mix (2.5 mM), 2 μL of 10× Ex Taq Buffer (Mg^{2+}), 1 μL of LB medium containing a positive colony, 0.5 μL of each primer (20 nmol/L), and 0.2 μL of Ex Taq DNA polymerase (Takara Biotechnology (Dalian) Co., Ltd.).

The PCR amplification cycle is presented in Table 3. The colony PCR conditions were as follows: 3 min initial denaturation at 95˚C; 30 cycles of denaturation at

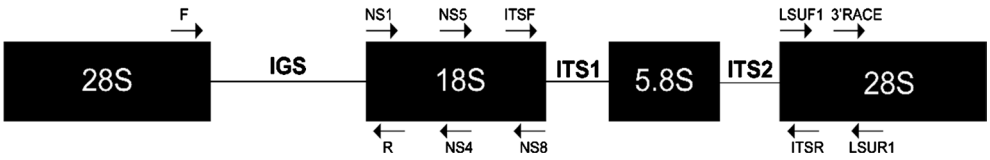

Figure 3. An overview of the sequence cloning method of the ribosomal RNA gene in *Blidingia minima*.

Table 2. Primers used for amplification in the present study.

Primer name	Gene product	Sequence (5' → 3')	Region and position	Source
NS1	18S rDNA	GTAGTCATATGCTTGTCTC	5' end (F)	[13]
NS4	18S rDNA	CTTCCGTCAATTCCTTTAAG	−1150 (R)	[13]
NS5	18S rDNA	AACTTAAAGGAATTGACGGAAG	−1150 (F)	[13]
NS8	18S rDNA	TCCGCAGGTTCACCTACGGA	3' end (R)	[13]
ITSF	ITS + 5.8S rDNA	GAGGCAATAACAGGTCTGTGATGC	5' end (F)	In this study
ITSR	ITS + 5.8S rDNA	GCTTATTGATATGCTTAAGTTCAGCG	3' end (R)	In this study
LSUF1	28S rDNA	GTTGAGGCTACCCGCTGAACTTA	5' end (F)	In this study
LSUR1	28S rDNA	CAGAAGATCATGGTCGGTCGATG	3' end (R)	In this study
GSP7	28S rDNA	CGCAAGGAAACTGACTGGTGGGA	5' end (F)	In this study
F	IGS	AGATAGGACGGGGTATTGTAAG	5' end (F)	In this study
R	IGS	GGATGTGGTAGCCGTTTCTCAG	3' end (R)	In this study

Table 3. PCR reaction profiles for amplifying regions of the ribosomal unit.

LSUF1/R1	GSP7(3' RACE)	ITSF/ITSR	F/R	IGSF1/R1
① 95˚C 3 min	① 94˚C 30 s	① 94˚C 3 min	① 95˚C 3 min	① 95˚C 3 min
② 94˚C 30 s	② 68˚C 30 s	② 94˚C 30 s	② 94˚C 30 s	② 94˚C 30 s
③ 48˚C 30 s	③ 72˚C 5 min	③ 53˚C 30 s	③ 55˚C 30 s	③ 56˚C 30 s
④ 72˚C 1 min	④ ①-③ 20 cycles	④ 72˚C 1.5 min	④ 72˚C 5 min	④ 72˚C 1.5 min
⑤ ②-④ 35 cycles		⑤ ②-④ 35 cycles	⑤ ②-④ 35 cycles	⑤ ②-④ 35 cycles
⑥ 72˚C 7 min		⑥ 72˚C 10 min	⑥ 72˚C 7 min	⑥ 72˚C 7 min

95˚C for 30 s, primer annealing at 55˚C for 30 s, and extension at 72˚C for 1 - 5 min; and a final extension step at 72˚C for 5 min. One percent agarose gel electrophoresis was used to analyse the PCR products.

The PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit Ver.4.0 (Takara Biotechnology (Dalian) Co., Ltd.), TA-cloned into the *pEASY*-T3 vector, and transformed into *Trans*1-T1 Phage Resistant Chemically Competent Cells (Beijing TransGen Biotech Co., Ltd.). Transformed cells were spread onto LB agar plates containing X-gal, Amp and IPTG, and the plates were then cultivated at 37˚C for at least 12 h. Positive colonies were picked and cultivated, and the transformed colonies were confirmed by colony PCR. Three positive recombinant colonies of each amplification product were sequenced using Sanger dideoxy sequencing by GENEWIZ Biotechnology Co. Ltd., Suzhou, China.

3. Sequence Alignment and Analysis

Nucleotide BLAST was used for sequence alignment with the database to confirm the amplification results. Tandem repeats within the IGS sequence were identified using the Tandem Repeat Finder [14]. Multiple sequence alignment analysis was carried out using the DNAMAN software. Phylogenetic analysis was conducted using MEGA version 5 software [15]. Sequences were aligned using the CLUSTALW program. Bootstrap values obtained after 1000 replications are shown on the branches. The numbers around the branches indicate bootstrap supports.

4. Results and Analyses

4.1. Amplification of the 18S rDNA, ITS + 5.8S rDNA, 28S rDNA and IGS Sequences

Total genomic DNA or cDNA was used to amplify the fragments of ribosomal RNA in *Blidingia minima* with the corresponding primers. The 18S rDNA was amplified with two pairs of primers (**Figure 4(a)**), and sequencing and splicing results indicated that the 18S rDNA sequence of *Blidingia minima* was 1759 bp in length. A single target band was successfully amplified with a pair of primers (**Figure 4(b)**), and the ITS sequence including 5.8S was 576 bp in length. Since it

was difficult to amplify the full-length 28S rDNA with only one pair of primers, 3' RACE technology combined with common PCR was applied to amplify this region. The amplified results were finally obtained and are shown in Figure 4(c) and Figure 4(d). After careful analysis, the complete length of the 28S rDNA sequence was determined to be 3282 bp. The primers for amplifying IGS sequences were designed based on the 18S rDNA and 28S rDNA sequences, and the amplified results are shown in Figure 4(e). Finally, through analysis and integration, we obtained the full-length 3059 bp IGS sequences. Each sequence region of the ribosomal RNA was submitted to GenBank with the accession numbers KY235302 (18S rDNA), KY235301 (ITS + 5.8S rDNA), KY401415 (28S rDNA), and KY235300 (IGS). The full-length sequence of the ribosomal RNA gene in *Blidingia minima* is 8676 bp.

4.2. Base Composition of the 18S rDNA, ITS + 5.8S rDNA, 28S rDNA and IGS Sequences

The base composition and GC content of each rDNA fragment were analysed, and the results showed that the C-base contents of the ITS and IGS sequences were the highest, and the G-base contents of the 18S rDNA and 28S rDNA sequences were the highest. The GC contents of the 18S rDNA, ITS + 5.8S rDNA, 28S rDNA and IGS sequences were 49.57%, 62.84%, 51.55% and 53.08%, respectively (Table 4).

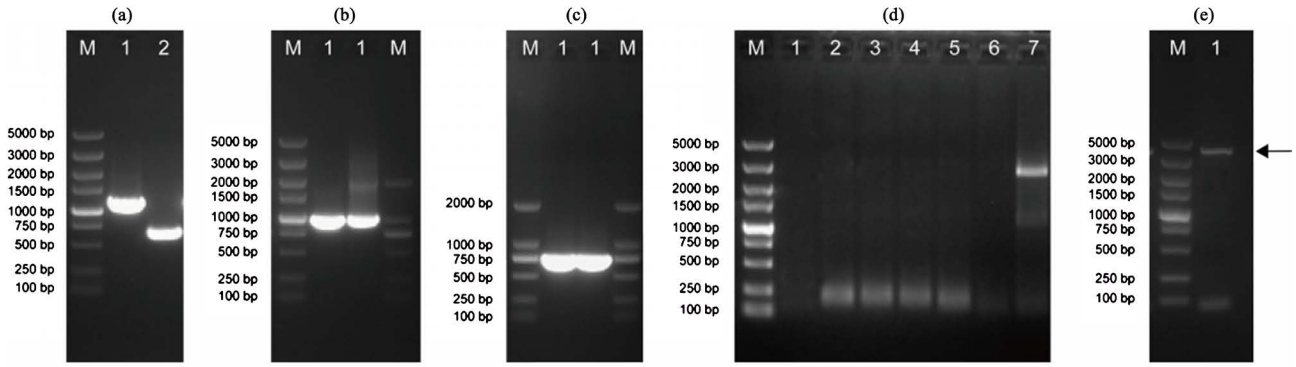

Figure 4. Gel electropherogram of PCR amplification products. (a) Lanes 1 and 2 represent the 18S rDNA sequence of *Blidingia minima.* (b) Lane 1 represents the ITS sequence of *Blidingia minima.* (c) Lane 1 represents the first portion of the 28S rDNA sequence in *Blidingia minima.* M: Takara DL2000 DNA marker (d) Lane 7 represents the second portion of the 28S rDNA sequence of *Blidingia minima* amplified by 3' RACE. (e) Lane 1 represents the IGS sequence of *Blidingia minima.* M: Takara DL5000 DNA marker.

Table 4. Base composition of each fragment of rDNA.

Fragments (bp)	The type of base				GC (%)
	A	G	C	T	
ITS + 5.8S	104	171	191	109	62.84
18S	441	488	384	446	49.57
28S	850	959	733	740	51.55
IGS	610	776	848	825	53.08

4.3. Phylogenetic Tree Analysis

Maximum likelihood phylogenetic trees were established based on the ITS sequences (Figure 5). Comparative analysis of the ITS sequences revealed that our amplified species were classified as *Blidingia minima* and distinguished from other green algae.

Maximum likelihood phylogenetic trees were established based on the IGS sequences (Figure 6) showed that the strains were divided into two big clades. The ZQ1 and ZQ2 strains formed one clade, other eight strains formed another big clade. This result shows that IGS is suitable for intraspecies relationship analysis.

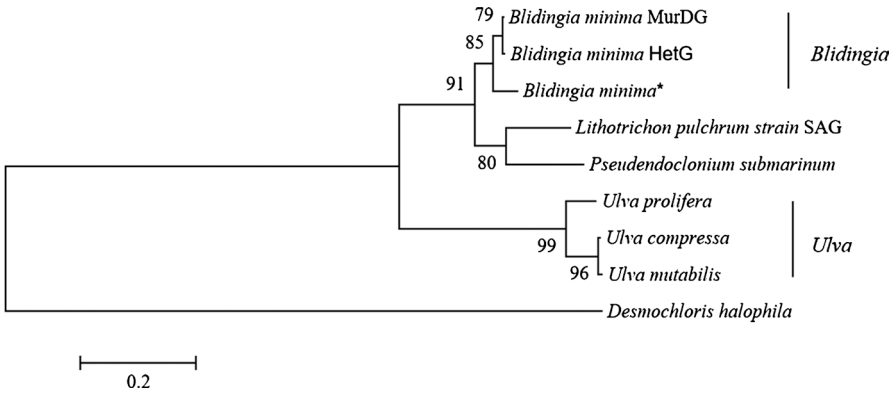

Figure 5. Maximum likelihood trees based on the ITS sequences of various green alage. Asterisks indicate samples from this study. The accession numbers of the sequences (not shown in figure) are *Blidingia minima* HetG (AF163102); *Blidingia minima* MurDG (AF163104); *Ulva mutabilis* (AEU256377); *Lithotrichon pulchrum* strain SAG (MF034614); *Pseudendoclonium submarinum* (MF034619); *Ulva prolifera* (AB830492); *Ulva compressa* (AB830495); *Desmochloris halophila* (HE610118).

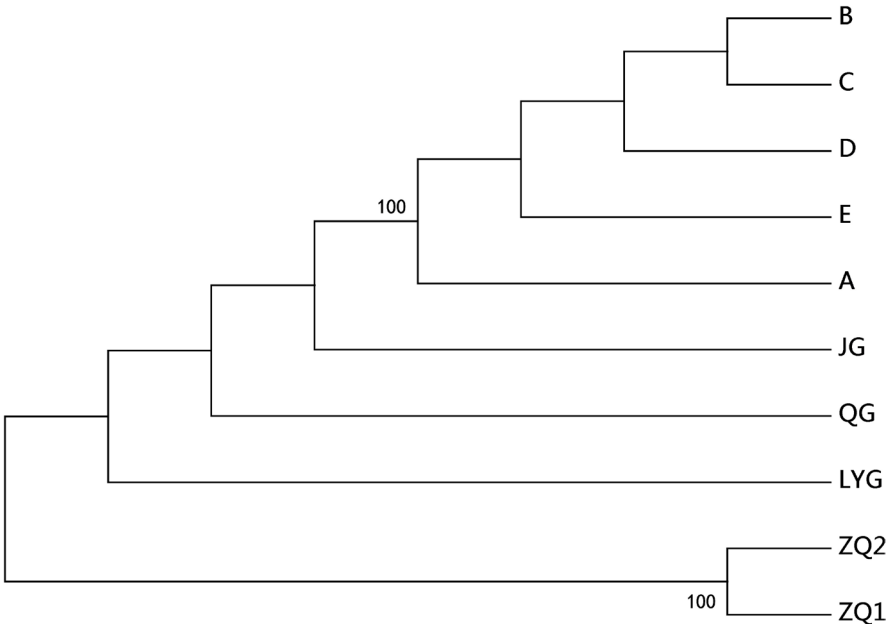

Figure 6. Maximum likelihood trees based on the IGS sequences of ten strains of *Blidingia minima*, abbreviations of strain names as in Table 1.

4.4. Analysis of Special Structures in the IGS

Two tandem repeats of different lengths at the 5' end of the IGS sequence were found by the Tandem Repeat Finder. In addition, short dyad and palindromic sequences were found in the 3' end and in the middle of the IGS sequence. The locations of specific structures in the IGS sequence are shown in Figure 7, and the detailed characteristics of these special structures are presented in Table 5.

4.5. IGS Sequence Similarity Analysis

The lengths of the IGS sequences of 10 strains were all about the same (Figure 8), and the sequences were relatively conserved. Multiple alignment of the IGS sequences of *Blidingia minima* from 10 different strains (Figure 9) showed multiple base differences, which indicated that the IGS sequences of the strains in different provinces were different, while the strains of the same province had only a few base differences.

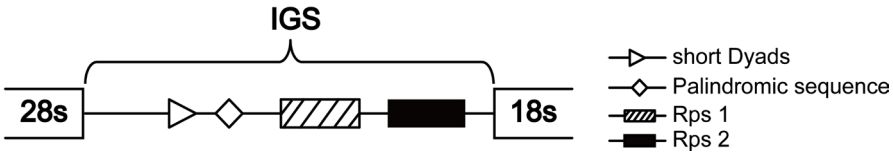

Figure 7. The structures of the IGS region in *Blidingia minima*.

Table 5. Characterization of special structures presented in the IGS sequence of *Blidingia minima*.

Species	Type of structure	Nucleotide position	Motif length [(bp)]	Motif Sequence	Copy number
Blidingia minima	RPS1	1145 - 1221	5	CTTTG	15
	RPS2	1333 - 1368	18	GCTGCTTCAATGTGCTTC	2
	Short Dyad	680 - 694	15	CTCTACCTCCATCTC	1
	Palindromic Sequence	701 - 716	16	GTAGAGGTACCTCTAC	1

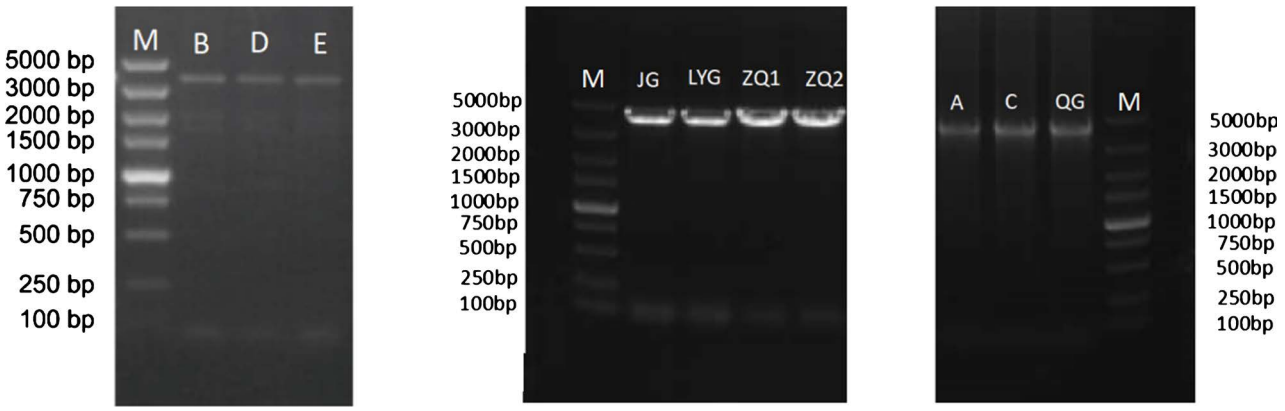

Figure 8. Gel electropherograms of PCR amplification products of *Blidingia minima* from the A, B, C, D, E, JG, LYG, ZQ1, ZQ2 and QG sites. M: Takara DL5000 DNA marker.

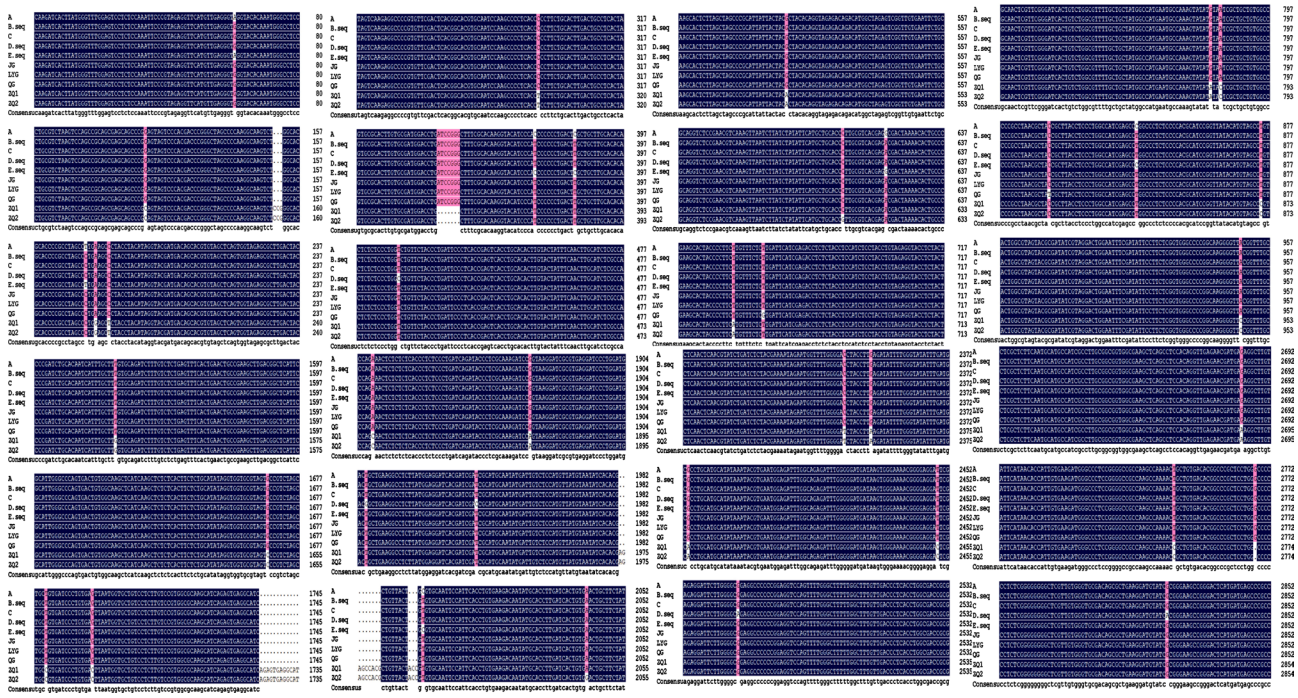

Figure 9. Four parts of alignment of IGS sequences from five strains of *Blidingia minima* which have obvious differences. Black indicates where base pairs of nucleotides of the five strains were all the same; red indicates where base pairs of nucleotides of one or two strains were different from the other strains; white indicates where base pairs of nucleotides of three or more strains were different from the other strains. Abbreviations of strain names as in Table 1.

5. Discussion

In eukaryotic organisms, the 18S rDNA, 5.8S rDNA, and 28S rDNA cluster as one transcript and are separated from the ITS and IGS. The nuclear ribosomal DNA is among the most conserved genes in living creatures (Hassouna *et al.*, 1984) [16]. Because different regions of nuclear ribosomal DNA have different functions, their evolutionary rates are different. Here, we carried out a series of genetic analyses based on the ITS and IGS sequences alone.

In this study, we obtained the full-length sequences of the 8676 bp ribosomal RNA gene (rDNA) in *Blidingia minima*, which included the 1759 bp 18S rDNA, 576 bp ITS + 5.8S rDNA, 3282 bp 28S rDNA, and 3059 bp IGS region. Reference [17] and [18] successfully obtained the complete ribosomal RNA genes of *Pyropia yezoensis* and Bangia (Bangiales, Rhodophyta). The total length of the ribosomal RNA gene of *Pyropia yezoensis* was approximately 13,650 bp and that of Bangia was 12,560 bp. Comparing their full-length ribosomal RNA genes revealed that the ribosomal RNA gene of *Blidingia minima* was much shorter than those of *Pyropia yezoensis* and *Bangia*, and the sequence length of each region was also very different. In the process of amplifying the ribosomal RNA gene, we could search for only a partial sequence of the 28S 5' end of the nucleic acid sequence in the NCBI library, and it was very difficult to amplify the complete 28S sequence and IGS sequence with only one pair of primers. Therefore, we amplified the known partial sequence by PCR and then amplified the 28S 3' end sequence by rapid amplifica-

tion using cDNA ends technology. Finally, through sequencing and splicing, a 3282 bp 28S sequence was obtained. In the process of IGS sequence amplification, due to the presence of some specific structures in the IGS sequence, more specific primers and stringent reaction conditions were required in the PCR. Fortunately, we finally obtained a pair of efficient primers and identified the best reaction conditions. The GC content of each unit of the ribosomal RNA sequence unit was analysed and found to be highest in the ITS regions and lowest in the 18S rDNA genes. The total GC content of the ribosomal RNA gene was 54.26%, which was in the range of 35% - 56% [19].

Given their high evolutionary rate, ITS sequences are expected to be suitable for detecting interspecific variation [20] [21]. We have made a phylogenetic tree of the amplified ITS sequences, and the results show that our amplified species are clustered together with *Blidingia minima*, whereas other algae are clustered into another branch, indicating that our expanded ITS can be used as an effective molecular marker to distinguish interspecies relations.

IGS sequences have been widely used as molecular markers, especially for intraspecies relationship analysis; for example, it has been used to distinguish Hordeum [22], Miscanthus [23], rice [24] [25] [26], and *Pyropia haitanensis* [27]. These regions can also contain functional sequences, such as spacers, promoters and enhancers, and transcription initiation and termination sites [28] [29]. Indeed, the IGS structure is attractive to study because of its importance in the transcription regulation of rRNA genes through the regulation of stop and start signals for transcription of the rDNA units that are located within the region [30] [31]. We compared the full sequence of IGS from 10 strains from different provinces. Strains in the same province were significantly different from IGS sequences in different provinces, which is consistent with the conclusion that the IGS is a highly variable region with a rapid evolutionary rate in *Blidingia minima.* Therefore, it can provide powerful classification and identification support at the species level and below.

6. Conclusion

In this paper, we first amplified the complete nuclear ribosomal DNA sequence of *Blidingia minima* and carried out a series of genetic analyses based on the ITS and IGS sequences. ITS can be effectively used as a molecular marker for interspecies identification. The phylogenetic tree shows that *Blidingia minima* can be distinguished from other green algae. We also used IGS to compare the homology of 10 different strains in the Yellow Sea. The results also show that IGS can provide important information for studying the intraspecific relationships and genetic diversity of *Blidingia minima*.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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