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The complete nuclear ribosomal DNA (nrDNA) cistron sequence of *Pyropia yezoensis* (Bangiales, Rhodophyta)

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Abstract The complete nuclear ribosomal DNA (nrDNA) cistron sequence of *Pyropia yezoensis* has been sequenced, of which each unit is composed of the intergenic spacer (IGS), the small-subunit (SSU) ribosomal RNA (rRNA) gene, the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, the internal transcribed spacer 2 (ITS2), and the large-subunit (LSU) rRNA gene. This cistron occurs in tandem repeats that can number in 100s to 1000s. Furthermore, in the analysis of the sequence data, we determined that introns were present in the small-subunit rRNA genes, for which the exon sequences (1834 bp) are almost identical. The intron numbers ranging from zero to two in the small-subunit rRNA genes are different between multiple individuals. However, the full length of the large-subunit rRNA genes is 4770 bp without any introns, and the 5.8S rRNA gene is 159 bp. Moreover, the 5′ and 3′ terminal sequences of the genic regions of the ribosomal cistron are extremely conservative even among various species in red alga. In addition, the range for ITS1 is 371–372 bp and for ITS2 is 532 to 535 bp. The IGS is 5984 bp in length and possesses upstream direct repeats, downstream repeats, and middle inverted repeats that make sequencing untoward. This is the first time that the complete nucleotide of the IGS region of *P. yezoensis* has been sequenced. The provision of the full length of nrDNA cistron will offer useful information to be widely utilized for identification, taxonomic relationships, and phylogenies in red algae.

Keywords *Pyropia yezoensis* · Bangiales · 5.8S rDNA · SSU rDNA · LSU rDNA · ITS · IGS

Introduction

The red algal order Bangiales has recently been revised, and the new genus *Pyropia* includes more than 75 species (Sutherland et al. 2011). This genus is commonly known as “zicai” in China, “nori” in Japan, and “kim” in Korea and has been utilized primarily as a food source for thousands of years in East and Southeast Asia (Niwa et al. 2008, 2009). In addition, *Pyropia* is one of the largest aquaculture industries in China, Japan, and Korea and, together with *Porphyra*, has an economic value of US$258 million in China in 2008 and US$1.3 billion per year worldwide (Blouin et al. 2011; He et al. 2013). *Pyropia yezoensis* is distributed along the coastal areas of Jiangsu and Shandong Province in China and is considered to be the most highly valued and economically important seaweed crop in East Asia (Nakamura et al. 2013). With the further research of cultivation, physiology, genetics, and genomics on marine plants, it has been observed that *P. yezoensis* has much more potential to be used as a model system for molecular genetics and other research fields similar to *Arabidopsis thaliana* (Saga and Kitade 2002; Sahoo et al. 2002; Matsuyama-Serisawa et al. 2007).

P. yezoensis, similar to other members of Bangiales, exhibits heteromorphic alternation of generations, and the gametophyte thallus is monecious and macroscopic. The gametophyte is the primary life history stage that is used for food as well as for scientific studies. The sporophytic stage, however, is primarily used for culturing and germplasm conservation (Iwasaki 1961). The morphological characteristics of the

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gametophyte thallus have been used for classical taxonomy for this genus, and these characteristics are comparatively simple and tend to vary according to habitat and other environmental conditions. Due to the lack of stable taxonomic characteristics, it is challenging to identify *Pyropia* to species (Brodie et al. 1998). With the rapid development of molecular biology, more and more investigators tend to adopt the molecular approaches to discriminate the various *Pyropia* species, which makes the identification and classification more efficient and accurate. In recent decades, the nuclear ribosomal DNA has been studied more for molecular classification system in marine algae (Patwary et al. 1998; Broom et al. 1999; Kunimoto et al. 1999a, b; Harper and Saunders 2001; Milstein and de Oliveira 2005; Hu et al. 2010; Saunders and Moore 2013).

In eukaryotes, the nuclear ribosomal DNA (nrDNA) is a multigene family. The rDNA transcription units consist of an intergenic spacer (IGS) [containing a non-transcribed spacer (NTS) region and an external transcribed spacer (ETS) region], the small-subunit (SSU) rDNA gene, internal transcribed spacer 1 (ITS1), the 5.8S rDNA gene, internal transcribed spacer 2 (ITS2), and the large-subunit (LSU) rDNA gene arrayed in this order and then tandemly repeated in clusters (Fig. 1) at the nucleolus organizer regions (NOR) of one or more chromosomes (Appels and Honeycutt 1986; Harper and Saunders 2001). These repeated clusters exhibit concerted evolution and do not evolve independently from each other, and the numerous cistrons maintain homogeneity (Zimmer et al. 1980; Harper and Saunders 2001; Alvarez and Wendel 2003). It is widely hypothesized that the mechanisms of concerted evolution are DNA recombination, repair, and replication, such as gene conversion and unequal crossing-over (Dover 1987; Worheide et al. 2004; Naidoo et al. 2013).

Due to different rates of evolution, these different regions of the ribosomal units have been used as molecular markers for phylogenetic analysis as well as germplasm identification. The SSU and LSU ribosomal RNA (rRNA) genes are conserved, so they have been selected as genetic markers to make discrimination at or above the species levels within various red algal lineages (Nelson et al. 2001; Gall and Saunders 2007). The complete nucleotide sequences of the SSU rDNA were used to determine nine *Porphyra* species by Kunimoto, and it was found that two classes of introns located upstream and downstream respectively were present in the *Porphyra* SSU rRNA gene (Kunimoto et al. 1999a), and the exon nucleotide sequences were identical in intraspecies (Kunimoto et al. 1999b). Because of the shortness of the 5.8S rRNA gene, it is rarely used by itself and generally combined with ITS regions to study intra- and interspecific evolution (Li et al. 2009; Chen et al. 2010). Moreover, the IGS region shows to be more variable and phylogenetically informative than the ITS, so it has been used for classification in intraspecies of different geographical distributions and among strains, even individuals (Dai et al. 2008; Li et al. 2010; Parvaresh et al. 2014).

In this paper, we provide the complete sequence of rDNA unit of *P. yezoensis* from Rudong, Jiangsu Province, which will provide more information for classification and identification in *Pyropia* species. In addition, the methodology outlined in this paper can be applied to obtain the entire rDNA cistron in other red algal taxa.

Materials and methods

The foliose gametophytes of *Pyropia yezoensis* strains used in this study were collected in 2012 from the cultivation center of Rudong in Jiangsu Province and frozen at −20 °C. The individual blades were identified as belonging to the species *P. yezoensis*, based on morphological characters. Prior to DNA extraction, samples were rehydrated in ddH_2O and also rinsed several times. DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) method (Yang et al. 1999). Initially, each thallus was separately ground with a pestle and mortar in liquid nitrogen until it became a very fine powder. Approximately 500 μL DNA extraction lysate [3 % CTAB, 0.1 mol L^{-1} EDTA (pH=8.0), 0.1 mol L^{-1} Tris-HCl (pH=8.0), 1.4 mol L^{-1} NaCl, 1 % PVP, 0.2 % (*v*/*v*) β-mercaptoethanol] and 5 μL protease K were added to the ground powder, after which the mixture was transferred to a

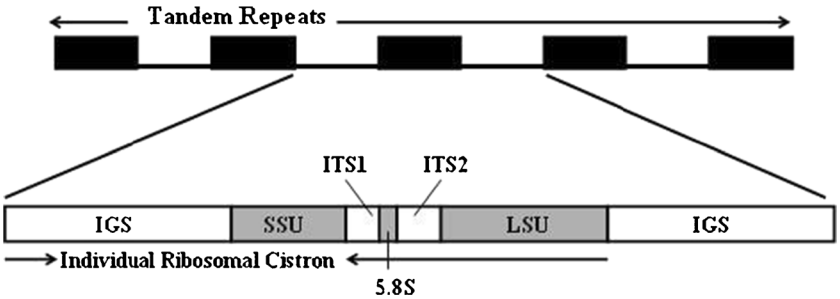

Fig. 1 Schematic diagram of the tandemly repeated ribosomal DNA (Harper and Saunders 2001). The *shaded regions* (*SSU*, *5.8S*, *LSU*) are separated by ITS1 and ITS2, which compose a transcription unit (a *black frame*). Two adjacent transcription units are separated by IGS (*a line between two black frames*)

Table 1 Primers used for PCR

Regions	Primers	Sequence 5′–3′	Position
SSU	SSUF	CAACCTGGTTGATCCTGCCAGT	18S: 1–22 (GenBank No. AB013177)
	SSUR	AATGATCCTTCCGCAGGTTCAC	18S: 2328–2349 (GenBank No. AB013177)
LSU	LSUF	CGGAGGAAAAGAAACTAACAAGGAT	28S: 1–25 (GenBank No. EF033597)
	LSUR	GGAAGAGCCGACATCGAAGGAT	28S: 3003–3024 (GenBank No. EF033597)
ITS	ITSF	GTAGGTGAACCTGCGGAAGGAT	18S: 1809–1830 (GenBank No. KJ578745)
	ITSR	CCCCATTTCAGACGGGATTTAT	28S: 137–158 (GenBank No. KJ578748)
IGS	IGS1F	GGAGGCTGGATAACGCTG	28S: 2069–2086 (GenBank No. JN104580)
	IGS1R	GCCATTCGCAGTTTCACG	18S: 85–102 (GenBank No. JN104582)
	IGS2F	TCCATACAACATAAACAGAGAGCG	IGS: 1101–1124 (GenBank No. KJ608640)
	IGS2R	TGGTGGGTCCTGAACGATT	18S: 811–829 (GenBank No. JN104582)
	IGS3F	TCTGGAGGGTTAGTTACTGGCAAAG	IGS: 646–670 (GenBank No. KJ608640)
	IGS3R	CTCCCTGATGAGTTACAGACCGAG	IGS: 4671–4694 (GenBank No. KJ608640)
	IGS4F	GCGTCGGCAAAAGGCACTCTGT	IGS: 1765–1785 (GenBank No. KJ608640)
	IGS4R	TATGAACCTGACCCCCATGAACC	IGS: 2593–2615 (GenBank No. KJ608640)
	IGS5F	AATTCGACATAGGGAGGAAGGGTT	IGS: 1922–1945 (GenBank No. KJ608640)
	IGS5R	ACGCGGTGAAGAGGCACAGTTT	IGS: 2401–2422 (GenBank No. KJ608640)

centrifuge tube and incubated at 60 °C for 2 h, with shaking well every 30 min. Following this, 200 μL 5 mol L^{-1} potassium acetate and an equal volume of chloroform/isoamyl alcohol (24:1) was added to the tube. The tube was then shaken to emulsify the liquid and then centrifuged at 8000×*g* for 10 min at 4 °C, and the supernatant was moved to a new tube. DNA in the supernatant was precipitated with two-thirds volume of glacial isopropanol by gently mixing and then centrifuging at 13,000×*g* for 20 min at 4 °C. The pellet was washed with 400 μL 70 % ethanol, air dried, and dissolved in 400 μL 1× TE buffer (0.01 mol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA). The solution was re-extracted once with chloroform. The purified DNA was eventually precipitated with dehydrated alcohol, air dried, and dissolved in 50 μL 0.1× TE buffer, used as a template for PCR amplification.

Regions of the ribosomal cistron were PCR amplified using oligonucleotide primers as shown in Table 1. The ITS1, 5.8S rDNA, and ITS2 were amplified as a single fragment with forward primer ITSF and ITSR. Due to the overlength and complex structures in the sequence of the IGS region, the partial LSU rDNA and IGS region were amplified as five fragments.

Genomic DNA was used as the template for each primer pair. The total volume for all PCR reactions was 50 μL, which contained 5 μL genomic DNA and per microliter concentration of DNA is 20 ng, 6 μL dNTP mixture (2.5 mM each), 5 μL 10× LA Taq Buffer II (Mg^{2+} plus) (TaKaRa), 1 μL of each primer (20 μM), and 0.5 μL LA Taq DNA polymerase (5 U μL^{-1}, TaKaRa). Each PCR reaction profile involves an initial denaturation, 30 cycles of denaturation, annealing, extension, and a final extension shown in Table 2.

Sequencing and analysis

The PCR amplification products were electrophoresized on 1 % agarose gels and purified using a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (Ver. 3.0). *pEASY*-T3 Cloning Kit and *Trans*1-T1 Phage Resistant Chemically Competent Cell (Beijing TransGen Biotech. Co., Ltd) were used for cloning the fragments according to the manufacturer's instructions. Three positive recombinant colonies of

Table 2 PCR reaction profiles for amplifying regions of the ribosomal unit

SSUF/R	LSUF/R	ITSF/R	IGS1F/R, IGS2F/R	IGS3F/R	IGS4F/R	IGS5F/R
① 95 °C 3 min	① 95 °C 3 min	① 95 °C 3 min	① 94 °C 5 min	① 95 °C 3 min	① 95 °C 3 min	① 95 °C 3 min
② 94 °C 30 s	② 94 °C 30 s	② 94 °C 30 s	② 98 °C 10 s	② 94 °C 30 s	② 94 °C 30 s	② 94 °C 30 s
③ 56 °C 30 s	③ 58 °C 30 s	③ 56 °C 30 s	③ 55 °C 30 s	③ 60 °C 30 s	③ 60 °C 30 s	③ 55 °C 30 s
④ 72 °C 3 min	④ 72 °C 5 min	④ 72 °C 2 min	④ 72 °C 2 min	④ 72 °C 7 min	④ 72 °C 3 min	④ 72 °C 40 s
⑤ ②–④ 30 cycles	⑤ ②–④ 30 cycles	⑤ ②–④ 30 cycles	⑤ ②–④ 30 cycles	⑤ ②–④ 45 cycles	⑤ ②–④ 30 cycles	⑤ ②–④ 30 cycles
⑥ 72 °C 7 min	⑥ 72 °C 7 min	⑥ 72 °C 7 min	⑥ 72 °C 7 min	⑥ 72 °C 7 min	⑥ 72 °C 7 min	⑥ 72 °C 7 min

Table 3 GenBank accession number of each region of the ribosomal cistron

	SSU rDNA	LSU rDNA	ITS region	IGS region
GenBank No.	KJ578745–KJ578747	KJ578748	KJ608639	KJ608640

each amplification product were sequenced using the Sanger method by Genewiz Biotechnology Co., Ltd. (Suzhou). Sequences were confirmed using BLASTN by comparison with available data in GenBank (Altschul et al. 1990). The identified sequences were aligned using megablast (bl2seq) for defining their boundaries and future manual sequence adjusting and splicing. Multiple sequence alignment was carried out by the software DNAMAN. Each region sequence of the ribosomal cistron was submitted to GenBank, and the accession numbers are shown in Table 3.

Results

SSU and LSU rDNA

Fragments of the entire SSU rDNA were PCR amplified from several individuals of *P. yezoensis* (Fig. 2a). The obtained sequences have 99 % identity with sequences of *P. yezoensis* published in the GenBank database. Due to the presence of introns, we found that three types of the SSU rDNA are present in the population of *P. yezoensis* collected from Rudong, Jiangsu Province, China (Fig. 3a), which were sequenced to be 3310 bp named type A, 2344 bp named type B, and 1834 bp named type C. However, the exon sequences of the SSU rDNA were almost identical with a nucleotide difference in type A, and the length is 1834 bp. Multiple sequence alignment indicated that the 5′ end and 3′ end of the upstream intron (510–511 bp) located at the nucleotide position 569 were AAC- and -TGG, and the 5′ end and 3′ end of the downstream one (965 bp) located at 2316 were TTC- and -ACG, respectively (Fig. 3a). In addition, two SSU rDNA sequences of *P. yezoensis* from Japan (GenBank No. AB013178 and AB013177) were analyzed (Fig. 3b), the exon length of which is 1832 bp.

The entire LSU rDNA fragments amplified from several individuals were the same in length (Fig. 2b). The amplified sequences having 99 % identity with the LSU rDNA sequences of *P. yezoensis* (GenBank No. KF501435 and JN104580) were confirmed. The LSU rDNA shared high homology (99 % sequence identity) between individuals of *P. yezoensis*, which just had four base differences.

ITS

Aligning two sequences to carry out homology analysis, we found that the 5′ and 3′ end nucleotide sequences of the amplified fragments were identical with the 3′ flanking region of the entire SSU and the 5′ flanking region of the entire LSU, respectively, so the sequence was confirmed to be the ITS. The ITS nucleotide sequences were almost identical (Fig. 2c) just with few base absence of A and one or two nucleotide differences, so the length varied from 1062 to 1066 bp. The length of 5.8S rDNA which is highly conservative is 159 bp.

IGS

The IGS sequence that was amplified with five primer pairs contains a large proportion of the 3′ end of the LSU rDNA and partial 5′ end of the SSU rDNA. The length of the complete IGS spacer was determined to be 5984 bp after excising the

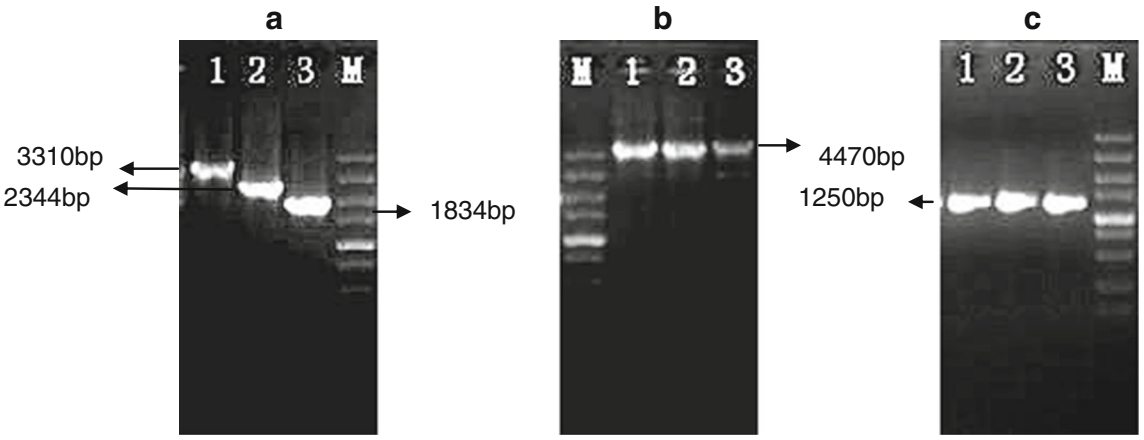

Fig. 2 Gel electrophoresis of PCR amplification products. Lane *M* shows the DL 5000 DNA marker (TaKaRa). The fragments were amplified from various individuals, the lengths of which are indicated by *arrows* and *corresponding numerals*. **a** PCR products of the SSU rDNA. *Lanes 1 to 3* show difference in length. **b** PCR products of the LSU rDNA. *Lanes 1 to 3* show identical fragment length (4770 bp). **c** PCR products of the ITS. The fragments are identical in size, which is indicated in *lanes 1* to *3*

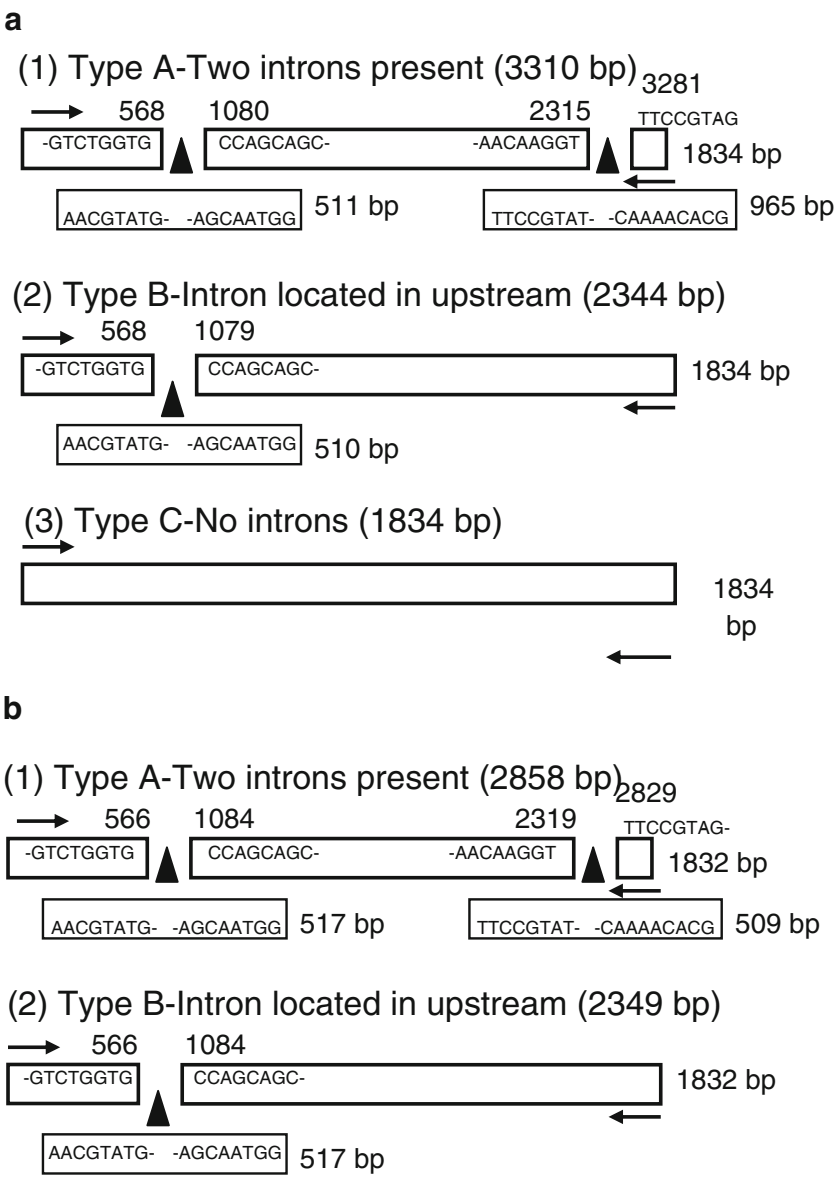

Fig. 3 Schematic representation of the structure of the SSU rDNA of *Pyropia yezoensis*. The *upper frames* represent exons, and the *lower frames* indicate introns. *Triangles* represent the inserted positions of introns. *Numbers above and below the frames* indicate the position of intron insertion and length of introns, respectively. Lengths of the entire SSU rDNA and exons are also indicated. **a** Structures of the SSU rDNA of *Pyropia yezoensis* used in this study. **b** Structures of the SSU rDNA of *Pyropia yezoensis* from Japan. The GenBank accession number of them is AB013178 for *(1)* and AB013177 for *(2)*

sequence regions that were homologous with the entire LSU and SSU rDNA. Sequence analysis certified that upstream direct repeats of which each unit was 74 bp, self-complementary structure, and downstream indirect repeats existed in the IGS region (Fig. 4).

Discussion

Nucleotide sequence of the SSU rDNA is one of the most efficient and reliable molecular markers for phylogenetic investigations at the higher taxonomic levels. The characteristics of introns described above are extremely conserved and highly consistent with results in the SSU rDNA of *Bangia* and *Pyropia* species reported by others (Müller et al. 2001; Kunimoto et al. 1999a). The downstream intron is the typical group I intron due to the presence of P1–P9 stem-loop

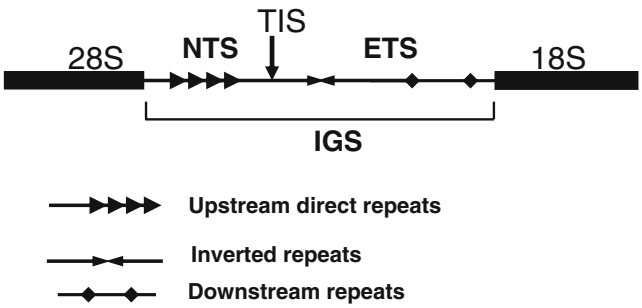

Fig. 4 Schematic representation of the IGS of the rDNA units of *Pyropia yezoensis*. *28S* end of the 28S rDNA, *18S* start of the 18S rDNA, *IGS* intergenic spacer region, *NTS* non-transcribed spacer, *TIS* transcription initiation site, *ETS* external transcribed spacer

domains; four conserved elements P, Q, R, and S; and 5′ and 3′ splice sites (Cech 1988; Ragan et al. 1993), and it is presumed that the upstream intron could be excised by self-cleavage in spite of some features dissimilar with group I intron. It shows that no introns are present in the SSU rDNA of type C, which is the first time to be reported in *P. yezoensis*.

Even though the number, position, and sequences of introns are different, exon sequences of the three individuals of *P. yezoensis* are almost identical and the length is 1834 bp. Exon sequences of the SSU rDNA (type A) have a base substitution compared with type B and C, but the ITS sequences of them have 99.53 % homology. The upstream introns of type A and type B just have the absence of one base. The ITS sequences of Japanese samples and our samples have 2.7 % variability. Moreover, the length and sequence of introns between them have considerable differences, almost 5 % differences. From these results, introns are not appropriate for discrimination of *Pyropia* species, but the features of introns may provide a reference that could be used to distinguish lineages. Moreover, nucleotide gaps in the SSU rDNA reveal larger variation than base substitutions, and a combination of SSU and ITS data seems to be appropriate for the study of phylogenetic intraspecies or interspecies relationships. According to the conclusions, we infer that the two populations of *P. yezoensis* discussed above are the distant relative subspecies or even different *Pyropia* species.

The 5′ end and 3′ end nucleotide sequences of the entire SSU rDNA are CAACCTGG- and -GGATCATT, respectively, which are highly conserved in *Pyropia* species (Kunimoto et al. 1999a). The conserved trait is also confirmed in the LSU rDNA by 5′ end and 3′ end sequence alignment of *Bangia atropurpurea* (GenBank No. AF419107), *Porphyra* sp. (GenBank No. EF033597), and *Porphyra purpurea* (GenBank No. EF033596). The conserved 5′ end and 3′ end nucleotide sequences are CGGAGGAA- and -GCTCTTCC, respectively, according to which primer pair LSUF/R using to amplify the entire LSU rDNA is designed. The amplified LSU rDNA sequence is considered to be the complete. The identical size of fragments amplified from different individuals of *P. yezoensis* indicates the absence of introns in the LSU rDNA. The LSU rDNA sequences are highly conserved among intra-species of *P. yezoensis* with just one or two base differences in 4770 bp. Because the full-length sequences of LSU rDNA were previously unknown, a partial LSU rDNA sequence was applied to study phylogenetic relationships at the levels of species and beyond (Harper and Saunders 2001; Erting et al. 2004: Saunders and Lehmkuhl 2005: Gall and Saunders 2010).

After sequence alignment, we found that the full sequence of regions amplified with five primer pairs from IGS1F/R to IGS5F/R had 99.79 % identity with most nucleotide sequences (2436 bp) of 5′ end of the entire LSU rDNA amplified by primer LSUF/R. The result certifies the correctness of the LSU rDNA and the IGS sequences due to the independent design of primers. The actual IGS region of which the length is 5984 bp was analyzed, and the presence of internal repetitive sequences with 74-bp nucleotides of each repeat and self-complementary structure was found, leading to the difficulty in sequencing (Fig. 4). The number of subrepeats is inappropriate for discrimination of *Pyropia* species, due to the difference within individuals of the same species. The IGS region is more variable than the ITS region; therefore, it is used for phylogenetic analysis in interspecies and intraspecies (Li et al. 2010; Onkendi and Moleleki 2013).

This is the first time that the complete nuclear ribosomal DNA cistron sequence has been obtained for *P. yezoensis*. The sequence will enrich GenBank and provide more information for phylogenetic analysis. Moreover, the approach and strategy will be useful for the study at various taxonomic levels in alga.

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