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Characterization and physical mapping of nuclear ribosomal RNA (rRNA) genes in the haploid gametophytes of *Saccharina japonica* (Phaeophyta)

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Abstract Nuclear tandemly repeated ribosomal RNA genes (18S-5.8S-25S rDNA and 5S rDNA) have been proven to be excellent cytogenetic markers for karyotype analysis of various higher plants by using the fluorescence in situ hybridization (FISH) technique. To illustrate physical mapping of these rDNAs in brown seaweed, *Saccharina japonica*, chromosomes, an approximately 5400-bp transcription unit of 18S-5.8S-25S rDNA was assembled by cloning the 25S rDNA after paired-end sequencing of two screened clones from a bacterial artificial chromosome library of kelp gametophytes. In contrast to the conserved 18S-5.8S-25S rDNA and ITS1 in *S. japonica*, the 245-bp ITS2 was variable in sequence. The cloned 5S rDNA revealed that the 120-bp conserved coding region was separated by a diverse intergenic spacer sequence that were 250, 445, 905, or 1335 bp in length. On average, the 18S-5.8S-25S rDNA of kelp female and male gametophytes had 45 and 41 copies per haploid genome, respectively, as detected by quantitative real-time PCR, whereas the 5S rDNA had 2590 and 2648 copies, respectively. Southern hybridization with labeled probes of 18S rDNA or 5S rDNA demonstrated that kelp gametophytes possessed only one locus each of the 18S-5.8S-25S or 5S rDNAs. This was further confirmed by FISH analysis using the same labeled probes, thus illustrating that 18S-5.8S-25S rDNA is located at the intercalary region of chromosome 23, whereas 5S rDNA at the sub-telomeric region of chromosomes 27. The localization of these rDNAs using the FISH technique has facilitated the identification of individual chromosomes and karyotype analysis of this kelp.

Keywords 18S-5.8S-25S rDNA · 5S rDNA · Copy number · Fluorescence in situ hybridization · Southern blot · Q-RT PCR

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Introduction

The previous cytogenetic reports on the economically significant brown seaweed, *Saccharina japonica* (Aresch.) C. E. Lane, C. Mayes, Druehl et G. W. Saunders (=*Laminaria japonica* Aresch.), have demonstrated that kelp chromosomes are not only very small (0.57–2.61 μm) in size but also nearly homomorphic in shape (Yabu 1973; Tai and Fang 1976, 1977; Yabu and Yasui 1991; Zhou et al. 2004; Liu et al. 2012a,b). These chromosomal features thus make it difficult to identify individual chromosomes and analyze the karyotype of brown seaweeds (Lewis 1996) by use of conventional cytogenetic techniques. However, using the sensitive fluorescence in situ hybridization (FISH) technique, especially with nuclear ribosomal RNA (rRNA) genes as probes, has been proven to be efficient for the investigation of species with small or similarly sized chromosomes (Leitch and Heslop-Harison, 1992; Garcia et al. 2012). Thus, this enables the identification and discrimination of different chromosomes within and between chromosome complements (Hamon et al. 2009).

The nuclear rRNA genes, which occur in nearly all of eukaryotes in the form of tandemly repeated arrays (Long and Dawid 1980; Rogers and Bendich 1987; Maluszynska et al. 1998; Volkov et al. 2007; Torres-Machorro et al. 2010; Garcia et al. 2012), are categorized into two different families. The major rRNA family, which constitutes the nucleolus organizer regions (NORs), contains the 18S, 5.8S, and 25S/26S/28S

rRNA genes that encode the 45S pre-ribosomal RNA as synthesized by RNA polymerase I (Mandal 1984; Sollner-Webb and Tower 1986; Hemleben and Zentgraf 1994; Paule and White 2000). The minor non-nucleolus forming 5S rRNA family, which is transcribed by RNA polymerase III (Mandal 1984; Sollner-Webb and Tower 1986; Paule and White 2000), contains a highly conserved 120-bp long coding region and a variable intergenic spacer (IGS) region (Long and Dawid 1980; Drouin and Moniz de Sá 1995; Barciszewska et al. 2000; Volkov et al. 2007). Due to their universal presence in eukaryotic genomes, the number, position, and structure of the 5S and 18S-5.8S-25S rDNA loci are considered important characteristics of a given species, genus, or group (Garcia et al. 2012). As we know, the sequences of 18S rDNA (Bi et al. 2009), internal transcribed spacer (ITS) 1 and ITS2 (Yotsukura et al. 1999), and 5S rDNA (Yotsukura et al. 2002) in *S. japonica* and other closely related species have been extensively characterized. However, the molecular organization of these two families of rDNAs, copy number, and physical mapping on chromosomes yet remain to be completed.

To characterize the rRNA genes in *S. japonica*, these were cloned by polymerase chain reaction (PCR), followed by the assembly of the gene structure based on their gene sequences. The copy number of rRNA genes in the haploid gametophytes of *S. japonica* was estimated by use of quantitative real-time PCR (Q-RT PCR), as previously employed in bacteria (Zeng and Jiao 2009), copepods (Zagoskin et al. 2010), fungi (Black et al. 2013), tea plants (Xu et al. 2015), and dinoflagellates (Saito et al. 2002; Galluzzi et al. 2004). Finally, the precise location of the 18S-5.8S-25S and 5S rRNA genes was determined by using the FISH technique. To our knowledge, this is the first report on the physical mapping of rDNAs in the kelp *S. japonica* or even in algae except for the dinoflagellates, *Alexandrium* spp. (Figueroa et al. 2014). These data will be useful for the construction of physical maps of chromosomes, the integration of genetic and physical maps, the discrimination of whole chromosomes for karyotypic analysis, the identification of U/V sex chromosomes (Bachtrog et al. 2011), and the comparison of chromosomal organization in phylogenetic studies on *S. japonica* and other related species in the future.

Materials and methods

Algal strain and culture

The Rongfu strain of *Saccharina japonica* was selected as the algal material in the present study. Female and male gametophyte clones germinated from zoospores were isolated according to cell size (about 20 μm for females and 10 μm for males) under a microscope and cultured in 500-mL flasks under vegetative growth conditions of 30 μmol photons m^{-2} s^{-1} at 17 ± 1 °C, with a photoperiod of 12 h: 12 h light: dark as previously described (Zhou and Wu 1998). During culture, the flasks were shaken several times by hand at intervals each day, and the Provasoli's enriched seawater (PES) medium (Starr and Zeikus 1993) was replaced every 2 weeks.

DNA extraction

Genomic DNA was extracted from freshly harvested gametophytes according to the modified cetyltrimethyl ammonium bromide (CTAB) method of Hu and Zhou (2001). The extracted DNA was dissolved in Tris-EDTA (TE) buffer which was composed of 10 mM Tris-HCl and 1 mM EDTA at pH = 8.0.

Cloning of the 18S-5.8S-25S and 5S rRNA genes

Amplification of the 18S rRNA gene using the previously designed primers 18S-F/18S-R (Bi et al. 2009) (Table 1) was conducted in a 25-μL reaction volume containing the template DNA (50 ng), Mg^{2+} (2.5 mM), *Taq* DNA polymerase (1 U), dNTPs (100 μM each), forward and reverse primers 18S-F/18S-R (0.2 μM each), and 1× reaction buffer. PCR was performed in a gradient Mastercycler (Eppendorf, Germany) and programmed as described by Hu and Zhou (2001) except for the use of 30 cycles of the reaction and annealing at 58 °C for 45 s. The amplified products were resolved on a 1.0% low-melting-point agarose gel for DNA recovery. The target product was purified using a UNIQ-10 column DNA gel extraction kit (Sangon, China) and ligated into a pMD19-T vector (TaKaRa, China). The constructed vector was subsequently transformed into *Escherichia coli* DH5α competent cells (Leihao, Shanghai, China), and several positive clones were screened and sent to Sangon (Shanghai, China) for sequencing. Because ITS sequences are generally less conserved (Coleman 2003; Calonje et al. 2009), more than 20 clones were sequenced following *S. japonica* ITS1 and ITS2 cloning.

According to the above-verified sequence of the 18S rRNA gene, one pair of primers 18S-F1/18S-R1 (Table 1) was designed by using the Primer Premier 5.0 software (http://www.premierbiosoft.com/primerdesign/index.html) and employed in PCR screening the constructed bacterial artificial chromosome (BAC) library of the kelp female gametophyte for the 18S-5.8S-25S rRNA genes. The PCR reaction system and program was the same as described earlier except for the template of the kelp genomic DNA, which was replaced by BAC clone DNA. Two clones (BAC Clones L10 and E18), which possibly contained the 18S-5.8S-25S rRNA genes, were screened. Based on the partial sequence of the 25S rRNA gene after paired-end sequencing of these two clones, one pair of primers 25S-F/25S-R (Table 1) was designed for cloning of the 25S rRNA gene. Furthermore, two pairs of primers, 18S-F2/ITS2-R and ITS1-F/25S-R1 (Table 1), were designed and used in cloning the ITS1-5.8S-ITS2 rRNA

Table 1 Oligonucleotide primers used for amplification and probe synthesis in the present study

Primer	Sequence (from 5′ to 3′)	Objective	Resource
DNA cloning			
18S-F 18S-R	ACCTGGTTGATCCTGCCAGT TCACCTACGGAAACCTTGT	18S rDNA cloning, probe synthesis and standard plasmid construction	Bi et al. (2009)
25S-F 25S-R	TAGACAGTAGATAGGGACAG ACTAAGCGGAGGAAAAGAAA	Cloning of partial sequence of 25S rDNA	This study
18S-F2 ITS2-R	CGGTTTTGTGGTGAATC GTTTCCAACGCTGACCCA	Cloning of 18S rDNA, ITS1, 5.8S and ITS2	This study
ITS1-F 25S-R1	AAAGCGGGTTCGTTCAAT CCAGTAACGGCGAGTGAAGC	Cloning of ITS1, 5.8S, ITS2 and 25S rDNA	This study
5Ss-F 5Ss-R	TATACATGCGCGCCAACTTG CCCCGTGGGTACGAGTAACA	5S rDNA cloning and standard plasmid construction	Yotsukura et al. (2002)
18S-F1 18S-R1	TCGGACGGTTTTGTGGTGA CCTTCCTTGGATGTGGTAGCC	Screening of 18S rDNA from a BAC library	This study
5S-F 5S-R	TAGTACTACGGTGGGGGACC CAGAGCGGACGGGATGTGGT	Screening of 5S rDNA from a BAC library	Yotsukura et al. (2002)
Quantitative real-time PCR			
q18SF q18SR	GTGAGGATTGACAGATTGAGAGC GTCACCGAAAGTCCCTCTAAGAA	Copy number estimate of 18S rDNA	This study
q5SF q5SR	ACGGCCATACCACGTCGAT GACGCCGCTTAACTTCACAGA	Copy number estimate of 5S rDNA	This study
q5ST	FAM-CACCACATCCCGTCCG-MGB		

genes of the kelp haploid gametophytes. The PCR reaction system and program was also the same as that for the 18S rRNA gene, except for the extension time used for amplification with the pairs of primers 18S-F1/18S-R1, 25S-F/25S-R, 18S-F2/ITS2-R, and ITS1-F/25S-R1 which was 1, 5, 4, and 4 min, respectively.

The genomic DNA of the 5S rRNA gene was amplified with the reported pair of primers, 5Ss-F/5Ss-R (Table 1) (Yotsukura et al. 2002). Each 25-μL PCR reaction system was composed of template DNA (50 ng), Mg^{2+} (2.5 mM), *Taq* DNA polymerase (1 U), dNTPs (100 μM), forward and reverse primers 5Ss-F/5Ss-R (0.2 μM each), and 1× reaction buffer. PCR amplification was programmed as described by Hu and Zhou (2001), with minor modifications, in which 30 cycles and annealing at 62 °C for 45 s were employed. Another pair of primers, 5S-F/5S-R (Table 1), was used to amplify the IGS of the *S. japonica* 5S rRNA gene. The PCR reaction system and program was the same as that for cloning the 5S rRNA gene, except for the use of a 2-min extension time. The amplified products were resolved on a 1.0% low-melting-point agarose gel for DNA recovery, cloning, and sequencing as earlier described.

Bioinformatics analysis

The DNAMAN (Lynnon Biosoft, USA) and BioEdit (Hall 1999) softwares were employed for sequence assembly, alignment, and restriction site analysis. BLASTP analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to obtain the homologous sequences of the 18S, 5.8S, and 25S rRNA genes from *Costaria costata*, *Saccharina angustata*, *Ectocarpus siliculosus*, and *Laminaria digitata*. The ITS and 5S rDNA sequences of several brown seaweeds were retrieved from NCBI. Supplementary Table 1 presents the details of these genes.

Copy number estimation of the rRNA genes using Q-RT PCR

Ribosomal RNA gene copies in *S. japonica* haploid gametophytes were estimated by using Q-RT PCR as previously described (Zagoskin et al. 2010; Xu et al. 2015). Prior to Q-RT PCR amplification, two vectors, pMD19-T18S and pMD19-T5S, which contained 1716-bp- and 120-bp-long 18S and 5S rDNAs, respectively, were constructed as references as described above. Both plasmid DNAs were extracted and digested by *Hin*dIII into linear plasmids for the construction of a calibration curve. This curve was plotted based on a series of 10-fold dilutions of digested pMD19-T18S (from approximately 30 ng to 3 pg) or pMD19-T5S (from 2.75×10^9 to 2.75×10^5 copies) DNA against the resulting fluorescence intensity (threshold cycle, C_T) as detected by Q-RT PCR. The copy number per fg plasmid DNA molecule (N_p) was calculated using the equation:

$N_p = N_A \times m/M$ (Zagoskin et al. 2010);

where N_A is the Avogadro constant, m is the DNA amount (i.e., 1 fg) for which the copy number is calculated, and M is

the molar mass of the plasmid. Because 1 bp of DNA corresponds to 660 g mol^{-1}, the N_p of the 18S rRNA gene is approximately 207 and that of the 5S rRNA gene is 315. Each plasmid DNA dilution was conducted in triplicate. The concentration of the initial plasmid DNA or the total *S. japonica* gametophyte DNA was determined by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

The primers used in the Q-RT PCR for the 18S-5.8S-25S rRNA (q18SF/q18SR) and 5S rRNA (q5SF/q5SR, q5ST) genes are also shown in Table 1, and the amplification of the 18S-5.8S-25S rRNA gene was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, USA) using the SYBR RT-PCR kit (TaKaRa) according to the manufacturer's recommendations. After predenaturation at 95 °C for 15 s, the amplification was programmed for 40 cycles of 94 °C for 5 s, 60 °C for 20 s, and a ramp of 0.5 °C/30 s to 95 °C. The Q-RT PCR reactions for 5S rDNA comprised the template DNA (200 ng), Mg^{2+} (50 μM), *Taq* DNA polymerase (5 U μL^{-1}), dNTPs (5 μM each), forward and reverse primers q5SF/q5SR (10 μM each), *TaqMan* probe q5ST (5 μM), and RNase-free H_2O to 20 μL. *TaqMan* probe q5ST was used because we could not design a specific pair of primers such as q5SF/q5SR (Table 1) for the 5S rRNA gene due to its too short insert (120 bp only) in the plasmid pMD19-T5S. This reaction program was as follows: 1 cycle of pre-denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. All Q-RT PCR reactions were performed in triplicate.

The copy number of rDNA in the kelp haploid genome was calculated using the following equation:

$N_r = N_m \times N_p \times C/k$ (Zagoskin et al. 2010);

where N_r is the 18S rDNA or 5S rDNA copy number, C is the size of the *S. japonica* haploid genome ($C = 0.5975$ pg) as estimated by Ye et al. (2015), N_m is the experimentally determined initial amount of the 18S or 5S rRNA gene templates, N_p is the same as earlier described, and k is the amount of genomic DNA template of the sample that was used in the Q-RT PCR.

Southern blot analysis

To obtain more information on the 18S-5.8S-25S and 5S rDNAs of the kelp gametophytes, Southern blot analysis was conducted as described by Ye et al. (2014). In this experiment, we chose 18S rDNA (synthesized by using the primer pair, 18S-F/18S-R, Table 1) and 5S rDNA (synthesized by using the primer pair, 5Ss-F/5Ss-R, Table 1) as probes. Aliquots of isolated DNA (approximately 2 μg per sample) were digested to completion at 37 °C for 12–14 h independently by using *Nco*I and *Sal*I. Agarose electrophoresis of the digested DNA samples, blotting onto a positively charged nylon membrane (Pall, USA), hybridization with the synthesized 18S and 5S rDNA probes, and signal visualization on an XBT-1 film (Kodak, USA) were performed as previously described (Ye et al. 2014).

FISH analysis

Chromosomes were prepared from *S. japonica* gametophytes according to previous studies (Liu et al. 2012a,b). Fresh female and male gametophytes of *S. japonica* were separately treated with 0.02% colchicine for 8–10 h at room temperature. After incubation, the samples were washed and then fixed in freshly prepared Carnoy's fixative solution (100% ethanol/acetic acid, 3:1, *v*/*v*) for 24 h (Schweizer 1976). Chromosome squashes were subsequently prepared following the enzymatic softening of material as described elsewhere (Liu et al. 2012b). After screening under a phase contrast microscope, the well-spread mitotic chromosomes were pretreated sequentially with 100 mg mL^{-1} of RNase A for 1 h and 0.25 mg mL^{-1} pepsin for 15 min (Lim et al. 2001), subjected to FISH detection, and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

The FISH experiment was conducted using two DNA probes, namely, 18S and 5S rDNAs, which were 1824-bp and 120-bp fragments labeled with digoxigenin (DIG)-12-dUTP (Roche, Switzerland) after PCR amplification from *S. japonica*. Hybridization was performed as described by Lim et al. (2001) with slight modifications. The slides were UV cross linked for 2 min, washed in 2 × SSC (3 × 5 min), and rinsed in a series of 70, 95, and 100% ethanol for 2 min each time and air dried. Approximately 10 μL of the hybridization mixture, which contained 50% formamide (*v*/*v*), 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7), 5% dextran sulfate (*w*/*v*), and 5 ng μL^{-1} of the probe, was added to the slide, covered with a slip of plastic sheet, heated to 100 °C in a water bath for 5 min, and then transferred to a 37 °C air oven for hybridization for 12 h. Post-hybridization washes of the slides were performed twice in a slightly more stringent solution of 2 × SSC at 42 °C in a shaker for 10 min to remove unbound, nonspecifically bound, or weakly hybridized probe. To detect the hybridization sites, the slides were washed with 4 × SSC and then blocked with 30 μL of 5% bovine serum (*w*/*v*) in PBS per slide, covered with plastic coverslips, and then incubated at 37 °C for 30 min. The two probes were detected by incubating with anti-digoxigenin-rhodamine conjugated to digoxigenin in a blocking reagent (Roche, Switzerland) at 37 °C for 1 h. After incubation, the slides were washed with 4 × SSC twice, each for 5 min (Osuji et al. 2006), and then counterstained with DAPI (2 μg mL^{-1}). Fluorescence images were captured using a Leica DM4000 epifluorescent microscope with an Orca ER camera and then processed using Adobe Photoshop 7.0 (http://www.adobe.com/products/photoshop.html) by uniformly adjusting the contrast and brightness (Liu et al. 2012a).

Results

Characterization of the 18S-5.8S-25S and 5S rRNA genes in *S. japonica*

Based on partial sequencing of two screened clones from the constructed BAC library of the female *S. japonica* gametophytes, several pairs of primers (Table 1) were designed to clone the coding sequence of 18S-5.8S-25S rRNA gene cluster via PCR amplification (Supplementary Fig. 1). One transcription unit (Fig. 1) of this gene cluster in *S. japonica* was assembled using the sequences of the amplified products. Approximately 5400 bp of the coding sequence of 18S-5.8S-25S rDNAs was confirmed by using other re-designed primers (data not shown) different from the used ones for gene cloning and was then deposited to NCBI (GenBank Accession No. KX827269). The length of the 18S rRNA gene was 1824 bp, and its sequence was identical to the reported 18S rDNA of the same species (GenBank Accession No. EU293553, Bi et al. 2009) and highly similar to those that were respectively cloned from *Laminaria digitata*, *Saccharina angustata*, *Costaria costata*, and *Ectocarpus siliculosus* (Supplementary Table 1).

The 5.8S rRNA gene of *S. japonica* was 160 bp in size and it was identical in size to those of *C. costata* (Bhattcharya and Druehl 1988) and *E. siliculosus* (Peters et al. 2010). Only two bases differed from that of *S. angustata*, *L. digitata*, *C. costata*, and *E. siliculosus*, all of which also belong to the same phylum Phaeophyta, thereby indicating the conservation of the 5.8S rDNA in various kelp species (Supplementary Table 1).

The 25S rRNA gene of *S. japonica* was 2891 bp in length, and sequence alignment with four other brown algal species (*C. costata*, *S. angustata*, *E. siliculosus*, and *L. digitata*, Supplementary Table 1) showed that the nucleotide arrangement of this gene was also highly conserved. The lowest similarity was 97%, which was observed between *S. japonica* and *E. siliculosus*.

Sequencing of ITS amplicons revealed that the ITS1 region was 238 bp in length and was identical to that of the other kelp species *S. augustata*, *S. religiosa*, and *S. ochotensis*, with only two different bases in *S. diabolia* and *S. longissma* (Supplementary Table 1), which was indicative of sequence conservation. Compared to ITS1, the cloned ITS2 of *S. japonica* was less conserved in sequence, with a 98% sequence similarity to each clone, although these were of the same length (245 bp).

The 5S rDNA of *S. japonica* was cloned by PCR amplification using previously reported primers (Table 1, Yotsukura et al. 2002). The size of the PCR products that were amplified by using the primer pair 5S-F2R2 ranged from 300 to 1500 bp (Supplementary Fig. 2). The four distinct bands were subsequently cloned and sequenced. Database searching result showed that each repetitive unit of 5S rDNA included a 120-bp coding region, which was similar to that reported by Yotsukura et al. (2002) but separated by a more diverse IGS (Fig. 1) as determined by sequencing. This variable IGS of *S. japonica* 5S rDNA was not only in sequence but also in length. Besides the reported length of about 250 bp (Yotsukura et al. 2002), this study has determined that the length of IGSs was 445, 905, and 1335 bp (Supplementary Fig. 2). This finding was confirmed by searching against the recently released kelp genome database by Ye et al. (2015). The IGSs detected in this study were deposited to NCBI (GenBank Accession Nos. KY440189–KY440192), and were characterized by a conserved TATA box that was located −27 to −25 bp upstream of the 120-bp coding sequence as reviewed by Volkov et al. (2004). The 5′ end of the IGSs also contained a block of T-rich sequence, thereby suggesting that this region could be involved in transcription termination as proposed by Korn (1982). Accordingly, these 445-bp, 905-bp, and 1335-bp 5S rDNA IGSs might be potentially used as molecular markers for discriminating between the Chinese and Japanese kelp populations.

Copy number estimation of 18S-5.8S-25S and 5S rRNA gene repeats

Upon the establishment of a standard Q-RT PCR protocol after optimization using various primers and PCR reagent concentrations, a standard curve (Supplementary Fig. 3) with the linearized pMD19-T18S DNA as a template was generated.

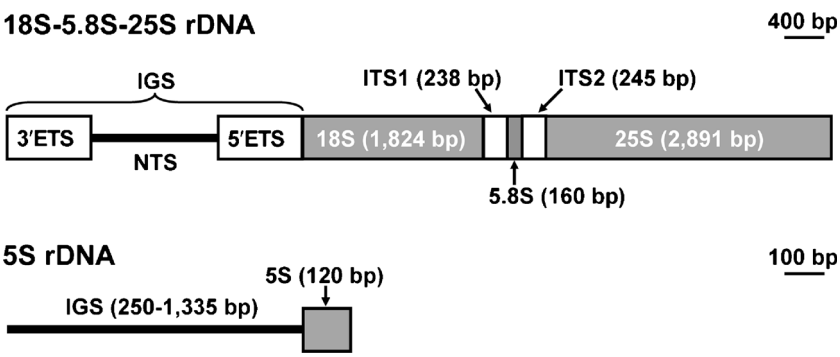

Fig. 1 Gene structure of a transcriptional unit of the 18S-5.8S-25S rDNA and 5S rDNA cloned from the *Saccharina japonica* gametophytes

Linear regression analysis revealed a positive relationship between the C_T values and the serially diluted plasmid DNA levels (R^2 = 0.999) (Supplementary Fig. 3). The C_T value of the *S. japonica* 18S rRNA gene in the haploid genome was also detected by Q-RT PCR (Table 2). By referring to the constructed standard curve and the recently reported genome size of *S. japonica* (Ye et al. 2015), the copy number of 18S-5.8S-25S rRNA gene in *S. japonica* female and male gametophytes was evaluated (Table 2). The copy number ranged from 39 to 51 (mean ± SD = 45 ± 7, n = 9) and from 22 to 56 (mean ± SD = 41 ± 15, n = 9) per haploid genome of female and male gametophytes, respectively (Table 2). Although the female and male gametophytes showed fluctuations in 18S-5.8S-25S rRNA gene copy number, these were not statistically different from each other (Student's t-test, P = 0.701 > 0.05).

Similar to the estimation for the 18S rRNA gene but using a designed *TaqMan* probe (q5ST, Table 1) and primers q5SF/q5SR (Table 1), a protocol for the quantification of the 5S rRNA gene copy number was established. A standard control curve between the C_T values and the series of 10-fold dilutions of copy number of pMD19-T5S (from 2.75 × 10^9 to 2.75 × 10^5 copies) with an R^2 = 0.999 was also constructed (Supplementary Fig. 4). By using this calibration curve, the 5S rRNA gene copy number in *S. japonica* gametophytes was calculated, which ranged from 1333 to 4404 (mean ± SD = 2590 ± 1399, n = 9) and from 1968 to 3314 (mean ± SD = 2648 ± 595, n = 9) per haploid genome of male and female gametophytes, respectively (Table 2). The female and male gametophytes showed no significant differences (Student's t-test, P = 0.957 > 0.05) in terms of copy number.

Locus and physical mapping of the 18S-5.8S-25S and 5S rRNA genes

To further investigate the locus of the nuclear rDNAs, genomic DNA that was extracted from *S. japonica* gametophytes was digested with two different restriction enzymes (*Sal*I and *Nco*I) and subjected to Southern blot analysis using labeled probes specific to the 1716-bp 18S or 212-bp 5S rRNA genes. Only one hybridization signal was observed in Fig. 2, implying that there might be one locus of these two genes in the *S. japonica* gametophyte genome that was digested by *Sal*I. When the restriction enzyme *Nco*I was used to digest the haploid genome of *S. japonica*, the same result was obtained for the 5S rRNA gene (Fig. 2, lower panel), whereas two signals (Fig. 2, upper panel) were observed for the 18S-5.8S-25S rRNA gene cluster. The intense band, which was about 3.5 kb in size (Fig. 2, upper panel), coincided with our predicted, which was cleaved by *Nco*I at both sites of 343 and 3980 bp downstream of the coding sequence of the 18S rRNA gene. The size of the minor signal was about 0.8 kb (Fig. 2, upper panel), which was suggestive of an *Nco*I cleavage site in the IGS sequence upstream of the 18S rRNA gene. Because the IGS sequence of the 18S-5.8S-25S rRNA genes was not cloned in this study, this cleavage site was not confirmed. Nevertheless, the results of Southern blotting analysis

Table 2 Copy number estimation for the 18S-5.8S-25S and 5S rRNA genes in the *Saccharina japonica* gametophytes

Gametophyte	Experiment times	C_T[a]	k[b] (ng)	Copy number
18S–5.8S-25S rRNA gene				
Male	1	13.436 ± 0.129 (n = 3)	118.8	22 ± 2
	2	12.679 ± 0.118 (n = 3)	100	44 ± 4
	3	12.662 ± 0.046 (n = 3)	79.8	56 ± 2
	Mean ± SD			41 ± 15
Female	1	12.943 ± 0.260 (n = 3)	95.6	39 ± 7
	2	12.468 ± 0.124 (n = 3)	113.4	45 ± 4
	3	12.665 ± 0.047 (n = 3)	86.8	51 ± 2
	Mean ± SD			45 ± 7
5S rRNA gene				
Male	1	18.658 ± 0.065 (n = 3)	10	4404 ± 204
	2	19.760 ± 0.035 (n = 3)	10	2033 ± 49
	3	20.604 ± 0.103 (n = 3)	10	1333 ± 94
	Mean ± SD			2590 ± 1399
Female	1	19.378 ± 0.084 (n = 3)	10	2661 ± 157
	2	19.064 ± 0.052 (n = 3)	10	3314 ± 123
	3	20.042 ± 0.099 (n = 3)	10	1968 ± 136
	Mean ± SD			2648 ± 595

[a] C_T or threshold cycle value is the cycle number at which the fluorescence is detected to significantly surpass the fluorescence threshold

[b] k is the amount (ng) of the sample genomic DNA template

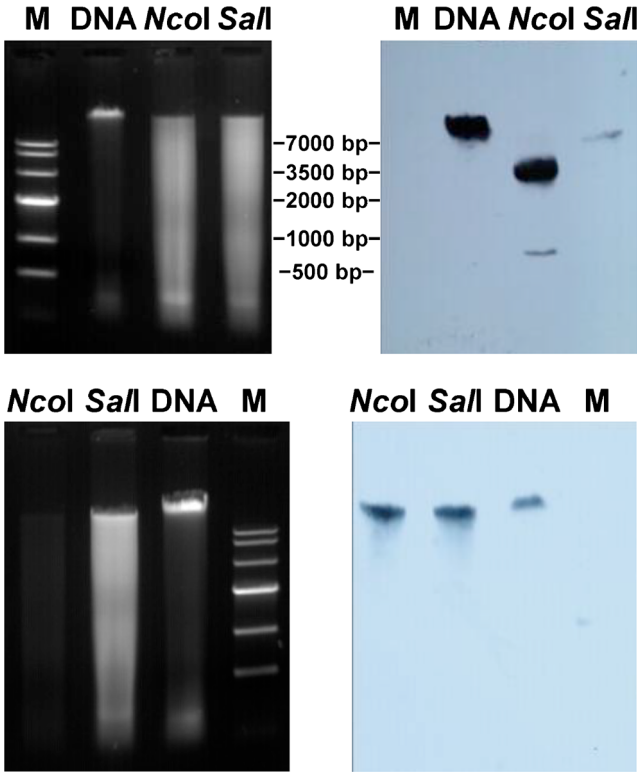

Fig. 2 Agarose electrophoresis profile (*left*) of *Sal*I-digested and *Nco*I-digested genomic DNA of the *Saccharina japonica* gametophytes and its corresponding Southern blot pattern (*right*) hybridized with the biotin-labeled 18S rDNA (*upper panel*) or 5S rDNA (*lower panel*). *M* Marker IV DNA standard, *DNA* undigested *Saccharina japonica* genomic DNA, *NcoI* genomic DNA digested by *Nco*I, *SalI* genomic DNA digested by *Sal*I

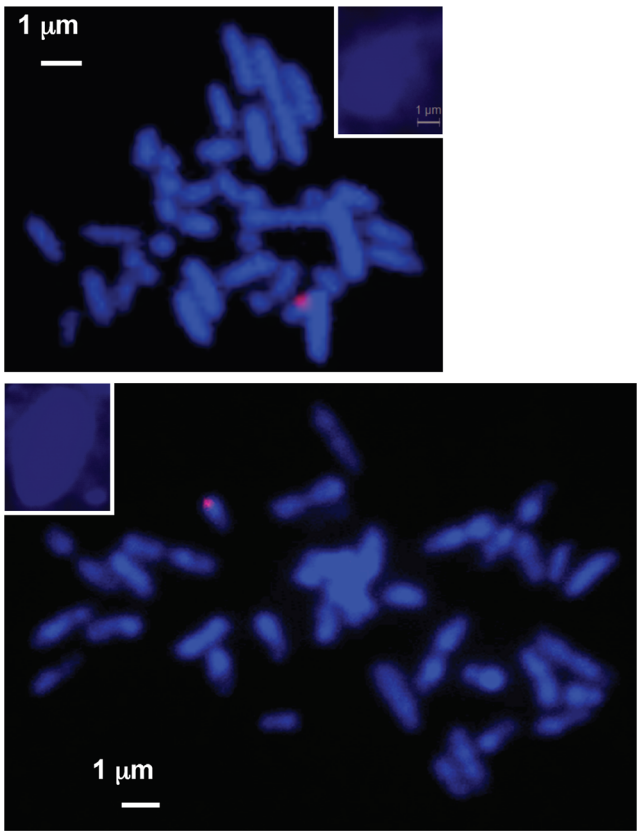

Fig. 3 FISH images of the labeled 18S rDNA marker (*upper panel*, *red*) or 5S rDNA marker (*lower panel*, *red*) on the *Saccharina japonica* metaphase chromosomes counterstained with DAPI (*blue*). The *inset* in each panel is an interphase nucleus of the kelp gametophytes showing the hybridization signals (*red*)

(Fig. 2) illustrated that only one locus of both the 18S-5.8S-25S and 5S rRNA genes was present in the haploid genome of the *S. japonica* gametophytes. Furthermore, no difference in rRNA gene locus between the kelp female and male gametophytes was observed by using Southern blot.

To determine the 18S-5.8S-25S and 5S rDNA loci on kelp chromosomes in both number and position, at least 100 metaphase nuclei of the *S. japonica* gametophytes were examined by using the FISH technique. The chromosome number of the haploid gametophytes was 31 (Fig. 3), which was in agreement with the recently reported results of Liu et al. (2012a,b). Rhodamine hybridization signals on the chromosomes counterstained by DAPI are shown in Fig. 3. Of the 20 examined metaphase nuclei that were examined to determine the locus of the 18S-5.8S-25S rRNA genes, 12 nuclei showed only one distinguishable fluorescent signal (Fig. 3, upper panel). On the other hand, 20 of the 30 examined nuclei showed one distinguishable 5S rRNA gene fluorescent signal (Fig. 3, lower panel). These FISH images cytogenetically demonstrated that both 18S-5.8S-25S and 5S rRNA genes were located at one locus on the kelp chromosomes, and these genes were thus encoded by the kelp nuclear genome as well. No difference in locus was observed between the female and male gametophytes by using the FISH technique.

The arrangement of the kelp chromosomes according to decreasing size (Fig. 4) showed that the 18S-5.8S-25S rDNA was located at the intercalary region of chromosome 23, as indicated by the hybridization of the labeled 18S rDNA probe, whereas the 5S rRNA gene was located at the subtelomeric region of chromosomes 27. The localization of the 18S-5.8S-25S and 5S rDNAs on different chromosomes supported the hypothesis that the latter was not linked to the former in *S. japonica* (Yotsukura et al. 2002).

Discussion

In addition to the reported sequences of 18S rDNA (Bi et al. 2009), ITS1 and ITS2 (Yotsukura et al. 1999), and 5S rDNA (Yotsukura et al. 2002), this study cloned the coding sequence of the 25S rDNA so as to complete the rDNA repeat unit in the haploid gametophytes of *S. japonica*. The 18S-5.8S-25S rRNA gene cluster is about 5400 bp long (Fig. 1), and all the sequences of the 18S rDNA, ITS1, 5.8S, and 25S rDNA

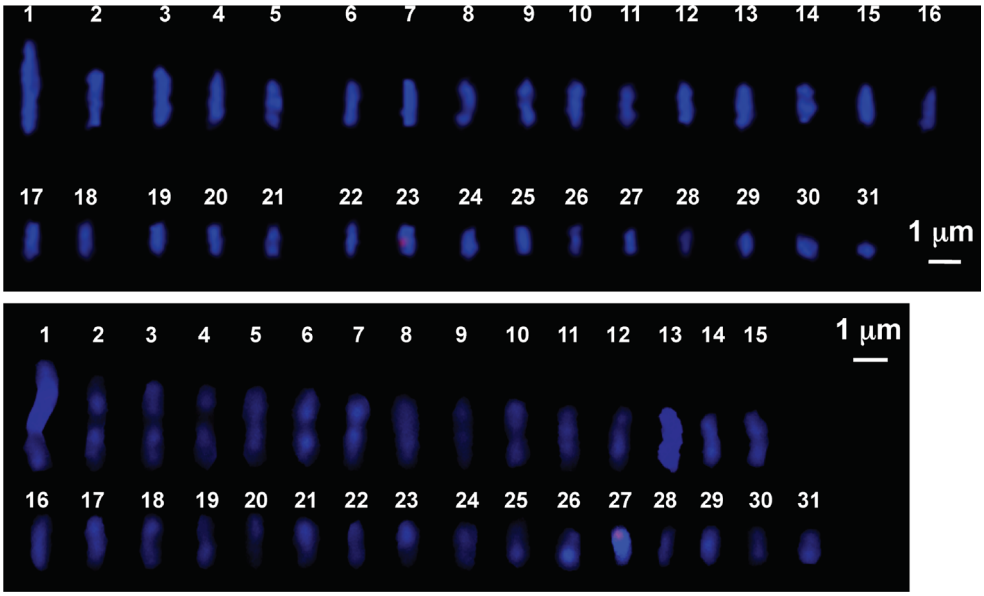

Fig. 4 FISH images of *Saccharina japonica* chromosomes in Fig. 3 arranged according to the decreasing length, showing the physical location of the 18S (*upper*) and the 5S (*lower*) rDNA sites

are highly conserved, whereas that of ITS2 varied. The ITS2 heterogeneity showed both inter-specific variations as suggested by Yotsukura et al. (1999), as well as intra-specific (within *S. japonica*) variations. Our findings also support the results of Coleman (2003) that there are variations among different ITS2 repeats that exist in a single eukaryote genome. This variation may be mainly due to nucleotide indels and substitutions in parents and then is inherited only in hybrids or polyploids as proposed by Buckler et al. (1997), Volkov et al. (2007), and Poczai and Hyvönen (2010). These findings thus reflect the possible hybrid of Rongfu kelp strain used in this study, which is consistent with the fact that this strain was cross-bred intra-specifically using a female gametophyte clone from Fujian (South of China) and a male gametophyte clone from the Strain Yuanza 10 (cultivated in Shandong, North of China) (Liao et al. 2010). The geographic distance of these two above-mentioned locations from north to south is about 1200 km, indicating the possibility of factors, such as temperature, to affect the genetic variations of its parents so that this hybrid could exhibit obvious heterosis (Liao et al. 2010). Therefore, our findings also imply that implications of ITS2 sequences in evolutionary studies should be performed with caution (Álvarez and Wendel 2003; Poczai and Hyvönen 2010; Wang et al. 2015).

The results of the present study support the findings of Rogers and Bendich (1987), Poczai and Hyvönen 2010, and Torres-Machorro et al. (2010) that the 18S-5.8S-25S rRNA gene cluster is separated by an IGS (Fig. 1). Although the kelp IGS sequence of this major rDNA could not be cloned in the present study, its length could be inferred from the Southern blot profiles (Fig. 2). In the upper panel of Fig. 2, the *Sal*I-digested genomic DNA hybridized with a labeled probe of 1716-bp 18S rDNA shows that the size of a repeat unit of the 18S-5.8S-25S rRNA gene cluster would be within the range of 7.0–8.0 kb. After the removal of the assembled 5400-bp coding sequence of the 18S-5.8S-25S rRNA gene cluster, the remaining 1.6–2.6 kb may highly likely be the IGS sequence. However, further studies that would validate our inferences are warranted.

In contrast to the unknown IGS of the major rDNA, sequencing of the IGS of 5S rDNA was completed in this study, thus unveiling the length and sequence heterogeneity of the kelp 5S rDNA (Supplementary Fig. 2). Interestingly, this particular heterogeneity was not observed in the Japanese populations of *S. japonica* (Yotsukura et al. 2002). Within a single genome, different size classes of 5S rDNA repeat units were detected in other organisms such as *Triticum* spp. (Baum and Appels 1992), *Populus deltoides* (Negi et al. 2002), and *Beta vulgaris* subsp. *maritima* (Turner and Brown 2005), but not in kelps. Volkov et al. (2007) suggested that different lengths of the 5S rDNA repeat units in the same genome possibly originated from duplications and rearrangements. Our current understanding of the mechanism of the 5S rDNA IGS inheritance is limited while compared to that of the 18S-5.8S-25S rDNA in plants, because the latter plays an important role in nucleolus construction (Volkov et al. 2007; Poczai and Hyvönen 2010). Based on the size of the cloned 5S rDNA IGS of *S. japonica*, we infer that the 445-bp IGS was generated from the 250-bp IGS by duplication, and the resulting 445-bp IGS had given rise to the 906-bp and 1335-bp IGSs using the same genomic mechanism. Consequently, we propose that this heterogeneity in the Chinese kelp population probably resulted from duplications and rearrangements of the Japanese kelp 250-bp IGS during acclimatization to the local sea environment in China, possibly since its colonization in the 1920s. However, further investigations in this proposal are needed.

To meet the enormous demand for rRNA in cells, the rDNA gene family occurs as tandem repeats in most

eukaryotes (Long and Dawid 1980; Rogers and Bendich 1987; Torres-Machorro et al. 2010). Higher plants usually have from 250 to 20,000 copies per haploid cell (Rogers and Bendich 1987), whereas several species of algae carry from 3 to 2000 copies per haploid cell (Table 3). By using the Q-RT PCR technique, a valid approach for dinoflagellate rRNA copy number estimation as described by both Saito et al. (2002) and Galluzzi et al. (2004), we determined that the 18S-5.8S-25S rDNA consists of 43 repeats, whereas the 5S comprises 2618 repeats (Table 2) on average in the haploid gametophytes of *S. japonica* regardless of their gender. This copy number is nearly within the range of available documented data on algae (Table 3). Nevertheless, it is necessary to point out that a huge variation occurs in each experiment (Table 2), probably resulting in a huge standard deviation, because different growth phases and environments have been reported to affect the copy number of rRNA genes in a plant (for review, Long and Dawid 1980). This might be the most plausible reason that in the same species, for example, *Euglena gracilis*, the reported copy number of rRNA genes differs in different documents (Table 3).

Prokopowich et al. (2003) provided the first convincing evidence of a strong positive relationship between genome size and rDNA copy number by surveying 94 species of animals and 68 species of plants. In contrast to this finding, the copy number of rRNA genes in *S. japonica* seems not very appropriate for its genome size of about 545 Mb (Ye et al. 2015), although the copy number still stands within the range of the available data from algae (Table 3). In these studies on the estimation of yeast rRNA gene copy number, Ide et al. (2010) and Kobayashi (2011) found that the loss of these repeats in yeast cells confers sensitivity to DNA damage caused by mutagens such as UV radiation. Taking this into consideration and the fact that *S. japonica* can survive under medium irradiance of no more than 30% of the water surface solar irradiance (Fei et al. 1989), which is generally accepted as the result of natural selection and adaptation to low light in the sub-littoral region (Häder and Figueroa 1997), the low copies of rDNA serves as a possible explanation why this particular kelp species is unable to tolerate high irradiance. Evidently, additional experimental evidence is necessary to further support this relationship.

In the present study, Southern blot (Fig. 2) and FISH (Fig. 3) analyses have indicated the presence of one single locus for the 18S-5.8S-25S rRNA genes is detectable in the haploid gametophytes of *S. japonica*. In the majority of organisms, the 18S-5.8S-25S rRNA genes are on chromosomes that associate with the nucleolus (Griffor et al. 1991; Volkov et al. 2007). Because there is only one prominent nucleolus in the kelp nucleus (Bouck 1965; Dai and Fang 1979), this locus is supposed to be confined to the nucleolus organizer region (NOR). Although the sites of the 18S-5.8S-25S rRNA genes showed a preferential distribution on the short arms of chromosomes (Roa and Guerra 2012), we were unable to determine whether this locus was located on the short arm of chromosome 23 due to the indistinguishable position of centromere (Figs. 3 and 4).

Unlike the nuclear 18S-5.8S-25S rRNA genes, the 5S rRNA gene is encoded also by plant chloroplast or

Table 3 The copy number of rRNA genes in several species of algae

Species	45S rDNA	5S rDNA	Genome size (Mb)	References
Saccharina japonica	43 (haploid)	2618 (haploid)	NA	This study
	NA	NA	545 (haploid)	Ye et al. (2015)
Acetabularia mediterranea	1900 (haploid)	NA	NA	Spring et al. (1978)
Alexandrium minutum	860–1284	NA	NA	Galluzzi et al. (2004)
	NA	NA	27,272 (haploid)	Figueroa and Bravo (2010)
Chlamydomonas reinhardtii	150 (haploid)	NA	NA	Howell (1972)
	3	3	121	Merchant et al. (2007)
Cyanidioschyzon merolae	3	3	16.6	Maruyama et al. (2004); Matsuzaki et al. (2004)
Euglena gracilis	1000	NA	NA	Scott (1973)
	800	740	NA	Gruol and Haselkorn (1976)
	2000 (haploid)	NA	NA	Curtis and Rawson (1981)
	NA	300 (haploid)	NA	Keller et al. (1992)
Ostreococcus tauri	4	4	12.56	Derelle et al. (2006)
Pfiesteria piscicida	100–200 (haploid)	NA	NA	Saito et al. (2002)
	NA	NA	5162 (haploid)	Parrow and Burkholder (2002)
Polytoma obtusum	375 (haploid)	NA	NA	Siu et al. (1976)

NA not available

mitochondrial genomes (Barciszewska et al. 2000; Valach et al. 2014). To avoid this possibility, the cloned 5S rRNA gene sequence from *S. japonica* was searched against the kelp chloroplast (GenBank Accession No. JQ405663, Wang et al. 2013) and mitochondrion (GenBank Accession No. AP011493, Yotsukura et al. 2010) genomic data on NCBI. No sequences homologous to our cloned 5S rRNA gene were identified, thereby suggesting that the 5S rRNA gene of *S. japonica* is encoded by the nuclear genome. This has been verified by the FISH image (Fig. 3, lower panel) of the kelp 5S rRNA gene.

The first report on the linkage between the 5S rRNA and 18S-5.8S-25S rRNA genes in the brown seaweed *Scytosiphon lomentaria* (Kawai et al. 1995) has sparked subsequent studies on other closely related species, and most of the studied Chromophyta, including brown seaweeds, have been shown to harbor a 5S-linked type of rDNA organization (Kawai et al. 1997). Nevertheless, PCR analysis has indicated that the 5S rRNA gene could be distinguished from the 18S-5.8S-25S rDNA in *S. japonica* (Yotsukura et al. 2002), and the FISH technique employed in this present study has shown that the 5S rRNA gene is located on chromosome 27, which is different from that of the 18S-5.8S-25S rRNA gene (Fig. 4). These findings are in agreement with the results of most plants (Garcia et al. 2012), wherein the use of dual-color FISH for co-localization of the 5S and 18S-5.8S-25S rDNAs on kelp chromosomes will illustrate the unlinked relationship between these two loci.

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