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Discrimination of *Porphyra* species based on small subunit ribosomal RNA gene sequence

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Abstract

The complete nucleotide sequences of ssu rRNA genes were determined for nine species of *Porphyra*. Ssu rRNA gene structure was classified into four types by the presence and absence of intron(s). Gene structure even differed within the same species. Exon nucleotide sequences were identical within the same species, but differed among species. Seventeen species of *Porphyra* were discriminated by comparing the sequences of these diversified regions, using the results of this study and previous studies.

Introduction

The genus *Porphyra* includes about 30 species in Japan (Ueda, 1932; Tanaka, 1952; Tokita, 1966), some of which are economically important as the edible 'Nori'. After the 1960s, many cultivars were produced by by 'Nori' farmers using repeated selection from 'Nori' farming nets . The most widely distributed cultivars are developed from *P. yezoensis*, with other cultivars from *P. tenera* frequently used in low salinity areas. These cultivars were formalized as *P. yezoensis* fo. *narawaensis* and *P. tenera* var. *tamatsuensis* based on the morphological characteristics used by Miura (1984, 1988). However, it is very difficult to define the species or forma of culture strains, due to the lack of suitable morphological features.

Because of commercial demands, a great deal of research has focused on the life cycle, ecology, physiology and biochemistry of *Porphyra* in Japan. In contrast, knowledge of systematics is limited due to the taxonomic difficulties, and no recent studies have defined morphological features that can be used to discriminate the species. Information on the molecular features of these species and formae may lead to the development of a simple and definitive method of discrimination.

An attempt to identify the species of *Porphyra* based on size and restriction-fragment-length characterization of nuclear small subunit ribosomal RNA genes indicated that the molecular diversity within the Bangiales and the traditional morphological taxonomic characters were insufficient for discrimination (Stiller & Waaland, 1993). Further, sequences of nuclear ssu rRNA genes of *Porphyra* species showed apparent divergence within a limited range of macromorphologies compared to other members within the orders *Bangiales, Gracilariales, Nemaliales, Acrochaetiales* and *Palmariale*s (Oliveira et al., 1995). Ragan et al. (1994) also investigated phylogenetic relationships within the genus *Porphyra* based on the ssu rRNA gene sequence. From the above information, we note that the nuclear ssu rRNA gene reveals apparent polymorpholism in a genus *Porphyra*, in contrast to other genera where the ssu rRNA gene is highly conserved. Regardless of the phylogenetic position of each *Porphyra* species, the above results indicate the possibility that species or forma can be discriminated with ssu rRNA gene polymorpholisms.

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Table 1. Details of *Porphyra* species examined in this study

Species		Collection data	Access number
Porphyra suborbiculata Kjellman	Kawatana, Yamaguchi	24 February 1997	AB013180
Porphyra dentata Kjellman	Koga, Fukuoka	24 January 1997	AB013183
Porphyra haitanensis Chang et Zheng	Uge, Ehime	27 February 1997	AB013181
Porphyra katadae Miura	Kawatana, Yamaguchi	27 February 1997	AB013184
Porphyra tenera Kjellman	Kawaura, Kumamoto	7 February 1997	AB01317
Porphyra tenera Kjellman	Shinwa, Kumamoto	7 February 1997	AB013175
Porphyra yezoensis Ueda	Ogatsu, Miyagi	1 April 1997	AB013178
Porphyra yezoensis Ueda	Hakodate, Hokkaido	11 March 1997	AB013177
Porphyra sp.	Shimonoseki, Yamaguchi	10 March 1997	AB013182
Porphyra pseudolinearis Ueda	Tohaku, Tottori	31 December 1996	AB013185
Porphyra umbilicalis Agardh	Nahant, Mass.	30 September 1996	AB013179

In this paper, we report 11 complete ssu rRNA gene sequences of 8 wild-collected *Porphyra* species, identified using morphological systematics, to provide a basic characteristics for their discrimination. For this study, representative thalli of the *Porphyra* species were collected to obtain preliminary nucleotide sequences of the ssu rRNA gene from *Porphyra* species.

Materials and methods

Identification of specimens

Thalli used in this study were wild-collected from the Japanese coast, except *P. haitanensis* and *P. umbilicalis*. *P. haitanensis* was obtained from culture nets from Ehime Prefecture. *P. umbilicalis* is a North Atlantic species and was used for comparison with already reported nucleotide sequences. Collection data are given in Table 1. Individual thalli identifications were performed according to the systematics keys for Japanese *Porphyra* species (Ueda, 1932) and the description(s) of each species.

DNA extraction, amplification and sequence analysis

Each thallus was ground in a silicone centrifuge tube with a silicone stick in liquid nitrogen until it became a very fine powder. DNA was extracted by using the cethyltrimethyl ammonium bromide (CTAB) method, and then purified with CsCl gradient ultracentrifugation (Mizukami et al., 1996).

Ssu rDNA was amplified into two overlapping pieces by using 4 oligonucleotide primers, F-1, R-2131, F-1527 and R-2833, as shown in Table 2. The end region was amplified with the internal transcribed spacer 1 (ITS1) with F-1527 and R-5.8S. The rDNA fragment was purified by using Easytrap ver. 2 (Takara

Table 2. Primers used for PCR amplification and sequencing

Primer	Sequence
F-1	5′-CAACCTGATTGATCCTGCCAGT-3′
F-397	5′-CTGAGAAACGGCTACCACAT-3′
F-925	5′-CAAAGGCGAACCTTCAGAGACT-3′
F-1092	5′-CGCGGTAATTCCAGCTCCAATAGCA-3′
F-1527[a]	5′-AGACGATCAGATACCGTCGTAG-3′
F-1652	5′-GGTCGCAAGGCTGAAACTTAAAGGA-3′
F-2078	5′-TCGTGCTGGGGATAGATCATTGCA-3′
F-2440	5′-CTGATACTGTGGTCGACAC-3′
R-5.8S[b]	5′-GCTGCGTTCTTCATCGATGC-3′
R-2833	5′-GATCCTTCTGCAGGTTCACCTA-3′
R-2138	5′-TATTCGGCGCAGGCTCATGACCT-3′
R-1132	5′-GTCCGACTACGAGCGTTTTAACTGC-3′
R-913	5′-TGGATTGGACTCTCCCTTAAGCTT-3′
R-308	5′-CCATCGAAAGTTGATAGGGCAG-3′
F-2816[c]	5′-TTAAGAGACAGTCGGGTCCCCT-3′
R-2836[c]	5′-CATGGGACTCTGCTTTCGCA-3′
R-2929[d]	5′-TCTCCAGCACCCACTTTTCAT-3′

'F′ and 'R′ represent primers for the forward and reverse strand respectively, while the following numbers designate the position of the 5′ nucleotide site on the forward strand of ssu rDNA from *P. yezoensis*, except where noted.
[a] Bird et al. (1990).
[b] Lindstrom et al. (1996).
Primers listed above were designed in our laboratory, except for those which were based on Bird et al. (1990) and Lindstrom et al. (1996).
[c] This primer is specific for *P. pseudolinearis* group I intron.
[d] This primer is specific for *P. dentata* group I intron.

Co. Ltd.). Sequence primers were designed from ssu rDNA sequence data of *Porphyra* species (Bird et al., 1990; Oliveira et al., 1995; Lindstrom et al., 1996), and primer walking through less-conserved region. Sequencing was carried out by the dideoxy chain-termination method using fluorescent terminators and an automated sequencer (model 373, ABI). All nucleotide sequences have been submitted to the DDBJ with the access numbers shown in Table 1. Ssu rDNA sequences obtained were aligned by the Higgins calculation method with the DINASIS ver. 3.4 application program (Teijin Co. Ltd.).

Results

Identification of specimens

All specimens had monostromatic structure. The microscopic serration present on the thalli margin was of 3 different types. One type was monoecious with carpogonium and spermatium dividing patterns of a/2,b/2,c/4 and a/4,b/4,c/4, respectively. The features of this thallus agreed with those of *P. suborbiculata* (Ueda, 1932). The other 2 thalli types were dioecious and the carpogonium and spermatium dividing patterns were both a/2,b/2,c/4 and a/4,b/4,c/8, respectively. However, the distribution of the carpogonium and spermatium differed in these two thalli types. The thallus, in which a carpogonium and a spermatium had developed separately in the upper and lower part, was identified as *P. haitanensis*. Thalli where carpogonium and a spermatium had developed on the edge of thallus was identified as *P. dentata*. These morphological features agreed with already reported features for *P. haitanensis* and *P. dentata* (Tseng et al., 1984; Fukuhara, 1968).

The six other thallus types had no marginal serration. One of these specimens showed a characteristic carpogonium and spermatium distribution, which were separated between right and left by a clear horizontal border. The carpogonium and spermatium dividing patterns were a/2,b/2,c/4 and a/4,b/4,c/4, respectively. These morphological features agreed with features already reported for *P. katadae* (Kito, 1966; Miura, 1968).

The two specimens without serration were both monoecious. Of these, the thallus identified as *P. tenera* had carpogonium and spermatium dividing patterns of a/2,b/2,c/2 and a/4,b/4,c/4, respectively, while the thallus identified as *P. yezoensis* had dividing patterns of a/2,b/2,c/4 and a/2,b/4,c/8, respectively. The morphological features of these 2 thalli agreed with previously reported features (Ueda, 1932; Miura, 1994).

The two remaining specimens were dioecious. The thallus identified as *P. pseudolinearis* had carpogonium and spermatium dividing patterns of a/2,b/4c/4 and a/4,b/4,c/8, respectively, while, the thallus with dividing patterns of a/2,b/2,c/2 and a/4,b/4,c/4, respectively, was not positively identified. This specimen showed similar morphological features to *P. angusta*, but we could not confirm the presence of a trichogyne, which is characteristic of *P. angusta* (Ueda, 1932; Fukuhara, 1968; Miura, 1970). We called this specimen *Porphyra* sp.

A *P. umbilicalis* thallus was collected from the North Atlantic coast at Nahant, Mass., for comparision with already reported nucleotide sequences. The thallus used in this study was monoecious and the distribution of the carpogonium and spermatium was separated at the horizontal border. The dividing patterns of the carpogonium and spermatium were a/2,b/4,c/4 and a/4,b/4c/8, respectively. The morphological features of this thallus agreed with previously reported features (Ueda, 1932; Kurogi & Yoshida, 1966).

Structure of ssu rRNA gene

DNA purified by CsCl gradient ultra-centrifugation could be routinely PCR-amplified, while unpurified DNA sometimes did not support PCR-amplification. The complete length of ssu rDNA ranged among from 1818 (*P. umbilicalis*) to 3764 (*P. tenera* Shinwa). This variation in nucleotide length among species mainly revealed the presence of intron(s), but exon length also varied slightly by up to 20 bp (*P. umbilicalis*, 1816 bp; *P. katadae* and *Porphyra* sp., 1836 bp). The intron regions were identified putatively by comparing the nucleotide sequences of ssu rDNAs of *Rhodophyta* (Hendriks et al., 1991) and *P. umbilicalis*. Four different ssu rRNA genes structures were found in these species: *P. umbilicalis* has no intron (Type A); *P. yezoensis* Hakodate and *P. suborbiculata* have an intron at the nucleotide position of 567–569 (Type B); *P. haitanensis* has an intron at the nucleotide position of 1808 (Type C); the remaining species have two introns at the nucleotide positions of 567–570 and 1803–1807 (Type D). Type D is a major structure, but differences in intron number and size were found even within the same species *(P. yezoensis*) (Figure 1).

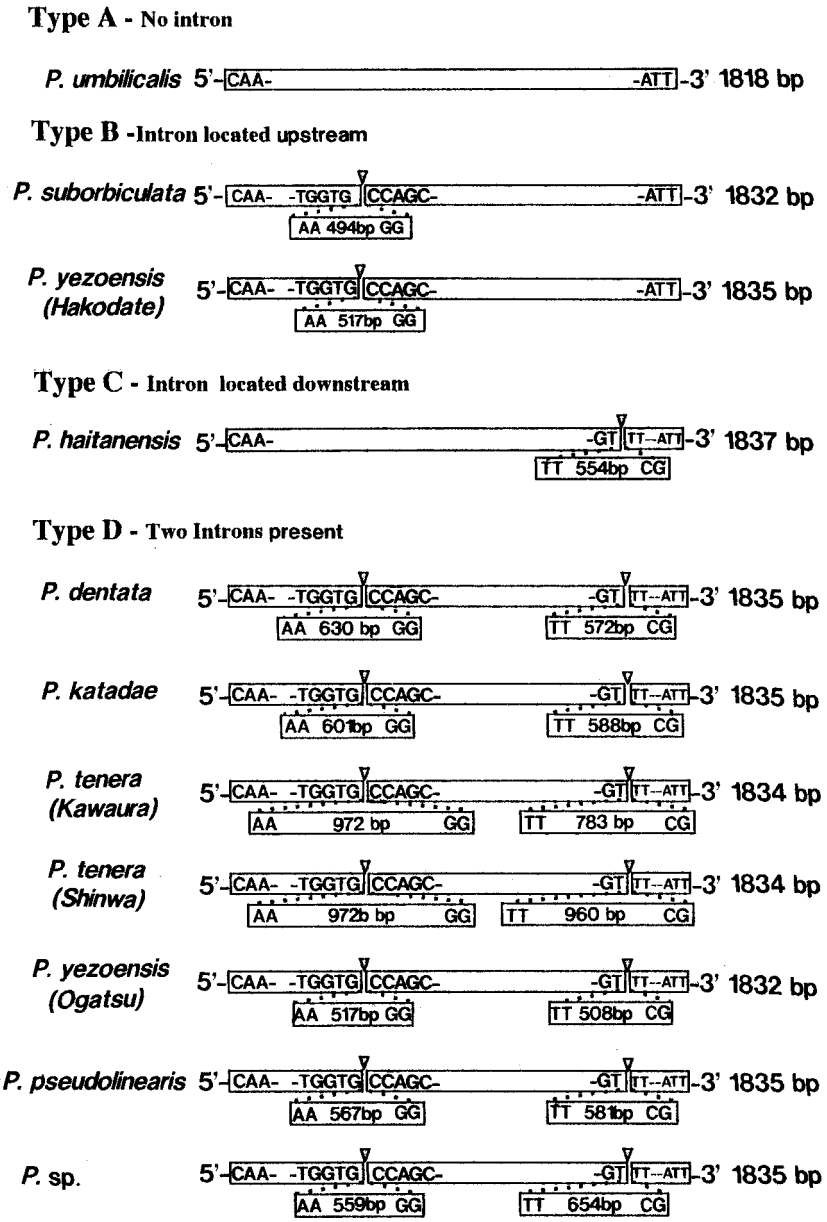

Figure 1. Structures of *Porphyra* ssu rRNA gene. The upper and lower flames represent exon and introns, respectively. Arrows represent the positions of introns insertion. Letters and numbers designated the partial sequences and number of nucleotide.

Exon sequence

The sequences of *Porphyra* species used were 89.0–99.6% homologous. The sequence alignment between *P. yezoensis* and *P. tenera* produced the highest homology score of 99.8%. *P. umbilicalis* was the least compatible with other species (89.0%). Within the same species, homology was 100%, regardless of the different intron number and length. Moreover, male and female *P. pseudolinearis*, which is dioecious species, were homologous (Data not shown). Highly conserved regions, at nucleotide positions of 1–70, 310–460, 550–650, 770–1080, 1120–1379, 1430–1550, 1660–1720 and 1800–1860 were found. Highly diversified regions were also detected at nucleotide positions of 120–140, 650–780, 1080–1110 and 1720–1770.

Intron sequences

Two classes of introns were presented in the *Porphyra* ssu rRNA gene, with one inserted in an upstream location at the nucleotide position 567–570. The location of intron insertion was the GTCTGGTG-CCAGCAGCC exon sequence in all DNA, and the 5′-end and 3′- end of introns were AAC- and -ATGG, respectively, in all these introns. A further intron was inserted downstream located at the nucleotide position 1803–1808. The location of intron insertion was the CAAGGT-TTCCGTA exon sequence in all DNA, and 5′- end and 3′- end of introns were TTCCGTA- and -ACG, respectively, in all these introns. The 5′ exon binding site of upstream located intron was not agreed to already reported intron classes, but the downstream intron have a 5′- and 3′- splicing site, exon binding site and core element as same as group I intron (Cech, 1990; Belfort, 1993). The length of introns located upstream varied from 494–972 nucleotides, while introns located downstream varied from 508–960 nucleotides. For *P. yezoensis* and *P. tenera*, the nucleotide sequences of upstream located introns were identical within each species, but the homology score between the two species was 51.3–73.1%. Beside, the sequence of downstream located intron varied within specimens of *P. tenera* (69.5% homologous), with homology scores among species ranging from 32.0–69.5%.

Discussion

The diversity in rDNA among the species revealed not only the presence of introns, but also gaps and substitutions in the exon (Figure 1 and Sequence). The exons of two *P. yezoensis* and two *P. tenera* thalli were homologous, respectively, but the presence of introns differed. *P. yezoensis* collected from Hokkaido prefecture had an intron in the upstream position, while *P. yezoensis* collected from Miyagi prefecture had an intron in both the upstream and downstream positions. Further, *P. tenera* collected from both Kawaura and Shinwa had two introns, with one intron identical between the two thalli, and the other differing to be only 69.5% homologious. These data indicate that thalli collected from different areas have different rDNA, even within the same species. The intron in an upstream location at the nucleotide position 567–570 is inserted just after CTGGTG and just before CCAGCA, and the intron in a downstream location at the nucleotide position 1803–1808 is inserted just after CAAGGT and just before TTCCGT, respectively, in all 10 thalli. The position of the intron insertion at the nucleotide position 1803–1808 was the same as found in *Porphyra spiralis* var. *amplifolia* (Oliviera and Ragan, 1994). This downstream introns had a 5′-splice site, 3′-splice site and conserved core elements as same as group I introns. The upstream introns were differed from group I introns in that the upstream flanking exon CTGGTG and 5′-end of intronic sequences AACCT. We putatively decided this site by comparison of already reported *Porphyra* ssu rRNA sequence data. Although the upstream introns have exon-intron pairing (P-1 like-stem), which include u-G pair and, 3′-splice site G as similar as group I intron, G of exon remained downstream of 5′-splicing site. Moreover, the conserved regions characteristic of group I intron P, Q, R and S, were dissimilar. We could not clarify the class of the upstream located introns. The nucleotide sequences flanking the upstream introns CTGGTG and CCAGCA are in highly conserved region and are present in all *Porphyra* ssu rRNA genes investigated. However, intron insertion at this position was found in the 9 Japanese thalli (Access numbers: *Porphyra spiralis* var. *amplifolia* L26175; *P. spiralis* var. *amplifolia* L26177; *P. umbilicalis* L36049; *P. umbilicalis* L26202; *P. purpurea* L26201; *P. leucosticta* L26199; *P. miniata* L26200; *P. acanthophora* L26197; *P. amplissima* L36048; *P. tenera* D86236; *P. tenera* D86237; *P. yezoensis* D79976). Furthermore, intron number(s) and sequences differed between individuals of the same species. From these results, introns were inappropriate for the discrimination of *Porphyra* species. However, introns number(s) and sequences may provide a means to trace cultivar lines.

Ssu rRNA gene exon lengths ranged from 1816–1835 nucleotides in 11 specimens of the 9 species examined in this study. These length were similar to those previously reported for ssu rRNA genes from *P. umbilicalis* (1818 nucleotides) and *P. acanthophora* (1845 nucleotides). Exons from 11 specimens were 89.0–100% homologous. The degree of sequence diversity differed among the species, while specimens of the same species had identical sequences. In the ssu rRNA genes, the highly conserved regions and the diversified regions occurred repeatedly. The diversified regions amplify the nucleotide sequence differences among the *Porphyra* species. The alignment of nucleotide positions 1070–1091 (in *P. suborbiculata*) indicated that the 9 species in this study and 8 previously studied species could be discriminated by comparing this region (Figure 2), except for *P.*

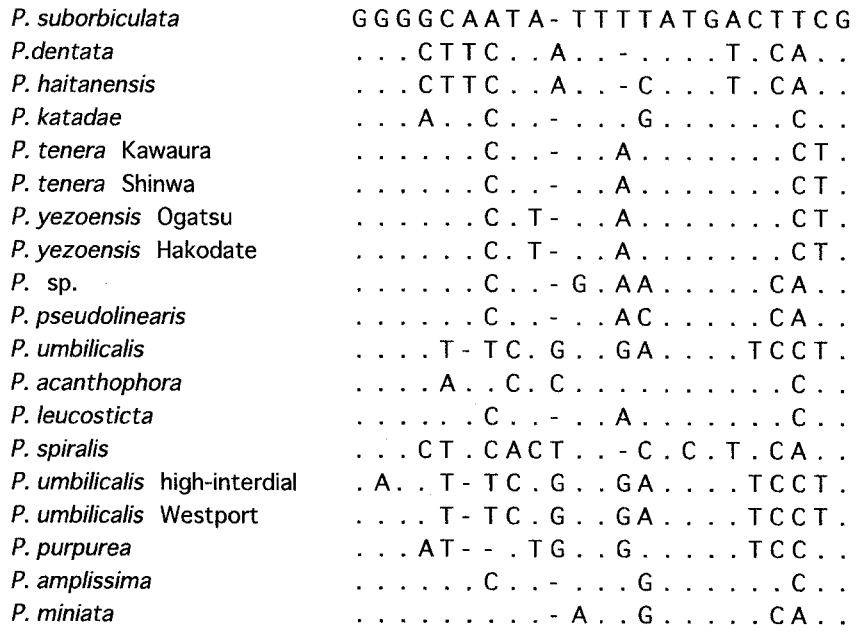

Figure 2. Alignment of nineteen *Porphyra* partial ssu rDNAs. The dots indicate the presence of the corresponding nucleotide in the ssu rDNA of *P. suborbiculata. P. suborbiculata* ssu rDNA nucleotide position 1070–1091 were used for the above alignment.

umbilicalis. Oliveira et al. (1995) reported the existence of 6 point substitutions between 2 strains of *P. umbilicalis* (Access number: *P. umbilicalis* Westport L36049; *P. umbilicalis* high-interdial L26202). The degree of diversity between two strains of *P. umbilicalis* was greater than that between *P. tenera* and *P. yezoensis* in this study, which had only 2 point substitutions and 2 point gaps in entire exon. In contrast, *P. umbilicalis* from Westport, and an individual thallus from Nahant, Mass., had identical ssu rDNA sequences.

These results raise the possibility that either the species level difference between *P. tenera* and *P. yezoensis* is hidden in mutations in the species or that the thallus samples have been contaminated by other species, which cannot be defined by morphological systematics. We will examine these possibilities using *P. yezoensis* in future research.

From the above results, we conclude that species discrimination was possible by contrasting the nucleotide sequences of the variant regions of ssu rDNAs in *Porphyra.*

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