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Molecular divergence and application of the ITS-5.8S rDNA and RUBISCO spacer in *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta)

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Abstract To select a reliable and sensitive method for discriminating strains of *Porphyra haitanensis*, the nucleotide sequence of the internal transcribed spacer 1 to internal transcribed spacer 2 regions (ITS-5.8S) of nuclear ribosomal DNA and the intergenic spacer region of RUBISCO were compared in five wild and five cultivated *Porphyra haitanensis* strains. Based on molecular analyses, sequences of ITS-5.8S (about 1,210 bp) could be divided into three regions: ITS1, 5.8S, and ITS2. The ITS1 and ITS2 sequences of each strain differed, even between individuals collected from the same site. In contrast, 5.8S rDNA and RUBISCO spacer sequences were identical among the ten *P. haitanensis* strains, although differences were found among different *Porphyra* species. Phylogenetic analysis also supported these conclusions. These sequence features of highly conserved regions and diversified regions that occurred repeatedly in ITS-5.8S could be useful in discriminating germplasm of *P. haitanensis* strains or *Porphyra* species. In contrast, the RUBISCO spacer is only suitable for identifying *Porphyra* species. New coupled primers were designed to amplify only the 5.8S rDNA and ITS2 region of *Porphyra*. The sequences of these amplified fragments can be readily used to identify germplasm or to perform phylogenetic analysis of *Porphyra* spp.

Keywords *Porphyra haitanensis* · Nuclear ribosomal DNA · Internal transcribed spacer · RUBISCO spacer · Molecular phylogeny

Abbreviations

ITS-5.8S	Nuclear ribosomal DNA of internal transcribed spacer 1 to internal transcribed spacer 2 region
RUBISCO spacer	Intergenic spacer region of RUBISCO
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
AFLP	Amplified fragment length polymorphism
SRAP	Sequence-related amplified polymorphism

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SSRs	Simple sequence repeats
SSU	Nuclear small subunit
rbcL	Plastid RUBISCO large subunit

Introduction

Porphyra, a genus of marine red algae, is an important economic marine crop, with an annual harvest of more than 130,000 t (dry weight) and a value of over US$2 billion. Today, the farming and processing of *Porphyra* have generated large seaweed industries in East Asian countries, including China, Japan, South Korea, and North Korea (Sahoo et al. 2002). Among *Porphyra* species, *P. haitanensis* is one of the most economically important. It has been widely cultivated along the coasts of South China, especially in the Fujian and Zhejiang Provinces.

In recent years, the production of *P. haitanensis* has constituted 75% of the total production of cultivated *Porphyra* in China (Zhang et al. 2005). With the advances of biotechnology and *Porphyra* farming, *Porphyra* cultivators are now carrying out intensive selections of *P. haitanensis* breed varieties and have selected many strains for cultivation in South China. For any cultivar, the correct identification of species or forma of the cultivated strains is necessary to ensure a well-bred cultivation and good production quality. However, because the gametophytic blade of *Porphyra* is morphologically simple and marked variations can occur as environmental conditions change, it is very difficult to precisely identify the species or forma of cultivated strains based only on their morphological characteristics. In addition, germplasm material has often been maintained as free-living conchocelis in laboratory subcultures. These conchocelis strains often cannot be definitively identified even using detailed morphological observations.

With new advances in molecular biology, molecular markers and DNA fingerprinting techniques have become routine for the identification and classification of many crops, including seaweeds. In cultivated *Porphyra*, the discrimination of strains has been attempted with restriction fragment length polymorphism (RFLP) analysis (Mizukami et al. 1996), random amplified polymorphic DNA (RAPD) analysis (Jia et al. 2000), amplified fragment length polymorphism (AFLP) analysis (Niwa et al. 2004; Sun et al. 2005), sequence-related amplified polymorphism (SRAP) analysis (Qiao et al. 2007; Xie et al. 2008), and simple sequence repeats (SSRs) analysis (Liu et al. 2005; Sun et al. 2006a). However, the limitations of these markers, including poor consistency, low reproducibility, labor intensiveness, or high cost, also limit their utilization to a significant degree.

Recently, nucleotide sequence data for the nuclear small subunit (SSU) rRNA gene, the ribosomal internal transcribed spacer (ITS), the plastid RUBISCO large subunit (rbcL), and the rbcL-rbcS intergenic spacer (RUBISCO spacer) have been used to determine the interspecific or intraspecific relationships of various *Porphyra* species (Freshwater et al. 1994; Ragan et al. 1994; Oliveira et al. 1994; Brodie et al. 1998; Woolcott and King 1998; Kunimoto et al. 1999a, b, 2003; Lindstrom and Fredericq 2003; Niwa and Aruga 2003, 2005a, b, 2008a, b; Xie et al. 2007). The superiority of these approaches is that they are based on popular and well-established sequencing technologies and can provide definitive results of nucleotide sequences (Kouduka et al. 2007).

In *Porphyra* species, Kunimoto et al. (2003) have suggested that the exon sequence of the SSU rRNA gene is an appropriately defined genetic marker for characterizing *Porphyra*

species and that the *Porphyra* intron sequences of the SSU rRNA gene and ITS1 are appropriate for cultivar identification. However, because of its complicated structure (including varied numbers of exons and introns) and its long sequence (Kunimoto et al. 1999a), the SSU rRNA gene is difficult to amplify by PCR and to sequence. Lindstrom and Fredericq (2003) reported that the rbcL gene has a better resolution than the SSU rRNA gene for elucidating species-level relationships in *Porphyra* species. However, the rbcL gene is inappropriate for elucidating intraspecific relationships in Porphyra species.

The nucleotide sequence of the ITS1 region and that of the RUBISCO spacer are assumed to have had substitutions many times more frequently than the rDNA exon region (Kunimoto et al. 1999b; Lindstrom and Fredericq 2003). Therefore, in present study, we have compared the nucleotide sequence of nuclear ribosomal DNA of the internal transcribed spacer 1 to internal transcribed spacer 2 region (ITS-5.8S) and of the plastid RUBISCO intergenic spacer region (RUBISCO spacer) for ten *P. haitanensis* strains. Our goal was to determine a reliable and sensitive method for discriminating strains of *P. haitanensis*.

Materials and methods

Materials

The free-living conchocelis (sporophyte phase of *Porphyra*) of ten strains of *P. haitanensis* were used in this study (Table 1). The cultivated strains of Q-1, Z-17, Z-26, and Z-61 of *P. haitanensis* have been selected and purified by the laboratory of germplasm improvements and applications in Jimei University, and all have been cultivated in a large scale on the coast of Fujian Province, China. Each strain has its own characteristics: Q-1 has high quality gametophytic blades, Z-17 grows rapidly, Z-26 can resist high temperatures, and the thallus of Z-61 is thick. The control cultivated strain (PXI) was a traditional cultivated strain from the coast of Fujian Province, China. The five wild strains were selected and

Table 1 Accession numbers of ITS-5.8S and RUBISCO spacer of *Porphyra haitanensis* analyzed in this study

Sample code	Origin	GenBank accession no	
		ITS-5.8S	RUBISCO spacer
Q-1	Cultivated strains	FJ217799	FJ217809
Z-17	Cultivated strains	FJ217800	FJ217810
Z-26	Cultivated strains	FJ217801	FJ217811
Z-61	Cultivated strains	FJ217802	FJ217812
PXI	Control cultivated strains	FJ217803	FJ217813
DD1	Selected and purified from Dadeng, Xiamen of Fujian Province	FJ217790	FJ611925
DD2	Selected and purified from Dadeng, Xiamen of Fujian Province	FJ217791	FJ687332
DS	Selected and purified from Dongshan of Fujian Province	FJ217792	FJ687333
JJS1	Selected and purified from Jiangjunshan, Pingtan of Fujian Province	FJ217795	FJ687334
JJS2	Selected and purified from Jiangjunshan, Pingtan of Fujian Province	FJ217796	FJ687335

purified from wild *P. haitanensis* populations off the coast of Fujian Province, China, and have been used as germplasm material in *P. haitanensis* breeding. All of the samples were stored in the laboratory of germplasm improvements and applications of *P. haitanensis* in Jimei University.

Stock cultures of the free-living conchocelis were maintained in the laboratory at 21 ± 1°C under 50–60 μmol photons m^{-2} s^{-1} (12L: 12D) provided by cool white fluorescent lamps. To obtain sufficient conchocelis clumps for DNA extraction, free-living conchocelis colonies were chopped into short fragments with a homogenizer and the conchocelis fragments were cultured in PES medium (Wang et al. 1986).

DNA extraction, amplification, and sequence analysis

DNA was isolated from the free-living conchocelis of each strain. The collected free-living conchocelis were ground into powder with high-speed homogenization, and DNA was extracted and purified by the method of CTAB (http://www.cimmyt.org/english/docs/manual/protocols/abc_amgl.pdf). DNA concentrations were determined with a DU-600 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and adjusted to 5 ng/μl for PCR amplification.

The regions of ITS-5.8S and RUBISCO spacer were amplified with the primers in Table 2.

For amplification of the two DNA fragments, the PCR mixture (25 μl) contained 2.5 μl 10× PCR Buffer,10 ng template DNA,2.5 mM Mg^{2+},1.5U *Taq* enzyme (Takara Biotechnology, Co., Ltd., Dalian, China),200 nM of each primer (Takara),200 μM dNTP (Takara), and sterile water. The amplifications were performed in a MT programmable thermal controller PTC-200 (MJ. Research, Inc.). The PCR program was 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 2 min, and finally 72°C for 10 min. To confirm the presence of amplified DNA fragments, PCR products were loaded onto 1.5% agarose gels and stained with ethidium bromide. PCR products were gel purified with a Takara agarose gel DNA purification kit (Takara) following the manufacturer's instructions. Sequencing was performed by Takara Biotechnology.

All of the novel sequence data were submitted to the GenBank. The accession numbers of ITS-5.8S and RUBISCO spacer are shown in Table 1.

Sequence identification, alignment, and phylogenetic analysis

Sequences were identified with *Porphyra* sequences existing in GenBank, using BLAST to confirm the boundary of each region.

The sequences determined in the present study and the additional corresponding sequences of other *Porphyra* species or strains, which were downloaded from GenBank

Table 2 The primers used for the amplification of the ITS-5.8S and RUBISCO spacer regions

Region	Forward primer (5′–3′)	Reverse primer (5′–3′)	Reference
ITS-5.8S	GGGATCCGTTTCCGT AGGTGAACCTGC	GGGATCCATATGCTTAA GTTCAGCGGGT	Goff and Moon (1993)
RUBISCO spacer	GACTCCAACAGCA AACATCTAG	TTAATA(T/C)CTAGCT CCTTCAGGC	Sun et al. (2006b)

Table 3 Details of *Porphyra* species analyzed in this study

Species	GenBank accession no		Reference
	5.8S	RUBISCO spacer	
P. yezoensis	AY368576(1)	AB287952(2)	(1) Hu et al. 2007 (2) Unpublished data
P. drewiana	AY766365	–	Milstein and Oliveira (2005)
P. spiralis	AY766366	–	Milstein and Oliveira (2005)
P. umbilicalis	AY322132	–	Unpublished data
P. suborbiculata	AF378665(1)	AB287943(2)	(1) Broom et al. 2002 (2) Unpublished data
P. katadae	AY368577	–	Hu et al. (2007)
P. oligospermatangia	AY368578	–	Hu et al. (2007)
P. dentata	–	AB287928	Unpublished data
P. pseudolinearis	–	AB287941	Unpublished data
P. tenera	–	AB118578	Unpublished data

(Table 3), were used in phylogenetic and genealogical analyses. The sequences were aligned using clustal X 1.83 (Thompson et al. 1997), and then refined by eyed. When all sequences had aligned, the base type that was in majority of the ten sequences seemed to be the intrinsic base in the variance positions. Phylogenetic relationships among these sequences were inferred using the neighbor-joining (NJ) method (Saitou and Nei 1987). The NJ method was performed with the MEGA4 (Tamura et al. 2007), and stability of the resulting groups was tested by bootstrap analysis over 1,000 replications.

Results

PCR amplification

Figures 1 and 2 show the PCR products of the ITS-5.8S and RUBISCO spacer, respectively, in ten *P. haitanensis* strains. A single band of amplified DNA was observed for each of the ten strains. The bands from the ten strains were of a similar size, at approximately 1.2 (ITS-5.8S) and 0.5 (RUBISCO spacer) kilobase pairs.

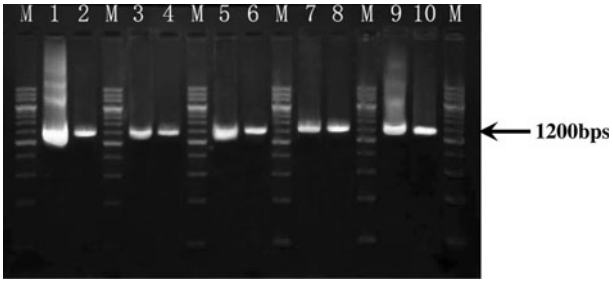

Fig. 1 PCR profile of the ITS-5.8S for ten *Porphyra haitanensis* strains. *Lane 1* Q-1, *lane 2* z-17, *lane 3* Z-26, *lane 4* Z-61, *lane 5* PXI, *lane 6* DD1, *lane 7* DD2, *lane 8* DS, *lane 9* JJS1, *lane 10* JJS2, *M* shows molecular weight markers

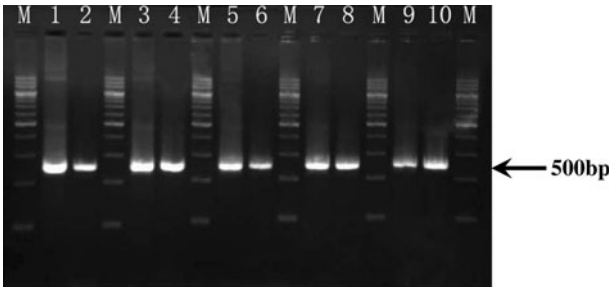

Fig. 2 PCR profile of the RUBISCO spacer for ten *Porphyra haitanensis* strains. *Lane 1* Q-1, *lane 2* z-17, *lane 3* Z-26, *lane 4* Z-61, *lane 5* PXI, *lane 6* DD1, *lane 7* DD2, *lane 8* DS, *lane 9* JJS1, *lane 10* JJS2, *M* shows the molecular weight markers

Nucleotide sequences and phylogenetic analyses of the ITS-5.8S

By multiple sequence alignments with existing *Porphyra* sequences in GenBank using BLAST, the structure and boundary of each part of ITS-5.8S of *P. haitanensis* could be defined. The two flanks of 5.8S had polyA and polyT sequences. Table 4 shows the details of ITS-5.8S of the ten *P. haitanensis* strains. The sequence length of ITS-5.8S ranged from 1,212 bps (PXI, DD1) to 1,221 bps (Q-1), and the base component of each sequence varied slightly. The 5.8S rDNA region of the ten strains was of an identical 160 bp size. The ITS1 and ITS2 region varied slightly by two or three bps between strains, due to the presence of indels, but were approximately 368 and 690 bp, respectively.

Table 5 shows the variable nucleotide positions of the ITS1 regions of the ten *P. haitanensis* strains by multiple sequence alignment. The sequence differences between the ten strains are shown in Table 6. The ITS1 sequences were all different from each other, and identical sequence pairs were not found among these strains, even between the strains that had been collected from the same site. Similar ITS1 sequences were found between Q-1 and Z-17, Z-17 and Z-61, Z-17 and JJS1, PXI and DS, PXI and JJS2, JJS1 and JJS2, which showed only one point change in 369 bp aligned sites. The maximum

Table 4 Detail of ITS-5.8S of ten *Porphyra haitanensis* strains

Strains	Sequence length (bp)	Base number				Length of ITS1 region	Length of ITS2 region
		A	*C*	*G*	*T*		
Q-1	1,221	304	292	314	311	369	692
Z-17	1,219	306	290	314	309	369	688
Z-26	1,218	301	293	316	308	368	690
Z-61	1,220	303	293	314	310	369	691
PXI	1,212	302	290	309	311	367	685
DD1	1,212	301	291	311	309	367	685
DD2	1,216	303	291	311	311	368	688
DS	1,215	302	291	312	310	368	687
JJS1	1,214	305	290	310	309	369	685
JJS2	1,217	303	292	310	312	368	689

The 5.8S rDNA region of the ten strains was of identical 160 bp size

Table 5 Variable nucleotide positions of the ITS1 region (369 bp) of ten *Porphyra haitanensis* strains by compressing alignments

Variance position	27	49	126	163	227	232	244	253	281	300	352	359	360	368	369
Base type	G	T	T	C	A	A	A	T	A	–	A	A	A	A	–
Q-1	T												G		A
Z-17													G		A
Z-26			G						G			G			
Z-61								C					G		A
PXI					–										
DD1				T	–	C	G								
DD2		C									T				
DS					–					G					
JJS1															A
JJS2															

Table 6 Absolute sequence differences (below diagonal) and % sequence divergence (above diagonal) of ITS1 sequences from ten *Porphyra haitanensis* strains

Strains	Q-1	Z-17	Z-26	Z-61	PXI	DD1	DD2	DS	JJS1	JJS2
Q-1		0.27	1.63	0.54	1.08	1.90	1.36	1.35	0.54	0.81
Z-17	1		1.36	0.27	0.81	1.63	1.08	1.08	0.27	0.54
Z-26	6	5		1.63	1.09	1.90	1.36	1.36	1.08	0.82
Z-61	2	1	6		1.08	1.90	1.36	1.35	0.54	0.81
PXI	4	3	4	4		0.72	0.72	0.27	0.54	0.27
DD1	7	6	7	7	3		1.63	1.09	1.36	1.09
DD2	5	4	5	5	3	6		1.08	0.81	0.54
DS	5	4	5	5	1	4	4		0.81	0.54
JJS1	2	1	4	2	2	5	3	3		0.27
JJS2	3	2	3	3	1	4	2	2	1	

difference in ITS1 sequences were found between DD1 and Q-1, Z-26, and Z-61, which showed 7 points of variance in 369 aligned sites. On the ten *P. haitanensis* strains, the ITS1 sequence divergence (%) was between 0.27 and 1.63, with an average divergence of 1.06.

Table 7 shows the variable nucleotide positions of the ITS2 region of the ten *P. haitanensis* strains by multiple sequence alignment. The sequence differences between the ten strains are shown in Table 8. The ITS2 sequences were also different from each other, and identical sequence pairs were not found among these ten strains. Similar ITS2 sequences were found between Q-1 and Z-17, Z-17 and Z-61, Q-1 and Z-61, which showed only 2 points of variance in 696 bp aligned sites. The maximum difference of the ITS2 sequences were found between DS and Z-26, which showed 21 points of variance in 696 aligned sites. For the ten *P. haitanensis* strains, the ITS2 sequence divergence (%) was between 0.29 and 3.03, with an average divergence of 1.66. The average % sequence divergence between the wild strains was 1.80, which was higher than that between the cultured strains at 0.36.

A phylogenetic tree (Fig. 3) was produced by neighbor-joining (NJ) analysis, based on the ITS2 sequence dataset of the ten *P. haitanensis* strains, using MEGA 4.0. In this tree, the ten *P. haitanensis* strains could be separated into three groups—one group that included the four cultivated strains and the second group that was composed of the five wild strains. The control cultivated strain (PXI) formed another clade.

Identical 5.8S rDNA region sequences were found for the ten *P. haitanensis* strains, but different sequences of 5.8S were found among different *Porphyra* species (Table 9). The divergence of eight species of *Porphyra* was between 0 and 14.1%. *P. drewiana* and *P. spiralis* had identical sequences in their 5.8S rDNA regions. The phylogenetic tree (Fig. 4) that was produced by NJ analysis based on the 5.8S rDNA sequence dataset indicated a clear separation of the *Porphyra* species, except for *P. drewiana* and *P. spiralis.*

Nucleotide sequences and phylogenetic analyses of the RUBISCO spacer region

Similar to the 5.8S rDNA region, identical RUBISCO spacer sequences were also found for the ten *P. haitanensis* strains, while different *Porphyra* species had different sequences for their RUBISCO spacers (Table 10). The divergence of six of the species of *Porphyra* was between 1.9 and 9.3%, and no identical sequences were found between the *Porphyra*

Table 7 Variable nucleotide positions of ITS2 region (696 bp) of ten *Porphyra haitanensis* strains by compressing alignments

Variance position	0	6	40	65	114	126	143	166	218	226	227	246	261	265
Base type	T	T	A	T	C	A	A	A	T	G	–	T	T	C
Q-1														
Z-17									–					
Z-26														
Z-61						T								
PXI									–	T				
DD1	–	C					G						C	G
DD2								G						
DS			G		T				–	T	G	G		
JJS1				C										
JJS2									–	T	G			

Variance position	266	317	378	386	402	406	407	429	430	470	471	506	572	573
Base type	G	T	C	C	C	T	G	–	–	–	–	G	–	A
Q-1										G				
Z-17														
Z-26		C												–
Z-61														
PXI				T										–
DD1	C					A	T							
DD2					G		T						A	
DS						A	T	T	T		G	A	A	–
JJS1			–											
JJS2								T	T		G			–

Table 7 continued

Variance position	594	595	596	597	607	608	673	678	679	680	693	696
Base type	–	–	–	–	A	C	G	A	C	T	T	C
Q-1	G	G	C	G								
Z-17	G	G	C	G								
Z-26	G	G	C	G								
Z-61	G	G	C	G								
PXI												
DD1						T	T					–
DD2												
DS					C			C	–	–	C	–
JJS1						T	T					–
JJS2												

Table 8 Absolute sequence differences (below the diagonal) and % sequence divergences (above the diagonal) of the ITS2 region from ten *Porphyra haitanensis* strains

Strains	Q-1	Z-17	Z-26	Z-61	PXI	DD1	DD2	DS	JJS1	JJS2
Q-1		0.29	0.43	0.29	1.16	2.17	1.30	2.59	1.45	1.15
Z-17	2		0.43	0.29	1.01	2.17	1.30	2.88	1.45	1.44
Z-26	3	3		0.43	1.01	2.32	1.45	3.03	1.59	1.30
Z-61	2	2	3		1.16	2.17	1.30	2.88	1.45	1.44
PXI	8	7	7	8		1.89	1.02	2.61	1.16	0.87
DD1	15	15	16	15	13		1.74	2.76	1.60	2.17
DD2	9	9	10	9	7	12		2.46	1.31	1.30
DS	18	20	21	20	18	19	17		2.61	2.03
JJS1	10	10	11	10	8	11	9	18		1.45
JJS2	8	10	9	10	6	15	9	14	10	

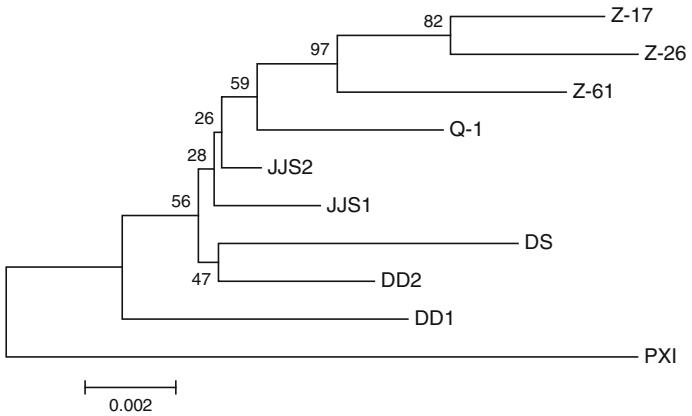

Fig. 3 Cladogram based on ITS2 sequences, showing the relationship between the ten strains of *Porphyra haitanensis*. The tree was constructed using the NJ method and bootstrap value calculation from 1,000 trees

species. The phylogenetic tree (Fig. 5) that was produced by NJ analysis based on the rbcS-RUBISCO spacer sequence dataset also showed a clear separation of the *Porphyra* species.

Discussion

In this study, we have addressed problems of strain identification of *P. haitanensis* using DNA sequencing of a small PCR product. Numerous papers have proposed that *Porphyra* strains of the same species can be discriminated by comparing the sequences of ITS1 regions (Kunimoto et al. 1999a, b, 2003; Niwa and Aruga 2003, 2005a, b, 2008a, b). Of the ten strains of *P. haitanensis* studied here, the sequences of the ITS-5.8S (about 1,210 bp) could be divided into three regions: ITS1, 5.8S and ITS2. The ITS sequence (including ITS1 and ITS2) of each strain was found to be different, and even differed between individuals collected from the same site. These sequences did tend to be very similar among all specimens, with variation scores $< 3.03\%$, as listed in Tables 6 and 8. This

Table 9 Sequence divergence (%) of 5.8S region of *Porphyra sp*

	P. suborbiculata	*P. umbilicalis*	*P. yezoensis*	*P. katadae*	*P. oligosperma*	*P. drewiana*	*P. spiralis*	*P. haitanensis*
P. suborbiculata								
P. umbilicalis	14.1							
P. yezoensis	11.4	13.8						
P. katadae	11.4	13.8	1.2					
P. oligospermatangia	11.4	13.2	1.9	1.2				
P. drewiana	12.1	13.2	11.2	10.6	10.6			
P. spiralis	12.1	13.2	11.2	10.6	10.6	0		
P. haitanensis	9.6	13.2	7.5	6.9	6.9	8.7	8.7	

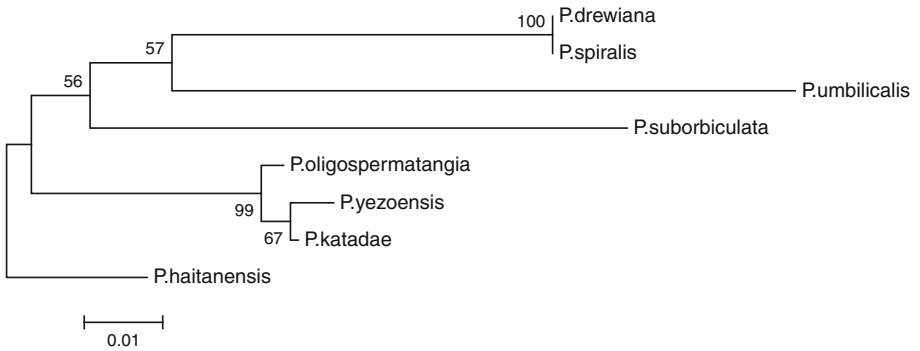

Fig. 4 Cladogram based on 5.8S sequences, showing the relationship between eight species of *Porphyra*. The tree was constructed by using the NJ method and bootstrap value calculation from 1,000 trees

Table 10 Sequence divergence (%) of RUBISCO spacer of *Porphyra sp*

	P. tenera	*P. haitanensis*	*P. dentata*	*P. pseudolinearis*	*P. suborbiculata*	*P. yezoensis*
P. tenera						
P. haitanensis	9.3					
P. dentata	6.6	5.0				
P. pseudolinearis	5.8	7.4	4.1			
P. suborbiculata	9.1	9.3	7.2	7.0		
P. yezoensis	1.9	8.9	7.4	5.8	8.7	

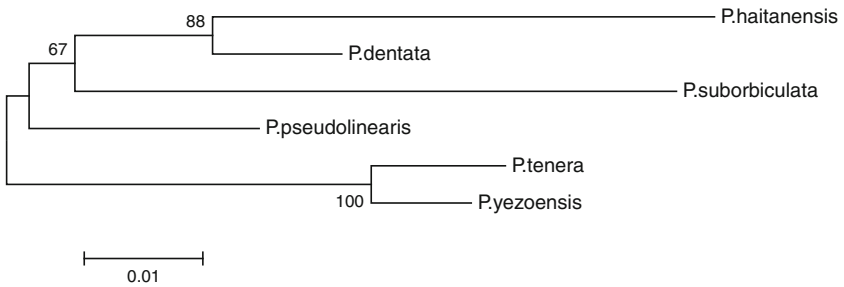

Fig. 5 Cladogram based on RUBISCO spacer sequences, showing the relationship between six species of *Porphyra*. The tree was constructed by using the NJ method and bootstrap value calculation from 1,000 trees

result is in accord with several previous published studies (Kunimoto et al. 1999a, b, 2003; Niwa and Aruga 2003, 2005a, b, 2008a, b). The molecular features reported here will be of practical use for discrimination and establishing the genetic relationships of *Porphyra* strains.

The sequence divergence (%) of the ITS1 region between strains was not similar to that seen for the ITS2 region, even between the same strains (Tables 6, 8). This difference was unpredictable. The ITS1 sequence length is about 368 bp, and only one or two base differences were noted between the ten *P. haitanensis* strains (Table 4). The sequence divergence (%) was between 0.27 and 1.63 (average was 1.06), and in many strains, only

one base substitution or gap difference was noted. The divergence among cultivated strains had less obvious differences than did the wild strains (Table 6).

For the ITS2, with a sequence length between 685 and 692 bp, the difference between strains was much greater than that seen for the ITS1. Sequence divergence (%) was between 0.29 and 3.03 (average was 1.66), which was also much higher than that of the ITS1 region. The divergence of ITS2 sequences among wild strains was clearly higher than that for cultivated strains (Table 8). Phylogenetic analysis based on the nucleotide sequences of ITS2 (Fig. 3) could clearly distinguish the wild and cultivated strains of *P. haitanensis*. The ITS2 region may, therefore, be more suitable than ITS1 for discriminating and establishing genetic relationships in *P. haitanensis* strains, as it offers a larger number of variable sites.

The 5.8S rDNA sequences were identical among the ten *P. haitanensis* strains. However, after blast analysis in GenBank, different sequences were found among different *Porphyra* species, except for *P. drewiana* and *P. spiralis*. This shows that the sequence of 5.8S differed among *Porphyra* species, while sequences from strains of the same species were identical. This sequence feature is similar to that seen for the SSU rDNA exon (Kunimoto et al. 1999a, b, 2003; Jones et al. 2004) and indicates that the nucleotide sequence of 5.8S RNA gene also can be used to discriminate between *Porphyra* species. The phylogenetic analysis clearly supported this conclusion (Fig. 4). The 5.8S RNA gene is only 160 bp lengths and has no introns, which makes it a much shorter and simpler gene than the SSU rDNA. We believe that the 5.8S RNA gene may, therefore, have a number of advantages over the SSU rDNA for the discrimination of *Porphyra* species.

Based on these results, one new set of coupled primers can be designed to amplify only the 5.8S rDNA and the ITS2 region of *Porphyra*. Sequences of the amplified fragments can then be easily used for germplasm identification and phylogenetic analysis of *Porphyra*. With the assistance of the Primer premier^{TM} 5.0 and oligo 6.0 software, the new couple primers 5′-TTTGTCTTATCCGCTTTGT-3′ and 5′-GTGGTCCTACCTGATTTGAG-3′ were designed for this study. Their Tm value was 54°C and the 936 bp amplified fragment included the 5.8S rDNA and the ITS2 region. The results of PCR amplification (Fig. 6) and sequence data of PCR products using the new couple primers showed that the new couple primers work reliably.

The result of multi-sequence alignment of the RUBISCO spacer was similar to that seen for the 5.8S rDNA Identical sequences were also found for the ten *P. haitanensis* strains, but different *Porphyra* species had different sequences (Table 10). The same result has also been reported for *P. yezoensis* strains (Niwa et al. 2008a). Therefore, the RUBISCO spacer is not suitable for the discrimination of *P. haitanensis* strains, but it is a valuable marker for discriminating different *Porphyra* species (Brodie et al. 1998). The variation values of 5.8S rDNA and RUBISCO spacer, listed in Tables 9 and 10, are not distinctly

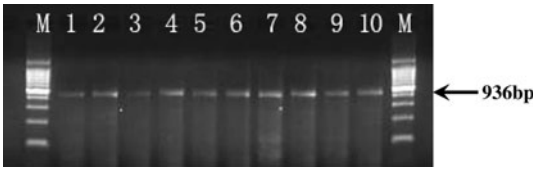

Fig. 6 PCR profile of the 5.8S-ITS2 for ten *Porphyra haitanensis* strains. *Lane 1* Q-1, *lane 2* z-17, *lane 3* Z-26, *lane 4* Z-61, *lane 5* PXI, *lane 6* DD1, *lane 7* DD2, *lane 8* DS, *lane 9* JJS1, *lane 10* JJS2, *M* shows molecular weight markers

different, so we cannot judge *a priori* which gene (5.8S rDNA or RUBISCO spacer) would be better suited for species identification in *Porphyra*.

From the above results, we can conclude that contrasting the nucleotide sequences of the 5.8S rDNA and the ITS2 regions would provide a reliable and sensitive method to discriminate *Porphyra* species or *P. haitanensis* strains. In contrast, the RUBISCO spacer is only suitable for identifying *Porphyra* species.

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