Note

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Intraspecific variation of gene structure in the mitochondrial large subunit ribosomal RNA and cytochrome c oxidase subunit 1 of *Pyropia yezoensis* (Bangiales, Rhodophyta)

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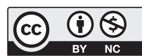
Red algal mitochondrial genomes (mtDNAs) can provide useful information on species identification. mtDNAs of *Pyropia* / *Porphyra* (Bangiales, Rhodophyta) have shown diverse variation in their size and gene structure. In particular, the introns and intronic open reading frames found in the ribosomal RNA large subunit gene (*rnl*) and cytochrome c oxidase subunit 1 gene (*cox*1) significantly vary the mitochondrial genome size in *Pyropia* / *Porphyra* species. In this study, we examined the exon / intron structure of *rnl* and *cox*1 genes of *Pyropia yezoensis* at the intraspecific level. The combined data of *rnl* and *cox*1 genes exhibited 12 genotypes for 40 *P. yezoensis* strains, based on the existence of introns. These genotypes were more effective to identify *P. yezoensis* strains in comparison to the traditional DNA barcode *cox*1 marker (5 haplotypes). Therefore, the variation in gene structure of *rnl* and *cox*1 can be a novel molecular marker to discriminate the strains of *Pyropia* species.

Key Words: *cox*1; intraspecific variation; intron; *Pyropia yezoensis*; *rnl*

INTRODUCTION

Pyropia species is one of the major seaweeds cultivated in Korea, Japan, and China (Niwa et al. 2004, Hwang et al. 2014). Strain identification at the intraspecific level of *Pyropia yezoensis* (Ueda) M. S. Hwang & H. G. Choi is important to the *Pyropia* aquaculture industry for the maintenance of aquaculture strains and for the development of new cultivars. Although various molecular markers have been developed to discriminate *P. yezoensis* at the inter / intraspecific levels (Niwa et al. 2004, 2005, Hwang et al. 2005, Park et al. 2008, Niwa and Kobiyama 2009), more efficient tools for precise discrimination are still required.

Red algal mitochondrial genomes (mtDNAs) have interesting genes and structural composition (Odintsova and Yurina 2002, Smith et al. 2012, Yang et al. 2015). Such genetic features have provided useful information for interpreting the evolutionary history of Bangiophycean species (Smith et al. 2012). Particularly, the gene composition and structural variations of mtDNAs of *Pyropia* species have provided useful genetic information at the inter / intraspecific levels (Hwang et al. 2013, 2014, Hughey et al. 2014).



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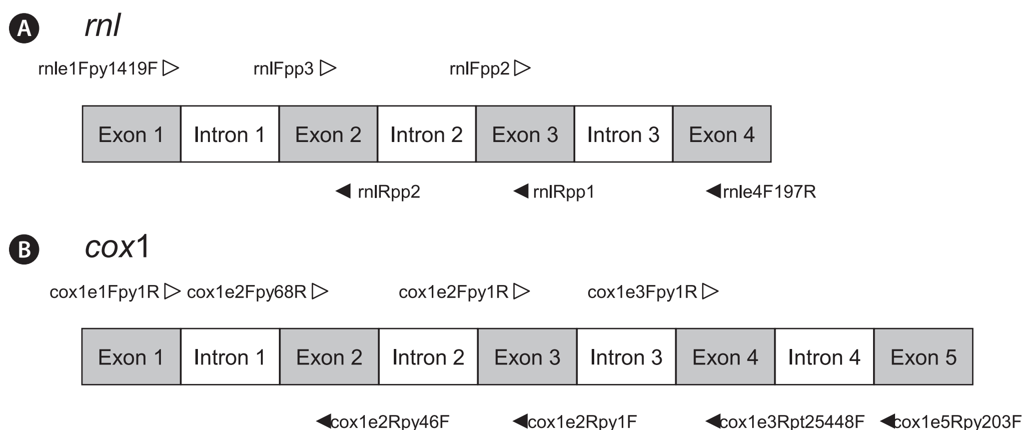

Fig. 1. Gene structure of *rnl* (A) and *cox*1 (B) regions and the primer binding map. The exon / intron structures were re-analyzed using Hwang et al. (2013, 2014) as reference.

The study of mtDNAs in *Pyropia* / *Porphyra* (Bangiales, Rhodophyta) showed that they exhibit diverse variation in genome size and genetic structure, and also that they contain higher number of introns and intronic open reading frames (ORFs) in comparison to other red algae (Hughey et al. 2014, Hwang et al. 2014, Yang et al. 2015). Specifically, structural variation in the exon / intron structure of the large subunit ribosomal RNA gene (*rnl*) and cytochrome c oxidase subunit 1 gene (*cox*1) was reported in *Pyropia* / *Porphyra* species (Hwang et al. 2013, 2014, Hughey et al. 2014). These genetic variations significantly affected the size of mitochondrial genome of *Pyropia* / *Porphyra* species (Hwang et al. 2013, 2014, Hughey et al. 2014); a similar phenomenon was observed in the brown alga, *Pylaiella littoralis* (Ikuta et al. 2008).

Hwang et al. (2013, 2014) and Kong et al. (2014) examined the complete genome of mtDNA of *P. tenera* and *P. yezoensis*, and explored the genetic variation of exon / intron structure and the phylogenetic relationship among the intronic ORFs. Hughey et al. (2014) also reported the presence of intronic ORFs and the size variations of mtDNA of *P. perforata*. In this study, we examined the exon / intron structure of *rnl* and *cox*1 of *P. yezoensis* at the intraspecific level. The presence / absence of intron patterns were examined among the different strains of *P. yezoensis*.

MATERIALS AND METHODS

We analyzed 40 strains of *P. yezoensis* deposited in the Seaweed Research Center (National Institute of Fisheries Science, Mokpo, Korea) (Table 1). Blades of 27 *P. yezoensis* strains were collected from Korean coastal regions or aquaculture farms, and the conchocelis filaments were induced and cultured in the Provasoli enrichment medium (Provasoli 1968) at 20°C under white fluorescent irradiation of 20 μmol photon $m^{-2} s^{-1}$ (14 L : 10 D cycle). Thirteen Japanese *P. yezoensis* strains were also analyzed (Table 1). The conchocelis filaments were used for the molecular analyses. Total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with an extension of incubation time by one hour.

To reveal the exon / intron structure of *rnl* and *cox*1 regions of *P. yezoensis*, we designed new primer sets having the binding sites on each exon region. These exon-primed intron-crossing (EPIC) markers showed high efficiency in revealing the exon / intron structure (Palumbi and Baker 1994, Bierne et al. 2000). Three and four primer sets were developed to reveal the genetic structure of *rnl* and *cox*1 regions, respectively (Tables 2 & 3, Fig. 1).

The mtDNAs of *P. tenera* (Kjellm.) N. Kikuchi, M. Miyata, M. S. Hwang & H. G. Choi (NC_021475, Hwang et al. 2013) and *P. yezoensis* (NC_017837, Kong et al. 2014; KF561997, Hwang et al. 2014) were used as reference sequences for the primer design. We performed long range polymerase chain reaction (LPCR) to amplify the exon / intron structure of *rnl* and *cox*1 genes (Hwang et al. 2013). LPCR was carried out in a 20 μL volume containing 10-50 ng of total genomic DNA, utilizing the LA *Taq* polymerase system (TaKaRa Bio, Shiga, Japan). The amplification conditions were as follows: 2 min at 94°C, followed by 30 cycles at 94°C for 10 s, 60°C for 30 s, and at 68°C for 5 min, with a final extension at 68°C for 7 min. Band patterns of LPCR products were analyzed by agarose gel electrophoresis.

Table 1. Details of the samples analyzed in this study

Species	Strain code	Genotype			Sample site	Sampling date
		*cox*1 haplotype	*rnl* and *cox*1 gene structure			
Korean *Pyropia yezoensis* (27 strains)	GB-1	C1	H1	R011C0100	Songdo, Pohang, Gyeongbuk	Feb 6, 2001
	GB-2	C2	H2[a]	R101C1101	Masanri, Pohang, Gyeongbuk	Apr 3, 2008
	GN-1	C3	H1	R011C0100	Neungpo, Geoje, Gyeongnam	Feb 5, 2001
	GN-2	C1	H3	R010C0100	Galmok, Tongyeong, Gyeongnam	Feb 7, 2001
	GN-3	C1	H3	R010C0100	Sachon, Namhae, Gyeongnam	Feb 29, 2004
	JN-1	C3	H4	R110C0001	Cheongsando, Wando, Jeonnam	Feb 19, 2004
	JN-2	C3	H5	R111C0000	Maenggoldo, Jindo, Jeonnam	Apr 1, 2007
	JN-3	C3	H6	R101C0101	Hajodo, Jindo, Jeonnam	Mar 19, 2004
	JN-4	C3	H7	R111C0001	Euisin, Jindo, Jeonnam	Feb 26, 2000
	JN-5	C3	H5	R111C0000	Songji-1, Haenam, Jeonnam	Feb 25, 2011
	JN-6	C3	H5	R111C0000	Songji-2, Haenam, Jeonnam	Mar 15, 2012
	JN-7	C3	H5	R111C0000	Songji-3, Haenam, Jeonnam	Feb 18, 2008
	JN-8	C3	H8	R111C0101	Songji-4, Haenam, Jeonnam	Feb 20, 2009
	JN-9	C3	H5	R111C0000	Songji-5, Haenam, Jeonnam	Feb 22, 2008
	JN-10	C3	H5	R111C0000	Imhado-1, Haenam, Jeonnam	Mar 10, 2014
	JN-11	C3	H5	R111C0000	Imhado-2, Haenam, Jeonnam	Mar 10, 2014
	JN-12	C3	H9	R111C0100	Hwawon, Haenam, Jeonnam	Mar 12, 2002
	JN-13	C3	H5	R111C0000	Madong-1, Muan, Jeonnam	Feb 27, 2013
	JN-14	C3	H5	R111C0000	Madong-2, Muan, Jeonnam	Feb 28, 2012
	JN-15	C3	H12	R101C0111	Dochodo, Sinan, Jeonnam	Mar 4, 2011
	JN-16	C3	H12	R101C0111	Bigeumdo, Sinan, Jeonnam	Mar 5, 2011
	JN-17	C1	H12	R101C0111	Heuksando, Sinan, Jeonnam	Jan 30, 2009
	JB-1	C3	H10	R101C0100	Wido-1, Buan, Jeonbuk	Apr 7, 2004
	JB-2	C3	H10	R101C0100	Wido-2, Buan, Jeonbuk	Apr 7, 2004
	JB-3	C3	H8	R111C0101	Munyeodo, Gunsan, Jeonbuk	Apr 8, 2004
	CN-1	C4	H11	R110C0101	Daecheon-1, Chungnam	Apr 22, 2004
	CN-2	C4	H11	R110C0101	Daecheon-2, Chungnam	Apr 22, 2004
Japanese *P. yezoensis* (13 strains)	JP-Tu1	C5	H12	R101C0111	Tu-1, Kisarazu, Chiba, Japan	Mar 9, 1974[b]
	JP-PYN	C5	H12	R101C0111	Aquaculture strain of *P. yezoensis* f. *narawaensis* from Japan	2000[c]
	JP-PY1	C5	H12	R101C0111	Same as above	2004[c]
	JP-PY2	C5	H12	R101C0111	Same as above	2004[c]
	JP-PY3	C5	H12	R101C0111	Same as above	2004[c]
	JP-PY4	C5	H12	R101C0111	Same as above	2008[c]
	JP-PY5	C5	H12	R101C0111	Same as above	2008[c]
	JP-PY6	C5	H12	R101C0111	Same as above	2007[c]
	JP-PY7	C5	H12	R101C0111	Same as above	2007[c]
	JP-PY8	C5	H12	R101C0111	Same as above	2007[c]
	JP-PY9	C5	H12	R101C0111	Same as above	2007[c]
	JP-PY10	C5	H12	R101C0111	Same as above	2007[c]
	JP-PY11	C5	H12	R101C0111	Same as above	2007[c]

The structural variation of the gene is represented as a code (R = *rnl*, C = *cox*1, 0 = intron absent, 1 = intron present).
GenBank accession numbers for five types of *cox*1 sequences (C1-C5, MF663741-MF663745).
Electrophoresis gel images were represented in the Supplementary Fig. S1.
[a]H2 genotype showed the different length in introns.
[b]This sample was obtained from Prof. Saga in Hokkaido University, Japan.
[c]These were obtained from a company that cultures free living conchocelis filaments of *Pyropia*.

Table 2. Details of the primers used for the amplification of *rnl* and *cox*1 regions

Gene region[a]	Forward primer	Sequence (5′ to 3′)	Reverse primer	Sequence (5′ to 3′)
rnl (e1)-(e2)	rnle1Fpy1419F	ACTCGGCAAATTTACTCCGTAC	rnlRpp2	CATGATAAATCTGTTATCCCTAGAG
rnl (e2)-(e3)	rnlFpp3	CCTCCTAAAGTGTAACGGAGGTG	rnlRpp1	ACTGTCTCACGACGTTCTGAACC
rnl (e3)-(e4)	rnlFpp2	GAACGTCGTGAGACAGTTCGGTC	rnle4F197R	GCAAGAAACAATACAACCGATACAC
*cox*1 (e1)-(e2)	cox1e1Fpy1R	CTCGACCAATCATAAAGATATAGG	cox1e2Rpy46F	AATACAGGATCGCCACCACC
*cox*1 (e2)-(e3)	cox1e2Fpy68R	TCAGGTGGTGGCGATCCTGT	cox1e2Rpy1F	ACCTATAGAAAGCATAGCATAAATC
*cox*1 (e3)-(e4)	cox1e2Fpy1R	ATGATTTATGCTATGCTTTCTATAGG	cox1e3Rpt25448F	AGCTAGTATAATACCAGTAAGTCC
*cox*1 (e4)-(e5)	cox1e3Fpy1R	TGGATTGCTACTATGTGAGAAGG	cox1e5Rpy203F	CTAAATAACGCAACATATGAACC

[a]We followed the results of Hwang et al. (2014) for nomenclature to present the structural variation.

We also determined the sequences of the *cox*1 in 40 *P. yezoensis* strains, which has been used as the standard target DNA region for DNA barcoding of red algae (Saunders 2005). To amplify the *cox*1 region, we designed a new primer, cox1e1R16F (5′-TGCCAAGACAGGTACTGCT-3′) having the binding position from 30738 to 30756 in *P. yezoensis* (NC_017837), located at the 5′ end of *cox*1 gene. The primer pair, cox1e1R16F (this study) and cox1e1Fpy1R (Hwang et al. 2013), was used for the amplification and sequencing of *cox*1 region in *P. yezoensis* samples. The amplification conditions were as follows: 3 min at 95°C, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were sequenced commerically (Genotech, Daejeon, Korea) and the sequences were assembled in Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Pairwise distances were calculated using the MEGA 6.0 program (Tamura et al. 2013).

RESULTS

PCR amplification of *cox*1 gene generated a 549 bp in all 40 samples, excluding the primer binding sites. Analysis of *cox*1 sequences revealed 4 haplotypes among the 27 Korean *P. yezoensis* strains (C1-C4) (Table 1). On the other hand, 13 strains of Japanese *P. yezoensis* shared the same haplotype (C5). The pairwise distance among the haplotypes ranged from 0.2 to 0.9% (1-5 bp).

We found four exons and three introns in the *rnl* in all of the sampled *P. yezoensis* strains, also reported in previous studies (Hwang et al. 2013, 2014); and five exons and four introns were revealed in the *cox*1 (Fig. 1). The results revealed five genotypes (R111, R110, R101, R011, and R010) in the *rnl* intron and six genotypes (C1101, C0111, C0101, C0100, C0001, and C0000) in the *cox*1 intron based on the existence of introns (Tables 1 & 3). Combined structural variations of *rnl* and *cox*1 exhibited 12 genotypes among the 40 *P. yezoensis* strains (Table 1). Korean *P. yezoensis* exhibited 12 genotypes (H1-H12) among 27 strains. The genotype 5 (H5) was dominant, and broadly distributed in nine Korean strains. All of the Japanese *P. yezoensis* strains had the same gene structure (H12) (Table 1).

DISCUSSION

The presence of introns and intronic ORFs is the main reason for variation in the size of the mitochondrial genome of *Pyropia* species. Hwang et al. (2013, 2014) was the first to report this intron structure from *P. tenera* and *P. yezoensis* in red algae. In this study, we examined the genetic features of intron structure of *rnl* and *cox*1 of *P. yezoensis* at the intraspecific level, and found 12 genetic types from 40 culture strains from Korea and Japan (Tables 1 & 3).

The sequences of mitochondrial *cox*1 region have been recommended as DNA barcode markers to identify animal and algal species, and the *cox*1 region exhibited high efficiency in describing species boundaries (Hebert et al. 2003, Saunders 2005). In this study, five haplotypes of the *cox*1 gene were revealed for 40 *P. yezoensis* strains (Table 1). Korean *P. yezoensis* exhibited 4 haplotypes

Table 3. Primer position and the predicted amplicon size

Gene region	PCR primer position[a]				Amplicon size (bp)	
	Primer	Binding site	Primer	Binding site	Intron (present)	Intron (absent)
rnl (e1)-(e2)	rnle1Fpy1419F	1,419-1,440	rnlRpp2	4,441-4,465	3,047	720
rnl (e2)-(e3)	rnlFpp3	4,260-4,282	rnlRpp1	4,583-4,605	2,842[b]	346
rnl (e3)-(e4)	rnlFpp2	4,589-4,611	rnle4F197R	7,174-7,198	2,610	120
*cox*1 (e1)-(e2)	cox1e1Fpy1R	31,306-31,329	cox1e2Rpy46F	30,658-30,677	3,258[c]	672
*cox*1 (e2)-(e3)	cox1e2Fpy68R	30,661-30,680	cox1e2Rpy1F	27,998-28,022	2,664	174
*cox*1 (e3)-(e4)	cox1e2Fpy1R	27,999-28,024	cox1e3Rpt25448F	25,448-25,471	2,577	261
*cox*1 (e4)-(e5)	cox1e3Fpy1R	25,536-25,558	cox1e5Rpy203F	22,773-22,795	2,786	425

PCR, polymerase chain reaction.

[a]The primer position and the predicted amplicon size was determined using the mitochondrial genome (mtDNA) of *Pyropia yezoensis* (NC_017837) as a reference.

[b]*Pyropia yezoensis* (KF561997, Hwang et al. 2014) was used as a reference, because *P. yezoensis* (NC_017837) does not have an intron between exon 2 (e2) and exon 3 (e3) of *rnl* gene (Hwang et al. 2014).

[c]*Pyropia tenera* (NC_021475) was used as a reference. *Pyropia yezoensis* (NC_01783, KF561997) does not have an intron between exon 1 (e1) and exon 2 (e2) of *cox*1 gene (Hwang et al. 2014).

(haplotypes C1-C4) for 27 strains with 0.2-0.9% pairwise genetic distances. All the 13 Japanese *P. yezoensis* strains had the same *cox*1 sequence (haplotype C5), and showed 0.2-0.7% pairwise distances from the Korean haplotypes.

Some of the *P. yezoensis* strains yielded the same *cox*1 sequence, but different introns (Table 1). The most variable haplotype C3 of *cox*1 was sub-divided into nine genotypes (H1, H4, H5, H6, H7, H8, H9, H10, and H12), using the combined *rnl* and *cox*1. The genotype C1 was subdivided into three genotypes (H1, H3, and H12). Therefore, the gene structure of the *rnl* and *cox*1 regions exhibited higher genetic resolution to discriminate *P. yezoensis* strains in comparison to the *cox*1 sequence variations in these samples.

Besides *cox*1 sequences, several genetic markers discriminating the *Pyropia* / *Porphyra* strains at the inter / intra-specific level, such as SSU ribosomal DNA (rDNA), ITS1, *rbc*L, and the RuBisCO spacer have been studied (Brodie et al. 1998, Lindstrom and Fredericq 2003, Hwang et al. 2005, Nelson et al. 2006, Niwa et al. 2009, Sutherland et al. 2011, Kucera and Saunders 2012, Mols-Mortensen et al. 2014, Guillemin et al. 2016). Nuclear SSU rDNA and *rbc*L sequences were shown to discriminate the Korean *P. yezoensis* strains from the Japanese *P. yezoensis* strains (Hwang et al. 2005). In this study, *cox*1 sequence analysis divided the 27 Korean *P. yezoensis* strains into four types; the presence / absence of introns in *rnl* and *cox*1 genes divided the 27 Korean *P. yezoensis* strains into 12 types; and the combination of both markers divided the Korean *P. yezoensis* strains into 14 types. These results implied that the combination of both markers was more useful in comparison to a single marker.

On the other hand, only one genotype was found in all the Japanese *P. yezoensis* strains, which was in contrast to the Korean strains. All Japanese *P. yezoensis* strains were aquaculture strains, except JP-Tu1. Niwa et al. (2008, 2009) reported the presence of three genotypes in 13 Japanese aquaculture strains of *P. yezoensis*; 11 strains had the same type, and each of the remaining two strains had a unique type based on ITS1 region analysis. The Japanese materials used in this study had only one genotype, which is the same type as that of *P. yezoensis* f. *narawaensis* reported by Niwa et al. (2008, 2009).

Theoretically, the presence / absence of introns can produce eight types in the *rnl* and 16 types in the *cox*1, and 128 types from the combination of *rnl* and *cox*1 of *P. yezoensis*. Additional genotypes of intron could be found across global samplings of *P. yezoensis* strains, including other *Pyropia* species in Korea, Japan, and China. In this study we found high intraspecific genetic variation in the *rnl* and *cox*1 genes, and showed that these two genes can better discriminate strains of *P. yezoensis*. These molecular markers can be useful to maintain the diversity of aquaculture strains and for the development of new cultivars.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. S1. Electrophoresis gel images of the genotypes of *rnl* and *cox*1 from *Pyropia yezoensis* strains. Two PCR markers were also loaded in the gel (GeneRuler 1 kb DNA ladder [left; ThermoFisher Scientific, USA] and GeneRuler 100 bp Plus DNA ladder [middle]) (http://www.e-algae.org).

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