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Article

DNA Barcoding and Intronic-ORF Structure Analyses of Cultivated *Pyropia yezoensis* in China: The Genetic Impact under Climate Change

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Abstract: *Pyropia yezoensis* is the most widely cultivated and economically important alga. Affected by climate change, the cultivation of *P. yezoensis* has gradually migrated to the northern coast of China, increasing from 6.8% in 2019 to 19.5% in 2023. To date, the genetic impact of northern migration on cultivation resources has not been assessed and analyzed extensively. Here, DNA barcoding (*rbc*L and *cox*1) and the presence/absence of intronic-ORFs in mitochondrial regions (*rnl* and *cox*1) were applied to investigate genetic diversity in 44 *P. yezoensis* specimens from 17 aquaculture farms in China, with comparisons to Korean and Japanese cultivated resources. The lower intraspecific variation was 0.31% for the *cox*1 gene and 0.14% for the *rbc*L gene, with three haplotypes, indicating that intensive selection and breeding during cultivation had narrowed the germplasm genetic variation. The intron structure of mitochondrial regions showed that the cultivated resources had 17 phenotypes, and the northern specimens shared 35.3% of genotypes with the southern specimens, indicating that the cultivated *P. yezoensis* is expanding its cultivation ranges through north migration. Even with lower genetic diversity, the northern area of cultivation had already developed 17.6% site-specific specimens. The genetic diversity of cultivated *P. yezoensis* from the Northwest Pacific is also discussed. Our work provides a preliminary framework for *P. yezoensis* breeding and cultivation under climate change.

Keywords: *Pyropia*; intraspecific variation; intronic-ORF; mitochondrial genome; DNA barcoding

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1. Introduction

The red algal genus *Pyropia (*formerly *Porphyra)* is a primitive photosynthetic eukaryote, which was classified in the new genus of Bangiales by Sutherland et al., in 2011 [1]. Although a recent study proposed a subdivision of *Pyropia* into six genera (*Calidia*, *Neoporphyra*, *Neopyropia*, *Uedaea*, *Porphyrella* and a redefined *Pyropia*) [2], the revision of *Pyropia* classification, with its potential complexity, remains ambiguous and requires more supporting genomic data [3,4]. *Pyropia* is an economically valuable red alga, which is commonly consumed as raw or processed food and is also a source of substances beneficial to human health [5]. *Pyropia yezoensis* is the most widely cultivated species in the aquaculture industry of the Northwest Pacific including Japan, Korea and China [6]. In China, the artificial cultivation of *P. yezoensis* was primarily developed on the coast of Nantong city (NT), Jiangsu Province (JS) and has become the main cultivation area of *P. yezoensis* since the 1970s. However, global warming in recent years has led to a continual increase in seawater temperature [7]. Affected by this, significant changes have taken place in the cultivation areas of *P. yezoensis*, and migration to the northern coast has gradually become a trend. Furthermore, other human factors, such as the prevention and control of green algae disasters and restrictions on sea area use, have also led *P. yezoensis* cultivation into a decline in JS and towards northern migration. Based on the data from Jiangsu Nori

Associate, the northern coast of JS in Lianyungang city (LYG) has replaced Nantong as the main cultivation area (>50%) since 2018 and cultivation has increased year by year, accounting for 76.6% in 2022. Meanwhile, the cultivation areas have been migrating to the more northern coast of China, including Shandong and Liaoning Provinces (SD and LN). And the proportions of cultivation area in the north (SD and LN) have increased rapidly in recent years, from 6.8% in 2019 to 19.5% in 2023 (Figure 1a). However, according to the trading price, the quality of northern cultivation resources is still lower than those from JS (Figure 1b). Therefore, in order to cope with changes in cultivation environments, more diverse *P. yezoensis* varieties or cultivars need to be developed [8]. Strain identification and genetic impact at the intraspecific level of *P. yezoensis* is important to the aquaculture industry for the development of new varieties or cultivars and for the maintenance of excellent strains under climate change [9]. Instead of relying on morphological features with phenotypic plasticity, molecular approaches are necessary to assess the diversity of *P. yezoensis* at intraspecific level.

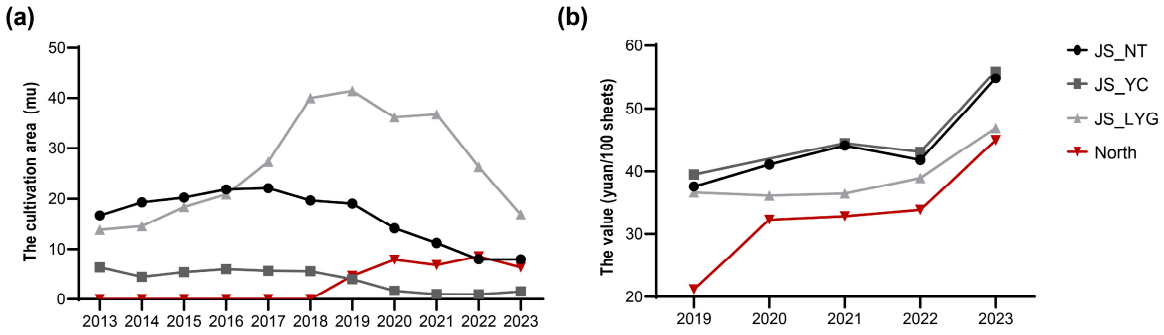

Figure 1. The cultivation area (**a**) and value (**b**) of *P. yezoensis* in China. The data were obtained from Jiangsu Nori Associate.

DNA barcoding is a rapid and efficient approach to studying the evolution, biogeography and systematics of red algae. The plastid RUBISCO large unit gene (*rbc*L) has been widely used to clarify the taxonomic position of *Pyropia* and to measure its intraspecific variation [10,11]. And the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) has been used as a supplementary marker to evaluate intra- and interspecific divergence and to assign *Pyropia* specimens [12–14]. The *cox*1 gene is an especailly reliable molecular marker for intraspecific study, revealing species relationships, population structure, and the hidden diversity of red algae [15]. With the development of genomics, red algal mitochondrial genomes (mtDNAs) can provide useful information for species identification. The mtDNAs of bladed Bangiales have shown diverse variation in their size and gene structures [16–18]. In particular, the presence or absence of introns and intronic open reading frames (intronic-ORFs) found in the ribosomal RNA large subunit gene (*rnl*) and *cox*1 significantly varied the mitochondrial genome size in *Pyropia* species, making it more effective in assessing genetic variation at the intraspecific level than using traditional DNA barcoding makers [9]. In *P. yezoensis*, the *rnl* and *cox*1 genes contained three and four introns with or without intronic-ORFs, separately [17]. Based on the present/absent intronic-ORFs structure of *rnl* and *cox*1, 12 genetic types were found in 40 cultivated strains of *P. yezoensis* from Korea and Japan, and only five haplotypes of the *cox*1 gene were revealed among these strains [9]. Therefore, the intronic-ORF structure of *rnl* and *cox*1 regions exhibited higher genetic resolution at the intraspecific level of *P. yezoensis*.

To respond and adapt to climate change, the species biodiversity, distribution, abundance, and population richness of seaweed resources will be affected [7]. With the north migration of *Pyropia* cultivation in China, the genetic diversity of current cultivation resources should be assessed and analyzed extensively to inform strategies for maintaining this economically important crop in the face of climate change. Here, 44 specimens of cultivated *P. yezoensis*, extensively collected from 17 different aquaculture farms in China,

were investigated using DNA barcoding (*rbc*L and *cox*1) and the presence/absence of intronic-ORF structure of the *rnl* and *cox*1 regions to elucidate genetic diversity at the intraspecific level and the genetic impact of climate change. Our work provides a preliminary framework for selecting and breeding suitable strains for *P. yezoensis* cultivation under climate change in the future.

2. Materials and Methods

2.1. The Collection and Culture of P. yezoensis

A total of 44 cultivated *P. yezoensis* specimens were collected from 17 aquaculture farms in Liaoning (LN), Shandong (SD) and Jiangsu (JS) provinces. These aquaculture farms were chosen to be in extensively different cities. The specimens from every farm were selected based on the different morphology of their blades, such as the length/width value, thickness or color. Three individuals with similar morphology from each specimen were chosen as three biological repeats. The letter of the specimen code referred to where it was collected from and the first number referred to the aquaculture farm, with farms arranged sequentially from the north to south. All specimens were preserved in the State Germplasm Bank for Porphyra at the Jiangsu Marine Fisheries Research Institute (Nantong, China). The blades of *P. yezoensis* were cultured in sterilized seawater. The growth conditions were 10 °C with a 12 h/12 h light/dark photoperiod under a light intensity of 100 μmol m^{-2} s^{-1}. A small tissue from the middle of the blades was used for genetic analysis.

2.2. The Amplification of DNA Barcoding

The genomic DNA of *P. yezoensis* was extracted using a Hi-DNAsecure Plant Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. The *rbc*L gene was amplified by using primers Kito-F1 (5′-ATGTCTCAATCCGTAGAATCA-3′) and JrSR (5′-AAGCCCCTTGTGTTAGTCTCAC-3′) [19]. And the amplification of the *cox*1 region was performed using the primer pairs GazF1 (5′-TCAACAAATCATAAAGATATTGG-3′) and GazR1 (5′-ACTTCTGGATGTCCAAAAAAYCA-3′) [20]. The PCR system was performed as previously described [21]. Amplification was under the following conditions: 3 min denaturation at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 45 °C for the *rbc*L gene and 56 °C for the *cox*1 region, 1.5 min extension at 72 °C for the *rbc*L gene and 30 s for the *cox*1 gene, and a final extension of 10 min at 72 °C. PCR products were sequenced by Sangon Biotech (Shanghai, China). The sequences of different phenotypes of *rbc*L and *cox*1 were deposited into the NCBI database (National Centre for Biotechnology Information).

2.3. The Cloning and Analyses of Intronic-ORF Structure

In the mitochondrial genome, *P. yezoensis* had four exons and three introns in the *rnl* gene, and five exons and four introns in the *cox*1 gene. The presence/absence of intronic-ORFs in the *rnl* and *cox*1 regions of *P. yezoensis* were amplified with three and four primer pairs, which were designed in the binding sites on each exon region [9]. The PCR was carried out in a 25 μL volume containing 50 ng of total genomic DNA using a LATaq polymerase system (TaKaRa Co. Ltd., Shiga, Japan). The amplification system and conditions were performed as described by Hwang et al., 2018 [9]. Band patterns of PCR products were analyzed using 1% agarose gel electrophoresis. Based on the amplicon size, the present and absent intronic-ORFs of the *rnl* and *cox*1 genes were marked with 1 and 0, separately. The intron structural variation was represented as a code RXXXCXXXX (R = *rnl*, C = *cox*1, X = 0/1, 0 = intronic-ORFs absent, 1 = intronic-ORFs present).

The phylogenies for intron structure were generated using the unweighted paired group method based on arithmetic averages (UPGMA). The unrooted UPGMA trees were reconstructed using MEGA 7.0 after alignment with ClustalX [22].

2.4. The Haplotype Diversity and Network Analysis

The *cox*1 sequences of cultivated *P. yezoensis* obtained from the present study were trimmed to 659 bp, and the sequences of 132 Chinese specimens, 27 Korean specimens and

13 Japanese specimens from Hwang et al. (2018) [9] were used for the haplotype analysis. The *rbc*L sequences of cultivated *P. yezoensis* were trimmed to1392 bp for the haplotype analysis, with 132 Chinese specimens, 8 Korean specimens and 7 Japanese specimens from GenBank. The sequence divergence of different haplotypes was analyzed using MEGA 7.0 with 1000 bootstrap replicates after alignment with ClustalX [22]. The diversity indices including the number of haplotypes (Nh), the number of parsimony informative sites (Np), haplotype diversity and nucleotide diversity were analyzed using DnaSP 6 [23]. The haplotype diversity of the intron structures of the *rnl* and *cox*1 genes was calculated using the Simpson function from the vagan package in R (version 4.2.3). To evaluate genetic relationships, haplotype networks were generated using POPART 1.7 with the TCS method [24]. The map of haplotypes and distribution of cultivated *P. yezoensis* was also generated using POPART 1.7 software.

3. Results

3.1. Genetic Diversity of cox1 Gene

The *cox*1 sequences of 44 specimens from *P. yezoensis* cultivated in China were obtained. Except for the S11 specimen, three repeats of the other 43 specimens all had the same *cox*1 sequences. The S11 specimen with three biological repeats had two different *cox*1 sequences, with 2 bp differences. Therefore, a total of 45 *cox*1 sequences were used to analyze the diversity indices (Table 1). The length of the *cox*1 sequenced region was 659 bp, and three parsimony informative sites were identified. According to the mitochondrial genome of *P. yezoensis* (KF561997) [17], the amplified sequence of *cox*1 in the present study was located in the first exon of the *cox*1 gene, in total, at 1158 bp. And the three parsimony informative sites occurred at positions 435, 603, and 702 of exon of the *cox*1 gene. According to the results of DnaSP 6, the 45 sequences generated three haplotypes (C1: $C^{435}C^{603}T^{702}$, C2: $C^{435}T^{603}C^{702}$, C3: $T^{435}C^{603}C^{702}$, GenBank accession numbers: OQ396573-OQ396575), with 0–3 bp intraspecific differences. Sequence divergence among the three haplotypes was from 0.20 to 0.31%. The amino acids of the three haplotypes were the same, indicating that the variable *cox*1 region differences of cultivated *P. yezoensis* were synonymous mutations (Table 2).

Table 1. Diversity indices of cultivated *Pyropia yezoensis* inferred from mitochondrial *cox*1 and plastid *rbc*L genes.

Location		Haplotype	Nh	Np	Haplotype Diversity	Nucleotide Diversity	References
*cox*1							
China	North (14)	C1/2/3	3	3	0.473	0.00144	This study
	JS (31)	C1/2/3	3	3	0.512	0.00156	
	All (45)	C1/2/3	3	3	0.569	0.00173	
Korea	27	C01/02/03/04	4	6	0.439	0.00165	Hwang et al., 2018 [9]
Japan	13	C2	-	-	-	-	
*rbc*L							
China	North (14)	R1/2/3	3	2	0.473	0.00042	This study
	JS (31)	R1/2/3	3	2	0.512	0.00040	
	All (45)	R1/2/3	3	2	0.569	0.00045	
Korea	8	R1/2/3	3	2	0.607	0.00049	GenBank
Japan	7	R2/3	2	1	0.476	0.00034	

Nh: Number of haplotypes. Np: Number of polymorphic sites. The *rbc*L sequences of Korean specimens were AB818919, DQ227860, DQ227861, DQ227862, DQ227863, DQ227864, DQ227865, DQ227866. The *rbc*L sequences of Japanese specimens were AB243204, AB243205, AB118587, AB118588, AB118589, AB118590, AB818917.

In cultivated *P. yezoensis* from China, haplotype C1 and C2 were shared among the LN, SD and JS specimens (Figure 2a). Haplotype C1 and C2 were found in the majority of the 45 specimens, accounting for 51.1% and 42.2% of all individuals. The haplotype C3 was

infrequently present in LN (n = 1) and JS (n = 2). The haplotype and nucleotide diversity indices showed that specimens from JS had higher genetic diversity than those from the north (SD and LN). In Korea, four haplotypes (C01-C04) were present in 27 cultivated *P. yezoensis* specimens. The C03 was the same as C3 from the present study, whereas other haplotypes were specific for Korean specimens (Table 2). A total of 13 specimens from Japan showed one haplotype, C2. According to the haplotype and nucleotide diversity indices, specimens from China had higher genetic diversity than those from Korea, followed by those from Japan (Table 1).

Table 2. The variation sites of *cox*1 and *rbc*L genes in different haplotypes of *P. yezoensis*.

Gene	Haplotypes	Variation Sites						
*cox*1	KF561997	TTT^{258}	CCT^{309}	AGT^{435}	GCT^{462}	ACG^{522}	TTC^{603}	GGC^{702}
China	C1			AGC				GGT
	C2			AGC			TTT	
	C3							
Korea	C01			AGC				
	C02	TTC	CCC	AGC		ACT		
	C03							
	C04			AGC	GCC		TTT	
	Amino acid *	Phe	Val	Ser	Ala	Thr	Phe	Gly
*rbc*L		GGT^{900}	TTC^{1335}					
	R1	GGC						
	R2							
	R3		TTT					
	Amino acid	Gly	Phe					

* Mitochondrial code was used with transl table = 4.

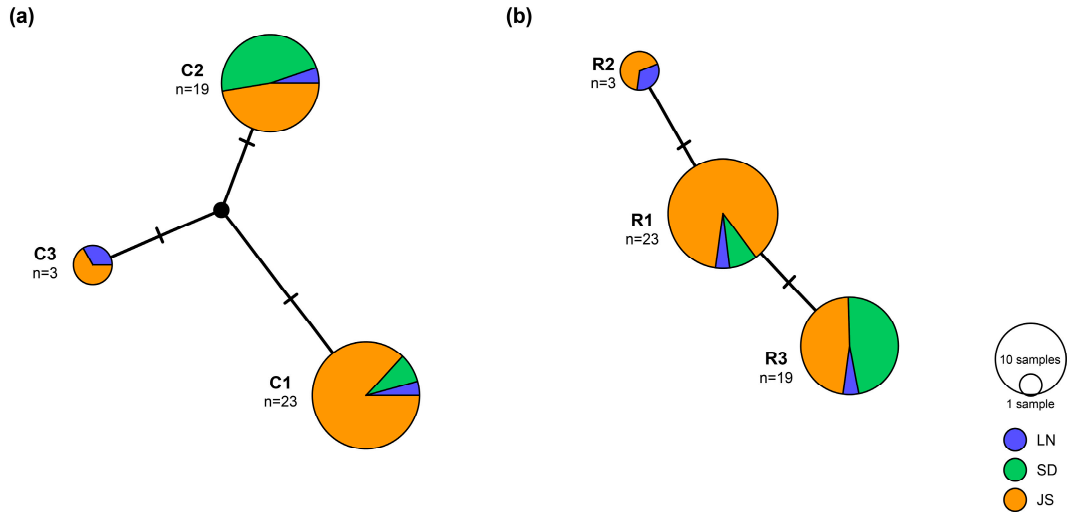

Figure 2. Haplotype network of cultivated *P. yezoensis* with *cox*1 gene (**a**) and *rbc*L gene (**b**).

3.2. Genetic Diversity of rbcL Gene

PCR amplification of the *rbc*L gene of cultivated *P. yezoensis* generated a full ORF with 1392 bp in all 44 specimens. Except for S11, the three repeats for the 43 specimens had the same *rbc*L sequence. The S11 specimen with three repeats had two different *rbc*L sequences, with 2 bp differences. In cultivated *P. yezoensis*, a total of 45 *rbc*L sequences from China were used to analyze the diversity indices (Table 1). When fully aligned, two parsimony informative sites of *rbc*L gene were identified. The parsimony informative sites occurred at positions 900 and 1335 of the *rbc*L gene. The 45 sequences could be organized into

three haplotypes (R1: $C^{900}C^{1335}$, R2: $T^{900}C^{1335}$, R3: $T^{900}T^{1335}$, GenBank accession numbers: OQ396570-OQ396572). Sequence divergence among haplotypes ranged from 0.07% to 0.14% (0–2 bp). And the amino acids of the three haplotypes were the same, indicating that the substitutions of *rbc*L gene in cultivated *P. yezoensis* were synonymous mutations (Table 2). In addition, the frequencies of haplotypes of the *rbc*L gene (R1, R2, R3) were in accordance with those of the *cox*1 gene (C1, C3, C2). And the incorporation of *cox*1 and *rbc*L genes presented three haplotypes R1C1, R2C3, R3C2 in the 44 specimens of cultivated *P. yezoensis* from China.

In cultivated *P. yezoensis* from China, haplotype R1 and R3 were shared among LN, SD and JS specimens (Figure 2b). The most abundant haplotypes, R1 and R3, accounted for 51.1% and 42.2% of all individuals. The haplotype R2 was infrequently present in LN (n = 1) and JS (n = 2). The haplotype and nucleotide diversity indices showed that specimens from JS had higher genetic diversity than the specimens from the north. The *rbc*L sequences of cultivated *P. yezoensis* analyzed in this study also included Korean and Japanese specimens. In cultivated *P. yezoensis* from Korea, three haplotypes (R1, R2, R3) were present in eight specimens. And seven specimens from Japan showed haplotypes R2 and R3. According to the haplotype and nucleotide diversity indices, specimens from Korea had a little greater genetic diversity than specimens from China, followed by those from Japan (Table 2).

3.3. The Intron Haplotype of Mitochondria

According to the presence/absence of the intronic-ORFs structure of the *rnl* and *cox*1 regions (Figure 3a), the cultivated *P. yezoensis* specimens showed four genotypes (R001, R011, R101, R111) in the *rnl* intron and nine genotypes (C0000, C0011, C0100, C0111, C1000, C1011, C1100, C1110, C1111) in the *cox*1 intron. Combined intron structural variations of *rnl* and *cox*1 exhibited 17 genotypes among 44 specimens, including H1–H17. Except S11 and S22, every specimen had unique intron structural variation. Two different structural variations of *rnl* and *cox*1 genes were shown in S11 (H3, H14) and S22 (H13, H17). Based on the presence and absence of intronic-ORF, the genotypes with close similarity matrices were clustered in groups with the UPGMA tree (Figure 3b).

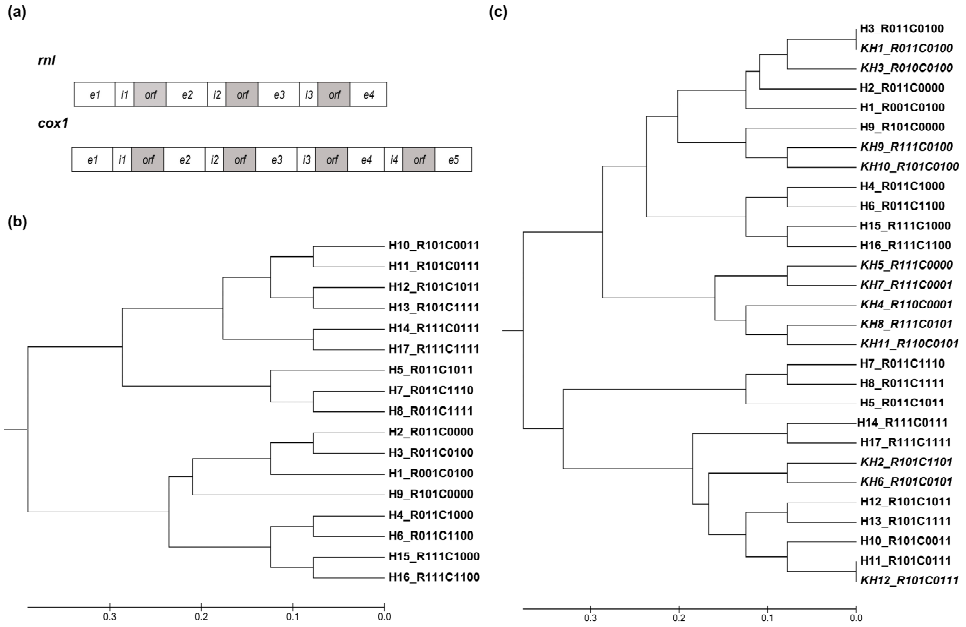

Figure 3. The UPGMA trees of cultivated *P. yezoensis* based on intronic-ORF structures. (**a**) The gene structure of exon (e), intron (i) and intronic-ORF (orf) in *rnl* and *cox*1 genes. (**b**) The UPGMA tree constructed with 17 haplotypes (H1-17) in the present study. (**c**) The UPGMA tree constructed with 17 haplotypes in the present study and 12 Korean and Japanese haplotypes (KH1-12).

In China, the three DL specimens exhibited three genotypes (H6, H13, H15). The SD specimens exhibited seven genotypes, among which three genotypes (H9, H10, H14) were site-specific, accounting for 17.6% of all genotypes. The diversity of JS specimens was the most, with fourteen genotypes; eight genotypes (H1, H2, H4, H5, H7, H8, H12, H16) were site-specific (47.1%), and six genotypes (H3, H6, H11, H13, H15, H17) were shared with the north specimens (35.3%) (Figure 4a). In JS, the genotype H6 was dominant (25.8%) and was broadly distributed in different aquaculture farms in Nantong City (Figure 5). In the 17 genotypes, the most abundant haplotype H13 was shared among DL, SD and JS, accounting for 10.9% of all individuals. The JS specimens shared H6 and H15 with DL and shared H3, H11 and H17 with SD (Figure 4a). Haplotype diversity indices showed that specimens from JS had high genetic diversity (0.884), followed by SD (0.833) and LN (0.667) (Table 3). The haplotype diversity of cultivated *P. yezoensis* from Korea and Japan were also analyzed, indicating that the genetic diversity of specimens from China (0.905) was slightly higher than those from Korea (0.842), followed by those from Japan (only 1 haplotype in 13 specimens). The UPGMA tree showed that China had two of the same haplotypes (H3 and H11) as the Korean and Japanese species (KH1 and KH12) (Figure 3c).

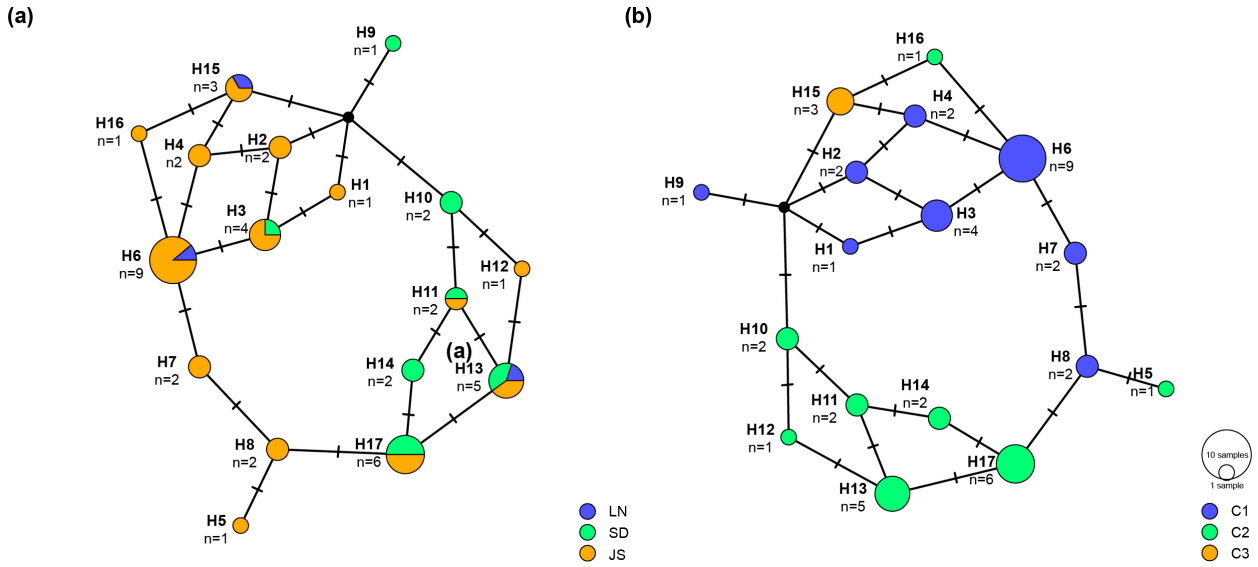

Figure 4. The haplotype network of cultivated *P. yezoensis* with intronic-ORF structures of *rnl* and *cox*1 genes. (**a**) The haplotype network constructed with 17 haplotypes based on intronic-ORF structures according to different cultivation locations (LN, SD and JS). (**b**) The haplotype network constructed with 17 haplotypes based on intronic-ORF structures according to different *cox*1 gene haplotypes (C1, C2 and C3).

Table 3. Diversity indices of cultivated *Pyropia yezoensis* inferred from the intron structures of *rnl* and *cox*1 regions.

Location		Haplotype	Nh	Site-Specific Nh	Haplotype Diversity	References
China	LN (3)	H6, 13, 15	3	0	0.667	This study
	SD (12)	H3, 9, 10, 11, 13, 14, 17	7	3	0.833	
	JS (31)	H1-8 11-13 15-17	14	8	0.884	
	All (46)	H1-17	17	-	0.905	
Korea	27	H1-12	12	-	0.842	Hwang et al., 2018 [9]
Japan	13	H12	1	-	-	

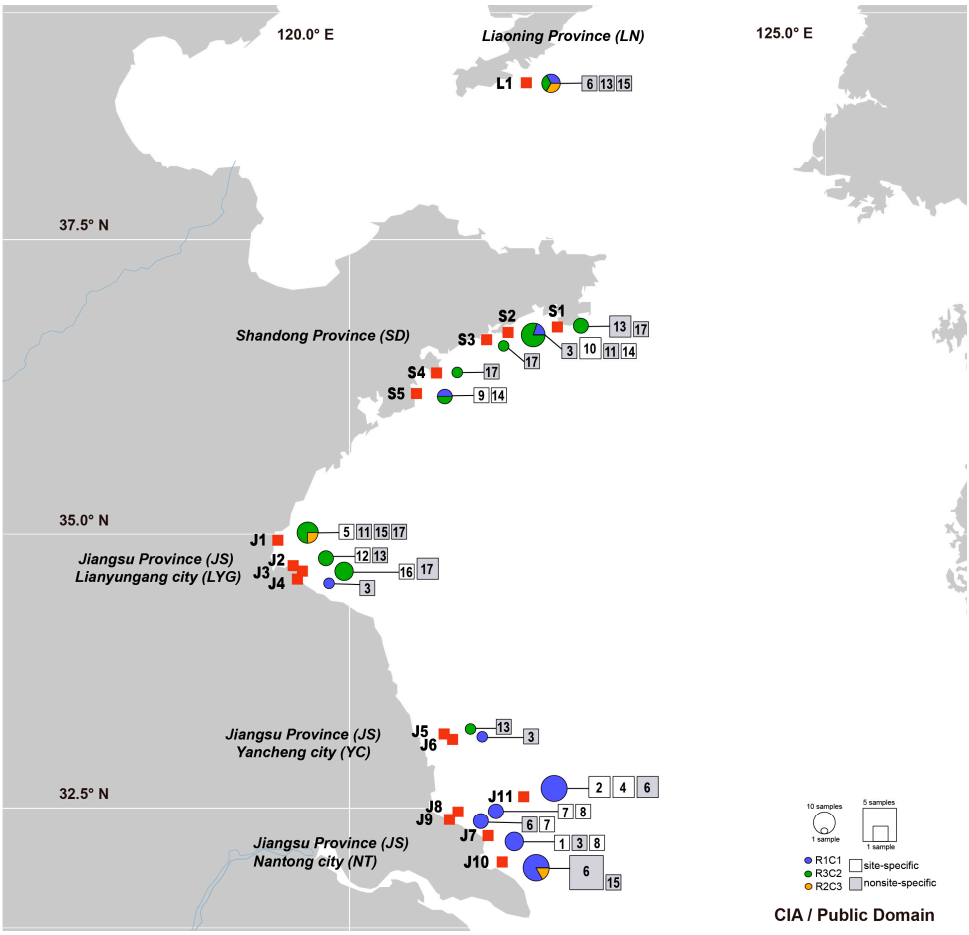

Figure 5. Cultivated *P. yezoensis* with different haplotypes in China. The red squares indicate the 17 aquaculture farms of *P. yezoensis*. The circles indicate three haplotypes based the DNA barcoding. The following squares indicate 17 haplotypes based on the intronic-ORF structure of *rnl* and *cox*1 regions.

The haplotype of the *cox*1 gene showed that the cultivated *P. yezoensis* from China exhibited three types (C1, C2 and C3). The results on intron structure showed that the same *cox*1 haplotype of *P. yezoensis* yielded different intron structures (Table 4). The C1 haplotype had six intron structures (C0000, C0100, C1000, C1100, C1110, C1111), C2 haplotype presented five intron structures (C0011, C0111, C1011, C1100, C1111), and C3 had only one intron structure (C1000). With the combined *rnl* and *cox*1 genes, the most variable haplotype C1 was sub-divided into eight genotypes (H1-4, H6-9), with 50% of all specimens. The haplotype of C2 was sub-divided into eight genotypes (H5, H10-14, H16-17), with 43.5% of specimens. And the haplotype C3 had only H15 genotype (n = 3) (Figure 4b).

Table 4. Details of the specimens analyzed in this study.

Code	Collection Location	Data	Barcode Haplotype	Intron Haplotype	
L11	Dalian, LN	31 December 2021	R3C2	R101C1111	H13
L12			R2C3	R111C1000	H15
L13			R1C1	R011C1100	H6
S11	Wendeng, SD	22 January 2022	R1C1	R011C0100	H3
			R3C2	R111C0111	H14
S12			R3C2	R101C0011	H10
S13			R3C2	R101C0011	H10
S14			R3C2	R101C0111	H11

Table 4. *Cont.*

Code	Collection Location	Data	Barcode Haplotype	Intron Haplotype	
S21	Rongcheng, SD	9 December 2020	R3C2	R101C1111	H13
S22			R3C2	R111C1111	H17
				R101C1111	H13
S3	Rushan, SD	8 December 2020	R3C2	R111C1111	H17
S4	Jimo, Qingdao, SD	10 December 2020	R3C2	R111C1111	H17
S51	Laoshan, Qingdao, SD	13 January 2021	R3C2	R111C0111	H14
S52			R1C1	R101C0000	H9
J11	Ganyu, Lianyungang, JS	7 December 2020	R3C2	R111C1111	H17
J12		16 November 2020	R3C2	R101C0111	H11
J13		16 November 2020	R3C2	R011C1011	H5
J14		16 November 2020	R2C3	R111C1000	H15
J21	Liandao, Lianyungang, JS	16 November 2020	R3C2	R101C1011	H12
J22		23 December 2021	R3C2	R101C1111	H13
J31	Gaogong Island, Lianyungang, JS	26 January 2021	R3C2	R111C1100	H16
J32		23 December 2021	R3C2	R111C1111	H17
J33		23 December 2021	R3C2	R111C1111	H17
J4	Haibing Avenue, Lianyungang, JS	18 March 2021	R1C1	R011C0100	H3
J5	Dafeng-1, Yancheng, JS	27 February 2019	R3C2	R101C1111	H13
J6	Dafeng-2, Yancheng, JS	8 March 2022	R1C1	R011C0100	H3
J71	Rudong-1, Nantong, JS	8 January 2022	R1C1	R011C0100	H3
J72			R1C1	R001C0100	H1
J73			R1C1	R011C1111	H8
J81	Rudong-2, Nantong, JS	20 February 2022	R1C1	R011C1110	H7
J82		20 January 2022	R1C1	R011C1111	H8
J91	Rudong-3, Nantong, JS	20 January 2022	R1C1	R011C1100	H6
J92		21 February 2022	R1C1	R011C1110	H7
J10-1	Haimen, Nantong, JS	10 March 2020	R2C3	R111C1000	H15
J10-2		16 January 2020	R1C1	R011C1100	H6
J10-3		16 January 2020	R1C1	R011C1100	H6
J10-4		16 January 2020	R1C1	R011C1100	H6
J10-5		19 March 2020	R1C1	R011C1100	H6
J10-6		27 February 2018	R1C1	R011C1100	H6
J11-1	Qidong, Nantong, JS	15 March 2021	R1C1	R011C1100	H6
J11-2			R1C1	R011C1000	H4
J11-3			R1C1	R011C1000	H4
J11-4			R1C1	R011C0000	H2
J11-5			R1C1	R011C1100	H6
J11-6			R1C1	R011C0000	H2

The barcode haplotype was represented as a combination of *rbc*L gene (R) and *cox*1 gene (C). The haplotypes of intron structural variation were represented as a code (R = *rnl*, C = *cox*1, 0 = intron absent, 1 = intron present).

4. Discussion

4.1. The Intraspecific Variation of Cultivated P. yezoensis with Gene Barcoding

Intraspecific studies of red algae have relied on DNA barcoding to address questions of systematics, biogeography or population genetics. Knowledge of the extent of intraspecific variation in *P. yezoensis* is necessary to rearrange these cultivation resources on the basis of genetic polymorphisms. In the present study, 44 specimens of cultivated *P. yezoensis* in China presented three haplotypes (R1C1, R2C3, R3C2) based on the *cox*1 and *rbc*L genes. The intraspecific variation of cultivated *P. yezoensis* was 0.31% for the *cox*1 gene and 0.14%

for the *rbc*L gene. In the genus *Pyropia*, the *cox*1 intraspecific nucleotide difference was 0–1.3%, and interspecific nucleotide differences ranged from 2.6 to 17.1% [12,20,25]. For the *rbc*L gene, the intraspecific divergence for *Pyropia* species was less than 1.0%, with a mean of 5.8% for interspecific divergence [19,26–28]. Our results for the *cox*1 and *rbc*L divergences of cultivated *P. yezoensis* in China were much lower than the intraspecific divergence of *Pyropia* from natural resources as previously reported [12,20,26,27]. The lower genetic variation of cultivated *Pyropia* was primarily due to seedling differences, and selection during cultivation had reduced the effective genetic size. Inbreeding and intensive selection during cultivation, which narrow the germplasm genetic base, reduce genetic diversity and promote adaptive divergence, have been reported in many plants and algae [29,30].

A comparison of *cox*1 with *rbc*L for *Pyropia* species revealed it was a more sensitive marker in revealing incipient speciation and cryptic diversity [25]. In the present study, analysis of the haplotype networks indicated that the haplotype number and diversity of cultivated *P. yezoensis* based on *cox*1 and *rbc*L genes were the same. However, higher nucleotide diversity was obtained from *cox*1 (0.00173) compared to the *rbc*L gene (0.00045). And the maximum intraspecific variation of *cox*1 (0.31%) in cultivated *P. yezoensis* was also higher than that of the *rbc*L gene (0.14%). The incorporation of the *rbc*L gene did not enhance the intraspecific genetic variation or the haplotype number. Hence, the *cox*1 marker was a more suitable marker for understanding the intraspecific variation and genetic diversity of cultivated *P. yezoensis.* Generally, the *cox*1 gene has revealed greater sequence divergence than the *rbc*L gene for identification of closely related red algal species, where the more conserved *rbc*L may be uninformative [31,32]. The evolution of the *cox*1 gene is sufficiently rapid to allow discrimination between closely related species and biogeographic subgroups within species of red algae [33]. However, relatively higher intraspecific variation of the *cox*1 gene may reduce accuracy for species identification [31,34]. In the present study, we recommend that *cox*1 is, overall, the best potential DNA barcode for cultivated *P. yezoensis*, whereas the combined mitochondrial-encoded *cox*1 and the plastid-encoded *rbc*L markers serve better as DNA barcodes encompassing the entire rhodophyte taxa [32,35].

4.2. The Intraspecific Variation of Cultivated P. yezoensis with Intronic-ORF Structure

In plants, mitochondrial genomes are highly dynamic, owing to gains and losses of repetitive noncoding DNA (intergenic spaces) and genetic elements (introns and transposable elements) throughout evolution, and they vary substantially among members of different genera or among species within the same genus [36]. In red algae, introns and intronic-ORFs are largely responsible for organellar genome expansion [37], leading to variation in the size of the mitochondrial genome of *Pyropia* species [17,38]. Based on the presence/absence of intronic-ORFs of *rnl* and *cox*1 genes, 27 cultivated Korean *P. yezoensis* strains exhibited 12 genetic types (H1-H12), and 13 Japanese strains showed 1 genotype (H12) [9]. In the present study, the intron structural variations of *rnl* and *cox*1 genes exhibited 17 genotypes among 44 specimens of cultivated *P. yezoensis* in China. And the same *cox*1 haplotype of *P. yezoensis* (C1-C3) yielded 1–6 different intron structures. Moreover, the haplotype diversity of the intron structure (0.905) was much more variable compared to DNA barcoding (0.569), which revealed higher variation for cultivated *P. yezoensis.* Therefore, the intron structure of the *rnl* and *cox*1 regions exhibited higher genetic resolution to discriminate *P. yezoensis* strains at the intraspecific level.

In the Bangiophycean species (*Pyropia*, *Bangia*, *Wildemania*), the *rnl* and *cox*1 introns both contain 1–3 copies, whereas they tend to be lost in most Florideophycean taxa, indicating that the *rnl* and *cox*1 introns may originate prior to the split of Bangiophyceae and Florideophyceae [16]. The mitochondrial *rnl* and *cox*1 genes of Bangiophyceae were extraordinarily similar to those found in the cyanobacteria and fungus, suggesting a recent lateral intron transfer from cyanobacteria or alpha-proteobacteria via mitochondrial primary endosymbiosis and targeted *rnl* and *cox*1 genes in red algae [39,40]. Because the intron did not reside in a vital gene, there would have been no selective pressure to conserve the

RNA secondary structure that is required for proper splicing [39]. Therefore, the evolution of intron variation is rapid enough to identify the intraspecies relationships in red algae.

4.3. The Genetic Impact of P. yezoensis under Climate Change in China

For species of red algae, intraspecific variation, which may arise through the interplay between environmental heterogeneity and adaptive variation, has consequences for the resilience of species to climate change [41]. Due to climate change, the abundance and composition of seaweed resources have changed over the past decades. Some species showed a significant extension in their distribution to the north, whereas some species decreased or even became extinct, mostly likely due to the increased seawater temperature [42]. For example, *P. tenera* has gradually disappeared from both natural habitats and aquaculture farms, which was previously one of the main cultivation species in Japan and Korea [43].

Climate-driven changes in habitat have an immediate effect on mariculture and the industry of seaweed. The future cultivation center of *Pyropia* was predicted to exhibit poleward and offshore shifts in the 2050s under climate change, especially sea surface temperature change [7]. Expansion of *Pyropia* farming northward and offshore is not simply a prediction, but a current cultivation practice in China. In the present study, the haplotype diversity of DNA barcoding and intronic-ORF structure of mtDNA genes both showed that the cultivated *P. yezoensis* in JS had higher genetic diversity than those in the north. However, based on the intronic-ORF structure, the northern specimens shared 35.3% of genotypes with JS specimens, indicating that the cultivated *P. yezoensis* were expanding their cultivation ranges along with north migration. Even with lower genetic diversity, the northern coast of cultivation had already developed 17.6% of site-specific specimens. Although we did not assess the direct impact of global climatic change on intraspecific genetic variation using ecological methods [44,45], the genetic diversity of cultivated *P. yezoensis* was affected by changing the cultivation location and seedlings. However, the seedlings for the northern coast were mainly prepared using a traditional selective breeding method by seedling producers, which partly led to instability in quantification in the northern cultivation of *P. yezoensis*. Based on the data from Jiangsu Nori Associate, the trading prices of northern *P. yezoensis* were 16.4% and 10.7% lower than those of the south in 2021 and 2022. With the migration to the north, different sea surface temperatures and solar light intensity conditions for *Pyropia* cultivation were present, leading to phenomena such as photoinhibition which usually occurs in SD [46]. However, there were only two certified new varieties of *P. yezoensis* in China (Sutong No.1 and No.2), which were more suitable to those cultivated in JS. So, more cultivars or varieties of *P. yezoensis* need to be developed to adapt to the cultivation environments of the northern coast.

4.4. The Genetic Diversity of P. yezoensis in Northwest Pacific

In the present study, the haplotype and nucleotide diversity index of the *cox*1 gene showed that the cultivated *P. yezoensis* in China had higher genetic diversity than in Korea and Japan. However, the haplotype and nucleotide diversity index of the *rbc*L gene had greater diversity in Korea, followed by China and Japan. Based on the intronic-ORF structure of the *rnl* and *cox*1 regions, the haplotype diversity of Chinese and Korean specimens had much higher genetic diversity than those from Japan, which had only one haplotype. These results indicate that cultivated *P. yezoensis* from China and Korea have more genetic diversity than those from Japan. The cultivation resources of *P. yezoensis* had the same haplotype as Korean specimens (H3), and also Japanese specimens (H11). The Korean strain (H3) was widely cultivated in SD and JS (LYG, YC, NT). And the Japanese strain (H11), *P. yezoensis* f. *narawaensis*, was also cultivated in SD and the north of JS (LYG). These results show that the cultivation resource in China is genetically diverse. In China, the fisherman bought conchocelis germplasm of *P. yezoensis* from different seedling producers, including some imported from Korea and Japan. Niwa et al. (2008, 2009) reported the presence of three genotypes in 13 Japanese aquaculture strains of *P. yezoensis*, 11 of which strains had one genotype in common with *P. yezoensis* f. *narawaensis* [10,47]. Moreover, the diversity of chloroplast and mitochondrial genomes of *P.*

yezoensis was lower in Japan than in China [48]. However, Japan had more registered cultivars of *P. yezoensis* than Korea and China. A total of 13 cultivars of *P. yezoensis* have been registered in Japan, and many cultivars are managed by seedling producers, which have already been in application [49]. Most of the Japanese cultivars were *P. yezoensis* f. *narawaensis*, which was selected in the 1960s and then subjected to selective breeding and provided large-sized, high-yielding cultivars for Japanese *Pyropia* aquaculture [49]. This explains why cultivated *P. yezoensis* in Japan had the lowest genetic diversity. Although the expansion of geographical sampling significantly increased intraspecific variation and genetic impact, the data from the present study offer a preliminary framework for future strain breeding of *Pyropia* in the Northwest Pacific.

However, the molecular markers for genetic diversity and genetic structure inferences still have limitations. Rapid progress in high-throughput sequencing technologies has provided an opportunity to infer genome-wide information, which provides thousands of genetic markers. Therefore, the detection of genome-wide markers needs to be analyzed, which can contribute to more targeted breeding efforts for *Pyropia* cultivation under climate change.

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