Fish Sci (2013) 79:865–875
DOI 10.1007/s12562-013-0661-2

ORIGINAL ARTICLE

Food Science and Technology

Origin identification of dried seaweed product "nori" by PCR–RFLP analysis of *Pyropia yezoensis* in the internal transcribed spacer ITS-1 region

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Received: 26 April 2013 / Accepted: 24 July 2013 / Published online: 23 August 2013

Abstract Nucleotide sequences in internal transcribed spacer (ITS)-1 region derived from dried nori products produced in Japan, China, and the Republic of Korea were compared. Thalli contained in the Japanese products were genetically homogenous, and their nucleotide sequences in ITS-1 were identical to those of the reference strains of *Pyropia yezoensis* f. *narawaensis*. In Chinese products, the thalli were related to *P. yezoensis* strain Minomiasakusa. In contrast, the thalli in the Korean products were genetically heterogeneous, and several different *P. yezoensis* strains and other *Pyropia* spp. were used for dried nori products. In some thalli produced in both China and Korea, the DNA sequences of the ITS-1 region were identical with that of Japan, suggesting that the cultivar strains might have been transplanted from Japan to China in recent years. The 432-bp-long nucleotide sequences in the ITS-1 region of thalli derived from Japanese origin were cleaved to two restriction fragments at 154 and 278 bp by cleavage of PCR-amplified products using *Msp*I. Conversely, almost all of the corresponding sequences derived from China and Korea were lacking *Msp*I or other restriction patterns, except for nori products from some areas that cultivate a closely related strain to the Japanese cultivar.

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Keywords Dried seaweed · Nori · *Pyropia yezoensis* · ITS-1 region · RFLP analysis · Origin identification

Introduction

"Nori" (edible seaweed, *Pyropia yezoensis*) is one of the most important aquaculture species in Japan [1]. Nori is cultivated on the surface of the sea; the thalli are cut, dried, pressed in a sheet, and then distributed as dried products [1]. Seaweed farms are distributed along the coastal area from southern to northern Japan [1]. Nori is also farmed in China and the Republic of Korea with the help of technological know-how and the transplantation of some strains of *P. yezoensis* from Japan [1]. *P. yezoensis* f. *narawaensis*, which has been established by selective breeding from cultivated populations, now dominates farms in Japan [2, 3].

Labeling is necessary for fresh marine products and processed foods to assist consumers in selecting foods according to the Law on Standardization and Proper Labeling of Agricultural and Forestry Products [Japan Agricultural Standard (JAS) Law]. For imported marine products, country-of-origin food labeling is required in the Japanese market. In addition to the import of nori products from the Republic of Korea, an import quota on nori products from China has been in place since 2005, and dried nori products are now imported from both countries. Therefore, biochemical and genetic techniques for origin identification are needed for dried nori products to certify whether country-of-origin food labeling is correct.

Molecular identification techniques of *Pyropia* spp. and *P. yezoensis* strains have been developed previously. DNA fingerprinting and PCR-based restriction fragment length polymorphism (RFLP) analysis have been conducted using

small subunit ribosomal RNA [4], internal transcribed spacer region (ITS) [2, 5–8], plastid ribulose bisphosphate carboxylase/oxygenase gene (RuBisCo) [6–8], and actin-related protein 4 gene [9]. Park et al. [10] reported genetic polymorphism within wild-collected *P. yezoensis* from Japan and Korea. In 2011, the red algal order Bangiales has been revised based on combined analyses of the nuclear SSU rRNA gene and the RuBisCo L gene [11]. Mitochondrial DNA was also used for the discrimination of 18 Japanese *Porphyra* and *Pyropia* species [12]. From these previous studies, Kunimoto et al. [2] and Niwa et al. [8] reported diverse nucleotide sequences in the ITS region among *P. yezoensis* wild-collected and culture strains. Therefore, to develop origin identification techniques for dried nori products, nucleotide sequences in the ITS region are required to determine and compare the thalli DNA extracted from dried nori products produced in Japan, Korea, and China. In *Pyropia* aquaculture, a mixed culture of several distinct cultivated strains is used for seed production to promote enhanced adaptation to different environmental situations. In addition, natural conchospores may be contaminated by cultivation nets in the sea. Therefore, a possibility existed that distinct DNA sequences derived from several cultured and wild strains might be detected in a single sheet of dried nori product. Thus, we isolated each thallus piece from dried nori products, and carefully determined its nucleotide sequence in the ITS region.

This study focused on differences between the nucleotide sequences in the ITS-1 region from the thalli in dried nori products produced in Japan, Korea, and China. According to the nucleotide differences between Japanese and the other two countries, we identified marker nucleotide sequences specific to dried nori products produced in Japan, Korea or China. In addition, we developed PCR–RFLP analysis methods for discriminating between the nori products produced in Japan, and those imported from Korea or China.

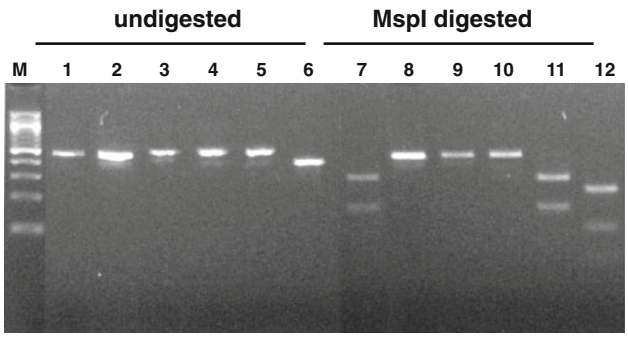

Fig. 1 Comparison of agarose gel electrophoresis patterns for polymerase chain reaction (PCR) products amplified with species-specific primers for the DNA sequence of the internal transcribed spacer (ITS)-1 region of *Pyropia yezoensis* and *Pyropia* spp. in dried nori products (*lanes 1–6*) and the subsequent restriction fragment length polymorphism analysis (*lanes 7–12*). *Lane M* 100-bp DNA ladder marker; *lanes 1* and *7 Pyropia yezoensis* Japan_Sub01; *lanes 2* and *8 Pyropia yezoensis* Japan_Sub02; *lanes 3* and *9 Pyropia yezoensis* China_Sub01; *lanes 4* and *10*: *Pyropia yezoensis* Korea_SubA01; *lanes 5* and *11 Pyropia yezoensis* Korea_SubB01; *lanes 6* and *12 Pyropia* sp. NRIFS-1. Note: *Pyropia yezoensis* Japan_Sub01 (154 and 278 bp), *Pyropia yezoensis* Korea_SubB01 (152 and 274 bp), and *Pyropia* sp. NRIFS-1 (96 and 252 bp) have two restriction fragments by cleavage of PCR-amplified products by *Msp*I

Fig. 2 Alignment of nucleotide sequences in the internal transcribed spacer (ITS)-1 region of *Pyropia yezoensis* thalli in dried nori products produced from Japan, China, and the Republic of Korea. The numbers on the *right* are the position of the nucleotides. Identical nucleotides to those of Japan_Sub-01 are indicated by *dots*. Gaps introduced to maximize the alignment are displayed by *dashes*. The *box* represents an *Msp*I site. The partial nucleotide sequence in the ITS-1 region of the Japan_Sub01 genotype identical to that of *P. yezoensis* f. *narawaensis* [5, 8] is underlined, and used for the phylogenetic analysis in Fig. 3. The nucleotide sequences determined in this study were deposited in the DDBJ/EMBL/GenBank DNA Database (Japan_Sub01: AB818905, Japan_Sub02: AB818906, China_Sub01: AB818907, China_Sub02: AB818908, Korea_SubA01: AB818909, Korea_SubA02: AB818910, Korea_SubA03: AB818911, Korea_SubA04: AB818912, Korea_SubB01: AB818913, Korea_SubB02: AB818914, Korea_SubB03: AB818915) ▶

Materials and methods

Materials

Dried nori products were obtained from Japan, China, and Korea between 2006 and 2008. Nori samples were stored with desiccant at −20 °C until use.

DNA extraction, PCR amplification, and sequencing

Nori sheets were cut into small pieces, and individual pieces were swollen and dissociated in distilled water. Each piece of thalli was put into a 1.5-ml tube and then stored at −20 °C until use. From 2 to 16 pieces were sampled from each nori sheet. One piece of thallus was thawed and ground with a mortar and pestle. Total DNA was extracted from the ground sample using the Illustra DNA Extraction Kit Phytopure (GE Healthcare, Wauwatosa, WI, USA) or DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The nuclear ITS-1 region was amplified using Premix Taq (*Ex*Taq Version: Takara Bio, Ohtsu, Japan) and 0.2 μM of the forward primer (CCGTA GGTGAACCTGCGGAAGGATCAT) and the reverse primer (CAAGATATCCACCGCTAAGAGTTGTAT). The PCR program was for 120 s at 96 °C, followed by 30–40 cycles of 15 s at 96 °C, 15 s at 55 °C, and 15 s at 72 °C, and finally 180 s at 72 °C. The products were treated with ExoSAP-IT (GE Healthcare) and then sequenced. The plastid RuBisCo and its spacer region were amplified with Premix Taq using 0.2 μM of the forward primer

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Japan_Sub01    TCACAAACTATTGACACAAACACACGCGAACCAAATCGTCCGCACAGGTGGTGGCAA-TG   59
Japan_Sub02    ..............................................CA........-..   59
China_Sub01    ..............................................CA........-..   59
China_Sub02    ..............................................CA........-..   59
Korea_SubA01   ..............................................CA........-..   59
Korea_SubA02   ..............................................CA........-..   59
Korea_SubA03   ..............................................CA........-..   59
Korea_SubA04   ..............................................CA........A..   60
Korea_SubB01   ..............................................CA........-..   59
Korea_SubB02   ..............................................CA........-..   59
Korea_SubB03   ..............................................CA........-..   59

Japan_Sub01    AAAGAGAGAATCTGCATGTCGCCTTTCGGGGTATAGCAAGCAGCACTCTTTTGCCATCGC  119
Japan_Sub02    .........C.....................--..............C.........  117
China_Sub01    .........C.....................--..............C.........  117
China_Sub02    .........C.....................--..............C.........  117
Korea_SubA01   ............T..................--...T..........C.........  117
Korea_SubA02   .........C.....................--..............C.........  117
Korea_SubA03   ..............................--..............C.........  117
Korea_SubA04   .........C.....................--..............C.........  118
Korea_SubB01   .........C.....................--..............C.........  117
Korea_SubB02   ..............................--..............C.A.......  117
Korea_SubB03   ..............................--..............C.A.......  117

Japan_Sub01    CTCTGTGCCGGGCGTAAATTCTCATTGAGAGGATGTGAGGGCACCACAGGAAGCTTTTCC  179
Japan_Sub02    ........A.........C.....................................  177
China_Sub01    ........A.........C.....................................  177
China_Sub02    ........A.........C.....................................  177
Korea_SubA01   ........A.........C.....................................  177
Korea_SubA02   ........A.........C.....................................  177
Korea_SubA03   ........A.........C.....................................  177
Korea_SubA04   ........A.........C.....................................  178
Korea_SubB01   .................C.....................................  177
Korea_SubB02   .................C..................A..................  177
Korea_SubB03   .................C.....................................  177

Japan_Sub01    ACAGGAAGTCGCCATCCTTCTCCCTCCACGGCGGCGCTTCTGTGCTTGGCAGTTTTTTTT  239
Japan_Sub02    ............................................................  237
China_Sub01    ............................................................  237
China_Sub02    ............................................................  237
Korea_SubA01   ..........T...................................................  237
Korea_SubA02   ..........T...................................................  237
Korea_SubA03   ..........T...................................................  237
Korea_SubA04   ............................................................  238
Korea_SubB01   ..........T...................................................  237
Korea_SubB02   ..........T...................................................  237
Korea_SubB03   ..........T...................................................  237

Japan_Sub01    TTTGCCTTCCAGGGAGGATGCCGCCAATGGAGCCCCATATAATATATACATCATCATATG  299
Japan_Sub02    .--......T.........................T....-.....................  294
China_Sub01    ---......T.........................T....-.....................  293
China_Sub02    ---......T.........................T....-.....................  293
Korea_SubA01   ---......T..........................-.....................  293
Korea_SubA02   ---......T..........................-.....................  293
Korea_SubA03   ---......T..........................-.....................  293
Korea_SubA04   ---......T.........................T....-.....................  294
Korea_SubB01   ---......T..........................-.....................  293
Korea_SubB02   ---......T..........................-.....................  293
Korea_SubB03   ---......T..........................-.....................  293

Japan_Sub01    CCCCTTTTTTTCTTAACCGCTTGCCAAAGCTTCTTCTATGAGGAGCTTGTGGGAAGACTG  359
Japan_Sub02    ............................................................  354
China_Sub01    ............................................................  353
China_Sub02    ............................................................  353
Korea_SubA01   ............................................................  353
Korea_SubA02   ............................................................  353
Korea_SubA03   ............................................................  353
Korea_SubA04   ............................................................  354
Korea_SubB01   ............................................................  353
Korea_SubB02   ............................................................  353
Korea_SubB03   ............................................................  353

Japan_Sub01    TCTCCATACAATAACAAA-G  378
Japan_Sub02    ..................-.  373
China_Sub01    ..................-.  372
China_Sub02    ..................A.  373
Korea_SubA01   ..................-.  372
Korea_SubA02   ..................-.  372
Korea_SubA03   ..................-.  372
Korea_SubA04   ..................-.  373
Korea_SubB01   ..................-.  372
Korea_SubB02   ..................-.  372
Korea_SubB03   ..................-.  372
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Table 1 Genotypes of the ITS-1 region in individual pieces of thalli in nori produced in Japan

Product ID	Producing area	Genotype												Total no.
		Japan_ Sub01	Japan_ Sub02	China_ Sub01&02	Korea_ SubA01	Korea_ SubA02	Korea_ SubA03	Korea_ SubA04	Korea_ SubB01	Korea_ SubB02	Korea_ SubB03	*Pyropia* sp. NRIFS-1	*Pyropia* sp. NRIFS-2	
06 J-001	Aichi	8	0	0	0	0	0	0	0	0	0	0	0	8
06 J-002	Mie	5	0	0	0	0	0	0	0	0	0	0	0	5
06 J-004	Kagawa	5	0	0	0	0	0	0	0	0	0	0	0	5
06 J-007	Kagawa	8	1	0	0	0	0	0	0	0	0	0	0	9
06 J-008	Saga	6	0	0	0	0	0	0	0	0	0	0	0	6
06 J-011	Saga	7	0	0	0	0	0	0	0	0	0	0	0	7
06 J-012	Saga	8	0	0	0	0	0	0	0	0	0	0	0	8
06 J-015	Hyougo	6	0	0	0	0	0	0	0	0	0	0	0	6
06 J-016	Hyougo	9	0	0	0	0	0	0	0	0	0	0	0	9
06 J-020	Hyougo	3	1	0	0	0	0	0	0	0	0	0	0	4
06 J-022	Chiba	8	0	0	0	0	0	0	0	0	0	0	0	8
06 J-026	Chiba	7	0	0	0	0	0	0	0	0	0	0	0	7
06 J-031	Chiba	1	0	0	0	0	0	0	0	0	0	0	0	1
06 J-032	Miyagi	3	0	0	0	0	0	0	0	0	0	0	0	3
06 J-033	Miyagi	3	0	0	0	0	0	0	0	0	0	0	0	3
06 J-034	Miyagi	1	0	0	0	0	0	0	0	0	0	0	0	1
08 J-012	Miyagi	2	0	0	0	0	0	0	0	0	0	0	0	2
Total no.		90	2	0	0	0	0	0	0	0	0	0	0	92

Table 2 Genotypes of the ITS-1 region in individual pieces of thalli in nori products produced in China

Product ID	Producing area	Genotype												Total no.
		Japan_ Sub01	Japan_ Sub02	China_ Sub01&02	Korea_ SubA01	Korea_ SubA02	Korea_ SubA03	Korea_ SubA04	Korea_ SubB01	Korea_ SubB02	Korea_ SubB03	*Pyropia* sp. NRIFS-1	*Pyropia* sp. NRIFS-2	
06C-001	Lianyungang	0	0	5	0	0	0	0	0	0	0	0	0	5
06C-002	Nantong	0	0	8	0	0	0	0	0	0	0	0	0	8
06C-004	Nantong	0	0	4	0	0	0	0	0	0	0	0	0	4
06C-007	Yancheng	0	0	5	0	0	0	0	0	0	0	0	0	5
06C-008	Nantong	0	0	8	0	0	0	0	0	0	0	0	0	8
06C-009	Nantong	0	0	6	0	0	0	0	0	0	0	0	0	6
06C-010	Nantong	0	0	8	0	0	0	0	0	0	0	0	0	8
06C-011	Lianyungang	0	0	7	0	0	0	0	0	0	0	0	0	7
06C-013	Nantong	0	0	5	0	0	0	0	0	0	0	0	0	5
06C-015	Nantong	0	0	4	0	0	0	0	0	0	0	0	0	4
07C-001	–	0	0	1	0	0	0	0	0	0	0	0	0	1
07C-002	–	0	0	8	0	0	0	0	0	0	0	0	0	8
07C-003	–	0	0	8	0	0	0	0	0	0	0	0	0	8
08C-001	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-002	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-003	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-004	–	0	1	2	0	0	0	0	0	0	0	0	0	3
08C-005	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-006	–	3	0	0	0	0	0	0	0	0	0	0	0	3
08C-007	–	3	0	0	0	0	0	0	0	0	0	0	0	3
08C-008	–	1	0	2	0	0	0	0	0	0	0	0	0	3
08C-009	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-010	–	0	1	2	0	0	0	0	0	0	0	0	0	3
08C-011	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-012	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-013	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-014	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-015	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-016	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-017	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-018	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-019	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-020	–	0	1	2	0	0	0	0	0	0	0	0	0	3
08C-021	–	0	1	2	0	0	0	0	0	0	0	0	0	3

Table 2 continued

Product ID	Producing area	Genotype												Total no.
		Japan_ Sub01	Japan_ Sub02	China_ Sub01&02	Korea_ SubA01	Korea_ SubA02	Korea_ SubA03	Korea_ SubA04	Korea_ SubB01	Korea_ SubB02	Korea_ SubB03	*Pyropia* sp. NRIFS-1	*Pyropia* sp. NRIFS-2	
08C-022	–	0	0	3	0	0	0	0	0	0	0	0	0	3
08C-023	–	0	1	2	0	0	0	0	0	0	0	0	0	3
08C-024	–	0	1	2	0	0	0	0	0	0	0	0	0	3
08C-025	QingDao	4	0	0	0	0	0	0	0	0	0	0	0	4
08C-026	QingDao	4	0	0	0	0	0	0	0	0	0	0	0	4
09C-001	–	0	1	2	0	0	0	0	0	0	0	0	0	3
09C-002	–	0	0	3	0	0	0	0	0	0	0	0	0	3
09C-003	–	0	1	2	0	0	0	0	0	0	0	0	0	3
09C-004	–	0	0	3	0	0	0	0	0	0	0	0	0	3
09C-005	–	0	0	3	0	0	0	0	0	0	0	0	0	3
09C-006	–	0	1	2	0	0	0	0	0	0	0	0	0	3
Total no.		15	9	151	0	0	0	0	0	0	0	0	0	175

Dashes represent nori products with unknown producing areas

(ATGTCTCAATCCGTAGAATCACG) and the reverse primer (TTAATATCTAGCTCCTTCAGGC) based on the small subunit ribosomal RNA gene and 5.8S ribosomal RNA gene, which are both highly conserved among *Pyropia* species. The PCR program was for 120 s at 96 °C, followed by 30–40 cycles of 15 s at 96 °C, 15 s at 55 °C, and 60 s at 72 °C, and finally 180 s at 72 °C. The products were treated with ExoSAP-IT and then sequenced. The products were sequenced using an ABI PRISM 3100 Genetic Analyzer or ABI PRISM 3130xl Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA, USA) with ABI PRISM BigDye Terminator v3.1. The nucleotide sequences in the ITS-1 region and the RubisCo gene in various *Pyropia* species were aligned using the CLUSTALW program [13] on the web site of the DNA Database Bank of Japan, and a phylogenetic tree was designed using the Treeview ppc version 1.6.6 program [14]. The nucleotide sequences determined in this study were deposited in the DDBJ/EMBL/GenBank DNA Database (accession numbers AB818905–AB818921).

Restriction fragment length polymorphism

Amplified PCR products of the ITS-1 region were digested with *Msp*I restriction enzyme (New England Biolabs Japan, Tokyo, Japan). A reaction mixture of 10 µl containing from 2 to 3 µl of PCR product, 5 units of *Msp*I, 1 µl of restriction enzyme buffer, and distilled water was incubated at 37 °C for at least 3 h. The reaction mixture was separated in a 3 % agarose gel containing ethidium bromide and photographed under ultraviolet light.

Results

Comparison of nucleotide sequences in the ITS-1 region of the dried nori products

Total DNA was extracted from the dried nori products produced in Japan, China, and Korea, and PCR amplification products were of similar size, approximately 430 bp long, except for some pieces from Korea (Fig. 1, lanes 1–6). The nucleotide sequences of the partial PCR fragment in the approximately 430-bp-long ITS-1 region were determined to identify DNA polymorphism in *Pyropia* species and *P. yezoensis* strains contained in the nori products (Fig. 2).

Almost all of the 343-bp-long partial nucleotide sequences in the ITS-1 region of 90 pieces of thalli derived from Japanese origin in the present study were identical to those of *P. yezoensis* f. *narawaensis*. This genotype was tentatively named "Japan_Sub01" in Table 1. In this sequence, we found an *Msp*I gene site at 126 bp as shown in Fig. 2, and two restriction fragments at 154 and 278 bp by

Table 3 Genotypes of the ITS-1 region in individual pieces of thalli in nori products produced in Korea

Product ID	Producing area	Genotype												Total no.
		Japan_ Sub1	Japan_ Sub2	China_ Sub01&02	Korea_ SubA1	Korea_ SubA2	Korea_ SubA3	Korea_ SubA4	Korea_ SubB1	Korea_ SubB2	Korea_ SubB3	*Pyropia* sp. NRIFS-1	*Pyropia* sp. NRIFS-2	
07 K-008	Pusan	8	0	0	0	0	0	0	0	0	0	0	0	8
07 K-010	Namdo	0	0	0	2	1	0	0	5	0	0	0	0	8
07 K-011	Sinan	0	0	0	0	0	0	0	2	0	0	6	0	8
07 K-012	Sinan	0	0	0	0	0	0	0	1	0	0	7	0	8
07 K-013	–	0	0	0	0	0	3	0	0	1	0	4	0	8
07 K-014	Pusan	8	0	0	0	0	0	0	0	0	0	0	0	8
07 K-015	Namhe	0	0	0	4	0	0	0	4	0	0	0	0	8
07 K-016	Gimhae	8	0	0	0	0	0	0	0	0	0	0	0	8
07 K-017	Nohwa	0	0	0	5	0	0	0	2	1	0	0	0	8
07 K-018	Chanfun	1	0	0	2	0	2	0	2	0	1	0	0	8
07 K-019	Gimhae	8	0	0	0	0	0	0	0	0	0	0	0	8
07 K-020	Sinan	0	0	0	0	0	1	0	4	1	0	2	0	8
07 K-021	Namdo	0	0	0	4	0	1	0	2	1	0	0	0	8
07 K-022	Haenam	2	0	0	2	0	0	0	2	1	0	1	0	8
07 K-023	Namdo	0	0	0	5	0	0	0	2	1	0	0	0	8
07 K-024	Chanfun	0	0	0	4	0	1	0	1	0	2	0	0	8
07 K-025	–	0	0	0	0	0	1	0	0	0	0	7	0	8
07 K-026	Namdo	0	0	0	5	0	2	0	1	0	0	0	0	8
07 K-027	Sinan	0	0	0	0	0	0	0	0	0	0	7	0	7
07 K-028	Chanfun	0	0	0	0	0	1	0	1	0	0	5	0	7
07 K-029	Pusan	8	0	0	0	0	0	0	0	0	0	0	0	8
07 K-030	Chanfun	0	0	0	6	0	1	0	0	0	0	0	1	8
07 K-031	Kofun	0	0	0	3	0	1	0	2	1	0	0	1	8
07 K-032	Gimhae	8	0	0	0	0	0	0	0	0	0	0	0	8
08 K-001	Sinan	0	0	0	1	0	0	0	0	0	0	6	0	7
08 K-002	Namdo	0	0	0	6	0	1	0	1	0	0	0	0	8
08 K-003	Pusan	8	0	0	0	0	0	0	0	0	0	0	0	8
08 K-004	Sinan	0	0	0	2	0	1	0	1	0	0	4	0	8
08 K-005	Kofun	1	0	1	0	0	2	1	1	0	0	1	0	7
08 K-006	Gimhae	8	0	0	0	0	0	0	0	0	0	0	0	8
08 K-008	Namdo	0	0	0	2	0	1	0	5	0	0	0	0	8
08 K-010	Mokpo	0	0	0	2	0	1	0	0	0	0	0	0	3
08 K-011	Pusan	0	0	0	3	0	0	0	2	2	1	0	0	8
08 K-012	Pusan	8	0	0	0	0	0	0	0	0	0	0	0	8
Total no.		76	0	1	58	1	20	1	41	9	4	50	2	263

Dashes represent the nori products with unknown producing areas

cleavage of PCR-amplified products using *Msp*I restriction enzymes. Therefore, we found close relationships between most of the cultured nori strains, except for two samples that were considered to be a wild strain ("Japan_Sub02"). The Japan_Sub02 genotype was detected in only one piece of thallus, as compared with 15 pieces having the genotype Japan_Sub01.

The dried nori products produced in China had two specific genotypes denoted "China_Sub01" and "China_-Sub02" marked by a single nucleotide deletion of the nucleotide sequence of the partial ITS-1 region of the *P. yezoensis* strain Minomiasakusa [2]. The China_Sub02 genotype had a single nucleotide insertion in the region of the 5.8S rRNA gene in the 426-bp long partial ITS-1 sequence in the China_Sub01 genotype. Among 160 pieces of thalli samples prepared from 54 dried nori products in China, 151 pieces had these Chinese-specific genotypes (Table 2). The remaining nine pieces had nucleotide sequences that were identical to Japan_Sub02.

The ITS-1 region was determined in 263 pieces of thalli samples prepared from 34 dried nori products produced in Korea. These 263 nucleotide sequences were classified into

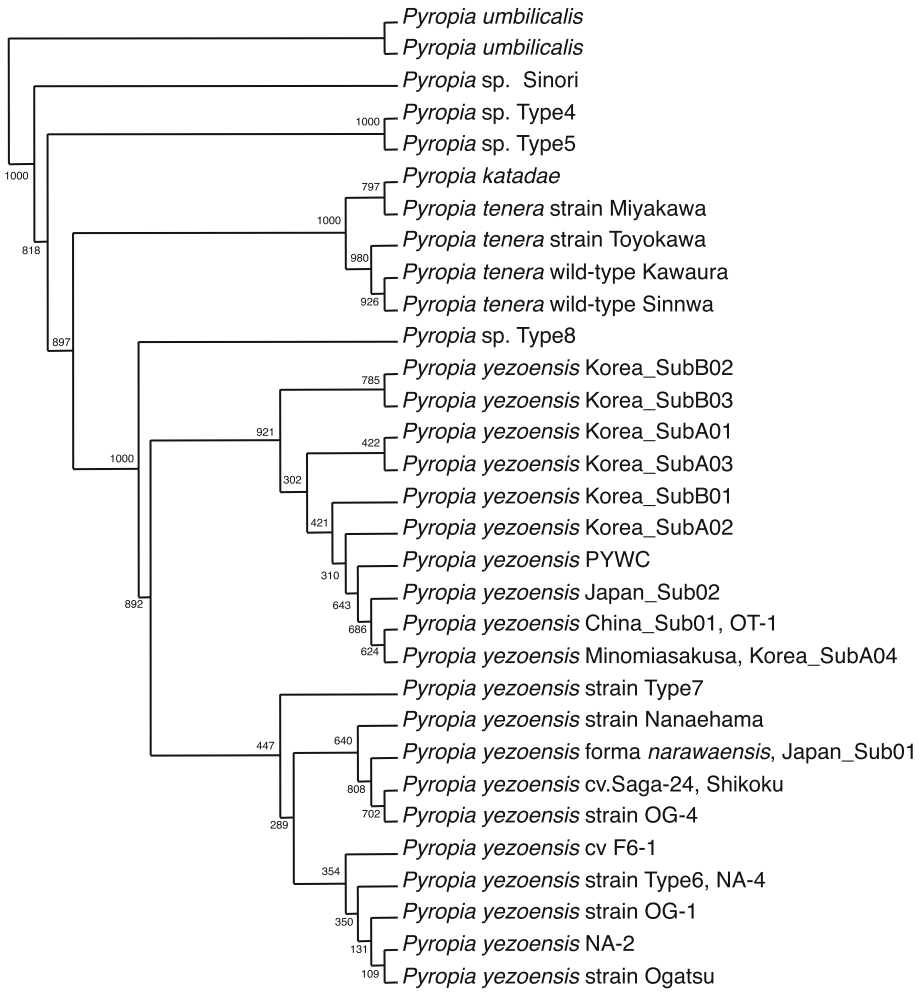

Fig. 3 Phylogenetic tree of the DNA sequence of the 343-bp-long partial ITS-1 region of *Pyropia yezoensis* and *Pyropia tenera* strains. *Pyropia umbilicalis* strains (DDBJ/GenBank/EMBL accession numbers AB017088 and AJ318959) are used as outgroups. The *scale* indicates the evolutionary distance of the base substitution per site. Bootstrap confidence values for the sequence groupings are indicated in the tree ($n = 1{,}000$). The nucleotide sequences determined in Japanese dried nori product (Japan_Sub01) and the *Pyropia yezoensis* cultivar strains (U-51, Saga-5, Sasiki, Oba-green, Fukuoka-,1 D-18-1, Ariake-1, Noma-1, Midorime and Saga103) were identical to the *Pyropia yezoensis* f. *narawaensis* reference sequence. *Pyropia yezoensis* type 6, type 7, type 8, Yunouraasakusa, Ogatsu-4, minohe, and Minomiasakusa are wild types or strains registered by Kunimoto et al. [2]. The DDBJ/GenBank/EMBL accession numbers were as follows: *Pyropia* sp. Sinori (AB017077), *Pyropia katadae* (AB017090), *Pyropia tenera* strain Miyakawa (AB100958), *Pyropia tenera* strain Toyokawa (AB100959), *Pyropia tenera* wt Kawaura (AB017073), *Pyropia tenera* wt Shinwa (AB017072), PYWC (DQ649354), strain OT-1 (AB365994), cv. F6-1(AB017083), strain NA-4 (AB017076), strain OG-2 (AB017079), strain OG-1 (AB017078), strain NA-2 (AB017075), strain Ogatsu (AB243203), cv. Shikoku (AB017087), cv. Saga-24 (AB019192), cv. Midorime (AB017084), strain OG-4 (AB017081)

11 genotypes of *P. yezoensis* strains: Japan_Sub01, China_Sub01&02, Korea_SubA01, Korea_SubA02, Korea_SubA03, Korea_SubA04, Korea_SubB01, Korea_SubB02, Korea_SubB03, and two genotypes of *Pyropia* sp. NRIFS-1 and NRIFS-2 that were not *P. yezoensis* (Table 3). The genotype Korea_SubA01 was identical to the nucleotide sequences of *P. yezoensis* wild strains NS003 and NS004 isolated from natural seeding on the southwest coast of Korea (accession numbers DQ227869 and DQ227870, respectively). Nucleotide sequences in the ITS-1 region of the genotypes Korea_SubB01, Korea_SubB02, and Korea_SubB03 were identical to those of *P. yezoensis* strains NS005 (accession no. DQ227871), NS002 (accession no. DQ227868), and NS001 (accession no. DQ227867) isolated from the natural seeds of wild strains in Korea, respectively. Thalli whose ITS-1 region's DNA sequences were identical to those in Japan (Japan_Sub01) or China (China_Sub01) were also detected (Table 3). These genotypes, China_Sub01-02, Korea_SubA01-A04, and Korea_SubB01-B03, closely resembled *P. yezoensis* strain Minomiasakusa, rather than resembling *P. yezoensis* strain f. *narawaensis*, in phylogenetic analysis (Fig. 3). In addition, two *Pyropia* spp. whose DNA sequences of the ITS-1 region completely differed from those of *P. yezoensis* strains were detected only in the dried nori products from Korea (Table 3). Among 263 pieces of thalli in the Korean products, 50 pieces contained the 348-bp-long segment of the ITS-1 region (*Pyropia* sp. NRIFS-1) and two pieces contained the approximately 350-bp-long segment of the ITS-1 region (*Pyropia* sp. NRIFS-2), whose nucleotide sequences differed from those of *P. yezoensis*. However, we could not determine these nucleotide sequences in the ITS-1 region of *Pyropia* sp. NRIFS-1 and NRIFS-2 because of highly repetitive nucleotide sequences. We also analyzed the nucleotide sequences of plastid RuBisCo genes.

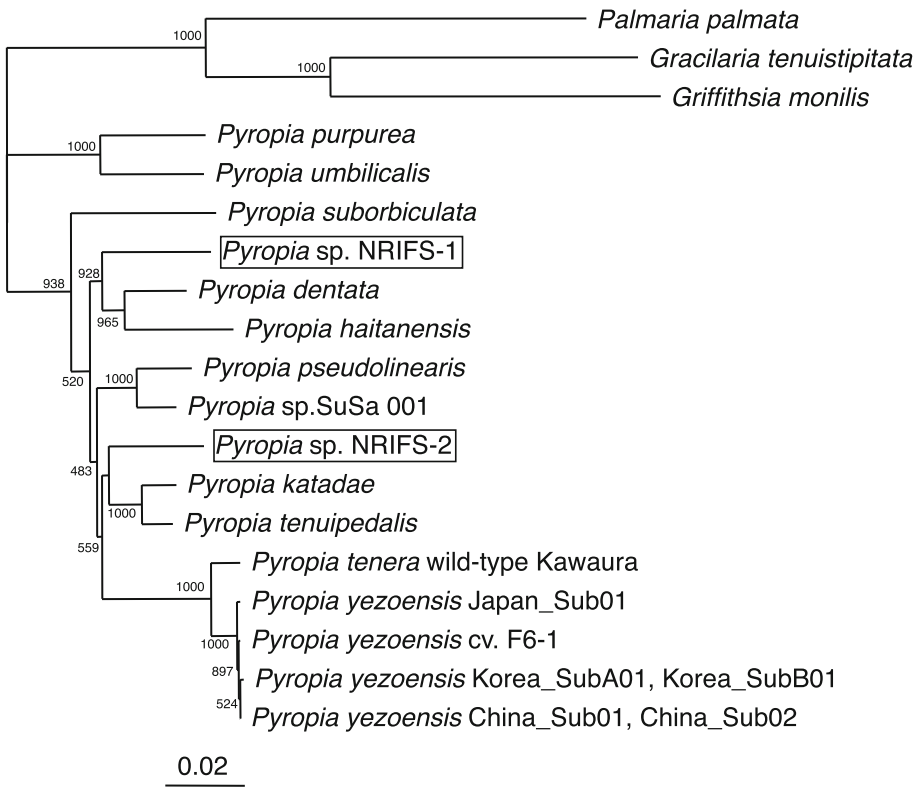

Fig. 4 Phylogenetic tree based on the partial nucleotide sequences of the ribulose bisphosphate carboxylase/oxygenase (RuBisCo) genes in *Pyropia* species. The *scale* indicates the evolutionary distance of the base substitution per site. RuBisCo genes of China_Sub01, China_Sub02, Korea_Sub01, and Korea_Sub02 were sequenced from thalli of nori products 07C-01, 08C-24, 07 K-30, and 07 K-31, respectively. *Palmaria palmata* (accession number PPU28421), *Griffithsia monilis* (accession number EU079379), and *Gracilaria tenuistipitata* (accession number AY673996) were used as outgroups. Bootstrap confidence values for the sequence groupings are indicated in the tree ($n = 1{,}000$). *Boxes* represent the *Pyropia* sp. NRIFS-1 and NRIFS-2 identified in the present study. The DDBJ/GenBank/EMBL accession numbers were as follows: *Pyropia yezoensis* Japan_Sub01 (AB818917), China_Sub01/China_Sub02 (AB818918), Korea_Sub01/Korea_Sub02 (AB818919), *Pyropia* sp. NRIFS-1 (AB818920), *Pyropia* sp. NRIFS-2 (AB818921), *Pyropia purpurea* (AJ634465), *Pyropia umbilicalis* (AB118584), *Pyropia suborbiculata* (AB118580), *Pyropia dentata* (AB118579), *Pyropia haitanensis* (AB118585), *Pyropia* sp. SuSa 01 (AB118586), *Pyropia katadae* (AB118583), *Pyropia tenuipedalis* (AB287951), *Pyropia tenera* wt Kawaura (AB118576), and *Pyropia yezoensis* cv F6-1 (AB118590)

Phylogenetic analysis based on the nucleotide sequences of the RuBisCo gene in the *Pyropia* genus showed that these sequences in *Pyropia* sp. NRIFS-1 and *Pyropia* sp. NRIFS-2 were included in a cluster of *Pyropia* spp. The nucleotide sequence for *Pyropia* sp. NRIFS-1 was closely related to those of *P. dentata* and *P. haitanensis* (Fig. 4), and identical by 95 and 94 %, respectively. The nucleotide sequence of *Pyropia* sp. NRIFS-2 was closely related to *P. katadae* and *P. tenuipedalis* (Fig. 4), and was identical by 96 and 96 %, respectively. By additional BLAST analyses using the RuBisCo L gene, the nucleotide sequence of *Pyropia* sp. NRIFS-1 was very similar to that of *P. seriata* that was isolated in Kumamoto, Japan (HQ687533) [11] with 99.8 % nucleotide identity. The nucleotide sequence of *Pyropia* sp. NRIFS-2 in the RuBisCo L gene was identical to those of *P. kuniedae* (HQ728200) and *P. pulchella* (GU046419) [11].

In contrast, nine dried nori products produced in Gimhae and Pusan (Table 3) contained only the thalli of the strain whose DNA sequence was identical with that from Japan (Japan_Sub01).

RFLP analysis

The nucleotide sequences showed two distinct RFLP patterns using *Msp*I that differed by origin. The genotype Japan_Sub01 contained a 432-bp-long DNA sequence with an *Msp*I site, resulting in 154 and 278-bp fragments isolated by RFLP (Fig. 1, lane 7). Other genotypes such as Japan_Sub02, China_Sub01, Korea_SubA01, Korea_SubA02, and Korea_SubA03, found in most of the dried nori products produced in China and Korea, contained a DNA sequence with no *Msp*I site (Fig. 1, lanes 8–10). Other genotypes, such as Korea_SubB01, Korea_SubB02, and Korea_SubB03, also contained a 426-bp-long DNA sequence with an *Msp*I site (Fig. 1, lane 11). In 116 dried nori products from Japan, PCR products with no *Msp*I site were detected in only two pieces of thalli from over 1,200 samples.

Discussion

The nucleotide sequences of the ITS region extracted from the thalli of dried nori products from Japan, China, and Korea were compared to discriminate between countries-of-origin for the food labeling of dried nori products. Differences in the nucleotide sequences of dried nori products from Japan, China, and Korea were identified, and genetic variation in *P. yezoensis* and other related species was characterized in these different country-of-origin products.

In Japanese products, the genetic variation of *P. yezoensis* was very low, and the nucleotide sequences in the ITS region of almost all products were identical to the genotype Japan_Sub01, which corresponded to the reference strains of *P. yezoensis* f. *narawaensis* (i.e., U-51, Saga-5, Sasiki, Fukuoka-1, Ariake-1, and Saga103) [5, 8]. The two exceptional cases in which we detected the genotype Japan_Sub02 may have been due to contamination by wild strains in the aquaculture field. In recent years, specific strains have been obtained by the selective breeding of *P. yezoensis* f. *narawaensis* in Japanese institutes and have been applied in commercial production [1–3]. Therefore, genetic homogeneity may be increased by the selective breeding of nori cultivar strains. In Chinese products, the cultivar strain that was closely related to the *P. yezoensis* strain Minomiasakusa, was detected in 41 of the 45 products used in this study. The Minomiasakusa strain was established from a wild strain in 1979 by a Japanese company (http://www.hinsyu.maff.go.jp/tokei/contents/14_2012kaiso.pdf). The genotypes China_Sub01 and China_Sub02 may have been derived from the Minomiasakusa strain and transplanted from Japan. On the other hand, three products produced in Qingdao had the genotype Japan_Sub01 identical to that of *P. yezoensis* f. *narawaensis*, suggesting that cultivar strains may have been transplanted from Japan to China in recent years.

In the Korean products, many genetically distinct genotypes of strains were detected. Four genotypes, Korea_SubA01, SubB01, SubB02, and SubB03, were identical to known cultivar strains of *P. yezoensis* isolated from natural seeds of Korea's southwest coast, according to the DNA database. These genotypes have never been found in Japanese products or cultivar and wild strains, suggesting they were transplanted from Japan in earlier years. Based on the partial nucleotide sequences in the RuBisCo L gene, the sequence of *Pyropia* sp. NRIFS-1 was closely related to that of *P. seriata* [11], and that of *Pyropia* sp. NRIFS-2 was identical to those of *P. kuniedae and P. pulchella* [11]. These were used in the Korean products, indicating that *Pyropia* species, other than *P. yezoensis*, are also cultured for the production of dried nori products. In addition, the genotype Japan_Sub01 of *P. yezoensis* was also used in the Korean products, suggesting that this genotype was transplanted from Japan in recent years. Recent studies characterized the phylogenetic relationship in *Pyropia* spp. by analyses of mitochondrial and plastid DNA [11, 12]. Therefore, mitochondrial and plastid DNA analysis may also be useful for species and origin identification of the Korean dried nori products.

This study showed that RFLP analysis of the ITS region by cleavage with *Msp*I is useful for the discrimination of Japanese domestic dried nori products versus those imported from China and Korea. However, some exceptional samples from the Chinese or Korean products showed similar RFLP patterns to Japanese products. In

such cases, multiple trace elemental analysis can support RFLP analysis by *Msp*I cleavage [15].

Acknowledgments The authors are grateful to Dr. Masahiko Kunimoto for helpful suggestions. We thank the Japan Fisheries Cooperatives, Japan Nori Association, National Federation of Nori and Clams Fisheries Cooperatives, Okayama Prefectural Fisheries Experiment Station, Saga Prefectural Ariake Fisheries Research and Development Center, Seikai National Fisheries Institute, Takaokaya Inc., and Fujimori-Shoten for providing the seaweed samples. This work was supported by funding from the Fisheries Agency, Japan.

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