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Genotype analysis of commercial products of the soft seaweed *Undaria pinnatifida* (Laminariales, Alariaceae)

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Abstract—Genotypes of commercial products of the soft seaweed, *Undaria pinnatifida*, or *wakame*, were examined to evaluate if genetic analyses can be used with the processed materials, and to compare the genotypes from various localities. A total of 56 kinds of the commercial products (raw, boiled, salted, dried), and 3 aquaculture strains were used to amplify partial fragments of the COIII and RubisCO genes by PCR, and their DNA nucleotide sequences were determined. There were 9 and 3 haplotypes detected for the COIII and RubisCO genes, respectively, allowing separation of the specimens into 11 genotypes. The two most common genotypes where found in 31 and 14 products, while the other 9 genotypes where only found in 1–4 products. These results suggest that the genotype analysis can be used for any form of *U. pinnatifida* products and will be useful to develop a method to maintain the commercial value of *U. pinnatifida* made along the Sanriku Coast.

Key words: COIII, genotype, RubisCO, Sanriku Coast, soft seaweed, traceability, wakame

Introduction

The soft seaweed, *Undaria pinnatifida* (Harvey) Suringar (Laminariales, Alariaceae), or *wakame* in Japanese, has been one of the most valuable aquatic resources cultivated along the Sanriku Coast in the northeastern region of Honshu, Japan. The annual production of *U. pinnatifida* by aquaculture was more than 150,000 tons in the 1970's, but decreased to about 60,000 tons due to an increase of imports from China and South Korea (Sato 2004). Recent analysis estimated the domestically produced *U. pinnatifida* to be about 25% of the total annual consumption of 240,000 tons in Japan (Japanese Ministry of Agriculture, Forestry and Fisheries 2010). The aquaculture production along the Sanriku Coast yields about 75% of the total domestic *U. pinnatifida* production, and its market prices are considerably higher than the other domestic and import products. The aquaculture of *U. pinnatifida* along the Sanriku Coast has been almost completely damaged by the *tsunami* disaster in March 2011, but has been recovered smoothly because of its relatively low cost for facilities, high availability of artificial seedlings and a rapid return in harvest with the benefit of an annual characteristic of *U. pinnatifida*. Accordingly, enhancement of the aquaculture of *U. pinnatifida* will directly contribute to the recovery of the fisheries industries along the Sanriku Coast.

U. pinnatifida from various localities is known to vary in its morphological characteristics, and the thick and flat leaf-blade is a key feature to give higher commercial value for the products (Ishikawa 1991, 1995, Ohno and Matsuoka 1993, Yamanaka and Akiyama 1993, Kim and Lee 1995, Choi et al. 2007). Water temperature and velocity during the aquaculture are one of the most significant factors that determine the growth rate and morphology of the blade (Nanba et al. 2011). In addition to phenotypic plasticity in morphology, the genetic background is also known to directly regulate the morphology of the blade in *U. pinnatifida* (Saito 1962, Kito et al. 1981, Taniguchi et al. 1981, Ishikawa 1991, 1995, Stuart et al. 1999).

In Japan, the aquaculture seeds of *U. pinnatifida* have been maintained at various levels of official institutes, fisheries cooperation or individual farmers. Especially, Iwate prefecture, possessing one major part of the Sanriku Coast, has been strictly maintained its domestic seeds by preventing both immigrants and emigrants. The disaster in 2011, however, had caused serious shortage of *U. pinnatifida* seeds for aquaculture in Japan, and it was likely that a certain amount of seeds from elsewhere had been introduced for aquaculture along the Sanriku Coast and caused a genetic pollution. Besides, *U. pinnatifida* has been expanding its distribution worldwide by artificial vectors both intentionally and unintentionally (Irigoyen et al. 2011). For example, their sporophytes can be transferred easily by draining of ballast water of transport ships, and *U. pinnatifida* has been listed in the list of the world's 100 worst invasive species (Lowe et al. 2000). Thus, it is important to know the current status of the genotype variation and distribution of *U. pinnatifida* including in the foreign countries whose export occupies a large

proportion of the consumption in Japan.

In addition, identification of different strains with genetic markers will be essential for breeding to enhance aquaculture, and also to develop traceability methods for commercial products to prevent camouflaging of imports (Endo et al. 2009). Accordingly, the present study examined the genotype of *U. pinnatifida* commercial products to evaluate if genetic analysis can be used with the processed products and to compare the genotypes of the products from various localities in East Asian countries.

Materials and Methods

Specimens

A total of 59 samples of *U. pinnatifida* specimens were examined in this study. The commercial products were obtained from local supermarkets (*n*=48) and restaurants (*n*=4) mostly in Kanagawa Prefecture, Japan in 2012 (Table 1). The packaged products from the supermarkets were originally produced by 24 companies, and were randomly assigned an identification number. The products purchased from the supermarkets were in various processed forms including dried (*n*=27), boiled (*n*=15), boiled and salted (*n*=8), and pickled (*n*=1). Their producing locations were Sanriku (*n*=27), others outside Sanriku in Japan (*n*=5), China (*n*=4), South Korea (*n*=2), and the remaining were unknown (*n*=21). We also examined cultivated specimens that were harvested in February 2013 at Okirai Bay, Iwate Prefecture (*n*=3) and Hayama Bay, Kanagawa Prefecture (*n*=1). In addition, aquaculture strains originated from the three localities of Taro and Okirai Bays, Iwate Prefecture, and Hayama Bay were also assessed (*n*=1 each). The two Iwate strains were collected from Taro and Okirai Bays in 2004 and 2005, respectively, and have been maintained alive in the laboratory by N. Nanba. The Hayama strain was provided by Dr. K. Yamaki of the Hayama Marine Science Laboratory, Kajima Technical Research Institute.

Genotype analysis

The specimens were rinsed with distilled water to remove contaminants such as salt and seasoning, and total DNA was extracted by a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. PCR was carried out to amplify partial fragments of the mitochondrial genome cytochrome oxidase unit III (COIII) and chloroplast genome ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) genes with an intergenic region. PCR primers employed were CAF4A and CAR4A (Kogame et al. 2005) for the COIII locus, and rbcL3F (Kogame and Matsuda, 2001) and RSPR (Kogame et al. 1999) for the RubisCO locus. A total 10 µL of reaction contained 1 µL of template DNA solution, 1 µL of 10× KOD Plus ver. 2 buffer (TOYOBO), 0.2 mM each dNTPs, 1.5 mM magnesium sulfate, 0.3 µM each sense and antisense primers, and 0.3U KOD Plus ver. 2 polymerase (TOYOBO). PCR was carried out for 40 cycles at 98 °C for 10 sec, 53 °C for the COIII or 55 °C for the RubisCO for 30 sec, and 68 °C for 1 min, followed by 68 °C for 7 minutes with iCycler (Bio-Rad) or TP600 (TaKaRa) thermal cyclers. The PCR products were visually observed by an electrophoresis with 0.8 % (w/v) agarose gel containing 1 ng/ml ethidium bromide in 44.5 mM Tris-borate, 1 mM EDTA (TBE) buffer. Excess primers and dNTPs were removed by precipitating the PCR products with 10 % (w/v) polyethylene glycol 8000 (Promega) in 10 mM magnesium chloride, and dissolved in 20 µl of 10 mM Tris-HCl, 1 mM EDTA (TE) buffer, pH 8.0. DNA nucleotide sequencing was performed for both 5′ and 3′ ends. A total of 10 µL of reaction consisted of 1 µl of the purified PCR product, 40 mM Tris-HCl, 1 mM magnesium chloride, 0.25 µl of the BigDye terminator v3.1 premixed solution (Applied Biosystems) and 0.25 µM sense or antisense primer. The reaction was carried out according to the manufacturer's protocol except the extension time was 1 minute and the number of cycles was 40 times. The labeled fragments were analyzed on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems). The DNA nucleotide sequences were assembled and further analyzed with Geneious ver. 6.1 (Biomatters). The DNA nucleotide sequences determined in the present study have been deposited in the DDBJ/EMBL/GenBank databases with accession nos. AB889527 to AB889538.

Results

All the 59 specimens examined in the present study were successfully amplified by the PCR, and the haplotypes were determined for both the COIII and RubisCO loci. The partial DNA nucleotide sequences of the COIII gene (448 base pairs) contained 8 sites of substitutions that were divided into 9 haplotypes (C1–C9; Fig. 1). The nucleotide substitutions of the COIII locus consisted of 7 sites of a transition (A/G and C/T; *n*=2 and 5, respectively) and 1 site of a transversion (G/T). There were variations at 2 sites in the 149 residues of deduced amino acid sequences of the COIII locus, which were isoleucine in the C6 and C7 types, and valine in the others (Fig. 1). The DNA nucleotide sequences of the RubisCO gene (464 base pairs) consisted of 3 haplotypes with the 2 sites of substitution being the transition and transversion (A/G and G/T; *n*=1 each) in the non-coding intergenic region (R1–R3; Fig. 2). No variation was detected in the deduced amino acid sequences of the RubisCO locus.

The frequency of each haplotype observed among the 59 specimens varied widely (Tables 1 and 2). The C1 and C6 haplotypes were present in 31 and 15 specimens, respectively, while the other COIII haplotypes were in less than 4 specimens. The C2, C5 and C9 types were each detected

Table 1. Commercial products and aquaculture strains of the soft seaweed *Undaria pinnatifida* examined in the present study.

Products	Source	Source ID	Preservation	Region	Country	C	R
Dried Wakame	SM	2	Dried	Iwate	Japan	1	1
Furikake	SM	9	Boiled	Iwate	Japan	1	1
BS Wakame	SM	12	Salted	Iwate	Japan	1	1
BS Wakame	SM	18	Salted	Iwate	Japan	1	1
Sashimi	SM	4	Boiled	Iwate	Japan	5	1
Sashimi	SM	32	Boiled	Iwate	Japan	9	1
BS Wakame	SM	27	Salted	Taro	Japan	1	1
Mekabu	SM	12	Boiled	Miyagi	Japan	1	1
Mekabu	SM	13	Boiled	Miyagi	Japan	1	1
BS Wakame	SM	3	Salted	Sanriku	Japan	1	1
Rice ball	SM	5	n/a	Sanriku	Japan	1	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	1	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	1	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	1	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	1	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	6	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	7	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	7	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	7	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	8	1
BS Wakame	SM	18	Salted	Sanriku	Japan	9	1
Dried Wakame	SM	24	Dried	Sanriku	Japan	9	1
Kuki Wakame	SM	18	Salted	Tokushima	Japan	1	1
BS Wakame	SM	11	Salted	Ariake	Japan	2	1
BS Wakame	SM	31	Salted	Suruga	Japan	3	3
Dried Wakame	SM	6	Dried		China	1	1
BS Wakame	SM	8	Dried		China	1	1
Dried Mekabu	SM	30	Dried		China	1	1
Dried Wakame	SM	24	Dried		China	9	2
Dried Wakame	SM	24	Dried		S. Korea	9	1
Kuki Wakame	SM	25	Salted		S. Korea	9	1
Miso soup	RT	2	n/a		n/a	1	1
Miso soup	RT	3	n/a		n/a	1	1
Pickled Mekabu	SM	10	Pickled		n/a	1	1
Miso soup	SM	14	Boiled		n/a	1	1
Furikake	SM	16	Dried		n/a	1	1
Instant noodle	SM	17	Dried		n/a	1	1
Instant noodle	SM	19	Dried		n/a	1	1
Rice ball	SM	20	n/a		n/a	1	1
Rice ball	SM	26	n/a		n/a	1	1
Instant noodle	SM	28	Dried		n/a	1	1
Instant noodle	SM	28	Salted		n/a	1	1
Furikake	SM	29	Boiled		n/a	1	1
Chinese noodle	RT	1	n/a		n/a	9	1
Japanese noodle	RT	4	n/a		n/a	9	1
Instant noodle	SM	1	Dried		n/a	9	1
Miso soup	SM	7	n/a		n/a	9	1
Furikake	SM	15	Dried		n/a	9	1
Instant noodle	SM	21	Dried		n/a	9	1
Instant soup	SM	22	Dried		n/a	9	1
Instant soup	SM	23	Dried		n/a	9	1
Furikake	SM	24	Dried		n/a	9	1
Gamate	KU		Intact	Okirai	Japan	6	1
Gamate	KU		Intact	Taro	Japan	1	1
Thallus	KU		Intact	Okirai	Japan	3	1
Thallus	KU		Intact	Okirai	Japan	1	1
Thallus	KU		Intact	Okirai	Japan	7	1
Sporophytes	KRI		Intact	Hayama	Japan	4	1
Thallus	KRI		Intact	Hayama	Japan	4	1

C, COIII locus; R, RubisCO locus; BS Wakame, Boiled and salted Wakame; SM, Supermarket; RT, Restaurant; KU, Kitasato University; KRI, Kajima Research Institute.

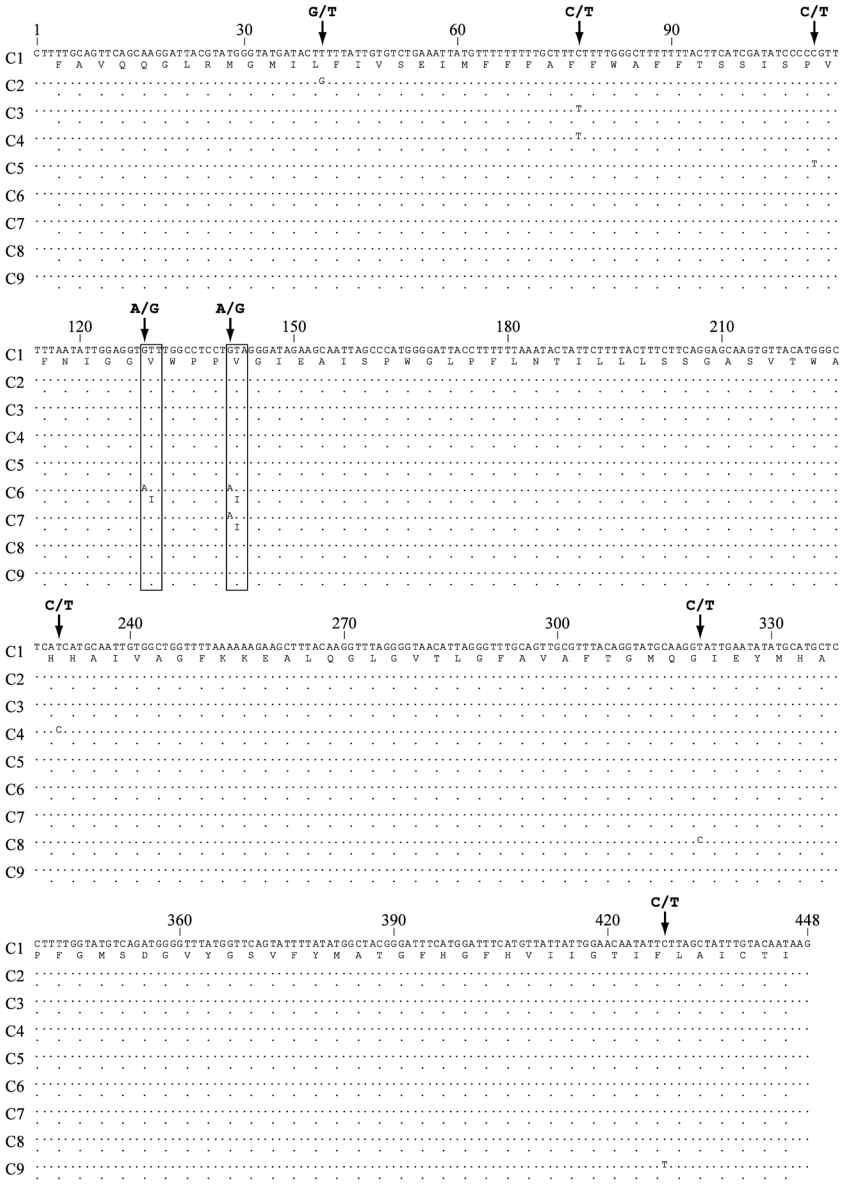

Fig. 1. The DNA nucleotide and deduced amino acid sequences of the 9 haplotypes (C1–C9) of the COIII gene found in the *U. pinnatifida* specimens examined in this study (*n*=59). Dots indicate the identical nucleotide or amino acid residues with those of the top line (C1). The nucleotide substitutions are shown with arrows, and the variations in the deduced amino acid sequences are shown with boxes.

from a single specimen. Further, for the RubisCO locus, the R2 and R3 types were each observed in a single specimen, and the other 57 specimens were all found to be the R1 type.

The 9 and 3 haplotypes, detected in the COIII and RubisCO loci, respectively, were considered together, and the 59 specimens were divided into 11 genotypes (Table 2). The most common type (C1R1) was present in 31 specimens including processed products from the Sanriku Coast (*n*=15), Tokushima (*n*=1), China (*n*=3), and unknown localities (*n*=12) (Table 2). Similarly, the second-most common type (C9R1) was present in the products from the Sanriku Coast (*n*=3), South Korea (*n*=2), and the unknown localities (*n*=9). On the other hand, among the remaining 9 genotypes observed in the present study, 5 types were unique to the products from the Sanriku Coast, and the other 4 types were unique to various localities except along the Sanriku Coast (Table 2). The C7R1, C6R1, C3R1, C5R1 and C8R1 types were found only from those cultivated along the Sanriku Coast (Table 2). The C4R1, C2R1, C3R3 and C9R2 types were found from products that originated from the Hayama Bay, Ariake Sea, Suruga Bay and China, respectively (Tables 1 and 2).

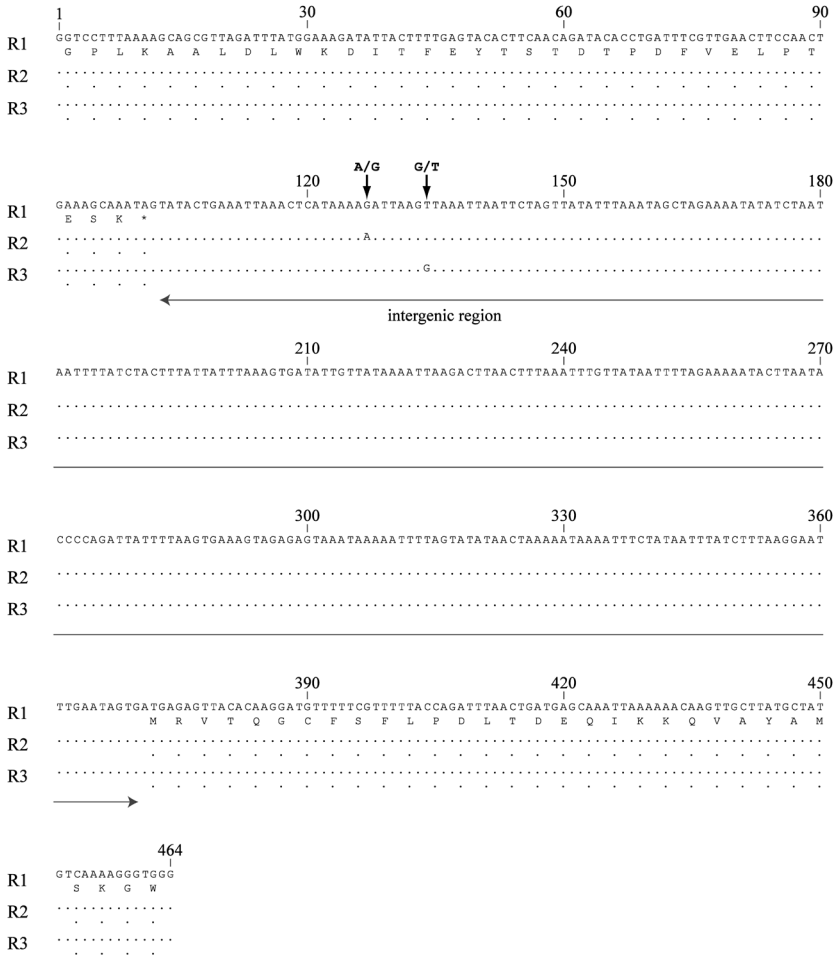

Fig. 2. The DNA nucleotide and deduced amino acid sequences of the 3 haplotypes (R1–R3) of the RubisCO genes with intergenic region found in the *U. pinnatifida* specimens examined in this study (*n*=59). The dots indicate the identical nucleotide or amino acid residues with the top line (R1). Two sites of the nucleotide substitutions are shown with vertical arrows. Horizontal arrows indicate the intergenic non-coding region (268 base pairs).

Table 2. Summary of the genotype analysis.

Genotype	*n*	Domestic		Import		Unknown
		Sanriku	Other	China	Korea	
C1R1	31	15	1	3		12
C9R1	14	3			2	9
C7R1	4	4				
C4R1	2		2			
C6R1	2	2				
C2R1	1		1			
C3R1	1	1				
C3R3	1		1			
C5R1	1	1				
C8R1	1	1				
C9R2	1			1		

Discussion

The most significant finding of this study was that the genotype analysis could be used for any processed forms of *U. pinnatifida* including boiled, salted and dried (Table 1). In addition, it was found that the commercial products could be classified by their genetic traits, and the frequencies of each genotype varied greatly: the two major genotypes occurred frequently, 52.5% and 23.7%, respectively, and the others were less than 7% (Table 2). Genotype analyses of *U. pinnatifida* have been carried out to study the ecological aspects (Muraoka and Saito 2005, Uwai et al. 2006a, b, 2007, Wang et al. 2006) and commercial products (Endo et al. 2009) of this species. Genetic variability of commercial *U. pinnatifida* products has been reported for the COI and ITS2 regions by Endo et al. (2009). For the COI locus, they found 5 haplotypes from 27 specimens from Japan, China and South Korea. All nucleotide substitutions were at the third position of the codons, and were synonymous substitutions (Endo et al. 2009). In contrast, for the COIII locus examined in the present study, two substitutions at the first position of the codons were non-synonymous substitutions (Fig. 1). These two haplotypes (C6 and C7) were both found only in the specimens from Okirai Bay of the Sanriku Coast (Tables 1 and 2)

and might be useful to distinguish *wakame* from that area. For the RubisCO loci, on the other hand, two out of three haplotypes were found only in single specimen from Suruga Bay and China (Table 2). This suggests that the RubisCO locus is fewer variable and thus less useful than the COIII locus to distinguish the commercial *U. pinnatifida* products.

Endo et al. (2009) suggested that Japanese aquaculture seeds might have been introduced into foreign aquaculture farms. Consistent with this, in this study we found that the products from China and South Korea included the same haplotypes as various products made in Japan (Table 2). On the other hand, one genotype (C9R2) was only detected in a product from China (Table 2). Although the sample collection of this study was not comprehensive, it was suggested that a certain genotype could be used to identify the imported products. The present study also examined some products with unknown origin (Table 1). Interestingly, all these products (n=21) were found to have the two major genotypes (Table 2). Information about original locality of these products was not available because they were sold as an ingredient or served at restaurants. They were likely from imported materials because of their significantly lower price than those domestically produced, and the statistics that three quarters of the annual consumption in Japan was from China and South Korea (Japanese Ministry of Agriculture, Forestry and Fisheries 2010).

The results obtained in the present study suggest that genotype analysis can be used with any type of currently circulating *U. pinnatifida* products, and the sensitive genetic markers will be useful to guarantee the origin of the products. The PCR genotyping can be completed in a few hours for a large number of specimens by employing a fluorescent-labeled probe (Minegishi et al. 2009). However, because *U. pinnatifida* at each locality could have been transferred to another place, both intentionally and unintentionally, genetic markers are not sufficient to clearly determine the place where the specimens were originally cultivated. Alternatively, stable isotopic composition analysis may be important because this method can distinguish the characteristics of water where the specimens grew (Suzuki et al. 2013). To efficiently clarify the origins of a large number of *U. pinnatifida* products in the market, a combination of genotyping and stable isotope analysis will likely be useful.

In conclusion, the present study showed the availability of genetic analysis for any *U. pinnatifida* products, and usefulness of two loci of the COIII and RubisCO to, at least partly, clarify the strain originated from the Sanriku Coast among the various products including those from foreign countries. It will be then important to develop more sensitive genetic markers to comprehensively distinguish the certain strain. So far, selective breeding to establish excellent aquaculture seeds has not yet been achieved in *U. pinnatifida*, but will be essential in the near future because of the high commercial value of the domestic product, especially those cultivated along the Sanriku Coast. Genotype analysis will be useful to help the selection of certain strains, and also to prevent camouflage of imported product in the market.

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