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Development of a rapid and accurate PCR-based detection method for commercially valuable green algal species

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Abstract We developed a rapid and accurate PCR-based inspection technique for individual detection of five commercially important green algal species, *Ulva linza, U. prolifera, U. intestinalis, U. compressa* and *Monostroma nitidum*, all of which are controlled as import quota items in Japan. This analytical method has solved the general problem of PCR-based detection methods of being defenseless against false-negatives by application of a duplex PCR technique with an internal control band generated by a set of universal primers for green algal species. Due to the detection of small-sized PCR products of <250 bp lengths, the analytical method would be useful for deeply processed products and completes its quick PCR amplification within 45 min. The validity of the developed analytical method was proved by a pre-test using a total of 129 samples (12 imported products and 31 Japanese commercial products). This analytical method is a powerful tool for screening of commercial green algal products to discover the five controlled algal species and will allow inspection authorities, including the Customs Service, to improve their examination systems in terms of simplification, rapidity and cost-effectiveness.

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Introduction

In globalized food trade, incorrect labeling or misdescription of foods or their ingredients often becomes a problem for proper implementation of national regulations. Higher premium foodstuffs generally tend to be targeted in food fraud, in which the originals are substituted by cheaper ones [1]. Quality control and traceability of food products are important to ensure compliance with food regulations as well as food safety and security for consumers [2] (Chemistry World: http://www.rsc.org/chemistryworld/Issues/2007/September/FightingFoodFraudWithScience.asp Accessed19 May 2014). Inspection authorities have to check a large number of samples rapidly to stop economic frauds and for protection of consumer rights, and therefore need fast, reliable and cost-effective analytical methods [3–5].

Seaweeds have traditionally been used as food in East-Asian countries, namely China, Japan and Korea, for several centuries. In recent years, seaweeds have widely been accepted in western countries as raw or semi-processed materials used in seafood products such as soup, drinks, etc. [6]. The worldwide production of aquatic algae reached 21 million tons in 2011 [7]. In Japan, green algal genera *Ulva, Enteromorpha* and *Monostroma* are generally used for human consumption in various forms, and the valuable green algal species, *Monostroma nitidum* and *Enteromorpha prolifera* (present species name, *Ulva prolifera*), are cultivated on a commercial scale [8, 9]. The labels of commercial green algal products in Japan often describe their raw materials as "aosa" for *Ulva*, "aonori"

for *Enteromorpha* or "hitoegusa" for *Monostroma.* Imported quantities of green algae of only the genera *Enteromorpha* and *Monostroma* and their processed varieties are strictly controlled under national law as import quota (IQ) items. Therefore, it is important for Japanese Customs Service to distinguish between green algal products of genera *Enteromorpha* or *Monostroma* and those of *Ulva* species. On the other hand, recent reclassification of green algae, proposing that the genus *Enteromorpha* should be merged into the genus *Ulva,* has made the matter more complicated [10–12].

Commercial green algal products are generally traded in the form of dried pieces, and have lost much of their distinctive morphological characteristics through manufacturing processes. Due to its higher applicability to chemical- or thermal-treated products and to smaller-sized samples, PCR-based analysis is now a mainstream analytical method for determining the ingredients of food products, in place of traditional protein-based methods such as protein-based electrophoresis and immunoassay [13–15]. The DNA barcoding project (Barcode of Life: http://www.barcodeoflife.org/ Accessed18 March 2014) strongly supports DNA sequencing-based identification [16, 17]. Herrero et al. [18] developed a species identification method for several kinds of seaweeds, using both an automated PCR-based restriction fragment length polymorphism (RFLP) analysis and a forensically informative nucleotide sequence (FINS) method. A recent study on species identification of Japanese commercial green algal products and imported green algal products into Japan using nuclear internal transcribed spacer region 2 (nrITS2) reported the detection of three controlled green algal species, *U. linza*, *U. prolifera* and *M. nitidum*, with findings of incorrect labeling for 19 of 44 products [19]. However, the above protocols are complicated and time consuming for routine inspections and require expensive equipment and reagents for the analytical procedures.

Under such circumstances, this study aimed to develop a simple, rapid, reliable and cost-effective PCR-based method for detection of five commercially important algal species (*U. linza*, *U. prolifera*, *U. intestinalis*, *U. compressa* and *M. nitidum*) in Japan including three import quota species previously identified from commercial products.

Materials and methods

Sample collection

Imported dried green algal products were collected at the customs inspection sites. Japanese commercial dried green algal products labeled as "aosa", "aonori" or "hitoegusa" were purchased at food stores in Japan. Imported algal products were, in most cases, presented in the form of small dried pieces of approximately 2–6 mm in size. Strains with code numbers ULC632, UPE21 [20] and C184 and C35 [12] were used as standard samples of *Ulva linza*, *U. prolifera*, *U. compressa* and *U. intestinales*, respectively. Standard specimens of *Monostroma nitidum* were provided from Mie University. All samples were stored at −20 °C until DNA extraction.

DNA extraction

For each product, total DNA was extracted from three different small tissue pieces. To facilitate the identification of each sample, the annotation "a", "b" or "c" was attached after the sample name. Each of the dried samples was reconstituted with sterile distilled water in a disposable Petri dish and then was separated into individual pieces. A wet piece of the separated sample was randomly chosen and placed in a micro-tube of 2.0 ml volume after removing attached water by clean tissue paper for laboratory use. Each piece of sample in the individual micro-tubes was thoroughly triturated with a disposable pestle in liquid nitrogen. Total DNA was extracted from each crushed sample using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the protocol provided from the manufacturer. The quality of the extracted DNA was confirmed by electrophoresis on 0.8 % agarose gel in TAE buffer with ethidium bromide (EtBr) under ultraviolet (UV) light. The extracted DNA was stored at 4 °C until polymerase chain reaction (PCR) assay.

Design of primers

For the primer design, a total of 66 nucleotide sequence data consisting of nrITS2 and its adjacent region, for 27 *Ulva* species (including the former *Enteromorpha* species) and three *Monostroma* species, were taken from GenBank and aligned by the Clustal W package in the MEGA 5 software [21]. Species-specific primers for two former *Enteromorpha* species, *U. compressa*, *U. intestinalis,* and for *M. nitidum,* were designed based on their species-specific nucleotide polymorphisms. However, a high similarity of the compared DNA region between two former *Enteromorpha* species, *U. linza* and *U. prolifera,* did not allow us to create their individual specific primers. Therefore, a specific primer common to those two species was designed within the region. A set of common primers covering a broad range of *Ulva* and *Monostroma* species was also designed at two conserved locations within nrITS2 and its adjacent region. The nucleotide sequences of the designed primers are shown in Table 1.

Table 1 Nucleotide sequences of universal primers for green algal species and species-specific primers for *Ulva* (the former *Enteromorpha*) and *Monostroma* species

Primer name	Sequence	Specificity
Universal primer		
Univ-F	5′-CTCTCGCAACGATGAAGAAC-3′	For *Ulva* and *Monstroma* species
Univ-R	5′-TATTCCGACGTGAGGCAG-3′	For *Ulva* and *Monstroma* species
Species-specific primer		
U_comp-R	5′-CTTCAGCCAGCCCGGCCGCAAGGACC-3′	Specific for *Ulva compressa*
U_intest-R	5′-CAGCCCGCGAGGGGCCGGCC-3′	Specific for *Ulva intestinalis*
U_linz_prol-R	5′-AGCCGGCCCGGGCGCCGAGGC-3′	Specific for *Ulva linza/prolifera*
M_nitid-F	5′-AACCAACGCACCCAACAAG-3′	Specific for *Monostroma nitidum*

Monoplex PCRs

Monoplex PCRs for detection of *U. linza*, *U. prolifera*, *U. intestinalis*, *U. compressa* and *M. nitidum* and for evaluation of the common primer set (univ-F and univ-R, in Table 1) were conducted in a total volume of 15 μl using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The reaction mixture contained 1 μl of template DNA (5 ng), 3 μl of dNTP mixture (2 mM each), 1× KOD FX buffer, 0.15 U of KOD FX DNA polymerase (TOY-OBO Co., Ltd., Osaka, Japan), 5 pmol of each forward and reverse primer, and sterilized water. The PCR was pro-gramed as follows: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 10 s and extension at 68 °C for 20 s. The amplified products were separated by electrophoresis with 2 % agarose gel in TAE buffer containing EtBr and visualized under UV light.

Duplex PCR systems

Following the results of the monoplex PCRs above, a duplex PCR system using three primers, combining one of the four species-specific primers with the common primer set (univ-F and univ-R, in Table 1), was developed. The common primer set, which covers a broad range of green algal species, was used to provide an internal amplification control for the duplex PCR system. For examination of the optimum duplex PCR conditions, amplification was con-ducted in a total volume of 15 μl containing 1 μl of tem-plate DNA (5 ng), 0.4 mM of dNTPs, 1× *Taq* buffer, 0.15 U of KOD FX DNA polymerase (TOYOBO Co., Ltd., Osaka, Japan) or 0.375 U of *Ex-Taq*^TM DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan), different amounts from 2.5 to 10 pmol of each primer, and sterilized water, using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The optimum thermal cycling conditions were also sought by changing the annealing temperature from 55 to 57 °C and the reaction time of each process, on the basis of the monoplex PCR conditions above.

Sequencing of the PCR products and pre-test samples

After separation by agarose gel electrophoresis, PCR pro-ducts amplified in evaluation tests for created species-specific primers were eluted from the gels by the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), accord-ing to the protocol provided from the manufacturer. The purified PCR products were sequenced with the ABI Big-Dye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA) using the same primers.

To authenticate the pre-test, species of commercial sam-ples used in this study were identified in advance by a phy-logenetic analysis after a sequencing analysis using a set of the nrITS2 primers 5.8S-F (5′-CTCTCAACAACGGATA TCT-3′) and 26S-R (5′-TGATATGCTTAAGTTCAGC-3′) [19, 22] (see Supplementary Figs. 1 and 2).

Standard specimens and commercial samples used in this study, together with the identified species names of the commercial samples, are listed in Table 2.

Results

Condition of extracted DNA from commercial green algal products

Commercial green algal samples used in this study were presented in a dried state. Manufacturing processes, especially drying processes, often cause the degradation of sample DNA. However, DNA extracted from the samples showed relatively good qualities, with lengths of more than 10 kbp, as exemplified in Fig. 1. On the other hand, our DNA extraction method provided a lower yield of DNA for the oldest sample (CN-1a in Fig. 1) than others. Even though the sampling quantity of CN-1a for DNA extraction was doubled, the subsequent PCR assay was less successful, due possibly to an increase of impurities interfering with the reaction [23] (data not shown).

Table 2 List of samples used in this study and identification results

| Species name/label description	Origin	Abbrev.	Common primer	M.nit	U.lin/prol	U.comp	U.inte	Duplex PCRs	ITS2 result	Accession no.
					Species-specific primer			Identifications		
Standard sample										
Ulva linza	Japan		+	–	+	–	–			AB933524
Ulva prolifera	Japan		+	–	+	–	–			AB298320
Ulva compressa	Japan		+	–	–	+	–			AB933525
Ulva intestinalis	Japan		+	–	–	–	+			AB933526
Monostroma nitidum	Japan		+	+	–	–	–			AB933331
Imported product										
Aosa	China	CN-1a	+	–	+	–	–	*U. linza/U.prolifera*	*U. linza/U.prolifera*	AB735240
		CN-1b,c	+	–	–	–	–	*other*	*U. pertusa*	AB735241, AB735242
Aosa	China	CN-2a-c	+	–	–	–	–	*other*	*U. pertusa*	AB735243, AB735244, AB735245
Aosa	China	CN-3a-c	+	–	–	–	–	*other*	*U. pertusa*	AB735246, AB735247, AB735248
Aosa	China	CN-4b	+	–	+	–	–	*U. linza/U. prolifera*	*U. linza/U. prolifera*	AB735250
		CN-4a,c	+	–	–	–	–	*other*	*U. pertusa*	AB735249, AB735251
Aosa	China	CN-5a-c	+	–	–	–	–	*other*	*U. pertusa*	AB735252, AB735253, AB735254
Aosa	China	CN-6a	+	–	+	–	–	*U. linza/U. prolifera*	*U. linza/U. prolifera*	AB735255
		CN-6b,c	+	–	–	–	–	*other*	*U. pertusa*	AB735256, AB735257
Aosa	China	CN-7a	+	–	–	–	–	*other*	*U. pertusa*	AB735258
		CN-7b,c	+	–	+	–	–	*U. linza/U. prolifera*	*U. linza/U. prolifera*	AB735259, AB735260
Aosa	China	CN-8a-c	+	–	–	–	–	*other*	*U. pertusa*	AB735261, AB735262, AB735263
Aosa	China	CN-9a	+	–	–	–	–	*other*	*U. fasciata*	AB735264
		CN-9b,c	+	–	–	–	–	*other*	*U. ohnoi or its related*	AB735265, AB735266
Aosa	China	CN-10a	+	–	–	–	–	*other*	*U. fasciata*	AB735267
		CN-10b,c	+	–	–	–	–	*other*	*U. ohnoi*	AB735268, AB735269
Aosa	Korea	KR-1a-c	+	–	–	–	–	*other*	*U. reticulata-related*	AB735366, AB735367, AB735368
Aosa	Korea	KR-2a,b	+	–	–	–	–	*other*	*U. pertusa*	AB735369, AB735370
		KR-2c	+	–	–	–	–	*other*	*U. reticulata-related*	AB735371
Commercial product in Japan										
Hitoegusa	Japan	JF-1a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735273, AB735274, AB735275
Hitoegusa	Japan	JF-2a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735276, AB735277, AB735278
Aosa	Japan	JM-1a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735300, AB735301, AB735302
Aosa	Japan	JM-2a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735303, AB735304, AB735305
Hitoegusa	Japan	JM-3a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735306, AB735307, AB735308
Aosa	Japan	JM-4a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735309, AB735310, AB735311
Hitoegusa	Japan	JM-5a-c	+	+	–	–	–	*M. nitidum*	*M. nitidum*	AB735312, AB735313, AB735314

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Table 2 continued

Species name/label description	Origin	Abbrev.	Common primer	Species-specific primer				Identifications		Accession no.
				M.nit	U.lin/prol	U.comp	U.inte	Duplex PCRs	ITS2 result	
Aosa	Japan	JK-1a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735279, AB735280, AB735281
Aosa	Japan	JK-2a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735297, AB735298, AB735299
Hitoegusa	Japan	JK-3a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735282, AB735283, AB735284
Aosa	Japan	JK-4a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735285, AB735286, AB735287
Hitoegusa	Japan	JK-5a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735288, AB735289, AB735290
Aosa	Japan	JK-6a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735291, AB735292, AB735293
Hitoegusa	Japan	JO-1a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735315, AB735316, AB735317
Aosa	Japan	JO-2a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735318, AB735319, AB735320
Hitoegusa	Japan	JO-3a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735321, AB735322, AB735323
Hitoegusa	Japan	JO-4a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735324, AB735325, AB735326
Hitoegusa	Japan	JO-5a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735327, AB735328, AB735329
Hitoegusa	Japan	JO-6a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735330, AB735331, AB735332
Hitoegusa	Japan	JO-7a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735333, AB735334, AB735335
Aosa	Japan	JO-8a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735336, AB735337, AB735338
Aosa	Japan	JO-9a-c	+	+	–	–	–	M. nitidum	M. nitidum	AB735339, AB735340, AB735341
Aosa	Japan	JS-1a	+	–	–	–	–	other	U. reticulata-related	AB735270
Aosa	Japan	JS-1b,c	+	–	–	–	–	other	U. ohnoi	AB735271, AB735272
Aosa	Japan	JC-1a-c	+	–	–	–	–	other	U. reticulata-related	AB735363, AB735364, AB735365
Aonori	Japan	JS-2a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735294, AB735295, AB735296
Hitoegusa	Japan	JH-1a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735342, AB735343, AB735344
Aonori	Japan	JH-2a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735345, AB735346, AB735347
Aonori	Japan	JS-3a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735348, AB735349, AB735350
Aonori	Japan	JC-2a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735360, AB735361, AB735362
Aonori	Japan	JC-3a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735357, AB735358, AB735359
Aonori	Japan	JC-4a-c	+	–	+	–	–	U. linza/U. prolifera	U. linza/U. prolifera	AB735351, AB735352, AB735353

"M.nit", "U.lin/prol" , "U.comp" and "U.inte" mean *Monostroma nitidum*, *Ulva linza/prolifera*, *U. compressa*, and *U. intestinalis*

Evaluation of the designs of species-specific primers

Standard specimens of *U. linza*, *U. prolifera*, *U. intestinalis*, *U. compressa* and *M. nitidum* were used for evaluation of our species-specific primers. As a result of examination under a PCR reaction, each of the species-specific primers, together with its paired-common primer (univ-F or univ-R, in Table 1), successfully detected its target species with a single band of 200 bp length for *Ulva*
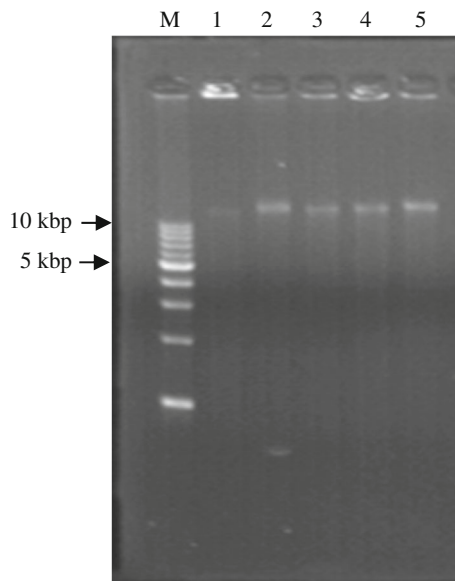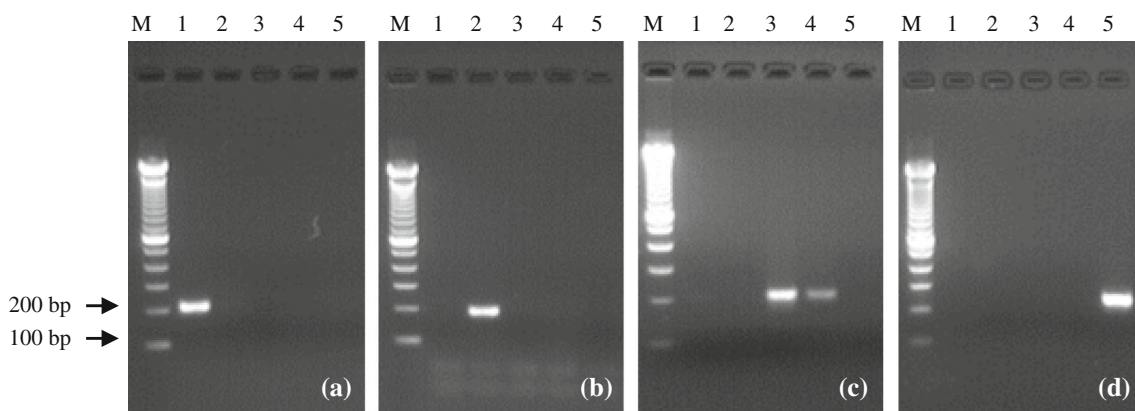
species and a single band of 220 bp length for *M. nitidum* (see Fig. 2). Each amplicon was eluted from the electrophoresis gels and sequenced with the PCR primers. In all cases, the obtained nucleotide sequences matched their original ITS2 sequences.

Optimization of duplex PCR system

First, the applicability of our common primers set (univ-F and univ-R, in Table 1) was checked using PCR with various commercial green algal products. In all the cases, the common primers set successfully worked for amplification of an expected partial 5.8S rRNA region of 110 bp length (see Fig. 3a), and therefore showed potential utility as an internal control for the duplex PCRs below.

Second, we tested four duplex PCRs for detection of five target algal species, *U. linza*, *U. prolifera*, *U. compressa*, *U. intestinalis* and *M. nitidum*. Their primer sets were respectively composed of combinations of the common primers set above and one of the four species-specific primers for *U. linza/prolifera*, *U. compressa*, *U. intestinalis* and *M. nitidum*. As the first step in examining the duplex PCR condition, two different *Taq* DNA polymerases, KOD FX DNA polymerase and *Ex-Taq*™ DNA polymerase, were separately used for amplification at different annealing temperatures from 55 to 57 °C. As a result, KOD FX DNA polymerase synthesized the expected DNA fragments and showed a better result at the annealing temperature of 57 °C, whereas *Ex-Taq*™ DNA polymerase did not produce any amplicon (data not shown).

Then, the duplex PCR (15 µl reaction volume) was optimized by changing the amount of each primer: 2.5, 5 and 10 pmol, under the KOD FX DNA polymerase


Fig. 1 Electropherogram on 0.8 % agarose gel of total DNAs extracted from imported or Japanese commercial green algal products. Samples loaded into *lanes* are: *1* CN-1a (2005), *2* KR-2a (2011), *3* JS-1a (2011), *4* JH-1a (2011), *5* JO-1a (2011), *M* 1-kbp DNA ladder marker. The numbers in parentheses after the sample abbreviations indicate the collection years

Fig. 2 Primer specificities for standard green algal species. Primer combinations are: **a** univ-F + U_comp-R, for *Ulva compressa*; **b** univ-F + U_intest-R, for *U. intestinalis*; **c** univ-F + U_linz_prol-R, for *U.* *linza/prolifera*; and **d** M_nitid-F + univ-R, for *Monostroma nitidum*. Samples loaded into *lanes* are: *1 U. compressa*, *2 U. intestinalis*, *3 U. linza*, *4 U. prolifera*, *5 M. nitidum*, *M* 100-bp DNA ladder marker

Fig. 3 Validation of internal control and specificity in duplex PCRs with the primer combinations of **a** univ-F and univ-R, for internal control; **b** univ-F, univ-R and U_comp-R, for *Ulva compressa*; **c** univ-F, univ-R and U_intest-R, for *U. intestinalis*; **d** univ-F, univ-R and U_linz_prol-R, for *U. linza/prolifera*; and **e** M_nitid-F, univ-F and univ-R, for *Monostroma nitidum*. Samples loaded into *lanes* are: *1 U. compressa* (standard), *2 U. intestinalis* (standard), *3 U. linza* (standard), *4 U. prolifera* (standard), *5 M. nitidum* (standard), *6* CN-4b (*U. linza/prolifera*), *7* CN-6a (*U. linza/prolifera*), *8* JH-1a (*U. linza/prolifera*), *9* JC-2a (*U. linza/prolifera*), *10* CN-7a (*U. pertusa*), *11* KR-2a (*U. pertusa*), *12* CN-9a (*U. fasciata*), *13* CN-10c (*U. ohnoi*), *14* KR-2c (*U. reticulata-related*), *15* JC-1a (*U. reticulata-related*), *16* to *20*, JO-1a, JO-2a, JO-3a, JO-4a and JO-5a, respectively (*M. nitidum*), *M* 100-bp DNA ladder marker. Species names in parentheses indicate the species identification results of a nrITS2-based phylogenetic analysis according to the method of Kawashima et al. [19] (see Supplementary Figs 1 and 2)
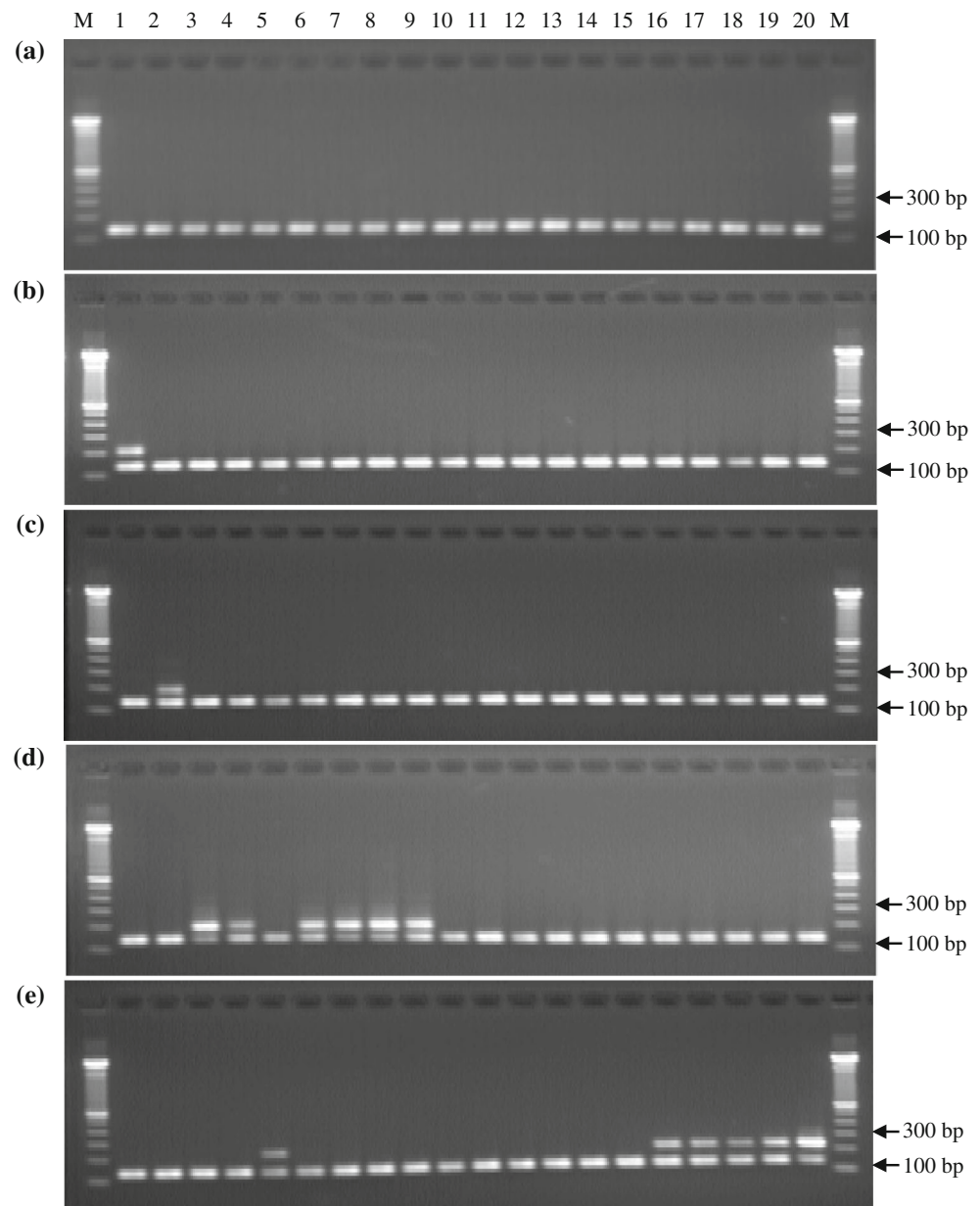
conditions at the annealing temperature of 57 °C. The optimized primer amounts for the duplex PCR (15 μl reaction volume) were, for *Ulva* species, species-specific primer of 5 pmol + the common primers (univ-F and univ-R) of 2.5 pmol each; and for *M. nitidum*, species-specific primer of 2.5 pmol + the common primers (univ-F and univ-R) of 2.5 pmol each. To improve the throughput of the duplex PCR system as much as possible, the thermal cycling program was shortened as follows: initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 7 s, and extension at 68 °C for 10 s. The optimized duplex PCRs clearly separated their target species from others by

showing two bands for positives and one band for negatives (see Fig. 3b–e).

Species identification by duplex PCR assay

To check the reliability of the developed duplex-PCRs, a pre-test using a total of 129 samples of green algal products (12 imported products and 31 Japanese commercial products) was conducted. As a result of the pre-test, the developed duplex-PCRs successfully detected the target species from the 129 samples without any inconsistency against ITS2-based sequencing identifications (see Table 2 and Supplementary Figs. 1 and 2).

Discussion

Our previous study [19] reported that nrITS2-based sequencing analysis followed by phylogenetic analysis could provide useful information for species identification of commercial green algal products and detected three import quota species, *U. linza*, *U. prolifera* and *M. nitidum*, from those samples, with findings of incorrect labeling for 19 of 44 products. On the other hand, these kinds of methods require users with higher biochemical knowledge and technical skills, together with expensive analytical instruments and reagents, in order to implement the protocols. These factors would inevitably become unfavorable for front-line inspection that has to handle a large number of samples rapidly and cost-effectively.

Taking into consideration the previous results above, we targeted five commercially important and import quota-controlled algal species (*U. linza*, *U. prolifera*, *U. intestinalis*, *U. compressa* and *M. nitidum*) which included the three algal species previously identified from commercial products, and developed four duplex-PCRs for detecting these in commercial green algal products for routine analysis purposes. Since the four duplex-PCRs work under the same thermal condition, they enable users to check commercial samples for five target algal species by executing only one thermal cycle program for about 45 min. This method gives clear test results, namely two bands for positives and one band for negatives. Furthermore, due to the small-sized PCR products of <250 bp lengths for detection, the duplex-PCR method is expected to have a higher applicability to deeply processed samples. For species identification, PCR-based detection methods, compared with sequencing-based methods or PCR–RFLP methods, generally follow simpler protocols and therefore allow users to reduce both operation time and costs. However, PCR-based detection methods with one paired primer, including real-time PCR methods, have a theoretical problem of not detecting false-negatives [24, 25]. To solve this false-negative problem, our analytical method using the duplex PCRs always shows an internal control band which covers a broad range of green algal species as an indicator of the success of the PCR reaction. False-positives resulting from contamination can be checked by performing PCR amplification with a negative control such as sterile water.

Our pre-test for the developed analytical method using a total of 129 samples of green algal products (12 imported products and 31 Japanese commercial products) was successfully conducted, as shown in Table 2. For the intra-species variations of the five target species, *U. linza*, *U. prolifera*, *U. intestinalis*, *U. compressa* and *M. nitidum*, we carefully analyzed a total of 30 nrITS2 sequences for design of their specific primers. Our duplex PCR method

allows front-line inspectors to conduct a screening test for commercial green algal products to find the five controlled algal species above easily, rapidly and cost-effectively. Accurate information concerning the raw materials of commercial green algal products is important not only for the Japanese Customs Service to ensure their proper control but also to enable general consumers to be aware of the safety and exact commercial value. We only performed an intra-laboratory test to validate this new method. Further validations such as inter-laboratory tests and repeatability tests will be needed prior to practical use.

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