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1 Rapid species identification of fresh and processed scallops
2 by multiplex PCR

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Abstract

Food control policies regarding to seafood label authenticity have become a global issue due to increased incidence of species substitution or mislabelling. Proper species-level identification in processed scallop products is hindered by the lack of morphological characters such as their valves. In order to identify four commercially important scallop species (*Argopecten purpuratus*, *A. irradians*, *Mizuhopecten yessoensis*, *Pecten albicans*) a species-specific multiplex PCR reaction is described herein. Novel reverse species-specific primers in combination with one universal forward primer designed to amplify a partial region of the mitochondrial 16S rRNA gene were assayed in fresh as well as in manufactured scallop samples. All PCR reactions showed a high specificity allowing an unambiguous species authentication.

Keywords Pectinidae • Scallop • Food control • 16S rRNA gene • Multiplex PCR

1. Introduction

Scallops are bivalve mollusks belonging to the family Pectinidae. They do not only play an important role in marine ecosystems but also have tremendous worldwide commercial importance. Scallop fisheries are distributed globally (Tracey and Lyle, 2011), being Japan the world largest scallop producer, with a total production of 565,600 tons in 2009 (FAO-Globefish, 2011). In Japan, *Mizuhopecten yessoensis* ("hotate-gai" or "Japanese scallop") is one of the most important fishery products (Sato et al., 2005) and is mainly cultured in Hokkaido prefecture. Another economically important scallop species in Japan is the baking scallop *Pecten albicans* ("itaya-gai"), which is less abundant but a substitute for the commonest *M. yessoensis*, especially in southern Japan (Wongso and Yamanaka, 1998). *M. yessoensis* naturally occurs also in the northern Korean Peninsula and the far east of Russia, and in 1982 was introduced into China to increase scallop cultivation (Hou et al., 2011). The Japanese scallop is now widely farmed in northern China, where it was accepted quickly due to its large size and high market value (He et al., 2012; Li, Xu, & Yu, 2007). The bay scallop *Argopecten irradians* is another commercially valuable species in China where it was introduced from America in 1982 (Zhang, He, Liu, Ma, & Li, 1986), and has become one of the most important aquaculture species (Li, Liu, Hu, Bao, & Zhang, 2007). The Peruvian scallop *A. purpuratus*, which is widely cultured in Peru and Chile, is a highly economically important aquaculture species and it has recently been introduced into China and hybridized with the bay scallop *A. irradians* (Wang et al., 2011). According to the Peruvian Association of Exporters (ADEX), during the year 2011, total exportation of *A. purpuratus* from Peru reached 10,409 m tons for a total value of

\$ 137 million (U.S.) dollars. France, United States and Belgium were the main destinations. In 2011, the European market was dominated by France, with 36,700 tons of scallops imported, followed by Spain (9,800 tons) Belgium and Germany (3,500 tons each; FAO-Globefish, 2012).

Globalization of the seafood market and increasing tendencies of international seafood trade are making governments around the world to enforce strict policies and new regulations (e.g. the European Council Regulation (EC) No 104/2000) in order to ensure food safety and prevent frauds (Santacarla et al., 2006; Zhan et al., 2008). Indeed, identification of food species is now a main concern not only for government entities and companies but also for consumers due to economic, regulatory, health and religious reasons. In Japan, food labelling of processed food is regulated according to the Law on Standardization and Proper Labelling of Agricultural and Forestry Products (JAS Law), product name, production site, and country of origin are required, particularly for imported products (Namikoshi, Takashima, Iguchi, Yanagimoto, & Yamashita, 2011). Seafood mislabelling is a very common issue; for example examinations performed during a nine-year period (1988-1997) by the National Marine Fisheries Service's National Seafood Inspection Laboratory (NSIL) showed that 37% of fish and 13% of other seafood were mislabelled (Buck, 2010). Manufactured scallop products are mostly commercialized without shells or in a processed form, consequently hampering species identification. According to FAO (2011), mislabelling import products (i.e. labelling Japanese scallop as USA domestic product) has been used fraudulently to obtain higher prices or to replace the product due to lack of supply. Furthermore, substituted and/or

mislabelled seafood is considered misbranded by the FDA (Food and Drug Administration) and is a violation of Federal law (FDA, 2011).

Advances in biomolecular methods have allowed the development of effective procedures for the identification of species in processed food. However, most of these procedures (e.g. FINS, PCR-RFLP, RAPD) require further secondary analyses (sequencing, endonuclease digestion, fingerprinting, phylogenetic analysis) resulting in extra time and cost. On the other hand, species-specific PCR technique has been proven to be faster, cheaper and more useful to control food authenticity and identify a large number of samples (Marshall, Johnstone, & Carr, 2007; Rodriguez et al., 2003; Wen, Hu, Zhang, & Fan, 2012). So far, nuclear markers such as microsatellite, ribosomal subunits, internal transcribed spacers (Fernández-Tajes, Freire, & Mendez, 2010; Lopez-Pinon, Insua, & Mendez, 2002; Bendezu, Slater, & Carney, 2005, Santaclara et al., 2006; Zhan et al., 2008), as well as mitochondrial markers such as COI, 16S rRNA, Cyt b genes (Bendezu et al., 2005; Colombo, Trezzi, Bernardi, Cantoni, & Renon, 2004; Jen, Yen, Liao, & Hwang, 2008; Marshall et al., 2007) have been used for the identification to species level in bivalve molluscs. However, it is well known that mitochondrial DNA is more abundant in cells than nuclear DNA. Moreover, intense heating with high-pressure conditions applied in food canning and drying process cause severe DNA degradation (Bellagamba, Moretti, Comincini, & Valfre, 2001; Pascoal, Prado, Calo, Cepeda, & Velasquez, 2005). These facts make mitochondrial DNA an easier and more effective target for PCR amplification, especially in case of tissues from heavily processed products, on which highly sensitive diagnostic procedures are required.

Consequently, we select a partial region of the mitochondrial 16S rRNA gene as a marker. Generally, the COI mitochondrial gene has been proposed as the more suitable marker for DNA barcoding among taxa (Hebert, Cywinska, Ball, & deWaard, 2003), due mainly to the existence of robust universal primers, and a relatively greater range of phylogenetic signal than other mitochondrial genes with an evolution rate three times greater than that of 12S or 16S rDNA (Feng, Li, & Zheng, 2011). However, it has been shown that the proposed universal primers for DNA barcoding are not applicable for species of the family Pectinidae (Feng et al., 2011). Moreover, it has been previously reported that mitochondrial 16S rRNA gene can be useful for the identification of bivalve species (Bendezu et al., 2005; Feng et al., 2011; Jen et al., 2008). In this study, we designed and evaluated the utility of novel species-specific oligonucleotides for the identification of four commercially important scallop species: *A. purpuratus*, *A. irradians*, *M. yessoensis* and *P. albicans*. In order to evaluate if processed food label is correct, the new primers were assayed in canned, frozen and boiled scallop products. The efficiency of the methodology and primers applied herein was increased by the application of multiplex PCR reaction designed to amplify one species-specific fragment of the mitochondrial 16S rRNA gene for each tested scallop species. Additionally, a longer specific fragment of the mitochondrial 12S rRNA gene was amplified using novel primers and used as common positive control in all multiplex PCR reactions.

2. Materials and methods

2.1. Materials

Fresh samples of *A. purpuratus* were collected in Independencia and Sechura Bay (Peru). *M. yessoensis* specimens were sampled in Funka Bay (Hokkaido, Japan) and *P. albicans* samples were collected in Shimoda Bay and Oki Island (Shimane, Japan). Samples of *A. irradians* were supplied by Dr. Linsheng Song (Institute of Oceanology, China). Approximately, 200 mg of adductor muscle was dissected, preserved in 95% ethanol and stored at -20 °C. In order to confirm the authenticity of the labelling of processed scallops, twelve samples were analyzed: three samples of canned *A. irradians* (labelled as “itaya-gai”), and three samples of canned *M. yessoensis* (labelled as “hotate-gai”) were purchased from a Japanese supermarket; three samples of boiled *A. irradians* (labelled as “itaya-gai” and imported from China), and three samples of frozen *A. purpuratus* (labelled as “concha de abanico”) were purchased from a Peruvian local market.

2.2. DNA extraction

From fresh samples, total genomic DNA was extracted using the standard phenol-chloroform protocol, quantified and adjusted to a concentration of 100 ng/μl for PCR reactions. DNA from processed scallop samples was extracted using the protocol described in Sokolov (2000) with minor modifications: briefly, 70 mg of tissue was soaked in distilled water for 10 minutes, transferred to a 1.5 ml microtube containing 400 μl of TNES buffer (10 mM Tris-HCl at pH7.4, 10 mM EDTA at pH 8, 125 mM NaCl, 0.5% SDS and 4M urea) and 10 μl of proteinase K (20mg/ml), mixed softly by vortexing and incubated for 2 h at 55 °C. Then, 75 ml of 3M sodium acetate (pH 5.2) was added and mixed by tube inverting. All samples were incubated on ice for 10 min and centrifuged at 13,500 rpm for 15

min. The supernatant was recovered and mixed with the same volume of phenol/chloroform/isoamyl alcohol (25:24:1) by tube inverting and centrifuged at 13,500 rpm for 15 min. The clear supernatant was recovered again mixed with an equal volume of isopropanol, incubated for 10 min at room temperature, and centrifuged at 13,500 rpm for 20 min. The pellet was washed twice with 70% ethanol, dried and dissolved in 100 µl of TE buffer (10mM Tris-HCl, 1mM EDTA at pH 8.0).

2.3. Primer design for species-specific mitochondrial 16S rRNA gene fragment and common mitochondrial 12S rRNA gene fragment amplification

The full mitochondrial 16S rRNA gene sequences for *M. yessoensis* (accession number: FJ595959), *A. irradians* (accession number: DQ665851), and *A. purpuratus* (accession number: HQ677600) were retrieved from GenBank database. Whereas for *P. albicans*, we determined its complete mitochondrial 16S rRNA gene sequence by “primer walking” strategy using the primer sets reported by Marín, Fujimoto, and Arai (2012) designed for the determination of the same gene in *A. purpuratus*, with two additional primers designed in this study: one specific primer Palb16S 5'-CTAAGTATAGCTCTTCGGTTGATG-3' and one degenerated primer ND1F 5'-TGTGCCGGAGCAGCttyccncgnta-3' (Table 1). PCR products were sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

To design species-specific primers for each scallop species, all complete mitochondrial 16S rRNA gene sequences were multi aligned using MEGA 5.05 software (Tamura et al., 2011). Genetic distances were calculated using the Kimura-2 parameter method. Based on interspecific variations, one species-

specific reverse primer was designed for each scallop (Table 2) according to the following criteria: a) all primers were at least 18 bp in length; b) at least three nucleotides from the extreme 3' of the primer end had to match perfectly with the complementary region of the target sequence; c) since the sterilization process in manufactured bivalves can fragment the DNA to sizes ranging from 200 to 700 bp (Fernández-Tajes et al., 2010), all primers were designed to amplify PCR products no larger than 700 bp (see Table 2). The forward primer 16S arl (Palumbi et al., 1991) was used as the common primer for all species in all PCR reactions (Table 2). Additionally, the complete mitochondrial 12S rRNA gene of *M. yessoensis* and *A. irradians* and a partial region of the same gene from *A. purpuratus* were multi aligned and two “universal” pectinid primers (12SpectU forward and reverse) were designed (Table 2) based on highly conserved regions. The fragments amplified by these novel primers were used as positive control in all multiplex PCR reactions.

2.4 Multiplex PCR amplification

Firstly, PCR amplification was standardized for each species-specific primer separately. To check the specificity of the species-specific primers, they were tested on both target and non-target species DNA. The optimum PCR conditions were the following: 100 ng of template DNA, 0.2 mM each for dNTPs, 1X Ex *Taq* buffer (TaKaRa, Japan), 0.25 μ M each for species-specific reverse and common 16S arl forward primers, 0.05 μ M each for 12SpectU forward and reverse primers, and 0.5 U of Ex *Taq* polymerase (TaKaRa, Japan) in a total volume of 20 μ l. Thermocycling conditions were: initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation for 15 s at 94 °C, annealing for 15 s

at 55 °C, and extension for 15 s at 72 °C, followed by a final extension for 7 min at 72 °C.

In order to set up a rapid and reliable species identification method from commercial scallop samples, a multiplex PCR reaction was performed. The common forward 16S rrl primer was pooled together in a single reaction with the three species-specific reverse primers for *A. irradians* (AirraR3 primer), *A. purpuratus* (ApurpR3 primer), and *M. yessoensis* (MyessoR4 primer). The positive control primers 12SpectU were used in all multiplex reactions. Different combinations of primer concentrations were tested. The optimum primer concentrations were: 0.5 µM for the common 16S rrl forward primer, 0.25 µM each for ApurpR3, AirraR3 and MyessoR4, and 0.1 µM each for 12SpectU forward and reverse primers. Except for primer concentrations, PCR conditions were the same as described above. All PCR products were electrophoresed for 35 min in a 1.5 % agarose gel, stained with ethidium bromide and visualized under UV light.

3. Results and discussion

After the alignment of the complete mitochondrial 16S rRNA gene from the scallop species studied herein, the lowest sequence divergence was found between *A. purpuratus* and *A. irradians* (14 %, 167 variable sites, data not shown). Nevertheless, this divergence allowed us to design a specific primer also for these species. In this study, species-specific primers in combination with one universal primer designed to amplify a partial fragment of the mitochondrial 16S rRNA gene were successfully assessed for a reliable identification at species level of fresh and processed scallop food samples. In the respective target species the pool consisting of the reverse species-specific primer in combination with the

forward common 16S arl primer and the universal 12SpectU forward and reverse control primers, gave one species-specific product (the mitochondrial 16S rRNA fragment) and one higher size band (the mitochondrial 12S rRNA gene fragment) amplified by the positive control primers (Fig. 1), as expected. Instead, PCR reactions containing non-target species DNA amplified only the positive control amplicon, as expected too.

The positive control fragment from mitochondrial 12S rRNA gene ranged from 616 to 660 bp, depending on species, (Fig. 1 and 2). It showed whether a failure in the amplification with species-specific primers in the absence of target DNA was truly due to the primer species-specificity or to a failure in the overall PCR reaction. Overall, the co-amplification of the positive control fragment was very consistent in all multiplex reactions that included target and non-target DNA.

No cross amplification (false positive) was observed when the species-specific primers were assayed with non-target species DNA (Fig. 1). Different PCR products were consistently amplified in fresh and processed samples of *A. irradians* (272 bp), *A. purpuratus* (344 bp) and *M. yessoensis* (425 bp) as reported in Fig. 2. All species-specific primers were successfully amplified in 29 fresh samples of *A. purpuratus*, 30 samples of *M. yessoensis*, 16 samples of *A. irradians*, and 9 samples of *P. albicans*, as well as in processed food (canned, boiled, frozen) samples of *A. purpuratus*, *A. irradians* and *M. yessoensis*. As for *P. albicans*, the complete nucleotide sequence of the mitochondrial 16S rRNA gene (1423 bp) was deposited in GenBank/DDBJ/EMBL DNA databases under the accession number JN896624. The species-specific primer designed for *P. albicans* (PalbiR2) in combination with the universal 16S arl primer produced a 270 bp size

amplicon. By using our species-specific primers, all commercial food samples labelled as “concha de abanico” (*A. purpuratus*, frozen) and “hotategai” (*M. yessoensis*, canned) were found to contain the species declared in their labels. Regarding to the product labelled as “itayagai”, three canned and three boiled samples (from Japan and China, respectively) were identified as *A. irradians* (bay scallop). The use of the same common name for different species is a frequent issue in marine processed food, especially when referred to products obtained from foreign species. In fact, the same vernacular name can be referred to different species or a single species may be diversely labelled depending on the region or country (Barbuto et al., 2010). For example, the baking scallop *P. albicans* is widely known as “itayagai” in Japan, but the bay scallop *A. irradians* is also known by the same vernacular name and sometimes referred as “America-itayagai” (Takahashi and Muroga, 2008). Our results thus suggested that Chinese bay scallop exported to Japan are sometimes labelled as “itayagai” due to unintended misconception, which may cause confusion among consumers. The use of similar names for different products introduced from one country to another where a market niche already exists makes it difficult to enforce rules, even if national food legislations indicate that the label must not mislead consumers (FAO-Globefish, 2012). However, it must be specified that a real misleading can be considered when there is a difference of quality traits among different species. Thus, some species can have the same commercial name if there are no reasons for giving them different names. Moreover, FDA generally recommends use of common name as the market name unless a common or usual name has been established by regulation or law.

This study highlights the importance of an accurate labelling of seafood products and the results demonstrated the specificity of the novel species-specific primers used and multiplex PCR technique for the forensic identification of scallop food products. This simple, inexpensive, timesaving, and powerful single step method can be reproduced easily by any basic molecular genetics laboratory. Progress in the area of authentication of traded seafood products requires the use of molecular tools to ensure a proper species identification, thus enhancing the application of effective food control regulations and consumer protection.

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447

448 Fig 1. Agarose gel electrophoresis of PCR products amplified using species-specific
449 primers in fresh scallop samples. Lanes 1a to 4a: 16Sar1/AirraR3. Lanes 1b to 4b:
450 16Sar1/ApurpR3. Lanes 1c to 4c: 16Sar1/MyessoR4. Lanes 1d to 4d:
451 16Sar1/PalbiR2. Lanes 1a, 1b, 1c, 1d: *A. irradians* DNA template. Lanes 2a, 2b, 2c,
452 2d: *A. purpuratus* DNA template. Lanes 3a, 3b, 3c, 3d: *M. yessoensis* DNA template.
453 Lanes 4a, 4b, 4c, 4d: *P. albicans* DNA template. M: 100 bp ladder, C-: negative
454 control.

455

456 Fig 2. Agarose gel electrophoresis of multiplex PCR products amplified using
457 species-specific primers in fresh and processed scallop samples. All species-
458 specific primers were included in every single reaction. Lanes 1 to 4: *A. irradians*
459 DNA templates (lanes 1 and 2: fresh, lanes 3 and 4: boiled). Lanes 5 to 8: *A.*
460 *purpuratus* DNA templates (lanes 5 and 6: fresh, lanes 7 and 8: frozen). Lanes 9 to
461 12: *M. yessoensis* DNA templates (lanes 9 and 10: fresh, lanes 11 and 12: canned).
462 A partial fragment of the mt 12S rRNA gene (616-660 bp) was used in all
463 multiplex PCR reactions as a positive control. M: 100 bp ladder, C-: negative
464 control.

465

Table 1. Primers used to determine the complete mitochondrial 16S rRNA gene sequence in *P. albicans*.

Primer name	Direction	Sequence (5'-3')	Source
16S arl	Forward	CGCCTGTTTAACAAAAACAT	Palumbi et al. (1991)
16S R	Reverse	CCGRTYTGAACCTCAGCTCACG	Puslednik and Serb (2008)
ND1F	Forward	TGTGCCGGAGCAGCttyccncgnta	This study
CO1AB	Reverse	GGTGCTGGGCAGCcayatnccngg	Marín et al. (2012)
Palb16S	Forward	CTAAGTATAGCTCTTCGGTTGATG	This study
16SCC	Forward	GCGTAATCCGTCTTGACAGT	Marín et al. (2012)

Table 2. Species-specific, common and universal primers used in multiplex PCR.

Species	Primer name	Sequence	Fragment size (bp)
<i>A. irradians</i>	AirraR3	5'-AAAGTAGCCTCCCACTATTCT-3'	272
<i>A. purpuratus</i>	ApurpR3	5'-GCTCACCAGCACTTAGAA-3'	344
<i>M. yessoensis</i>	MyessoR4	5'-CGTATCCGTTTAGTGATTA-3'	425
<i>P. albicans</i>	PalbiR2	5'-GCCTTGGAAGTTCCCAATAACT-3'	270
Forward common	16S arl	5'-CGCCTGTTTAACAAAAACAT-3'	–
	12SpectUF forward	5'-AGTCCAACCAGGTGCCAGCA-3'	–
	12SpectUR reverse	5'-GAGAGCGACGGGCAGTTTGT-3'	–