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Rapid species identification of fresh and processed scallops 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*, *Argopecten 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.

1. Introduction

Scallops are bivalve molluscs 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 & 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* ("hotategai" 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* ("itayagai"), which is less abundant but a substitute for the commonest *M. yessoensis*, especially in southern Japan (Wongso & 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 *Argopecten 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 (9800 tons) Belgium and Germany (3500 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 (Santaclara 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, 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

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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 (Bendezu, Slater, & Carney, 2005; Fernández-Tajes, Freire, & Mendez, 2010; Lopez-Pinon, Insua, & Mendez, 2002; 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 (Shizuoka, Japan) and Oki Islands (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 analysed: three samples of canned *A. irradians* (labelled as "itayagai"), and three samples of canned *M. yessoensis* (labelled as "hotategai") were purchased from a Japanese supermarket; three samples of boiled *A. irradians* (labelled as "itayagai" 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 min, 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 4 M urea) and 10 μl of proteinase K (20 mg/ml), mixed softly by vortexing and incubated for 2 h at 55 °C. Then, 75 ml of 3 M 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 (10 mM Tris–HCl, 1 mM 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 (2013) designed for the determination of the same gene in *A. purpuratus*, with two additional primers designed in this study: one specific primer Palb16S 5′-CTAAGTATAGCTCT TCGGTTGATG-3′ and one degenerated primer ND1F 5′-TGTGCCGG AGCAGCttyccncgnta-3′ (Table 1). PCR products were sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

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	CCGRTYTGAACTCAGCTCACG	Puslednik and Serb (2008)
ND1F	Forward	TGTGCCGGAGCAGCttyccncgnta	This study
CO1AB	Reverse	GGTGCTGGGCAGCcayatnccngg	Marín et al. (2013)
Palb16S	Forward	CTAAGTATAGCTCTTCGGTTGATG	This study
16SCC	Forward	GCGTAATCCGTCTTGACAGT	Marín et al. (2013)