Specific Real-time PCR Detection of Monkfish Ingredients

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This study was to establish the optimal real-time PCR method for specific identification of puffer fish and monkfish. In this study, we designed the specific primers and probes for fish 12s and monkfish cytochrome oxidase subunit I (COI), and used them for specific identification of fish and monkfish. Our results showed that the specific primers and probes for monkfish COI could specifically identify 3 monkfish species, respectively, from total 42 samples of aquatic products. Moreover, the primers and probes for COI were very sensitive to detect 0.01% *Lophius litulon* content in the mixed sample of *Theragra chalcogramma* and *Lophius litulon*. Therefore, this method is simple, practical, sensitive and specific; is helpful and useful to identify the products of fish and monkfish.

Keywords: monkfish, real-time PCR, detection

Introduction

The aquatic products are important sources of high protein food, and the business on aquatic products is an important part of globe economy. There are a variety of edible aquatic products, which mainly include 25 populations in international trade (Voorhees *et al.*, 2008). According to U.S. Food and Drug Administration (FDA), over 1700 products are included in the list of aquatic products. As the development of global trade and improvement of seafood processing, some seafood companies usually labeled low value seafood with high value seafood to get high profit, for example, the farm-raised trout was mislabeled with the atlantic salmon, the tilapia and dolphin fishes were mislabeled with the red snapper, etc. (Jacquet and Pauly 2008). According to a 9-year report from National Seafood Inspection Laboratory (NSIL), 37 percent of fish products and 13 percent of other seafood products were mislabeled¹⁾. Recent market investigations in Europe and North America showed that the mislabeling rate for raw materials of aquatic products was 15 – 43% (Wong and Hanner 2008; Espineira *et al.*, 2009), while the mislabeling rate for red snapper products was as high as 75% (Marko *et al.*, 2004). Except for economic loss, the mislabeling has raised serious problems in the protection of endangered species, food safety and economic order.

Identification of aquatic products has become an important issue for their processing companies. The European Union has implemented regulations to force manufacturers to provide the official commercial and scientific name, the origin, and the production method for all fishery and aquaculture products. The US FDA did not allow the evangelical makeover in aquatic products. Since December, 2011, Canadian Food Inspection Agency and importers employed the DNA testing on seafood to avoid the mislabeling of high value species.

To avoid the fraudulent behavior and ensure food safety, it is necessary to identify the materials of aquatic products. Although it is easy to identify the species of unprocessed aquatic products according to their different appearance and morphological characters, it is necessary to use the DNA testing method to identify the processed aquatic products, such as, minced fish, grilled fish fillet, sashimi, canned fish, fish powder, etc. In the present study, we employed the gene databases from European Molecular Biology Laboratory (EMBL), BOL and FISHTTRACE to compare the specific genes for the puffer fish and monkfish. Among candidate genes, we selected the cytochrome oxidase subunit I (COI) as the biomarkers for the monkfish, respectively; while the 12s gene was used the biomarker for fish. The specific primers and probes for above two genes were designed, and the conditions for real-time PCR were optimized to specifically amplify the target gene in different samples.

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Materials and Methods

Samples Total 42 samples listed in Table 1 were used in this study, which include 3 monkfish species (*Lophius litulon*, *Lophiomus setigerus*, *Lophius americanus*); 9 puffer fishes (*Lagocephalus inermis*, *Lagocephalus lagocephalus*, *Takifugu fasciatus*, *Takifugu xanthopterus*, *Takifugu oblongus*, *Takifugu rubripes*, *Gastrophysus gloveri*, *Gastrophysus lunaris*, *Gastrophysus spadiceus*); 24 other fish species; 1 *Tremoctopus violaceus*; 1 snow crab (*Chionoecetes bairdi*); 2 shrimp species; 2 shellfish species. The samples of cooked fish-ball, fish head can, surimi, dried fish floss, roasted fish fillet made from monkfish were supplied by Dalian Ocean Fishery Group Ocean.

DNA extraction DNA from samples was isolated using revised high salt method (Marko *et al.*, 2004). Briefly, samples were grinded with liquid nitrogen and then 150 mg samples were mixed with 400 μ L TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA), 40 μ L 10% SDS, and 8 μ L 20 mg/mL proteinase K. The mixture was digested at 55 – 65°C for 4 h and mixed with 300 μ L 6 mol/L NaCl. After centrifugation at 12000 rpm/min for 30 min, the supernatant was collected and mixed with the same volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) for 1 min. The mixture was centrifuged at 12000 rpm/min for 10 min, then the supernatant was collected and mixed with the same volume of chloroform/isoamyl alcohol (24:1, v/v). The supernatant was collected after centrifugation and mixed with the same volume of isopropyl alcohol and one-tenth volume of 3 M NaAc

for 30 min on ice, followed by centrifugation at 12000 rpm/min for 10 min. The DNA pellet was washed with 800 μ L 70% ethanol and dried out at 56°C. The DNA was dissolved in 50 μ L TE buffer and stored at –20°C. The concentration from 10 μ g/mL – 100 μ g/mL and A260/A280 ratio from 1.7 – 1.9 were taken as the DNA templates of real-time PCR.

Design of primers and probes The specific primers and probes for fish 12s and monkfish COI were designed by DNAMAN 8.0 software, and the sequences of primers and probes were listed in Table 2. For design of primers and probes, the consistence of annealing temperature and similarity of GC content were considered. The primers and probes were synthesized by Takara Biotechnology (Dalian) Co., Ltd.

Real-time PCR The PCR was performed in a 25 μ L reaction mixture containing 2 μ L DNA (10 μ g/mL–100 μ g/mL), 1 μ L forward primer (10 μ mol/L), 1 μ L reverse primer (10 μ mol/L), 1 μ L probe (5 μ mol/L), 0.5 μ L Taq DNA polymerase (5 U/ μ L), 1 μ L dNTP (10 μ mol/L), 2.5 μ L 10 $\times$ PCR buffer, 16 μ L dH₂O. The PCR reaction was programmed at 1 cycle of 95°C for 10 s and 40 cycles of 95°C for 5 s, 52°C for 10 s and 72°C for 34 s.

Experiments for specificity and sensitivity of detection method The DNA from all samples were used for the specific detection of fish 12s and monkfish COI, while the DNA samples from *Lophius litulon* and *Theragra chalcogramma* were used for the sensitivity detection of COI primers. For the sensitivity detection experiment, the samples for *Lophius litulon* and *Theragra chalcogramma* were minced and dried

Table 1. Name of samples.

No.	sample name	No.	sample name
1	<i>Lophius litulon</i>	22	<i>Epinephelus awoara</i>
2	<i>Lophiomus setigerus</i>	23	<i>Sardinella longiceps</i>
3	<i>Lophius americanus</i>	24	<i>Epinephelus coioides</i>
4	<i>Lagocephalus inermis</i>	25	<i>Pseudosciaena crocea</i>
5	<i>Lagocephalus lagocephalus</i>	26	<i>Pseudosciaena polyatics</i>
6	<i>Takifugu fasciatus</i>	27	<i>pentagonian tooth fish</i>
7	<i>Takifugu xanthopterus</i>	28	<i>Reinhardtius hippoglossoides</i>
8	<i>Takifugu oblongus</i>	29	<i>Cyprinus carpio</i>
9	<i>Takifugu rubripes</i>	30	<i>Tilapia nilotica</i>
10	<i>Gastrophysus gloveri</i>	31	<i>Argyrosomus argentatus</i>
11	<i>Gastrophysus lunaris</i>	32	<i>Mylopharyngodon piceus</i>
12	<i>Gastrophysus spadiceus</i>	33	<i>Pleuronectes platessa</i>
13	<i>Gadus macrocephalus</i>	34	<i>Schilbe intermedius</i>
14	<i>Melanogrammus aeglefinus</i>	35	<i>Regalecus glesne</i>
15	<i>Theragra chalcogramma</i>	36	<i>Heemisanx prognathus</i> Regan
16	<i>Scomber scombrus</i>	37	<i>Tremoctopus violaceus</i>
17	<i>Clupea harengus</i> L.	38	<i>Chionoecetes bairdi</i>
18	<i>Anoplopoma fimbria</i>	39	<i>Pandalus borealis</i>
19	<i>Thunnus alalunga</i>	40	<i>prawn</i>
20	<i>Oncorhynchus</i>	41	<i>Mercenaria mercenaria</i> Linnaeus
21	<i>Epinephelus akaara</i>	42	<i>patinopecten yessoensis</i>

Table 2. Sequences of primers and probes.

gene name		sequences	product length (bp)
fish 12s	forward primer	5'-GCCCAATATACGAAAGTAG-3'	217
	reverse primer	5'-GGGTTATCGGTTCTAGAA-3'	
	probe	5'-FAM-CTAAGGCACCGTCAGGTCCT-Eclipse-3'	
monkfish COI	forward primer	5'-CTCTTAGGGGATGACCA-3'	162
	reverse primer	5'-CCGATCATAATTGGTATAACC-3'	
	probe	5'-FAM-CGTTATTGTTACCGCACATGCC-TAMRA-3'	

out overnight at 80°C. Then all samples were grinded into powder, and we added different ratios (100%, 10%, 1%, 0.5%, 0.1%, 0.01%, 0.001% (w/w)) of the *Lophius litulon* powder into the *Theragra chalcogramma* powder. The DNA from mixed samples was extracted and used as the template for the sensitivity detection of COI primers by real-time PCR. All experiments were repeated for 6 times.

Results

Amplification of fish 12s by real-time PCR All samples were used to amplify the fish 12s using specific primers and probe and result was shown in Fig. 1. Using real-time PCR, the growth curve for fish 12s was obtained in all 36 fish samples, while negative result was found in water control and DNA samples from 6 other seafood products including *Tremoctopus violaceus*, *Chionoecetes bairdi*, *Pandalus borealis*, prawn, *Mercenaria mercenaria* Linnaeus, and *patinopecten yessoensis*. These results suggest that the primers for fish 12s are good for identifying the fish species and their processing

products.

Specific amplification of monkfish COI using real-time PCR The specific primers and probe for monkfish COI were used to identify the monkfish species in all 42 samples and results were shown in Fig. 2. We found the growth curve for COI was only detected in DNA samples from 3 monkfish species (*Lophius litulon*, *Lophiomus setigerus*, *Lophius americanus*), while negative results were obtained from 39 other DNA samples and water empty control. These data suggest that the primers and probe for COI are specific to identify the monkfish species. All the specific tests were repeated 6 times. Meanwhile, the repeatability of results were all fine.

Sensitivity of detection method Since the processing procedure may affect the DNA quality of fish samples and some retailers may have fraudulent behavior in some processed aquatic products by mixing low value seafood with high value seafood, these situations need a sensitive method to identify the exact components of processed products. To

Fig. 1. Illustration for specific amplification of fish 12s using real-time PCR.

Fig. 2. Specific amplification of monkfish COI DNA fragment using real-time PCR.

Fig. 3. Sensitivity of real-time PCR method for monkfish COI.

1, 100% *Lophius litulon* powder; 2, 10% *Lophius litulon* powder; 3, 1% *Lophius litulon* powder; 4, 0.5% *Lophius litulon* powder; 5, 0.1% *Lophius litulon* powder; 6, 0.01% *Lophius litulon* powder.

identify the sensitivity of this method, here we added different ratios (100%, 10%, 1%, 0.5%, 0.1%, 0.01%, 0.001%) of *Lophius litulon* powder into the *Theragra chalcogramma* powder and detected the DNA of monkfish COI in different mixed samples. As shown in Fig. 3, we found the specific band of monkfish COI was detected in all mixed samples except 0.001% *Lophius litulon* powder, suggesting that this method for monkfish COI is very sensitive to detect as low as 0.01% *Lophius litulon* powder in the mixed powder. From

the DNA extraction to real-time PCR amplification, all the specific tests were repeated 6 times. Only 0.001% monkfish powder appeared positive results 1 times, and negative results 5 times. The test results of other samples of different concentration were all positive.

Real food testing The food made by fish is more delicious and convenient to eat. In order to verify the effectiveness of this method, the samples of cooked fish-ball, fish head can, surimi, dried fish floss, roasted fish fillet made from

monkfish were used as test specimens. All of the results were positive, which demonstrated the effectiveness of this method.

Discussion

Because the authentication of aquatic products is helpful to maintain fair trade and protect food safety and endangered species, many researchers work in this field and have developed some applicable methods. Espineira *et al.* (2008a, 2008b, 2009) developed a method for the authentication of sapper, tilapia, scombroid, anglerfish, flatfish and salmon, by means of FINS (Forensically Informative Nucleotide Sequencing) technique, which is based on PCR followed by a phylogenetic analysis. DNA barcoding project is an important part of FINS technology, which is a global program that uses the COI sequences to identify the species. To date, this method can accurately identify 98% of marine fish species and 93% of freshwater fish species (Ward *et al.*, 2009). Some researchers used the PCR-RFLP (restriction fragment length polymorphism) technology to identify the processed products of salmon, trout, anchovy and puffer fish (Rasmussen *et al.*, 2009; Rea *et al.*, 2009; Hsieh *et al.*, 2010), while the PCR-SSCP (single strand conformation polymorphism) method was used to identify the species of salmon, sardine, eel, tunny, skipjack, sturgeon, etc (Rehbein *et al.*, 1997; Rehbein *et al.*, 1999; Rehbein *et al.*, 2002). In addition, Lowenstein *et al.* (2009) used the RAPD (random amplification of polymorphic DNA) technology for genetic analysis and identification. Recent studies reported that the multiplex PCR method was successfully used to identify shark, flatfish, skipjack, mackerel, salmon and trout (Lin and Wang, 2008; Mendonca *et al.*, 2010; Rasmussen Hellberg *et al.*, 2010; Catanese *et al.*, 2010). In 2002, Taylor *et al.* developed a multiplex, single tube (TaqMan) PCR assay for the identification of three commercially important gadoid species, the cod (*Gadus morhua* L.), the haddock (*Melanogrammus aeglefinus* L.) and the whiting (*Merlangius merlangus* L.) (Taylor *et al.*, 2002). After that, researchers successfully employed TaqMan MGB probes to identify eel, tunny, salmon and trout, and Taqman LNA probes for identifying sea bream and American redfish (Itoi *et al.*, 2005; Lopez and Pardo 2005; Mort *et al.*, 2008). In 2004, BioMérieux announced the launch of FoodExpert-ID that is the first high-density DNA chip for food and animal feed testing and can detect the raw materials and processed products from 33 different species of vertebrate, including 15 fish species. Chisholm *et al.* employed the bioMérieux FoodExpert-ID system to identify the species of food products and verified its applicability (Chisholm *et al.*, 2008). In addition, Kochzius *et al.* created a "Fish Chip" to differentiate 11 important fish species from European seas (Kochzius *et al.*, 2008).

Each method has its own advantages and disadvantages: PCR sequencing is not remarkable in speed and cost; RCP-RFLP is time-consuming due to many analysis steps; high sensitivity of SSCP requires high repeatability and consistency of experiments; the disadvantage of PCR-RAPD is its repeatability, which disadvantage is more severe when the DNA sample is less or degraded (Rego *et al.*, 2002). In addition, the AFLP method is very time-consuming so as to affect its large-scale application. Compare to other methods, the DNA chip is very costly and slow to analyze samples. Although the specific primers for specific species limit the application of real-time PCR method to general species, this method has the characteristics of low cost and easy manipulation, is extensively used for identifying the specific species from mixed samples.

It is difficult to differentiate the fillet and fish paste made from monkfish by morphological character. To ensure food safety, it is very imperative to select the appropriate gene and develop a practical and feasible real-time PCR method to identify the products of monkfish. In the present study, the real-time PCR methods are very specific, sensitive, simple, quick, and practical, and provide effective tools for authentication of the monkfish species.

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