Journal of Food and Drug Analysis, Vol. 20, No. 4, 2012, Pages 839-843 doi:10.6227/jfda.2012200413

Identification of Species in Commercial Frozen Shrimp Meat in Taiwan

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(Received: September 2, 2011; Accepted: May 31, 2012)

ABSTRACT

Attempts were made to identify species in commercial frozen shrimp meat samples collected in Taiwan. First, approximately 20 fresh specimens of each of the 10 common commercial shrimp species were identified by morphological methods. A 401 to 407-bp fragment of the cytochrome *b* gene was amplified from the shrimp meat by polymerase chain reaction (PCR) using the primer pair UCYTB151F/270R. By comparing the gene sequences and PCR-restriction fragment length polymorphism (RFLP) maps obtained using the endonucleases *Alu* I, *Ssp* I and *Hae* III, the 10 species of shrimp could be successfully differentiated. The PCR-RFLP method was proved applicable for the species identification of 10 marketed shrimp samples.

Key words: PCR-RFLP, shrimp, sulfur dioxide, boric acid, species identification, primer, cytochrome *b* gene

INTRODUCTION

Methods used to identify shrimp species include traditional morphological identification, electrophoresis, liquid chromatography, immunoassay, isoelectric focusing, and biological technologies[1,2]. Among these, the most promising and reliable approach is the use of DNA biomarkers because these techniques are easily adapted into routine surveys[3] and can also be applied to identify the species present in raw material and to identify the adulteration of processed products. As a result of the rapid development of molecular biology techniques, DNA biomarkers have become important in species identification[4].

Because mitochondrial DNA (mtDNA) is highly conserved[5] and is often used to analyze molecular evolution. In recent years, it has been applied to detect the adulteration of market products[6,7]. The cytochrome *b* gene (*Cyt b*) is a functional gene between the $tRNA^{Glu}$ and $tRNA^{Thr}$ genes in mtDNA, which encodes a component of cytochrome *c* oxidoreductase, a complex enzyme involved in oxidative phosphorylation. Many studies on the vertebrate *Cyt b* gene have been focused on inheritance and evolution[8].

At present, DNA-related techniques are being utilized to authenticate species in market products, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, species-specific PCR, PCR-random amplified polymorphic DNA (RAPD) and PCR-single strand conformation polymorphism (SSCP)[9,10]. Due to its high sensitivity, speed and simplicity, PCR-RFLP has often been applied to identify the adulteration of food products[11-13]. This technique is based on the specificity of a gene sequence, and PCR can be employed to amplify a particular sequence from the processed products. PCR techniques followed by sequence analysis have been applied for the identification of the species contained in toxic and nontoxic processed puffer fish products[14-16]. Recently, PCR-RFLP was also applied in the identification of shrimp species[12,17]. In this study, PCR-based sequence analysis has been employed to establish a restriction map for shrimp species. Commercial frozen shrimp meat samples collected from markets in Taiwan were examined.

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MATERIALS AND METHODS

I. *Samples*

Fresh samples from 10 species of commercial shrimp (about 20 specimens per species) were collected from different seafood markets in Keelung and Taipei, Northern Taiwan. These species were identified by co-author Dr. Tan-Zen Chen, Professor of the Institute of Marine Biology, National Taiwan Ocean University, according to traditional morphological methods. These shrimp species included

brine shrimp *Penaeus monodon, Aristaeomorpha foliacea, Metapenaeus ensis, Solenocera melantho, Metapenaeopsis harbata, Penaeus japonicus, Parapenaeus fissuroides, Litopenaeus vannamei* and *Sergia lucens,* and freshwater shrimp *Macrobrachium rosenbergii.* These shrimps were eviscerated, and their meat samples were kept at -20°C for DNA extraction. The other 10 frozen shrimp meat samples (about 300 g for each sample) were collected from supermarkets and mall markets in northern Taiwan, and were also kept at -20°C for DNA extraction.

II. *DNA Extraction*

DNA extraction was performed using magnetic beads (Chemagic DNA Tissue 10 Kit, Chemagen, Baesweiler, Germany). Approximately 0.2 g of each sample was homogenized with lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 10% SDS, 0.2 M NaCl), and 100 μL of 10 mg/mL proteinase K (Amereses, Solon, OH, USA) was added, followed by incubation at 55°C for 4 h with shaking. After lysis was completed, the magnetic beads were added and slightly shaked. At the end of incubation, magnetic beads with bound DNA were recovered using a magnetic separator. The mixture was then washed twice with washing buffer supported by Chemagen (Baesweiler, Germany). Finally, the magnetic beads were removed from the solution and the genomic DNA was eluted with 50 μL of elution buffer.

III. *PCR Primers*

The set of primers (UCYTB151F/270R) described in a previous report[18] was used for PCR amplification of each shrimp species and sample. The sequences of the primers are as follows: UCYTB151F: 5'-TGTGGRGCNACYGTWATYACTAA-3'; 270R: 5'-AANAGGAARTAYCAYTCNGGYTG-3'.

IV. *PCR Amplification*

PCR amplification was performed in a total volume of 50 μL. Each reaction mixture contained 100 ng of extracted template DNA, 0.2 μM of each primer, 200 μM of each dNTP, 2 U Pro *Taq* DNA polymerase (Promega, Madison, WI, USA), and PCR buffer (20 mM Tris-HCl, 15 mM $MgCl_2$, 10% Triton X-100, 0.1 mM EDTA, 1 mM DDT and 50% glycerol).

PCR was carried out in a Gene-Amp PCR system 2400 (Perkin-Elmer, Foster, CA, USA) programmed to perform a denaturation step at 94°C for 4 min, followed by 40 cycles at 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. The final extension step was at 72°C for 6 min, and then products were stored at 4°C.

V. *PCR Product Analysis and Sequencing*

PCR products (5 μL) were loaded onto a 2% agarose gel (containing Healthview DNA dye, 0.02 μL/mL) in TBE buffer and electrophoresed at 100 V for 40 min. DNA bands were detected under ultraviolet light and photographed by Image Master VDS (Pharmacia Biotech, Piscataway, NJ, USA). The products were sequenced at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI Prism BigDye Terminator B3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 377 sequencer (Applied Biosystems).

VI. *Restriction Site Analysis of PCR Products*

For restriction enzyme analysis of the *Cyt b* gene region, sites for the endonucleases *Alu* I, *Hae* III and *Ssp* I (Promega) were found in our sequences from the GCG system by uploading our sequences and testing for restriction analysis of the amplified PCR products. The amplified DNA fragments of the PCR products were used without purification. Digestions were performed with 5 μL of amplified DNA, 5 U enzyme, and a 1 : 10 dilution of the manufacturer's recommended 10X digestion buffer. Digestion mixtures were incubated at 37°C for 6 h. The results were analyzed by electrophoresis performed on a 3% agarose gel (containing Healthview DNA dye, 0.02 μL/mL) in TBE buffer under 100 V for 45 min and then analyzed as described above.

RESULTS

The PCR primers UCYTB151F/270R were found to be able to specifically amplify the partial mtDNA fragments of the *Cyt b* gene from the 10 fresh shrimp species studied. After DNA sequencing, the fragments from different shrimp species were obtained with lengths of 401 bp for *P. fissuroides*, 403 bp for *L. vannamei*, 404 bp for *P. japonicus*, 405 bp for *A. foliacea, M. ensis and M. barbata*, 406 bp for *P. monodon*, *S. melantho* and *M. rosenbergii*, and 407 bp for *S. lucens*. The

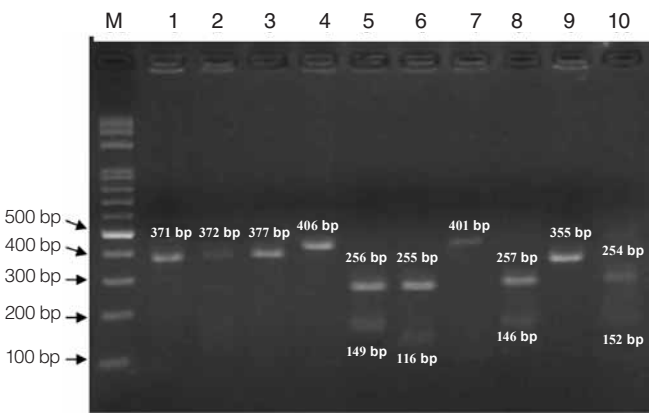

Figure 1. Electrophoretic analysis of the PCR products of fresh shrimp meat digested with *Alu* I on 3% agarose gel. M, 100-bp ladder; lane 1, *Penaeus monodon*; lane 2, *Aristaeomorpha foliacea*; lane 3, *Metapenaeus ensis*; lane 4, *Solenocera melantho*; lane 5, *Metapenaeopsis barbata*; lane 6, *Penaeus japonicus*; lane 7, *Parapenaeus fissuroides*; lane 8, *Litopenaeus vannamei*; lane 9, *Sergia lucens*; lane 10, *Macrobrachium rosenbergii*.

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five *cyt-b* mitochondrial sequences available for *P. fissuroides* (accession number: bankit 1322661), *A. foliacea* (accession number: bankit 1322734), *M. ensis* (accession number: bankit 1322745), *M. barbata* (accession number: bankit 1325277), *S. melantho* (accession number: bankit 1325271), *S. lucens* (accession number: bankit 1322777) have been deposited into the GenBank and other shrimp accession numbers have already existed in the GenBank. Furthermore, we also observed that the pair of primers UCYTB151F/270R were useful to amplify the partial *Cyt b* gene even when these shrimp samples were heated at 100 and 121°C for 15 and 30 min, respectively, representing conditions in which the samples were subjected to either heating or sterile processing under which their *Cyt b* genes should be less degraded.

The PCR products from the 10 species of fresh shrimp samples were digested by the endonucleases *Alu* I, *SspI* and *Hae III*, and the results are shown in Figure 1-3. The products from *S. melantho* and *P. fissuroides* had no cutting site, while those of *P. monodon*, *A. foliacea*, *M. ensis*, *M. barbata*, *L. vannamei* and *M. rosenbergii* had one cutting site, and *P. japonicus* had two cutting sites. After digestion by the endonuclease *Alu* I (Figure 1), different groups became evident, including a first group comprised of *P. monodon*, *A. foliacea*, *M. ensis*, a second group of *S. melanth* and *P. fissuroides*, and a third group of *M. barbata, L. vannamei, M. rosenbergii*, *P. japonicas* and *S. lucens*. When PCR products from *P. fissuroides* and *S. melantho* were digested with endonuclease *Ssp* I, *P. fissuroides* had one cutting site and *S. melantho* no cutting site (Figure 2). Finally, when the PCR products of the first group and the third group were separately digested with the endonuclease *Hae* III, the three shrimp species in each group could be clarified (Figure 3). In conclusion, these 10 species of shrimp can be differentiated by digestion of their PCR products using the endonucleases *Alu* I*, Ssp* I and *Hae* III stepwise (Table 1).

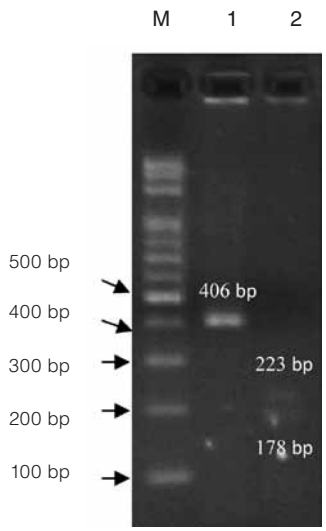

Figure 2. Electrophoretic analysis of the PCR products of fresh shrimp meat digested with *Ssp* I on 3% agarose gel. M, 100-bp ladder; lane 1, *Solenocera melantho*; lane 2, *Parapenaeus fissuroides*.

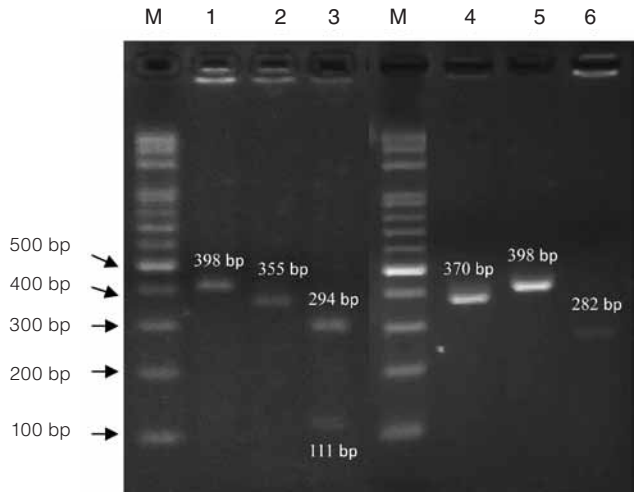

Figure 3. Electrophoretic analysis of the PCR products of fresh shrimp meat digested with *Hae* III on 3% agarose gel. M, 100-bp ladder; lane 1, *Penaeus monodon*; lane 2, *Aristaeomorpha foliacea*; lane 3, *Metapenaeus ensis*; lane 4, *Metapenaeopsis barbata*; lane 5, *Litopenaeus vannamei*; lane 6, *Macrobrachium rosenbergii*.

Table 1. Summary of restriction fragments differentiating between ten shrimp species after digestion of PCR products using the primers UCYTB151F/270R

Family	Species name	PCR product (bp)	Restriction enzymes/Fragments (bp)		
			Alu I (Stage I)	*Hae* III (Stage II)	*Ssp* I (Stage III)
Penaeidae	*Penaeus monodon*	406	35 + 371	8 + 398	
	Aristaeomorpha foliacea	405	33 + 372	50 + 355	
	Metapenaeus ensis	405	28 + 377	111 + 294	
	Solenocera melantho	406	406		406
	Metapenaeopsis barbata	405	149 + 256	35 + 370	
	Penaeus japonicus	404	33 + 116 + 255		
	Parapenaeus fissuroides	401	401		178 + 223
	Litopenaeus vannamei	403	146 + 257	5 + 398	
Sergestidae	*Sergia lucens*	407	52 + 355		
Palaemonidae	*Macrobrachium rosenbergii*	406	152 + 254	8 + 29 + 87 + 282	

Table 2. Species of 10 frozen shrimp samples collected from different commercial markets determined by using DNA sequence analysis and the PCR-RFLP method

No.	Labeled name	DNA sequence analysis	PCR-RFLP method
1	*Penaeus monodon*	*P. monodon*	*P. monodon*
2	*Litopenaeus vannamei*	*L. vannamei*	*L. vannamei*
3	*Litopenaeus vannamei*	*L. vannamei*	*L. vannamei*
4	shrimp	*L. vannamei*	*L. vannamei*
5	shrimp	*L. vannamei*	*L. vannamei*
6	shrimp	*L. vannamei*	*L. vannamei*
7	*Parapenaeus fissuroides*	*P. fissuroides*	*P. fissuroides*
8	shrimp	*P. fissuroides*	*P. fissuroides*
9	*Litopenaeus vannamei*	*L. vannamei*	*L. vannamei*
10	*Litopenaeus vannamei*	*L. vannamei*	*L. vannamei*

The PCR primers UCYTB151F/270R were able to specifically amplify the partial mtDNA fragments of the *Cyt b* gene from the 10 frozen shrimp meat samples collected from supermarkets and mall markets. After DNA sequence and PCR-RFLP analyses, the species present in these 10 frozen shrimp meat samples were identified as *Penaeus monodon, Litopenaeus vannamei* and *Parapenaeus fissuroides*. Six out of the ten tested frozen shrimp meat samples were labeled as the same shrimp species as we identified in the samples. The other unlabeled samples were identified as *L. vannamei* in three tested samples and *P. fissuroides* in one tested sample (Table 2).

DISCUSSION

As reported by previous investigators, the reasons that the *Cyt* b gene is a good molecular marker for examining species contained in seafood products include: (1) it is highly conserved; (2) mitochondrial DNA in total cell nucleic acid is abundant; (3) its preparation results in more effective PCR amplification[19,20]. PCR-RFLP is also a quick and effective method to identify the species in seafood products[2,21,22]. Here, we found that the primers UCYTB151F/270R were able to successfully amplify the partial *Cyt* b gene from 10 shrimp species that are common and important in the markets of Taiwan. Furthermore, direct sequence analysis and restriction enzyme analysis can be applied to test the actual species contained in frozen shrimp meat products.

The frozen shrimp meat products tested were found to be made of the cheaper shrimp species *P. monodon, L. vannamei* and *P. fissuroides*. Among these three shrimp species, the price of *P. fissuroides* was the lowest in Taiwan, and the samples with no label were determined to be *P. fissuroides* in this study.

Pascoal *et al.* reported the other PCR-RFLP method could identify the shrimp species. Their primers could amplify a small *cyt-b* fragment (175-184 bp) and differentiate 6 shrimp species only. In this study, PCR-RFLP method using UCYTB151F/270R primer could amplify 401-407 bp fragment of 10 shrimp species. Hence our method could differentiate more shrimp species. Our PCR-RFLP method focused on species identification of frozen shrimp products. The application of this method to the shrimp species and other shellfish such as crab needs to be studied further. Merritt *et al.* reported that these primers could be usable for several species of shellfish. Meanwhile, how to quantitatively identify the amount of specific shrimp species meat also needs further study.

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