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Bighead Carp Meat Adulterated in Commercial Milkfish Ball Products

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ABSTRACT

Attempts were made to identify the species of raw materials of milkfish ball products. A 466-bp fragment could be amplified by polymerase chain reaction (PCR) using a pair of primer (L14735/H15149ad). Sequences of cytochrome b gene (*cyt b*) were determined in 3 fish species including milkfish (*Chanos chanos*), bighead carp (*Hypophthalmichthys nobilis*) and grass carp (*Ctenopharyngodon idella*). These three fish species contained distinguishable sequences. In addition, RFLP analysis of the PCR products with endonuclease *Hpa*II showed unique restriction maps for these 3 fish species. Based on gene sequencing and PCR-RFLP analyses, we have identified one commercial milkfish ball be intentionally adulterated with bighead carp.

Key words: cytochrome b gene, milkfish, bighead carp, grass carp, PCR-RFLP

INTRODUCTION

Milkfish, one of the most important economic cultural fish in Taiwan, is unique in flavor, high nutrient and popularity worldwide. A great deal of manufactures of milkfish such as fish paste, canned food and fish ball, are exported. Milkfish, *Chanos chanos*, is the major species used for processed seafood product[(1)]. Owing to the price of the raw material raised, some manufacturers use cheaper fish such as bighead carp (*Hypophthalmichthys robilis*) and grass carp (*Ctenopharyngodon idella*) to reduce the cost. Therefore, it is necessary to develop a quick examination technique to test the adulteration of raw materials in processed seafood products.

So far several methods for testing adulteration have been reported, including near-infrared spectroscopy, immunoassay, gas chromatography, liquid chromatography/mass spectrometry and molecular biology technique[(2-4)]. Among them, the most promising and reliable one is DNA technique due to that DNA technique is easily adapted into routine surveys[(5)]. It can also be applied to identify the species of raw material and the adulteration of processed products. As a result of fast development of molecular biology, DNA biomarker becomes important in species identification[(6)].

The mtDNA is highly conservative[(7)] and often used to analyze the molecular evolution. In recent years, it has been applied to detect adulteration of market products[(8,9)]. Cytochrome b gene (*cyt* b) is a functional gene between $tRNA^{Glu}$ and $tRNA^{Thr}$ genes in mtDNA, and plays a role in encoding partial cytochrome c oxidoreductase, a complex enzyme in oxidative phosphorylation. Many researches about vertebrate *cyt* b gene were focused on the inheritance and evolution[(10)].

At present, DNA moleculars are utilized to authenticate specific species, 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)[(11,12)]. Due to the high sensitivity, speed and simplicity, PCR-RFLP was often applied to identify the adulteration of food products[(13,14)]. The principle of this technique is based on the specificity of gene sequence. PCR can be employed to amplify particular partial sequence and sequence analysis can be used to identify the species in the raw material of processed products. PCR technique and sequence analysis have been applied to the identification of the species of toxic and nontoxic puffer fish processed products[(15-17)]. In this study, we used PCR and sequence analysis to establish the restriction map of three species of *Ch. chanos*, *H. nobilis* and *Ct. idella*. The commercial milkfish ball products collected from supermarkets in Taiwan were examined for the evidence of adulteration.

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MATERIALS AND METHODS

I. *Sample Materials*

Three specimens for each fish species including milkfish *Chanos chanos*, bighead carp *Hypophthalmichthys nobilis* and grass carp *Ctenopharyngodon idella* were collected from traditional markets in Keelung. Each specimen was skinned and stored at -20°C for DNA extraction. Seven commercial milkfish ball products (300 g/package) were purchased from supermarkets in Taipei, Keelung and Kaohsiung. Each milkfish ball sample was mixed and stored at -20°C until further analysis.

II. *DNA Extraction*

DNA extraction was performed by the method of binding magnetic beads (Chemagic DNA Tissue 10 Kit, Chemagen, Baesweiler, Germany). Briefly, about 0.2 g of sample was homogenized with lysis buffer (50 mM of 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, Ohio, USA) was added. The sample was incubated at 55°C for 4 hr with shaking, and adding magnetic beads after lysis was completed. After incubation, magnetic beads with bound DNA were recovered using a magnetic separator. The mixture was washed twice with different washing buffer. Ultimately, the magnetic beads were removed from the solution and the genomic DNA was eluted with 50 µL of elution buffer.

III. *PCR Primer*

The set of primers (L14735 and H15149ad), reported by Wolf *et al.*[18] was used for PCR amplification of each fish species and samples, L14735: 5'-AAAAACCACCGTTGTTATTCAACTA-3', H15149ad: 5'-GCTCCTCAGAATGACATTTGTCCTCA-3'.

IV. *PCR Amplification*

The PCR amplification were performed in a total volume of 50 µL. Each reaction mixture contained 100 ng extracted template DNA, 0.2 µM of each primer, 200 µM of each dNTP, 2 units of Pro *Taq* DNA polymerase (Promega, Wisconsin, 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).

The PCR was carried out in a Gene-Amp PCR system 2400 (Perkin-Elmer, Foster, California, USA) programmed to perform a denaturation step at 95°C for 10 min, followed by 40 cycles including of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C. The last extension step was at 72°C for 10 min and stored at 4°C.

V. *PCR Product Analysis and Sequencing*

PCR product (5 µL) was loaded onto 1.5% agarose gel (containing Healthview DNA dye, 0.02 µL/mL) in TBE buffer and electrophoresed at 50 V for 50 min. The DNA band was detected under ultraviolet light and photographed by Image Master VDS (Pharmacia Biotech, Piscataway, New Jersey, USA) and the products were sequenced at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) in an ABI Prism 377sequencer (Applied Biosystems, Foster City, California, USA).

VI. *Restriction Site Analysis of PCR Products*

For the restriction enzyme analysis of the *cyt b* region, the endonuclease *Hpa*II (Promega, Madison, Wisconsin, USA) was searched from the GCG system by uploading our sequences and testing for restriction analysis of the amplified PCR products. The amplified DNA fragment of PCR products was used without purification to obtain the final result. Digestions were performed in 5 µL of amplified DNA, 5 units of enzyme, and 1: 10 dilution of the manufacturer's recommended 10 X digestion buffer. Digestion mixtures were incubated for 6 hr at 37°C. The result was analyzed by electrophoresis performed on a 3% agarose gel (containing Healthview DNA dye, 0.02 µL/mL) in TBE buffer at 50 V for 120 min and analyzed as described above.

VII. *Detection Limit Experiment*

Here we imitated the actual situation of adulteration of bighead carp/grass carp in milkfish, the rate of adulteration was tested as follows: 0%, 1%, 5%, 10%, 20%, 50%, 75% and 100%. The protocol of PCR-RFLP to PCR products by using primer pair L14735 and H15149ad is stated as above.

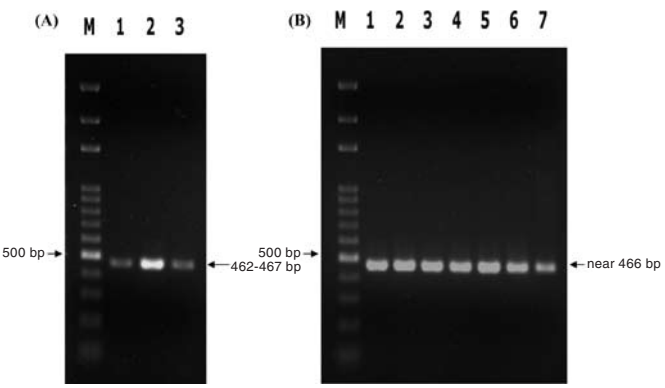

Figure 1. 1.5% agarose gel electrophoresis result of the partial cytochrome b gene PCR product. (A) lane M: MW Bio 100 bp ladder; lane 1: *Ch. chanos* meat; lane 2: *H. nobilis* meat; lane 3: *Ct. idella* meat. (B) lane 1-7: number of commercial milkfish ball sample collected from supermarkets.

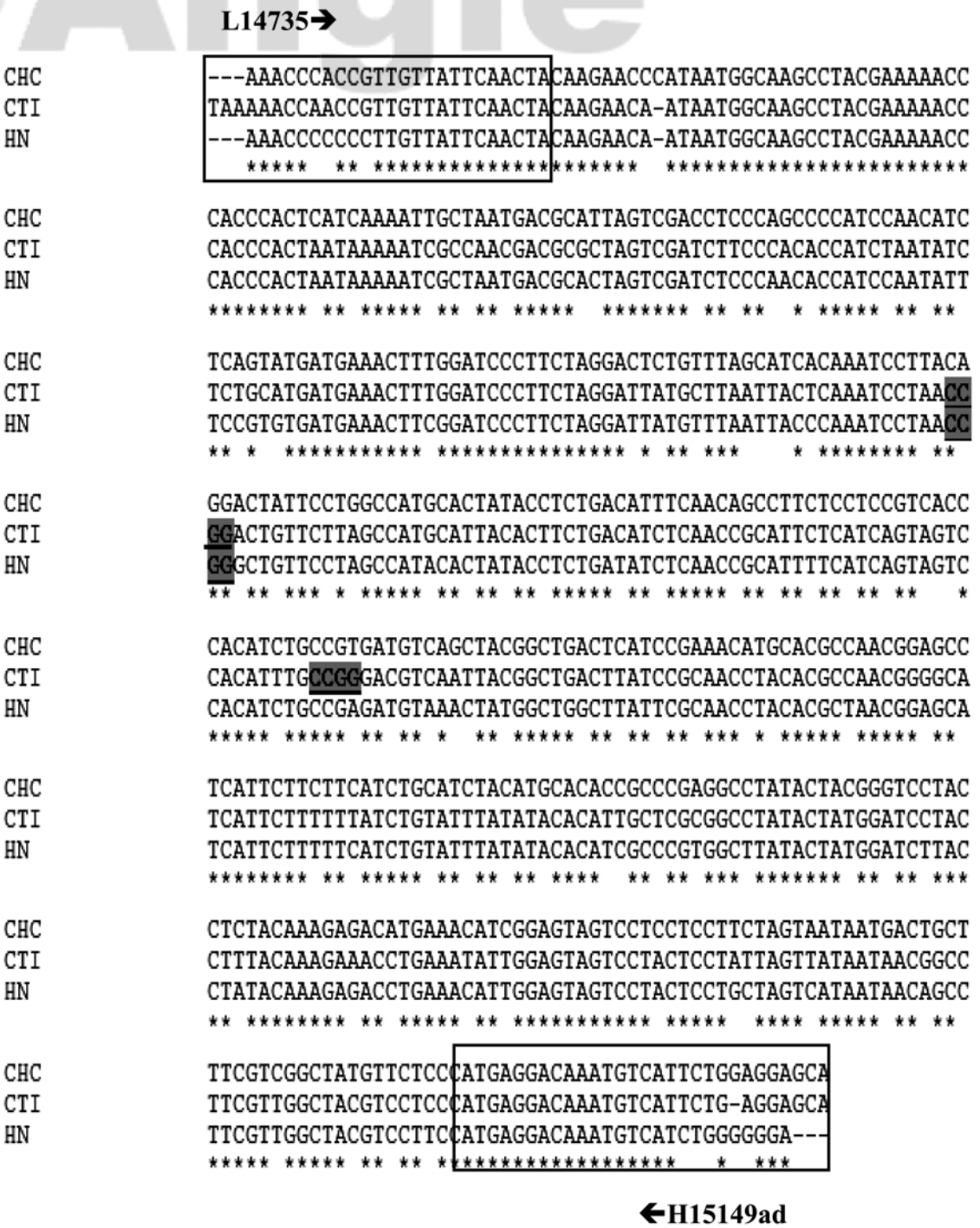

Figure 2. Comparison of the *cyt b* partial gene of three fish species by using the set of primer L14735 and H15149ad. Samples in lane are as follows: CHC: *Ch. chanos*; CTI: *Ct. idella.*; HN: *H. nobilis.* Primer site is cited in frame and cutting sites of *Hpa*II is underlighted in figure.

RESULTS

The PCR products of 3 fish species are shown in Figure 1A. By amplifying partial *cyt b* gene with the primers L14735 and H15149ad, the partial mtDNA fragments of *cyt b* gene were obtained as 466 bp, 462 bp and 467 bp for milkfish, bighead carp and grass carp, respectively. The sequences of each partial mtDNA fragment from three different fish species are shown in Figure 2.

These sequences were used to find their homologue in Genbank and genes with accession No. of AB054133 (*Chanos chanos*), EU343733 (*Hypophthalmichthys nobilis*) and EU391390 (*Ctenopharyngodon idella*) were found. The PCR products of 7 commercial milkfish ball samples

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from supermarkets are presented in Figure 1B. All of 7 samples were amplified successfully, and samples No. 1-6 were sequenced and identified as *Ch. chanos*. But sample No. 7 could not be sequenced and it was proposed that more than one kind of DNA template existed in sample No. 7. PCR products of three fish species and 7 commercial milkfish ball samples were digested by the endonuclease *Hpa*II and the result are shown in Table 1. The segment of *Ch. chanos* had no cutting site, but that of *H. nobilis* and *Ct. idella* had one and two cutting sites, respectively (Figure 3A). After examining the adulteration of 7 commercial milkfish ball samples, samples No. 1-6 had no cutting site, so there were no adulteration of bighead carp meat or grass carp meat. However, sample No. 7 showed three band in gel electrophoresis, including 466 bp, 285 bp and 177 bp (Figure 3B), indicating that the adulteration of bighead carp meat was found in sample No. 7. The meats of milkfish and bighead carp were identified in the raw materials of this milkfish ball.

The sensitivity for detecting adulteration of bighead carp meat and grass carp meat in milkfish meat by PCR-RFLP was determined. The result indicated that the adulteration of more than 10% bighead carp meat to milkfish meat was detectable (Figure 4A). The adulteration of grass carp meat in milkfish meat was difficult to detect (Figure 4B). It is concluded that the primer pair L14735 and H15149ad was suitable for amplifying *cyt* b gene fragments of bighead carp meat and milkfish meat. Once the adulteration of grass carp meat is found in milkfish meat, a suitable PCR-RFLP method needs to be established.

DISCUSSIONS

As described by many investigators, the *cyt b* gene is a good molecular marker for examining adulteration of seafood products for several reasons. Most of all, it is very conservative and high abundance of mitochondrial DNA in total cell nucleic acid preparations results in more effective PCR amplification[19,20]. The PCR-RFLP method can be quick and effective method to detect adulteration of processed products, e.g. smoking salmon[21], dressed toasted eel[22] and commercial dried bonito products[23]. Here, we found that direct sequence analysis and restriction enzyme can be applicable to test the adulteration of bighead carp and grass carp in milkfish ball product.

In Taiwan, milkfish is an important aquaculture fish and can be manufactured into fish ball, dried fish fillet

Table 1. The restriction enzyme cutting result of *cyt b* gene of the sample purchased from supermarkets

Source of PCR product	*Hpa*II enzyme cut (base pairs)	Species identified by *Hpa*II system
Ch. chanos	466	
H. nobilis	285 + 177	
Ct. idella	70 + 180 + 217	
sample No.1	466	*Ch. chanos*
sample No.2	466	*Ch. chanos*
sample No.3	466	*Ch. chanos*
sample No.4	466	*Ch. chanos*
sample No.5	466	*Ch. chanos*
sample No.6	466	*Ch. chanos*
sample No.7	466 + 285 + 177	*Ch. chanos* + *H. nobilis*

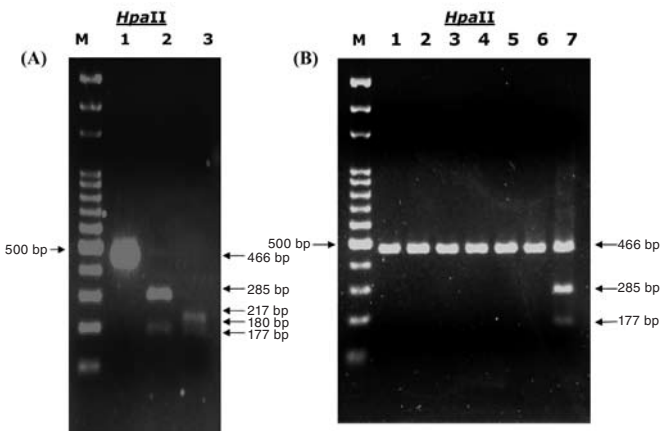

Figure 3. 3.0% agarose gel electrophoresis result of the partial *cyt b* gene PCR product cutting by restriction enzyme *Hpa*II. (A) lane M: MW Bio 100 bp ladder; lane 1: *Ch. chanos* meat; lane 2: *H. nobilis* meat; lane 3: *Ct. idella* meat. (B) lane 1-7: number of commercial milkfish ball samples collected from supermarkets.

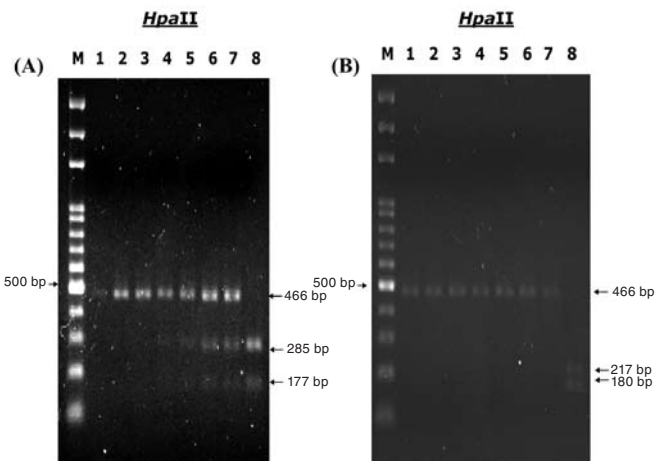

Figure 4. 3.0% agarose gel electrophoresis results of the detection sensitivity experiment of partial *cyt b* gene PCR product cutting by restriction enzyme *Hpa*II. (A) lane M: MW Bio 100 bp ladder; lane 1-8: the adulteration rate of bighead carp in milkfish: 0, 1, 5, 10, 20, 50, 75 and 100%, respectively. (B) lane M: MW Bio 100 bp ladder; lane 1-8: the adulteration rate of grass carp in milkfish: 0, 1, 5, 10, 20, 50, 75 and 100%, respectively.

and dried fish shred. Recently, milkfish price is rising and some manufacturers adulterate cheaper fish meat to lower the cost. Judging from above analysis data, the adulteration of bighead fish has been evidenced in commercial milkfish ball products collected from Kaohsiung. In addition, it is suggested that PCR-RFLP method is an effective way to detect the adulteration of bighead carp or grass carp in milkfish ball products. However, a more suitable primer pair for grass carp meat is needed for further study. To prevent other cheap fish meats from adulteration in the milkfish ball products, the *cyt b* gene data of other fish species are also needed to be studied. Meanwhile, the government must strengthen the screening of the labeled fish species for the reliability of specific fish products.

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