Authentication of consumer fraud in Taiwanese fish products by molecular trace evidence and forensically informative nucleotide sequencing

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ABSTRACT

An inexpensive, efficient, and reliable molecular method of authenticating commercial puffer fish-based products was developed to determine the origin of adulterated fish products and for food regulatory control. This system is based on molecular trace evidence obtained using a specific primer set with a short gene marker located on the mitochondrial *cytochrome b* sequence region. The method was successfully tested and validated in 12 specimens of puffer fish, simulated products, and 50 commercial samples. Fourteen percent of the collected commercial products were found to be puffer fish-based. However, of these 14%, 28% were identified as toxic varieties by further analysis with forensically informative nucleotide sequencing (FINS) and BLAST methods. These results reveal that the developed method is a rapid and efficient tool to unequivocally identify puffer fish, which may aid in the prevention of consumer fraud or mislabeling of fish products.

1. Introduction

During the last few decades, demand for fish products has increased significantly. Currently, the three most popular fish products in Taiwan are dry-dressed fish fillet, dressed fish powder, and canned fish. Due to an increase in popularity, consumption rate, and high prices for fish products, fish sellers may substitute less expensive and sometimes toxic fish (e.g., puffer fish) for more expensive fish such as ayu sweetfish, malabar grouper, cod, unicorn filefish, and horse mackerel. Consequently, two or three cases of puffer fish poisoning resulting from this practice are reported annually (Hwang & Noguchi, 2007). Therefore, the need for improving consumer knowledge on the hygiene, quality, and origin of food has increased significantly.

In Taiwan, only two nontoxic puffers, *Lagocephalus gloveri* and *Lagocephalus wheeleri*, are allowed to be sold in fish markets and to be used as provision of materials for dry-dressed fish fillets; however, these are not approved for use in dressed fish powder, canned fish, or other products (Department of Health, 1997). Because the morphological characteristics of fish are partially or completely lost after processing, identifying different species from commercial fish products is particularly important for consumers. This is also important with respect to issues related to allergies (Wal, 2001), toxicity (Hsieh et al., 2010), the protection of endangered species (Yan et al., 2005), the protection of consumer rights, and the objection to using certain species on either ethical or religious grounds (Calvo, Zaragoza, & Osta, 2001).

Therefore, several methods have been developed for species identification of fish products (Teletchea, 2009). Protein-based electrophoretic and immunological techniques, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Chen & Hwang, 2002), native or urea isoelectric focusing (IEF) (Mackie et al., 2000), two-dimensional electrophoresis (2-DE) (Chen, Shiau, Wei, & Hwang, 2004), and enzyme-linked immunosorbent assay (ELISA) (Huang, Marshall, Kao, Otwell, & Wei, 1995), have been successfully used. DNA-based methods, such as random amplified polymorphic DNA (RAPD) (Calvo et al., 2001), polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (Hsieh et al., 2010), real-time PCR (Jones, Oliver, Deeds, & Yancy, 2010), and forensically informative nucleotide sequencing (FINS) (Chen, Hsieh, & Hwang, 2012), employed for fish species authentication have also shown relevant results.

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The FINS technique was first reported by Bartlett and Davidson (1992), who proposed the genetic identification of species using phylogenetic analysis. Chan, Ling, Shaw, Chiu, and But (2012) indicated that FINS analysis could be used to accurately identify the species. FINS is a rapid, trustworthy, and reproducible procedure. Similar to BLAST analysis, it involves sequencing amplified DNA fragments. The DNA regions of mitochondrial DNA (mtDNA), such as regions 12S and 16S rRNA, *cytochrome c* subunit I (COI), and especially *cytochrome b* (*cyt b*), are usually employed as a species-determining target in FINS analysis (Ardura, Pola, Linde, & Garcia-Vazquez, 2010; Lago, Vieites, & Espiñeira, 2012; Santaclara, Espiñeira, & Vieites, 2007; Wen et al., 2010).

In this study, a simple, cheap, efficient, and reliable authentication method was developed for puffer fish-based products by using species-specific primers with a novel and short gene region. Overall, the aims of this study were three-fold. First, we sought to identify a gene marker region that processed food producers could employ to protect the originality of and/or add value to their products. Second, we aimed to establish a gene marker system based on the sequence of either the *cyt b* gene or the COI gene that could be applied to the detection of fraudulent and/or unintentional mislabeling of processed fish products. Third, it was our hope that successful application of this method would encourage the Taiwanese government to improve regulations for banning the use of cheaper and potentially hazardous species in fish products for the protection of consumers' rights and to avoid unfair competition.

2. Materials and methods

2.1. Materials

Sixty specimens of each of the following puffer fish species were obtained from a fishing pier in Ilan County, eastern Taiwan: *Takifugu niphobles*, *Takifugu oblongus*, *Takifugu pardalis*, *Takifugu poecilonotus*, *Takifugu stictonotus*, *Takifugu vermicularis*, *Takifugu xanthopterus*, *Lagocephalus inermis*, *Lagocephalus lunaris*, *Lagocephalus wheeleri*, *Lagocephalus gloveri*, and *Sphoeroides pachygaster*. The 20 most homogeneous species identified from GenBank (National Center for Biotechnology Information, NCBI), by using the BLAST (http://blast.ncbi.nlm.nih.gov) tool for sequences analysis, were also collected and compared. Upon collection, the fish were immediately transferred on ice to the laboratory at Asia University, Taichung, Taiwan. Fresh muscle of each sample was used for DNA extraction.

All of the tested products were simulated and produced using commercial methods in our laboratory. Fillets (fresh meat of the above-mentioned 12 puffer fish species) were first immersed in a seasoning solution (20% sugar, 0.8% salt, and 0.3% monosodium glutamate) with a ratio of fish to solution 1:2 for 12 h. For dry-dressed fish fillet samples, treated fillets were then dried with hot air for 2 h at 50 °C. For canned fish samples, treated fillets were sterilized in an autoclave at 121 °C for 80 min. For fry-dressed fish powder samples, the meats were mixed with soya sauce and cooked at 100 °C until the textured/flavored products were fully processed.

The mixtures of simulated dry-dressed fish fillet products were also prepared with 0%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 20%, and 100% ratios from *L. gloveri* to *A. monoceros*. The 0% mixture of simulated product was made completely from *A. monoceros*, and was used as the negative control. The 100% mixture of simulated products was made completely from *L. gloveri*, and was used as the positive control.

Commercial products were purchased from fishery companies in Ilan, Penghu, and Kaohsiung counties. These samples included 17 dry-dressed fish fillet products (Numbered 1–17), 16 canned fish products (Numbered 18–33), and 17 fry-dressed fish powder products (Numbered 34–50). All 50 of the samples were labeled, and the manufacturer's confirmed that the samples were derived from *Aluterus monoceros*.

2.2. DNA extraction

Total genomic DNA was extracted from the samples using a MasterPure™ DNA Purification Kit (Epicentre Biotechnologies; Madison, WI, USA) according to the manufacturer's protocol. Final pellets were allowed to dry at 25 °C and were then resuspended in 20 μL of sterile distilled water prior to analysis. DNA quantity was determined by measuring the absorbance at 260 nm, and DNA quality was determined by determining the ratio of absorbance at 260/280 nm using a NanoPhotometer™ spectrophotometer (IMPLEN; München, Germany). The extracted DNA was also electrophoresed on a 0.8% agarose gel containing ethidium bromide, visualized, and photographed under ultraviolet trans-illumination before PCR amplification. All extracted DNA were appropriately labeled and stored at −20 °C for subsequent tasks.

The DNA mixtures of fresh meats were also prepared with 0%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, and 100% ratios from *L. gloveri* to *A. monoceros*. The 0% DNA mixture was made completely from *A. monoceros*, and was used as the negative control. The 100% DNA mixture was made completely from *L. gloveri*, and was used as the positive control.

2.3. PCR amplification of the complete mitochondrial cyt b gene fragment and COI gene fragment

The PCR amplification reactions were conducted using Fast-Run™ Taq Master Mix Kit (Protech Technology Co.; Taipei, Taiwan). PCR was carried out in a GeneAmp PCR System 2400 (PE Applied Biosystems; Foster City, CA, USA) with an initial denaturation step at 94 °C for 2 min, followed by 30 cycles consisting of 30 s at 95 °C, 30 s at 50–60 °C, and 1 min at 72 °C. A final extension for 10 min at 72 °C was also included. The primer sequences used for PCR amplification are summarized in Fig. 1.

The products of PCR amplification were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, and were visualized and photographed under ultraviolet trans-illumination before cleanup and sequencing.

2.4. Gene marker region selection and Primers designed

Having completed a comparison of the *cyt b* and COI gene sequences of 12 puffer fish species and to 20 other related species identified from GenBank, a new primer set (TLS-LC and TLS-HC) was designed from two conserved regions (base pair (bp) numbers 674–695; bp numbers 796–817) close to the 3′ end of the *cyt b* gene. At the same time, a mostly variable region (100 bp) was also identified between two conserved regions, which exhibited over 3 genotypes at the same nucleotide site among 12 puffer fish species. The selected gene marker region was therefore 144 bp, and could only be amplified by the species-specific primer set (TLS-LC and TLS-HC) from the 12 puffer fish species.

2.5. Cloning and sequencing analysis

The PCR products were obtained using a Micro-Elute DNA Clean/Extraction Kit (GeneMark Technology Co., Tainan, Taiwan). Purified PCR products from all samples were cloned into PCR 2.1-TOPO Vector, and then introduced into *Escherichia coli* competent cells (TOPO TA Cloning Kit, Invitrogen; Carlsbad, CA, USA). Several successfully established clones were sent to Mission Biotech Co. (Taipei, Taiwan) for sequencing using the above-mentioned primers with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer/Applied Biosystems Division; Foster City, CA, USA) in an ABI PRISM 377-96 DNA Sequencer (Perkin Elmer/Applied Biosystems Division). Sequence analysis was performed using the Genetics Computer Group Wisconsin Package, Version 11.1.2 (2007).