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ESTABLISHMENT OF A MOLECULAR BASED METHOD FOR THE IDENTIFICATION OF SKIPJACK TUNA (*Katsuwonus pelamis*) IN LARGE SCALE FISH PROCESSING INDUSTRY

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Abstract

Skipjack tuna (*Katsuwonus pelamis*) is most often sold as canned light tuna and is the most common species found in tuna cans. In Sri Lanka differentiation of tuna species prior to processing is achieved through morphological identification, which is not a reliable method. Since the quality and market value of tuna products differ from species to species, a fraudulent replacement of valuable species with less valuable ones may occur. This has become a major limitation in fishery industry in order to reach products to the international market. Therefore, the objective of the current study was to establish a molecular based diagnostic method to differentiate skipjack tuna from other tuna species commonly found in Sri Lanka. Genomic DNA of skipjack tuna (*K. pelamis*), yellowfin tuna (*Thunnus albacares*) and bigeye tuna (*Thunnus obesus*) were extracted from the muscle tissues. Amplification of DNA from tuna samples were carried using genus specific primers which flank at 558 bp region of Cytochrome b gene. The amplified DNA products of tuna species were digested with ScaI restriction enzyme. The pattern restriction fragments evidence that products having band sizes of 215 bp and 343 bp were detected only from *T. albacares* (n= 10) and *T. obesus* (n= 10) while, *K. pelamis* (n= 10) was remained as an indigestive product (558 bp). Therefore, this can be used to differentiate *K. pelamis* from the other tuna species which are commonly found in Sri Lanka.

Keywords: Tuna species, DNA extraction, polymerase chain reaction, restriction enzyme digestion

INTRODUCTION

Tuna fishery industry is a valuable global trade in which is becoming species of special economic interest to many countries bordering the Western Indian Ocean including Sri Lanka. Tunas enjoy a very good export market as sashimi or loins (Subasinghe, 2004). In Sri Lanka, this industry has been identified as a rapid growing area which has a great potential to earn for earning foreign exchange to the country by reaching the export market. There are three major species such as yellowfin tuna (*Thunnus albacares*), bigeye tuna (*Thunnus obesus*) and skipjack tuna (*Katsuwonus pelamis*) are highly abundant in the Sri Lankan sea. Skipjack is the most common species of canned and pouched light meat tuna available on the US market. Skipjack is the best choice among these light tuna options for lowering the risk of mercury exposure. Skipjack tuna identification is traditionally based on external morphological features, including body shape, pattern of colors, scale size and count, number and relative position of fins, number and type of fin rays, or various relative measurements of body parts (Fabrice, 2009).

The authenticity of food labeling is a serious issue that involves not only the consumer but also the manufacturers and everyone involved in the food chain (Michelini *et al.*, 2007). There is no proper method to confirm the identification of tuna species after it had been processed. It has become a limitation to reach the international markets, which is on high demand. In Sri Lanka, differentiation of tuna species before the processing is done through morphological

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identification, which is not a reliable method. The main difficulties in fish species identification especially in processed fish derive from many factors, such as the absence of morphological features (e.g., fins, head, and skin) in the processed product, the denaturation of proteins or other material important for the analysis during heat treatments and finally the presence of contaminants (e.g., olive and other vegetal oils), which may interfere with the analysis (Michelini *et al.,* 2007). Frozen tuna are far more difficult to distinguish due to the fin damage, discoloration, skin abrasion and distortion or crushing during the storage process. But even at small sizes, each species has distinct coloration, body markings and body morphologies that allow rapid visual keys to positive identification (Itano, 2005).

Therefore, the current study will focus on developing a molecular based diagnostic method to detect species identity of tuna species in order to facilitate the fish processing industries and fish exporters by making the test available. The development of analytical methods for fish species identification may help detecting and avoiding unintentional/intentional substitution of different fish species and thus enforce labeling regulations. Thus the objective of the present study was to develop a PCR based assay to differentiate *Katsuwonus pelamis* (skipjack tuna) from other tuna species.

MATERIALS AND METHODS

Collection of Samples

Raw samples belong to *T. albacares* (yellowfin), *T. obesus* (bigeye) and *K. pelamis* (skipjack) tuna, which were certified, by the National Aquatic Resources Research and Development Agency (NARA), Sri Lanka were obtained as positive controls. Processed samples of *K. pelamis* were obtained from leading fish processing industries in Sri Lanka. To avoid contaminations, all the tissue samples were collected using sterile blades and packed in labeled sealed polythene. All these samples were kept at 4 °C until those are taken to the research laboratory. Then all the muscle samples were stored at -20 °C until used for DNA extraction.

Extraction of Genomic DNA from Tuna Samples

A tissue sample (200 mg) of each species was taken in to a micro-centrifuge tube. The tissues were incubated with 500 µL of STE buffer and 500 µL of 5M Ammonium Acetate at 65 °C for one hour. The incubated samples were homogenized using sterile mortars and pestles followed by incubated at 55 °C for one hour and centrifugation, at 14000 rpm for 5 minutes at 4 °C. The DNA was extracted using 500 µL of the supernatant by phenol-chloroform method (Coen *et al.,* 1982). The extracted DNA was re-suspended in TE buffer and kept at -20 °C.

Primer Selection

The sequences of the mitochondrial *cytochrome b* gene for genus *Thunnus* (Table 1) were targeted for the PCR amplification (Michelini *et al.,* 2007).

Table 1: Sequences genus specific primers used for PCR protocol

Name of the primer	Sequence (5' – 3')
Forward (TnaF)	CAGGACTATTCCTCGCAA TACA
Reverse (TnaR)	CGAAACCAAGGAGGTCT TTGTA

Amplification of the Genomic DNA Using Polymerase Chain Reaction (PCR)

The PCR amplifications were conducted using family specific primers for *Scombridae spp* (TnaF/TnaR) in 15 µL of solution containing 0.6 µL each of Forward (TnaF- 5 µM) and Reverse primer (TnaR -5 µM), 4.5 µL of Master mix [10× PCR buffer + $MgCl_2$ (50 mM) + dNTPs- all four types (100 mM)], 0.6 µL of Taq polymerase (2 U/µL), 7.7 µL PCR water and 1.0 µL of DNA template. The cycling conditions were hot start at 95 °C for 60 seconds followed by 30 cycles each of denaturation at 95 °C for 60 seconds, annealing for 52 °C for 60 seconds, and extension at 68 °C for 60 seconds, followed by the final extension at 68 °C for 5 minutes.

Validation of Results by Restriction Enzyme Digestion

Restriction enzymes digestions were performed to differentiate the two tuna species using purified PCR product. The following restriction enzyme was selected for the digestion by analyzing the genomic sequences of *K. pelamis*, *T. obesus* and *T. albacares* (Table 2).

Table 2: Restriction enzymes used for restriction enzyme digestion

Name of the restriction enzyme	Restriction site (5' – 3')	Accession number	Species differentiated
*Sac*I	AGT▼ACT TCA▲TGA	NC_014059.1	*T. albacares* *T. obesus* *K. pelamis*

Agarose Gel Electrophoresis

Agarose powder (Promega) was used for the preparation of gels. The 1 % agarose gels which stained with Ethidium Bromide (0.5 µg/mL) were prepared. Amplified PCR products were loaded with Promega 100 bp DNA ladder and gel electrophoresis was carried out at 70 V for 60 minutes to get a good separation in the amplified products. Following the gel electrophoresis, the migrated DNA was visualized and documented using gel documentation apparatus and Image Lab 3.0 software protocol (GelDoc ™XR+). The results obtained from the gel electrophoresis were compared with the 100 bp DNA ladder.

RESULTS

Amplification of Tuna Samples

As depicted in the gel photograph, a specific band of 558 bp was identified which is the genus specific band size for *Scombrida* species.

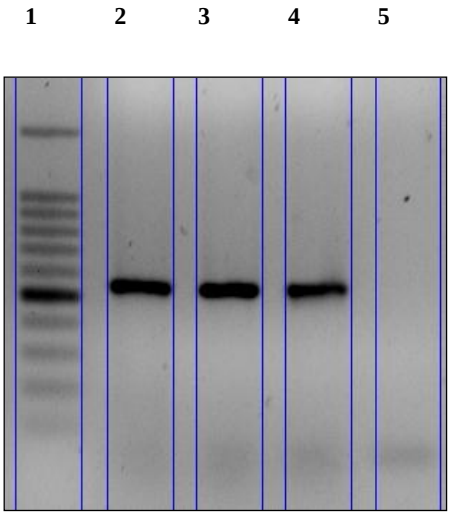

Figure 1. Amplified of tuna samples using family specific primer. (Lane 1: 100 bp DNA ladder; Lane 2: T. albacares; Lane 3: T. obesus; Lane 4: K. pelamis; Lane 5: Negative control - PCR water)

Digestion of the PCR Product of Tuna Samples with *Sca*I Restriction Enzymes

The pattern restriction fragments evidence that products having band sizes of 215 bp and 343 bp were detected only from T. albacares and T. obesus while, K. pelamis was remained as an indigestive product (558 bp). Therefore, restriction enzyme digestion was successful achieved and *K. pelamis* species were differentiated according to the specific sizes of bands (Figure 2).

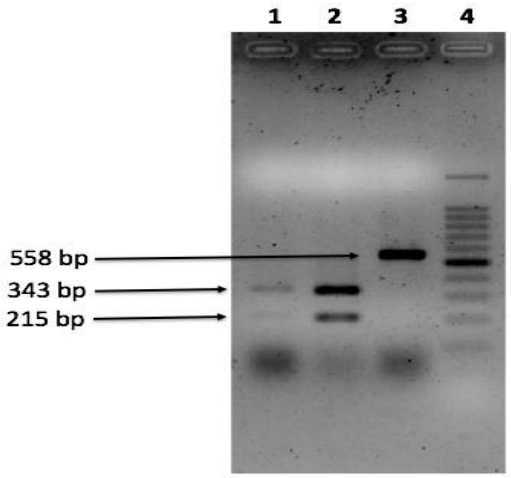

Figure 2. Restriction enzyme digestion of positive tuna samples using ScaI enzyme (Lane 1: T. albacares; Lane 2: T. obesus; Lane 3: K. pelamis; Lane 4: 100 bp DNA ladder Lane).

DISCUSSION

A PCR based diagnostic method to differentiate *K. pelamis* (skipjack tuna) has been developed in this study. The template DNA for PCR was prepared by the method devised by Nishiguchi *et al* (2002) with some modifications. Sequences of the DNA amplified by PCR were digested from restriction enzymes to differentiate two tuna species. Muscle tissues of *K. pelamis (n=10), T. obesus* (n= 10) and *T. albacares* (n= 10) were analyzed to evaluate this molecular assay. Developed and tested four STE buffers, which were modified its composition in order to obtain the maximum DNA quantity. The STE Buffer which contain 0.4 % SDS (pH 7.2) and 0.001 moldm^{-3} EDTA (pH 8.0) resulted higher DNA quantity than other modifications. Therefore, it was used throughout the extractions. DNA isolation was optimized with tuna samples for the temperature and time of incubation. The incubation was performed at 55 °C and 65 °C for each temperature the incubation was performed at 17 hours (overnight) and one hour. The incubation performed at 65 °C for one hour was identified as the best incubation temperature for the DNA extraction.

*Stu*I restriction enzyme was used to digest the PCR products of *T. albacares.* It was failed to digest the PCR product of *T. albacares* due to a difference in the sequence available in the NCBI database form genomic and mitochondrial cytochrome b gene (figure 3). Therefore, an alternative restriction enzyme (*Sca*I) was used to overcome this condition.

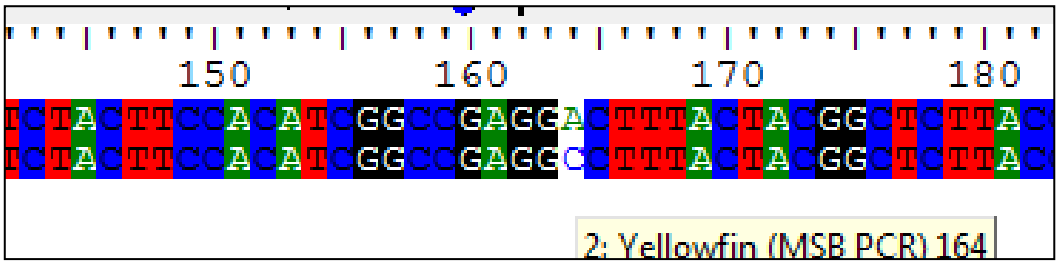

Figure 3. Genomic and mitochondrial cytochrome b gene sequence of T. albacares

CONCLUSION

In conclusion, this study carries a reliable approach to identify and distinguish *K. pelamis* from the other tuna species commonly found in Sri Lanka.

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