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Primer Effectiveness Test for DNA Authentication of Skipjack Tuna (*Katsuwonus pelamis*) Using Multiplex PCR

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Abstract

Species substitution in processed fishery products remains a major concern for food authenticity and consumer protection, particularly in tuna commodities where mislabeling is frequently reported. Reliable molecular tools are therefore needed to ensure accurate species identification and support food fraud prevention. This study aimed to develop a novel species-specific primer for the molecular authentication of skipjack tuna (*Katsuwonus pelamis*) and assess its application in a multiplex PCR assay. Three primer candidates targeting mitochondrial DNA regions (12S rRNA and NADH4) were designed and evaluated through in silico BLAST analysis and singleplex PCR. Among the three primer candidates, only one NADH4-based primer pair generated the expected 475 bp amplicon and showed specific amplification of *K. pelamis* without cross-amplification in non-target species. The selected primer was combined with published primers for *Thunnus albacares* and *Thunnus alalunga* in a multiplex PCR assay. Reliable amplification was obtained for *K. pelamis* and *T. albacares*, while amplification of *T. alalunga* was less consistent under multiplex conditions, suggesting that further optimization may improve assay performance. Sensitivity testing demonstrated successful DNA detection at concentrations as low as 0.1 ng/µL. Application of the multiplex PCR assay to processed fish meatballs successfully detected *K. pelamis* DNA in a complex food matrix. These findings indicate that the developed NADH4-based primer is a reliable molecular marker for *K. pelamis* authentication. Furthermore, the multiplex PCR approach shows strong potential as a sensitive tool for food authenticity verification and food fraud detection in processed fishery products.

Keywords : *DNA authentication, multiplex PCR, processed fish products, specific primer.*

1. INTRODUCTION

Recently, issues related to species identification in fish and fishery products have become an increasingly serious concern [1]. Seafood fraud generally occurs due to the deliberate mislabeling of fish species and inappropriate labeling of fishery products, both fresh and processed. Tuna is one of the most sought-after fish species [2] and has a high consumption rate. However, because tuna is often marketed in vacuum-packed pieces or canned products, its morphological characteristics are difficult to recognize, making accurate species identification quite challenging [3]. Furthermore, each tuna species has varying quality and price. This situation opens up opportunities for fraudulent practices through substitution with cheaper species (e.g., Katsuwonus pelamis) [1].

Species authentication can be performed using protein- or DNA-based approaches. However, protein-based methods are vulnerable to denaturation during processing, while DNA is more stable and therefore provides higher resolution in species identification. Research by [4] compared the SDS-PAGE (Protein) and DNA barcoding methods to test the authenticity of tuna. SDS-PAGE showed protein damage in processed tuna, making it less accurate for identification. In contrast, the DNA method, using

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PCR electrophoresis, successfully identified tuna steaks and canned tuna as *T. albacares*, consistent with the product labels.

The success of DNA detection in authenticating processed products depends heavily on the use of primers, which are short sequences of nucleotide bases specifically designed to bind to specific regions of the target DNA. These primers serve to initiate the DNA replication process in the polymerase chain reaction (PCR), allowing the amplification of specific DNA fragments from the species being identified. Target gene selection, such as Cytochrome C Oxidase Subunit I (COI) and Cytochrome b (cyt b), is a critical factor in determining successful authentication.

Research by [5] used the COI gene with DNA barcoding to identify *Pangasius macronema*. and (*Pangasius hypophthalmus*, with a genetic relationship of 95% to P. macronema from Vietnam and 100% to *P. hypophthalmus* from Africa and Thailand, respectively. [6] used cyt b gene-based primers to detect janitor fish (*Pterygoplichthys pardalis*) DNA in siomai, with 10 of 28 samples showing a 496 bp DNA band. The results of the sequencing of nine samples containing janitor fish DNA and one sample of gourami (*Osphronemus goramy*) DNA revealed the potential use of inappropriate raw materials.

Multiplex PCR is a variant of the PCR technique that allows the simultaneous amplification of two or more target DNA fragments in a single reaction using multiple primer pairs. This method has proven efficient because it saves laboratory time and resources without compromising the accuracy of the test results. The effectiveness of this technique was demonstrated in a study by [7] who used multiplex PCR with five primer sets to simultaneously identify five tuna species, demonstrating high accuracy in detecting species in commercial tuna products. To accurately identify species using PCR, primers specific to each DNA target are required.

Therefore, this study aimed to design specific primers and test their effectiveness using the multiplex PCR (mPCR) method to detect a mixture of fish species in fresh and processed samples. Validation was conducted in silico and in vitro to provide a comprehensive picture of primary performance. The results are expected to support efforts to prevent raw material counterfeiting, strengthen food oversight, and increase consumer confidence.

2. METHOD

2.1 Time and place

The research was conducted in June–July 2025 at the Central Laboratory of Andalas University.

2.2 Materials and equipment

The materials used included fresh fish samples, namely skipjack tuna (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) obtained from the Pasia Kandang Mutiara Morning Market and Gaung Fish Market, Teluk Bayur (Padang, West Sumatra), as well as albacore tuna (*Thunnus alalunga*) purchased online. Other materials used included designed primers, ddH_2O, nuclease-free water, the Quick-DNA Miniprep Plus Kit (Zymo Research), and DreamTaq 2X Master Mix.

The main equipment used included a thermal cycler, NanoPhotometer® NP80 (Implen), a gel electrophoresis system (Accuris MyGel Mini) with documentation using the iBright CL1500 Imaging System, micropipettes of various capacities, a centrifuge, and a vortex.

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2.3 Research Design

Table 1. Research design

No	Activity	Output
1	Sample Preparation	Muscle tissue (~25 mg) was collected from one specimen each of *Katsuwonus pelamis*, *Thunnus albacares*, and *Thunnus alalunga*. Each tissue sample was placed into a sterile 1.5 mL microcentrifuge tube and stored at −20°C until DNA extraction. In addition, one processed fish meatball sample was included to evaluate the applicability of the multiplex PCR assay for processed fish products.
2	Primer Design	Primer design results in the form of primer sequences specific to the target gene to be amplified.
3	DNA Extraction	DNA extraction results in the form of pure DNA samples.
4	PCR	PCR amplification results in the form of abundant DNA fragments, identified based on their size and the target gene being amplified.
5	Gel Electrophoresis	Visualization of PCR amplification results in the form of separate bands based on the size of the DNA fragments.

2.4 Primer Design and In Silico Specificity Analysis

Species-specific primers for *Katsuwonus pelamis* were designed based on the mitochondrial NADH4 gene sequence (GenBank accession number NC_005316.1) using Primer3 software. To enable multiplex PCR analysis, primer pairs specific for *Thunnus albacares* and *Thunnus alalunga* were adopted from Lee et al. (2022). The selected primers were evaluated for their basic characteristics, including primer sequence, target gene, melting temperature (Tm), and expected amplicon size. The primer information is summarized in Table 2.

The specificity of the designed *Katsuwonus pelamis* primer was initially evaluated *in silico* using the NCBI BLASTn program against the GenBank nucleotide database. Primer specificity was assessed based on sequence identity, query coverage, and the absence of significant matches with non-target species. The selected primer pair was subsequently validated experimentally using multiplex PCR together with the published primer pairs for *Thunnus albacares* and *Thunnus alalunga*. Multiplex PCR amplification was performed under the following thermal cycling conditions: an initial denaturation at 95°C for 60 s, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 300 s.

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Table 2. Characteristics of primers used in this study

Primer	Sequence (5'–3')	Target gene	Amplicon size (bp)	Tm (°C)	Source
K-Pel 12S 1303F	GGC TTT ATG ACC CCT GAC CC	12S	976	60.11	This Study
K-Pel 12S 2278R	TTT TGC CAC AGA GAC GGG TT			60.11	
K-Pel NADH 4 11287F	CCC TCG GAC ACA GCC TAA TC	NADH4	475	60.00	This Study
K-Pel NADH 4 11761R	GGG GCG GAG TAT TGA AGT GT			55.00	
K-Pel N4 11743F	CAC TTC AAT ACT CCG CCC CT	NADH4	300	55.00	This Study
K-Pel N4 12042R	GGA GGC AGA TTG AGC CTG TT			55.00	
K-Pel CYTB 16103F	CCC CTG ACG TAG AAT CAG CC	Cyt b	237	NR	Ref: (Lee et al., 2022).
K-Pel CYTB 16340R	TGC AAG TGG GAA GAA GAT G			NR	
T-Alb N4 11301F	CAT GAT TGC CCA CGG ACT TA	NADH4	126	NR	Ref: (Lee et al., 2022).
T-Alb N4 11427R	TGT TGT TAT AAG GGG CAG C			NR	
T-Ala CYTB 15937F	GTT TCG TGA TCC TGC TAG TG	Cyt b	177	NR	Ref: (Lee et al., 2022).
T-Ala CYTB 16114R	CCT CCT AGT TTG TTG GAA TAG AT			NR	

*NR = Not reported in the original publication (Lee et al., 2022).

2.5 GEL ELECTROPHORESIS

Gel electrophoresis was performed using 2% agarose gel in 1× TBE solution with SYBR Safe dye. A 5 µL PCR product and DNA marker were added to the gel wells and run under standard conditions. Results were visualized using the iBright Imaging System. DNA fragments were separated based on size, with smaller fragments migrating faster than larger ones. Band size was determined by

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comparing their migration position to a DNA ladder marker, and a positive result was considered if the DNA band appeared at a position corresponding to the size of the target gene.

3. RESULT

3.1 DNA isolation

DNA isolation is a crucial initial step in molecular analysis to obtain high-quality genomic DNA from fish tissue. In this study, DNA isolation was performed using the Zymo Research DNA Extraction Kit. The procedure followed the kit's standard protocol, which includes cell lysis, DNA binding to the column membrane, washing, and elution.

Table 3. DNA Isolation Results.

Sample	Concentration DNA	Absorbance Ratio A260/A280	Absorbance Ratio A260/A230
Katsuwonus pelamis	2.10	2.100	0.038
Thunnus albacares	3.50	1.795	0.041
Thunnus alalunga	4.20	1.787	0.147

Measurements of DNA purity and concentration from the three fish species showed varying results. The *K. pelamis* sample had an A260/A280 ratio of 2.100, with a DNA concentration of 2.10 ng/µL. The *T. albacares* sample had an A260/A280 ratio of 1.795 and a DNA concentration of 3.50 ng/µL, while the *T. alalunga* sample had an A260/A280 ratio of 1.787 and the highest DNA concentration of 4.20 ng/µL.

Although the extracted DNA concentrations (2.10–4.20 ng/µL) were considerably lower than the commonly recommended range of 10–100 ng/µL for PCR, successful amplification was still achieved. This result suggests that PCR performance was not determined solely by DNA concentration but also by DNA quality, primer specificity, and optimized amplification conditions. The A260/A280 ratios (1.795–2.100) indicated that the extracted DNA was of acceptable purity with minimal protein contamination, thereby supporting efficient amplification. Furthermore, the use of highly specific primers together with 30 PCR cycles likely enhanced the detection of low-copy DNA templates, enabling successful amplification despite the relatively low DNA concentration. These findings indicate that the developed assay is sufficiently sensitive to amplify low-abundance DNA, although higher DNA concentrations may further improve amplification efficiency and reproducibility[8]. A ratio below 1.8 indicates possible protein contamination, while a value above 2.0 may indicate RNA contamination [9].

A ratio that is too low, such as 1.787 and 1.795, indicates possible protein contamination, while a value of 2.100 indicates the possible presence of RNA. This indicates that the extraction process was not optimal, particularly during the tissue destruction stage or that the enzyme incubation was incomplete, resulting in inadequate separation of DNA from interfering compounds. Although the DNA concentration and purity did not fully meet standards, PCR was still performed to evaluate the effect of contaminants on DNA polymerase activity and ensure the possibility of amplification.

3.2 Testing on processed products

Fish Meatball samples were ground and then extracted using the Zymo Research DNA Extraction Kit for DNA isolation. This was followed by DNA concentration and purity measurements using the NanoPhotometer® NP80. The results were as follows in Table 3.

DNA concentration measurements from the meatball sample showed a value of 66.15 ng/µL, which is considered high and within the acceptable range for PCR reactions. However, purity measurements indicated contamination. An A260/A280 ratio of 0.977 indicated possible protein contamination, while an A260/A230 ratio of 0.033 indicated the presence of interfering compounds such as salt, polysaccharides, or organic solvents.

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Table 3. DNA Isolation Results of Processed Products

Sample	Concentration DNA	Absorbance Ratio A260/A280	Absorbance Ratio A260/A230
Fish Meatball	66.15	0.977	0.033

The low DNA purity was likely caused by a suboptimal isolation process, resulting in protein, buffer residue, or components from the fish meatball ingredients, such as fat and spices, remaining in the isolation. These compounds can inhibit DNA polymerase activity, disrupting the formation of the primer-template complex.

After confirming the DNA concentration and purity, the next step was electrophoresis to observe the PCR amplification results.

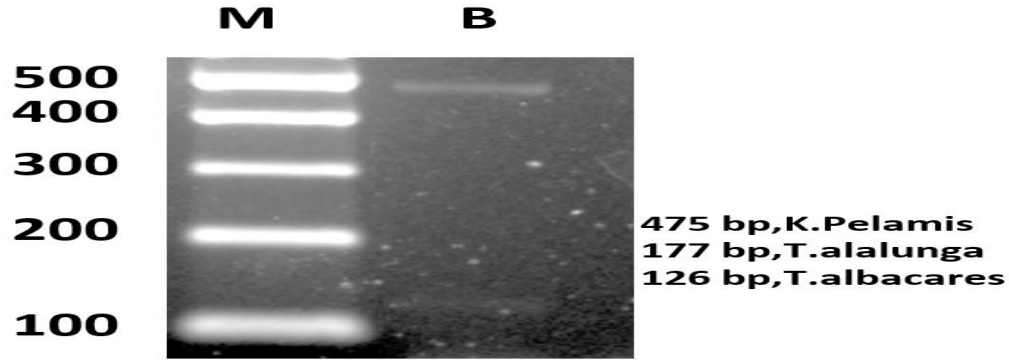

Figure 1. Multiplex PCR amplification of DNA extracted from fish meatball samples. Lane M: DNA ladder. Lane B: fish meatball sample. The expected amplicon sizes were 475 bp for *Katsuwonus pelamis*, 177 bp for *Thunnus alalunga*, and 126 bp for *Thunnus albacares*. The electrophoresis results showed amplification bands corresponding to *K. pelamis* (475 bp) and *T. albacares* (126 bp), whereas no amplification band was observed for *T. alalunga* (177 bp).

Electrophoresis of the multiplex PCR products from the fish meatball sample (lane B) revealed two distinct DNA bands corresponding to the expected amplicon sizes of *K. pelamis* (475 bp) and *T. albacares* (126 bp). The *K. pelamis* band exhibited stronger amplification intensity than the *T. albacares* band. No amplification corresponding to the expected 177 bp fragment of *T. alalunga* was observed under the optimized multiplex PCR conditions.

The absence of the *T. alalunga* amplicon may be associated with several factors affecting multiplex PCR performance. Amplification imbalance in multiplex PCR may occur due to differences in primer concentration, primer amplification efficiency, and thermocycling conditions, resulting in preferential amplification of certain targets over others[10]. In addition, low template DNA quality or degraded DNA may further reduce amplification success under multiplex PCR conditions. However, these possibilities were not specifically evaluated in the present study and therefore require further investigation.

4. DISCUSSION

In this study, tuna species authentication was carried out through a series of stages, starting from DNA isolation of fresh fish samples, followed by in silico and in vitro specificity tests, multiplex PCR optimization, sensitivity evaluation, and application to processed fishery products. The results demonstrated that multiplex PCR is a promising molecular tool for the authentication of skipjack tuna (*Katsuwonus pelamis*) in both raw and processed products.

Although the extracted DNA exhibited relatively low concentrations, the purity values were sufficiently close to the recommended range for PCR amplification. This finding indicates that the developed multiplex PCR assay was sufficiently robust to amplify target DNA despite the presence of potential contaminants introduced during the extraction process. DNA purity is an important factor

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affecting PCR performance because contaminants may inhibit polymerase activity and reduce amplification efficiency [8]. Nevertheless, previous studies have shown that successful PCR amplification can still be achieved when sufficient intact target DNA remains available, even if DNA quality is not optimal [11]. These findings suggest that amplification success was supported not only by DNA quality but also by the optimized primer design and PCR conditions employed in this study. Therefore, the successful amplification obtained in this study suggests that the developed assay has good potential for routine molecular identification of tuna species.

The successful amplification confirmed that the designed primer set possessed adequate specificity for multiplex PCR analysis. Primer specificity is essential because multiple primer pairs are simultaneously present in a single reaction, increasing the possibility of non-specific amplification. The primers developed in this study target mitochondrial DNA regions, which are widely recognized as reliable molecular markers for fish species identification because of their high copy number, maternal inheritance, and sufficient sequence variation among species [6], [7]. These findings are consistent with previous reports demonstrating that mitochondrial DNA-based multiplex PCR provides reliable discrimination among tuna species for seafood authentication [7].

The multiplex PCR approach used in this study offers several advantages over conventional singleplex PCR. By simultaneously amplifying multiple DNA targets in one reaction, multiplex PCR reduces reagent consumption, analysis time, and laboratory workload. Lee et al. [7] reported similar advantages when identifying five tuna species simultaneously using multiplex PCR. The present study further confirms that multiplex PCR can effectively distinguish tuna species and detect skipjack tuna DNA in mixed-species samples. The successful amplification obtained in this study indicates that the selected PCR conditions were appropriate for simultaneous detection of multiple target species. One of the most important factors influencing multiplex PCR performance is annealing temperature, as it determines the specificity of primer binding to the target DNA sequence. In this study, the annealing temperature was optimized, and 60°C was selected as the optimal condition because it produced clear and specific amplification of the target fragments without detectable non-specific products. Annealing temperatures that are too low may allow primers to bind to non-target DNA sequences, whereas excessively high temperatures can prevent amplification by reducing primer-template hybridization efficiency [12]. Therefore, optimization of PCR conditions, particularly annealing temperature and primer concentration, is essential to ensure amplification specificity and reproducibility in multiplex PCR assays, as primer concentration and cycling temperature are among the most critical parameters influencing multiplex PCR performance [13].

Sensitivity testing demonstrated that the optimized primers could detect DNA concentrations as low as 0.1 ng/µL. This result indicates high analytical sensitivity and suggests that the developed assay can identify target species even when DNA concentrations are very low due to processing, degradation, or dilution within mixed products. The ability to detect trace amounts of DNA is essential in food authentication because thermal processing, mechanical treatment, and ingredient mixing frequently result in DNA fragmentation. Similar findings have been reported in processed food authentication studies where PCR-based methods successfully detected target species despite significant DNA degradation [14].

The application of the multiplex PCR assay to processed fish products further demonstrated its practical value. The fish meatball sample showed a high DNA concentration (66.150 ng/µL) but poor purity, with A260/A280 and A260/A230 ratios indicating substantial contamination from proteins and other compounds. Processed products often contain fats, spices, carbohydrates, and additives that may co-extract with DNA and inhibit PCR reactions. Despite these challenges, successful amplification of *K. pelamis* and *T. albacares* DNA was achieved, demonstrating the robustness of the developed method. The presence of visible amplification bands suggests that the PCR system was able to overcome the inhibitory effects of contaminants commonly found in processed food matrices.

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Interestingly, amplification of *T. alalunga* was not observed in the processed product sample. Several factors may explain this result. First, the concentration of *T. alalunga* DNA may have been below the detection limit due to low abundance in the sample. Second, DNA degradation caused by processing conditions such as heating, grinding, and storage may have reduced the availability of amplifiable template DNA. Third, competition among primer pairs during multiplex PCR may have favored amplification of more abundant targets, resulting in preferential amplification of *K. pelamis* and *T. albacares*. Similar preferential amplification has been reported in multiplex PCR systems, where differences in primer performance and reaction optimization may result in unbalanced amplification among target fragments [15].

Overall, the results indicate that the designed primers possess adequate specificity and sensitivity for skipjack tuna authentication. Although DNA purity and amplification balance remain challenges in multiplex PCR analysis, the method successfully identified target species under various sample conditions. Further optimization of primer concentrations, PCR conditions, DNA extraction procedures, and validation using additional biological replicates is recommended to improve the robustness of the multiplex PCR assay and expand its application for the authentication of additional tuna species and processed seafood products.

5. CONCLUSION

Based on the research conducted, the following conclusions can be drawn: The developed multiplex PCR successfully amplified target DNA with a sensitivity of up to 0.1 ng/µL in both fresh fish samples and processed products, despite indications of contamination in the DNA isolation results. The detected contamination did not inhibit amplification, and the method was able to specifically identify *K. pelamis* and *T. albacares*. However, *T. alalunga* was not detected in mixed-species samples, which was possibly due to factors such as low DNA abundance, DNA degradation during processing, or primer competition effects in multiplex amplification. Further validation, such as spiking experiments using known concentrations of *T. alalunga* DNA into different sample matrices, is required to determine the specific cause of non-detection. Overall, this method has proven efficient and applicable for fish species authentication and has great potential for detecting adulteration of raw materials in processed fishery products.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper. The authors have no financial, personal, or professional relationships that could have influenced the research, authorship, or publication of this study.

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