Development and optimization of PCR assay to identify fish fraud

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Method Article

Keywords: PCR assay, Salmo, Gadus, Oreochromis, COX1, Sardinella

Posted Date: October 25th, 2023

DOI: https://doi.org/10.21203/rs.3.pex-2333/v1

Abstract

Fish fraud, characterized by intentional mislabeling or substitution of fish species for economic gain, poses significant risks to public health, food safety, and the integrity of the seafood industry. Effective detection methods are crucial to combat this fraudulent activity. This protocol outlines the development of a novel PCR assay, utilizing a new set of primers optimized for the precise identification of species frequently involved in frauds within the Brazilian fisheries sector, namely salmon, tilapia, and cod. The primer sets were designed based on a comprehensive dataset of mitochondrial DNA sequences, specifically the locus COX1 (cytochrome c oxidase subunit I). By implementing this robust PCR-based approach, the Brazilian fisheries sector can significantly enhance traceability and authenticity verification in their supply chain. Consumers' confidence will be safeguarded, and fair business practices can be promoted, ultimately fostering sustainability in the industry. This assay offers a practical and effective tool to tackle fish fraud and support efforts to maintain the integrity of the fisheries sector in Brazil.

Introduction

Presently, one of the foremost challenges confronting processors, traders, and, above all, consumers pertain to the issue of fraud within the fish product market. This predicament is exacerbated by the sheer diversity of fish species in existence and the inclination of certain traders to capitalize on the relatively limited knowledge of Brazilian consumers regarding fish consumption.

In the realm of the fisheries sector, recent research led by Pollack et al. (2018) has highlighted a spectrum of challenges. These encompass issues such as inaccurate labeling, deceptive activities, and the substitution of products—collectively acting as barriers to the market's expansion. Labeling errors often arise due to the striking similarity between various fish species in terms of appearance, structure, texture, flavor, and other morphological traits. Additionally, situations occur where lower-quality fish are blended or incorrectly presented as high-value species, all with the aim of commanding premium prices. This practice extends to species with marginal commercial significance, as documented by Cawthorn et al. (2012). These fraudulent practices require the implementation of strict control measures to safeguard the fisheries sector.

A crucial aspect of this endeavor is the introduction of initiatives that enhance public awareness and create effective authentication programs. These programs play a pivotal role in detecting and deterring the mislabeling of fish products, as underscored by Ali et al. (2018). To address these challenges, a potential solution lies in the adoption of molecular methodologies for fish species identification. This approach circumvents the limitations associated with morphology-based identification methods and the shortage of local expertise in fish species classification, as pointed out by Di Pinto et al. (2015).

Among these molecular techniques, DNA barcoding emerges as a promising tool. This method utilizes distinct DNA sequences that exhibit minimal variation within a species while demonstrating significant diversity across different species. DNA barcoding holds practical significance for ensuring food

traceability, as highlighted by Galimberti et al. (2013). Notably, DNA extraction can be conducted from processed meat products, enabling analyses at various stages of the production chain (Khaksar et al., 2015).

Numerous DNA biomarkers have been proposed for the identification of fish species. The DNA barcoding approach is remarkably robust and can be used at any point in the production process (Nicolè et al., 2011). However, acquiring adequate amounts of nuclear DNA from raw or processed meat can be challenging in some situations, and its use is relatively restricted compared to organelle DNA (Asif and Cannon, 2005). The COXI gene emerges as an excellent choice for identifying fish species. A multitude of studies have confirmed its usefulness in the domains of systematics and molecular ecology in various biological groups (Dona et al., 2015; Nzelu et al., 2015; Staffen et al., 2017; Weigt et al., 2012). Furthermore, the regions of the COXI gene have been fundamental in phylogenetic and population analyses of fish species (Bingpeng et al., 2018).

In sum, the COXI locus holds a prominent role in studies of fish species identification through DNA barcoding (ISO/DIS 17174), largely owing to its status as the optimal mitochondrial gene for phylogenetic investigations due to its protein structure and function. The deliberately slow-evolving codon positions and variable domains within the COXI offer a robust foundation for unraveling phylogenetic diversity.

In Brazil, noteworthy fraudulent practices encompass employing diverse species of salted fish, particularly *Gadus macrocephalus*, being presented as *G. morhua* (cod); the substitution of *Sardinella brasiliensis* with other *Clupeidae* species; the use of salmon trout (*Oncorhynchus clarkii*) as a substitute for true salmon (*Salmo salar*); and the deceptive replacement of fish like Panga, Alaska pollock, halibut, and hake with the genuine "Linguado".

The objective of this study was to develop primers that facilitate both the amplification and sequencing of the *COX1* locus in fish. Additionally, the aim was to refine the overall identification assay. The motivation behind developing this test is rooted in the inadequacies observed in the existing primer sets from the literature. These primers often presented challenges due to their extensive length and significant degree of degeneracy, causing amplification to become problematic at times. Furthermore, a notable drawback was the size of the resultant amplicons, which averaged around 650 base pairs. There was a distinct need for primers that better suited the context of fish fraud in Brazil, meaning primers that were customized to accommodate the intricacies of both intra and interspecific diversity inherent to the key species that are susceptible to fraudulent practices within the Brazilian fish market.

To design the primer pairs, a search for sequences of the *COXI (=COX1*) locus in GenBank for 10 species related to fraud in Brazil (*Xiphias gladius, Sardinella maderensis, Limanda aspera, Prionace glauca, Salmo salar, Oreochromis niloticus, Gadus chalcogrammus, Gadus morhua, Gadus ogac*, and *Gadus macrocephalus*). A total of 826 sequences were retrieved from GenBank (July 1, 2023).

An initial curation based on multiple alignments was performed using the ClustalW algorithm in BioEdit 7.2.5 (Hall, 1999), where very short sequences that prevented obtaining higher quality alignments were removed. The analysis involved sequence alignment, using ClustalW (Thompson et al., 1994), and a visual search for regions that would allow the design of primers to accommodate all the intra and interspecific diversity of this set of 10 species.

The designed primers were checked for their specificity/uniqueness against the GenBank database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Melting temperature, formation of secondary structures and other parameters were analyzed in silico using Oligoanalyzer 3.1 (https://www.idtdna.com/calc/analyzer). Primer sequences are shown in Table 1 (SM-1). Primers were created for amplification via PCR (COX1ITAL-F/ COX1ITAL-R), and additional internal primers for improved sequencing quality (COX1-INTER-F/ COX1-INTER-R).

Reagents

PureLink™ Genomic DNA Mini Kit (Invitrogen™)

Liquid nitrogen-N2

dNTPS (2,5 mM) (Invitrogen™)

Primers (2 mM stock – see Tables 1 and 2)

Agarose

UltraPure™ TBE Buffer, 1X (Invitrogen™)

Ethidium Bromide

ExoSAP-IT Express (Applied Biosystems™)

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™)

BigDye XTerminator Purification Kit (Applied Biosystems™)

Equipment

70 ºC water bath

Veriti™ Thermal Cycler, 96-well (Applied Biosystems™)

SeqStudio Genetic Analyzer (Applied Biosystems™)

NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific™)

Procedure

1. About 5 mg of tissue from each sample was macerated with the aid of liquid nitrogen and mortar, until the material was completely pulverized.

2. The macerated material was subjected to genomic DNA extraction using the PureLink™ Genomic DNA Mini Kit (Invitrogen™), according to the manufacturer's protocol.

3. The DNA obtained was subjected to quality analysis and quantification in the Nanodrop 2000 device. (Thermo Scientific™).

4. The pair of primers used for amplification were those developed in-house, as described above. The sequences of the primer pairs are shown in Table 1 (SM-1). The PCR assay was conducted under the following conditions: Amplifications were conducted on a Veriti™ thermal cycler (Applied Biosystems, CA, USA) using a 25 µL reaction volume containing 10 ng of DNA template, 1× PCR buffer, 3 mM of MgCl2, 0.25 mM of dNTP, 0.4 µM of each PCR primer, and 1 U of Taq DNA polymerase. The mixture was subjected to the following amplification program: initial denaturation at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 °C for 40 s, annealing at 57 °C for 40 s and extension at 72 °C for 1,5 min, and a final elongation for 5 min at 72 °C.

5. The amplicon products were evaluated in agarose gel electrophoresis (1%), stained with eitdeum bromide and photographed under uv light. The generated amplicons had a size of about 1,500 bp, as expected theoretical size (Supplementary Material 2-SM2).

6. The amplification products were purified with the aid of ExoSAP-IT Express (Applied Biosystems™), according to the manufacturer's protocol.

7. Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit in the Veriti thermal cycler, according to the manufacturer's protocol.

8. The sequencing reaction was subjected to capillary electrophoresis on the SeqStudio genetic analyzer.

9. Sequences were analyzed for quality and subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch#).

10. Additionally, a maximum likelihood phylogenetic tree was constructed in the program MEGA 11.

Troubleshooting

Time Taken

Anticipated Results

The primers are designed to provide clean, single-band PCR products for 10 species (*in silico*), *Xiphias gladius, Sardinella maderensis, Limanda aspera, Prionace glauca, Salmo salar, Oreochromis niloticus, Gadus chalcogrammus, Gadus morhua, Gadus ogac*, and *Gadus macrocephalus*. The generated amplicons demonstrated the effectiveness of the primer sets both for the PCR reaction and for sequencing. The primers offered an easy and efficient amplification for 4 species (*in vitro*): *Salmo salar, Oreochromis niloticus, Gadus morhua* and *Gadus macrocephalus*. The obtained sequences demonstrated good quality, with the generation of a fragment of about 1, 500 bp. Based on the sequences obtained, it was possible to identify the species at the level of the samples, as shown in Figure 1.

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Acknowledgements

The authors would like to thank the technical and administrative staff of the Instituto de Tecnologia de Alimentos (ITAL) for the infrastructure provided to carry out the study. We acknowledge the funding agency Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Process 17/50349-0).