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APPLICABILITY OF DNA MINI-BARCODING FOR SPECIES IDENTIFICATION IN PROCESSED SEAFOOD

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1. Introduction

Global seafood supply chains are highly vulnerable to economic and sanitary fraud [1]. Substitution of high-value species with lower-cost alternatives harms consumers and poses public health risks, such as exposure to unidentified allergens or toxins [2]. In the Brazilian regulatory context, the Ministry of Fisheries and Aquaculture (MPA) Ordinance No. 532, dated September 23, 2025 establishes guidelines for official seafood nomenclature, the enforcement of which requires high-precision taxonomic identification methods [3]. The incompatibility between the declared species and the one identified in the final product constitutes both a sanitary and economic violation, justifying the need for sensitive molecular tools capable of operating in highly complex processed matrices. DNA Barcoding (COI gene) is the taxonomic reference [4], but industrial processing such as salting and drying promotes DNA fragmentation through hydrolytic and oxidative degradation [5]. This makes standard 650-bp amplification unfeasible. To circumvent this limitation, mini-barcodes (100–300 bp) within the COI region offer a robust alternative [6]. Given this, rigorous validation is required. This study evaluates a mini-COI system, comparing *in silico* Primer-BLAST specificity with *in vitro* performance to enhance laboratory capacity in combating fraud and ensuring regulatory compliance

2. Experiment

Fish species identification was performed using a molecular approach, as detailed below.

Primer design: A universal primer pair was selected based on in silico multiple sequence alignment to amplify a short fragment (mini-barcode) of the COI gene, with an expected amplicon size of 295 bp, optimized for degraded DNA samples. Primer specificity was assessed using NCBI's Primer-BLAST to ensure specific amplification of the target region. The primer pair miniCOI-F (5′–ATCACAAAGACATTGGCACCCT–3′) and miniCOI-R (5′–AATGAAGGGGGGAGGAGTCAGAA–3′) consists of 22 and 23 nucleotides, with GC contents of 45% and 52% and melting temperatures (Tm) of 54°C and 57°C, respectively. Primers were synthesized by Invitrogen.

Sample collection: Fish products (*Gadus morhua*; desalted and salted-dried) were sourced in Chapecó, SC, Brazil. Samples were homogenized (min. 50 g) under sterile conditions and ~50 mg aliquots were stored at -20 °C until DNA extraction.

DNA extraction: Genomic DNA was extracted using the SureFood® PREP Basic kit (R-Biopharm), per manufacturer instructions. Concentration and purity ($A_{260/280}$ and $A_{260/230}$) were quantified via NanoDrop spectrophotometry.

PCR amplification of miniCOI and purification: A 295-bp fragment from the 5′ COI region was amplified by PCR. Reactions (25 µL total volume) followed optimized conditions based on the original miniCOI protocol [7]. PCR was performed on a QuantStudio™ 6 Pro Real-Time PCR System (Applied Biosystems) under standard cycling conditions (initial denaturation, 37 amplification cycles, and final extension), and products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) to remove residual contaminants.

Gel electrophoresis and visualization: PCR amplification success was verified by agarose gel electrophoresis. PCR products were resolved on a 1.0% agarose gel prepared in a 1× TAE buffer and stained with SYBR™ Safe DNA Gel Stain (Invitrogen). A GeneRuler 100 bp DNA Ladder (Thermo Scientific) was used as a molecular size marker to confirm the expected amplicon size. A total of 5 µL of each PCR product was loaded onto the gel. Electrophoresis was performed at 100 V for 60 min. Following electrophoresis, gels were visualized under UV illumination using a transilluminator (Loccus), and images were documented.

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Sequencing and post-sequencing analysis: MiniCOI amplicons were Sanger sequenced on an ABI 3730 DNA Analyzer (bidirectional), with chromatograms processed in TaxoScan to trim, align, and generate consensus sequence, which were compared in the BOLD Identification System for species identification. Identification was considered reliable when sequence similarity was ≥ 99%.

3. Results and Discussions

The observed absorbance ratios indicated good DNA purity, suitable for downstream molecular analyses. Quantification and purity results for the desalted and salted-dried samples are presented in Table 1.

Table 1. DNA quantification and purity of analyzed samples.

Sample identification	DNA (ng/µL)	A260/280	A260/230
Desalted cod	240.5	2.11	2.23
Salted-dried cod	85.7	2.09	1.95

Purified amplicons were quantified using a NanoDrop, yielding DNA concentrations of 39.0 ng/µL and 16.59 ng/µL for the analyzed samples. Successful amplification of the miniCOI region was confirmed by agarose gel electrophoresis, with samples showing visible bands at the expected size of approximately 295 bp (Figure 1), indicating adequate DNA integrity for sequencing.

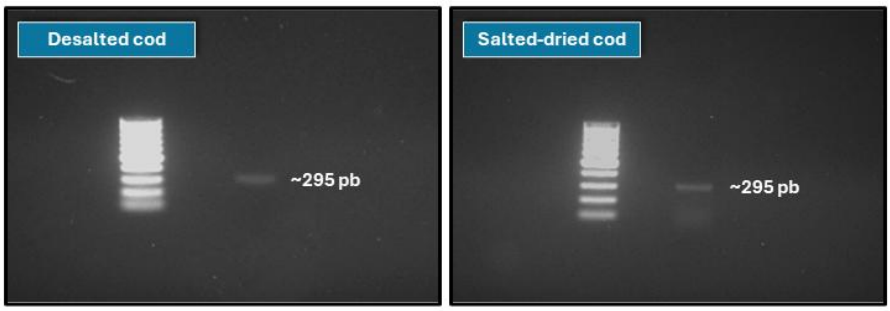

Fig. 1. *Agarose gel of miniCOI PCR products (~295 bp) with 1000 bp ladder* .

Consensus sequences of 242 and 245 bp were obtained for the analyzed samples, respectively. Both samples were identified as *Gadus morhua* with 99% sequence similarity, demonstrating that even reduced-length miniCOI fragments provide reliable species-level resolution. Results align with Brazilian regulations [3], which limit the label "bacalhau" to *G. morhua*, *G. macrocephalus*, or *G. ogac*. This study confirms that mini-COI fragments provide reliable species-level resolution in processed seafood for regulatory compliance.

The miniCOI approach proved reliable for fish species identification in processed matrices, with the 295 bp fragment overcoming DNA degradation limitations in salted and dried cod where conventional barcoding is unfeasible. The results support mini-barcoding as an effective strategy for species authentication and fraud detection, and highlight that tools such as TaxoScan can enhance laboratory efficiency and strengthen prevention of species substitution, ensuring consumer safety.

4. References

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