A retail market survey on fish fraud from Southern Italy using DNA barcoding

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Abstract

Consumption of seafood, which includes both wild and aquaculture products, has increased several-fold during the last 50 years. Species substitution, in which low-value fish are replaced with high-value fish, is one of the prominent phenomena happening in the international seafood trade and the leading cause of fraud in the fishery sector, leading to both economic and health concerns. In this study, DNA barcoding was employed to identify 78 fishery product samples collected from markets and supermarkets located in the Apulia region (Southern Italy) at the genus or species level. Non-compliance between the species detected and the species declared in the label was detected in 5 (6.41%) samples. This study highlights the need for further investigations regarding the traceability and assessment of food product authentication. Indeed, accurate taxonomic assignment and a robust traceability system are essential tools for tackling food adulteration problems, providing transparency, and protecting food safety.

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Introduction

Due to increasing health consciousness and awareness of consumers towards the beneficial effects of fish consumption, its demand is increasing gradually over the decades. Global demand for fish and seafood products increased considerably in 2022 compared to 2005; according to Food and Agriculture Organization (FAO) data, 184,1 million metric tons were consumed in 2022 and 141.5 million in 2005 (FAO, 2020 and 2023). Additionally, according to the latest FAO report, "The State of World Fisheries and Aquaculture 2024", global aquatic animal production reached a historic high in 2022, totaling 223.2 million tons, marking a 4.4% increase compared to 2020 (FAO, 2024). Market demand for fish and fish products is growing rapidly across all continents worldwide for several reasons. Among other reasons, the availability of ready-to-eat and ready-to-cook products is a key factor. The fact that aquaculture products are considered environmentally friendly with a lower carbon footprint compared to beef and pork (Nijdam *et al.*, 2012) is another reason; additionally, fisheries are acceptable in different religions and cultures. Despite its increasing popularity, seafood is one of the prominent products associated with food fraud; authentication studies and market monitoring of commercial products showed fish products are more vulnerable to mislabeling than other consumer goods (Chang *et al.*, 2016; Kappel and Schröder, 2016; Pardo *et al.*, 2016). This can lead to both commercial and sanitary problems. Cases of seafood fraud are reported in almost all countries, although their rate can vary. Mislabeling was detected in 50% of fish products in Germany (Kappel and Schröder, 2016; Kappel *et al.*, 2017), 22% in India (Nagalakshmi *et al.*, 2016), and 24% in South Brazil (Carvalho *et al.*, 2014; Di Pinto *et al.*, 2015). Almost 80% of commercial fish fillets were mislabeled in Italy (Castigliego *et al.*, 2015; Di Pinto *et al.*, 2015; Di Pinto *et al.*, 2016; Paracchini *et al.*, 2017) and several other countries. The European Union enforced Regulation (EU)

1379/2013, which requires fish economic operators to provide certain information on the labels of fish products. This information includes the commercial and scientific designation names, the production method (catch or breeding), the origin (the FAO fishing area for sea products and the name of the country for breeding products), and the fishing gear used (Xiong, D'Amico *et al*., 2016). Canned seafood products, shellfish, crustaceans, seafood salads, and breaded products are subject to a separate regulation; information that must be reported is defined in Article 19 of Legislative Decree 231/2017. Similar regulations are in force in many countries; however, despite these regulations widespread seafood mislabeling has been identified in United States and Canada (Hanner *et al.*, 2011; Willette *et al.*, 2017), in Europe (Di Pinto *et al.*, 2016), in Asia (Sultana *et al.*, 2018) and south Africa (Cawthorn and Hoffman, 2017). These cases of mislabeling highlight the need for stringent control measures to generate efficient species identification (OCEANA, 2021). Food fraud mainly involves the adulteration of food through mislabeling, but it also presents considerable safety risks for consumers. In particular, public health is compromised when toxic fish species are sold as non-toxic alternatives or when freshwater fish from contaminated sources are substituted for marine species (Goyal *et al.*, 2021) or when consumers are exposed to undeclared allergens. The impact of food fraud includes loss of consumer confidence in the food industry and in the effectiveness of competent authorities responsible for official food controls. In fact, intestinal disorders (*e.g*., Keriorrhea) may arise from consumption of escolar fish (Ling *et al.*, 2009; Caballero-Mateos *et al.*, 2018); in addition, severe food intoxication or fatal cases due to substitution of pufferfish with monkfish are reported (Cohen *et al.*, 2009).

Morphological identification of fish species is possible under raw or lightly processed conditions, but the taxonomical assignment becomes more difficult during processing, such as battering, crumbling, mincing, and cooking. Many methods based on protein biomarkers (electrophoretic method, immunological, and chromatography-based techniques) have been proposed for fish species identification in different seafood products. Some of these methods are quite successful in raw fish speciation, but most of them are not suitable for routine commercial fish control. Heat treatments induce protein degradation; in addition, some proteins lose biological properties after animal death, which is the main limitation to their effectiveness (Piskatá *et al.*, 2007; Angers *et al.*, 2017). DNA-based methods provide a better alternative to protein-based methods (Ali *et al.*, 2016; Angers *et al.*, 2017). Greater stability, cellular uniformity, and polymorphism of DNA biomarkers make these methods more reliable. DNA barcoding is one of the most widely accepted and successful methods for species identification in plants, as well as the animal kingdom (Sultana *et al.*, 2018; Adibah *et al.*, 2020). This approach uses various gene target regions to "fingerprint" or uniquely identify fish species. DNA authentication is based on the sequencing of mitochondrial genes such as *cytochrome c oxidase subunit 1* (*COI*) and *cytochrome b* (*CytB*), and it has been widely applied in various branches of biological sciences, including seafood identification (Hebert and Gregory, 2005; Leahy, 2021). Genetic markers are selected because they generally show high interspecies polymorphism and low intraspecies polymorphism (Rasmussen and Morrissey, 2009). Availability of DNA barcode libraries and databases such as the Barcode of Life Data Systems (BOLD) has greatly simplified the expansion of the DNA barcoding system as an effective means of fish speciation. The BOLD database includes more than 10,000 fish out of an expected 30,000 fish species with detailed information on the origin and location of the COI Voucher specimen. The aim of this study was to use DNA barcoding to investigate the prevalence of mislabeling of fish products purchased from retail markets located in the Apulia region (southern Italy).

Materials and Methods

Sample collection

During 2019-2021, a total of 78 samples of fish products from national and international brands were collected from supermarkets, fishmongers, and other local retail stores in the Apulia region (Southern Italy). All samples (fishes, cephalopods, and crustaceans) belonged to different manufacturing lots, including fresh (raw), frozen, or processed. From each sample, 25 g of tissue was stored at -20°C until the analysis. The samples include 39 fillets, 12 fresh fish cubes, 8 fish burgers, 8 flavoring products, 6 nugget samples, 3 sushi dishes, and 2 canned products (*Supplementary Table 1*).

DNA extraction and quantification

Total genomic DNA was extracted from fish tissue with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. 25 mg tissue was taken from each sample and transferred to a 1.5 mL Eppendorf tube. Lysis of tissue was done using the lysis buffer supplied with the kit. After incubation and two washing steps, DNA was eluted using 200 µL of AE elution buffer (Qiagen, Hilden, Germany). For each sample, the DNA concentration was estimated by a Qubit Fluorometer using Qubit ds DNA HS Assay (ThermoFisher Scientific, Waltham, MA, USA) and stored at 4°C for polymerase chain reaction (PCR) amplification.

Polymerase chain reaction amplification

The universal primers targeting *COI* and *CytB* mitochondrial

Table 1. Sequence of universal primers used for amplification.

ID primer	Primer Sequence (5'-3')	Length	Gene	Reference
CYTB 1	5'-CCATCCAACTCTCAGCATG ATGAAA-3'	25 bp	*CytB*	(Pepe *et al.*, 2005)
CYTB2	5'-GCCCCTCAGAATGATATTTGTCCTCA-3'	26 bp	*CytB*	(Pepe *et al.*, 2005)
Fish-F1	5'-TCAACCAACCACAAAGACATTGGCAC-3'	26 bp	*COI*	(Ward *et al.*, 2005)
Fish-R1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	26 bp	*COI*	(Ward *et al.*, 2005)
Fish-F2	5'-TCGACTAATCATAAAGATATCGGCAC-3'	26 bp	*COI*	(Camacho-Oliveira *et al.*, 2020)
Fish-R2	5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'	26 bp	*COI*	(Camacho-Oliveira *et al.*, 2020)

COI, cytochrome c oxidase subunit 1; CytB, cytochrome b.

genes were used for amplification. Specifically, we used two sets of primers to amplify two distinct regions of *COI*, which amplify a gene sequence of approximately 650 base pairs (bp) and *CytB* targeting a 359 bp region of mitochondrial DNA (Table 1) (Pepe *et al*., 2005; Ward *et al*., 2005; Camacho-Oliveira *et al*., 2020). PCR amplifications were performed with a 25 µL reaction mixture consisting of 3 µL of genomic DNA, 2.5 µL of 10XPCR Buffer, 0.25 µL of Platinum® Taq DNA Polymerase (Thermo Fisher Scientific) (5 U/µL), 0.5 µL of 10 mM dNTPs mix, and 50 pmol/µL of each primer. The PCR reactions were performed with an initial denaturation step at 94 °C for 2 min followed by 40 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 68°C for 30 sec, with a final extension at 68°C for 5 min). The PCR products were analyzed using the QIAxcel advanced system (Qiagen, Hilden, Germany). Before Sanger sequencing, PCR products were purified using the enzyme kit "Exo-SAP IT" (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Sequencing and detecting

Sequence reactions were carried out using the "Big Dye Terminator v.3.1 Cycle Sequencing Ready Reaction" (Life Technologies) according to the manufacturer's protocol. The sequenced products were separated with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), imported and assembled in Bionumerics 7.6 software (Applied Maths, Saint Marteen-Latem, Belgium). Sequences were used as queries against a specialized DNA barcode databank, Bold System Identification Engine (http://www.boldsystems.org/), and a primary nucleotide database, NCBI (National Center for Biotechnology Information) (www.ncbi.nlm.nih.gov). While the first resource mainly consisted of barcode gene marker sequences with curated taxonomical nomenclature, "non-redundant" NCBI sequence collection represents the largest known public bioinformatic resource, containing highly heterogeneous sequence data (from gene fragments to whole genomes). Both databanks are valuable resources for sequence taxonomical assignment, although NCBI is characterized by a constant update and a more powerful and customizable DNA similarity search engine.

Results

Only samples with sequences showing high percentages of identity were considered, and the two databases (NCBI and BOLD systems) were compared for both regions of the *COI*. Out of 78 analyzed products, 5 samples (6.41 %) were assigned to a different species compared to the one declared (Table 1). In detail, blue shark (*Prionace glauca*) was found instead of squid (*Loligo opalescens* sample 13); in samples 14, 15, 16, and 17, haddock (*Melanogrammus aeglefinus*) were observed in place of swordfish (*Xiphias gladius*). All these samples were packaged frozen products. Main results for the species identification are visualized in Figure 1, with mislabeled products (and declared species) in red color scale, while the proportion of samples with consistent species labeling is in green color scale (Figure 1).

The highest percentage of pairwise aligned consensus sequences was blasted in NCBI to compare the similarity scores against the BOLD database. 63 samples amplified with Fish F1-R1 primers showed a high percentage of identity; the *COI* F2-R2 region allowed 12 samples to be identified with a high percentage; only 9 samples were analyzed with primers that amplified the *CytB* gene portion. Five samples showed a high percentage of identity with both *CytB* primers and the *COI* gene region.

Discussion and Conclusions

The global impact of fish fraud

Several studies demonstrated the vulnerability of the fish supply chain to fish fraud, particularly species substitution and mislabeling (Fox *et al.*, 2018; Munguia-Vega *et al*., 2022). An investigation was conducted by INTERPOL_EUROPOL, which demonstrated fish fraud as the third-highest risk category of food vulnerable to fraud (EUROPOL, 2016; Paolacci *et al.*, 2021). Various kinds of processed fish products like fish balls, fish nuggets, fish fingers, canned tuna, fish chips, fish burgers, and sandwiches are prepared from some costly fish species like salmon, tuna, sardines, mackerel, shrimps, crabs, and cod (Nagalakshmi *et al.*, 2016). Replacement of costly fish with some less expensive ones, such as tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), milkfish (*Chanos chanos*), and mud cap (*Cirrhinus molitorella*), is reported (Hubert *et al.*, 2015; Nagalakshmi *et al.*, 2016; Xiong, Guardone, *et al.*, 2016). In addition, in recent years, ready-to-cook fish products have gained huge popularity due to lack of time and busy life in urban areas, so these fish products have received a lot of attention as a stable, attractive, and economically beneficial commodity in international food trade (Hebert and Gregory, 2005). In 2021, a Guardian Seascape analysis of 44 recent surveys of more than 9000 seafood samples from restaurants, fishmongers, and supermarkets in more than 30 countries was conducted and found that 36% samples were mislabelled, exposing a large amount of seafood fraud on a global scale (Leahy, 2021). All studies conducted indicate that species substitution and mislabeling are serious problems in the international fish trade. There are many other studies conducted in other countries like Italy (Di Pinto *et al.*, 2016; Filonzi *et al.*, 2021), Germany (Kappel and Schröder, 2016; Kappel *et al.*, 2017), India (Nagalakshmi *et al.*, 2016), South Brazil (Carvalho *et al.*, 2014; Di Pinto *et al.*, 2015), which show

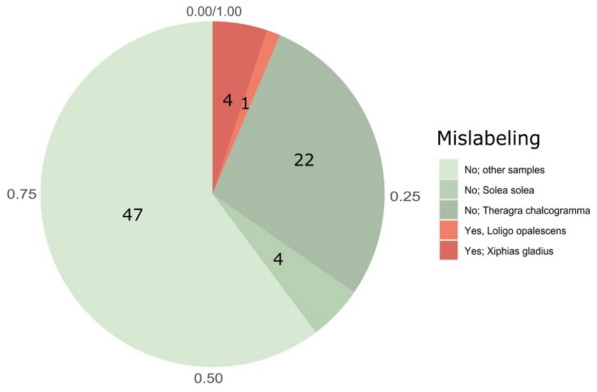

Figure 1. Pie chart for species labeling consistency between declared and detected species among fish products. Red color scale areas: mislabeled products with declared species and sample size (number within chart); green color scale areas: consistently labeled products. Two example subsets are shown, *i.e.,* products composed of *Theragra chalcogramma* and *Solea solea*.

the concerns related to fish fraud (Di Pinto *et al.*, 2016; Staats *et al.*, 2016).

The role of DNA barcoding

DNA barcoding is one of the most widely used molecular tools to investigate species substitution and mislabeling in various fish products (Venuti *et al*., 2021). Several researchers applied the DNA barcoding tool to investigate species substitution and mislabeling in various parts of the world. Filonzi *et al.* (2010) reported substitution of halibut (*Renharditus hippoglossus*) with pangasius (*Pangasianodon hipophthalmus*). Maralit *et al*. (2013) observed substitution of gindara or sablefish (*Anoplopoma fimbria*) with escolar (*Lepidocybium flavobrunneum*), while Warner (2018) identified red snapper (*Lutjanus campechanus*) replaced with tilefish (*Malacanthidae* spp.). In 2016, large-scale mislabeling (85%) was observed in products purchased from e-commerce platforms (Xiong, D'Amico, *et al.*, 2016). These studies highlight the widespread occurrence of species substitution and labeling inaccuracies in seafood products worldwide. They also emphasize the critical role of precise molecular techniques in ensuring transparency and quality within the seafood market, helping to prevent economic losses and safeguard consumer health. While molecular diagnosis techniques are now largely implemented for research or evaluation/comparison purposes, there is a need to standardize these methodologies and create a global legislative framework for their routine practical application.

In our study, out of 78 tested samples, 5 were found to be non-compliant with respect to the identification reported on the label; in addition, some unusual results were observed. For example, we found haddock (*Melanogrammus aeglefinus*) instead of swordfish (*Xiphias gladius*). It is important to consider that the samples where species substitution was found are anatomically distinct from each other, but they were processed matrices, specifically nuggets, resulting in the loss of their morphological characteristics. From a sanitary point of view, mislabeling can produce food poisoning, sometimes severe or fatal, when poisonous fish, such as those belonging to the Tetraodontidae or Diodontidae families, are marketed as common fish; a study from Italy reported a case in which squids were replaced with pufferfish (Armani *et al.*, 2015). In addition, severe and fatal cases of tetrodotoxin poisoning due to puffer fish consumption were reported from Israel and Bangladesh (Islam *et al.*, 2011). Such incidents indicate severe risks associated with mislabeling and species substitution of fish, which also hampers the trust of consumers in the market. A slight food poisoning, characterized by gastrointestinal disorders and keriorrheas, is associated with the consumption of *Ruvettus pretiosus* and other fish collective named "oil fish" (Bentur *et al.*, 2008; Ling *et al.*, 2009). These fish show a high amount of indigestible, heat-resistant wax esters known as gempylotoxins (Bentur *et al.*, 2008; Ling *et al.*, 2009). The EU legislation allows their market but impose to report on the label that the product could cause gastrointestinal disorders (Regulation (CE) 1021/2008). International regulations focus on marine resource management, traceability, and labeling to prevent illegal fishing, species substitution and ensure product quality. In this regard, consumer education is essential to promote informed and sustainable choices, encouraging understanding of proper labeling and responsible fish species, and contributing to fraud prevention.

Challenges in DNA barcoding of processed fish products

Recent advancements in DNA-based techniques have shown greater capability to authenticate even highly processed fish products. One of the most difficult tasks for such techniques is to extract good quality DNA from those food products because many ingredients used in fish processing act as PCR inhibitors, which can hamper DNA amplification (Adibah *et al.*, 2020). Although DNA can be isolated it may be possible that inhibitors present in the sample still interfere with PCR by disrupting or even completely inhibiting the activity of DNA polymerase (Di Pinto *et al.*, 2007). For species identification using the *COI* gene, the use of both GenBank and the BOLD database is recommended. Our experience suggests that the databases show a very similar percentage of identities between them. The major difference is that the BOLD system was developed with specimens in good condition and contains only validated sequences that can be used for identification. BOLD system results identify species by the degree of nucleotide variation, with similar species having a divergence value of less than 1 (Nicole *et al.*, 2012). However, if no match with the BOLD system database is found, it is possible to look for an alternative NCBI BLAST analysis, which displays a list of species that are more like the query sequence, as well as a BIT score, which estimates the percent identity and E value. The *COI* gene has the largest taxonomic presence in the NCBI nucleotide database.

The genetic strategies proposed in this work have shown efficient results for biological tissue identification of seafood products. Species identification based on the two gene markers (*COI* and *Cytb*) is a relatively simple and efficient tool for the authentication of seafood products. However, we must consider that the technique of DNA barcoding, although it proves successful in the analysis of products composed of a single species, has often proved complex when applied to multispecies products, because the method used (Sanger sequencing) aims to sequence only the DNA of the most abundant species in the sample (Rasmussen and Morrissey, 2009). In this way, lesser species are not identified, and this limitation poses a risk if the species belong to particularly allergenic categories such as crustaceans and mollusks.

Future directions in seafood traceability and fraud prevention

In this regard, next-generation sequencing techniques would represent an innovative and promising approach for the identification of species in complex matrices. Indeed, the DNA metabarcoding approach allows simultaneous amplification of gene portions of any organism potentially present in the test sample, making use of appropriately designed universal primers (Mottola *et al*., 2022; Miya *et al*., 2022). In fact, although traditional Sanger sequencing technology is the most widely used technique for species identification, it is not suitable for analyzing processed samples that may contain more than one species. In addition, the next-generation sequencing approach allows the use of a larger number of databases and software useful for *in silico* analysis. Therefore, this new strategy opens the way also for quantitative studies of fish mixtures.

Finally, it is essential to improve the traceability and regulation of seafood products by educating consumers to make responsible choices and recognize traceable and sustainable products; enforcing clear labeling with information on species, origin, fishing methods, and processing; promoting sustainable certifications; standardizing traceability and labeling standards internationally; and adopting advanced technologies, such as DNA barcoding and metabarcoding, to ensure traceability and prevent fraud in the seafood sector.

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Online supplementary material:
Supplementary Table 1. Summary results for analyzed samples.