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Efficiency of DNA Mini-Barcoding to Assess Mislabelling in Commercial Fish Products in Italy: An Overview of the Last Decade

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Abstract: The problem of fish traceability in processed products is still an important issue in food safety. Major attention is nowadays dedicated to consumer health and prevention of possible frauds regulated by national and international laws. For this reason, a technical approach is fundamental in revealing mislabelling at different levels. In particular, the use of genetic markers has been standardized and DNA barcoding is considered the gold-standard strategy to examine and prevent species substitution. Considering the richness of available DNA databases, it is nowadays possible to rapidly reach a reliable taxonomy at the species level. Among different approaches, an innovative method based on DNA mini barcoding has recently been proposed at an international level. Starting from this evidence, we herein illustrate an investigation dealing with the evolution of this topic in Italy over the last decade. The molecular analysis of 71 commercial fish samples based on mini-COI sequencing with two different primer sets reached an amplification success rate of 87.3 and 97.2%. The investigation revealed four major frauds (5.8%) and four minor ones (5.8%). Results highlighted a decrease in incorrect labeling in Italy from 32% to 11.6% over the last decade, although a recurrent involvement of “endangered” species sensu IUCN was still observed.

Keywords: food frauds; conservation; molecular genetics; mitochondrial DNA; cytochrome oxidase; biodiversity

1. Introduction

The consumption of seafood products has increased all over the world during the last 50 years as demonstrated by data issued by the United Nations Food and Agriculture Organization (FAO) [1,2] that estimated the value of fish commerce to be over hundreds of billion dollars each year. Considering the importance of fish trade in the globalization era, consistent monitoring of the production chain focusing on technological developments, handling, processing and distribution by global networks is, therefore, necessary [3]. Nowadays, food quality and safety issues are crucial points for consumers, also considering the frequency of fish species substitutions. Basic consequences may be health problems that occur primarily through the consumption of cryptic species coming from contaminated areas or able to trigger allergy problems [4]. Despite financial fraud still being the main issue [5], major attention must be dedicated to such cryptic species as those belonging to the genera Pangasius, Salmo and Tilapia whose aquaculture exploitation makes them easy substitutes for wild species [4].

Precautionary measures are, therefore, necessary, particularly for products that are not visually recognizable at sight and are indistinguishable on a morphological basis after processing. Deliberate mislabelling and replacement of high-value species with cheaper ones is an Economically Motivated Adulteration (EMA) and is considered as fraud [5]. In a...
report paper published by the European Parliament in 2013, seafood was identified as the second most likely group of food to be subject to fraud, following olive oil [6].

Although the European Union labeling law (EU Regulation No. 1379/2013) [7] requests appropriate species traceability and labeling (scientific binomial nomenclature based on genus and species together with the common name), the identification of processed species is often difficult. Many scientists are working with innovative technologies to assess taxa identification and authenticity. Several molecular methods have been proposed to identify the correct species, from the use of single-protein or species-specific DNA sequences to modern genomic approaches. Among the wide variety of DNA methods nowadays available, the choice is mainly influenced by use simplicity and affordable costs in relation to the product value [8].

In recent years, the gold-standard strategy to examine and prevent species fraud has been DNA barcoding, a fast and cost-effective method for correct classification at a species level [9]. The original approach is based on the sequence analysis of a 650-bp mitochondrial DNA fragment. The cytochrome c oxidase I (COI) gene is the favorite sequence to act as a “barcode” to identify and delineate the animal lifeform [10]. Although the analysis of long DNA fragments is complex due to degradation along the different steps from production to analysis, most of the mtDNA-based studies have analyzed the full-length COI barcode [11,12]. Since the first applications of mitochondrial DNA barcoding [13], and then through constant methodological improvements [14], the most recent advances have led to an innovative approach based on mini-barcodes [15–17].

The methodology refers to the analysis of short DNA fragments and it can be applied in different fields of systematics (from museum collection research to forensic applications). COI mini-barcoding is, therefore, useful to assess the correct taxonomy in processed products. This is particularly the case with commercial fish, for which the correct preservation under stable refrigerated conditions is a major issue during fishing, transportation and distribution. In addition, fish processing for preservation is the major cause of DNA degradation. Consequently, molecular analyses to reveal species substitution may face DNA degradation limitations and, therefore, become biased by technical questions. The possibility of analyzing short DNA fragments such as COI mini-barcoding seems to solve this issue. In the field of ichthyology, the application of mini-barcoding is nowadays possible thanks to the availability of specific databases accounting for millions of COI sequences. To give a general idea, BOLD database reports cover more than 24,000 barcoded species among Actinopterygii and Elasmobranchii. On the other hand, it is noteworthy to observe that data redundancy may generate confusion or technical biases due to contradictory attribution within a genus level.

Starting from past experience of the application of COI and CytB barcoding, besides the use of additional markers [18], for the identification of species substitution in fish products [19], a new investigation was carried out to verify the usefulness of COI mini-barcoding as an innovative methodology to substitute for classic barcoding. The research was focused on sampling and analyzing the same taxa purchased in the same department stores to assess the evolution of cryptic species mislabeling after a decade, also considering the application of new European regulations [7]. The suitability of COI mini-barcoding and the evolution of the mislabeling issue are herein discussed, also considering some critical ecological, taxonomic and commercial aspects that have been arisen by experts based on their judgments.

2. Materials and Methods

Fresh and frozen commercial fish products were acquired in 10 different department stores located in the Emilia Romagna region during 2020 and 2021. The different department stores belonged to the major brands fully distributed over the entire country, with their own national fish provider. In this way, although executed locally, the research had national coverage.
A small sample of the edible tissue of approximately 50 mg was collected and fixed in absolute ethanol under refrigerated conditions. Samples were stored at $-20^\circ C$ to be processed within one week of purchasing. A total number of 71 specimens belonging to 27 putative seawater species were analyzed. The samples dataset was prepared, listing the declared names and areas of fishing. The entire dataset is reported in Table 1. It is noteworthy to observe that four samples (MB43, MB64, MB66, MB71) did not report the origin in the label either as a specific FAO fishing area or a summary geographic declared one.

Table 1. Detailed description of analyzed samples and information reported in the labels (common name, declared scientific name and fishing area). “N/A” (not available) refers to lacking label information.

| Sample ID | Geographical Origin | Common Name | Declared Scientific Name |
|-----------|---------------------|-------------|--------------------------|
| MB01      | Southeast Atlantic Ocean | Deep-water Cape hake | *Merluccius capensis* e/o *M. paradoxus* |
| MB02      | Northeast Atlantic Ocean | Pink salmon | *Oncorhynchus gorbuscha* |
| MB03      | Northeast Atlantic Ocean—Iceland seabed | Goldblotch cod | *Gadus morhua* |
| MB04      | East Central Atlantic Ocean | Pink salmon | *Epinephelus costae* |
| MB05      | Northeast Atlantic Ocean | Swordfish | *Xiphius gladius* |
| MB06      | Eastern Indian Ocean | Angolan dentex | *Dentex angolensis* |
| MB07      | East Central Atlantic Ocean | Atlantic cod | *Gadus morhua* |
| MB08      | Northeast Atlantic Ocean—Baltic Sea | Pink salmon | *Oncorhynchus gorbuscha* |
| MB09      | Northeast Pacific Ocean | Atlantic cod | *Gadus morhua* |
| MB10      | Northeast Atlantic Ocean—Baltic Sea | Blue shark | *Prionace glauca* |
| MB11      | Southeast Atlantic Ocean | Haddock | *Melanogrammus aeglefinus* |
| MB12      | Northeast Atlantic Ocean | Pink salmon | *Oncorhynchus gorbuscha* |
| MB13      | Northeast Pacific Ocean | Goldblotch grouper | *Epinephelus costae* |
| MB14      | East Central Atlantic Ocean | Haddock | *Melanogrammus aeglefinus* |
| MB15      | Northeast Atlantic Ocean | Deep-water Cape hake | *Merluccius capensis* e/o *M. paradoxus* |
| MB16      | Southeast Atlantic Ocean | Atlantic cod | *Gadus morhua* |
| MB17      | Northeast Atlantic Ocean—Iceland seabed | Deep-water Cape hake | *Merluccius capensis* e/o *M. paradoxus* |
| MB18      | Southeast Atlantic Ocean | Skipjack tuna | *Katsuwonus pelamis* |
| MB19      | Central Western Pacific Ocean | Angolan dentex | *Dentex angolensis* |
| MB20      | East Central Atlantic Ocean | Pink salmon | *Oncorhynchus gorbuscha* |
| MB21      | Pacific Ocean Northeast or North West | Atlantic cod | *Gadus morhua* |
| MB22      | Northwest Atlantic Ocean | North Pacific hake | *Merluccius productus* |
| MB23      | Northeast Pacific Ocean | Swordfish | *Xiphias gladius* |
| MB24      | Southeast Atlantic Ocean | Skipjack tuna | *Katsuwonus pelamis* |
| MB25      | Central Western Pacific Ocean | North Pacific hake | *Merluccius productus* |
| MB26      | Northeast Pacific Ocean | Atlanto-East Pacific Ocean | *Gadus morhua* |
| MB27      | Northeast Atlantic Ocean—Iceland seabed | Atlantic cod | *N/A* |
| MB28      | East Central Atlantic Ocean | Angolan dentex | *N/A* |
| MB29      | Northeast Atlantic Ocean | Beaked redfish | *N/A* |
| MB30      | Pacific Ocean Northeast or North West | Alaska pollock | *Theragra chalcogramma* |
| MB31      | Northeast Atlantic Ocean | Atlantic cod | *Gadus morhua* |
| MB32      | Pacific Ocean Northeast or North West | Pink salmon | *Oncorhynchus gorbuscha* |
| MB33      | Pacific Ocean Northeast or North West | Alaska pollock | *Theragra chalcogramma* |
| MB34      | Central or Southeast Pacific Ocean | Swordfish | *Xiphias gladius* |
| MB35      | Southeast Atlantic Ocean | Blue shark | *Prionace glauca* |
| MB36      | Southeast Atlantic Ocean | Haddock | *Melanogrammus aeglefinus* |
| MB37      | Atlantic Ocean | Tub gurnard | *Cleidionichthys lucerna* |
| MB38      | Northeast Atlantic Ocean | Spiny turbot | *Psetta spp.* |
| MB39      | East Central Atlantic Ocean | Swordfish | *Xiphias gladius* |
| MB40      | East Central Atlantic Ocean | Leerfish | *Lueha amia* |
| MB41      | Western Mediterranean | Deep-water Cape hake | *Merluccius capensis* e/o *M. paradoxus* |
| MB42      | Southeast Atlantic Ocean | Alaska pollock | *Theragra chalcogramma* |
| MB43      | N/A | Porbeagle | *Lamna nasus* |
| MB44      | Northwest Atlantic Ocean | European plaice | *Pleuronectes platessa* |
| MB45      | Northeast Atlantic Ocean | Yellowfin tuna | *Thunnus albacares* |
| MB46      | Indian Ocean | Nile perch | *Lates niloticus* |
| MB47      | Lake Victoria | Picked dogfish | *Squalus acanthias* |
| MB48      | Northwest Atlantic Ocean | Golden redfish | *Sebastes norvegicus* |
| MB49      | Northeast Atlantic Ocean | Bearded brotula | *Brotula barbata* |
| MB50      | East Central Atlantic Ocean | European plaice | *Pleuronectes platessa* |
| MB51      | Northeast Atlantic Ocean | Shortfin mako | *Isurus oxyrinchus* |
| MB52      | East Central Atlantic Ocean | Nile perch | *Lates niloticus* |
| MB53      | Lake Victoria, Tanzania | Porbeagle | *Lamna nasus* |
| MB54      | East Central Atlantic Ocean | Porbeagle | *Lamna nasus* |
Table 1. Cont.

| Sample ID | Geographical Origin        | Common Name        | Declared Scientific Name          |
|-----------|----------------------------|--------------------|-----------------------------------|
| MB55      | North Sea                  | Smooth-hound       | *Mustelus mustelus*               |
| MB56      | Northwest Atlantic Ocean   | Halibut            | *Reinhardtius hippoglossoides*    |
| MB57      | Unreported                 | Alaska pollock     | *Theragra chalcogramma*           |
| MB58      | Northeast Atlantic Ocean   | European plaice    | *Pleuronectes platessa*           |
| MB59      | Iceland seabed             | Beaked redfish     | *Sebastes mentella*               |
| MB60      | Pacific Ocean Southwest    | Shortfin mako      | *Isurus oxyrinchus*               |
| MB61      | Northeast Atlantic Ocean   | Atlantic cod       | *Gadus morhua*                    |
| MB62      | Norwegian Sea              | Saithe             | *Pollachius virens*               |
| MB63      | Northeast Atlantic Ocean   | Swordfish          | *Xiphias gladius*                 |
| MB64      | North Sea                  | European plaice    | *Pleuronectes platessa*           |
| MB65      | Norwegian Sea              | Porbeagle          | *Gadus morhua*                    |
| MB66      | Northwest Atlantic Ocean   | Atlantic cod       | *Gadus morhua*                    |
| MB67      | North Sea                  | Pacific cod        | *Gadus macrocephalus*             |
| MB68      | Pacific Ocean Northwest    | Smooth-hound       | *Mustelus mustelus*               |
| MB69      | Northeast Atlantic Ocean   | Atlantic cod       | *Gadus morhua*                    |
| MB70      | N/A                        | Alaska pollock     | N/A                               |

Genomic DNA was extracted and purified from about 10 mg of ethanol-preserved samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Purified DNA was evaluated by means of 1% agarose gel electrophoresis. No samples were discharged due to bad DNA quality although some of them were considered borderline (as outlined in the Results section).

Two different sets of primers were tested. The first pair of universal primers, Fish_miniFW: ATCACAAAGACATTGGCACCCT and Fish_miniRV: AATGAAGGGGGGAGGAGTCAGAA, specifically proposed by Sultana et al. [17] for fish species, were used and are herein named “Fish_Mini”. A 295 bp fragment of the mitochondrial COI gene was amplified through polymerase chain reaction (PCR) amplification using a Bio-Rad T100 Thermal Cycler. The cycling conditions were 95 °C for 10 min, followed by 34 cycles of 95 °C for 45 s, 57 °C for 45 s, 72 °C for 45 s and a final step at 72 °C for 10 min.

The second set of primers was tested following the protocol suggested by Shokralla et al. [15]. Amplicons of 226 base pairs were obtained using a primer pair originally called Mini_SH-E. The two primers, “Fish_miniE”_F 5′-CACGACGCTTGGTAAACGCACAYAACAYAAAGAYATIGGCG-AC-3′ (forward) and Fish_miniE_R 5′-GGATAACAAATTTCACACAGGTCAC-3′ (reverse), were chosen and are herein referred to as “Fish_miniE”. The PCR was set as follows: 34 cycles of 45 s at 95 °C, 45 s at 67 °C, and 45 s at 72 °C, after an initial 10 min denaturation step at 95 °C and a final extension at 72 °C for 10 min.

The chemical conditions for both approaches were the following: a reaction volume of 20 µL—containing 1 U of GoTaq Polymerase (Promega, Madison, WI, USA), Mg2+ 1.5 mM, dNTPs 0.2 mM and 10 pmol of each primer—was used.

Amplicons were separated by 2.5% agarose gel electrophoresis and purified using the Qiagen MinElute PCR Purification Kit. The quality of the purified sample (1 µL) was visualized in 1.5% agarose gel that yielded clear bands. DNA quantity was evaluated using the Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen, Waltham, MA, USA) with a Qubit 3.0 Fluorometer (Life Technologies, Waltham, MA, USA). COI sequencing of both amplified regions was performed by the MACROGEN Europe service (Amsterdam, The Netherlands). Twenty percent of the samples were locally reanalyzed using a CEQ8000 DNA Analysis System (Beckman Coulter, Milan, Italy) based on capillary electrophoresis. The analytical conditions are reported in detail in Filonzi et al. [19].

The obtained sequences were manually corrected using MEGA 7.0 [20] and compared with those available in the genomic databases of GenBank using the BLAST service and BOLD (Barcode of Life Data System). In both cases, the species level was assigned when the identity rate was greater than 98% considering either BLAST or BOLD analyses [21]. The accession numbers of selected reference sequences displaying the highest identity score are available on request.
3. Results

Data concerning 71 analyzed samples are reported in Table 2. Considering the two different primer sets, “Fish_miniE” [15] successfully amplified 62 samples out of 71 (87.3% success rate) while “Fish_mini” [17] displayed a positive result in 69 of 71 samples (97.2%). In our dataset, four samples (MB28, MB29, MB66, MB71) did not have a clear scientific name (5.6%) and species comparison was executed based on common names. Similarly, four additional samples (MB43, MB64, MB66, MB71) did not report the origin on the label either as a specific FAO fishing area or a geographic declared region (5.6%).

Table 2. Scientific names and identity percentage values of each sample are provided both for BLAST and BOLD alignment analysis. Major frauds: mislabeling at genus level; minor frauds: different species within the same genus. Major species substitutions in bold; minor substitutions underlined; N/A: Not Available.

| Sample ID | Declared Scientific Name | GenBank Result (BLAST) | % Identity GenBank | BOLD Result | % Identity BOLD |
|-----------|---------------------------|------------------------|--------------------|-------------|----------------|
| MB01      | Merluccius capensis e/o M. paradoxus | Merluccius paradoxus | 99.1               | Merluccius paradoxus | 98.9 |
| MB02      | Oncorhynchus gorbuscha     | Oncorhynchus gorbuscha | 98.3               | Oncorhynchus gorbuscha | 97.7 |
| MB03      | Gadus morhua               | Gadus morhua           | 98.6               | Gadus morhua           | 98.4 |
| MB04      | Epinephelus costae         | Epinephelus costae    | 98.3               | Epinephelus costae    | 99.1 |
| MB05      | Oncorhynchus gorbuscha     | Oncorhynchus gorbuscha | 97.7               | Oncorhynchus gorbuscha | 98.0 |
| MB06      | Xiphias gladius            | Xiphias gladius      | 100                | Xiphias gladius      | 100  |
| MB07      | Dentex angolensis          | Dentex moroccanus    | 99.1               | Dentex macroptalmus   | 100  |
| MB08      | Gadus morhua               | Gadus morhua          | 98.3               | Gadus morhua          | 98.1 |
| MB09      | Oncorhynchus gorbuscha     | Oncorhynchus gorbuscha | 98.6               | Oncorhynchus gorbuscha | 100 |
| MB10      | Gadus morhua               | Gadus morhua          | 98.3               | Gadus morhua          | 98.0 |
| MB11      | Prionace glauca            | Prionace glauca      | 99.1               | Prionace glauca      | 100  |
| MB12      | Melanogrammus aeglefinus   | Melanogrammus aeglefinus | 99.1             | Melanogrammus aeglefinus | 100 |
| MB13      | Oncorhynchus gorbuscha     | Oncorhynchus gorbuscha | 99.5               | Oncorhynchus gorbuscha | 100 |
| MB14      | Epinephelus costae         | Epinephelus costae   | 98.2               | Epinephelus costae   | 99.1 |
| MB15      | Melanogrammus aeglefinus   | Melanogrammus aeglefinus | 99.5             | Melanogrammus aeglefinus | 100 |
| MB16      | Merluccius capensis e/o M. paradoxus | Merluccius paradoxus | 98.2               | Merluccius paradoxus | 99.1 |
| MB17      | Gadus morhua               | Gadus morhua          | 98.2               | Gadus morhua          | 100  |
| MB18      | Merluccius capensis e/o M. paradoxus | Merluccius paradoxus | 100                | Merluccius paradoxus | 100  |
| MB19      | Katsuwonus pelamis         | Thunnus thynnus      | 98.7               | Thunnus maccoyii     | 100  |
| MB20      | Dentex angolensis          | Dentex moroccanus    | 96.9               | Dentex macroptalmus   | 99.4 |
| MB21      | Oncorhynchus gorbuscha     | Oncorhynchus gorbuscha | 98.8               | Oncorhynchus gorbuscha | 100 |
| MB22      | Gadus morhua               | Gadus morhua          | 99.1               | Gadus morhua          | 98.7 |
| MB23      | Merluccius productus       | Merluccius productus  | 98.8               | Merluccius productus  | 100  |
| MB24      | Xiphias gladius            | Xiphias gladius      | 98.7               | Xiphias gladius      | 100  |
| MB25      | Katsuwonus pelamis         | Thunnus thynnus      | 99.2               | Thunnus maccoyii     | 100  |
| MB26      | Merluccius productus       | Merluccius productus  | 99.1               | Merluccius productus  | 100  |
| MB27      | Gadus morhua               | Gadus morhua          | 99.1               | Gadus morhua          | 100  |
| MB28      | N/A                       | Dentex angolenses    | 99.1               | Dentex angolenses    | 100  |
| MB29      | N/A                       | Sebastes mentella    | 99.1               | Sebastes mentella    | 99.5 |
| Sample ID | Declared Scientific Name | GenBank Result (BLAST) | % Identity GenBank | BOLD Result | % Identity BOLD |
|-----------|--------------------------|------------------------|--------------------|-------------|----------------|
| MB30      | Theragra chalcogramma    | Theragra chalcogramma  | 99.5               | Gadus chalcogrammus | 100           |
| MB31      | Gadus morhua             | Gadus morhua           | 99.4               | Gadus morhua   | 98.8          |
| MB32      | Oncorhynchus gorbuscha   | Oncorhynchus gorbuscha | 99.5               | Oncorhynchus gorbuscha | 100           |
| MB33      | Theragra chalcogramma    | Theragra chalcogramma  | 99.5               | Gadus chalcogrammus | 100           |
| MB34      | Xiphias gladius          | Xiphias gladius        | 99.5               | Xiphias gladius | 100           |
| MB35      | Priacne glauca           | Priacne glauca         | 99.5               | Priacne glauca  | 99.4          |
| MB36      | Xiphias gladius          | Xiphias gladius        | 100                | Xiphias gladius | 100           |
| MB37      | Sebastes norvegicus      | Sebastes norvegicus    | 99.1               | Sebastes viviparus | 100          |
| MB38      | Chelidonichthys lucerna  | Merluccius paradoxus   | 98.7               | Merluccius paradoxus | 99.1          |
| MB39      | Psetodes spp.            | Psetodes bennetti      | 99.1               | Psetodes bennetti | 99.5          |
| MB40      | Xiphias gladius          | Xiphias gladius        | 100                | Xiphias gladius | 100           |
| MB41      | Lichia amia              | Lichia amia            | 99.1               | Lichia amia    | 99.0          |
| MB42      | Merluccius capensis e/o M. paradoxus | Merluccius paradoxus | 99.1 | Merluccius paradoxus | 98.1 |
| MB43      | Theragra chalcogramma    | Theragra chalcogramma  | 99.1               | Gadus chalcogrammus | 100           |
| MB44      | Lamna nasus              | Isurus oxyrinchus      | 99.5               | Isurus oxyrinchus | 99.5          |
| MB45      | Pleuronectes platessa    | Pleuronectes platessa  | 100                | Pleuronectes platessa | 99.1 |
| MB46      | Thunnus albacares        | Thunnus albacares      | 99.6               | Thunnus albacares | 99.1          |
| MB47      | Lates niloticus          | Lates niloticus        | 100                | Lates niloticus | 100           |
| MB48      | Squalus acanthias        | Squalus acanthias      | 99.1               | Squalus acanthias | 98.2          |
| MB49      | Sebastes norvegicus      | Sebastes norvegicus    | 99.1               | Sebastes viviparus | 99.5          |
| MB50      | Brotula barbata          | Brotula barbata        | 98.3               | Brotula multibarbata | 99.5 |
| MB51      | Pleuronectes platessa    | Pleuronectes platessa  | 99.1               | Pleuronectes platessa | 99.5 |
| MB52      | Isurus oxyrinchus        | Isurus oxyrinchus      | 99.2               | Isurus oxyrinchus | 99.5          |
| MB53      | Lates niloticus          | Lates niloticus        | 98.2               | Lates niloticus | 97.1          |
| MB54      | Isurus oxyrinchus        | Isurus oxyrinchus      | 99.5               | Isurus oxyrinchus | 99.5          |
| MB55      | Mustelus mustelus        | Mustelus asterias      | 98.3               | Mustelus asterias | 98.3 |
| MB56      | Reinhardtius hippoclossoides | Reinhardtius hippoclossoides | 99.1 | Reinhardtius hippoclossoides | 99.5 |
| MB57      | Theragra chalcogramma    | Theragra chalcogramma  | 98.7               | Gadus chalcogrammus | 99.4          |
| MB58      | Pleuronectes platessa    | Pleuronectes platessa  | 98.3               | Pleuronectes platessa | 99.1 |
| MB59      | Sebastes mentella        | Sebastes mentella      | 99.1               | Sebastes viviparus | 99.5          |
| MB60      | Pollachius virens        | Pollachius virens      | 99.1               | Pollachius virens | 100           |
| MB61      | Isurus oxyrinchus        | Isurus oxyrinchus      | 99.1               | Isurus oxyrinchus | 99.4          |
| MB62      | Gadus morhua             | Gadus morhua           | 99.5               | Gadus morhua   | 99.5          |
| MB63      | Pollachius virens        | Pollachius virens      | 99.5               | Pollachius virens | 100           |
| MB64      | Xiphias gladius          | Xiphias gladius        | 98.8               | Xiphias gladius | 100           |
| MB65      | Pleuronectes platessa    | Pleuronectes platessa  | 99.1               | Pleuronectes platessa | 99.5 |
| MB66      | N/A                      | N/A                    | N/A                | N/A           | N/A           |
| MB67      | Gadus morhua             | Gadus morhua           | 96.5               | Gadus morhua   | 98.6          |
| MB68      | Mustelus mustelus        | Mustelus asterias      | 98.1               | Mustelus asterias | 97.5          |
| MB69      | Gadus macrocephalus      | N/A                    | N/A                | N/A           | N/A           |
| MB70      | Gadus morhua             | Gadus morhua           | 99.1               | Gadus morhua   | 97.8          |
| MB71      | N/A                      | Theragra chalcogramma  | 99.1               | Gadus chalcogrammus | 98.3 |

**Table 2. Cont.**
The sequencing results and their comparison with available genomic databases allowed the recognition and identification of various fish species present in the analyzed commercial fish products. The obtained identity scores ranged between 96.5 and 100% in BLAST and 97.1 and 100% in BOLD. Mismatches between BLAST and BOLD were appropriately corrected by integrating either result. In fact, according to the 98% threshold value for species identification [21], three samples were discharged after using BLAST but all three of them were recovered after BOLD matching. Similarly, four products displayed a low identity score using BOLD but they were recovered using BLAST.

Among the 69 samples successfully attributed, a total number of eight species substitutions were detected (11.6%). In particular, four major frauds (MB19, MB25, MB38, MB44) related to mislabeling at the genus level (5.8%) and four minor (MB07, MB20, MB55, MB68) ones concerning different species within the same genus were detected (5.8%). The single names and substitutions are fully described in Table 2. Among the four major frauds, two samples labeled as *Katsuwonus pelamis* (MB19 and MB25) turned out to be either *Thunnus thynnus* (GenBank) or *Thunnus maccoyii* (BOLD). Similarly, *Chelidonichthys lucerna* (BAR38) was found to be *Merluccius paradoxus* (GenBank and BOLD), and *Lamna nasus* (MB44) was *Ixurus oxyrinchus* (GenBank and BOLD). Concerning minor frauds, the four labeled species displayed lower identity percentages in BLAST and BOLD within the same genus: MB07 (respectively 97.1% and 99.1%), MB20 (96.4% and 97.5%), MB55 (97.1% for both databases), MB68 (97.5% and 97.1%).

Discrepancies between GenBank and BOLD were also evaluated and were evident in eight samples (MB07, MB19, MB20, MB25, MB37, MB49, MB50, MB59) (11.6%).

Interestingly, among the eight species substitutions, application of the IUCN index [22] revealed three Critically Endangered (CR) (MB19, MB25, MB44), two Near Threatened (NT) (MB55, MB68), two Least Concern (LC) (MB07, MB20) and one Not Evaluated (MB38) species.

4. Discussion

The mini-barcoding approach based on analysis of short COI fragments proved to be a useful methodology to define the correct taxonomy of commercial fish products. Among previously proposed primer sets, a choice was made based on the most reliable ones [15,17], and “Fish_miniE” showed a lower success rate than “Fish_mini”. In particular, the lower success of “Fish_miniE” (87.3%) was consistent with the one original report of 88.6% by Shokralla et al. [15], which was determined in a lower number of samples compared to our work. In fact, the authors correctly classified 39 of 44 samples, which is almost half the number of samples determined in our dataset. On the other hand, the experimental success of “Fish_mini” (97.2%) was in accordance with similar recent works (93.2%) based on longer sequences [23], and thus confirmed the reliability of the mini-barcoding methodology.

Although new markers are emerging for charismatic fish species and add technological improvements to this topic [24–26], the need for data-rich databases is still important, particularly in the case of widespread investigations at a national level. From this point of view, the BOLD database is considered better performing than BLAST; however, integration of different databanks is fundamental to reaching a reliable attribution. Consistency between databases should be expected but that is not always the case. On the other hand, a correct assessment at the species level is sometimes difficult due to contradictory identity scores and variable species names released by different databases. This aspect can generate trivial attributions, particularly whenever labeling does not report the scientific name or the appropriate fishing area, as happened in a limited number of our samples. Discrepancies between GenBank and BOLD had already emerged in the past and were evaluated by Sultana et al. [17], who evidenced that BOLD records were available for only 10,000 species. This number has recently rapidly increased to over 24,000 fish species; therefore, ambiguity problems should nowadays be limited to intraspecific variability and population diversity. Although intraspecific genetic diversity should be scarce using COI,
the need for a continuous update of databases is important to reach a wide coverage of different populations over a large geographic scale.

In this research, the intervention of expert judgment was, therefore, necessary to evaluate genetic results that had to be integrated with sampling information useful to determine species-specific ecological characteristics (in particular, reported species name coupled to the area of origin, whenever available). To better clarify this concept, that was the case of sample MB19, which was labeled as Pacific *Katsuwonus pelamis*. BLAST and BOLD analyses returned two different Bluefin tuna species (*Thunnus thynnus* and *T. maccoyii*), therefore, revealing a mislabeled sample. Expert judgment was important in assigning the sample to Southern Bluefin Tuna rather than to Atlantic Bluefin Tuna according to both the identity score and the area of origin, the former species being a Pacific taxon consistent with the declared fishing zone.

Similarly, sample MB38 was not properly considered a fraud but rather hypothesized as an involuntary substitution that happened during delivery at the moment of purchasing. In fact, *Chelidonichthys lucerna* and *Merluccius paradoxus* have completely different meat colors and morphologies, even after processing, in their fillet appearance. Fillets of both species were also close to each other in the exposition counter. The product was classified anyway as a major fraud since the final delivery still belongs to the entire traceability process.

From a general point of view, the research evidenced 5.8% major frauds and 5.8% minor ones. Major and minor frauds were considered in relation to the taxonomic level of erroneous labeling. A final percentage of 11.6% of species substitutions were, therefore, discovered. It must be remarked that data are greatly variable on a world scale. As a matter of comparison, mislabeling was detected in 9.3% of seafood products in Germany [27], 24% in South Brazil [28] and 22% in India [29]. The results of Di Pinto et al. [30], based on molecular investigations, revealed an incredible occurrence of 82% of incorrect species declaration in fish fillet products. In some cases, the results could be biased by the product choice at the time of purchasing; this may be relevant whenever fillets are selectively chosen among suspected or evident frauds. In our investigation, fresh and frozen products were randomly bought with no particular attention to species or brands. Independent of local data, our results were in agreement with a recently published paper assuming that the most credible average mislabeling rate at the product level is 8% [31].

Besides the generalized assessment of multiple specimens, the experimental design considered a long time comparison starting from the previous work by Filonzi et al. [19] (see Figure 1). Our previous study [19] was among the first highlighting a high occurrence of incorrect species declaration in fillet fish products, underlying the strong trend towards seafood mislabeling in the Italian retail sector [30,32–35]. The past work [19] revealed incorrect labeling in 22 of 69 samples (32%), 18 of which were serious frauds (26.1%) from both the financial and nutritional points of view. The final aim of this new investigation was undoubtedly to assess the new trend after a decade of technical innovation and market surveillance. Results have evidenced a decrease in incorrect labeling in Italy from 32 to 11.6% over the last ten to eleven years. In particular, major frauds decreased from 26.1% to 5.8%. Data were in the low end of mislabeling rates reported in the literature [23]. It is noteworthy to observe that to minimize and prevent seafood frauds, proper regulations were issued in Europe and recommended on other continents. For example, fish labeling with both common and scientific names to be included on the product label together with FAO fishing area is nowadays mandatory and has been the current practice in Italy over the last decade. Similarly, Mariani et al. [36] also observed a sudden reduction of seafood mislabeling in Europe due to recent efforts in legislation governance with a positive impact on the entire commercial chain. Compared to data reported in this interesting publication [36], our percentages are in strict agreement with those obtained in several other European countries and fill the gap concerning the lack of data about Italy.
Figure 1. Comparison of percentages of species substitutions, both as major and minor frauds, assessed in 2010 and in the present work.

Nonetheless, another major issue is the illegal sacrifice and trade of endangered species widely protected by an international fishing ban. Fraudulent substitutions seem to continue, particularly in sharks and tunas, despite apparently increased control since the first published papers focusing on the problem [19,33]. Interestingly, application of the IUCN index [22] among eight species substitutions revealed three Critically Endangered (CR), two Near Threatened (NT), two Least Concern (LC) and one Not Evaluated species. This aspect is also related to conservation biology problems, rather than just the food safety system, and will have to be furtherly monitored using mini-barcoding, eventually widened to new genes [24]. In this respect Mini-barcoding could be considered as a sensitive application tool or applied to specific taxa highlighted as very often mislabeled and as leading to important health problems [37].

5. Conclusions

Mini-barcoding is a valuable tool to assess seafood species substitution, particularly in the case of processed products, displaying a high success rate. Despite DNA degradation, which may limit its diagnostic reliability, the majority of samples were correctly classified using BOLD and BLAST databases when supported by expert evaluation to solve cryptic nomenclature cases or apparent database redundancies. Although a general decreasing trend in fish species substitutions over the last decade was observed, consistent with similar trends in other European countries, a continuous update of datasets is important to reach a wide coverage of different species and populations. In particular, special attention should be focused on critically endangered species sensu IUCN whose involvement in mislabeling is recurrent and suggests still existing inappropriate control processes at different levels.

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