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A fish market survey using a novel PCR-sequencing-based protocols for the identification of commercial significant fish species

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ABSTRACT

This study developed a simple, specific, and affordable PCR-sequencing-*COI* gene-based protocol for the simultaneous identification of some important commercial fish species: *Merluccius merluccius*, *Lates niloticus*, *Gadus morhua*, *Ruvettus pretiosus*, *Pangasianodon hypophthalmus*, *Epinephelus* spp. For this study, a local market survey on fish was carried out to evaluate the application of labelling laws and to detect fraudulent actions using the developed PCR protocols. Ten specimens of each fish species of interest were obtained from wholesale fishery plants and were utilized for the protocol development. DNA was extracted from the individual samples and quantified. DNA isolates were subjected to end-point PCR and the PCR products were sequenced. For the identification of fish species, novel species-specific primers were developed by the program "Primer Express 3.0" and by the software "Primer-BLAST" to amplify fragments of 200 bp, 250 bp, 300 and 562 bp, 350 bp, 400 bp and 522 bp within the *COI* gene for *M. merluccius*, *L. niloticus*, *G. morhua*, *R. pretiosus*, *P. hypophthalmus*, *Epinephelus* spp., respectively. Single PCR was performed using DNA isolates and developed primers for each fish species of interest. After sequencing, the isolates were compared with the selected sequences of the COI gene and showed a similarity ranging from 99 to 100%. Among 43 samples obtained for the survey, 19 (44.2%) were mislabelled, with 18 (41.9%) mislabelled samples from local fisheries and fish marketplaces and 1 (2.32%) from hypermarket stores. Among fish samples purchased at local fisheries and fish marketplaces, fraudulent actions were observed more frequently in fish slices (100%) than fish fillets (65%). Regarding fish fillets, out of four samples labelled as grouper, three were *L. niloticus* and one *P. hypophthalmus*. Two fillets marketed as cod were substituted with *L. niloticus*. Five samples labelled as "fillet" and two samples labelled as "perch" were identified as *P. hypophthalmus*. Regarding fish slices, all samples marketed as grouper (*E. marginatus*) were slices of *R. pretiosus*. The single case of mislabelling detected from fishery products purchased at hypermarket stores was a sample of "Spinycheek grouper" (*Epinephelus diacanthus*) that was indicated on label as "Grouper" (*Epinephelus marginatus*). In summary, our work highlights the need for continuous surveillance of the commercialization of fishery products, to reduce the number of fraud cases that happen in the market. Furthermore, our protocols based on PCR techniques could be useful for quality control of fresh finfish and to strengthen controls on the most frequent fraudulent actions of marketed fishery products.

Keywords: fishery products, fish frauds, Multiplex PCR, *COI* gene

INTRODUCTION

The seafood consumption has increased several folds during the last 50 years, including wild and aquaculture products **[1]**. The species substitution in which low value fish is replaced with high value ones is a prominent phenomenon in the international seafood trade and is a leading cause of fraud in the fishery sector, leading to economic and health concerns. Fishery products present a valid alternative to other types of animal-origin food (terrestrial animal meat, eggs, dairy products, etc.) especially for their high digestibility due to the lower presence of connective tissues and lipid components **[2]**, **[3]**, **[4]**. Despite its increasing popularity, seafood is one of the prominent products associated with food frauds. Authentication studies and market monitoring of commercial

products show that fish products are more vulnerable to mislabelling than other consumer goods **[5]**, **[6]**. This phenomenon regards both the acquisition of fishery products traditionally and new products, for example fillets, slices, fish burger, "ready to cook" breaded products, or „ready to eat products". In this situation, fish are not easily identifiable from a phenotypic perspective with the increase in commercial and sanitary frauds. Victims of such frauds can be both consumers and the fishery industry. In fact, cases of seafood fraud are reported in almost all countries although the rate can vary. Mislabelling was detected in 50% fish products in Germany **[6-8]**, 22% seafood products in India **[9]**, 24% in South Brazil **[10]**, **[11]** and almost 80% commercial fish fillets in Italy **[11]**, **[12]**, **[13]**, **[14]** and several other countries. The EU enforced the Regulation (EU) 2013/1379 that lay down to the fish economic operators to report on the label of the fish products some information such as the commercial and scientific designation name, the production method (catch or breeding), its origin (the FAO fishing area for sea products and the name of the Country for breeding products) and the fishing gear **[15]**. Similar regulations are in force in many countries; however, despite these regulations, widespread seafood mislabelling has been identified in the United States and Canada **[16]**, **[17]**, in Europe **[14]**, in Asia **[18]** and south Africa **[19]** indicating the need for stringent control measures to generate efficient species identification **[20]**. The identification of species represents a key aspect both for food control and food safety and it is an important tool to ascertain frauds. DNA-based identification methods present several advantages over protein analysis, including increased specificity, sensitivity, and reliable performance with processed samples. In fact, DNA molecules are more resistant and thermo-stable than proteins. For the simultaneous amplification of many targets of interest, Multiplex-PCR is often performed using more than one pair of primers in one reaction **[21]**. Multiplex-PCR can produce considerable savings of time and effort within the laboratory **[22]**, **[23]**.

This study developed a set of original primers for the molecular identification of valuable fish species, using the PCR. To test the suitability of the developed protocols, a local market survey was done. The final objective of the study was to provide multiplex-PCR-based protocols suitable for the quality and safety assessment of some valuable fishery products.

Scientific Hypothesis

Designing set of primers for the molecular identification of valuable fish species and its validation by PCR and authenticate various fish species sold in the market.

MATERIAL AND METHODOLOGY

Samples

Ten specimens of each fish species of interest (*Merluccius merluccius*, *Lates niloticus*, *Gadus morhua, Ruvettus pretiosus*, *Pangasianodon hypophthalmus*, *Epinephelus* spp.) were obtained from wholesale fishery plants and were utilized for the protocol development. Forty-three fishery products were purchased in some cities located in Apulia Region (Southern Italy). Among these, 18 samples (42%) were obtained at four hypermarket stores and 25 samples (58%) at five fisheries and at six local markets. All samples were stored at -20 °C until analysed.

Chemicals

All chemicals were purchased by IZSPB were of analytical grade.

Animals and Biological Material

Samples purchased at hypermarket stores consisted of 6 fish skewers containing Nile perch (labelled as *Lates niloticus*), 2 breaded hake fillets (labelled as *Merluccius merluccius*), 2 fish burgers (labelled as *Gadus morhua*), 1 cod fillet (labelled as *G. morhua*), 1 breaded Nile perch fillet (labelled as *L. niloticus*), 3 Nile perch fillets (labelled *L. niloticus*), 2 salted cod fishes (labelled as *G. morhua*) and 1 grouper fillet (labelled as *Epinephelus marginatus*).

Instruments

The PCR machine, electrophoresis apparatus, weighing balance, microcentrifuge, laminar air flow, were used in this research.

Laboratory Methods

DNA extraction, PCR, gel electrophoresis and sequencing were used.

Description of the Experiment

Sample preparation: The fish samples were collected from different stores. 50-100 g samples were cut and stored at -20 °C until analysed. 20-25 mg tissue were taken from stored sample for DNA extraction and PCR analysis.

Number of samples analyzed: 43

Number of repeated analyses: two repetitions.

Number of experiment replication: two repetitions.

Design of the experiment: To develop novel protocols based on the PCR for the genetic identification of some significant commercial fish species, we created specific primers for the identification of the following: *Merluccius merluccius, Lates niloticus, Gadus morhua, Ruvettus pretiosus, Pangasianodon hypophthalmus* and *Epinephelus* spp. Mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene was selected to identify fish species. This genetic fragment presents very low intraspecific variability, thus permitting the unequivocal identification of fish species. Then, we applied the developed protocols to a local survey and ascertained fishery products' correct labelling at local retail outlets.

Primer design: *Merluccius merluccius, Lates niloticus, Gadus morhua, Ruvettus pretiosus, Pangasianodon hypophthalmus* and *Epinephelus* spp. were the six fish species of interest subjected to the study. Mitochondrial cytochrome oxidase subunit 1 (*COI*) gene was used to identify the above fish species. For each fish species, *COI* sequences were obtained from the GenBank database and aligned and compared by the program BioEdit. The primers were developed via two methods. Firstly, species-specific primers to amplify fragments of 200 bp, 250 bp, 300 bp, 350 bp and 400 bp within the COI gene for *M. merluccius, L. niloticus, G. morhua, R. pretiosus, P. hypophthalmus*, respectively, were designed by the program "Primer Express 3.0". The program "Primer Express 3.0" was set according to the parameters reported in Table 1. Secondly, *COI*, FASTA sequences for *Epinephelus* spp. and *G. morhua* were inserted in the software "Primer – BLAST" to develop primers to amplify fragments of 522 bp and 562 bp, respectively. The software "Primer – BLAST" was set to create primers according to the parameters reported in Table 2.

Table 1 Parameters inserted in the software "Primer Express 3.0" in order to obtain a pair of primers for the identification of *M. merluccius, L. niloticus, G. morhua, R. pretiosus, P. hypophthalmus*.

Parameter	Value
Primer Tm	
Min Primer Tm	58
Max Primer Tm	60
Max difference in Tm of two primers	2
Primer GC Content	
Min Primer % GC Content	30
Max Primer % GC Content	80
Max Primer 3' GC's	2
Primer 3' End Length	5
Primer 3' GC Clamp Residues	0
Primer Length	
Min Primer Length	9
Max Primer Length	40
Optimal Primer Length	20
Primer Composition	
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniqueness	
Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
Amplicon	
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	200 (variable)
Max Amplified Region Length	400 (variable)
Penalty	close to zero

Table 2 Parameters inserted in the software "Primer – BLAST" to obtain a pair of primers for the identification of *Epinephelus* spp. and *Gadus morhua*.

Parameter	Value
PCR product lenght	
Min product lenght	500
Max product lenght	600
Primer melting temperatures (T_m)	
Min Primer Tm	57
Optimum Primer Tm	58
Max Primer Tm	59
Max Tm difference	1

Sample collection and DNA Extraction: Forty-three fishery products were purchased in some cities located in Apulia Region (Southern Italy). Among these, 18 samples (42%) were obtained at four hypermarket stores and 25 samples (58%) at five fisheries and at six local market. Samples purchased at hypermarket stores consisted of 6 fish skewers containing Nile perch (labelled as *L. niloticus*), 2 breaded hake fillets (labelled as *M. merluccius*), 2 fish burgers (labelled as *G. morhua*), 1 cod fillet (labelled as *G. morhua*), 1 breaded Nile perch fillet (labelled as *L. niloticus*), 3 Nile perch fillets (labelled *L. niloticus*), 2 salted cod fishes (labelled as *G. morhua*) and 1 grouper fillet (labelled as *Epinephelus marginatus*). Samples purchased at fisheries and fish marketplaces consisted of 20 fish fillets and 5 fish slices. Regarding fish fillets, four were labelled as grouper, two as cod, three as Nile perch, four as striped catfish, five reported as "fillet" from local fisheries and fish marketplaces and two as "perch" (both without the indication of fish species). All fish slices were labelled as grouper (Table 7). After collection samples were subjected to DNA extraction with NucleoSpin Tissue Kit (Macherey-Nagel). All DNA samples were quantified (about 20 ng/μL) by Nanodrop (Thermo Scientific) and subjected to PCR with original species-specific primers developed for the identification of fish species. Primers were commercially synthesized by Sigma Aldrich (Milan, Italy). Primers were diluted to a final concentration of 100 nM. PCR primers for each fish species of interest were created. Both methods developed two pairs of primers for G. morhua (Table 3).

Table 3 Original species-specific primers developed for the identification of fish species.

Method	Fish species	Primers sequences	Length (bp)	Product size (bp)
Primer Express 3.0	*Merluccius merluccius*	FWD 5'- ATAATTGGAGGCTTCGGAAACTG -3' RVS 5'- CCAGCGTGGGCAAGATTACT -3'	23 20	200
Primer Express 3.0	*Lates niloticus*	FWD 5'- GGAGCTGGAACCGGTTGAA -3' RVS 5'- CAGCTAAGACTGGGAGGGAAAG -3'	19 22	250
Primer Express 3.0	*Gadus morhua*	FWD 5'- GGTGCACTTCTTGGTGATGATC -3' RVS 5'- ATCAACAGATGCCCCAGCAT -3'	22 20	300
Primer Express 3.0	*Ruvettus pretiosus*	FWD 5'- CGGCACATGCCTTCGTAATAA -3' RVS 5'- GGCTGCGGGTTTCATATTAATAA -3'	21 23	350
Primer Express 3.0	*Pangasiodon hypophthalmus*	FWD 5'- CCTTCTAGGCGACGACCAAA -3' RVS 5'- ATATTGTGAAATTGCTGGTGGTTTT -3'	20 25	400
Primer – BLAST	*Epinephelus* spp.	FWD 5'- TCTTGTATTTGGTGCCTGGG -3' RVS 5'- ACTGCTGTAATTAGGACGGC -3'	20 20	522
Primer – BLAST	*Gadus morhua*	FWD 5'- TCTCGTATTTGGTGCCTGAG -3' RVS 5'- GATACCAGCTGCTAAGACGG -3'	20 20	562

Polymerase chain reaction (PCR): All samples were subjected to end-point PCR in a Thermal Cycler Eppendorf. The PCR mixture (total volume 25 μL) contained 1X PCR buffer containing 1.5 mM $MgCl_2$ (20 nm Tris-HCl pH 8.4, 50 mm KCl), 0.2 mM dNTPs, 0.5 μM of each primer, 2 U of Hot Start II DNA Polymerase (Thermo Scientific) and approximately 5 ng of DNA (Table 4). PCR conditions were 98 °C for 30 s, 34 cycles of 98 °C for 5 s, 58 °C for 30 s, and 72 °C for 15 s, with a final extension at 72 °C for 1 min (Table 5).

Table 4 PCR Master Mix for the identification of fish species of interest.

Reaction Component	Final Concentration	Amount for each Reaction
Water	-----	16.85 μL
PCR Buffer	1X	5 μL
dNTP's	0.2 mM	0.5 μL
Primer Forward	0.5 μM	0.5 μL
Primer Reverse	0.5 μM	0.5 μL
Taq DNA Polymerase	2 U	0.15 μL
DNA	-----	1.5 μL
		Final Volume: 25 μL

Table 5 PCR Amplification Program performed in a Thermal Cycler Eppendorf.

Step	Temperature (°C)	Time	Number of Cycle
Initial Denaturation	98	30 sec	1
Denaturation	98	5 sec	29
Annealing	58	30 sec	
Extension	72	15 sec	
Final Extension	72	1 min	1

The PCR amplicons were analysed by agarose gel electrophoresis by using a horizontal 2% (wt/vol) agarose gel in lX TBE buffer (pH 8.3; 0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA) and with 0.003% (wt/vol) ethidium bromide for DNA staining. PCR products were mixed with a sample buffer of 1X TBE and then applied to each well. Gel ran in 1X TBE buffer at 200 V for 30 min. The DNA marker used was Amplisize molecular ruler, 50% GC content, 50-2000 bp, 10 bands (Bio Rad, Hercules, Spain). The PCR products were visualized and photographed by a Gel Doc XR+ System transilluminator (Bio Rad, Milan, Italy).

Sequencing: PCR products were purified using Montage PCR filter units (Millipore, Milan, Italy) and sequenced by BigDye 3.1 Ready reaction mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (Tables 4 and 5). Sequences were imported and assembled with the BioNumerics 7.5 software (Applied Maths, Saint-Martens-Latem, Belgium) and searched for homologous sequences by BLAST search analysis (http://www.ncbi.nlm.nih.gov).

Multiplex-PCRs: Primers were developed to obtain amplicons with different lengths (at least 50 base pairs). Duplex and Triplex PCR protocols were developed to simultaneously analyse more fish species using the designed primers with several combinations (Table 6).

Table 6 Combinations of Duplex and Triplex PCR protocols by using the developed primers.

Multiplex-PCR	Fish Species	Amplicon Length
Duplex-PCR	*Lates niloticus*	250 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Lates niloticus*	250 bp
	Gadus morhua	300 bp
Duplex-PCR	*Merluccius merluccius*	200 bp
	Gadus morhua	562 bp
Duplex-PCR	*Ruvettus pretiosus*	350 bp
	Gadus morhua	562 bp
Duplex-PCR	*Ruvettus pretiosus*	350 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Pangasianodon hypophthalmus*	400 bp
	Epinephelus spp.	522 bp
Duplex-PCR	*Pangasianodon hypophthalmus*	400 bp
	Gadus morhua	562 bp
Triplex-PCR	*Merluccius Merluccius*	200 bp
	Lates niloticus	250 bp
	Pangasianodon hypophthalmus	400 bp

Table 6 Cont.

Multiplex-PCR	Fish Species	Amplicon Length
	Merluccius merluccius	200 bp
Triplex-PCR	*Ruvettus pretiosus*	350 bp
	Epinephelus spp.	522 bp
	Lates niloticus	250 bp
Triplex-PCR	*Pangasianodon hypophthalmus*	400 bp
	Gadus morhua	562 bp
	Lates niloticus	250 bp
Triplex-PCR	*Ruvettus pretiosus*	350 bp
	Epinephelus spp.	522 bp

Specificity tests: Single PCRs were performed using the designated primers for each fish species of interest with the DNA extracted from the non-target fish species (negative controls).

Statistical Analysis

Statistical Analysis is not required for this study.

RESULTS AND DISCUSSION

Specificity of the developed protocols

PCR assay allowed the detection of DNA extracted from all specimens of each fish species of interest, giving fragments of the expected length. At the end of the running, the electrophoresis agarose gel showed a clear separation of amplicons due to their different sizes (Figures 1, 2, 3, 4 and 5). Single PCRs performed for the specificity tests gave the expected results. After sequencing, the isolates were compared with the selected sequences of *COI* gene and showed a similarity ranging from 99 to 100%. Grouper samples subjected to Epinephelus spp. authentication, showed 97.5% homology to Epinephelus costae GenBank entry (KM077928.1) and 100% homology to Epinephelus marginatus GenBank entry (KC500692.1). The results of the experiment are shown in Table 1 and Figure 1.

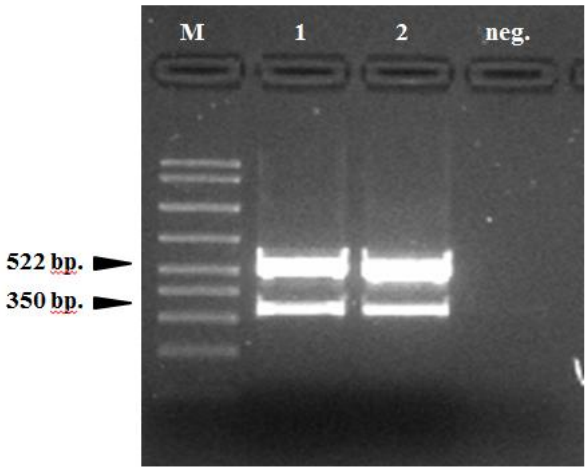

Figure 1 Gel electrophoresis of PCR products obtained from Duplex PCR assays (lanes 1 and 2) for identification of *Ruvettus pretiosus* (350 bp) and *Epinephelus* spp. (522 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane (neg.): negative control.

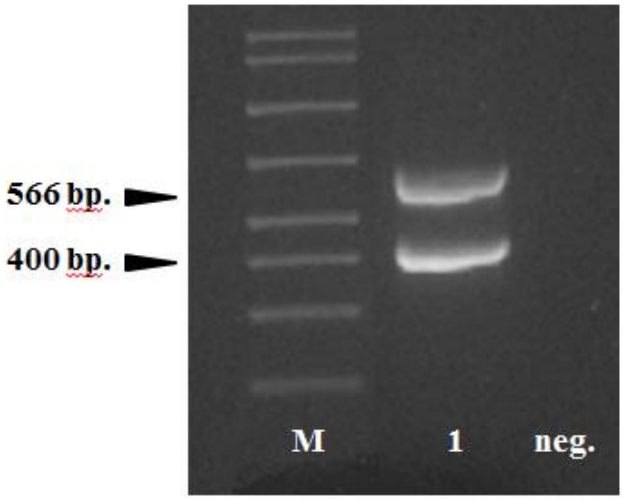

Figure 2 Gel electrophoresis of PCR products obtained from Duplex PCR assays (lane 1) for identification of *Pangasianodon hypophthalmus* (400 bp.) and *Gadus morhua* (566 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative control.

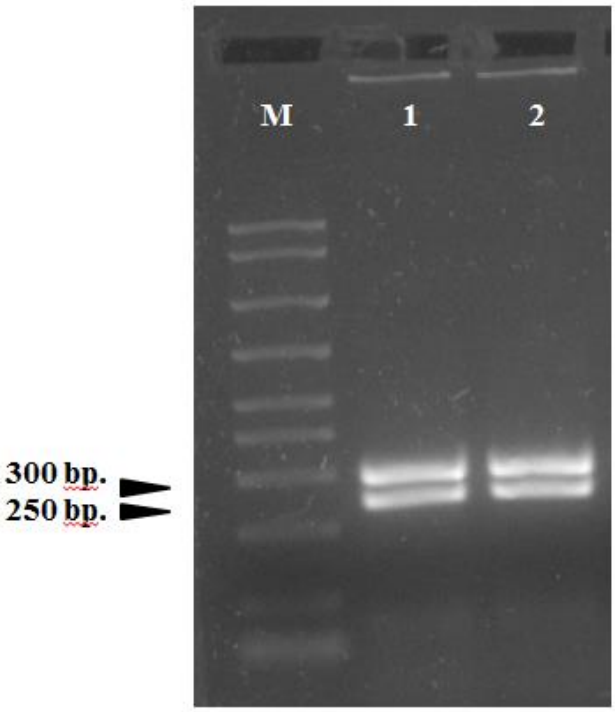

Figure 3 Gel electrophoresis of PCR products obtained from Duplex PCR assays (lanes 1 and 2) for identification of *Lates niloticus* (250 bp.) and *Gadus morhua* (300 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad).

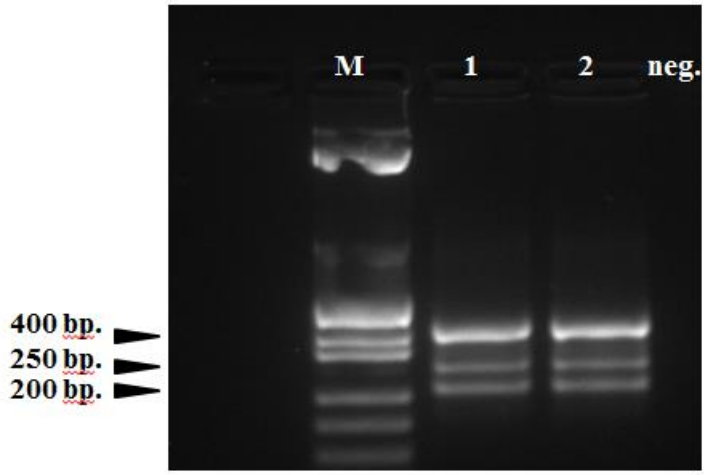

Figure 4 Gel electrophoresis of PCR products obtained from Triplex PCR assays (lanes 1 and 2)for identification of *Merluccius merluccius* (200 bp.), *Lates niloticus* (250 bp.) and *Pangasianodon hypophthalmus* (400 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative control.

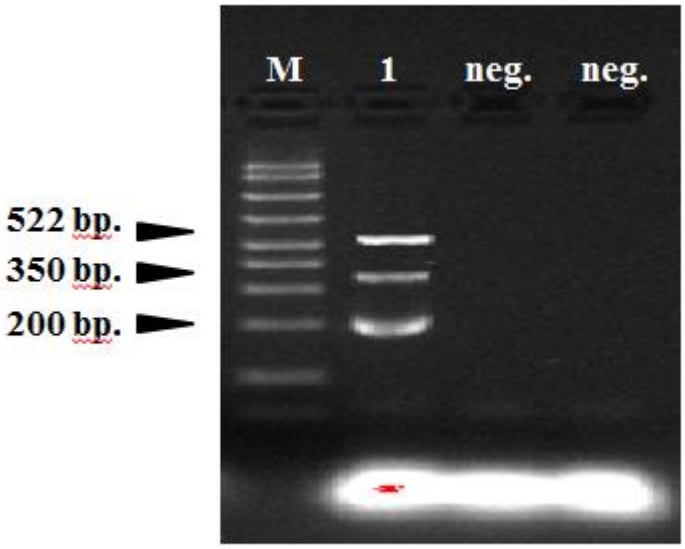

Figure 5 Gel electrophoresis of PCR products obtained from Triplex PCR assays (lane 1) for identification of *Merluccius merluccius* (200 bp.), *Ruvettus pretiosus* (350 bp.) and *Epinephelus* spp. (522 bp.). Lane M: AmpliSize™ Molecular Ruler (50–2000-bp ladder; Bio-Rad). Lane neg.: negative controls.

Identification of the samples used for the survey

Overall, out of 43 fish samples analysed, 19 (44.2%) resulted mislabelled, with 18 (41.9%) mislabelled samples from local fisheries and marketplaces and 1 (2.32%) from hypermarket stores (Table 7). As Regarding fish samples purchased at hypermarket stores, all cod samples tested positive for *G. morhua* showing an amplicon of 562 bp; all Nile perch samples tested positive for *L. niloticus* showing an amplicon of 250 bp; all hake samples tested positive for *M. merluccius* showing an amplicon of 200 bp; the grouper sample tested positive for *Epinephelus* spp. showing an amplicon of 522 bp. To ascertain the existence of false positives, identifications were confirmed by sequencing. After sequencing, *Epinephelus* spp. isolates showed 100% homology to *Epinephelus diacanthus* GenBank entry (EF609520.1). Out of 25 fish samples purchased at fisheries and fish marketplaces, 18 (72%) were mislabelled. Cases of mislabelling regarded more fish slices (100%) than fillets (65%). Regarding fish fillets, three Nile perch fillets (15%) and four striped catfish fillets (20%) were correctly labelled. The DNA analysis on the remaining fillets showed that thirteen samples were mislabelled (65%). All

samples marketed as grouper fillets showed fraudulent actions. In fact, out of four samples labelled as grouper, three (75%) tested positive for L. niloticus showing an amplicon of 250 bp., and one (25%) positive for *P. hypophthalmus* showing an amplicon of 400 bp. Both cod fillets (100%) resulted to be *L. niloticus*, showing an amplicon of 250 bp. The 5 samples from local fisheries and fish marketplaces labelled as "fillet" and the 2 samples labelled as "perch" were identified as *P. hypophthalmus* showing an amplicon of 400 bp (Table7). As regards grouper slices, all samples (100%) showed fraudulent species substitutions; in fact, *R. pretiosus* was marketed as grouper (amplicon of 350 bp.).

Table 7 Results of the survey on the application of the labelling laws and for the detection of fraudulent actions.

Retail outlet	Fishery products	N.	Species labelled	Species identified by PCR	Result
Hypermarket stores	Fish skewer	6	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Fillet	2	Hake (*Merluccius merluccius*)	*Merluccius merluccius*	Correctly labelled
Hypermarket stores	Fish burger	2	Cod (*Gadus morhua*)	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Cod *(Gadus morhua)*	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Fillet	3	Nile perch (*Lates niloticus*)	*Lates niloticus*	Correctly labelled
Hypermarket stores	Salted fish	2	Cod (*Gadus morhua*)	*Gadus morhua*	Correctly labelled
Hypermarket stores	Fillet	1	Grouper (*Epinephelus marginatus*)	*Epinephelus diacanthus*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	4	Grouper	*Lates niloticus (75%)* *Pangasius hypophthalmus (25%)*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	2	Cod	*Lates niloticus*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	3	Nile perch	*Lates niloticus*	Correctly labelled
Local fisheries and fish marketplaces	Fillet	4	Striped catfish	*Pangasius hypophthalmus*	Correctly labelled
Local fisheries and fish marketplaces	Fillet	5	Reported as "fillet"	*Pangasius hypophthalmus*	**Mislabelled**
Local fisheries and fish marketplaces	Fillet	2	Perch	*Pangasius hypophthalmus*	**Mislabelled**
Local fisheries and fish marketplaces	Fish slices	5	Grouper	*Ruvettus pretiosus*	**Mislabelled**

Recently, several studies have demonstrated the vulnerability of the fish supply chain to fish fraud, particularly species substitution and mislabelling **[24]**. An investigation was done by INTERPOL_EUROPOL which demonstrated fish fraud as 3rd highest risk category of food vulnerable to fraud **[25]**, **[26]**. In the fish sector, the identification of fish species throughout the production chain is of main importance, even if fishery products have already been processed. In fact, there are different ways to purchase fish and fishery products: whole, fillets, slices, skewers or mixed with other species for gastronomic dishes (seafood salad, risotto mix, fish fingers, etc.). Furthermore, the presence of similar fish species, but very different from a nutritional and organoleptic perspective, is more frequent. Currently, commercial fishery products in Europe come from all parts of the world, meaning that accurate species identification is not always easy. In this situation, both sanitary and quality control and product traceability could be obstructed because fish are not easily identifiable, with the increase in commercial (*aliud pro alio*) and sanitary frauds (commercialisation of toxic organisms). Further, food poisoning due to the consumption of toxic fishery products belonging to *Tetradontidae*, *Molidae*, *Diodontidae* and *Canthigasteridae* families may occur **[27]**, although their marketing is forbidden by European Regulations (EC Reg. 853/2004). For example, oilfish (*Ruvettus pretiosus*) is seldom marketed in conformity with the current EU Regulation (EC Reg. 1021/08) and it is often commercialized in place of the most popular, expensive, and precious species, such as grouper (*Epinephelus* spp.). The problem of fraudulent actions in the commercialization of foods is strongly felt at the European Union level; in fact, recently a recommendation was enacted in the need to establish a "coordinated plan of supervision designed to determine the prevalence of fraudulent practices in the marketing of certain foodstuffs", including fishery products (EU Recommendation n. 1558 – 12 March 2015).

EU enforced the Regulation (EU) 1379/2013 that lay down to the fish economic operators to report on the label of the fish products some information such as the commercial and scientific designation name, the production method (catch or breeding), its origin (the FAO fishing area for sea products and the name of the Country for breeding products) and the fishing gear **[28]**. The objective of this regularity policy was to generate safe supply for consumers and the food processing industry as well as to give consumers a more detailed information about food products to protect fraud, prevent illegal fishing and promote sustainable aquaculture. Similar regulations are in force in many countries; however, despite these regulations, widespread seafood mislabelling has been identified in the United States and Canada **[16]**, **[17]**, In Europe **[14]**, Asia **[9]** and south Africa **[19]** indicating the need for stringent control measures to generate efficient species identification **[20]**. In fact, in 2016, Oceana published a major report by reviewing more than 200 published studies across 55 countries and found 20% mislabelling in catering and related sectors **[20]**. In 2021, Oceana Canada observed 46% mislabelling in seafood products, which is just 1% less compared to a study conducted during 2017-2019 **[29]**. In 2021, a Guardian Seascape analysis of 44 recent surveys of more than 9,000 seafood samples from restaurants, fishmongers, and supermarkets in more than 30 countries conducted and found that 36% samples were mislabelled, exposing a large amount of seafood fraud at global scale **[30]**. All studies conducted indicate that species substitution and mislabelling are serious problems in international fish trade. Other studies conducted in countries like Italy **[31]**, **[32]** Germany **[6]**, **[7]**, India **[9]**, South Brazil **[10]**, **[11]** show the concerns related to fish fraud **[33]**, **[34]**, **[35]**.

The development of PCR protocols has allowed a rapid and specific response for identifying fish species. In fact, the time required from the arrival of the fish sample to the end of the analysis was about 6-8 h. Thanks to the development of Duplex and Triplex PCR protocols, additional information may be gained from a single test run with considerable saving of time, reagents, and efforts within the laboratory. Furthermore, the applicability of the assay to commercial fishery products has been demonstrated. In fact, in our survey, of the 43 investigated samples, we detected 19 (44.2%) mislabelled samples. Most of the mislabelled samples derived from local fisheries and marketplaces (41.9%) and one sample (2.32%) from hypermarket stores. Our findings are similar to the results obtained from a national seafood fraud investigation carried on in the United States from 2010–2012. In this survey, out of 1200 seafood samples from 674 retail outlets in 21 States, DNA testing found that one-third (33 per cent) were mislabelled **[36]**. Forty-four per cent of the retail outlets visited sold mislabelled fish. Also, a recent Italian investigation revealed numerous commercial frauds; for example, Cutarelli et al., found that a sample marketed as "frozen grouper fillet" was made from halibut (*Hippoglossus hippoglossus*) instead of grouper (*E. marginatus*) **[37]**. Given consumers' high demand for grouper, the prices at the subsequent wholesale and retail market levels are also high relative to other finfish species. Additionally, the importation of large quantities of grouper from many foreign sources must meet the ever-growing demand for grouper. The strong demand for grouper and its high market value, which continues to be evident in the market, is also a motivation for economic frauds. The most prevalent economic fraud associated with grouper is the selling of a cheaper finfish as grouper. In fact, the most common types of mislabelling among the grouper samples collected in the US were substitutions with farmed Asian striped catfish (*Pangasianodon hypophthalmus*), freshwater perch (*Macquaria novemaculeata*), weakfish (*Cynoscion regalis*), bream (*Abramis brama*), and king mackerel (*Scomberomorus cavalla*). It is important to underline that grouper is a precious fish species often an item of fraud; in fact, when grouper is sold as fillet, its main features completely disappear, and its identity cannot be established on the basis of morphological features **[38]**.

A survey carried out by the Eurofishmarket (www.ilfattoalimentare.it) showed that around 15% of fresh/frozen grouper fillets sold on the market belonged to other species. These facts are strongly confirmed in our survey, in fact, we found that all samples marketed as grouper slices (*E. marginatus*) were slices of *R. pretiosus*. Such fraud could be considered both a commercial and a sanitary fraud because *R. pretiosus* is a fish known for its potential dangerousness for consumer. In fact, *R. pretiosus*, also known as "oilfish," is a deep-sea fish that stores a large amount of wax esters in its body for buoyancy control. In humans the accumulation of the indigestible wax esters in the rectum through the consumption of these fish produces discharges or leakage per rectum as orange or brownish green oil, but without noticeable loss of water; this response is called keriorrhea **[39]**. Outbreaks of keriorrhea have been repeatedly reported across continents. In the EU, the marketing of *R. pretiosus* is regulated by the EC Reg. 1021/08 (EC Reg. 1021/08). According to this regulation, food business operators must sell oilfish products in packaged form and provide information on label to the consumer about their gastrointestinal adverse effects.

In conclusion, our method based on PCR constitutes an effective molecular tool for detecting fraudulent substitution of fish species of interest applicable to raw finfish. These protocols could be applied to both quality control and official sanitary control of fishery products and to help the anti-fraud actions control fishery products' traceability and labelling.

CONCLUSION

The seafood industry is one of the imported traded products globally. Due to several health benefits, availability, less religious concerns, and possibility options, its demand and consumption increased exponentially. Due to increasing trade value, it is continuously vulnerable to frauds where costly fish can be replaced with cheap fishes, particularly in products where morphological identification is lost. Fish frauds may have health and environmental concerns. It must be authenticated before serving customers. Due to the limitations of protein-based methods, DNA-based methods like multiplex PCR provide a better alternative for species identification and tracking food fraud. It can help food control authorities to ensure food safety and the rights of consumers.

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