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DOCTORAL THESIS ON "**SPECIES IDENTIFICATION AND FOOD FRAUD ANALYSIS IN COMMERCIAL FISH PRODUCTS THROUGH DNA- BASED METHODS**"

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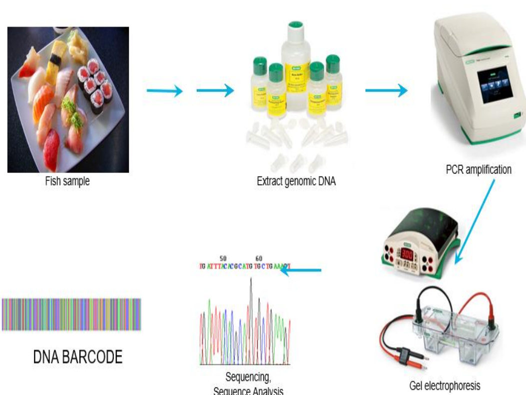

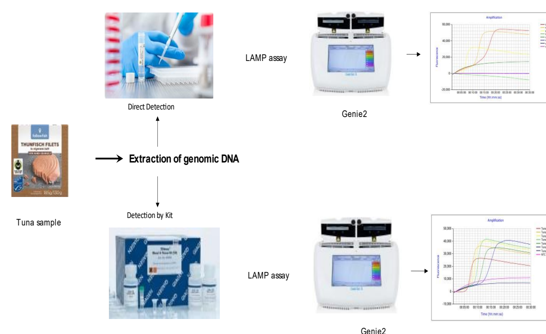

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ACRONYMS

aa – amino acid

AFLP – amplified fragment length polymorphism

BLAST – basic local alignment search tool

BOLD – Barcode of Life Database

bp – base pair

COI – cytochrome oxidase, subunit i

Ct – cycle threshold

Cob – apocytochrome b

Cytb – cytochrome b

DBPCFC – double-blind placebo-controlled food challenge

DNA – deoxyribonucleic acid

EAACI – European academy of allergology and clinical immunology

ELISA – enzyme-linked immunosorbent assay

EU – European Union

FAO – Food and Agriculture Organization of the United Nations

FINS – forensically informative nucleotide sequencing

FISH-BOL – Fish Barcode of Life Initiative

GMO – genetically modified organisms

HRM – high resolution melting

IEF – isoelectric focusing

ITS-1 – nuclear internal-transcribed spacer 1

IUIS – International Union of Immunological Societies

LC-ESI-IT – liquid chromatography with electrospray ionization-ion trap

LC-MS – liquid chromatography mass spectrometry

LFD – lateral flow device

LNA – locked nucleic acid

LOAEL – lowest observed adverse effect level

LOD – limit of detection

LOQ – limit of quantification

MS – mass spectrometry

NCBI – National Center for Biotechnology Information

NGS – next-generation sequencing

PCR – polymerase chain reaction

PDB – Protein Data Bank

PPi – pyrophosphate

PUFA – polyunsaturated fatty acids

RAPD – random amplified polymorphic DNA

RFLP – restriction fragment length polymorphism

RNA – ribonucleic acid

SDS-PAGE – sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SNP – single-nucleotide polymorphism

SSCP - -single-stranded conformational polymorphism

Ta – temperature of annealing

Tm – temperature of melting

USA – United States of America

WHO – World Health Organization

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Chapter 1. A retail market survey on fish frauds from Southern Italy

STATE OF THE ART

1. General Introduction

Food fraud is act of deceiving the consumers which is mostly done for financial gains, and it is executed when food is illegally placed on the market for customers [1]. Food fraud includes growing criminal activities like mislabeling, over glazing, substitution, counterfeiting, dilution, and adulteration. Food fraud not only causes deception to consumers, but it also causes food safety risks for consumers. Public health is greatly undermined when nontoxic fish species are marketed with toxic varieties. Public health is also affected when farmed or freshwater species from polluted watercourse is replaced for wild marine fish. Food fraud can lead to decrease of consumer confidence in food industry and mistrust in effectiveness of government food control authorities. Past decade have witnessed some notorious food fraud scandals like horsemeat scandal in Europe which can damage national reputation, with unwanted attention need on the safety, quality and authenticity of all exported food items in the International food supply chain [2]. Food fraud including mislabeling and species substitution is major concern within sea food industry.

Fish fraud is one of the most widely malpractice food fraud happening in the global food market [3, 4]. Fish fraud is done when fish is purposefully placed on the market for economic gain with the intention of deceiving the consumers [5, 6]. Fish fraud can be executed at multiple points along with fish supply chain. The most widely executed fish fraud is mislabeling and species substitution. On minimum scale it also occurs when fish is over breaded causing deception to consumers regarding nature of fishery products. Sometime misuse of water binding agent is also done which is also a fraudulent activity leading to increase the weight of products causing economic benefit by selling additional water substituted for fish.

Species substitution is done when low value or less desirable fish is replaced in place of more expensive fish varieties. Fraudulent marketing of farmed salmon as wild captured fish is an example of fish fraud. The flesh part of many fish is quite similar in appearance, texture, and taste, but it is difficult to differentiate such fish species once they are processed or prepared for

consumption and presented with flavoring in sauces or in batter. Sometimes to avoid tax and save money high value fish is marketed as low value fish which is also a kind of food fraud. Sometimes species substitution is done to hide the geographical origin of fish or to conceal an illegally harvested species or hide species from a protected area [7].

Recently several studies are published which has demonstrated the vulnerability of fish supply chain to fish fraud, particularly species substitution and mislabeling [8]. Unfortunately, most of the fish fraud studies were conducted in developed countries, much less is known about fish fraud problems in developing countries. In 2015, an investigation was caused by INTERPOL_EUROPOL which demonstrated fish fraud as 3rd highest risk category of food vulnerable to fraud. Similar study in 2013 conducted by European countries put fish in 2nd most vulnerable category. Report published in several journals and media in last decades have provided adequate proof that mislabeling and species substitution is worldwide problem in both national and international market for fish and fishery products [9]. Mislabeling can be executed at any point of the seafood supply chain. It can be executed at the level of processing, distribution, retail, and catering. In 2016, Oceana published a major report by reviewing more than 200 published studies across 55 countries and found 20% mislabeling in catering and related sectors [9, 10]. All studies conducted indicates species substitution and mislabeling are serious problems in international fish trade.

Species substitution and mislabeling is difficult when morphological features are no longer visible particularly when heads, tails and fins of fish are removed, and fish is processed into ready to cook breaded products, fillets, or battered products or when it is highly processed in pre prepared fish meals [11]. With the advancement of molecular detection methods, like DNA barcoding and Next Generation Sequencing (NGS) greater transparency in fish marketing chain can be expected. Traceability of fish is prime factor in countering fish fraud, applying food safety laws and maintaining high standards of sustainable fishery management. Fish traceability is also very important for maintaining quality and standard of fish products improving health of consumers. One of the key obstacles in handling fish fraud is making an agreement on list of common names which are liked with scientific nomenclature. This is the principal step by any national government towards introducing official fish fraud control programs.

Managing fish fraud is daunting task for national authorities as no single government has the regularity power to take this challenge. Also, no single food law or regulation can directly handle all aspects of food fraud. It is cumulative effort undertaken by national food regularity authorities, custom import authorities, border control agencies and special cell within the national police force. Proper coordination between all these agencies is required for effective regulations. Official food control programs must be strengthened more effectively by making new regulations about fish fraud, increasing enforcement methods which can inhibit landing and market access for products from illegal, unreported, and unregulated fisheries, introduction of monitoring and surveillance methods for assessing the degree of compliance with fish labeling regulations and upgrading laboratory detection methods based on molecular techniques.

An effective science-based fish traceability system is needed to meet present demand of international fish marketing chain for countering food fraud effectively. The system must have capacity to identify fish species and its geographical origin, and it must also differentiate between wild captured and farmed products. It must also be able to discriminate fresh and frozen fish and many different forms of processed fish products currently traded in international market. The traceability methods must be able to track fish from point of harvest to the consumer's plate. The present traceability system is mostly dependent on paper trails that contains data about geographical origin, species, and registration details of vessel. There is need of alternative traceability system based on scientific principles and verified by independent scientific and analytical methodologies to track fish and fish products throughout the marketing chain. Analytical tools based on molecular methods like DNA barcoding need to be adopted by food control authorities for identification of fish species.

Standardization of analytical method is needed for global access to a reference database with reliable data on genetic primer based on scientific names. To control fraudulent malpractices involved in fish fraud greater cooperation and coordination between food control authorities and law enforcement agencies is required both at national and international level. Strengthening of food laws and regulations with proportionate penalties to criminal infringement is required. Fish labeling must provide sufficient information for consumers so that they are able to make informed choices about the product they purchase. Food inspectors and laboratory staff

must be well trained for new analytical techniques of species identification. These methods required further improvement before their use in routine official food control programs.

DNA barcoding based on mitochondrial cytochrome c oxidase subunit 1 has been quite effective and reliable method for identification of fish species. It has certain limitation as its application in the identification of geographical origin of fish is not very successful. Hence to identify the origin or provenance of fish catches NGS and other advanced genetic methods has been proposed.

The food industry needs to adopt latest analytical methods to protect against fraudulent malpractices in the food supply chain. Food safety management system needs to include vulnerability and threat assessment to analyze risks and to put control and preventive strategies in place. Routine periodic analysis assessment for species authentication and method for validating traceability documentation need to be implemented. System for fish fraud vulnerability assessment needs to be implemented within its supply chain to control measures to minimize risks of receiving fraudulent or adulterated raw material or ingredients.

The Codex Alimentarius Commission with associated countries must take charge to effective control these fraudulent activities. It should develop international guidelines for identification, management, and mitigation of fraudulent practices in food trade and to develop guidelines to standardized food safety management system for fish and vulnerability assessment.

In this survey, out of 90 tested samples, 10 (11.1%) were found showing noncompliance with the label. Sample no 35 and 36 were found to be shark (*Prionace glauca*) instead of squid (Table 6). In sample no 41 squid was found in place of haddock (*Melanogrammus aeglefinus*). In sample n. 159 *Ruvettus pretiosus* was found instead of grouper (*Epinephelus* spp.). In samples 37,38,39 and 40 haddock (*Melanogrammus aeglefinus*) were observed in place of swordfish (*Xiphias gladius*) (Table 6). Sample 50 and 83 also falls in food fraud cases.

2. Frauds

Fraud is a false representation of a matter of fact - whether by words or by conduct, by false or misleading allegations, or by concealment of what should have been disclosed - that deceives

and is intended to deceive another so that the individual will act upon it to her or his legal injury [12]. Frauds are characterised by voluntariness, aimed directly or indirectly to subtract a value to a business and are mainly perpetrated for profit to the benefit of those who commit them. In food safety, fraud is considered an act or deception that is configured in a decrease in the value of the goods, economic or nutritional. It is performed by the manufacturer or seller with the modification of the characteristics of food, making it different from the one agreed. Food frauds are practiced with several illegal conduct to adulterate, counterfeit, substitute and alter food products with the ultimate goal to benefit.

Food frauds can be divided in two types:

> "Sanitary fraud", also called toxic fraud, they are a threat to consumers' health causing harm.
>
> "Commercial fraud", they damage the economic interests of the consumer without causing, necessarily, harm [13].

In addition, according to the effects on composition and/or the external aspects, frauds are distinguished as: fraud inherent product quality and fraud regarding the marketing of foods.

Frauds inherent product quality are:

- ❖ Alterations: are changes in the composition and organoleptic characteristics of food caused by degenerative phenomena for bad or prolonged storage.
- ❖ Adulterations: are changes in the natural composition of a food product, due to voluntary and unreported addiction or subtraction of some components, in order to obtain an economic profit. These frauds have both negative commercial and nutritional impact. Moreover, adulterations may expose the consumer to health risks such as allergic reactions.
- ❖ Sophistications: are voluntary changes in natural composition of a food product by the addition of foreign substances, or the substitution of one or more of its elements with substances of lower quality and value, or by the addition of chemical substances not allowed by the laws. These frauds are practiced in order to improve its appearance or to cover its defects.

Frauds regarding the marketing of foods are:

- Falsifications: are fraudulent operations which consist in the replacement of a food with another.
- Counterfeiting: is a fraudulent action which consists in labelling products using a name different from the real one, usually of a high-value product.

The most common frauds which involve fishery products are:

1) substitution of a high-value fish species with a less expensive or lower quality alternative ("*aliud pro alio*"). Some examples are exchange of flying squid (*Todarodes sagittatus*) for common squid (*Loligo vulgaris*), blue whiting (*Micromesistius potassou*) for hake (*Merluccius merluccius)*, scaldfish (*Arnoglossus* spp.) for sole (*Solea vulgaris*), but mostly takes place on slice and on fillets, where the recognition becomes more difficult.
2) Mislabelling or fraudulent substitution of fishery products with toxic puffer fish. An example is the exchange of puffer fish (fam. Tetraodontidae) for monkfish (*Lophius piscatorius*).
3) The marketing of defrosted fishery products as fresh ones. European Regulations oblige to declare on label if fish is “defrosted”; otherwise, the product is intended sold as fresh.
4) The marketing of farmed fish as wild caught ones (EC Reg. 1224/2009[14].
5) False *rigor mortis* obtained with the cold, in the refrigerator a few hours before of sale.
6) The marketing of fishery products with histamine content in excess of what is permitted (EC Reg. 2073/2005)[14, 15].
7) Bad state of preservation of fish.
8) Use of additives permitted beyond the set limit or not permitted by law (EC Reg. 1129/2011)[14].
9) Import of fishery products subject to specific sanitary prohibitions (EC Reg. 853/04; EC Reg. 625/2017).

2.1. Food Fraud

Food fraud is not considered a new phenomenon but recently it has drawn attention due to increase in international trade and being a billion-dollar industry, it is quite vulnerable for

fraudulent activities. The notorious horsemeat scandal in 2013 bring the food fraud phenomenon in limelight as it exposed the vulnerability of the international food supply chain to organized criminal offenses. Now major initiatives are underway by several countries to combat these malpractices. To reduce the phenomenon national, international, and regional food fraud network and platforms have been developed which can show the food fraud information and foster cooperation. Many international organizations like Europol (Europol, 2017), the Food Fraud Database of the United States Pharmacopeial Convention (USP) (United States Pharmacopeial Convention, 2018), and the Food Fraud Network of the European Commission (European Commission, 2018a) are cooperating in these activities. Specialist units and dedicated task force are made by several countries for strengthening food control system to counter food fraud cases (Food Safety Authority of Ireland, 2017; Food Standards Agency, 2016; United States Food and Drug Administration, 2017a). To deal with criminal aspects of food fraud official food control programs are now routinely undertaken. Several dedicated units in law enforcement agencies are made for criminal investigation associated with food fraud. Effective collaborations are needed between several government agencies such as food control authorities, excise and custom department and the national police force to target food fraud activities at national level.

There are several ways by which food fraud is committed. Most commonly when food is illegally placed on the market with the objective of deceiving the customers for financial gains. Mostly it is an act of defrauding food consumers for financial gain by substituting or providing substandard food products. Any criminal act which affects the safety and authenticity of food is called a food crime.

Several food safety risks are associated with food fraud cases. Consumer's health is main threat which can be compromised in most cases. This happened when adulteration of infant formula milk with melamine is done in 2008 and also when lead chromate was used for enhancing the color of turmeric [16]. Nutritional quality of food is also compromised during food fraud activities particularly in cases where milk or fruit juices are diluted with water or sugar solution. Consumers trust in integrity of the food supply chain is severely hampered as it happened in the cases of horsemeat scandal in which lower value horsemeat was replaced with beef in processed

meat products on the market in European Union. Food fraud can cause several economic losses and it can damage national reputation in the global food market.

2.2. Fish Fraud

Fish has always been appreciated by consumers for its organoleptic properties and because is easy to prepare and to cook. Eating fish is part of the cultural traditions of many people. In fact, in some populations, seafood is a major source of food and essential nutrients. 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 a lower presence of connective tissues and lipid component [17]. Fish is a key component of a healthy diet and the consumption of about 1-2 servings of seafood per week has been associated with many benefits. From a nutritional point of view, fish is a source of energy and protein with high biological value, balanced in composition in essential amino acids, rich in methionine and lysine. This underlines the importance of fish not only for the diet of the industrialised countries, but also for the supply of the poorest people, which is often based on the tuber or cereal consumption in which amino acids are limited. Seafood also provides important nutrients such as *n*-3 long-chain polyunsaturated fatty acids (LC *n*-3 PUFA), in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) synthesized from α-linolenic acid (ALA), that is a component of dietary patterns associated with good health [17, 18]. The consumption of fish, because of the beneficial effects of the LC *n*-3 PUFA on the cardiovascular system, may decrease the risk of cardiovascular disease (CVD) and may have an effect on the molecular, cellular and whole-body pathogenic processes of atherosclerosis and thrombosis [18]. Data derived from prospective epidemiological studies and secondary prevention trials conducted in subjects at high risk of coronary heart disease (CHD) also support the conclusion that these fatty acids protect against CHD [18, 19]. Moreover, many studies have shown beneficial effects of increased LC *n*-3 PUFA intake on CHD mortality [19]. Health benefits have been also observed during pregnancy and lactation. High levels of fish consumption, and of EPA and DHA, have been suggested to be responsible for the reported longer gestation rates and higher birth weights [20]. On the contrary, low consumption of fish can be considered as a strong risk factor for preterm delivery

and low birth weight [20]. Adequate intakes of DHA, between 100 - 200 mg per day, have been estimated for pregnant and lactating women to accommodate the needs of their infants for deposition of DHA in the brain and retina [20, 21]. So, this is linked with better visual acuity in early life because of the recently established role for DHA in GTP-dependent signal transduction pathways involved in vision [21, 22]. Moreover, several studies have reported the effects of consuming increased amounts of dietary α-LNA on the fatty acid composition of plasma or cell lipids [23]. Fish presents high levels of minerals (calcium, iron, iodine, selenium, phosphorus and fluorine), vitamin A, vitamin E and vitamins of B group [22]. An iodine-deficient diet can cause a wide spectrum of illnesses, including goitre and mental retardation. In fact, maternal iodine deficiency during pregnancy can cause foetal iodine deficiency, which impairs early brain development with consequent physical and mental retardation and lower cognitive and motor performance in later life.

Despite of countless benefits, fish consumption may address safety aspects, for example hazards related to contamination with biological hazards (parasites, bacteria, viruses, protozoan) and chemicals, mainly heavy metals, pesticides, dioxins, furans, polychlorinated biphenyls and brominated flame retardants. In fact, attempts to increase consumption of fish appear to be undermined by the risk to expose consumers to contamination with mercury and methyl mercury. High mercury levels are directly associated with the risk of myocardial infarction and may reduce the cardio-protective effect of fish intake. Mercury, cadmium, and lead concentrations were determined in various fishery products (fishes, cephalopod molluscs, and crustaceans) imported into Italy from many European and non-European coastal countries [24].

Further, food poisoning due to consumption of toxic fishery products belonging to Tetradontidae, Molidae, Diodontidae, Canthigasteridae and Gempylidae families may occur [25], despite their marketing is forbidden by European Regulations (EC Reg. 853/2004; EC Reg. 2074/2005). For these reasons, a careful risk analysis is required to protect consumer's health, in conformity with EU food hygiene regulations (EC Reg. 178/2002). So, the only way to guarantee an acceptable level of safety and quality of these products is to optimise and control production procedures, also because consumers have become more exigent in terms of quality. Quality in

fish sector is a complex concept involving a whole range of factors which for the consumer include for example: safety, nutritional quality, availability, convenience and integrity, freshness, eating quality and the obvious physical attributes of the species, size and product type.

Fish industry is one of the major sectors vulnerable to fraud when fish is deliberately placed on the market for economic benefit with the intention of deceiving the consumers. Fish fraud can happen due to number of reasons, it can be due to simple misunderstanding of regulations to intentional deception of consumers for financial gains or to though illegal laundering of harvested fish and by making false documents for trading. Misleading claims about the product can affect consumer's perception about the product regarding its premium quality. When someone is making claim that tuna is caught by pole and line when it is in fact caught from a purse seine fishery is also a kind of fish fraud. Fish fraud can be done in various ways either from deliberate mislabeling and species substitution to "short -weighting" of products. Overglazing or overbreading are also kind of fish fraud. Overglazing was done more often to high value products like scallops or peeled shrimps and in prawns. Sometimes weight of products is also increased by undeclared use of water binding agents. Use of sodium tripolyphosphate (E541) can make the weight gain up to 50 percent, when it was used in processing of Vietnamese pangasius (World Fishing and Aquaculture, 2010). This additive can be used up to 5% legally in processed fish in USA and Europe, its overuse can cause substantial financial and economic gain by defrauding the customers. Other malpractice observed in fish fraud is quality enhancement of fish which can alter the appearance of fish presenting it as being superior quality than original. Carbon monoxide (CO) is frequently used for this type of malpractices. It is often used for enhancing or maintain the color of fish flesh during frozen storage of fish products. It is already banned in some countries or so its use must be displayed on the label.

Most common type of fish fraud observed is species substitution. In many fraudulent activities low value or less desirable fish species is substituted with more expensive fish species. Fraudulent replacement of pangasius as more-valuable white fleshed species is quite common. The flesh of several fish species is quite similar in terms of taste, texture, and morphology but once they are processed or prepared for consumption preserved with flavoring in sauce or in batter, it is quite difficult to identify or differentiate such species. Sometimes species substitution

was done to avoid taxation, and, in such cases, higher valued species is marketed as lower value species. The other objective is to conceal the geographical origin of fish, hiding of an illegally harvested protected species or catching fish from protected area.

Whatever the way by which this fraudulent activity occurs fish fraud is illegal and threat to public health. It decreases the confidence of the consumer in the market, and it can have serious consequence for fishery management and fish industry besides economic, social, and environmental costs.

2.3. **FISH AND FISHERY PRODUCTS CONSUMPTION**

2.3.1. World fisheries and aquaculture production and utilisation

In the last five decades, world fish production has steadily grown. This growth proceeds at the same time with food fish supply increasing at an average annual rate of 3.1 per cent, outpacing world population growth at 1.6 per cent. World *per capita* apparent fish consumption increased from an average of 9 kg in the 1960s to 20.5kg in 2018 [26]. This remarkable development has been driven by a combination of population growth, rising incomes and urbanisation, and allowed by the strong expansion and development of fish production and distribution channels.

In 2018, global fish production confirmed the huge volumes involved in this sector with 179 million tonnes, of which 156 million tonnes (87.2%) was directly used for human consumption, while the remaining part was used to produce fish meal and fish oil. Moreover, global fish production consisted of 96.4 million tonnes captured by commercial fishing in wild fisheries, plus 82.1 million tonnes produced by fish farms [26]. Aquaculture accounted for 46 percent of the total production and 52 percent of fish for human consumption.

Total fish production is expected to expand from 179 million tons in 2018 to 204 million tons in 2030. Aquaculture production is anticipated to reach 109 million tons in 2030, an increase of 32 percent (26 million tons) over 2018. Yet, the average annual growth rate of aquaculture should slow from 4.6 percent in 2007–2018 to 2.3 percent in 2019–2030 [26].

2.3.2. FISH UTILIZATION AND PROCESSING

In 2018, about 88 percent of the 179 million tons of total fish production was utilized for direct human consumption, while the remaining 12% was used for non-food purposes. In 2018, live, fresh, or chilled fish represented the biggest share of fish utilized for direct human consumption (44%). A significant but declining proportion of world fisheries production is processed into fishmeal and fish oil. Fishmeal and fish oil are still considered the most nutritious and most digestible ingredients for farmed fish. However, their inclusion rates in compound feeds for aquaculture have declined. Fish oil represents the richest available source of long-chain polyunsaturated fatty acids (PUFAs), with several health benefits. A large part of fishmeal and fish oil, estimated at 25–35%, is produced from the by-products of fish processing which earlier often discarded or used as direct feed, in silage or in fertilizers. Other aquatic organisms, including seaweeds and aquatic plants, are the subject of encouraging experimentation and pilot projects for use in medicine, cosmetics, water treatment, food industry and as biofuels. In fisheries and aquaculture, it is expected that 35% of the global harvest is either lost or wasted every year. Effective fish loss and waste reduction need proper policies, regulatory frameworks, capacity building, services, and infrastructure, as well as physical access to markets. Reducing fish loss and waste can ease pressure on fish stocks and contribute to improving resource sustainability as well as food security.

2.3.3. FISH CONSUMPTION

In the period 1961–2017, the average annual growth rate of total food fish consumption increased at 3.1 %, outpacing annual population growth rate (1.6 %). In *per capita* terms, food fish consumption rose from 9.0 kg (live weight equivalent) in 1961 to 20.3 kg in 2017. Preliminary estimates for *per capita* fish consumption in 2018 currently stand at 20.5 kg. The term “food fish” refers to fish available for human consumption, thus excluding fish for non-food uses. The term “consumption” refers to apparent consumption, which is the average food available for consumption, which, for several reasons (for example, waste at the household level), is not equal to food intake. Production, but also by a combination of many other factors: technological developments, rising incomes worldwide, reductions in loss and waste, and increased awareness

of the health benefits of fish. Fish provided an average of only about 35 calories *per capita* per day in 2017, exceeding 100 calories *per capita* per day in countries where a preference for fish has developed and endured traditionally (e.g., Iceland) and where alternative proteins are not easily accessible (e.g. small island developing States [SIDS]). The dietary contribution of fish is more significant in terms of high-quality animal proteins, PUFAs and micronutrients of fundamental importance for diversified and healthy diets. In 2017, fish accounted for about 17 % of total animal protein, and 7 % of all proteins, consumed globally. Moreover, fish provided about 3.3 billion people with almost 20 percent of their average per capita intake of animal protein. At the regional and continental levels, the lowest *per capita* fish consumption occurs in Africa, where it peaked at 10.5 kg in 2014 and then declined to 9.9 kg in 2017. Low fish consumption in sub-Saharan Africa is the result of several interconnected factors, including among others: population increasing at a higher rate than food fish supply; stagnation of fish production because of pressure on capture fisheries resources; and a poorly developed aquaculture sector.

At the global level, since 2016, aquaculture has been the main source of fish available for human consumption. In 2018, this share was 52 %, a figure that can be expected to continue to increase in the long term. Aquaculture has expanded fish availability to regions and countries with otherwise limited or no access to the cultured species, often at cheaper prices, leading to improved nutrition and food security.

2.3.4. FISH TRADE AND PRODUCTS

In 2018, 67 million tons of fish (live weight equivalent) were traded internationally, equating to almost 38% of all fish caught or farmed worldwide. In the same year, 221 States and territories reported some fish trading activity. The total export value of USD 164 billion recorded in 2018 represented almost 11% of the export value of agricultural products.

From 1976 to 2018, the value of global exports of fish and fish products increased at an annual rate of 8 percent in nominal terms and of 4 percent in real terms. Available estimates for 2019 suggest that total trade value contracted by about 2 percent in both quantity and value compared with the previous year. In addition to being by far the major fish producer, China has also been the main exporter of fish and fish products since 2002. Since 2004, Norway has been the second major exporter, now followed by Viet Nam. While developed markets still dominate

fish imports with the European Union followed by the United States of America and Japan, the importance of developing countries as consumers as well as producers of fish and fish products has been steadily increasing. In 2018, fish imports by developing countries represented 31 percent of the global total by value and 49 percent by quantity (live weight). Over 90 percent of the quantity (live weight equivalent) of trade in fish and fish products consisted of processed products (i.e., excluding live and fresh whole fish) in 2018, with frozen products representing the highest share. About 78 percent of the quantity exported consisted of products destined for human consumption.

Table 1. World fisheries and aquaculture production and utilisation (FAO, 2020).

	1986-1995	1996 - 2005	2006-2015	2016	2017	2018
Production			**(million tonnes)**			
Capture						
Inland	6.4	8.3	10.6	11.4	11.9	12
Marine	80,5	83	79.3	78.3	81.2	84.4
Total capture	**86.9**	**91.3**	**89.8**	**89.6**	**93.1**	**96.4**
Aquaculture						
Inland	8.6	19.8	36.8	48	49.6	51.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8
Total aquaculture	**14.9**	**34.2**	**59.7**	**76.5**	**79.5**	**82.1**
Total world fishery and aquaculture	**101.8**	**125.6**	**149.5**	**166.1**	**172.7**	**178.5**

Utilisation						
Human Consumption	71.8	98.5	129.2	148.2	152.9	156.4
Non-food Uses	29.9	27.1	20.3	17.9	19.7	22.2
Population (Billions)	5.4	6.2	7.0	7.5	7.5	7.6
Per Capita apparent consumption (kg)	13.4	15.9	18.4	19.9	20.3	20.5

2.3.5. Capture fisheries production

Global capture production has increase in the last years. In fact, in 2016 and 2017, global fishery production in marine waters was 89.6 million tonnes and 93.1 million tonnes, respectively. Moreover, in 2018 global capture production was about 96.4 million tonnes confirming as the highest ever. In last few years, 18 countries caught more than an average of one million tonnes per year, accounting for more than 76 %of global marine catches [26]. Eleven of these countries are in Asia (also including the Russian Federation, which fishes much more in the Pacific than in the Atlantic). The increased production in 2018 was mostly motivated by marine capture fisheries, whose production increased to 84.4 million tons in 2018 [26] . The top seven capture producers are China, Indonesia, Peru, India, the Russian Federation, the United States of America, and Viet Na which) accounted for almost 50 percent of total global capture production.

Although Asian countries, mainly Philippines and the Republic of Korea, have shown considerable increases in marine catches in the last 10 years, Japan, Russian Federation, India, Malaysia and Thailand have registered decreases. However, marine catches submitted to FAO by Myanmar, Viet Nam, Indonesia, and China have shown continuous growth with astonishing decadal increases (e.g., Myanmar up 121 per cent, and Viet Nam up 47 per cent).

China is, by far, the largest exporter of fish and fishery products. However, since 2011, it has become the world's third-largest importing country, after the United States of America and Japan. As regards the European Union (Member Organisation), it may be considered as the largest market for imported fish and fishery products, also because of its decreasing capture production.

2.3.6. Inland aquaculture and mariculture

World aquaculture production continues to grow, although at a slowing rate. According to the latest available statistics collected by FAO, in 2018 world aquaculture production recorded another all-time high of 114.5 million tonnes (live weight equivalent), including 82.1 million tonnes of food fish and 32.4million tonnes of aquatic algae and 26 000 tons of ornamental seashells and pearls [26]. The total farm gate sale value of USD 263.6 billion was obtained in 2018 by world aquaculture. The farming of aquatic animals in 2018 was dominated by finfish (54.3 million tons). Fed aquaculture production (57 million tons) has overpowered the non-fed subsector in world aquaculture. World aquaculture production of farmed aquatic animals grew on average at 5.3 percent per year in the period 2001–2018, whereas the growth was only 4 percent in 2017 and 3.2 percent in 2018. The recent low growth rate was caused by the slowdown in China, the largest producer. In 2018, inland aquaculture produced 51.3 million tons of aquatic animals, accounting for 62.5% of the world's farmed food fish production, as compared with 57.9 percent in 2000. Mariculture and coastal aquaculture collectively produced 30.8 million tons of aquatic animals in 2018. Despite technological developments in marine finfish aquaculture, marine and coastal aquaculture produce currently many more mollusks than finfish and crustaceans. Aquaculture development and production distribution are imbalanced, in fact, about 89 per cent of world aquaculture production by volume come from Asia. The major aquaculture producing countries are Egypt, Chile, India, Indonesia, Viet Nam, Bangladesh, and Norway. The development of aquaculture has made a great contribution to the supply of food fish for consumption, mainly in the world's most populous countries such as China, India, Indonesia, Pakistan, Bangladesh, and Japan. Among major producing countries, Egypt, Chile,

India, Indonesia, Viet Nam, Bangladesh, and Norway have consolidated their share in regional or world production to varying degree over the past two decade.

2.3.7. Fish consumption in Italy

Italy, home of the Mediterranean diet, has always set his eating habits on fish consumption. It is important to say that Italy has two fishery products registered with PDO and PGI protection mark, such as "*acciughe sotto sale del Mar Ligure*" (PDO) and "*tinca gobba dorata del pianalto di Poirino*" (PGI).

In past years, fish consumption at national level has recorded positive trends compared to 2014 (+ 4.3%). Mainly, this increase regards north-western and central regions of Italy, though the highest consumption has been recorded in South of Italy (33%) [27]. This rise has showed a difference in sale between modern and traditional distribution channels. In fact, consumers have mainly purchased fishery products at hypermarkets and at discount supermarkets (79.8%) rather than at traditional fisheries (20.2%) [27]. This is also due to the rising share of supermarkets in the retail of seafood products which increases their availability, leading to increase the consumption.

It is noticeable that the consumption shifts away from traditional fresh fish towards other products. In fact, processed products (fresh and defrosted both packaged and unpackaged) seem to be preferred by consumers in line with a more and more frenetic lifestyle. This increase in consumption of convenience products is possible because people have less and less time to spare for meal preparation. The category of dry, salted and smoked products has grown more, also thanks to smoked salmon, a product appreciated by a growing number of consumers in every period of the year.

An increase in demand for fish has been added to the growth of consumption. Foreign demand has appeared more dynamic than in 2013. In fact, exports have recorded an overall amount of 135,000 tonnes, with an increase of 6.2% and an export value grown by 7.3% [27].The growth was mainly derived from increased supplies to the European countries (116,000 tonnes) such as Spain, France and Germany, with an export volume grown by 9.6%. Fishery products that have been mainly exported are canned tuna, bivalve molluscs, and fresh and chilled sardine [27].

At the same time, imports have increased both in volume (+ 5.7%) and value (+ 6.2%). Out of 975,000 tonnes of imported fish, 56.4% (550,000 tonnes) come from European countries; mainly canned tuna, frozen squids and octopus, bivalve molluscs from Spain, Netherlands, and Greece [27]. In conclusion, in past year national fish trade balance has shown a clear difference between imports and exports confirming that Italy is within the first ten positions of the world as importation.

Italy is a net importer of fish and fish products. Between 2008 and 2018, exports decreased by a total of 1%, while imports increased by 16%. In 2018, Italy produced 0.3 million tons of fish (including mollusks and crustaceans), with a value of USD 1658.4 million. 32% of this value came from aquaculture and 68% from fisheries (that is, the capture of wild resources). Between 2008 and 2018, the quantity produced decreased by 10%, while its value decreased by 15%.

2.4. Scale and Global Incidence of Fish Fraud

Fish frauds can involve many species and can happen anywhere. They may occur in the wholesale fish markets, but more frequently in the retail sale of fish markets, from street vendors, and in supermarkets or in restaurants. Mislabelling and fraudulent substitution for certain fish species is rampant and widespread. Recently, Oceana's report showed findings from one of the largest fish fraud investigations in the world, performed over a two-year period to determine the prevalence of mislabelled fish sold by 674 retailers in the U.S. such as sushi venues, grocery stores and restaurants. DNA analysis of 1215 fish samples from 21 States revealed that one-third were mislabelled. Forty-four per cent of the retail outlets visited sold mislabelled fish [28]. However, mislabelling rates varied greatly depending on the type of fish purchased. Among finfish categories, grouper (*Epinephelus* spp.) and Atlantic cod (*Gadus morhua*) were chosen as species of interest and 26% and 30%, respectively, were found to be mislabelled [28].

As regards Atlantic cod, food products from *Gadidae* fish species are often subjected to frauds also because they are sold commercially in many forms, including fresh/frozen fillets, frozen fillet blocks, surimi blocks, salt-cured or smoked, fish sticks, canned fish, and roe. Increases in the international trade of these processed fishery products have also increased the feasibility of fish species substitution, especially due to the similar appearance of many gadoids. According to data from Oceana's report, fish species marketed or mislabelled as Atlantic cod were mainly

Pacific cod (*Gadus macrocephalus*), tilapia (*Oreochromis aureus*), Asian catfish (*Pangasianodon hypophthalmus*), white hake (*Urophycis tenuis*), red drum (*Sciaenops ocellatus*), pacific halibut (*Hippoglossus stenolepis*) and haddock (*Melanogrammus aeglefinus*) [28]**.** Asian catfish (*P. hypophthalmus*) is widely exported due to great acceptability, affordable cost, and the white colour of the meat, which can replace expensive white fishes such as cod and grouper. Currently, catfish fillets have been exported to over 80 countries worldwide including Netherlands, Germany, and United States, which demand mainly frozen fillets without skin and bone [29, 30]. In this situation, the value of frozen catfish, when sold as grouper, quadruples, as does the loss to consumers [31]. In addition, typical cases of mislabelling that involve catfish and frequently occur at retailers are the marketing of frozen fillets as fresh. Beyond fraudulent actions, the consumption of catfish fillets may represent a serious health risk because of its possibility to contain chemical contaminants [32]. In fact, catfish is primarily farmed in Vietnam along the Mekong River, a body of water that has become polluted in many areas due to increases in unregulated mining activity and anthropogenic run-off [33]. Moreover, Vietnamese fish farming regulations are often less stringent than European laws. Although this situation, many authors report that chemical quality parameters regarding frozen catfish fillets are below the recommended limits established by regulations [34, 35]. Even if, in a study carried on in Brazil, 50% of imported frozen catfish fillets have demonstrated methyl mercury concentrations over 0.5 mg kg^{-1} [32].

Growing problems of fraudulent substitution for grouper and cod products in the production and distribution chain are involving oilfish (*Ruvettus pretiosus*) of the Gempylidae family. Approximately 20% of oilfish's wet weight consists of indigestible lipids (wax esters), which, two hours after the ingestion, have been found to cause keriorrhea and other acute gastrointestinal symptoms, such as abdominal cramps, nausea, headache, and vomiting in susceptible subjects [36, 37]. Oilfish is usually mislabelled as cod or grouper, either intentionally or accidentally. Under these circumstances, outbreaks of keriorrhea associated with consumption of oilfish have been repeatedly reported in several continents [37, 38]. Oilfish is of low commercial values because of their kerriorrheic properties and is considered as ‘‘not suitable for catering’’ or even banned from sale in various countries. European Union has issued special

guidelines toward trading and consumption of oilfish. In conformity with EC Reg. 1021/08, fresh, prepared, frozen and processed fishery products belonging to the family Gempylidae, in particular *Ruvettus pretiosus*, may only be placed on the market in wrapped/packaged form and must be appropriately labelled to provide information to the consumer on preparation/cooking methods and on the risk related to the presence of substances with adverse gastrointestinal effects. The scientific names of the fishery products and the common names must appear on the label.

Hake (*Merluccius merluccius*) is also implicated in cases of mislabelling. It is often marketed as cod and as grouper for its similar organoleptic and morphological characteristics [39, 40]. The marked differences in price and marketability between these species increase the opportunities for their fraudulent commercial substitution. So, numerous studies have been carried out on protein and DNA sequence analysis for a correct identification of these fish species [40, 41]. Another example of substitution of fish species may be the case of Nile perch (*Lates niloticus*) fillets which are frequently marketed as grouper (*Epinephelus* spp.) [42]. Due to its lower value, Nile perch is often labelled as grouper and sold at a higher price, because of the higher popularity and quality of the latter species. In the last years, Asensio (2008) have analysed 37 grouper meals collected at the restaurant industry and 70 fillets labelled as grouper at the retailers for the identification of fish species [43, 44]. Out of 37 purported grouper meals served at cafeterias (school and university) and restaurants, only 9 (24%) were determined to contain authentic grouper (*Epinephelus* spp.). In the case of commercial fish fillets, only 12 (17%) were determined to be grouper (*E. marginatus*). Of the remaining fillets, 34 (48,5%) were determined to be Nile perch [44].

An investigation made about food fraud cases carried out around 57 countries and coordinated by INTERPOL-Europol in 2015 has clearly indicated vulnerability of fish supply chain[45]. The report placed the fish as 3rd highest risk category of product vulnerable to food fraud[45]. European parliament placed fish as 2nd most vulnerable category of product having chances of fraud among food items traded internationally [46]. The report published by various international organizations indicated fish fraud as serious problem in global food supply chain.

Although fish fraud is not a new phenomenon in food sector the number of reported cases has increased significantly in recent years particularly species substitution and mislabeling at global level. According to a major report published by Oceana in 2016, species substitution and mislabeling occurred at every step of fish supply chain either at the point of landing through processing, distribution, retail or catering [9, 10]. Fraudulent activities done in the process of import and export of fish were also reported. The Oceana report was based on study conducted in 55 countries and reviewing 200 published research articles across the world. According to the report around 20 % of all fish samples tested were mislabeled. An investigation carried out about mislabeling in Canadian restaurants and grocery stores have found more than half of samples were mislabeled [47]. Most of the studies conducted on the retail and catering sector of the marketing chain. In one interesting study mislabeling incidents of last 5 years were summarized by Pardo et al 2016 [48] and according to the study around 30 % samples were mislabeled , the majority of frauds were detected from the restaurants and takeaways. Past few years observed several publications based on seafood fraud and mislabeling around the world. Almost all studied indicated large scale species substitution and mislabeling in seafood industry which is a serious threat to public health and food safety.

In USA one study conducted to check labeling of red snapper have found that around 75% of samples were mislabeled [49]. In another study conducted in various restaurants in three regions of USA have observed around 17% mislabeling in fish served to the customers [50]. A study about authenticity of fish species sold in sushi restaurants was conducted around 4 years in USA and 47% mislabeling detected in that study [51]. Another study about fish fraud conducted through DNA Barcoding observed 33% mislabeling in 21 states of USA, the fish sample were collected from different retail outlets such as restaurants and grocery stores. In one Canadian study done by Canadian center of DNA Barcoding in five metropolitan areas to check authenticity of fish in retail sectors have observed 41% mislabeling in fish products [52]. The study was part of Fish Barcode of life project for generating reference database of barcodes obtained from cytochrome c oxidase subunit I (*COI)* gene. The objective of Fish BOL project was to facilitate rapid, accurate, perfect and cost-effective nucleic acid-based identification of fish species [53].

In 2015 following the horsemeat scandal the EU initiated and coordinated control program across all member countries to check the extent of mislabeling in white fish market [EU2015]. Around 4000 samples were analyzed in this initiative across 29 countries in which 94% compliance with respect to mislabeling regulation observed. Many other small studies are carried in member countries to check mislabeling and species identification. In 2017, research carried out by Italian authorities to check labeling compliance of imported fishery products has found 22.5 percent mislabeling in these imported products [54, 55]. Cephalopod based products displayed the most cases of mislabeling (43.8%). Other major products showing mislabeling are crustacean (17%), and fish (14%), imported from China, Vietnam, and Thailand.

Tantillo *et al.* in 2015 studied the malpractices of mislabeling in varieties of fish fillets from different supermarkets in Southern part of Italy and found 42.8% of fillets (sole, salmon, plaice and hake) products were mislabeled, 46.4 percent of plaice fillet where being substituted with *Pangasianodon* spp. [56]. Similar kind of study is also conducted by Di Pinto *et al.,* in 2015 by DNA Barcoding in which he observed 82 percent (64/200) mislabeling in different fish fillet samples[2]. In one large study done in Sardinia region of Italy between 2009-2014 to check mislabeling and species at retail level, more than 3000 samples of fresh fishery products were investigated in which 30% incidence of non-compliance observed [57].

One study about hake products in Spanish and Greek market identified 30% substitution with African fish species [2, 58, 59]. Two low valued fish species like Sardines and farmed catfish were used in making surimi products which was marketed from India, China, and Singapore. The study was conducted by DNA Barcoding [60]. Endangered species was also found in one sample which suggests testing of fishery products though DNA Barcoding where classical morphological method of identification is not effective.

Detection of mislabeling is sushi bars and restaurants were conducted across UK and North Ireland in which 10% species substitution with tuna, eel and white fish species was observed [61]. DNA Barcoding was successfully used to check fish species in two convenience products like fish fingers and fish sticks, in which around 1.5% of fish fingers were found to be mislabeled [62]. High level of species compliance with labeling regulations is observed in a survey of the authenticity

of white fish in supermarkets in UK and Northern Ireland. The study observed 94% sample compliance with information on the label [6]. Low level of species substitution was probably observed due to high level of industry and public awareness about fish fraud which occurs due to enforcement of strict labeling regulation and media coverage.

In France, a comprehensive survey was made in which around 371 fresh and farmed fish meals containing 55 commercial fish species were analyzed [63]. It was found 3.7% species substitution in this study. They observed zero mislabeling in frozen fillets or in industrially prepared meals. Mislabeling was mostly observed in fish mongers and restaurant in which 5 species (bluefin tuna, cod, yellowfin tuna, sole and seabream) were substituted with cheaper one.

A national survey conducted in Ireland by food safety authorities about authenticity of fish species marketed in restaurants, retail stores and takeaways and surprisingly one fifth of products tested were found to be mislabeled [64]. One of mislabeled products was sold as cod but it was found to be made up of pollock, smelt or other cheaper fish species. Around three quarter of smoked fish products sold in takeaways were mislabeled. A warning to sea food companies about fish labeling regulation were issued by food safety authorities of Ireland. Now food authenticity check is part of routine national food surveillance programs in Ireland and since 2012 almost negligible cases of fraudulent labeling detected in Ireland [65-67].

A lot of study based on DNA barcoding was done in Brazil, South America. High level of mislabeling and species substitution was detected in one study [68]. In 2017, DNA barcoding was selected as standard method to check authenticity of processed fish produced by Federal government of Brazil [69].

Species substitution and mislabeling of fish products were also detected in Africa. In South Africa one study was done in which 149 fish samples were collected from different restaurants and retail stores in three provinces of the country, and around 18% mislabeling was detected [70]. According to results obtained 9% of samples collected from wholesalers and 31% products collected from retail where identified as different species to one mentioned at the point of sale [70]. A high level of species substitution and mislabeling was found in different fish fillets sold in

in Egyptian market [71]. An interesting study was done to check composition of aquaculture feed in Egypt through DNA metabarcoding. In this study 46% of all fish species detected were either overfished or their stocks were in decline [72].

Asian countries also face the problem of mislabeling and substitution in fish products. In 2016, a study was conducted in Malaysia in which 16% of fresh, raw or commercially processed fish were found to be mislabeled [73]. Studies conducted through DNA barcoding in China revealed several fraudulent activities in fish products [74-76]. A survey to check authenticity of fish maws (dried, salted swim bladders) in Chinese seafood market revealed around 53% mislabeling in commercial species substitution with low valued species [77]. Similar study to check authenticity of fish products implemented in Taiwan detected 70% mislabeling in different products [78].

In India, a study was conducted through DNA barcoding to check authenticity of fresh and processed fish from the domestic market, and 22% mislabeling was detected in this study [79]. An interesting study was done through DNA barcoding to verify shark species from dried fins confiscated from a vessel fishing illegally in Australian waters [80]. They observed that fins were from 27 different shark and ray species, some of which comes from endangered species.

A study done in Indonesia based on DNA barcoding of fish products in different retail stores and fish ports with the help of *COI* and nuclear rhodopsin gene fragment found mislabeling in some fish products and species substitution with endangered species [81].

The large-scale fish frauds including species substitution and mislabeling in international fish supply chain is cause of great concern and it is widely spread in many countries. Traditional identification methods of fish species are not effective in cases where fish were processed as fillets and other ready to eat products. For providing definitive identification of fish to species level DNA barcoding is most well-defined method. It also provides science-based system for linking scientific nomenclature with approved common fish names. It needs international recognition to authenticate official food control programs for countering fish fraud, mislabeling and species substitution worldwide in fish supply chain, many countries are adopting this method to tackle this this issue.

Table 2. Examples of some recent scientific articles regarding seafood authenticity surveys

Seafood samples/ species groups	Samples	Country	% Mislabeling	Reference
Packaged frozen fishery products (e.g. Gadidae, Merluccidae)	120	Italy	5	[82]
Convenience seafood products (Gadidae, Merluccidae)	54	Italy	35	[83]
Processed seafood products including fish (e.g.*Salmonidae*), surimi, sushi, bivalves (e.g. *Ostreidae*)	62	Malaysia	16	[73]
Products commercialized as cod (e.g. Gadidae, Tetraodontidae)	52	China	60	[84]
Frozen products sold as sablefish (e.g. Anoplopomatidae, Nototheniidae)	42	China	86	[85]
Processed fish (e.g. Salmonidae) and crustacean products (e.g. Penaeidae)	100	India	22	[86]
Frozen seafood: fish (e.g. Gadidae, Merluccidae), crustaceans (e.g. Penaeidae and Solenoceridae), bivalves (e.g. Mytilidae)	60	Portugal	19	[84]
Fish (e.g. Salmonidae, Gadidae), crustaceans (e.g. Penaeidae)	118	Germany	6	[87]
Typical sushi fish (e.g. Scombridae, Salmonidae)	364	USA	47	[88]
Fish (e.g. Ophidiidae, Scombridae, Gadidae, Merluccidae)	255	Brazil	17	[89]
Sushi seafood (e.g.Salmonidae, Scombridae)	185	Italy	3	[90]
Sushi	115	UK	10	[61]
Salmonid products (e.g. Salmonidae	111	Canada	6	[91, 92]
Processed cephalopods products including cuttlefish, octopus, squid	95	China	2	[93]

Fishery products including fish (e.g. Merluccidae), cephalopods (e.g. Sepiida), crustaceans (e.g. Penaeidae)	277	Italy	22	[94]
Codfish products (e.g. Gadidae)	43-53 (each country)	2 19 4 7 59	UK Denmark Sweden Canada Estonia	[95]

Table 3. Examples of commonly substituted seafood

True identity	Common Substitution	Reference
Rockfish	Red snapper (*Lutjanus campechanus*)	[96]
Grouper	Gilt-head bream (*S. aurata*), perch (*Lates spp.),* king mackerel (*Scomberomorus cavalla*), whitefin weakfish (*Cynoscion albus*), speckled hind *(Epinephelus drummondhay*i), tilefish (*Malacanthidae spp*.)	[97, 98]
Sea bass	Toothfish (*Dissostichus spp*.), tilapia (*Oreochromis niloticus*), croaker (*Argyrosomus regius*), Pangasius *spp*	[52, 97]
Cod	Walleye pollock (*G. chalcogrammus*), haddock (*Melanogrammus aeglefinus*), whiting (*Merluccius productus*), Liparis spp., threadfin slickhead (*Talismania bifurcata*), rockfish (*Sebastes spp.),* tilapia (*Oreochromis spp.),* Pangasius spp., Pacific halibut (*Hippoglossus stenolepis*)	[52, 99-101]
Caviar	Northern pike (*Esox lucius*), lumpfish roe (*Cyclopterus lumpus*), paddlefish roe (*Polyodon spathula*), kaluga (*Huso dauricus*), beluga (*Huso huso*)	[52, 102]
Salmon	Other salmon, trout, tuna	[52, 103]
Black drum	Red drum (red fish)	[98]
Tuna	Other tuna (*Thunnus spp*.), mozambique tilapia (*Oreochromis mossambicus*), Japanese amberjack (*S. quinqueradiata*), escolar (*Lepidocybium flavobrunneum*), banded rudderfish (*Seriola zonata*), salmon (*S. salar*), yellowtail amberjack (*Seriola lalandi*)	[97, 100, 103, 104]

Hake	Barracuda (*Sphyraena spp.),* Patagonian grenadier (*Macruronus magellanicus*), swai (*Pangasianodon hypophthalmus*), Pacific grenadier (*Coryphaenoides acrolepis*), European bass (*Dicentrarchus labrax*), Patagonian toothfish (*Dissostichus eleginoides*), saithe (*Pollachius virens)*	[100, 103, 105, 106]
Flounder	Northern rock sole (*Lepidopsetta polyxystra*), *Pangasianodon sp.,* Alaska plaice (*Pleuronectes quadrituberculatus*), Indian Ocean spiny halibut (*Psettodes erumei*)	[98, 101, 103, 107]

2.5. Impact of Fish fraud on Public Health

Although fish fraud is global phenomenon proliferated in lot of countries most of the fraudulent cases do not pose any major threat to public health. There are some exceptional cases in which public health is really in danger and some cases of death are also reported. The cases are particularly more threatening when fish species that are nontoxic is replaced with toxic species. Public health also adversely affected when farmed fish from polluted watercourses are replaced with marine fishes. Some of naturally occurring toxic fish species responsible for serious form of food poisoning and even sometimes death includes species of puffer fish, scombroid fish, escolar or oilfish and ciguatoxic fish.

Puffer fish (*Lagocephalus sceleratus* and other species) contain a powerful neurotoxin called tetrodotoxin which can cause paralysis and even death due to muscular paralysis, respiratory depression, and circulatory failure. There are reported some cases of puffer fish fatalities in Bangladesh which are executed by ignorance, unscrupulous marketing by intermediaries in the fish supply chain and species replacement. Weak food control, poor regulations and consumer awareness can aggravate the problem of fish fraud. In 2008, inland population of Bangladesh without knowing the fact consumed pufferfish sold at local market in which 17 people died [108]. Similar phenomenon was also occurred in Khulna in 2002 leading to 8 deaths. In 2016 also. five people died due to consumption of puffer fish In Sylhet [109]. DNA barcoding was successfully applied in identification of toxic fish species which were substituted. In a study of ethnic fish on Italian market in 2015 samples labelled as squid were identified as toxic species of puffer fish [110]. In 2007, in USA two cases of puffer fish poisoning detected after consumption of

incorrectly labelled monkfish [111]. In Italy, a survey was conducted about authentication of fish fillets in commercial market in which 32% mislabeling was detected [112]. Some species of escolar or oilfish (*Ruvettus pretiosus* and *Lepidocybium flavobrunneum*) which contain waxy esters and difficult to digest are responsible for gastrointestinal disorders whit anal leakage and oily orange diarrhea as symptoms[113]. These waxy fish species belong to family Gempylidae and the waxy esters are mentioned as gempylotoxins [114]. Consumption of certain reef fish species from tropical and subtropical climate can cause Ciguatera fish poisoning. These fishes contain naturally occurring ciguatoxins [115, 116]. Species substitution and mislabeling can also occur when aquaculture species are replaced for wild capture species. Farmed fishes are exposed to environmental contaminants such as heavy metals which increase risks of contamination of farmed fish and such species are harmful for consumption [117, 118] Lot of antibiotics are used in fish farming and these residues may also pose danger to public health emphasizing the link between species authentication and food safety.

Other cases of substituted also detected in which Mediterranean grouper being replaced with Nile tilapia (*Oreochromis niloticus*), gurnard (*Chelidonichthys cuculus),* substituted with Nile perch and halibut (*Hippoglossus*) with pangasius (*Pangasianodon).* In one study conducted in Philippines the substitution of gindara or sablefish (*Anoplopoma fimbria*) with escolar (*Lepidocybium flavobrunneum)* was reported [119]. In one study done in 2013 in USA red snapper (*Lutjanus campechanus*) was found to be replaced tilefish (*Branchiostegus*) [97]; tile fish contains high mercury level so pregnant women and young children must avoid its consumptions according to guidelines by USFDA. In 2016, Pardo *et al*., published detailed reports about impact of species substitution on public health and warned about consequences of fish fraud [48].

2.6. Economic Impact of Seafood Fraud

There is significant economic loss associated with seafood fraud, although there is lack of data regarding actual estimate [120, 121] . The total first sale value of fishery and aquaculture production around the world be around US$362 billion globally in 2016 [122]. This section is highly prone to fraud. According to one estimate in 2011 in US if 2% of the declared weight of seafood purchased annually by consensus in the US was ice the annual loss to consumer would

amount to US $ 1.6 billion per year [123]. The replacement of a lower priced fish for higher valued fish may have significant economic consequences for the seafood industry and consumers [85, 124]. In 2010 Filonzi *et al*., investigated various seafood product in Italy and described the commercial value of the declared seafood species to be in the range of 19.90-40 euros/kg, compared to 8.90-11.20 euros/kg for the substituted varieties [124]. In one estimate in Canada by Oceana 74% of mislabeled seafood products to be listed as more expensive variety than the actual fish which was being sold including whiting (CND $ 7.33/kg), sold as Atlantic cod (CND $ 33.33/kg). Atlantic Salmon (CND $ 37.66 kg) sold as Sockeye salmon (CND $ 101.69/kg) and catfish (CND $ 11.64/kg) sold as sea bass (CND $ 113.88/kg) [125]. Product mislabeling and seafood substitution can hamper the reputation of the seafood industry culminating in economic losses due to unfavorable consumer participation and lack a demand for seafood products [120, 121]. Furthermore, seafood fraud can lead to low pricing for products with high market demand and prevent legitimate producers from receiving their fare market share.

3. Traceability of fish products

Traceability is synonymous with total transparency, and it is the knowledge of every stage and treatment performed for the food production. The fishing industry has aspects of considerable complexity about the food supply chain that makes it even more necessary, though more difficult, the creation of full transparency. It is important to protect and to inform the consumer giving right information about fish and fishery products. For example, if fish was caught or farmed, when and where it was caught (country of origin) or, especially for fishery products, whether it was fresh or frozen and thawed. So, in fishery sector the traceability and labelling are an imperative and urgent needs of the market. Traceability, in fact, is increasingly becoming a requirement in major fish importing countries. It can safeguard public health and demonstrate that fish has been caught legally from a sustainably managed fishery or produced in an approved aquaculture facility. European laws on food safety and traceability of fish and fishery products are:

- ❖ Council Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law,

establishing the European Food Safety Authority and laying down procedures in matters of food safety [126].

- ❖ Regulation (EU) No. 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products [127]
- ❖ Regulation (EU) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers [128].
- ❖ Regulation (EU) No. 1420/2013 of 17 December 2013 informing consumers about fishery and aquaculture products [129].
- ❖ Council Regulation (EC) No. 1224/2009 of 20 November 2009 establishing a community control system for ensuring compliance with the rules of the common fisheries policy. Regulation (EU) No. 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No. 1224/2009 [130]

Moreover, it is important to include the principles of Community Regulations regarding the hygiene of foodstuffs such as EC Reg. 852/2004 [131], the hygiene for food of animal origin such as EC Reg. 853/2004 [132] and organisation of official controls on products of animal origin intended for human such as EU Reg. 625/2017.

4. LABELLING

In food sector, traceability is guaranteed by the label. Labelling ensures the consumer about the right correspondence of the product and about its hygienic and sanitary features. It has not to attribute to the product effects or properties which it does not possess. So, label is the only real tool which the manufacturer and retailer uses to communicate food information to consumers. Since 13 December 2014, a new food labelling European Regulation has been come into force. It is EU Reg. 1169/2011 [128]. The Regulation defines the term ‘labelling’ as follows: “any words, particulars, trademarks, brand name, pictorial matter or symbol relating to a food and placed on any packaging, document, notice, label, ring or collar accompanying or referring to such food”.EU Reg. 1169/2011 brings general and nutritional information together to simplify and consolidate existing labelling legislation. It applies to food business operators at all stages of

the food chain. Thanks to the new law, consumers can receive clearer and more comprehensive information on food content, and they can make a more accurate choice about what they purchase. For these reasons, food and beverage manufacturers and retailers have updated their labels to comply with the new Regulation. In accordance with EU Reg. 1169/2011, food information that must be provided on label are the following:

- The name of the food.
- The list of ingredients.
- Any ingredient or processing aid listed in Annex II or derived from a substance or product listed in Annex II causing allergies or intolerances used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form.
- The quantity of certain ingredients or categories of ingredients.
- The net quantity of the food.
- The date of minimum durability or the 'use by' date.
- Any special storage conditions and/or conditions of use.
- The name or business name and address of the food business operator.
- The country of origin or place of provenance.
- Instructions for use where it would be difficult to make appropriate use of the food in the absence of such instructions.
- With respect to beverages containing more than 1.2% by volume of alcohol, the actual alcoholic strength by volume.
- A nutrition declaration (it becomes mandatory for packaged foods from 13 December 2016).

4.1. LABELLING OF FISH AND FISHERY PRODUCTS

The mandatory information about labelling of fish and fishery products must comply with the EU Reg. 1379/2013 [127]. The information on label must be updated considering the requirements of EU Reg. 1169/2011 and the lists of denominations of the fish species of commercial interest in Italian D.M. Mi.P.A.F. 22/09/2017. Regulations specify that labels of different fishery products, such as prepacked and non-prepacked products, must indicate appropriated information

(European Commission, 2014). The mandatory information to be reported on label of non-prepacked products are the following:

a) the commercial designation of the species and its scientific name. These names must match those on the official list drawn up and published by each EU country. This information need not accompany the name of the food, although it must be shown on billboards or posters.
(b) the production method, by the following words "… caught …" or "… caught in freshwater …" or "… farmed …".
(c) the area where the product was caught or farmed, and the category of fishing gear used in capture of fisheries. The catch area for fish caught at sea is the FAO area, sub-area or division where the fish were caught. Fish caught in freshwater must display both the name of the body of water (river, lake, etc.) and the country where the product was caught. Farmed fish (aquaculture) must display the country of production. Wild fish must display one of the following fishing gear categories used to catch the fish: 'seines', 'trawls', 'gillnets and similar nets', 'surrounding nets and lift nets', 'hooks and lines', 'dredges', and 'pots and traps.
(d) whether the product has been defrosted. This information is not necessary if fishery and aquaculture products: are ingredients present in the final product; or have been previously frozen for health safety purposes; or have been defrosted before smoking, salting, cooking, pickling, drying or a combination of these processes; or are foods for which freezing is a technologically necessary step.
(e) the date of minimum durability, where appropriate. It corresponds to the 'best before' date or 'best before end' date. For all non-prepacked products, products prepacked for direct sale or on sales premises at the consumer's request, EU countries can decide whether to adopt national rules stipulating that the 'best before' or the 'use by' date should be displayed.

As regards allergens, for non-prepacked products, information is also mandatory, as required by EU Reg. 1169/2011. However, European countries can adopt national measures about the 'means by which this information is provided. Moreover, where no list of ingredients exists, the presence of allergens must be indicated as follows: 'contains…'. This information is not required when the food name clearly refers to allergen(s).

As regards the prepacked products, different information must be reported on label, in particular:

- whether the product has been defrosted, this information must accompany the commercial name.
- For the date of minimum durability, all pre-packed products which are not highly perishable must display the 'best before' date. By contrast, highly perishable products should display the 'use by' date.
- For allergens, a clear reference to the name of any allergens should be included in the list of ingredients. This should appear in a typeset (e.g. font style, or background colour) which clearly distinguishes it from the rest of the list of ingredients.

Additional requirements for prepacked products must be provided, in addition to that listed above. For example:

- List of ingredients: a list of all ingredients in descending order of weight should be displayed next to 'Ingredients'. This is not necessary for single-ingredient foods that have the same name as the ingredient.
- Quantity of ingredients: this must be expressed as a percentage. This must be shown when the ingredient appears in the name of the food, is emphasised on the labelling, and is essential to characterise a food.
- Net quantity: this must be expressed in grams or kilograms. The drained net weight of the food must also be shown where a solid food is in a liquid medium (also frozen or quick-frozen). If the food has been glazed, the declared net weight of the food must exclude the glaze.
- Conditions for storage and use: any special storage conditions and/or conditions of use must be shown.
- Name or business name and address of the food business operator: the name and address of the food operator responsible for the food information, and under whose name the food is marketed, should be displayed.
- Instructions for use: only if needed.

- Nutrition declaration: only from 13 December 2016.
- ‘Date of freezing’ or ‘Date of first freezing’: this requirement only applies to unprocessed products. The date must be indicated as follows: ‘Frozen on day/month/year’.
- Identification mark: the name of the country, the approval number of the establishment where production takes place and the abbreviation EC, or its translation in other EU languages, must be shown when the product is produced in Europe (EC Reg. 853/04) [132, 133]. For imported products, only the name of the country and the approval number of the establishment are mandatory.
- Date of packaging: this date must be shown for live bivalve molluscs. This date must comprise at least the day and the month.

The mandatory information must be available and easily accessible. In contrary case, an increase of commercial (*aliud pro alio*) and sanitary (commercialisation of toxic organisms) frauds could occur.

5. IDENTIFICATION OF FISH SPECIES

Identification of species represents a key aspect of biodiversity studies, but also for food control and food safety. As regards to fishery products, identification of species is an important tool to ascertain frauds. In fact, fishery products and olive oil, are subjected to fraud more than any other food [134]. In the last years, there has been an increase in fish and seafood consumption at a national level, also thanks to growing amount of fishery products given by zootechnical sector and international trade. This growth regards both the acquisition of fishery products in a traditional way and new products, for example fillets, slices, fish burger, “ready to cook” breaded products or RTE products. In this contest, fish is not easily identifiable from a phenotypic point of view with the increase of commercial and sanitary frauds. Victims of these kind of frauds can be both consumers and fishery industries. Traditionally, fish species identification is based on morphological features of fish, such as number and position of fins, morphology of scales and other dermal structures, body shape, various measurements of body parts, pigmentation, and colour patterns. In some cases, these morphological features are not useful for identification and differentiation purposes, even with whole specimens, because fish

can show both considerable intraspecific variations and small differences between species. So, the necessity of an excellent authentication of fish and seafood species has led to develop new methods. The new techniques for an efficient identification of fish species are based on separation and characterisation of specific proteins and on deoxyribonucleic acid (DNA) analysis. The methods based on protein analysis use electrophoretic techniques, such as isoelectric focusing (IEF), capillary electrophoresis (CE), high performance liquid chromatography (HPLC) or immunoassay systems, such as Enzyme-Linked Immuno-Sorbent Assay (ELISA) [44, 135, 136]. These methods are generally reliable for use with fresh and frozen tissue but not with other conditions. In fact, proteins lose their biological activity after animal deaths [137]. Moreover, proteins are heat-labile and become irreversibly denatured when the flesh is cooked. In fact, heat processing or drying can destroy the biochemical properties and structural integrity of proteins, making analysis impossible [138]. 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. Furthermore, DNA can provide much more information than proteins; it is due to the degeneracy of the genetic code and the existence of noncoding regions. Besides, DNA is present in all cells of an organism, so it could be retrieved from many substrates. The main DNA-based methods applied to the fish species identification in the last decade are eleven. Some of them, namely PCR-SSCP (Single Strand Conformation Polymorphism), PCR-RAPD (Random Amplified Polymorphic DNA), PCR-DGGE (Denaturing Gradient Gel Electrophoresis), PCR-ALFP (Amplified Fragment Length Polymorphism), and cloning and sequencing, seem to be abandoned today [139]. On the contrary, six techniques are more and more used nowadays; they are PCR-RFLP, PCR-sequencing, PCR-specific primers, Real-time PCR, microarray technology and DNA-barcoding [98].

Some of the methods are discussed briefly in following sections.

6. DNA Barcoding as an effective tool to detect fish fraud

Traditional methods of species identification based on morphological feature is not sufficient when fish is processed into ready to eat breaded or battered products or when fish is converted

into fillet form, or when fish is highly processed in prepared fish meals. Traditional method of species identification is also of very little help when species content of fish feed is assessed or in products such as surimi are consumed. The morphological features of fish such as head, tail and skin which help in identification is lost when it is processed making it difficult to identify by traditional means. Advancement in molecular identification techniques based on DNA like DNA Barcoding and Next Generation Sequencing (NGS) has provided hope for increased transparency in international fish trade and supply chain. Fish traceability is very important to detect fish fraud or implementing regulations about food safety and maintaining high standard of sustainable fishery management. Traceability is also very important to ensure the quality of fish products and minimizing health risk of consumers. Presently most successful traceability method for definitive identification of fish species is DNA Barcoding which is based on short sequences from the mitochondrial cytochrome C oxidase subunit 1 (*COI*) region. After proper standardization and accreditation, it can be used in official food control laboratories as a routine testing technology. Although there is enormous improvement in DNA based molecular detection methods, its routine application in fish fraud detection and traceability is far from being fully established. This is concluded by the joint research Centre of the European Commission on its report on tracing illegal activities in the fishery sector [140].

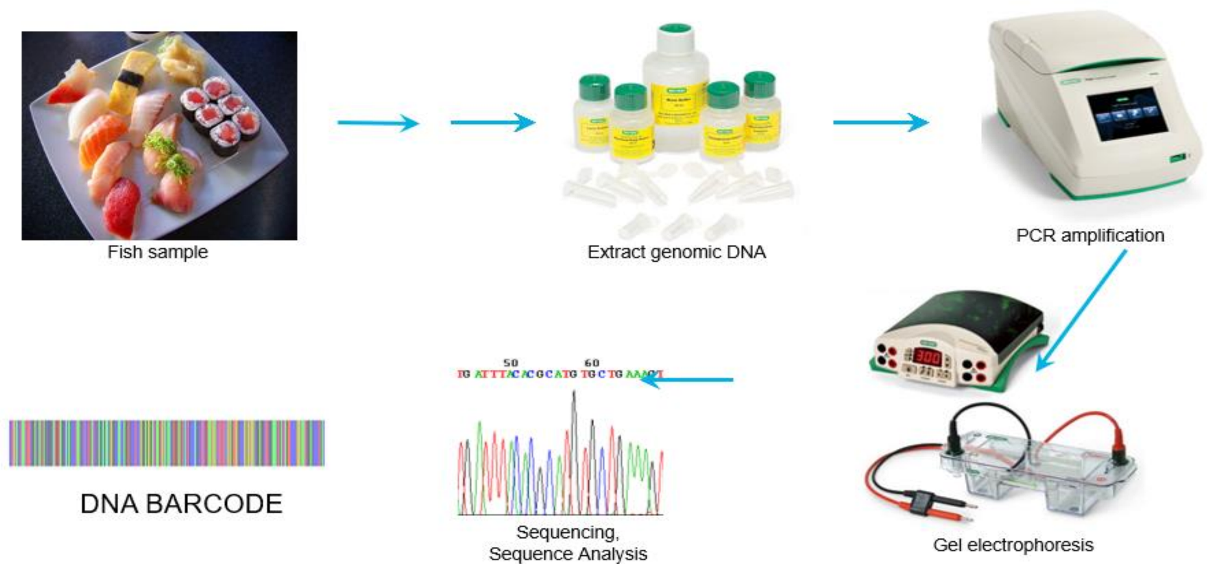
DNA barcoding is urgently needed for authentication and certification of fish products. Importance of this method is demonstrated by Marine Stewardship Council (MSC), by detection of fish products labelled as Patagonian toothfish and marketed as “Chilean sea bass”. Using DNA testing method, 8% of the fish with MSC certification labels were in fact other species [141]. DNA barcoding was also effectively used for identification of processed fins from internationally endangered shark species [142]. In this study DNA minibarcoding approach is successfully implemented for identification of fins in processed shark fin soup. A survey about mislabeling in tuna products was done in Spain for around one year with DNA barcoding approach. Also, they observed that species substitution starts from suppliers with 40% mislabeling in products observed at that level. The mislabeling was increased up to 58% at fish mongers and 62% at restaurants [55]. Now lot of people are using e-commerce platform for purchasing food products where there is more potential of species substitution and mislabeling. An interesting study was

conducted about authentication of fishery products sold at online platform in China; surprisingly they observed that 85% of sample investigated by DNA barcoding were mislabeled [74-76]. For effective food authentication system to combat fish fraud the companies should require a certificate of analysis based on DNA barcoding with every business transaction. The technique must be applied routinely in European meat sectors where laboratory certification granting the authenticity of meat was product was given before it is available to supermarkets and meat processors.

Although DNA barcoding applications using like mt *COI* gene has been quite successful in identification of fish at species level it has certain limitation particular in the identification of geographical origin of fish species. Advanced molecular techniques like NGS and other advanced methods have reduced the limitation to identify the origin or provenance of fish catches. The approaches need further refinement before applying in traditional food control programs. This advanced molecular methods are successfully applied to identify river of origin of wild caught salmon [143]. Fish Pop Trace project funded by European commission have developed advanced methods which can have potential of simultaneous testing of fish species and provenance [144].

DNA barcoding is a rapid and reliable method for identification of fish species for detecting food malpractices and ideal tool for regulating control purposes. Developing countries need technical assistance to integrate this system into their food control programs. Integration of these technologies need technical expertise and advance food laboratory capacity. Standardization and accreditation of these methods is needed and to harmonize DNA database for confirmation of these barcodes. In one article published by Clark *et al.,* in 2015 have explained importance of DNA barcodes for identification of fish and challenges available for integrating this technique into national food control surveillance. Molecular detection of fish species is rapidly developing diagnostic area: a handheld genetic sensor has been quite successfully implemented for identification of grouper and it compared favorably with standard laboratory methods [145].

Figure 1. DNA barcoding Workflow



7. Review of molecular biological methods for fish and crustacean species authentication

7.1. Protein based methods

Although nucleic acid-based methods mostly used for species identification in fish and seafood products, some well executed methods like Isoelectric Focusing (IEF) are still valid due to their simplicity and cost effectiveness. Additionally, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF–MS) applied for species identification in several organism is now widely applied for seafood species identification also.

7.1.1. Isoelectric focusing (IEF)

In IEF proteins are separated in polyacrylamide gel with a pH gradient [146, 147]. After running on polyacrylamide gel proteins are visualized with staining and the verification is done by comparing the pattern of reference species on the same gel [148]. It is an cost effective and simple method for fish and seafood species identification [146]. IEF is used for species identification of 14 commercial important shrimp species of the order Decapoda [146]. Wide variety of fish species were also identified by this method [149, 150]. Although it is successfully applied in several cases it is not a perfect solution for processed seafood due to lack of stability

of some proteins during thermal processing [148]. In case of closely related species, it must be examined that at least one is the target of analysis and produces identical or highly similar band patters [151, 152]. Its application in fish species identification is limited and mostly used as a screening tool for fish species identification [153, 154]. Due to its limited application and quite time-consuming protocol, MALDI TOF MS offers quicker protein-based alternative for species identification which also requires further preparation steps.

7.1.2 Matrix-assisted laser desorption/ionization (MALDI) Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS)

MALDI TOF is quite rapid non- targeted protein-based technology. It works on co-crystallization of the samples (whole cell or extracted protein) on the target plate with an energy absorbing matrix. With assistance of the matrix, a pulsed laser desorbs and ionizes the sample. Furthermore, the charged ions generated from the sample are accelerated by a strong electric field and separated according to charge to mass ration and are measured with time-of-flight mass spectrometry. The generated protein spectra on chromatogram are compared with reference database of proteins for identification of species [155, 156]. The technique is already well established for identification of bacteria and fungi [157-160]. It is also quite successfully executed for authentication of several metazoan species such as insects [156, 161, 162], mammals [163], mollusks [164], fish [165-167] as well crustaceans [168-170]. Although being quite rapid and efficient it has also some limitations as for the several group of animals there is lack of standard protocol and there are limited studies regarding influences of fat content, storage temperature and level of food processing. In 2014, Stephan *et al.,* has used formic acid and chloroform methanol for removal of fats for protein preparation of fresh and frozen tissues for identification and mislabeling of *Placopecten magellanicus* as *Pecten maximus* [164]. In 2013, Salla *et al,* identified skeletal muscle for 6 shrimps to species level irrespective of their storage condition (fresh or frozen) with the application of Trifluoroacetic acid (TFA) for protein preparation [171]. MALDI TOF was also successfully used for discrimination of 11 calanoid copepod species [crustacean] from the tissues fixed in ethanol on different developmental stages without preceding protein preparation [169].

Volta *et al,* 2012 used frozen muscle and liver in formic acid for comparing protein spectra for three fish species (*Alosa agone, Coregonus macrophthalmus* and *Rutilus rutilus*). According to him both tissues were suitable for discrimination of three fish species. In one study proteins are prepared for analysis with TFA from frozen muscle tissues from 25 fish species of the order *Perciformes, Gadiformes* and *Pleuronectiformes*. This protein preparation was successfully used to discrimination genera and also able to differentiate species within the genus *Merluccius* [166]. In another study researcher used protein spectra prepared with TFA of frozen fillets and developed a MALDI TOF MS database of 54 fish species. They also studied the impact of contamination of the fillets with bacterial proteins on identification of fish species [167].

Despite of several applications like all other protein-based methods species identified through MALDI TOF has some limitations that proteins are less thermostable. Additionally, like DNA Barcoding, a comprehensive available reference database is also required for its success, but MALDI TOF display several advantages over IEF, and several other DNA based methods as it requires only few and simple preparation steps along with short analysis time [164, 167, 172].

7.2. DNA-based methods

Nucleic acid-based detection methods offer several advantages over protein-based methods. It is independent of sample origin as all cells of the organism contains DNA and they are also independent of developmental stage of organism (from eggs to adults). They provide higher information content (down even to population) and they are more suitable for detection of processed samples due to higher thermal stability of nucleic acid compared to proteins [139, 160, 173]. Therefore, most of the analytical methods used for species identification in seafood is based on DNA, in particular PCR –RFLP and DNA sequencing-based methods (like DNA barcoding). Additionally, loop-mediated isothermal amplification (LAMP) is also providing an alternative approach for species detection in several organisms.

7.2.1. PCR-RFLP

Endonucleases are enzyme which recognizes specific restriction site of an amplified fragment and digest them into smaller fragments of different size and length. PCR-RFLP is based on several endonucleases. These smaller fragments can be separated by gel electrophoresis and visualized

by staining [139, 174]. PCR RFLP is simple method and quite easier to execute and it is cheaper than DNA Sequencing based methods [175, 176]. PCR-RFLP methods was successfully used for detection of 17 prawn and shrimp species by targeting 16S ribosomal RNA /tRNA [177]; similar approach was also used for authentication of commercially important shrimp species in India [160]. PCR-RFLP method is also used for fish species identification. It was used for detection of flat fish species [175, 178], and authentication of Snappers [179], and eel species based on the *cytb* or the 16S ribosomal RNA [180-182]. Despite of several applications PCR- RFLP is not error free due to intraspecific variability and incomplete enzymatic digestion, it also does not provide sample information as provided by Sequencing methods.

7.2.2. **Species Specific PCR**

Species specific PCR is a targeted approach which implies species specific PCR primers for amplifying regions of DNA from particular species [148]. For making species specific PCR assay nucleic acid sequences of various species are compared for determination of nucleotide regions that varies among the species. With this information primers were designed to bind to DNA from the target species only amplify it using PCR [183]. The amplicons can be detected using gel electrophoresis. In many cases multiple sets of species-specific fragments of different length were combined in a multiplex PCR approach which targets species specific fragments of different length which can separated using gel electrophoresis. This approach is used to detect several seafood species including shrimp [184], weakfish (*Cynoscion acoupa*) [185], croaker (*Cynoscion leiarchus* and *Plagioscion squamosissimus*) [68], gemfish (*Ruvettus pretiosus* and *Lepidocybium flavobrunneum*) [186], anglerfish (*Lophius* spp.) [187] and tilefish (*Branchiostegus japonicus* and *Branchiostegus albus*) [188]. Species specific PCR is also used for sensitivity testing and identification of yellow drum (*Nibea albiflora*) in in mixed fresh surimi products with detection limit of less than 0.5% [189].

Species specific PCR is more useful than PCR RFLP because it is more quick, need less material and allow simplified detection [190]. It is also used for testing of mixed species products because short generic region of different lengths can be targeted [191]. The drawback includes need of developing specific primers for each target species which may be time consuming and the limited

range of species that can be detected with a given assay. Several methods rely on species specific primers including RT PCR and isothermal amplification methods Loop mediated Isothermal Amplification (LAMP), high resolution melting (HRM).

7.2.3. Real Time PCR

RT PCR is quite successful method for quick and specific detection of seafood species [192]. The method involves the combination of species-specific PCR with addition of fluorescent technology that enables detection of species as amplification is progressing. The most common fluorescence dye used with real time PCR is SYBR green which binds to small grooves of dsDNA [192, 193]. Another more specific fluorescent technology involves use of species-specific probes like Taq Man probes which binds to specific region of DNA and hydrolyzed during PCR amplification. In Taq Man approach probes are clubbed with reporter fluorescent dye at 5' end of quencher dye at the 3'end. In the presence of target sequence probes got cleaved during amplification releasing fluorescence signal which is further analyzed and compared to standards [176]. The advancement in technology in development of specialized products have made further improvement in the specificity of probes for use in RT PCR. Some approaches like incorporation of minor grove binder of (MGB) or locked nucleic acid are developed to increase melting temperatures which leads to shortening of primers and displaying more effective sequence specificity [194].

RT PCR has been widely applied for authentication of seafood species [195-197]. Most of these assays target mitochondrial gene including 16s rRNA, cytochrome c oxidase subunit 1 (*COI*) and cytochrome b (*cytB*). A RT PCR assay was developed targeting the *16S rRNA* which successfully differentiated Nile and wreck fish from grouper species [198]. There are several other studies in which RT PCR was used targeting 16 S ribosomal RNA for authentication of seafood species containing cod, sole, pollock, hake [39, 195]. Real time assay based on *COI* gene was applied for differentiation of numerous fish species including cod, salmon, trout, swordfish, and grouper [190, 199-201]. RT PCR based on *cytB* gene has been successfully applied for species detection in tuna, including big eye tuna and yellowfin tuna [202, 203]. The same approach was used for identification of crab, ling, tilapia, pollock and cod [204, 205]. Recently, Hulley *et a*l.,

developed and validated primer sets corresponding to smallmouth bass *(Micropterus dolomieu)* and *round whitefish (Prosopium cylindraceum)*, having detection limit of 0.03 and 0.1-1.0 ng of DNA respectively [196].

The advantage associated with RT PCR is that it allows rapid identification of seafood species because fluorescence can be detected in real time and no *post* PCR sample processing steps like gel electrophoresis is required. Additional advantage include greater sensitivity compared to conventional PCR, minimum laboratory preparation requirement and computer generated results [174]. The assay target short DNA fragments (<300 bp) and is effective with variety of seafood products including those which are highly processed and contain mixture of species. This method has potential for large scale implementation within the seafood supply chain with portable on site instrumentation and analysis [183]. Sometimes these assays are difficult to develop particularly in multiplex approach also the use of specific fluorescent probes cab be costly. Also, the high sensitivity of the assay increases the potential for false positive resulting from cross contamination of samples and or processing equipment.

7.2.4. **High resolution melting curve (HRM)**

In this method there is amplification of the target gene with subsequent melting of amplicon. It is done in the presence of saturation dye which enables the discrimination of small sequence variation among target sequences. The melting temperature (Tm) of nucleic acid is influenced by the sequence as well as number of guanine and cytosine (GC) content of the nucleotide. The main specificity which makes HRM a better choice is that it can be performed in real time in a close tube allowing for rapid detection of even single base variants and detection of small insertions or deletions. The exact difference at sequence is not determined, but if needed the amplicon can be subsequently sequenced and differences can be identified. HRM is quite successful in differentiation of pathogens, spoilage microorganism, to discriminate among genetically modified organism [206, 207] and detection of food allergens [208]. It is quite effective in the field of adulteration and authenticity checks and has got lot of attention for that as economic consequence of adulteration is quite high.

Tomas *et.al,* 2017 has successfully discriminated the more valuable Atlantic cod (*Gadus morhua*) with commonly less valuable species pacific cod (*Gadus macrocephalus*) by application of short amplicon HRM Analysis [209]. Similar approaches were also successful for other fishes [210], and for several plant species and their replacement and/or adulteration with closely related ones. Other application includes detection of putatively toxic species [211, 212] and also wine [213]. Subsequently HRM has played successful role in safeguarding the quality designation of European Union, e.g., the "protected designation of origin (PDO) or the "protected geographical indication" [211].

Three molecular approaches, DNA Barcoding, SNP and HRM has provided powerful methods for screening and discrimination among closely related species. HRM offers several advantages for species discrimination as it can provide very accurate results and it is quite fast, reliable, cheap and one of the high throughputs even after DNA is degraded. In case of products containing mixture of species, although it cannot provide a response in terms of identification, it can elucidate differences among samples. In case of product having mixed samples or complex samples the most useful method is NGS which seems to be analytical solution of future and can be valuable tool in the case of food fraud detection.

7.2.5. DNA Microarrays

Microarray technology is not very popular choice for species identification and till now very few studies are reported when it is used for such purpose like when specimen is in poor condition or comprised of very limited material. Marine organism and their complex development stages are quite cumbersome to identify by morphological features and nucleic acid-based methods provide powerful options. DNA microarrays have been used as potential technology for the identification of fish species with the use of three mitochondrial genes *16 S rRNA* (16s), *cytochrome b* (*cytB*) and *cytochrome oxidase subunit I* (*COI*) for the identification of fish species by combining techniques of DNA barcoding and microarrays [214, 215]. It is evident that mainly *16 S rRNA* gene is suitable for designing oligonucleotide probes [215]. Recently a less expensive modification in microarray was done. The technique is like earlier, but the spots are larger (about 1mm as compared to 250 µ). It can be visually interpreted without a microscope and radioisotope

as well as fluorescent label can also be used for the same. The technology provides useful alternative to isoelectric focusing, 2-dimensional gel electrophoresis, protein capillary electrophoresis, HPLC and ELISA. One limitation with protein based analytical tools in species identification is the susceptibility to protein denaturation due to physical, chemical, and industrial treatments. Compared to other non-protein based bimolecular methods liked peptide bimolecular mass spectrometry and low lipid based biomolecules, low cost density (LCD) microarrays displayed higher sensitivity and are not affected by cooking steps [216] .

7.2.6. Sanger Sequencing

DNA barcoding and Forensically Informative Nucleotide Sequencing (FINS) are the two major sequencing based methods applied for seafood identification [217]. Both techniques depend on Sanger sequencing of a standardized gene target which display little divergence within species and higher variation between species. These methods need reference database which contains sequences from specimen that can be used comparing to the sequences isolated from unknown organism which enable species identification. DNA barcoding method is highly informative, reliable widely and successful [101, 218-220]. In 2011 USFDA published and implemented DNA barcoding protocol for the regulatory identification of fish species [221].

The main region targeted by sequencing-based identification methods (DNA Barcoding) are mitochondrial cytochrome b and cytochrome oxidase subunit 1 (*COI*). For FINS, cytochrome b is mostly used while for DNA barcoding *COI* is preferred [217]. For identification of species in seafood mitochondrial DNA is preferred due to high mutation rates and availability of multiple copies [138]. Nuclear DNA is also recognized species identification marker which is used instead or in conjugation with mitochondrial DNA when hybridization and introgression occurs within specific seafood and fish population. In one study intron less nuclear rhodopsin gene has been successfully used in combination with mitochondrial for targeting tuna imported in Indonesia [222].

Regions of DNA or barcodes which are 400-700 base pairs in length (bp) in length are targeted by sequencing-based methods for identification of species in seafood products. However, in processed fish products the integrity of DNA is reduced which can present challenges

for species identification. In case of processed fish products shorter region of DNA (150-350 bp) also called "mini barcode" are used [223, 224]. These mini-barcode regions are quite successfully utilized in several researches and even applied for cooking methods [225]. Although DNA barcoding based on sequencing methods is quite successful it has certain limitation, it is unable to simultaneously identify multiple species in seafood products, sometime useless or expensive and time-consuming cloning method included. In case of product containing mixed fish species, alternative method such as RTPCR and high throughput sequencing (HTS) may be utilized.

A limitation for DNA base identification method is dependence on reference database for sequence information. Although public database contains a large amount of sequence information there are sometimes incomplete reference libraries for a given species and errors such as misidentification of a specimen.

7.2.6.1. COI gene

Animal mitochondrial DNA (mtDNA) contains one major noncoding region, thirteen protein-coding genes, twenty-two genes coding for transfer ribonucleic acid (tRNA), and two genes coding for ribosomal RNA (rRNA) [226]. This presents many advantages. Mainly, mtDNA is small, easy to extract and does not undergo genetic recombination [226-228]. The mtDNA is much expanded for using as a marker in species identification. The genes such as *D-loop*, *cytochrome b* and *cytochrome c oxidase* are more usual for this aim [229]. Cytochrome *c* oxidase subunit 1 (*COI*) DNA size is around 1548 bp which has 70.2% total T content [230]. In addition, *COI* encodes Cytochrome *c* oxidase (*COX*), the terminal complex of the mitochondrial respiratory chain, which catalyses the electron transfer from reduced cytochrome *c* to oxygen [231]. There are three different subunits of cytochrome *c* oxidase, *COI, COII* and *COIII*. *COI* is the largest one and the most conserved among them. For these reasons, *COI* is one of the most used gene for species identification on some of the search engines such as GenBank BLAST and BOLD [232]. *COI* gene has been used for the identification of many fish species, such as Alaska skates (*Amblyraja, Bathyraja* and *Raja* spp.) [233], Indian sciaenids (*Otolithes cuvieri, Otolithes ruber, Johnius dussumieri, Johnius elongatus, Johnieops vogleri, Otolithoides biauritus* and *Protonibea diacanthus*) [234], 17 members of the family *Scombridae* common to the western Atlantic Ocean

[235], 58 seafood samples in Italy [236], scombrid juvenile in an area off the Kona Coast of Hawaii Island [235] and tuna species (genus *Thunnus*) [237].

Cytochrome subunit oxidase 1 (*COI)* is most widely used genetic marker for identification of seafood species and mostly used in DNA barcoding methodology [217]. DNA barcodes used for species identification are short standardized nucleic acid (DNA) sequence which are relatively conserved within species and display divergence between the species. The 650 bp region of *COI* is considered as standard DNA barcode used for species identification in animal species including fish. DNA barcoding for seafood is assisted by large searchable reference database of *COI* barcode data collected through FISH BOL initiative, which contain sequences from wide range of fish species [183]. DNA barcoding by using *COI* gene is quite successful in fish differentiation with unambiguous detection of 93% of freshwater species and 98% of marine species [173].

COI gene is used in many countries for species identification of fish like salmon, halibut, sole, cod, hake and many more [71, 73, 78, 89, 105, 238]. It was used for detection of fish fraud (mislabeling) in Canada in which they observed 41% mislabeling sold products. The most common mislabeled species are snapper, seabream, and cod. In other DNA barcoding study with the help of *COI* in Malaysia, 16% mislabeling detected with the observed mislabeled species included are mackerel and walleye Pollock [73]. The *COI* gene was also applied to detect mislabeling of seafood species in South Africa where 18% mislabeling detected containing seabass, yellowfin tuna, seabream and barracuda [238].

Although 650 bp *COI* region was effectively used in DNA barcoding for species identification in many fish species, it has limitation with highly processed fish products where there are more chances of DNA degradation [223]. To solve this, mini barcode which target <350 bp region of *COI* was tested in various seafood authentication studies [239]. Shokralla *et al.,* used DNA mini-barcode targeting 127-314 bp fragments of *COI* for species identification of fish in processed products [223]. The approach was quite successful in species identification (93%) for a combination of six mini barcodes and a maximum success rate of 88.6% for an individual primer set (SH-E). In an interesting study when assessment of various cooking temperature on fish

species identification was done the mini SH-E primer set allowed an overall success rate of 92% compared to 90% with full barcoding [225].

7.2.6.2. *Cytochrome b*

Beside *COI* another mitochondrial gene *cytB* is highly successful for species identification in various seafood species [217]. Generally a 464 bp region *cytB* is commonly used for assigning a set of universal primers which amplified across a range of seafood species although shorter region of the gene are also successfully targeted [217]. Various studies are conducted across the world to identify fish species using the *cytB* gene including cod, grouper, halibut, sole, hake and many more [124, 151, 152, 240]. Several other studies combined *cytB* with other molecular marker such as *COI, 16S rRNA* and mitochondrial control region. Filonzi *et, al*. 2010 identified mislabeling in many commercial fish products sold in Italy through sequencing of a 360 bp region of *cytB* gene combined with 600 bp region of *COI* [124]. Mislabeling was detected in 32 % of the products with common mislabeled species are tilapia substituted with grouper, flounder replaced with sole, and haddock replaced with Atlantic cod. In Germany substitution of common sole in restaurant was studied with application of 413 bp fragments of the *cytB* gene with supplemental investigation by application of *COI* and *16S rRNA* [152]. Mislabeling observed in in this study was quite high (57%) in products sold as common sole with replacement found with flatfish and catfish. Sotelo *et.al.,* in 2018 used *cytB* (176-464bp) in combination with *COI* (650 bp) and mitochondrial control region (150-450 bp) for species identification in various tuna products collected from 6 European countries [241]. Overall, 6.79% of mislabeling was observed in various tuna products including fresh, frozen, canned, and other forms (dried, roe). The most mislabeling in fresh and frozen samples observed in Bluefin tuna substituted with big eye tuna or yellowfin**.**

7.2.6.3. *16S r RNA*

Despite lower rate of divergence than *COI* and *cytB, 16S r RNA* gene is quite effective tool for species identification [239]. Gene coding for ribosomal RNA like 16S rRNA RNA contains specific insertions and deletion that can impact the alignment of sequences [242]. Compared to *COI* and *cytB* fewer studies are done with *16S rRNA* as gene for species identification in seafood species [198, 243, 244]. Species identification analysis in seafood species was conducted by Di Finizio *et*

al., in which they differentiated Walleye Pollock, cod, whiting and Ling. Other application in which 16S rRNA marker was differentiated successfully grouper from perch and ocean perch from redfish [198, 245]. Species differentiation of several abalone species with *16S rRNA* marker was done by Klingbunga *et al.,* in 2003 which are able to differentiate three species [244]. Application of *16S rRNA* for seafood identification was also implemented to canned pet food in which universal primers targeting 118-213 bp fragments of *16S r RNA* genomes were produced relating to various families of fish [239]. In summary around 80% of the samples were identified at species level while remaining 20% samples could only be identified at genus level.

Table 4. Comparison of various DNA-based methods used for seafood authentication

Method	**Brief description**	**Advantages**	**Disadvantages**
PCR-RFLP	PCR with universal primers followed by restriction enzyme digest an	Relatively inexpensive; well established; minimal equipment required	Difficult for multi-species authentication; time consuming due to post-PCR step
Species-specific PCR	PCR with species-specific primers followed by electrophoresis	Relatively simple and rapid; primers can be multiplexed; minimal equipment required	Use of separate primers for each species targeted; potential for cross-reactivity
Microarrays	PCR with universal primers followed by hybridization to species-specific probes on a microarray	Allows for multi-species identification	Complex to develop; multiple post-PCR steps required; requires advanced instrumentation, costly
Real-time PCR	PCR with species-specific primers and use of fluorescence for detection in real-time	Rapid technique with no post-PCR steps; highly sensitive; primers can be multiplexed; some instruments are portable	Use of separate primers for each species targeted; potential for cross-reactivity; probes can be expensive; requires specific instrumentation
PCR-ELISA	PCR with species-specific primers followed by hybridization to species specific probes and enzymatic detection	Relatively sensitive; minimal equipment required	Requires multiple post-PCR steps; potential for cross contamination

High-resolution melting (HRM)	PCR with universal primers and dsDNA-binding dyes followed by melt curve analysis	Relatively simple procedure; minimal post-PCR analysis time, low cost	Cannot differentiate species with minimal genetic differences; requires real-time PCR instrumentation with HRM capabilities
Droplet digital PCR	PCR carried out in separate droplets with universal primers and species-specific probes	Allows for quantification; does not require post-PCR steps; minimal amplification bias	Requires specific instrumentation and design of species-specific probes
Isothermal amplification (LAMP)	Isothermal amplification with species-specific primers followed by detection with various methods	Rapid; simple to perform; relatively portable	Requires development of specific primers; rapid detection with fluorescence increases the cost of assay
Sanger sequencing	PCR with universal primers followed by cycle sequencing and analysis	Detection of a broad range of species; high information content	Time-consuming and labor-intensive procedure; cannot identify multiple species in a sample; requires advanced instrumentation, costly
High-throughput sequencing	Template preparation followed by sequencing of multiple DN	Information-rich; in-depth sequencing	Requires advanced instrumentation; complex data analysis; time consuming and labor intensive, relatively costly

*The table was modified from Silva *et, al*. 2021 [98]

8. Emerging DNA based methods for Seafood authentication

There are many other techniques used for species identification in seafood which includes RAPD, PCR-SSCP, and AFLP. These techniques are rarely used for seafood species identification now. Some Emerging methods are now in much demand.

8.1. Droplet Digital PCR

Droplet digital PCR (ddPCR) is an advanced method for quantification of target DNA molecule in the given sample- The method contains a PCR, mixture distributed in around 20,000 droplets

by use of droplet generator. Here every droplet work as an independent PCR [246]. A fluorescent hydrolysis probe specific for the target species are used to confirm amplicons within each droplet [247]. After the confirmation of amplicons, the number of positive and negative reactions are recorded. The concentration of target DNA are measured from the ratio of positive reactions to the total number of reactions analyzed [248],

Till now very few studies are conducted quantification analysis of fish species in seafood products with application of ddPCR. In 2019 the ,method was applied for quantification of walleye pollock in surimi-based products, The method was successful to identify the proportion of pollock in 60% of commercial samples tested [246], Other studies in which ddPCR was applied for identification of fish including silver pomfret, , crab, crayfish and lobster[249, 250]. Cao *et, al.* in 2020 used this method for identification of silver pomfret in varying amounts with golden pompano (*Trachinotus ovatus*) and observed sensitivity limit of 0.1% [249]. The method is also successfully used for eDNA testing of fish in applications like estimating diversity of fish species and its biomass. In summary ddPCR is an accurate and effective method not influenced by amplification efficiency bias and does not require post PCR laboratory steps. One disadvantage associated with ddPCR is that the method required dedicated instrumentation for carrying out ddPCR and development of species-specific probe which can be labor intensive.

8.2. PCR ELISA

PCR ELISA is a technique involving immunodetection assay targeting DNA. In this method amplification with species specific primers are performed and labelling of sample DNA with digoxigenin. The reaction is followed by the hybridization of the amplified labelled DNA with specific probes. The amplicon was detected by anti-DIG-peroxidase conjugate which develop a blue green color visible by eye or measured using spectrophotometer [251]. The method is used for identification of cod, Nile perch, grouper, ling and tuna species [44, 252, 253]. In 2014, a species-specific PCR-ELISA assay was developed for the differentiation of Alaska pollock, ling and Atlantic cod considering *cytB* and *ND4* genes. This is highly sensitive method, it offers 100 fold greater sensitivity compared to the traditional gel based methods detecting as low as 123pg of DNA template [253]. PCR -ELISA offers several advantages as it is applicable to fresh, frozen or

processed products and requirements of minimum equipment for testing [253], It can rapidly and effectively measure results and visually allows for rapid throughput and overall analysis. The drawback associated with this method are time consuming procedures, risk of contamination and cost associated with material and equipment [254].

8.3. PCR-Lateral Flow Dipstick Assay (LFDA)

PCR-Lateral Flow Dipstick Assay (LFDA) is almost equivalent to PCR-ELISA but the PCR products were identified with dipstick in place of fluorescence [255]. There are 2 steps involved in this method. In step one a specific DNA fragment was amplified with labels such as biotin and 6-FAM. In next step PCR products are detected using a dipstick which are available commercially with variety of reagents and includes three zones, the sample application zone, the test zone, and the control zone. Gold nanoparticles are linked with antibodies in sample application zone which recognizes 6-FAM in the labelled DNA molecule. When this is moved to test zone, they are captured by the biotin ligand. Finally the control zone contains antibodies which identify the anti-6-FAM present in the nanoparticles [255]. The results can be seen with under 5 minutes of loading the PCR products into the dipsticks. Despite of huge potential for seafood identification through this technique very few studies are conducted till now [256].

PCR-LFDA is used to differentiate four common species in seafood products which contains Atlantic cod, Pacific cod, walleye pollock, and ling [256]. The method was able to differentiate all 31 seafood samples and displayed a mislabeling rate of 22-6%. It also identified multiple species in given sample which was hard to detect by sanger sequencing. The rapid analysis time and simplified work flow make it suitable for species identification [256], although its limitation is that it relies on species-specific primers and visual analysis can produce different results depending on the observers.

8.4. High-throughput sequencing (HTS)

The steps involved in NGS and HTS platform includes combination of template preparation ,sequencing and imaging and genome alignment and assembly methods [257]. HTS technologies can sequence multiple DNA molecule in parallel, allowing for hundreds of millions of DNA molecules to be sequenced in one run. These methods are quite different for the identification

and differentiation of species as it provides information -rich, in-depth testing option [151]. HTS method provide edge over traditional sanger sequencing methods due to the ability to acquire a large amount of data. In this method whole genome of various organism can be analyzed to provide important information related to authentication of several species. Various HTS method such as pyrosequencing, illumine, Ion Torrent was used for species identification in seafood products [103, 258, 259].

8.4.1. Pyrosequencing

Pyrosequencing which was discovered in 2005 was the first HTS technology which provide ligand-based detection after the release of pyrophosphate from DNA. After release of pyrophosphate specific peaks that relate to a single base pair [258]. In one study 25 whole and processed fish samples were analyzed targeting the 16 S r RNA gene with pyrosequencing with 80% success rate. Species detected include Northern rock sole (*Lepidopsetta polyxystra*) and European anchovy (*Engraulis encrasicolus*). The method is also used successfully for species identification in bivalves, applying both the 16S rRNA and *COI* mitochondrial regions [260]. In this study 15 different bivalve species were successfully differentiated. The advantage of this method are its long reads (1kb max) which is easy to map along with relatively faster run time (23h) [261]. The disadvantage includes low read count (∼200,000) and costly equipment needed for testing.

8.4.2. Illumina

Illumina, a big success in sequencing-based studies which was introduced in 2006. It involves immobilizing DNA fragments on a glass chip for PCR amplification [259]. The method contains a flow cell having a library of DNA fragments followed by followed by amplification of sequence clusters [151]. Illumina offers the highest throughput per run and the lowest cost per base in comparison to Sanger sequencing. HiSeq X Ten is the most advanced instrument Illumina offers. It has potential to generate up to 1.8 Terabytes of sequence per run [261]. Illumina also offers Mi seq system for benchtop analysis having capacity to produce 25 million reads of up to 600bp each [261]. In 2017, Kappel et al used Illumina MiSeq for analyzing nine known tuna fish mixtures varying from one to three species with the *cytB* marker segments BDR (131 bp) and BMID (126 bp). Overrepresentation of *skipjack tuna* compared to the *Thunnus* species was reported in this

study. They also observed BDR having a higher average read number (372,363) compared to BMID (359,103). They were able to detect mixtures as low as 1% tissue of a specific species, The major advantage with Illumina include high-throughput and low per-base cost, while disadvantages of this method include sample loading, including poor data resulting from overloading [261].

8.4.3. Ion torrent-personal genomic machine

Makers of pyrosequencing platform also introduced ion torrent sequencing machine to increase the efficiency of HTS analysis [261]. It involves detection of hydrogen ion released from DNA after polymerization with the help of semiconductor chip. It differs from Illumina and Roche pyrosequencing methods as this platform does not require optical detection of incorporated nucleotides culminating in higher speed, lower cost, and smaller instrument size [205, 262]. The method is very effective in detection of species in mixed fish products such as fish ball, fish cakes, cooked meals, and cod cakes [103]. In one study this method was used targeting both *cytB* and *COI* genes for detection of fish species in mixed products. Mislabeling in 4 out of 22 fish products were reported and in 23% products more than one species detected [103]. In another study 37% mislabeling rate was found after analysis of several fish based products originating from Spain, Germany, France, and China [262]. Faster run time (∼2–5h) and broad range of applications makes it more successful and also does not require optical scanning or fluorescent nucleotides [261]. However, the major disadvantage is the presence of high error rates in homopolymers, which are sequences of consecutive identical bases. Since homopolymers are prone to insertion and deletion mutations, erroneous over- and under-calls can happen during sequencing analysis and produce poor results [263].

8.5. Metabarcoding

DNA barcoding involves amplification of certain Nucleic acid (DNA) region by PCR followed by sequencing of the amplified amplicons [242, 264]. The species identification is performed by comparing the obtained sequences to a reference database for fish species. Fish barcode of life campaign (FISH BOL) is an initiative for species identification of fish which already complied the specific sequence region of the *COI* gene for more than 10 0000 specimen representing more than 10000 species[173]. Sanger sequencing is the main arsenal for DNA barcoding, but it does

not produce a reliable output when the sequencing reaction contains a mixture of different DNA. To overcome this limitation other sequencing platforms are needed for simultaneous identification of multiple species in a complex sample. Other approaches are also triad like cloning individual PCR fragments for separate sanger sequencing [72, 91, 265].

Another alternative is use of NGS which has the capacity to sequence thousands of thousands DNA strands in parallel. This advantage provides these techniques outstanding sensitivity allowing evaluation of the fish diversity in different environment by collecting, amplifying and sequencing environmental DNA (eDNA) [266, 267].

Several techniques were currently applied to control fish fraud along the supply chain using adaptation of the mitochondrial target used for traditional barcoding [72, 223, 239]. An important modification contains trimming the length of amplified region used as barcode to accommodate one of the limitations of NGS which can read much shorter sequences compared to sanger sequencing method. This limitation can cause loss of discriminating power specially for closely related species [239]. For more efficiency additional barcode candidate in regions with different evolution history are needed to broaden the specificity of barcoding regions.

8.6. Nanopore Sequencing

Nanopore Sequencing (NGS) is new milestone in genomic research. Due to its small size and low cost the oxford MinION sequencing is drawing growing interest in the genomic community especially for surveillance of pathogens and clinical diagnostic due to real time nature of this sequencing platform. There are several applications of Nanopore for barcoding. Species identification include-

- Nucleic acid (DNA/RNA) in remote locations
- Specific identification and molecular evolution having environmental importance e.g., new nitrogen fixing bacteria.
- Metagenomics analysis of complex samples which include environmental or biological samples. Examples are infection control and pathogen detection, microbiome analysis, study of relative abundance.

- Taxonomy classification and relative abundance studies.

NGS is quite efficient in detecting viruses, bacteria, and parasites in clinical samples and in hospital environment [268-273]. Sequencing of such pathogenic microorganism help in identification and surveillance of host pathogens interactions, study of diagnostic targets, response to vaccine and evolution of pathogens [273]. Nanopore MinION is arsenal in diagnostic area which provide substantial advantage in read length, portability, and time to pathogen identification. Its clinical application is already proved in study of Chikungunya virus [268], Hepatitis C virus [268], Salmonella enterica [272], Salmonella Typhimurium [274] as well as study of antibiotic resistance gene [275]. Although Nanopore sequencing is quite successful for identification of bacteria or virus species, its application in study of eukaryotic species is not much explored.

The limitation of NGS is that the data generated currently have single read accuracy of around 92% [276] and the current algorithm are not equipped for handling long sequencing data with error rates above a few percent. The method for improving long read sequences produced by Nanopore are ongoing [277]. Goodwin et al, demonstrated how single molecule long read data generated by the oxford MinION can successfully use to compliment short read data for creating highly continuous genome assemblies in case of small eukaryotic genome (Saccharomyces cerevisiae) [277]). Recently there is lot of improvement in read length, base modification detection, base-call accuracies, and throughput for Nanopore sequencing which is likely to continue further.

8.7. Deep learning

This is growing field where the potential of machine learning algorithm to scan and learn from image and video collections to automatically identify the species when presented with the image of unknown organism is used. There are ongoing efforts in the development of cross language information retrieval tool including imageCLEF and lifeCLEF [278]. These methods are not intrusive, it need only the availability of media and internet connection to analyses image of whole, eventually live fishes. In one study recognition of fish species was done from underwater video sequences, even in very bad light and water conditions [279]. The accuracy of this method

was assumed to be between 90% and 95%. There are more efforts to improve it further with possibility to increase the performance by color information and more sophisticated algorithm like better and accurate loss functions.

Another study proposed a technique for image regeneration of underwater images dependent on Faster R-CNN (Region-based convolutional Neural Network) running on state of art hardware GPU NVIDIA Tesla K20 [280]. The results were promising in terms on speed and accuracy.

Other approaches were identification of fish out of sea such as in fish markets or inside fishing vessels. A smartphone app was developed for taking picture of fish which can be sent to cloud server for image processing and recognitions [281]. The result will be available directly to the smartphone. Further improvement can be done with the possibility of recognizing the image using a database without the requirement to send a request to the cloud server.

Other more intensive or intrusive approaches were also used, identification of fish species based on the morphological features of the otolith outline counter which are part of the inner ear of fishes [282]. In deep learning approaches the algorithm learns the unique shape of different species, depending on the tracing and testing image sects, achieves the identification of different species and fishes. The accuracy of the automated techniques is between 92% to 96% which may increase with bigger training sets.

Figure 2. DNA based methods for Seafood authentication

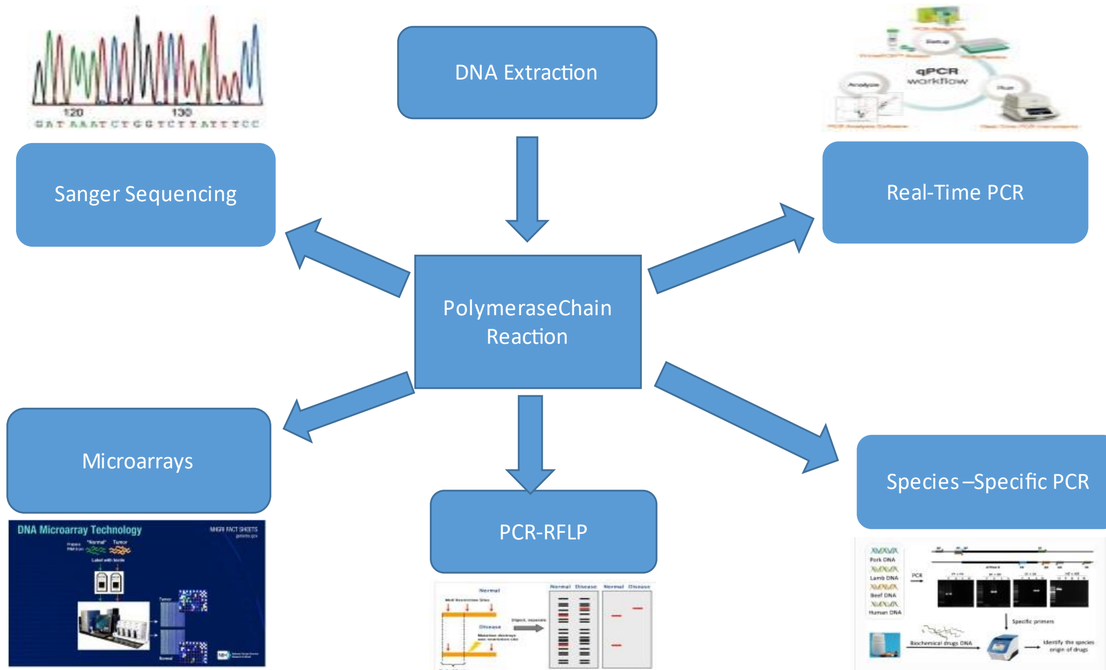
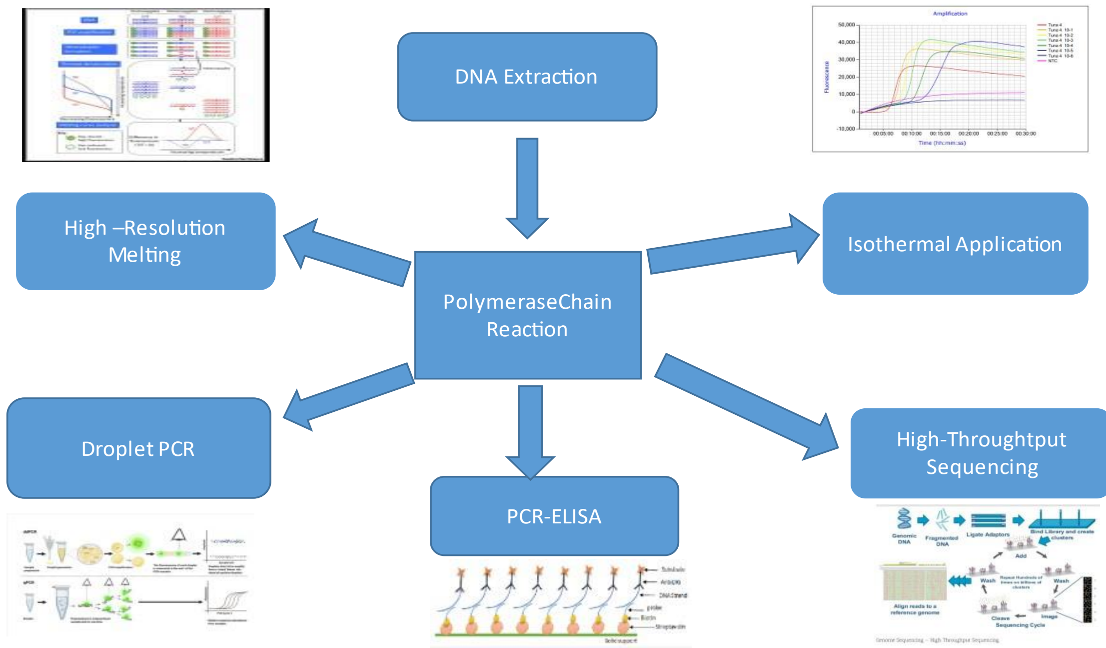


Figure 3. Emerging DNA based methods for Seafood authentication



9. Aim of the thesis

Globalization of food supply chains has led to an increased uncertainty of the origin and safety of fish-based products. Barcoding can be used to validate the labelling of products and to trace their origin. “Fish fraud” has been discovered across the globe. Barcoding can also trace fish species as there can be human health hazards related to consumption of fish.

The study evaluated the applicability of the mitochondrial genes cytochrome b (*cytB*), and cytochrome oxidase subunit I (*COI*) for the identification of fish and processed fish product by “DNA barcoding". In the study, universal primers for mitochondrial *cytB* were used to discriminate fish species in raw and processed forms. The barcode primers were cross tested against collected raw fish species and processed fish product. For this project different varieties of fish samples were collected from different supermarket and of different companies. DNA was isolated from all samples and amplified by PCR; the most intense amplified product was chosen for Sanger Sequencing. After sequencing, the sequences were matched with NCBI BLAST and FISH BOL. After obtaining the results species were identified and matched with the labelling of the products. The final objective of the study was to apply the developed protocols for quality and safety purposes in the field of food control.

Another objective of the thesis was development of loop-mediated isothermal amplification (LAMP) assay for rapid and direct screening of yellowfin tuna (*Thunnus albacares*) in commercial fish products. This is discussed in chapter2.

10.Materials and methods

10.1. Sample collection

During 2019 – 2021 a total of 91 samples of fish products from national and international brand were collected from retail stores in Apulia region (Southern Italy). All samples (fishes, cephalopods, and crustaceans) belonged to different manufacturing lots, included fresh (raw), freezing, or processed. From each sample 25 g of tissue were stored at -20°C until the analysis.

The samples include fillets, fresh fish cubes, canned samples, flavoring products, fish burgers, fish sandwiches, nuggets, and sushi (Table 6).

10.2. Isolation and DNA

DNA extraction was done with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). 20-30 mg tissue was taken from each sample and transferred to 1.5 mL Eppendorf tube. Lysis of tissue was done with lysis buffer supplied with kit. After incubation and two washing steps DNA was eluted using AE elution buffer (Qiagen, Hilden, Germany). The purity of extracted DNA was checked by spectrophotometer measurements using Nanodrop (Thermoficher scientific USA). Extracted DNA were stored at 4 °C for PCR amplification.

10.3. PCR amplification

Primers are strands of short nucleic acid sequences (generally about 20 base pairs) that serve as a starting point for DNA synthesis. In order to generate target DNA amplicons, visualised by agarose gel electrophoresis, primers need to match the beginning and the end of the DNA fragment to be amplified. Reaction occurs under suitable stringent conditions, involving DNA polymerase, specific *Tm* (melting temperature) and DNA extracted from a given species. Besides, to preclude the possibility of false positive or negative results, appropriate controls should be included (the lack of amplified fragment on the gel may be due to technical problems rather than due to the absence of the target DNA). For the simultaneous amplification of many targets of interest, Multiplex-PCR and DNA barcoding is often performed using universal or more than one pair of primers in one reaction tube.

This DNA-based technique has been applied to the identification of numerous species of fish and seafood, including gadoids [283, 284], flatfish [178, 285], salmonids [286, 287], swordfish [288], scombroids [289], sardines and anchovies [290, 291], eels [289], mollusks [244, 292], tuna [293] and many more.

The universal primers targeting cytochrome oxidase subunit I and cytochrome b were used for nucleic acid amplification. The primers are based on cytochrome c oxidase subunit 1 (*CO1*) targeting ~700 bp region and cytochrome b targeting 359 bp region of mitochondrial DNA (Table 5). PCR amplification was performed with a 25µl reaction mixture consisting of 3 µl of genomic

DNA, 2.5 µl 0f 10XPCR Buffer, 2 U of enzyme Taq Polymerase, 10 mM dNTPs mix and 50pmol/µl of each primer. All the primers used in this study are listed in Table 1. PCR amplifications done with thermal cycler (Bio-Rad, Germany) with 25 µL as the final volume. The PCR reaction was performed with an initial denaturation step at 95 °C for 1 min followed by 40 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 68 °C for 1 min, with a final extension at 68 °C for 5 min). PCR products were purified with a Qiagen QIAquick kit according to the manufacturer's protocol. PCR, electrophoresis was done for amplified samples through QIAxcel advanced system (Qiagen, Hilden, Germany).

Table 5. Sequence of Universal Primers used for Amplification

Designation	**Primer Sequence (5'-3')**	**Length**	**Gene**	**Reference**
CYTB l	CCATCCAACTCTCAGCATG ATGAAA	25bp	*cytB*	[294]
CYTB2	GCCCCTCAGAATGATATTTGTCCTCA	26bp	*cytB*	[294]
Fish-F1	TCA-ACC-AAC-CAC-AAA-GAC-ATT-GGC-AC	26bp	*cytochrome c oxidase subunit 1 (COI)*	[295]
Fish-R1	TAG-ACT-TCT-GGG-TGG-CCA-AAG-AAT-CA	26 bp	*cytochrome c oxidase subunit 1 (COI)*	[295]

10.4. Sequencing and detection of food fraud

Sanger sequencing was used to carry out the analysis in most products. Sequence reactions were carried out using Big Dye 3.1 Ready reaction mix (Life Technologies) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Life Technologies). Fish species in collected samples were detected based on sequencing results using BOLD (FISH-BOL) (https://www.boldsystems.org/) and GenBank (ID BLAST) (https://www.ncbi.nlm.nih.gov/genbank/). All amplified sequences were compared with available sequences in the Barcode of Life Data System (BOLD) and GenBank database. The highest similarity percentage of queried sequences with references sequences were determined. The sequences which have 97-100% similarity with database sequences were identified as the

respective species. The identified species were matched with labeling on products for detecting food fraud.

10.5. PROTOCOLS

DNA Isolation Protocol [DNA Extraction by QIAGEN Spin-Column kit]-

- Cut 25 mg of Fish tissue and place in 1.5ml micro centrifuge tube.
- Add 180 µL of ATL buffer.
- Add 20 µL proteinase K. Mix by vertexing,
- Incubate at 56° until completely lysed.
- Add 200 µL AL buffer vortex and add 200 µL Ethanol.
- Pipet the mixture into a Dneasy Mini spin column in a 2 ml collection tube.
- Centrifuge at 8000 rpm for 1 mi. Discard flow through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µL AW1 buffer. Centrifuge for 1min at 8000rpm. Discard flow through and collection tube.
- Place the spin column in a new 2 ml collection tube. Add 500 µL AW2 buffer. Centrifuge for 3 min at 15000rpm. Discard flow through and collection tube.
- Transfer the spin column to new 1.5ml micro centrifuge tube. Add 200 µL AE buffer for elution. Incubate for 1 min at room temperature.
- Centrifuge at 8000 rpm for 1 min and repeat it for maximum yield.

DNA sequencing-

- After PCR Electrophoresis will be done by QIAxcel capillary electrophoresis system.
- PCR products will be purified using a PCR purification kit (Qiagen).
- The purified PCR products will be sequenced in both directions using the Big Dye® Terminator v3.1 cycle sequencing kit chemistry in an ABI PRISM 96-capillary 3730xl genetic analyzer (Applied Biosystems, USA) using both the forward and reverse primers.
- Sequences will be retrieved after completion of sequencing

Sanger Sequencing Protocol-

- ✓ Take 96 well plates

- ✓ Take Sephadex resins in separate plate
- ✓ Invert filled resin plate on 96 well sequencing plate
- ✓ Add 300 µl of dH_2O in each 96 well plate
- ✓ Keep it for around 2 hours (between 1.10 hour to 1.5 hours)
- ✓ Centrifuge it for 3 min at 2800 rpm

  (Balance it with another plate)
- ✓ Take new sequencing plate with cover
- ✓ Transfer 10 µl sample to sequencing plate
- ✓ Give shot spin to all samples
- ✓ Centrifuge again for 3 min at 2800 rpm

  (Balance it with another plate)
- ✓ Add 10 µl formamide to each well with autosampler
- ✓ Put it on PCR machine for denaturation for 5 minutes
- ✓ Make sequence file on machine and import it for run
- ✓ Set for sequencing Run

DNA Estimation by Qubit® 3.0 Fluorimeter

- ✓ Working Solution =199 µl Buffer + 1 µl dye
- ✓ Add 197 µl Working Solution + 3 µl DNA
- ✓ Vortex
- ✓ Leave it for 2 minutes
- ✓ Estimate the DNA (ng/ µl)

Data analysis

- ▶ Fish species in different commercial fish products will be identified based on sequencing results using BOLD and GenBank database search engines.
- ▶ The highest percentage of pairwise identity of consensus sequences will be blasted in NCBI to compare the similarity scores against BOLD-IDS.
- ▶ The results will be matched with the label of the sample

11.Results

11.1. Sample collection and topology

The samples were collected from stores like hypermarkets and fishmongers. DNA was successfully extracted from all samples. After application of DNA samples with PCR, electrophoresis was done for amplified samples through QIAxcel Advanced system (Qiagen, Hilden, Germany). It is an advance system avoiding time consuming gel preparation steps and giving results in as early as 10 minutes. The most intense samples were selected purified and sequenced with genetic analyzer. Sequence reactions were carried out using BigDye 3.1 Ready reaction mix (Life Technologies) according to the manufacturer's instructions. The sequenced products were separated with a 3130 Genetic Analyzer (Life Technologies).

11.2. Fish Detection using BOLD and GenBank database

Fish species in collected samples were detected based on sequencing results using BOLD (FISH-BOL) and GenBank (ID BLAST). The highest percentage of pairwise aligned consensus sequences was blasted in NCBI to compare the similarity scores against BOLD-IDS [79]. All amplified sequences were compared with available sequences in the Barcode of Life Data System (BOLD) and GenBank database. The highest similarity percentage of queried sequences with references sequences were determined. The sequences which have 97-100% similarity with database sequences were identified as the respective species. The identified species were matched with labeling on products for detecting food fraud.

The highest percentage of pairwise aligned consensus sequences was blasted in NCBI to compare the similarity scores against BOLD-IDS [79]. Out of 90 products analysed mismatches were observed in 10 samples (from sample no 35 to 41 and sample no 50,83 and 159) (table 6), 3 samples (sample no 5,6 and 43) identified with both FISH BOL and GenBank (ID BLAST) while 14 samples (B1-B14) identified with only NCBI BLAST (ID BLAST based on *cytB*) while 79 samples (F1-F79) were identified with only FISH BOL (based on *COI*). All together 11.1 % samples were mislabeled among the samples analysed. In sample no 35 and 36 blue shark (*Prionace glauca*) were found instead of squid, in sample no 41 it was replaced with haddock (*Melanogrammus aeglefinus*). In sample 37,38,39 and 40 haddock (*Melanogrammus*

aeglefinus) were observed in place of Swordfish (*Xiphias gladius*) (Table 6). Samples 50 fraud is also considered a fraud because the Italian law (DM n. 19105 – 2017) states that the name "Acciuga" must be attributed only to the species *Engraulis engrasicolus*. *E. rigens* must be named as "Acciuga del Pacifico". Sample 83 is a commercial fraud. According to the Italian law, Triglia are the fish included in the genera *Mulls*; *Pseudopeneus prayensis* must be named as "*Triglia atlantica*". One sample (sample no 159) labelled as grouper (*Epinephelus* spp.) was identified as oil fish (*Ruvettus pretiosus*).

Table 6. Results of analyzed samples

S.N	Sample No	Sample Name (Local)	Sample Name (English)	Scientific Name	ID BLAST	ID FISH BOL	Fraud
1	1	Filetto di platessa	*Plaice fillets*	*Pleuronectes platessa*		*Pleuronectes platessa (F1)*	N0
2	2	Alici panate	*Breaded anchovies*	*Engraulis encrasicolus*	*Engraulis encrasicolus (B1)*		NO
3	5	Filetto di nasello	*Hake fillet*	*Merluccius merluccius*	*Merluccius hubbsi (B2)*	*Merluccius hubbsi (F2)*	NO
4	6	Filetto di Merluzzo di Alaska	*Alaskan cod fillet*	*Gadus chalcogrammus*	*Gadus calcogrammus (B3)*	*Gadus calcogrammus (F3)*	NO
5	10	Bastoncini di pesce	*Fish sticks*	*Gadus chalcogrammus*		*Gadus calcogrammus (F4)*	NO
6	11	Bastoncini di pesce	*Fish sticks*	*Macronus novaezelandiae*		*Macronus novaezelandiae (F5)*	NO
7	12	Filetto di merluzzo di Alaska	*Alaskan cod fillet*	*Gadus chalcogrammus*		*Gadus calcogrammus (F6)*	NO
8	13	Schlemmerfilet di Alaska (pesce impanato)	*Alaska schlemmerfilet (breaded fish)*	*Gadus chalcogrammus*		*Gadus calcogrammus (F7)*	NO
9	14	Chele di surimi	*Claws of surimi*	*Merluccius merluccius*	*Merluccius merluccius (B4)*		NO
10	18	*Melanogrammus aeglefinus*	*Melanogrammus aeglefinus*	*Melanogrammus aeglefinus*		*Melanogrammus aeglefinus (F8)*	NO
11	31	Filetti di merluzzo bastoncini	*Cod fillets fillets*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F9)*	NO
12	32	Filetti di merluzzo di Alaska	*Alaskan cod fillets*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F10)*	NO
13	33	Filetti di merluzzo di Alaska con patate e rosmarino	*Alaskan cod fillets with potatoes and rosemary*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F11)*	NO
14	35	Calamaro	*Squid*	*Loligo opalescens*		*Prionace glauca (F12)*	YES
15	36	Calamaro	*Squid*	*Loligo opalescens*	*Prionace glauca B5*		YES
16	37	Pesce spada	*Sword fish*	*Xiphias gladius*		*Melanogrammus aeglefinus (?) (F13)*	YES
17	38	Pesce spada	*Sword fish*	*Xiphias gladius*		*Melanogrammus aeglefinus (?) (F14)*	YES
18	39	Pesce spada	*Sword fish*	*Xiphias gladius*	*Melanogrammus aeglefinus (B6)*		YES

19	40	Pesce spada	*Sword fish*	*Xiphias gladius*		*Melanogrammus aeglefinus (F15)*	YES
20	41	Calamaro	Squid	*Loligo opalescens*		*Melanogrammus aeglefinus (F16)*	YES
21	42	Sushi NYC Suami	Traditional sushi	*Salmon*	*Salmo salar (B7)*		NO
22	43	Sushi NYC Suami	Traditional sushi	*Salmon*	*Salmo salar (B8)*	*Salmo salar (F17)*	NO
23	47	Sushi NYC Roma	Traditional sushi	*Litopenaeus vannamei*		*Litopenaeus vannamei (F18)*	NO
24	48	Filetti di alici marinate	*Marinated anchovy fillets*	*Engraulis encrasicolus*	*Engraulis encrasicolus (B9)*		NO
25	50	Filetti di acciughe	*Anchovy fillets*	*Engraulis encrasicolus*		*Engraulis rigens (F19)*	YES
26	52	Filetti di alici marinate	*Marinated anchovy fillets*	*Engraulis encrasicolus*	*Engraulis encrasicolus (B10)*		
27	53	Bastoncini di surimi	*Surimi sticks*	*Priacanthus prolixus*		*Priacanthus prolixus/Chordata actinopterygii (F20)*	NO
28	54	Bastoncini di pesce	*Surimi sticks*	*Gadus morhua*	*Gadus morhua (B11)*		No
29	55	Fishburger di merluzzo d'alaska	*Alaskan cod fishburger*	*Gadus chalcogrammus*		*Gadus calcogrammus (F21)*	N0
30	56	Filetto di merluzzo di Alaska	*Frozen skinless Alaskan cod fillet*	*Gadus chalcogrammus*		*Gadus calcogrammus (F22)*	NO
31	57	Filetto di merluzzo di Alaska senza pelle congelato	*Frozen skinless Alaskan cod fillet*	*Gadus chalcogrammus*		*Gadus calcogrammus (F23)*	NO
32	58	Filetti di rombo	*Turbot fillet*	*Paralichtys patagonicus*		*Paralichtys patagonicus (F24)*	NO
33	59	Filetti di cernia indopacifica	*Indo-Pacific grouper fillets*	*Hyporthodus octofasciatus*		*Hyporthodus octofasciatus (F25)*	NO
34	60	Nuggets merluzzo di Alaska	*Alaska cod nuggets*	*Gadus chalcogrammus*		*Gadus calcogrammus (F26)*	NO
35	61	Bocconcini merluzzo	*cod morsels*	*Pollachius virens*		*Pollachius virens (F27)*	NO
36	62	Croccole, filetti di merluzzo croccante	*Crispy cod fillets*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F28)*	NO
37	63	Polpette di pesce	*Fish balls*	*Pollachius virens*		*Pollachius virens (F29)*	NO
38	64	Bastoncini	*Fish sticks*	*Merluccius* spp.		*Merluccius* spp. *(F30)*	NO
39	67	Spunti al salmone	*Salmon slices*	*Oncorhynchus gorbuscha*	*0ncorhynchus gorbuscha (B12)*		NO
40	75	Filetto di platessa	*Plaice fillet*	*Pleuronectes platessa*		*Pleuronectes platessa (F31)*	
41	76	Fishburger di ricciola con radicchio e peperoncino	Amberjack fishburger with radicchio and chilli	*Seriola*		*Seriola quinqueradiata (F32)*	No
42	78	Merluzzetti del pacifico	*Pacific cod*	*Merluccius gayi*		*Merluccius gayi (F33)*	NO
43	79	Tonno rosso	*Bluefin tuna*	*Thunnus thynnus*		*Thunnus thynnus (F34)*	NO
44	83	Fietti di triglia	*Mullet fillets*	*Mullus* spp		*Pseudupeneus prayensis (F35)*	YES
45	84	Filetti di sogliola	*Sole fillets*	*Solea solea*		*Actinoperygii* sp.*(F36)*	NO
46	86	Cuore di merluzzo	*Cod heart*	*Gadus morhua*		*Gadus morhua (F37)*	NO
47	90	Bistecchine di mare	*Sea steaks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F38)*	NO
48	92	Burger di pesce spada	*Swordfish burger*	*Xiphias gladius*		*Xiphias gladius (F39)*	NO
49	93	Palombo spellato	*Dogfish*	*Mustelus mustelus*		*Mustelus asterias (F40)*	NO
50	94	Burger di merluzzo nature	*Cod burger*	*Gadus morhua*		*Gadus morhua (F41)*	NO
51	95	Filetto di platessa	*Plaice fillet*	*Pleuronectes platessa*		*Pleuronectes platessa (F42)*	NO
52	96	Filetto di merluzzo	*Cod fillet*	*Gadus morhua*		*Gadus morhua (F43)*	NO
53	97	Sogliola	*Sole*	*Solea solea*		*Solea solea (F44)*	NO
54	99	Sogliola	*Sole*	*Solea solea*		*Solea solea (F45)*	NO
55	100	Sogliola	*Sole*	*Solea solea*		*Solea solea(F46)*	NO
56	101	Chele di surimi	*Surimi*	*Merluccius* spp	*Merluccius productus (B13)*		NO

57	102	Bastoncini di merluzzo	*Cod sticks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F47)*	NO
58	106	Chele di surimi	*Surimi*		*Merluccius productus (B14)*		NO
59	115	Fantasie di merluzzo panate, prefritte e congelate	*Breaded, pre-fried and frozen cod fantasies*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F48)*	NO
60	116	Filetti di tonno a pinne gialle	*Yellow fin tuna fillets*	*Thunnus albacares*		*Thunnus albacares (F49)*	NO
61	117	Filetti di tonno a pinne gialle	*Yellow fin tuna fillets*	*Thunnus albacares*		*Thunnus albacares (F50)*	NO
62	118	Bastoncini di pesce	*Fish sticks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F51)*	NO
63	120	Fishburger filetti di merluzzo	*Fishburger cod fillets*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F52)*	NO
64	122	Merluzzo atlantico	*Atlantic cod (cod)*	*Merluccius hubbsi*		*Merluccius hubbsi (F53)*	NO
65	126	Merluzzo atlantico	*Atlantic cod (cod)*			*Merluccius hubbsi (F54)*	NO
66	127	Fish burger di salmone	*Salmon fish burger*	*Salmo salar*		*Salmo salar (F55)*	NO
67	128	Filetti di salmone	*Salmon fillets*	*Oncorhynchus gorbuscha*		*0ncorhynchus gorbuscha (F56)*	NO
68	129	Pesce spada	*Swordfish*	*Xiphias gladius*		*Xiphias gladius (F57)*	NO
69	132	Bistecchine di mare	*Sea steaks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F58)*	NO
70	134	Merluzzetti atlantici	*Atlantic cod*	*Merluccius hubbsi*		*Merluccius hubbsi (F59)*	NO
71	136	Tranci di pesce spada	*Swordfish steaks*	*Xiphias gladius*		*Xiphias gladius (F60)*	NO
72	137	Burger di tonno	*Tuna burger*	*Thunnus albacares*		*Thunnus albacares (F61)*	NO
73	139	Bistecchine di mare	*Sea steaks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F62)*	NO
74	140	Merluzzetti atlantici	*Atlantic cod*	*Merluccius hubbsi*		*Merluccius hubbsi (F63)*	NO
75	141	Bastoncini di surimi	*Surimi sticks*	*Merluccius* spp.		*Merluccius spp(F64)*	NO
76	143	Filetti grigliati di merluzzo d'Alaska	*Grilled Alaskan cod fillets*	*Theragra chalcogramma*		*Theragra chalcogramma (F65)*	NO
77	146	Tranci di pesce spada	*Swordfish steaks*	*Xiphias gladius*		*Xiphias gladius (F66)*	NO
78	148	Tranci di verdesca	*Slices of blue shark*	*Prionace glauca*		*Prionace glauca (F67)*	NO
79	150	Filetti grigliati di merluzzo d'Alaska	*Grilled Alaskan cod fillets*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F68)*	NO
80	151	Bistecchine di mare	*Sea steaks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F69)*	NO
81	154	Tranci di verdesca	*Slices of blue shark*	*Prionace glauca*		*Prionace glauca (F70)*	NO
82	155	Fish burger di merluzzo d'Alaska	*Alaskan cod fish burger*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F71)*	NO
83	158	Burger di tonno	*Tuna Burger*	*Thunnus albacares*		*Thunnus albacares (F72)*	NO
84	159	Cernia	*Grouper*	*Epinephelus* spp.		*Ruvettus pretiosus (F73)*	YES
85	160	Bistecchine di mare	*Sea steaks*	*Gadus chalcogrammus*		*Gadus chalcogrammus (F74)*	NO
86	161	Filetto di merluzzo dell'oceano	*Ocean cod fillet*	*Merluccius capensis*		*Merluccius capensis (F75)*	NO
87	163	pesce spada	*Swordfish*	*Xiphias gladius*		*Xiphias gladius (F76)*	NO
88	164	Pesce spada	*Swordfish*	*Xiphias gladius*		*Xiphias gladius (F77)*	NO
89	165	Filetti di platessa	*Plaice fillets*	*Pleuronectes platessa*		*Pleuronectes platessa (F78)*	NO
90	166	Merluzzetti del pacifico	*Pacific cod*	*Merluccius gayi*		*Merluccius gayi (F79)*	NO

12. Discussion

In fish sector, the identification of fish species throughout the production chain is of main importance, even if fishery products have been already 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 point of view, is more frequent. In this regard, at present, 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 seem to be obstructed, because fish is not easily identifiable, with the increase of commercial (*aliud pro alio*) and sanitary frauds (commercialisation of toxic organisms). For example**,** oilfish (*Ruvettus pretiosus*) is seldom marketed in conformity with current EU Regulation (EC Reg. 2008 n.1021) and it is often commercialised in place of the most popular, expensive, and precious species, such as grouper (*Epinephelus* spp.). The problem of fraudulent actions in the commercialisation of foods is strongly felt at European Union level; in fact, recently a recommendation was enacted on 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).

In 2015, an investigation was caused by INTERPOL_EUROPOL which demonstrated fish fraud as 3rd highest risk category of food vulnerable to fraud [296, 297]. Mislabeling can be executed at any point of the fish production chain, at processing, distribution, retail, and catering level. In 2016, Oceana published a major report by reviewing more than 200 published studies across 55 countries and found 20% mislabeling in catering and related sectors [9, 10]. In 2021 Oceana Canada observed 46 % mislabeling in seafood products which is just 1% less compared to study conducted during 2017-2019 [298]. 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 large amount of seafood fraud at global scale [299]. All studies conducted indicates species substitution and mislabeling are serious problems in international fish trade.

The loss of morphological feature encourages replacement of higher priced fish species by the lower priced one in finished fish products causing consumer dissatisfaction, economic loss, and health hazard [300]. In 2015 another report was published in Italy in which squids are replaced with pufferfish[110]. Some fatal cases of puffer fish poisoning were also reported in Bangladesh [301]. Such incidents indicate severe risks associated with mislabeling and species substitution of fish which also hampers trust of consumers in the market.

Proper labeling and description and their subsequent control are very important for ensuring commercial exchanges, food safety and imposing proper price on these products. 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 [79]. In addition, in recent years ready to be cooked fish products has gained huge popularity due to lack of time and busy life in urban areas. So ready to cook fish products and some processed forms of fish has received lot of attention as a stable, attractive and economically beneficial commodity in International Food Trade [242]. Due to increasing world trade and popularity these costly fish are quite vulnerable to fraud. There is replacement with some less expensive fish such as tilapia (*Oreochromis niloticus)*, common carp (*Cyprinus carpio*), milkfish (*Chanos chanos*), mud cap (*Cirrhinus molitorella***)** etc. [79, 85, 302]. Xiong *et al.,* in 2016 conducted a study in China reported that some economically imported fish species like cod, tuna and salmon displayed lack of national mandatory regulation for fishery products which badly impact seafood industry, right of consumers as well as preservation of fish stocks [85]. Oceana published a major report in 2016 by reviewing more than 200 published studies across 55 countries and found 20% mislabeling in catering and related sectors [9, 10]. In 2021 Oceana Canada reported 46% mislabeling in seafood products which is slightly less compared to study conducted during previous year [298]. 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 large amount of seafood fraud at global scale [299]. There are many other studies conducted in other countries like Italy [82, 124] Germany[152, 303], India [79], South Brazil [59, 300] which show the concerns related with fish fraud [82, 304, 305].

DNA barcoding is now one of the most widely used molecular too to investigate species substitution and mislabeling in various fish products. Several researchers applied DNA barcoding tool to investigate the species substitution and mislabeling in various parts of the world. Filonzi *et al.,* in 2010 reported substitution of halibut (*Renharditus hippoglossus*) with *Pangasius* (*Pangasianodon hipophthalmus)* [124]. Maralit *et, al.* (2013) observed substitution of gindara or sablefish (*Anoplopoma fimbria*) with escolar *(Lepidocybium flavobrunneum)*, [119] while Warner and colleagues in 2013 red snapper (*Lutjanus campechanus*) being replaced with tilefish (*Malacanthidae spp.)* [306]. Recently large scale mislabeling [85%] was observed in products purchased from e commerce platforms [307].

In our study, out of 90 tested samples 10 (11.1%) were found showing noncompliance with the label; in addition, some unusual results were observed: as sample no 35 and 36 were found to be shark (*Prionace glauca*) instead of squid (Table 6). In sample no 41 squid was found in place of haddock (*Melanogrammus aeglefinus*). In sample n. 159 *Ruvettus pretiosus was found instead of* grouper (*Epinephelus* spp.). In samples 37,38,39 and 40 haddock (*Melanogrammus aeglefinus*) were observed in place of swordfish (*Xiphias gladius*) (Table 6). Sample 50 and 83 also falls in food fraud. Samples 50 fraud is considered a fraud because the Italian law (DM n. 19105 – 2017) states that the name “Acciuga” must be attributed only to the species *E. engrasicolus. E. rigens* must be named as *Acciuga del Pacifico*. Sample 83 is a commercial fraud as according to the Italian law, “Triglia” are the fish included in the genera *Mulls*; *Pseudopeneus prayensis* must be named as “*Triglia atlantica*”. Our findings also confirm 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 mislabeled [97]. Forty-four per cent of the retail outlets visited sold mislabeled 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 of halibut (*Hippoglossus hippoglossus*) instead of grouper (*E. marginatus*)[236]. Given the high demand for grouper by consumers, 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, as well as its high market value, which continues to be evident in the market, is also a motivation for economic fraud. The most prevalent economic fraud associated with grouper is the selling of a cheaper finfish as grouper. In fact, the most common types of mislabeling among the grouper samples collected in the US were substitutions with farmed Asian 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 [42].

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. In our survey one sample (sample no 159) marketed as grouper (*Epinephelus* spp.) was identified as *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 numerous wax esters in its body for buoyancy control; 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 [308]. Outbreaks of keriorrhea have been repeatedly reported across continents. In the EU, the marketing of *R. pretiosus* is regulated by the EC Reg. 2008 n.1021. According to this Regulation, food business operators are obliged to sell oilfish products in packaged form and to provide information on label to the consumer about their gastrointestinal adverse effects.

We also observed less species replacement on other products tested, this may be due to the low number of tested samples and to the restricted area of sample collection due to Covid-19 pandemic which occurs during the sample collection period. From sanitary point of view, mislabeling can produce food poisoning, sometime severe or fatal, when poisonous fish, such as those belong Tetraodontidae or Diodontidae families, are marketed as commonly fish: a study from Italy reported a case in which squids were replaced with pufferfish [110]. In addition, severe and fatal cases of tetrodotoxin poisoning due to puffer fish consumption were reported from

Israel and Bangladesh, respectively [301]. Such incidents indicate severe risks associated with mislabeling and species substitution of fish which also hampers trust of consumers in the market.

Recent advancement in DNA based techniques has shown greater capability to authenticate even highly processed fish products. One of the difficult task in this techniques is to extract good quality DNA from those food products because many ingredients used in fish processing acts as PCR inhibitors which can hamper DNA amplification in PCR [309]. Although DNA was successfully extracted from all collected samples using "Qiagen DNAesy blood and tissue kit" according to protocol provided with product. Samples having A260/A280 ratio between 1.7-2.0 are selected for amplification as suggested by Piskata *et.al,* 2017 [310]. There may be chances of DNA degradation due to extreme heat exposed during processing or physical and chemical treatment during fish processing. In cases DNA were not amplified during PCR cycling which suggests presence of PCR inhibitors in these extracted samples. 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 [311].

For species identification using *COI* and cytB gene use of both GenBank and BOLD (FISH BOL) database suggested. It is preferable to BOLD search first because it this database was developed with voucher specimen and contains only validated sequences which can be used for identification purpose [312]. BOLD results identify species by the degree of nucleotide variation with similar species having a divergence value of less than 1 [313]. If there is no match with BOLD database one can look for alternative NCBI BLAST analysis which display a list of species which are more like the query sequence as well as BIT score which estimates the percent identity and E value *COI* gene has the largest taxonomic presence in NCBI nucleotide database. Here, out of 90 products analyzed discrepancy is observed in 10 samples (samples 35-41 and 50, 83 &159), 3 samples (no 5,6 and 43) are identified with both FISH BOL and GenBank (ID BLAST) while 14 samples (B1-B14) identified with only NCBI BLAST (ID BLAST based on *cytB)* while 79 samples (F1-F79) were identified with only FISH BOL (based on *COI*). Although DNA Barcoding is most successful method for species identification which is quite standardized. Similar and affordable submission size sequence (200-300bp) must be encouraged for identification of processed fish products. In summary, our method based on DNA barcoding 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 controlling the traceability and labeling of fishery products.

13. Conclusion

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 fraud where costly fish can be replaced with cheap fishes particularly in products where morphological identification is lost. The fish fraud may have health concerns as well as environmental concerns. It must be authenticated before serving to customers. Due to limitation of protein-based methods DNA barcoding has emerged as most suitable method for species identification and tracking food fraud. DNA barcoding is based on some genes such as *cytB* and *COI* with the application of sequencing methods. Due to its growing success, it must be proposed by food control authorities to ensure food safety and right of consumers.

Mitigating the sea food fraud risk is not an easy task for any country as no single government agency can regulate the fish fraud cases, and no single food law can directly address all aspects of food fraud. These are cumulative efforts taken by official food regulatory authorities, custom authorities, border control agencies and specialist bodies within national police force.

Number of key mandatory steps and close association between different government agencies are needed to combat fish fraud at national level. An effective scientific fish traceability system is needed which can identify the fish species, the geographical origin which can differentiate between wild capture and farmed products. The system must be effective in identification of fresh and frozen fish and harness different forms of processed fish that are currently traded.

Making an agreed list of common names linked to scientific nomenclature is one of the main challenges to countries facing fish fraud incidents. For any national government this is the principal step in introducing official fish fraud control measures, many countries are adopting the

approach and established such lists. A list of common names, scientific names and market names are published by USFDA that are mandatory for commercial trades (USFDA, 2018). Similar kind of list is also published by Canadian Food Initiative agencies. EU recommended all its associate countries to draw and publish list of commercial designation and scientific names for fish and aquaculture products in commercial trade (EU, 2013b).

Several countries have updated the commercial list of fish species with FISH BOL information system database (FAO, 2018). The main problem appear in uniform nomenclature of fish names is that different species may have the same common names in different countries, or the same species may have different names in the same language in different regions in same country.

The introduction of mandatory labeling requirement for fish and fish products is major step in addressing concerns about fish fraud. The EU has adopted the most comprehensive regulatory method for labeling of fish and fishery products. The most important principle was applied on food labeling information is that it must not mislead the consumers specially with respect to food characteristics. The labeling must provide all relevant information which must be honest and accurate with respect to property, identity, composition durability, quantity, country of origin or place of provenance and method of manufacturer or production.

There is some additional information which is also necessary to provide about fishery and aquaculture products traded in EU member countries mentioned in article 9 and 10 of regulation (EU) 1169/2011 on the provision of food information to consumers.

To ensure an effective food control programs and to fight fish fraud and for following fish traceability for point of catch to consumption, proper fish labeling information is very much needed. FAO has proposed 12 major fishing areas which are important for controlling Illegal Unreported and Unregulated (IUU) fisheries and this is reference standard for geographical catchment areas where fish is farmed.

The 3rd important proposal in countering fish fraud cases is to strengthen official food control system. This can be done by introduction of regulations to counter fish fraud, application of monitoring and surveillance measures for controlling and assessing the degree of compliance with fish labeling regulation. The other way is by application of laboratory detection methods

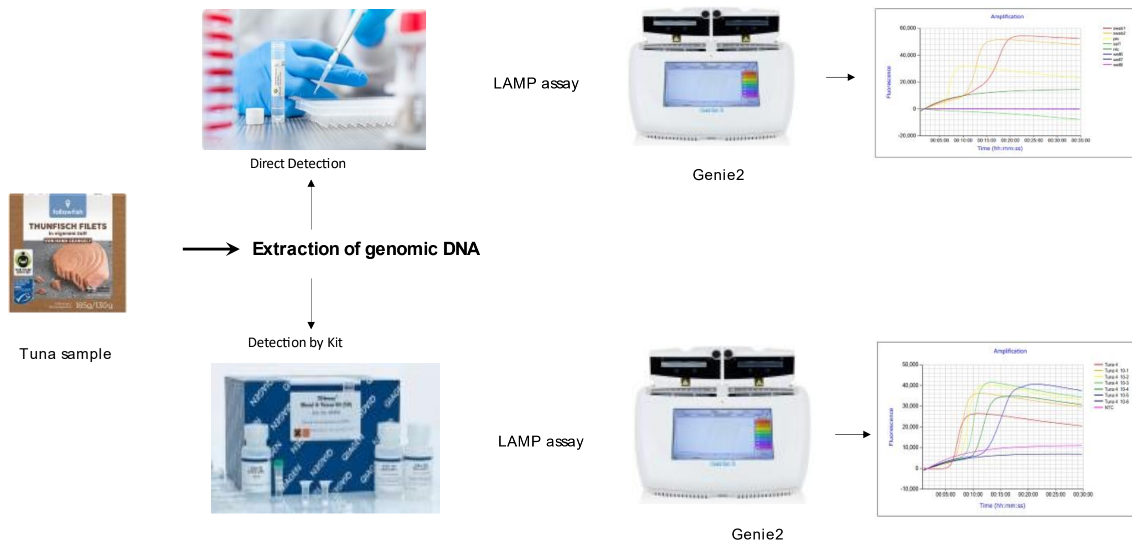
based on DNA Barcoding and enforcement of regulations in the event of the detection of non-compliance. Food control authorities need to authenticate and validate traceability system based on a documentary paper trail to implement effective food control program and to mitigate fish fraud.

Chapter 2. Development of Loop-Mediated Isothermal Amplification (LAMP) assay for rapid and direct screening of yellowfin tuna (*Thunnus albacares*) in commercial fish products

1. Summary

Tuna is one of the most widely consumed fish in the European market, which is available in various consumable options. Among them *Thunnus albacares* also called yellowfin tuna is a delicacy and consumed by millions of people around the world. It is quite costly compared to other tuna species available in the market. Due to its cost and demand, it is more vulnerable to fraud in which low-cost tuna or other fish varieties can be replaced for economic gain. In this study a Loop Mediated Isothermal Amplification (LAMP) assay was developed and validated targeting mitochondrial *cytochrome b* gene for quick and direct detection of *T. albacares* which is a valuable species of tuna. The specificity was confirmed with 18 targets (*T. albacares*) and 18 non- target samples (other fish species). The analytical sensitivity observed in this assay was 540 fg. Further, the detection of *T.s albacares* was also confirmed by gel electrophoresis. Most importantly, detection time of as early as 7 minutes was observed for this species. In addition, a simple and direct swab method without nucleic acid extraction by using MSwab was also performed for this assay. The MSwab method can detect the target species in 13 minutes. Therefore, with high specificity and sensitivity this novel LAMP assay can be used for the identification of *T. albacares* as a rapid screening method, and it can provide a valuable portable and quick option for food control authorities.

Fig 4. Workflow of the assay for yellowfin tuna (*Thunnus albacares*) detection



2. **Literature**

Isothermal amplification is an emerging technique which amplified a specific region of DNA at constant temperature. The various isothermal techniques used for seafood species identification include RT-NASBA and LAMP[314-316]. In RT-NASBA three specific enzymes were used avian myeloblastosis virus (AMV) reverse transcriptase (RT), RNase H, and T7 DNA dependent RNA polymerase (DdRp) [317]. The technique is quite similar to PCR as both methods amplify specific nucleic acid sequence via enzymatic reactions, the only difference is that RT-NASBA is performed under isothermal conditions and targets RNA [314]. The results are detected with a molecular beacon hybridizing with target region and emitted a florescent signal detected in real time[316]. Although the technique was mostly used in detection of microorganisms, it is successfully used for differentiating 29 out of 35 grouper species from several fresh and cooked fish samples by targeting the *16S rRNA* gene [314].

Loop Mediated Isothermal Amplification (LAMP) is novel nucleic acid amplification assay with several advantage compared to conventional PCR which requires several steps and more time. The techniques were developed by Notomi *et. al,* in 2000 and it is highly specific and rapid which avoid several temperatures profiles; rather it uses isothermal condition for amplification

[318]. Two sets of primers and a DNA polymerase with high displacement activity is needed for this assay. There is need of two outer primers called F3 and B3 and two inner primers named FIB and BIP (forward inner primer and backward inner primer). Six distinct genomics regions (F1c, F2c, F3c, B2c, B1c, and B3c) are target of the two primer pairs for providing high specificity. F3c and B3c regions are targeted by F3 and B3 primers while F1c and F2c are targeted by FIP primers, the other specific region B1c and B2c are target of the primer BIP. The role of outer primers is limited to initial step of the reaction in combination with inner primers. The inner primers play dual role targeting the sense and antisense strand, one function is to extend the reaction while the other function is self-priming. The DNA polymerase is responsible for initiation of three step amplifications [133]. The assay can be divided by three steps in which starting material is produced by first step and second step is of amplification cycle, third step elongates and recycles. In the initial step both primers are used while during second and third steps only inner primers needed for the reaction. The target sequence is tripled every half cycle. Addition of loop primers can increase the exponential amplification and can also reduce the analysis time to less than 30 minutes. The target region of loop primers is between F2c F1c and the B1c B2c regions, although it is not required for LAMP assay. End point detection can be done by product separation using agarose gel electrophoresis, or techniques such as visual detection using DNA binding dyes such as SYBR green, detection of turbidity by precipitation of magnesium. Additionally, melting curve analysis and subsequent real time fluorescence detection can be done with instruments like real-time cyclers or real time fluorimeters tailored for isothermal application [319, 320]. LAMP application is successfully seen in detection of several viruses and pathogens [321, 322], besides detection of plant species in herbal products [323, 324] . Now it is widely used in food sector for food fraud detection including identification of fish species. Due to advantage of high specificity and short analysis time LAMP assay provide useful alternative for PCR based methods in detection of poultry and mammalian species detection ([325, 326]. The assay is successfully applied for species detection in seafood using *COI* as target gene for detection of squid [327]. LAMP assay is also used for discrimination of Atlantic cod (*Gadus morhua*) from Pacific cod (*Gadus macrocephalus*) and pollock (*Gadus chalcogrammus*), based on *cyt*B as target gene, for the analysis of frozen and smoked fish fillets [315]. LAMP assays provide several advantages over

conventional PCR based methods as it is less affected by inhibitors and it provide more sensitivity [322, 328]. The instrument is easy to handle and portable, so it provides advantage of on-site analysis. In summary LAMP assay is highly specific, cost effective, simple and time efficient as it avoids many temperatures profile like conventional PCR.

3. Introduction

Tuna belongs to the *Thunnus* tribe (scombridae) family, and it is one of the most popular delicacies among marine fish around the world. Tunas are very large species widely distributed in oceanic environments. It is consumed in various forms either as fresh or canned products. The European Union included several close members of the genus *Thunnus* in the category of “Tuna”. The closed members included in the group “Tuna” are Bluefin tuna (*Thunnus thynnus*), bigeye tuna (*Thunnus obesus*), yellowfin tuna (*Thunnus albacares*). Other species like longfin tuna (*Thunnus alalunga*) and skipjack tuna (*Katsuwonus pelamis*) are also included in commercial designation of Tuna.

Tuna is considered as a healthy source of animal proteins, omega 3 fatty acids, bioactive nitrogen compounds which are an ideal parameter for balanced and quality nutrition [329, 330]. The total catch of several *Thunnus* species in 2018 was around 5.61 million tonnes. Among these, contributions of yellowfin tuna (*T. albacares*) and skipjack tuna (*K. pelamis*) are the most common. The worldwide output related to both species in 2018 are 1.46 and 3.16 million tonnes respectively [331]. The capture of Bluefin Tuna was not constant during the past several years, yet in the year 2018 it surpassed to 60,000 tonnes [331].

Due to its bulky size it is not possible to sell it as such so they are usually traded in portion or fillet forms, most of them are used for canning and making sashimi and sushi products [332]. Due to the characteristic texture, taste and flavour, canned tuna products available either in oil or brine is a delicacy for consumers [333]. Skipjack tuna (*K, pelamis),* yellowfin tuna (*T. albacares*) and big eye tuna (*T. obesus*) are the most widely used species in canned products [334]. There are other forms also where tuna is consumed as food of choice. They can be served as fillets or alternatively offered as a floss product after being dried, flavoured, roasted, and rolled to make such products. These floss products are easier to digest and provide suitable alternatives for

infants, kids and older people [335, 336]. Prices for different tuna species vary according to species and popularity. The prices are regulated by species of tuna, trade performance and the ultimate use. The market price of raw tuna which is mostly used in sashimi and sushi is quite high. Sashimi preparation requires high quality fish and usually it is the most demanding and expensive option in the market [332]. Compared to fresh and raw tuna, quality requirements are not very stringent for cooked, canned, frozen, and smoked tuna products. Prices for skipjack tuna are much lower than yellowfin and big eye tuna probably due to lesser demand in the market. Due to variation in prices and tax imposed by the European Union on imported products attention has been given to methods which can identify canned fish species according to EU regulations (4). Several methods are available for species identification in fish species [337, 338]. Raw fish products can be easily identified by method which depends on availability of water soluble proteins in the fish species [339]. The detection of processed tuna products is quite cumbersome and needs alternative methods. Steps like canning, smoking, and cooking involve intense heat treatment which can cause irreversible loss of water and affects solubility of proteins hampering the identification process [340]. In these methods proteins which are soluble in water are isolated from fish tissues and compared with those of legitimate species for the setting up the identity. Methods based on antibody-antigen interaction can provide suitable alternatives, but till now only a handful of immunoassays have been made, and none of the immunoassays are available for large scale application for routine analysis. Precision characteristics also depend on cell types which also vary according to organ or tissue which express different proteins. So, due to limitations associated with proteins as ideal candidate for species detection it is preferable to use DNA instead of proteins. Many successful DNA based amplification methods are available for species detection in fish products like PCR –RFLP[341], PCR-SSCP[342], real time PCR [343], sequencing of PCR amplified mitochondrial DNA [98, 344]. Use of multiplex PCR is another method which can amplify multiple DNA targets in in a single reaction tube and the amplified amplicons are identified by gel electrophoresis with respective band size [42, 345].

For species identification the choice of mitochondrial DNA scores higher over DNA of nuclear origin. Several factors like relatively high abundance in cell making detection easier and because mitochondria remain robust during processing steps makes it a preferable choice for

species identification. Majority of nucleic acid-based identification methods analyse sequences derived from the gene encoding *cytochrome b* located on the mitochondrial genome. Mitochondrial DNA displays high intraspecific variability, which can help in species-specific primer designing. Most of the time it is preferable to do a preliminary analysis for evaluation of the detection of intraspecific polymorphism in individuals of the same species [346].

Although sequencing-based approaches are quite successful in species identification, they need costly and bulky instrumentation and sophisticated research labs, which is not an ideal choice for a quick and economically effective identification method for routine screening directly by the food safety and industrial unit authorities.

The PCR based application techniques also requires costly and bulky equipment and also need longer time to pursue, limiting their utility for on site or rapid diagnosis [347]. From the past few years a nucleic acid amplification technique gained popularity called loop-mediated isothermal amplification (LAMP), which is now widely used in food legitimacy testing for quick identification [348]. LAMP assay uses four specifically designed primers that recognises six regions on target gene sequences producing exceptionally high sensitivity and specificity. Additionally, the complete identification process can be performed in less than 60 minutes, even as low as 10 minutes under isothermal conditions (constant temp. of 60-65). In LAMP reaction initial denaturation steps are not required and there is no need for temperature change throughout the assay. In the last few years many studies have been performed using LAMP assay for food authentication including eel [349], salmon[350, 351], cod [315] ,tuna [352], ostrich meat [353] etc.

The aim of present study was to develop a new LAMP assay for direct detection of yellowfin tuna (*Thunnus albacares)* in commercial fish products targeting the mitochondrial cytochrome b gene.

4. Material and Methods

4.1. Sample collection

Thirty-six samples were collected from different supermarkets in Hannover city. Among them 18 samples are of *T. albacares* and 18 are from other fish species. The samples include Tuna steaks and canned samples. The samples were stored at -20 °C while canned products have been kept unopened at room temperature.

4.2. DNA extraction

DNA extraction was done with the DNeasy Blood &Tissue Kit (Qiagen, Hilden, Germany). 25-40 mg tissue was taken from each sample and transferred to a 1.5 mL tube. Lysis of tissue was done with a lysis buffer supplied with kit. After incubation and two washing steps DNA was eluted with 100 µl AE elution buffer. The purity of extracted DNA was checked by spectrophotometer measurements using Nanodrop (Thermo Fisher scientific, VWR International GmbH, Darmstadt, Germany). Extracted DNA was stored at 4°C for future experiments.

4.3. Designing of species-specific LAMP primers set

Primers for LAMP assay was designed with mitochondrial *cytochrome b* genes of *T. albacares.* The sequence of mitochondrial *cytochrome b* gene having accession number JN086153.1 was retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned using NCBI and primer-binding sites were selected to ensure coverage of all sequences. Alignments were also done with genetic similar non- target species by selecting *cytochrome b* sequences to ensure that there will be sufficient mismatch in primer binding sites between non-target species.

Six oligonucleotide primers based on mitochondrial *cytochrome b* gene were designed with the help of LAMP Designer software, ver. 1.10 (PREMIER Biosoft, CA, USA). The primer set consists of two outer primers (Forward primer F3 and backward primer B3), and two inner primers (forward inner primer FIP, and backward inner primer BIP). Additionally, two loop primers (forward loop primers LF, and backward loop primers LB) are also designed to accelerate the LAMP reaction (Table 7). The designed LAMP primers for *T. albacares* were synthesized by Eurofins Genomics (Ebersberg, Germany). The designed oligonucleotide primers were also tested for their specificity through NCBI BLAST.

Table7. Sequence of oligonucleotide primers used for LAMP assay

Designation	Primer Sequence	Length	Tm [°C]	GC %	Position
Tuna F3	ATACGCAATTCTTCGGTCC	19 bp	54.51	47	831
Tuna B3	TTGTTCTCAGCTCAGCCT	18 bp	53.69	50	1124
Tuna LF	GAAGTGTGCAGGAAGGGAA	19 bp	56.67	53	929
Tuna LB	GCGGAACAGCCCTTCATTA	19 bp	56.67	53	1027
Tuna FIP	TGGTCGGAATGTTAGAGTT CGCAGCCTCCATCCTTGTA CTT	41 bp	73.45	49	-
Tuna BIP	TGCAGACGTAGCCATTCTT ACCAGGCTACTTGGCCGAT AA	40 bp	73.55	50	-

Table 8. LAMP assay results for target species

S.N	Sample ID	Species	Amplification (mm:ss)	Anneal (C°)
1	T3	*Thunnus albacares*	7:45	85.5
2	T4	*Thunnus albacares*	7:00	85.4
3	T5	*Thunnus albacares*	7:45	85.5
4	T6	*Thunnus albacares*	7:30	85.3
5	T7	*Thunnus albacares*	7:15	85.4
6	T8	*Thunnus albacares*	7:30	85.3
7	T25	*Thunnus albacares*	8:45	85.4
8	T26	*Thunnus albacares*	9:00	85.5
9	T27	*Thunnus albacares*	9:30	85.5
10	T28	*Thunnus albacares*	9:30	85.5
11	T29	*Thunnus albacares*	10:00	85.1
12	T30	*Thunnus albacares*	9:30	85.4
13	T31	*Thunnus albacares*	9:30	85.4
14	T32	*Thunnus albacares*	9:15	85.4

15	T34	*Thunnus albacares*	10:00	85.3
16	T35	*Thunnus albacares*	10:00	85.3
17	T37	*Thunnus albacares*	10:00	85.4
18	T38	*Thunnus albacares*	9:45	85.4

Table 9. LAMP assay results for non- target species

S. N	Sample ID	Species	Amplification	Anneal (C°)
1	Ant1	*Oncorhynchus keta* 1	--	--
2	ANT2	*Oncorhynchus keta 2*	--	--
3	ANT3	*Salmo salar*3	--	--
4	ANT4	*Oncorhynchus keta 4*	--	--
5	ANT5	*Gadus morhua 1*	--	--
6	ANT6	*Gadus morhua 2*	--	--
7	ANT7	*Gadus morhua3*	--	--
8	ANT8	*Gadus morhua4*	--	--
9	ANT9	*Gadus morhua*5	--	--
10	ANT10	*Gadus chalcogrammus2*	--	--
11	ANT11	*Gadus chalcogrammus2*	--	--
12	ANT12	*Gadus chalcogrammus3*	--	--
13	T15	*Katsuwonus pelamis*	--	--
14	T16	*Katsuwonus pelamis*	--	--
15	T17	*Katsuwonus pelamis*	--	--
16	T18	*Katsuwonus pelamis*	--	--
17	T20	*Katsuwonus pelamis*	--	--
18	T23	*Katsuwonus pelamis*	--	--

4.4. LAMP assay

LAMP assay was executed in the total volume of 25 µl. The reaction mixture consists of 15 µl of optigene Isothermal Master Mix, 2.5 µl of 10x Standard primer mix and 2.5 µl of Nuclease free water, finally adding 5 µl of template DNA. 10 X Standard primer mix were prepared by mixing F3 (0.2µM), B3 (0.2µM), LF (0.2µM), BF (0.4µM), FIP (0.8µM) and BIP (0.4µM) according to instruction provided by Optigene (http://www.optigene.co.uk/support/). The LAMP reaction was performed at 65°C for 30-40 minutes with melting curve analysis (annealing curve 98°C–

80°C, ramping at 0.05 per min) in a portable real-time fluorimeter (Genie II®, Optigene, Horsham, UK) (Table 8 and9).

4.5. Gel Electrophoresis

The detection of *T. albacares* was confirmed with gel agarose electrophoresis. 2 % gel was prepared with agarose powder (Universal, VWR International GmbH, Darmstadt, Germany) with GelRed™ (Biotium, Eching ,Germany) as staining agent. Gel electrophoresis was done at 5 volts/cm for 90 minutes to observe a clear separation in the amplified product. After electrophoresis, the DNA was visualized and documented with gel doc (Gel Doc EZ Imager, Bio-Rad, München, Germany). The obtained results were compared with DNA ladders of different sizes (Quantitas, Biozyme, Hessisch Oldendorf, Germany).

4.6. Direct Detection by MSwab

For a quick and simple processing step for detection of *T. albacares*, MSwab® method was performed. The dry and sterile swabs (Copan, Brescia, Italia) were applied straight on steaks of *T. albacares*. The dry swab was picked from the supplier packet and scrubbed and rolled firmly several times over the surface of Tuna steak samples. The swab was dipped into an MSwab® buffer tube provided by supplier, inserting the swab head inside the tube. The tube containing a rubbed swab was shaken many times manually without any Vortexing and incubation period. After proper mixing 5 µl of the buffer was used directly for the LAMP reaction as a template in the reaction master mix.

4.7. Analytic sensitivity of the *Thunnus albacares* LAMP assay

Sensitivity of LAMP assay was calculated by 10-fold serial dilution of *T. albacares* DNA (10^{-0} to 10^{-6}). The dilution contains 54 ng/µl to 0.54 pg/µl of DNA. The dilution was made in the AE buffer (Qiagen). Each dilution was run in three parallels to confirm the sensitivity test.

5.Results

5.1. Specificity and the analytical sensitivity of the LAMP assay

The specificity of the LAMP assay was tested with DNA from 18 target species *T. albacares* and 18 other fish species (non-target species) (Table 8 and 9). All *T. albacares* samples were amplified, while no amplification was detected with 18 non-target species. Amplification of non-target species were also confirmed with their respective primers. The amplification reaction was performed with Genie II®. Melting curve analysis by Genie II® (also called anneal curve) showed no significant differences among the different *T. albacares* samples. A melting temperature of 85.5 °C (±.08) was observed for the specific *T. albacares* amplicons (Figure 5).

The analytical sensitivity and amplification rate of the LAMP assay were determined based on serial dilutions (10^{-1} to 10^{-6}). The amount of DNA ranging from 54 ng/µl (10^{-0}) to 540 fg/µl (10^{-6}) was successfully detected with the assay. The analytical sensitivity observed for *T. albacares* was 540 fg/µl in the LAMP assay (table 10, figure 6). 100% detection probability observed in this assay. This concentration can be identified by a detection time of approximately 13 minutes (±1min).

Table 10. Analytical sensitivity of the LAMP assay using serial dilution of *T. albacares* DNA.

	Sample	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-5}	10^{-5}	10^{-6}
	Amount	(54 ng/µl)	(5.4 ng/µl)	(0.54ng/µl)	(54pg/µl)	(5.4pg/µl)	(0.54 pg/µl)	(0.054pg/µl)
Amplification (mm:ss)	Dilution 1 (run1)	7:15	8:00	9:00	10:15	11:45	14:30	-
Amplification (mm:ss)	Dilution 2 (run2)	7:30	8:15	9:30	10.45	12:15	13:15	-
Amplification (mm:ss)	Dilution 3 (run3)	6:45	7:30	8:45	9:45	11:45	13:45	-
mean	-	6.97	7.82	8.92	10.1	11.68	13.63	-
SD±		0.45	0.45	0.43	0.51	0.41	0.60	-

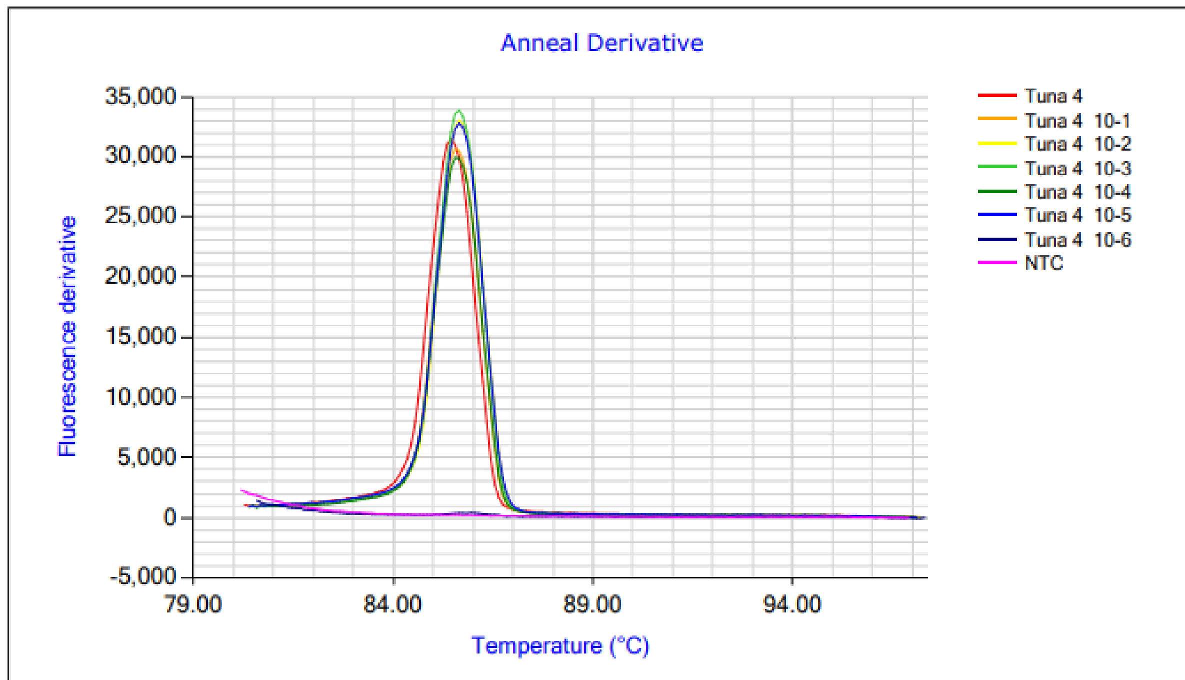


Figure5. The anneal curve reactions of different *T. albacares* reference DNA. The assay shows a melting temperature of 85.5 °C (±.08)

5.2. Direct Detection using MSwab

To avoid time consuming DNA extraction methods a quick and simple sample preparation method was adopted. For this approach application of MSwab method used. The method observed a clear fluorescence curve with detection time of as early as 13 minutes (fig 7). The DNA extracted by the DNeasy Tissue Kit showed detection time of as early as 7:30 minute (fig 6). All reactions showed an annealing temperature of 86.5 °C (±.08).

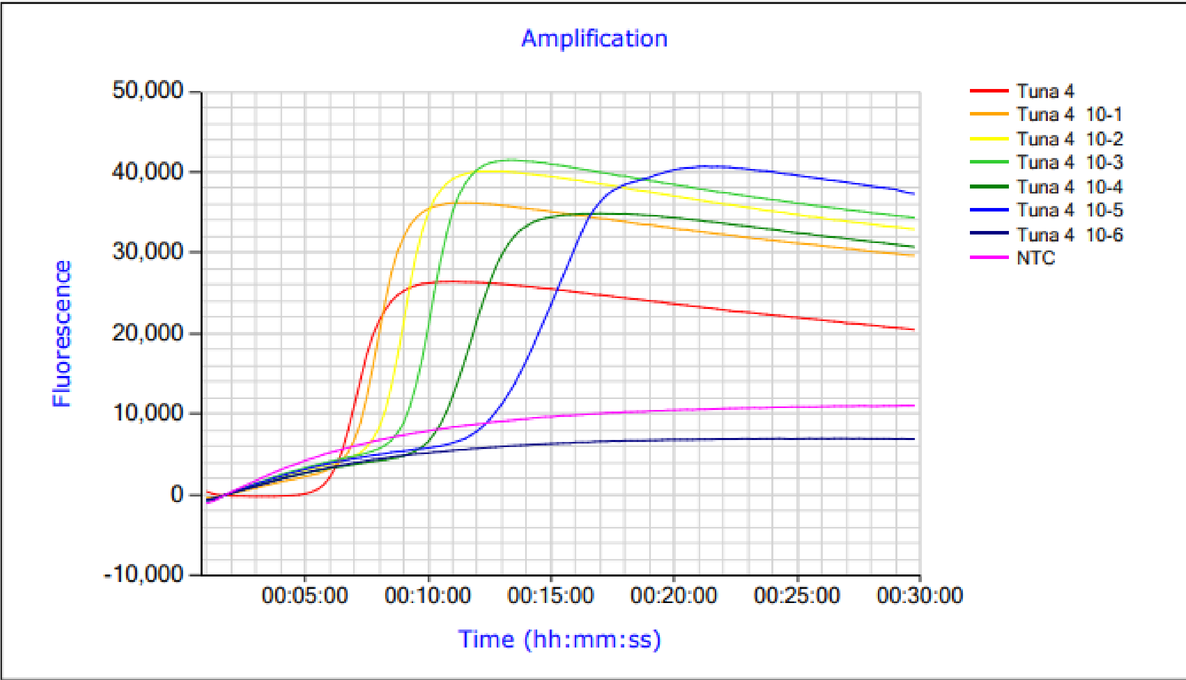


Figure 6. Amplification profile of *T. albacares* LAMP assay with different dilutions. T4 is one of the *T. albacares* reference DNA.

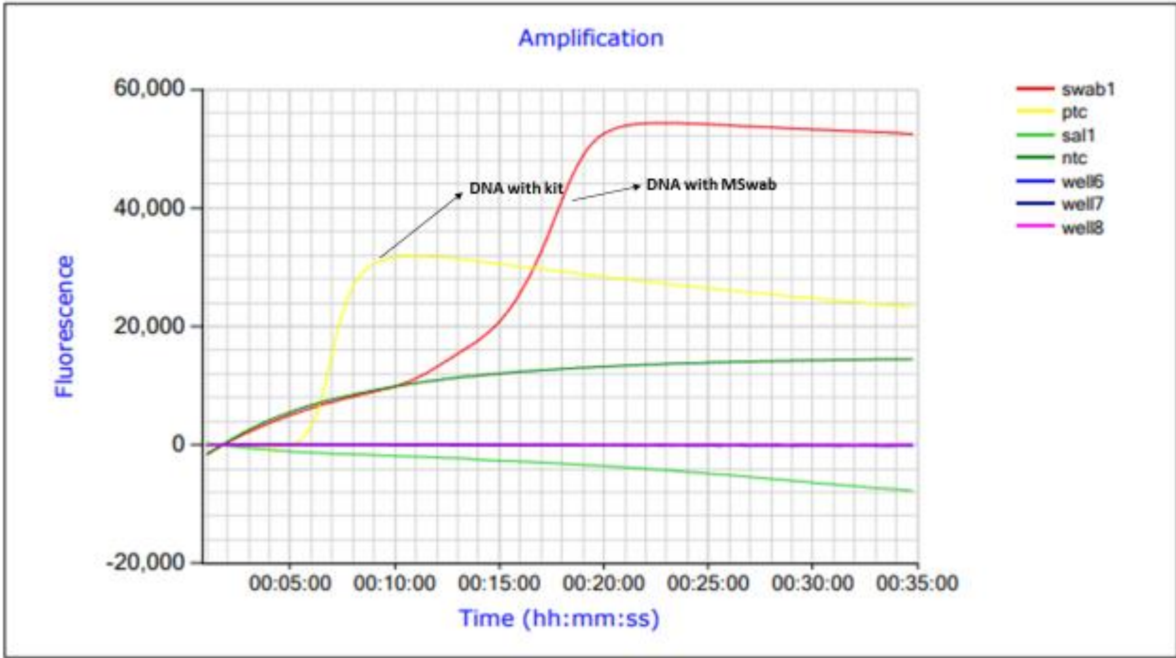


Figure 7. Amplification profile of *T. albacares* LAMP assay with simple extraction (MSwab) and comparison with Qiagen Kit extraction.

5.3. Detection by Gel Electrophoresis

Gel electrophoresis was performed with amplicons for all dilution (10^{-1} to 10^{-5}). DNA band from all diluted amplicons clearly visible after the run. Markers of 2000 bp to 50 bp size was used (fig 8)

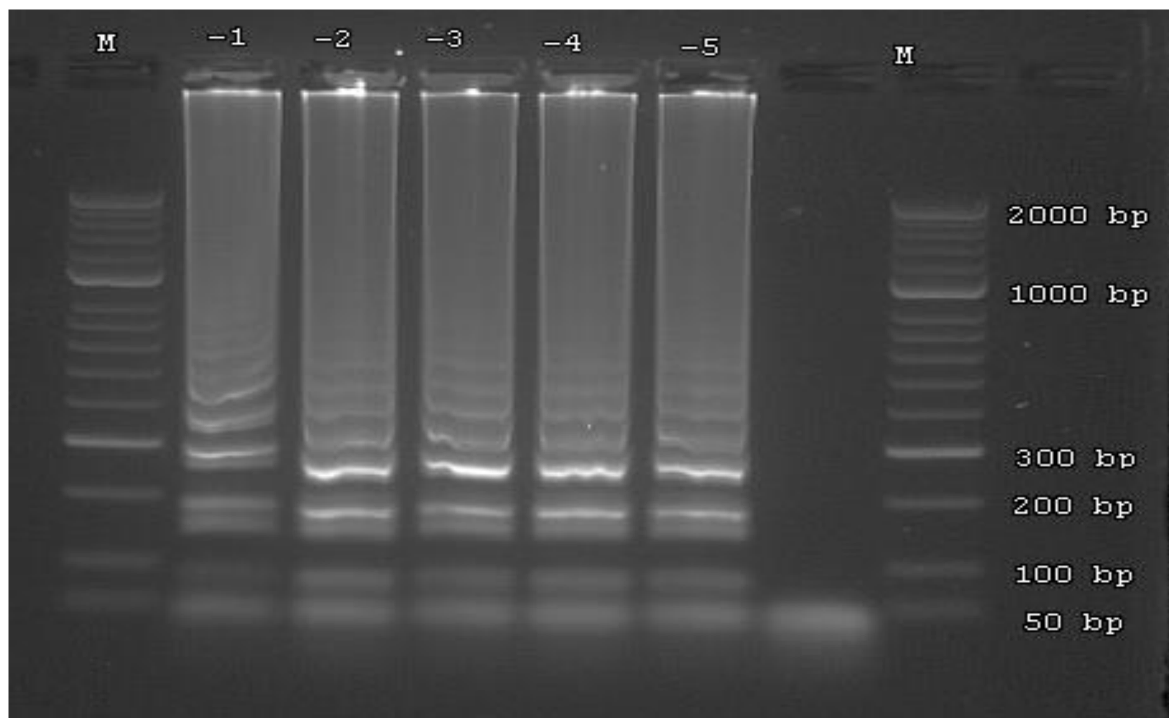


Figure 8. Confirmation of amplified DNA with Gel Electrophoresis. All diluted samples were detected with gel electrophoresis.

6.Discussion

Proper identification of fish species is very important for food safety, public health, and prevention of food fraud. Due to growing economic value species substitution in fish products are quite rampant leading to health risks and economic loss. According to European and German law, all types of meat products must be correctly labelled with proper nomenclature. According to European Regulation related to food traceability -Reg. (EC)178/200, it is mandatory that origin of all raw materials must be detected readily at all stages of meat processing.

According to EU Regulation, (EC) 1379/2013, seafood labelling requires to include the commercial and scientific names of the fish species [74]. The incorporation of scientific names

instead of common names on fish products make it possible to allow appropriate taxes to be imposed to products and make tracking easier for regulatory compliance and prevention of food fraud. The regulation makes border inspection efficient by permitting early detection of seafood items, which is suggested by the convention on international trade related to endangered species of wild fauna and flora.

There are many molecular methods available for fish species detection. Among them, LAMP has gained a lot of popularity in recent decades. LAMP is one of the most widely researched methods for species identification in plants [354, 355], animals [325, 353, 356-358] and bacteria and herbal medicine [220]. It is quite successful in various applications and appears to be an efficient substitute to the PCR based detection methods not only in food fraud detection [359], but also in diagnosis of microorganisms including bacteria [360-362] and viruses [363, 364]. LAMP is also used in identification of several fish species [349-351], but in our survey its application in detection of *T. albacares* is not yet reported. Although other methods are available for its detection. Most species identification in done by PCR-RFLP [353, 365], species specific conventional PCR or real time PCR [343, 365, 366]. In most of the methods *cytochrome b* gene is used for species identification. Although these methods are quite successful, several limitations associated with them forced scientists to look for other diagnostic assays that are quick, cost effective and portable. LAMP is one of the successful alternatives, which is quite robust and provides portability options for onsite application.

Due to its relatively simple operation manual and portability option, LAMP assay has been among the most successful molecular detection methods and provides a better substitute to PCR based methods in food legitimacy testing [367]. The most challenging task towards establishing a LAMP assay is the design of species specific primers [315]. Both nuclear DNA and mitochondrial DNA are successfully used for primer designing for fish species identification, although use of mitochondrial DNA is more preferred due to availability of multiple copies , high evolving speed and maternal inheritance [337]. The most targeted mitochondrial genes for species identification includes *cytochrome b*, *12SrRNA,16SrRNA* and *Cytochrome c oxidase subunit I* (*COI*) [368].

In this study, all *T. albacares* samples collected from different supermarkets were positively amplified using newly developed LAMP assay. None of the other fish species used for control purposes displayed any cross-reaction with the developed assays. The high specificity of LAMP assay for authentication of *T. albacares* is due to successful design of primer which ensures sufficient mismatches with non-target species [365]. Ideally 6-8 mismatches per primer set are required, and mismatch at 3′ end of B2 region of BIP and the 5′ end of the B1c region of BIP is desirable to stop LAMP auto –cycling [315]. To ensure specificity the set of six primers with eight binding sites must hybridize correctly to their target gene sequence before DNA amplification occurs.

The analytical sensitivity observed in this assay was less than 1pg DNA. It is due to the use of six primers designed with LAMP designer, which ensures proper mismatches with the other fish species even within the tuna family. Due to use of lop primers more amplicons were produced during the amplification cycle, however the higher reproduction of the target amplicon is associated with an increased possibility of cross contamination of subsequent samples of aerosolized products [369]. To avoid the risk of cross contamination the present assay used a closed system tube (Genie II®) in which reaction vessels was never opened either during the reaction or after the reaction. Another advantage of this real time fluorimeter compared to conventional gel electrophoresis systems is the semi quantitative result of analysis of different meat products including fish.

For specificity testing the respective non-target species were also cross confirmed with their counterpart primers designed by Primer explorer 5. Sensitivity test of LAMP assay was done by 10-fold serial dilution of *T. albacares* (54ng-540fg) and the lowest concentration of DNA which can be detected with the LAMP assay was selected as sensitivity. The minimum amount of detectable DNA found was 540 fg (Table 10) which was further confirmed by gel electrophoresis (figure 8). The LAMP assay is quite robust in the presence of PCR inhibitor substances like salt, spices and oil as it was able to detect DNA isolated from various processed and canned products [370]. The quick and short amplification time and low susceptibility towards inhibitors compared with conventional PCR are noteworthy properties for LAMP assay in the investigation of food samples in restaurants and in the canned products [370, 371].

Another important parameter achieved with this assay is simple and direct detection of *T. albacares* without using any kit or time-consuming DNA extraction process. MSwab supplied by Copan, Italy, was used for direct and simple detection of target species. The method gives positive results without undergoing a tedious DNA extraction process. However, the amplification time was longer than DNA extracted with kits (figure 7). Overall, this is much less time taking than conventional PCR. The assay was able to detect the target species in 13 minutes with direct swab methods. In case of the kit-based method the detection time obtained was 7 minute (figure 7). Despite slightly longer amplification time with MSwab method compared to kit the assay provided an accurate identification within 20 minutes using portable equipment. The application of Genie II® in food analysis will allow the food safety authorities to carry out the test in the field, in the restaurant, retail shops and in processing plants. The other advantage associated with LAMP technology is lower cost for instrumentation which can be combined readily with real time fluorimeter like Genie II®.

7.Conclusion

The LAMP assay provides a simple, rapid and reliable method of identification for *T. albacares.* The other advantage is that without undergoing a complex DNA extraction process it can detect the target species in less than 20 minutes. The method can be used successfully for identification of inappropriately labelled fish products directly at restaurants or at retail level.

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