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ISAAC – Innovative SeAfood AuthentiCation: DNA-Based Solutions to Promote Safety and Sustainability in the Global Supply Chain

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"We shouldn't lose hope. It can be tempting to give up when confronted with the scale of humanity's consumption and the speed with which are changing the climate and losing the natural world.

But nature is our greatest ally."

- Sir David Attenborough

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Abstract

In recent years, processed seafood products have become increasingly appreciated by consumers as a practical, affordable, and widely accessible way to benefit from the nutritional properties of fish. However, the processed seafood sector is characterized by highly fragmented and globalized supply chains which, combined with the loss of species-specific morphological traits during processing, facilitates the occurrence of fraudulent practices, such as the substitution or the inclusion of undeclared species, particularly in mixed fish products. In this context, strengthening systems for processed seafood authenticity, safety, and transparency has become increasingly critical. DNA metabarcoding, made possible by the advent of high-throughput sequencing (HTS) technologies, currently stands as the only molecular approach capable of reliably authenticating potentially multi-species seafood products. In the absence of standardized analytical protocols, targeted methodological evaluations are needed to guide its implementation and fully utilize its potential. Within this framework, the objective of the ISAAC – Innovative SeAfood AuthentiCation doctoral thesis was to contribute to the development of effective DNA metabarcoding strategies for the authentication of processed seafood products. *In-silico* primer assessments, followed by *in-vitro* applications, provided insights that support the definition of robust multi-marker approaches. Species composition analyses of products sourced from European retailers and in different supply chain stages revealed frequent mismanagement of raw materials by global processors, the presence of harmful species, and gaps in labelling regulations. Considering the outcomes of this thesis, DNA metabarcoding for seafood authenticity can be considered as a sufficiently mature approach to be adopted by various stakeholders across the supply chain, particularly when based on the "gold-standard" Illumina platform. While further methodological refinements may still enhance its performance in the coming years, DNA metabarcoding already represents a robust solution to ensure food safety, prevent species substitution fraud, and contribute to ongoing efforts toward the sustainable use of marine resources.

Preface

Species substitution is a critical food safety issue, with implications ranging from trade relations to consumer trust. The seafood supply chain is particularly vulnerable to species substitution due to the astounding diversity of exploited aquatic species interplaying with extensive product processing, the presence of long, complex supply networks involving multiple intermediaries, economic incentives for mislabelling, and inconsistent regulatory monitoring. These factors not only compromise the transparency of supply chains and hinder the implementation of effective "From boat to plate" traceability strategies, but also undermine efforts to achieve sustainable fisheries management, which relies on accurate species identification and reliable reporting. As a result, current traceability and tracking systems remain fragile, making effective monitoring particularly challenging. The significant number of mislabelling cases reported is the result of increased efforts to study the phenomenon and to detect cases which would have remained undetected without such investigation. Nevertheless, comprehensive and comparable statistics on the prevalence of species substitution remain scarce, partly due to the lack of harmonised monitoring frameworks. In this context, the adoption of advanced molecular analytical tools has become essential for verifying species authenticity of processed products from both official control authorities and industry itself to implement an effective Food Safety Management System.

1. Introduction

1.1. Blue foods

Aquatic organisms form a critical part of the human food chain, with significant socio-economic and cultural value in many regions of the world, contributing to food security, nutrition and poverty mitigation (Kron, 2014; Tigchelaar et al., 2022). Seafood - also known as blue foods - includes all marine and freshwater animals, whether wild or farmed, including species of major commercial interest such as fishes, crustaceans (e.g., prawns, crabs, lobsters), molluscs – including bivalves (e.g., clams, mussels and oysters) and cephalopods (e.g., octopus, squid and cuttlefish) – as well as echinoderms, cnidarians, and various other marine animal groups spread worldwide (Naylor et al., 2021). The biodiversity underpinning human nutrition is immensely vast and complex, requiring periodical monitoring to ensure that changes in its trends are recorded (Garibaldi and Busilacchi, 2002; Blasco et al., 2020). From a nutritional perspective, seafood is an excellent source of macro and micronutrients, such as long-chain polyunsaturated omega-3 fatty acids, proteins, vitamins and minerals, including vitamin D, vitamin B12, selenium, phosphorus and calcium, strongly associated with health benefits, support brain and nervous system function, and anti-inflammatory properties (Khalili Tilami and Sampels, 2018; Aakre et al., 2019). In 2022, global fisheries and aquaculture production reached a record 223.2 million tonnes, largely driven by the recent expansion of aquaculture, with future projections suggesting a further 10% increase in 2032 (FAO, 2024). Recent data indicate that apparent per capita consumption of seafood is estimated to be 20.7 kg in 2022 (FAO, 2024), reflecting continued global demand for these nutritionally valuable products. The consequent impact of this enormous and constant demand for natural resources has led to increasing exploitation of fish stocks worldwide, with overfishing having a historically unprecedented effect on the health of some fish stocks, as for the notable decline of cod in the North

Atlantic (Pauly et al., 2002). Beyond that, other practices such as bycatch and Illegal, Unreported and Unregulated (IUU) fishing have a direct impact on the conservation of marine resources often leading to some of them being included into the Red List of the International Union of Conservation Nature (IUCN) or even contributing to biodiversity loss (WWF, 2019). These considerations led the Organization of the United Nations (ONU) to include, among the 17 Sustainable Development Goals (SDGs), the conservation and sustainable management of oceans, seas and marine resources (Goal 14) delineated in the 2030 Agenda for Sustainable Development. In the fishing industry, sustainability is considered achieved when not too many adult individuals are caught, ensuring that enough remain in the water to sustain the population across generations (Fromentin et al., 2023). Progress towards this goal remains modest, with global annual trends indicating that the proportion of fish stocks within biologically sustainable levels continues to decline steadily[1]. Nevertheless, recent changes in these negative trends have been observed in certain regions, thanks to the development of specific management plans, such as for some demersal stocks in the Western Mediterranean Sea (European Commission, 2024) and in the United States of America waters (NOAA, 2023). Indeed, various measures to promote sustainable practices and mitigate the environmental impact of overfishing are being adopted, including the implementation of stricter fishing quotas, the development of marine protected areas, and the promotion of aquaculture as a sustainable alternative to wild-caught seafood[2]. In addition, efforts to reduce by-catch, the unintentional capture of non-target species, have been intensified through a combination of regulatory measures and the engagement of non-governmental organisations (NGOs) (NOAA), 2016)[3]. These initiatives have played a crucial role in raising awareness on sustainable fishing practices within the seafood industry and among consumers, driving the success of voluntary certifications, such as the Marine Stewardship Council (MSC) (Travaille et al., 2019). Also, technological advancements such as blockchain traceability systems have been employed to ensure transparency and accountability throughout the seafood supply chain (Blaha and Katafono, 2020).

[1]https://www.fao.org/sustainable-development-goals-data-portal/data/indicators/1441-fish-stocks-sustainability/en

[2]https://www.fao.org/gfcm/news/detail/en/c/1733981/

[3]https://www.fao.org/flw-in-fish-value-chains/resources/articles/the-role-of-eu-laws-and-policies-in-bycatch-reduction/en/

1.2. The Seafood Supply Chain: Challenges and Strategies

Millions of people worldwide rely on fishing as their primary livelihood, harvesting a vast array of species across all the world's oceans, ranging from small-scale independent fishers to industrial trawlers. China remains the leading global producer and exporter of seafood, followed by Norway and Vietnam, whereas the European Union stands as the largest importer of aquatic animal products, primarily salmonids, cods, shrimp, tuna, and squid. The degree of industrialization, economic development, and consumer lifestyles across different regions significantly influence how aquatic foods are marketed. In high-income countries, there is a notable preference for high-value, ready-to-eat seafood products, whereas in low-income countries, the market is predominantly oriented toward lower-value species that can be freshly sourced or processed for long-term storage/trade (FAO, 2024). Beyond these influences, the processing and preservation of seafood have an ancient origin, primarily driven by the need to extend shelf life for long-distance trade, considering the intrinsically highly perishable nature of fish, as exemplified by the traditional fermented fish sauce of ancient Rome, the *garum* (Grainger, 2020). Over the past decades, significant advancements in processing, refrigeration, freezing, and transportation technologies have not only played a pivotal role in extending the shelf life of fishery products, but have also facilitated the globalization of seafood trade, enabling processed seafood to reach diverse markets worldwide (Hassoun et al., 2022; Rabiepour et al., 2024). The selection of fish species used in processed seafood products varies globally, influenced by regional preferences, availability, and culinary traditions. For instance, in the United States and Europe, numerous species of high commercial value from Salmonidae, Scombridae, and Gadidae families are widely consumed and predominantly processed into smoked, canned, or breaded fillets, respectively (Granata et al., 2012). Similarly, Clupeidae and Engraulidae (e.g., herring and anchovies) are highly valued in diverse preparations - including pickling, salting, and fermentation - and take parts in traditional cuisines across Latin America, Southeast Asia, and many Europeans countries (Stefánsson et al., 2000; Narzary et al., 2021; dos Santos Fogaça et al., 2023). In Asia, commonly processed seafood species belong to the Nemipteridae, Sauridae, Mullidae, and Priacanthidae families, serve as primary raw materials for surimi production, a

highly popular commodity in the Asian seafood industry (Pangsorn et al., 2007). Beyond regional preferences and culinary traditions, globalization has expanded the demand for these products worldwide, driving their integration into international markets (Gephart and Pace, 2015). Processed seafood refers to aquatic organisms that have undergone various preparation techniques, including filleting, mincing, cooking, salting, smoking, drying, marinating, and/or enrichment with additional ingredients (e.g., oil, vegetables, eggs, jellies, starches, and flavour enhancers) to produce ready-to-cook or ready-to-eat foods. The growing demand for these products reflects broader shifts in global dietary habits, where convenience, accessibility, and culinary diversity play an increasingly central role in food consumption patterns. Typical examples of modern convenience seafood products include canned fish, surimi, breaded crab claws, breaded or buttered fillets, nuggets, fish cakes, and fish burgers. These products facilitate meal preparation and are primarily distributed through mass retail channels, including grocery stores, supermarkets, hypermarkets, and online platforms, providing consumers with convenient access to these goods (European Commission, 2021). However, the point of sale represents only the final stage of a highly complex supply chain, and few are as intricate as those involving seafood, particularly if processed (Hopkins et al., 2024). From primary producers, such as fishermen and aquaculture farmers, raw materials undergo multiple stages of processing and distribution, often involving international trade interactions: seafoods are, indeed, transferred to primary processors, wholesalers, secondary processors, dealers, distributors, and end buyers before reaching the final consumer (Fox et al., 2018; Shehata et al., 2018). Thus, the vast diversity of species processed and handled by global stakeholders makes this sector one of the most vulnerable to mistakes and food crimes. The interconnected and interdependent nature of the global food system requires an integrated strategy to effectively mitigate fraud risks (Smith et al., 2017). Food companies are responsible for ensuring the quality and safety of their products, and bear responsibility for food fraud, even when they are themselves the victim. In response to these challenges, food industry standards have evolved significantly in recent years, establishing guidelines and best practices to prevent and mitigate food fraud risks. One of the most widely adopted industry frameworks, the Global Food Safety Initiative (GFSI), mandates explicit requirements for food fraud prevention, including the assessment of food fraud

vulnerabilities and the implementation of control plans [4]. This is also incorporated into certification programs, such as BRCGS, FSSC 22000, and IFS, facilitating global harmonization of food safety standards (Robson et al., 2021; Cavelius et al., 2023). In this way, the application of a Vulnerability Assessment and Critical Control Point (VACCP) approach becomes an integral part of a company's Food Safety Management System, enabling the implementation of its own preventive measures, including the integration of innovative analytical techniques such as molecular testing (Butler et al., 2021).

This standard, however, offers general guidance for the food industry across all supply chains, whereas sector-specific guidelines could further facilitate the implementation of targeted countermeasures (Robson et al., 2021). For instance, in the specific case of the seafood industry, which relies on a wider range of species, most of which caught from the wild, an alternative strategy to ensuring traceability throughout the supply chain is to voluntarily adhere to sustainability certification schemes. Eco-labels such as the Marine Stewardship Council (MSC, for wild-caught

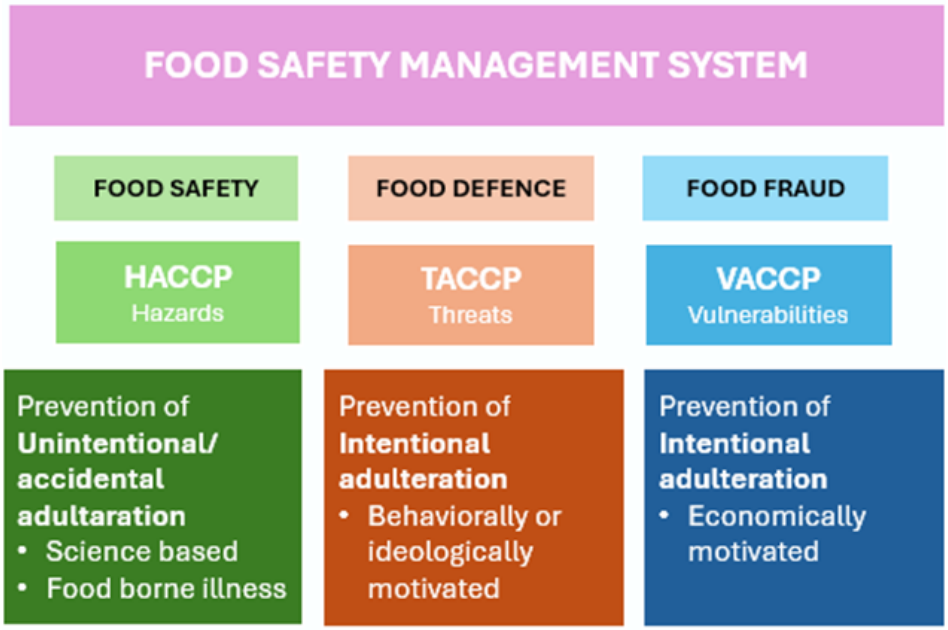

Figure 1.1.: Structure of a Food Safety Management System (FSMS), emphasising the integration of HACCP, TACCP and VACCP to address hazards, threats and vulnerabilities related to food safety, defence and fraud throughout the supply chain.

[4]https://mygfsi.com/wp-content/uploads/2019/09/Food-Fraud-GFSI-Technical-Document.pdf

fish resources) and the Aquaculture Stewardship Council (ASC, for certified farmed products) not only require that their products comply with strict sustainability criteria, but can also help ensure that the Chain of Custody (CoC) is respected and carefully monitored from the moment of harvest to the final sale to consumers (Jardim and Currey, 2023). These CoC standards concisely require compliance with the following five principles:

- Principle 1: certified products are purchased from a certified supplier;
- Principle 2: certified products are clearly identifiable;
- Principle 3: certified products are separated from non-certified;
- Principle 4: certified products are traceable, and volumes are recorded;
- Principle 5: management system addresses all requirements of the Standard.

Figure 1.2.: Official logos of the MSC (Marine Stewardship Council) and ASC (Aquaculture Stewardship Council) certification schemes

1.3. Food Frauds and Authenticity Implications

Food quality is a multifaceted concept that encompasses sensory attributes, nutritional value, safety, and authenticity. A product is considered authentic when its intrinsic characteristics align with its declared attributes, which may include geographical origin, production methods, ingredient composition, and ethical or environmental claims (Haynes et al., 2019). While the food industry and regulatory frameworks have traditionally focused on safety concerns related to pathogens, chemical contaminants, and physical hazards, food authenticity and frauds have emerged as relatively recent, yet increasingly complex, challenges, with both economic and safety implications (Kwasi Bannor et al., 2023). Indeed, non-authentic products not only mislead consumers through commercial fraud (e.g., misrepresentation of ingredients or origin) but may also introduce potential health hazards,

constituting safety fraud (Marvin et al., 2016; European Food Safety Authority (EFSA), 2018; Q. Li et al., 2019). Globally, olive oil, wine, honey, dairy, seafood, and herbs are among the most frequently reported food products subject to fraud, with each category vulnerable to different types of adulteration and deceptive practices (Everstine et al., 2024). Despite the widespread occurrence of food fraud, a globally standardized definition remains absent. Academics have proposed various definitions, all converging on a core element: the intent to gain a financial advantage (J. W. Spink, 2019). Economically motivated adulteration (EMA) causes annual financial losses estimated between 8 and 12 billion euros, while also eroding consumer trust in the food supply chain. The European Commission outlined four main fraud categories, each with specific subtypes, providing a structured framework for addressing and mitigating fraudulent activities in the food sector [5]:

1. **Adulteration**: intentional addition of a foreign or inferior quality substance or element; by replacing a more valuable substance or element with less valuable or inert ingredients.

    a) Substitution: process of replacing a nutrient (e.g., proteins substituted by fat), an ingredient (e.g., high value oil substituted with lower value oil, authorised GMO product not labelled as GMO), a food or part of a food (e.g., high value fish species substituted with lower value fish species when selling processed products).
    b) Dilution: process of mixing a high-value liquid ingredient with a liquid of lower value to expand the total volume and reduce the concentration (e.g., addition of water to fruit juice or to milk).
    c) Removal: removing a constituent that should have been present in the product (e.g., removing of essential oils from herbs and spices sold as whole spice; removing piperine from pepper; removing omega 3 components from fish).
    d) Unapproved/undeclared enhancement: adding unapproved and undeclared compounds to food products in order to enhance their perceived quality attributes (e.g., melamine in milk; use of unauthorised chemicals in spices).
    e) Unapproved/undeclared treatment, process or product (e.g., pesticides,

[5] https://knowledge4policy.ec.europa.eu/food-fraud-quality/topic/food-fraud_en

growth promoters; handling, packaging, transport, storage; decontamination; veterinary medicine; GMO).

f) Concealment: process of hiding the low quality of food ingredients or products (e.g., products treated with food improvement agents or unauthorised additives).

2. **Counterfeit**: Intellectual Property Right (IPR) infringement, including any aspects of the genuine product or packaging being replicated, for instance the process of copying the brand name for economic gain.
3. **Document forgery**: The process of creating, adapting, or imitating documents such as certificates, passports and other identification, administrative documents.
4. **Grey market**: Production, theft, and diversion involving unauthorised sales channels for products (e.g., traceability issues, smuggling, illegal import, production and trade, protected species (CITES), Illegal/uncertain/unregulated area (IUU), Unapproved and unregistered establishment.
5. **Misdescription/mislabelling/misbranding**: Placing of explicit false/misleading/deceptive information on packaging for economic gain (e.g., expiry/production date, nutrition/health claims, geographical claims, quality terms, animal welfare, method of manufacture/production/capture/breeding techniques).

Seafoods are particularly vulnerable to authenticity issues, as seafood species substitution can also involuntarily happen among similar looking congeneric species, that complicate their accurate identification (FAO, 2022). Common crimes in the seafood sector include species substitution, fishery substitution, Illegal, unreported and unregulated (IUU) substitution, species adulteration, chain of custody abuse, catch method fraud, undeclared product extension, modern day slavery, animal welfare, illegal processing and illegal or unauthorized international trade (Fox et al., 2018; Lawrence et al., 2022).

In the seafood sector, species substitution is the most frequent type of fraud reported in the literature (M. Pardo et al., 2016; FAO, 2018). However, a meta-analysis of various food fraud incident notification platforms did not confirm this trend, suggesting that inadequate detection methods used by competent authorities may hinder their ability to regulate, control and prosecute instances of mislabelling (Lawrence et al., 2022). Species substitution is primarily driven by economic in-

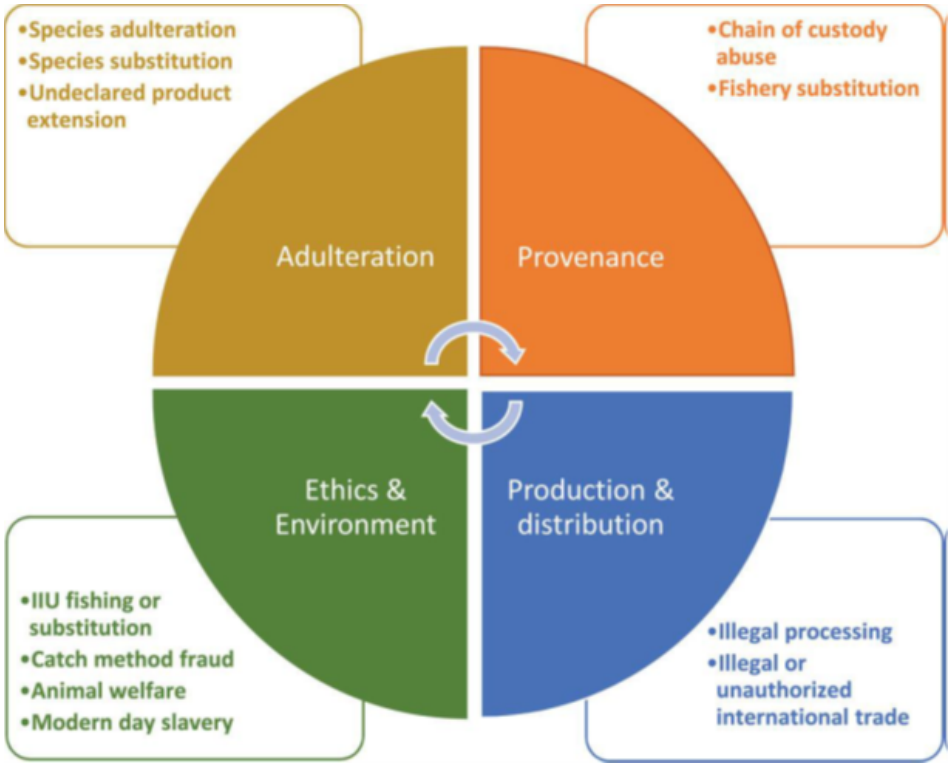

Figure 1.3.: Schematic representation of the 11 types of seafood fraud, classified into four main categories, as described by Lawrence et al., 2022

centives, as substituting a high-value species with a cheaper alternative allows unscrupulous operators to maximize their profits. The complexity and globalization of the seafood supply chain complicate this issue, creating opportunities for fraudulent activities to go undetected (Luque and Donlan, 2019). Beyond economic implications, such unfair practices can pose serious health risks to consumers (J. Spink and Moyer, 2011). For example, cases have been reported where toxic species, such as members of the Tetraodontidae family, have been placed on the market through mislabelled products (N. Li et al., 2015; Giusti et al., 2018; Piredda et al., 2022). These species contain tetrodotoxin (TTX), a potent neurotoxin produced by symbiotic bacteria in fish, and their trade and associated risk of poisoning from the consumption have traditionally been linked to imported tropical species (Cohen et al., 2009). However, climate change and Lessepsian migrations have led to a rising presence of TTX-associated species in the Mediterranean Sea (Sabatino et al., 2024). Ciguatera is another seafood-borne food poisoning linked to the consumption of tropical fish species, such as those belonging to *Lutjanus* sp., which

have bioaccumulated ciguatoxins, lipophilic neurotoxins of dinoflagellate origin (Friedman et al., 2017; Loeffler and Spielmeyer, 2024). Several outbreaks in Europe have been correlated to imported mislabelled congeneric fish species with high ciguatera risk (Friedemann, 2019, Kusche and Hanel, 2021). Seafood contains some of the highest risk allergens, with molluscs, fish and crustaceans being major allergy causes. Failure to disclose their presence is a violation of the EU food labelling Regulation (EU) 1169/2011, which requires clear labelling to protect allergic consumers. Food safety risks are further exacerbated when seafoods originate from IUU fishing activities, which bypass regulations, traceability requirements, and undermines sustainability, making it difficult to monitor the safety and legality of the products entering the market (Borit and Olsen, 2012). Indeed, the absence of control increases the chances of seafood originating from contaminated waters or involving species that are not subject to proper health controls. A major concern is the introduction of harmful contaminants, including dioxins, dioxin-like PCBs, and heavy metals, into the human food chain (FAO & WHO, 2024). Moreover, certain marine organisms with long life cycles (e.g., sharks, tunas, swordfish) are particularly inclined to bioaccumulation of contaminants (biomagnification phenomenon), making it advisable for vulnerable consumers, such as pregnant women and children, to limit their consumption (European Food Safety Authority (EFSA), 2018). Beyond health concerns, the preference for certain species, may be influenced by ethical and dietary choices. In this context, consumers have the fundamental right to access safe products and make informed choices that align with their health, values and dietary preference or requirements (FAO, 2018).

1.4. The European Union's Legislative Framework and Efforts Against Food Fraud

The European Union (EU) shared competences with its Member States in several key areas, with a particular emphasis placed on consumer protection, as outlined in Article 38 of the Charter of Fundamental Rights of the European Union and by Article 12 of the Treaty on the Functioning of the European Union (TFEU, 2010). This legal foundation underscores the EU's commitment to safeguarding consumers' interests across various sectors, ensuring transparency, fairness, and public health. The EU, thanks to a robust body of legislation to ensure the safety of food and

feed, is considered a world leader in providing the highest food safety standards. EU policy has undergone significant reform since the early 2000s when, following a series of zoonotic outbreaks, the EU became aware of the close link between human health and food consumption (Sarno et al., 2021). The need to ensure healthy and safe food throughout the production chain and to maintain high quality standards for food, whether produced in the EU or imported, led the EU to introduce a harmonised approach to food safety in all Member States, the Food Hygiene Package and the development of the "farm to fork" approach[6]. Regulation (EC) 178/2002, known as the "*General Food Law*", has been a revolutionary force that established the European Food Safety Authority (EFSA), introduced traceability at all stages of food supply chain and required Food Business Operators (FBOs) to put in place, among other measures, systems and procedures to be able to identify the suppliers of their products (tracking). Indeed, "tracing" refers to the capability to follow the path of a food product from production to distribution, while "tracking" denotes the ability to identify the past locations of a food product. Also, the Regulation (EU) 1169/2011 on the general food labelling requires FBOs to declare on food product labels several details about the goods they sell to make consumer informed about the food they consume, supporting their awareness and confidence. In the specific case of certain seafood product categories (e.g., whole fish, fillets, minced, smoked fresh and frozen products), the EU has introduced Regulation (EU) No. 1379/2013, which mandates the inclusion of key information on seafood labels, such as the scientific name of the species, the commercial designation, the FAO fishing area, the production method (wild-caught or farmed), and whether the product has been previously frozen. However, in the case of processed seafood products (e.g., canned, pickled, breaded) it is generally sufficient to list the presence of fish among the ingredients, following the indication of the general food labelling Regulation. In addition to the various regulations that the EU has adopted to ensure food safety and hygiene, attention has recently been placed on promoting fair trade practices and integrity. Interestingly, Article 9(2) of Regulation (EU) 625/2017 established for the first time the requirement for competent authorities to carry out regular official controls to detect deliberate non-compliance through misleading or fraudulent practices. In this direction, Regulation (EU) 1715/2019 has introduced the EU "Food Fraud Network", composed of the Commission,

[6]https://www.europarl.europa.eu/factsheets/en/sheet/51/food-safety

EUROPOL, and the liaison bodies designated by the Member States, with the specific purpose of facilitating the exchange of information on fraud notifications. Thus, any "*suspected intentional action by businesses or individuals with the aim of deceiving purchasers and obtaining undue advantage therefrom, in violation of the rules [...] of Regulation (EU) 625/2017*" must be reported within the i-RASFF (Rapid Alert System for Food and Feed) electronic system as a "fraud notification". This definition makes it possible to identify the key elements that characterise agri-food fraud for the EU, such as the violation of its rules, the deception of consumers, the undue advantage and the intention - i.e., when several factors suggest the voluntary nature of food operators to take advantage of consumers. To this end, the same Regulation establishes EU reference centres for the authenticity and integrity of the agri-food chain. Also, the EU is assisted by the European Anti-Fraud Office (OLAF), which since 1999 has been investigating matters relating to fraud, corruption, and other illegal activities affecting the financial interests of the EU, including in the agriculture sector (OLAF, 2023).
In Italy, a key role in ensuring compliance with food regulations and protecting consumer rights is played by the Ministry of Agriculture, Food Sovereignty and Forestry (MASAF). A central part of this effort is carried out by its Department of the Central Inspectorate for the Repression of Fraud in Agri-Food Products (ICQRF), one of the national body responsible for monitoring the agri-food sector, alongside the Ministry of Health (e.g., ASL and NAS). As a member of the Food Fraud Network (FFN), the ICQRF supports cross-border cooperation between EU Member States for food and feed controls under Regulation (EU) 625/2017. With 32 territorial offices and 6 laboratories, its main tasks include carrying out official controls (inspections, chemical analyses, audits and monitoring of e-commerce), combating unfair commercial practices in agricultural and food supply chains and imposing administrative sanctions. The ICQRF also takes part in OPSON, an international operation co-ordinated by INTERPOL and EUROPOL, which aims to combat the counterfeiting of food products and to ensure the authenticity of products across the Europeans borders (ICQRF, 2024).

Regarding official controls, the European Parliament's 2014 resolution highlighted the potential use of DNA analysis in meat and fish products to detect species substitution fraud. However, to date, no validated method for species authentication in animal-derived products has been officially recognized as a reference method

Figure 1.4.: Official logo of the ICQRF-MASAF, one of the Italian authorities responsible for quality control and fraud prevention in the agri-food sector

under any European Regulation . Due to their innovative and opportunistic nature, fraudsters are skilled at exploiting weaknesses in both supply chains and official control systems, therefore, the EU collaborate with several research institutions to carry out investigations and point out analytical methods in the field of food integrity and authenticity (M. Á. Pardo et al., 2018; Haynes et al., 2019).

1.5. Innovative Molecular tools

Seafood species can be taxonomically recognized by fisheries inspectors following the FAO guidelines on the morphological features of fish species, being the only recognized method in the EU for fish species identification. For processed seafoods, the lack of these diagnostic elements exposes this food category to errors or deliberate malpractice that cannot be easily detected. Therefore, various techniques have been developed to verify species authenticity in processed foods, including chromatography, spectroscopy, machine learning-based approaches (Medina et al., 2019; Z. Deng et al., 2024). However, DNA analysis remains the gold standard for species authentication in meat, fish, dairy, and plant-based products, offering superior stability under food-processing conditions (Dooley et al., 2005; Naaum and Hanner, 2015), and remarkable, progressive technological advancement coupled with steadily decreasing costs. Several species-specific molecular techniques have been developed for this purpose, enabling the detection of expected species within a given product. Among these, real-time PCR (qPCR) is widely used, along with

Loop-Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA), which offer rapid and highly sensitive species identification (J. Zhang et al., 2025). Furthermore, droplet digital PCR (ddPCR) has been introduced to detect even trace amounts of DNA, while the CRISPR/Cas system represents a recent and promising advancement in species authentication (R. Deng et al., 2024, C. Cai et al., 2025). However, to overcome the limitation of targeted species detection, DNA barcoding is the most widely adopted approach, particularly for detecting species substitution in fish fillets given the high biodiversity of marine species. This method is particularly effective for single-species products, as it produces a single genetic sequence corresponding to the amplified DNA region. The cytochrome c oxidase subunit I (COI) gene, approximately 650 base pairs (bp) long, is the standard marker for molecular species identification, as proposed by Hebert et al., 2003 for the classification of animal species. COI is preferred due to its high interspecies variability and low intraspecies variation, making it an ideal target for species differentiation. While the FishTrace consortium proposed the cytochrome b gene for DNA barcoding of teleost fish, a subsequent EU-funded project demonstrated the superior performance of the COI gene and produced a specific Standard Operating Procedure (SOP) for fish species identification (Sevilla et al., 2007; Griffiths et al., 2014). DNA barcoding has been extensively used to detect fish fillets substitution events in several countries worldwide (D. D. Miller and Mariani, 2010; Galal-Khallaf et al., 2014; Di Pinto et al., 2015; Xiong et al., 2019; Liou et al., 2020). Additionally, considering the possible presence of degraded DNA in processed products, the use of shorter DNA regions, called mini barcoding, have been successfully applied (Shokralla et al., 2015; Mottola, Piredda, Catanese, Lorusso, et al., 2022). Once the DNA sequence is obtained, it can be compared against genomic sequence databases, such as the Barcode of Life Data System (BOLD) and GenBank (on NCBI), providing an accurate species identification of the analysed tissue (Antil et al., 2023). In cases of species substitution, sequencing can identify the substituted species, allowing at least a tentative interpretation to distinguish between accidental mislabelling and deliberate fraud. Indeed, it is important to highlight that genetic evidence alone is often not sufficient to determine whether a case of species substitution constitutes intentional fraud. Additional elements, such as supply chain documentation, patterns of economic gain, and repeated occurrences, must also be considered to support such an assessment. This

approach has been made possible by first-generation sequencing technologies, such as Sanger sequencing, which provides a single long-read DNA sequence per sample. Innovative solutions to overcome costs and time required for DNA sequencing include the development of a portable, closed-tube barcoding system for fish species identification, called FASTFISH-ID^{TM} (Naaum et al., 2021).

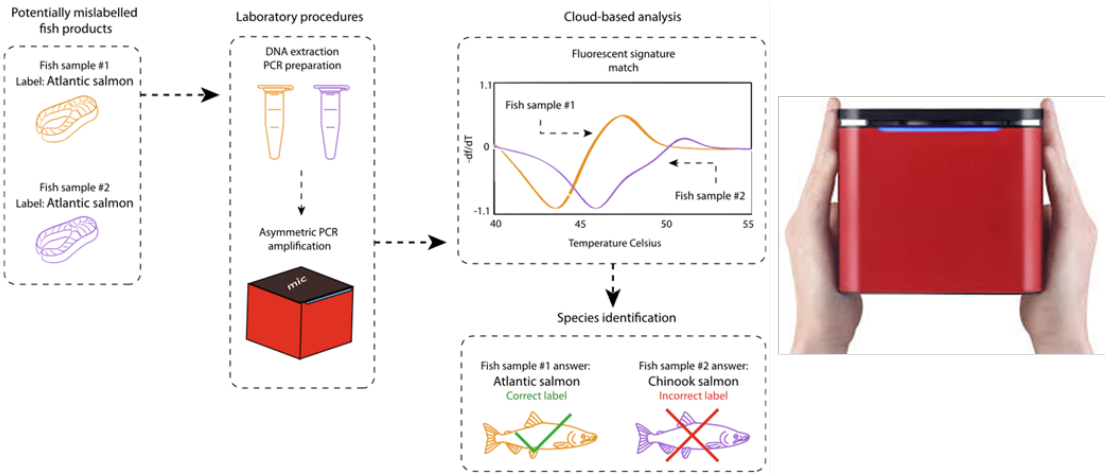

Figure 1.5.: Experimental workflow and principles of species identification using the FASTFISH-ID system from the original paper Naaum et al., 2021. The image on the right shows the handheld qPCR device (Bio Molecular Systems) employed for the analysis.

However, all these tools have intrinsic limitations when applied to highly processed foods, where multiple animal species may be present simultaneously, often mixed or ground together (Staats et al., 2016). The advent of High-Throughput Sequencing (HTS) platforms in the mid-2000s revolutionized the field, enabling an unprecedented increase in sequencing data output through the parallel processing of millions of reads. Unlike Sanger sequencing, the HTS allows for the simultaneous sequencing of multiple different DNA filaments within a single sample, producing the list of all the species included. This technological advancement led to the development of a new approach that integrates the principles of DNA barcoding with HTS technologies, known as DNA metabarcoding (Franco et al., 2021). DNA metabarcoding has already been applied to the authentication of various processed food products demonstrating its great potential (Hawkins et al., 2015; Dobrovolny et al., 2019; Bruno et al., 2019). This approach not only enables the identification of species listed on product labels but also allows for the detection of undeclared species, which, in some cases, have completely replaced the labelled species (Piredda et al., 2022), undeclared allergens as well as detecting traces of species indicative

of poor hygiene conditions in food production facilities (Mottola et al., 2024; Pan et al., 2020). Despite its advantages, the DNA metabarcoding approach requires further optimization for each food matrices, as several methodological choices can significantly influence the results (Giusti et al., 2024). As extensively discussed in the next Chapters, one of the most critical decision involves the selection of the molecular marker(s) and related primer pairs to be applied for the intermediate PCR phase, which must target conserved gene regions, to ensure the detection of all the species present in that product, high sequence variation to allow species-level discrimination, and a fragment length compatible with the sequencing platform used (Arulandhu et al., 2017). A range of bioinformatic tools has been developed to support this selection process, including ecoPCR (G. F. Ficetola et al., 2010), RESCRIPt (Robeson et al., 2021) and CRABS (Jeunen et al., 2023), which was introduced more recently. These tools can be used to create curated reference databases and execute in silico PCR simulations to evaluate primer–template affinities across a wide range of taxa. Although they provide a quicker and more cost-effective alternative to empirical testing, the results should be considered preliminary screening of primer universality. The fragment length is particularly relevant, as HTS platforms, also referred to as Next-Generation Sequencing (NGS), include short-reads and long-reads sequencers. Second-generation sequencing technologies, including platforms from Illumina and Thermo Fisher Scientific, allow sequencing of DNA fragments up to $\sim$ 600 base pairs. In general, Illumina platforms (e.g., MiSeq, NextSeq) perform paired-end sequencing, generating two reads per fragment - one from each end - which contributes to higher data accuracy. In contrast, Thermo Fisher Scientific platforms (e.g., Ion PGM, Ion S5), based on Ion Torrent technology, perform single-end sequencing, generating one read per fragment. Third-generation sequencing platforms, such as Pacific Biosciences and Oxford Nanopore Technologies, enable the sequencing of significantly longer DNA fragments using single-molecule, real-time approaches. In the context of DNA metabarcoding applied to processed seafood authentication, these long-read technologies could facilitate the reading of full-length barcode regions improving taxonomic resolution. Beyond fragment length, each sequencing technology, and related machines, differs in several key aspects, including the number of bases sequenced per run, the complexity of library preparation, the time required for sequencing, the overall cost of analysis, the error rate, the bioinformatics analysis

necessary for data processing, and the space required for the instrument (Hu et al., 2021).

1.5.1. Ion Torrent platform

Ion Torrent sequencing is a semiconductor-based technology that detects DNA polymerization events through changes in pH. The sequencing process begins when millions of short DNA fragments, ligated to adapters, are subjected to emulsion PCR (ePCR). During the ePCR, each fragment is clonally amplified into an individual bead, ensuring that each bead is coated with multiple identical copies of a single DNA molecule for generating a strong signal during sequencing. Once amplification is complete, the DNA-coated beads are deposited into microwells on a semiconductor sequencing chip, which serves as the core detection platform for Ion Torrent technology. The sequencing reaction is initiated by flooding the chip with one of the four deoxyribonucleotide triphosphates (dNTPs). Unlike traditional sequencing methods (i.e., Sanger sequencing) that rely on optical fluorescence detection, Ion Torrent sequencing identifies nucleotide incorporation electrochemically. When a nucleotide is incorporated into the complementary DNA strand, a hydrogen ion (H^+) is released as a byproduct of the polymerization reaction. This ion release alters the pH of the solution within the well. A proton-sensitive layer below the wells measures the pH fluctuations and converts them into a voltage signal, which is then digitally recorded to determine which nucleotide was incorporated. This process occurs simultaneously in millions of microwells, allowing for massively parallel sequencing, which significantly enhances throughput and speed. Every 15 seconds, a different nucleotide is introduced, and the cycle is repeated until the sequencing process is complete. Unlike fluorescence-based NGS methods, Ion Torrent sequencing eliminates the need for lasers, fluorophores, and cameras, making the system cost-effective and highly scalable.

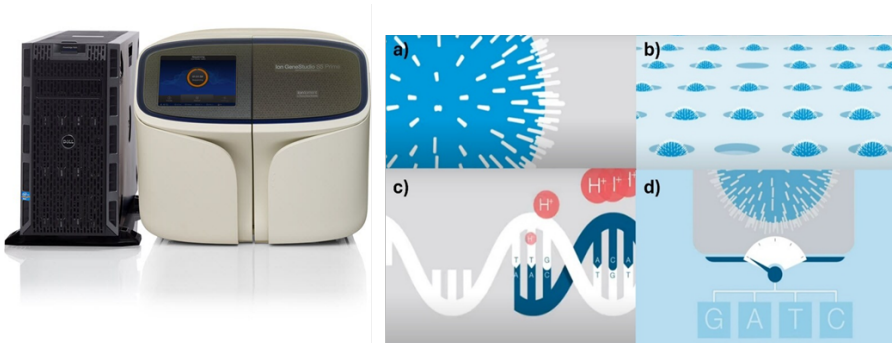

Figure 1.6.: The Ion GeneStudio™ S5 Prime System platform and the schematic representation of the Ion Torrent sequencing process, edited from Thermo Fisher website.

1.5.2. Illumina platform

Illumina sequencing follows a three-step process consisting of library preparation, cluster generation, and sequencing. Library preparation begins with the ligation of adapters to the ends of DNA fragments, enabling their subsequent amplification and sequencing. These adapters contain essential functional regions, including sequencing initiation sites, barcodes for samples indexing, and sequences complementary to those on the flow cell surface, allowing hybridization. Indeed, during cluster generation, library fragments hybridize to the flow cell surface, where each fragment undergoes clonal amplification to form clusters of identical copies. Following attachment, the polymerase enzyme synthesizes a complementary strand, and the newly generated strand subsequently hybridizes to the second type of oligonucleotide on the flow cell surface, producing two identical copies of the fragment. This repeated amplification cycle results in clonal amplification of all library fragments, ensuring high sequencing accuracy and read depth. After amplification, reverse strands are selectively removed, leaving only forward strands available for sequencing. The sequencing process begins with the extension of the first sequencing primer, initiating the first read. Each cycle introduces a mixture of fluorescently labelled nucleotides, from which only a single nucleotide complementary to the template strand is incorporated. After incorporation, the clusters are excited by a light source, emitting a characteristic fluorescent signal. This process, known as Sequencing by Synthesis (SBS), ensures high base-calling accuracy and the number of sequencing cycles determines the final read length, while the emission wavelength and signal intensity define the base call. Upon completion of the first read, the sequencing product is washed away. Following index sequencing, the template folds over and hybridizes

to the second oligonucleotide on the flow cell, allowing polymerase extension to create a double-stranded bridge. This bridge is then linearized, with the original strand selectively removed, leaving only the reverse strand that can be sequenced (paired-end sequencing mechanism) (Westbury, 2018).

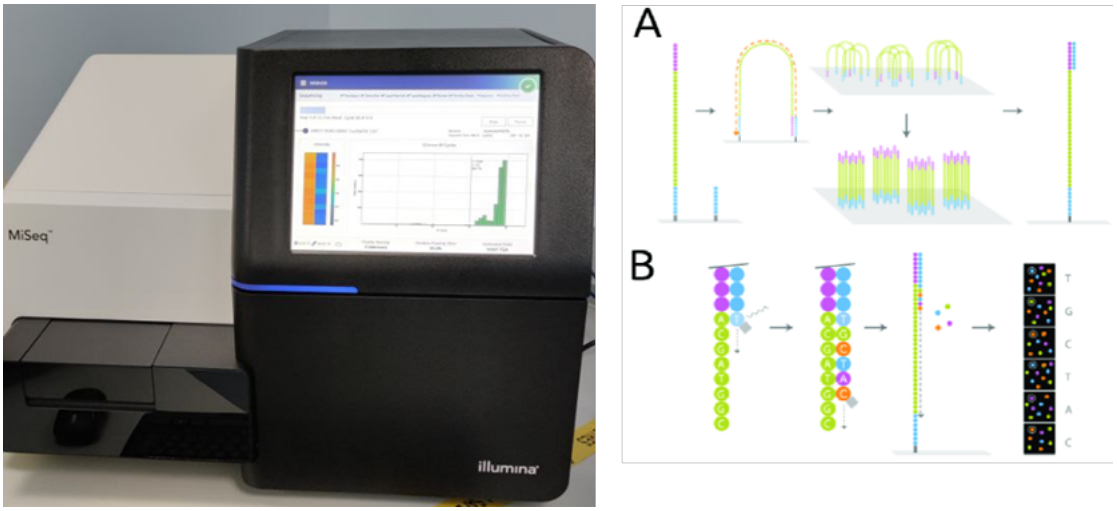

Figure 1.7.: The MiSeq platform and the Illumina sequencing process from Westbury, 2018.

1.5.3. (Re)-Emerging Sequencing-by-Synthesis Platforms

Beyond Illumina and Ion Torrent, pyrosequencing represents another sequencing-by-synthesis approach. Initially developed by 454 Life Sciences and later commercialized by Roche, the 454 platform was the first NGS system based on pyrosequencing technology. This method relies on a cascade of enzymatic reactions in which DNA polymerase incorporates complementary nucleotides into the growing DNA strand, releasing pyrophosphate (PPi) molecules. ATP sulfurylase then converts PPi into ATP, which fuels the luciferase-mediated conversion of luciferin to oxyluciferin, resulting in light emission that is detected in real time by the instrument. Although Roche officially discontinued the 454 platform in 2016, Qiagen has since developed new instruments based on the same sequencing chemistry, such as PyroMark Q24, PyroMark Q96 ID, and PyroMark Q48 Autoprep, which deliver short reads (up to $\sim$ 140 bp, depending on the specific assay) but high accuracy[7].

A completely novel sequencing-by-synthesis chemistry has been introduced by

[7]https://www.qiagen.com/us/product-categories/discovery-and-translational-research/pyrosequencing

Element Biosciences through the AVITI™ System, designed to combine high-quality data with low reagent consumption and reduced costs. At the core of this platform are Avidites, multivalent structures composed of a central core and flexible arms carrying complementary nucleotides. These Avidites are transiently bind to the DNA template by the DNA polymerase. A high-resolution camera records the fluorescent signal associated with the Avidite, identifying the base present at the active site. Following base identification, the Avidite is removed, and the corresponding unlabelled nucleotide is incorporated. Currently, the highest-output flow cell configuration supports read lengths of up to 2 × 300 bp, emerging as a strong competitor to Illumina[8].

[8]https://dnatech.ucdavis.edu/element-biosciences-aviti-sequencing https://www.elementbiosciences.com/products/aviti

1.5.4. Oxford Nanopore Technologies platform

Oxford Nanopore Technologies (ONT) sequencing enables real-time, single-molecule DNA sequencing by detecting changes in ionic current as nucleotides pass through a nanopore. The sequencing process necessitates a specific library preparation, which involves end-repair, adaptor ligation, and barcodes ligation for indexing samples. At the core of this platform there is a synthetic nanopore, a nanoscale engineered channel that originate from a staphylococcal a-hemolysin (aHL) protein pore that generates a stable ionic current across the pore (Kasianowicz et al., 1996). The ONT flow cells are equipped with hundreds to thousands of nanopores. Once the library is loaded, during sequencing, DNA molecules are guided toward the nanopore by a motor protein, which "unzips" the double-stranded DNA and regulates the translocation speed, ensuring optimal signal resolution. As nucleotides pass through the nanopore, they partially block the ionic flow, causing discrete disruptions in electrical current. These fluctuations produce a unique electrical signal, known as a "squiggle" that is then decoded into a nucleotide sequence by an integrated basecalling software. The most significant advantages of Oxford Nanopore platforms are their device portability (e.g., MinION) and their ability to generate long reads making them particularly useful for a wide range of applications (Byrne et al., 2017; Bruels et al., 2022; Amarasinghe et al., 2020)[9].

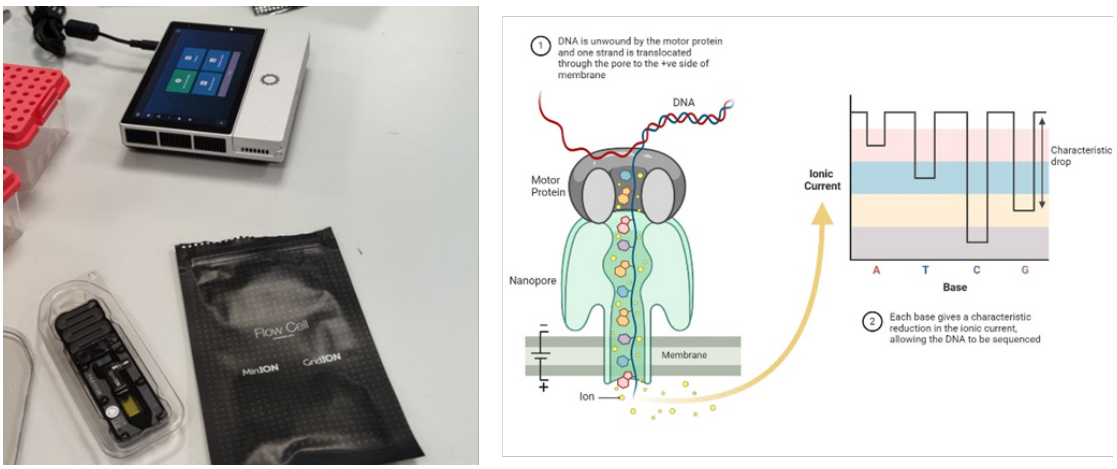

Figure 1.8.: The Mk1C MinION portable device and the principle of Nanopore Sequencing (https://microbenotes.com/oxford-nanopore-sequencing/).

[9]https://nanoporetech.com/blog/how-oxford-nanopore-sequencing-works

1.6. Aims and objectives

In August 2021 the University of Bari Aldo Moro was funded by the Italian Ministry of Education, University and Research (MUR) and the "PON Research and Innovation 2014-2020 - Education and Research for Recovery REACT-EU" scheme, with the aim of developing PhD programs on "Innovation" (Action IV.4) and "Green" (Action IV.5) topics involving companies within the research activities. Specifically, the *ISAAC – Innovative SeAfood AuthentiCation* doctoral project – under the "Innovation" theme, and promoted by the Department of Veterinary Medicine – aimed to contribute to the understanding and application of advanced molecular techniques for the authentication of processed seafood products. To this end, each chapter of this thesis examines a specific aspect of the DNA metabarcoding approach for species authentication in processed seafood, ranging from preliminary *in-silico* testing of suitable primer pairs to their practical applications using dual-marker strategies, utilizing various NGS platforms. Specifically, the doctoral research began with a review focusing on molecular techniques developed for seafood authentication, with a specific case study on mackerel species. This included the compilation of a comprehensive list of primer pairs, for the detection of marine species, previously described in literature (**Chapter 2**). A study was then carried out to *in-silico* evaluate the previously selected primer pairs in terms of taxonomic coverage against commercially important marine organisms as defined by the FAO (**Chapter 3**). Subsequently, two of these primer pairs were chosen for a multi-marker study aimed at verifying the authenticity of declared single species processed fish products sold on the Italian market. This research was conducted in collaboration with the ICQRF laboratory located in Modena (Italy), that was responsible for the Ion Torrent Sequencing (**Chapter 4**). Afterward, two additional primer pairs were employed to assess the species authenticity of convenience seafood sold in three European countries. Laboratory procedures, including library preparations and the subsequent Illumina sequencing, of this study were personally conducted during my six months placement at Liverpool John Moores University (United Kingdom) under the supervision of Professor Stefano Mariani (**Chapter 5**). Simultaneously, Nanopore sequencing was performed on the same samples to evaluate its feasibility for the molecular authentication of processed seafood products by comparing its performance with Illumina-based results (**Chapter 6**).

2. Revisiting molecular techniques for the authentication of mackerels in commercial products: Approaches to prevent seafood fraud

This chapter was published in *Trends in Food Science and Technology*

Authors: **Lucilia Lorusso**, Anna Mottola, Roberta Piredda, Angela Di Pinto, Chiara Intermite, Lucia Ranieri, Gaetano Catanese.

L. Lorusso and A. Mottola contributed equally to this work.

2.1. Abstract

Background: Seafood fraud involves mislabelling and false claims. To avoid this deceptive practice, authentication techniques, including DNA-based methods, have been developed. These techniques detect fraud, ensure accurate labelling, and maintain product integrity, aiding in species identification, verifying origins, and promoting industry transparency.

Scope and approach: This review aims to provide a detailed list of approaches for the authenticity of mackerel species due to the prevalence of misllabeling and substitution. Utilizing techniques like DNA, we can identify *Scomber* species by analyzing specific genetic markers. This work advances the evaluation of existing molecular markers through *in-silico* simulations, while also purposing novel primers designed for a more accurate authentication of processed mackerel products.

Key Findings and Conclusion: With advances in techniques, we are seeing significant advances in food authentication methods. DNA barcoding has seen substantial evolution, thanks to improvements in databases and NGS sequencing technologies. The advent of DNA metabarcoding is also revolutionizing species identification in highly processed seafoods. Using more comprehensive primers in Metabarcoding can enhance the assessment of scombrid products. In recent times, SNP-based approaches with microfluidic enrichment, DNA-based biosensors and microbiome sequencing techniques have emerged as key tools for assessing the authenticity and identifying the origins of seafood products.

2.2. Introduction

Mackerels (*Scomber* spp.), belonging to the family Scombridae, include different species of pelagic fish that are among the most widely fished and traded species, widely used in the food industry to produce fresh, frozen and preserved products. They possess high nutritional value with high content in protein and highly unsaturated fatty acids and therefore mackerels have considerable economic importance for global fishing industry supporting many coastal communities as a source of income for fishermen and an export product. As with other foods, mackerel products can be found on the market in different formats and categories: a) Ready-to-process: fresh mackerel for immediate cooking or freezing; b) Ready-to-use: preserved mackerel such as canned (the product with the highest commercial interest) in natural or oil (sometimes prepared with other ingredients such as onion, tomato, etc.), smoked varieties or Sushi; c) Ready-to-cook: frozen mackerel needing only cooking (products containing mackerel such as Burger Paella,.... etc.); d) Ready-to-eat: pre-prepared and packaged mackerel dishes with a 5-21 days shelf life; and e) Ready-to-heat: vacuum-packed mackerel dishes requiring heating, with a 10-20 days shelf life when refrigerated or frozen. Each category necessitates specific storage conditions to maintain quality and safety.

Due to the important commercial value and the complexity of the seafood supply chains, species substitution of mackerels in market products can often occur. The various species of mackerel exhibit distinct organoleptic characteristics influenced by factors like different species, chemical composition and habitat. These disparities often reflect in market prices, with certain species esteemed higher or sought after more. However, pricing fluctuations can also stem from factors such as regional availability, fishing seasons, and market dynamics.

Aliud pro alio fraud often involves substitution with fish species less expensive or derived from Illegal, Unreported and Unregulated fishing (IUU) with consequences for food safety, and marine resource sustainability (FAO, 2018). Species identification of mackerels (mainly genus *Scomber*) in fresh products is mostly based on morphological identification, such as observation of the lateral line, pattern, and body color, but phenotypes can change with growth, environmental condition making morphological identification difficult. Moreover, after industry processing when distinctive external traits are removed, morphological identification is impossible

(Infante et al., 2006).

In this context, the application of DNA-based techniques is considered effective to accurately determine the species of seafood and the use of nucleotide sequence "DNA barcode" for taxa identification of animal and vegetal species (Hebert et al., 2003) is almost very common. Although a fragment of the cytochrome oxidase subunit I (COI) mitochondrial gene is recognized as the official barcoding region marker for metazoans, its universality in primer sites is often limited as in some taxonomic groups, its rate of evolution is slow, hindering the discrimination of closely related fish species. Thus, the careful choice of a molecular marker and the length of the DNA fragment play a crucial role in species authentication and in the identification of possible food frauds. These factors affect the specificity, sensitivity and accuracy of molecular analyses, enabling the presence or absence of specific species in seafood products to be confidently identified. The choice of highly specific markers for a certain seafood species guarantees the specificity of the amplification, which means that only DNA from that species is amplified and analyzed. This helps to avoid false positives or ambiguous results that could occur if DNA fragments from other species present in the sample are amplified. Choosing the appropriate genetic markers and DNA fragments can also help to detect any cross-contamination or mix of species present in the commercial products (Lago et al., 2011; Armani et al., 2013; Giusti et al., 2017; Mottola, Piredda, Catanese, Giorelli, et al., 2022; Piredda et al., 2022). In some cases, species may be genetically similar and so the use of highly discriminating molecular markers allows closely related species to be accurately differentiated. This is especially important to avoid confusion between species which could easily be mistaken or mixed. Therefore, the length of the amplified DNA fragments can also influence the quality and robustness of the sequencing. However, DNA fragments that are too long may not be obtained due to DNA degradation as a consequence of chemical-physical treatments for product preservation as processing, defrosting, and product deterioration (Quinteiro et al., 1998; Infante et al., 2004). Degradation in heavily processed seafood can produce fragments smaller than 350 bp, often averaging between 100 and 200 bp (Quinteiro et al., 1998). To mitigate degradation issues, researchers typically analyze amplicons smaller than 200 bp. These shorter gene targets, referred to as "mini barcodes" (Hajibabaei and McKenna, 2012; Shokralla et al., 2015), ranging from 100 to 300 bp, facilitate sequencing-based species identification. However, fragments that are

too short, or obtained from molecular markers of highly conserved DNA regions across species, could hardly show a sufficient number of nucleotide positions capable of accurate and reliable species identification.
In this review, were described the status on the application of DNA barcode markers for mackerel's species authentication in fresh and processed market products, focusing on suitable barcode loci, from the classical Sanger to the advanced next generation sequencing (NGS), assessing their effectiveness, accuracy and limitations. In addition, different European legislations were compared to examine the requirements and practices related to seafood labelling across different jurisdictions/Countries.

2.2.1. Mackerel Species

Mackerels include several species of fish. However, although about 21 species in the family Scombridae are commonly called mackerel in the world, the main recognized species with commercial importance, known also as the "true mackerels", belong to the genus *Scomber* are four (**Figure 2.1**):

- *Scomber scombrus* (Mackerel or Atlantic mackerel)[1] has an elongated spindle-shaped body, with a bluish green back and silvery flanks. It has dark "S" shaped stripes on its upper part and a black spot near the gills. It is found mainly in the North Atlantic Ocean. Its distribution extends from North America and Europe to the Mediterranean Sea and the Black Sea. It is especially abundant in coastal waters and in areas with cold, nutrient-rich currents (B. B. Collette and Nauen, 1983).
- *Scomber japonicus* (Pacific chub mackerel)[2], similar to Atlantic mackerel, but is usually slightly larger. It is dark blue on the back and silver on the flanks. It also features irregular stripes on its upper part. It is found in the Indian and Pacific oceans. In the Pacific region, it is particularly common in the Sea

[1]https://fish-commercial-names.ec.europa.eu/fish-names/species/scomber-scombrus_en?fao-code=MAC

[2]https://fish-commercial-names.ec.europa.eu/fish-names/species/scomber-japonicus_en?fao-code=MAS#commdes

of Japan, hence its common name. Also, it is found in coastal areas of Asia. The distribution of the Pacific chub mackerel covers a wide range of latitudes and climates, adapting to different oceanic conditions (B. B. Collette and Nauen, 1983).

- *Scomber colias* (Atlantic chub mackerel or Tinker mackerel)[3] is found mainly in the Mediterranean Sea and the Black Sea. Its distribution is not limited to these areas but also to some regions of the eastern and western Atlantic. The name *S. colias* has been used as an older synonym of *S. japonicus*. However, after recent taxonomic studies it has proposed that *S. colias* should be considered as a different species (B. Collette, 1999; Infante and Manchado, 2006; Catanese, Manchado, and Infante, 2010.
- *Scomber australasicus* (Blue mackerel, Spotted chub mackerel or Australian mackerel)[4] has an elongated spindle-shaped body, similar to other species in the *Scomber* genus. It usually has a dark blue back and silver flanks with dark stripes resembling the letters "V" or "W". It can reach a considerable size, reaching more than 60 centimeters in length. It is found in the waters of the Indian and Pacific Oceans, mainly in the Australasian region. Its distribution ranges from the southeastern Indian Ocean to the southwestern Pacific, including Australia, New Zealand, the Polynesian islands, and parts of Asia Indo-West Pacific: Red Sea, Persian Gulf (Baker and Collette, 1998). Like other mackerel species, Australian mackerel is a migratory fish that moves in search of food and breeding grounds. Its fishing is regulated in many countries to guarantee its sustainability and conservation.

The genus *Scomber* has undergone taxonomic revisions of its species over time. In fact, as already mentioned, the nomenclature *S. colias* was considered during many years as a synonym of *S. japonicus* (B. B. Collette and Nauen, 1983; Castro Hernandez and Santana Ortega, 2000). However, although morphologically very similar, different studies have highlighted the high molecular divergences existing between the mackerels living in the Atlantic-Mediterranean and those living in the Indo-Pacific (B. Collette, 1999). These differences finally led to the recognition of

[3]https://fish-commercial-names.ec.europa.eu/fish-names/species/scomber-colias_en?fao-code=MAZ

[4]https://fish-commercial-names.ec.europa.eu/fish-names/species/scomber-australasicus_en?fao-code=MAA

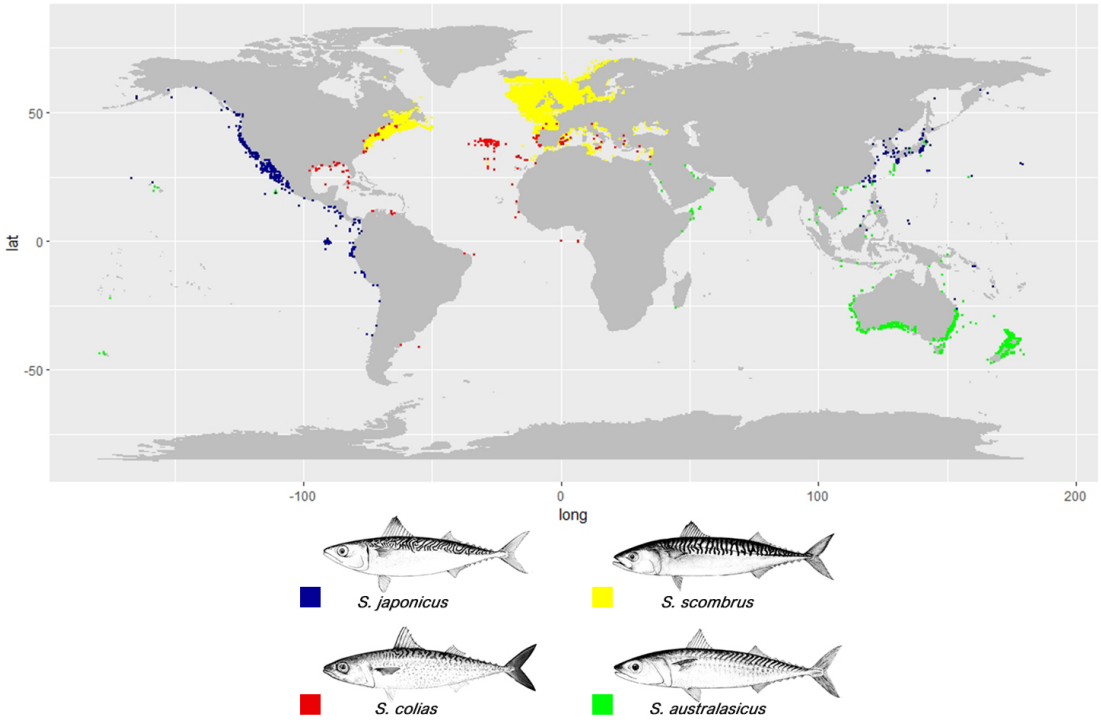

Figure 2.1.: World distribution of allopatric and sympatric species traced on the information available on AquaMaps, and morphology (from FAO species catalogue, Vol. 2 Scombrids of the World [5]) of the "true mackerels" *Scomber* species

two distinct species, accepting *S. colias* and *S. japonicus* as independent scientific names and definitive nomenclature (Infante et al., 2007; Catanese, Manchado, and Infante, 2010).

Each species has specific adaptations that allow them to inhabit their respective geographic areas. These adaptations are related to climatic conditions, food availability and other environmental factors specific to each region (Olafsdottir et al., 2018; Domínguez-Petit et al., 2022). Their ecological role and economic importance may vary depending on the region and local fishing activity. It is important to mention that although *S. colias* is not as well known or as widely distributed as Atlantic mackerel, it is still a relevant species within its range (B. Collette, 1999).

2.2.2. Global trade

Throughout history, species like mackerel have been vital for fishing and consumption, given their migratory habits in pursuit of food and breeding grounds. Recognizing their economic and ecological significance, global efforts have been made to manage and conserve mackerel populations, ensuring sustainability and protection.

Between 1950 and 2020, global mackerel fisheries experienced significant fluctuations. In recent decades, many countries have implemented fisheries management strategies to safeguard fish stocks, including mackerel. These measures encompass fishing quotas, seasonal restrictions, size limits, and marine protected areas. During this period, mackerel catches surged in regions like the North Atlantic, driven by rising global demand for fresh and preserved fish, expanding fishing fleets, particularly for Atlantic and Pacific Chub mackerel, which exceeded 4,500,000 tons in the early 1980s (**Figure 2.2**).

In terms of commercial products, mackerel are marketed in several forms including fresh or refrigerated, prepared frozen and canned. The global canned mackerel market was valued at $771.7 million in 2021. In recent years, top mackerel exporters, including China, Thailand, Vietnam, Denmark, and Morocco, have ranged from 86,379 to 11,119 tons, generating revenues between $244,467M and $60M[7]. European suppliers supply 84% to European manufacturers, but major importers include

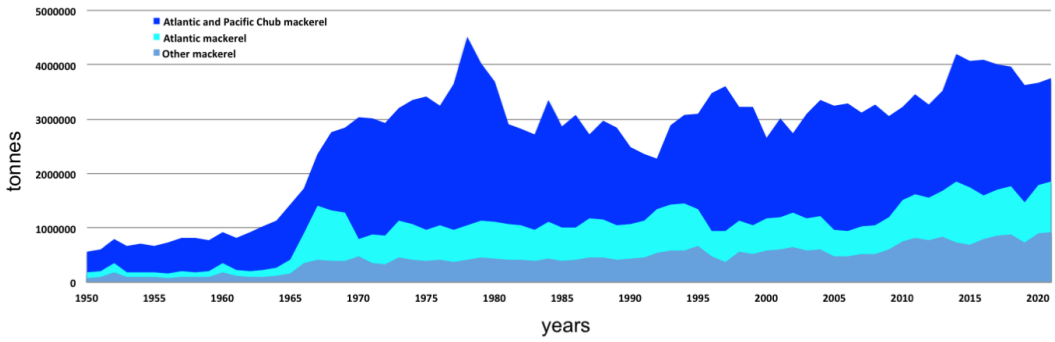

Figure 2.2.: Mackerel global captures from 1950 to 2010. Data from FAO[6].

[7]https://wits.worldbank.org/trade/comtrade/en/country/ALL/year/2019/tradeflow/Exports/partner/WLD/product/160415

Italy, Spain, Portugal, Germany and the United Kingdom (Scientific, Technical and Economic Committee for Fisheries (STECF), 2019). In 2021, EU mackerel exports peaked at 180,169 tons, though average prices dropped by 3% to 1.69 EUR/kg. However, export prices surged by around 20% in the first eight months of 2022, fueled by strong demand from Asian and African markets, signaling a promising outlook for the industry (EUMOFA, Directorate-General for Maritime Affairs and Fisheries, 2023). Due to the continuous increase in world catches, the International Council for the Exploration of the Sea (ICES) proposed a 7% reduction in Atlantic mackerel quotas for 2022, suggesting a limit of 794,920 tons. However, similar to the previous year, the coastal states involved in Atlantic mackerel fishing failed to reach a mutual agreement on quotas. By the conclusion of September 2022, Norway, Iceland, the Faroe Islands, and the UK, collectively, managed to land 3% more mackerel than in the corresponding period of 2020 (EUMOFA, Directorate-General for Maritime Affairs and Fisheries, 2023).

2.2.3. Seafood labelling legislations

Seafood labelling is a key element to protect consumers' interests, health and to ensure the sustainability of stock exploitation (Paolacci et al., 2021). In order to provide consumers accurate information about fish and fisheries that are buying, several legislative tools have been proposed at global scale.
In Europe, the Regulations (EU) No.1169/2011 and (EU) No 1379/2013 are the legislative tools that specify the mandatory information that must be present on seafood labels. In particular, the Article 35 of Reg. (EU) 1379/2013 establishes that the commercial designation, scientific name, production method, geographical area, category of fishing gear used in the capture of the species and whether previously frozen must be reported on each label. Furthermore, Article 39 of the Regulation allows voluntary inclusion of information on aquaculture and fishery products' catch/harvest dates, landing details, fishing gear, vessel flag state, environmental, ethical, social aspects, production techniques, and nutritional content on product labels. Regarding the commercial designation and the scientific name, the Article 37 of the Regulation requires each Member State to draw up, publish and update, in response to the expansion of the variety of fish species on the market, a list of

official commercial names associated with scientific names[8].

Even if the Regulation (EU) 1379/2013 strives to standardize seafood labelling in Europe, the absence of a unified EU list linking official commercial names with scientific names can lead to confusion and inconsistent enforcement of control measures (Griffiths et al., 2014). In the specific case of the genus *Scomber*, among 27 EU countries, only 8 national commercial designations list (Latvia, Estonia, Denmark, Slovakia, Spain, Portugal, Italy and Germany) include all the "true mackerels" species *S. scombrus*, *S. japonicus*, *S. colias* and *S. australasicus*. The lists of the other countries include two or three of the abovementioned species. For instance, Hungary, Romania, Ireland, Bulgaria, Belgium and Malta lists include only the species *S. scombrus* and *S. japonicus*; Czech Republic and Austria lists only include the species *S. japonicus* and *S. australasicus*. In other cases, the national lists include two or three species of the genus *Scomber* associated to their respective commercial names, while allowing the sale of all *Scomber* genus species, leading to inconsistency as is the case of Cyprus where *Scomber* spp. can be sold under the commercial name "Scomber mackerels nei", in Slovenia "skuše", Croatia "plavice, skušovke" and in Lithuania "paprastosios skumbrės". Another important difference existing between the different national lists is the commercial designation associated to *S. colias* and *S. japonicus*. Indeed, in 5/27 lists, these species are associated with the same commercial designation, as is the case of Hungary (közönséges makréla), Luxemburg (maquereau espagnol), France (maquereau espagnole), Croatia (lokarda, plavica), Spain (caballa del sur), Portugal (Cavala) and Italy (Lanzardo o Sgombro occhione), while in other countries such species are associated with a distinct commercial name. Another example of differences between the national list regards the Germany list where the same commercial designation "Makrele" is attributed to *S. scombrus* and *S. japonicus*, or the Austria list where *S. australasicus* and *S. japonicus* could be sold as "Spanische Makrele".

In contrast to the strict seafood labelling regulations imposed by the European Union (EU), extra EU countries labelling rules are often lenient, merely requiring a minimal 'common name' for seafood products. For instance, in UK the Fish Labelling (England) Regulations 2010 (n. 420), improved with the Fish Labelling Regulations 2013 (n. 1768), generally follow the EU directives containing rules on what information must be provided to the consumer, but the Lists of commercial

[8]https://fish-commercial-names.ec.europa.eu/fish-names/member-states_en

designations includes all species of *Scomber* as the commercial name of Mackerel. In Canada, the Fish Inspection Regulations require specific labelling including the common name to identify the product, net quantity, country of origin, if the seafood is imported, date marking, if the product has a limited shelf life and finally, nutritional and allergen information[9]. In the U.S., the Federal Government worked to provide consistent and scientifically sound recommendations to industry and consumers about acceptable market names for seafood sold in interstate commerce. This advice was consolidated in 1988 when The Seafood List was first published by the Food and Drug Administration (FDA) in cooperation with the National Marine Fisheries Service to provide a source of names that would facilitate consistency and order in the U.S. market place and reduce confusion among consumers. The Fish list was then revised through the years until the last update in January 2023[10].
In Japan, food labelling is regulated by two key laws: The Food Sanitation Law of 1947, aimed at safeguarding public health, and the Japanese Agricultural Standard Law of 1950, which focuses on product quality and labelling standards. Since March 2000, fresh food products have been required to include details such as the product's name, specific name, and place or country of origin on their labels. For processed foods, additional information such as the product's name, processor's name and address, list of ingredients and additives, manufacturing date, expiry date, storage instructions, and place or country of origin must be provided. The global seafood supply chain exposes seafood products to be associated to ambiguous names in seafood marketplaces, suggesting that global seafood labelling legislations should first be harmonized by rejecting vague 'umbrella' terms and adopting a 'one species, one code' approach. In addition, global labelling legislations should be aligned to require scientific names on seafood products, along with additional criteria (geographical origin, processing history, production and harvesting methods) to promote consumer choice and effective 'boat-to-plate' traceability (Cawthorn et al., 2021).

[9]https://inspection.canada.ca/food-labels/labeling/industry/fish/eng/1630326254350/1630326374266

[10]https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=SeafoodList

2.2.4. Species substitution

Similar to categorizations used for *Merluccius* spp. substitutions (Blanco-Fernandez et al., 2021), mackerel substitutions can be classified into three groups: "Different genus" involving species outside the *Scomber* genus; "Allopatric" where the same species with no overlapping geographical distribution is substituted; and "Sympatric" indicating species with overlapping distribution. Sympatric substitutions may arise from sorting errors aboard vessels, especially in mixed fisheries. Conversely, allopatric or non-Mackerel species substitutions are more likely deliberate. Traditional morphological identification relies on key morpho-anatomical characteristics for species authentication of fresh products, but muscle tissues both in fresh and processed seafood often lack distinguishing features, making it challenging to differentiate congeneric species, as in the case of *Scomber* species. Mackerel substitutions vary based on product type, geographical area, and catching season. Species like *S. colias* and *S. japonicus*, morphologically similar but do not inhabit the same geographical areas, may be exchanged. Similarly, *S. colias* and *S. scombrus* in the Atlantic-Mediterranean and *S. japonicus* and *S. australasicus* in the Indo-Pacific might be substituted despite phenotypic differences, because they are fished in the same areas. Processed mackerels are often indistinguishable due to flesh similarity, leading to mislabelling (Infante et al., 2006). In fact, Mottola, Piredda, Catanese, Lorusso, et al., 2022 found mislabeling in 45% of canned mackerel. Generally, fraudulent substitutions, especially in processed mackerels, involve relatively inexpensive species like *Rastrelliger*, *Scomberomorus*, or fish from Carangidae and rarely Clupeidae or *Lepidocybium* genera. The substitutions, in particular cases, can include species of *Thunnus* (with higher commercial value), but it seems to be only in particular cases for facilitating the commercialization of illegally caught species (Gordoa et al., 2017; X. Zhang, Tinacci, et al., 2024). The lack of comprehensive studies or data sources makes it difficult to determine the extent of species mislabelling within the mackerel market, hindering understanding of the economic losses and impact on the fishing industry, necessitating dedicated research to quantify the frequency and consequences of this issue.

2.3. Scomber genetic diversity

Molecular genetic analysis plays an important role for enhancing the scientific basis for fisheries management and conserving marine resources by aiding in species identification and understanding evolutionary differences among fish populations. The status of mackerel stocks varies depending on the species, with some showing extensive long-distance migrations and mixing across ocean basins (i.e., between Mediterranean and Atlantic or Indian and Pacific Oceans), suggesting a panmictic status, while others exhibit localized movements and genetic differentiation indicative of diverged subpopulations (Zardoya et al., 2004; Yan et al., 2015; Olafsdottir et al., 2018). Nevertheless, despite the genetic complexities observed in mackerel populations across diverse regions and environments, the focus of this review was to highlight identifying and authenticating species at a molecular genetic level, with a specific emphasis on ensuring accurate species authentication in commercial products. Genetic research on the genus *Scomber* has primarily focused on mitochondrial DNA regions and microsatellite analysis to explore genetic variation. However, as already mentioned, for many years *S. colias* was considered synonymous with *S. japonicus* (Castro Hernandez and Santana Ortega, 2000) until studies using mitochondrial Control Region (D-loop), cytochrome b (Cytb), and nuclear 5S rDNA revealed genetic distinctions between *S. colias*, in the Atlantic-Mediterranean and *S. japonicus* in the Indo-Pacific regions (Scoles et al., 1998, B. Collette, 1999; Infante et al., 2007). Similarly, Catanese et al., 2007 found significant genetic differences between *S. colias* and *S. japonicus* based on the ATCO region, a section of the mitochondrial ATP synthase subunit 6 (ATPase6) and Cytochrome oxidase subunit I (COI) genes. Subsequent research, utilizing complete mitochondrial genomes, confirmed the monophyletic origin of *S. colias* in relation to *S. japonicus*, but supported definitively the classification of *S. colias* and *S. japonicus* species as separate entities (Catanese, Manchado, and Infante, 2010).

However, the historical confusion of the Atlantic-Mediterranean *S. colias* which was often misidentified as *S. japonicus*, has highlighted the potential for confusion in genetic databases such as GenBank. Before the recognition as two distinct species, Zardoya et al., 2004 investigated the D-loop mitochondrial DNA from samples collected in the Atlantic and Mediterranean regions. Their research identified structure differences among *S. japonicus* populations (at that time considered

synonymous with *S. colias*) even if gene flow was observed between basins and among *S. scombrus* populations, in which a distinct east-west genetic structured axis was observed in the Mediterranean Sea. Similarly, Quinteiro, 2011 found significant inter- and intra-oceanic differentiation in *S. japonicus*, but considering it as a species with a global distribution.

The general phylogenetic relationships of *Scomber* species were further studied using markers such as COI, Cytb, D-Loop and 5S rDNA (Cheng et al., 2011). This study confirmed the monophyly of the *Scomber* genus and revealed that *S. japonicus* is genetically closer to *S. australasicus* than to *S. colias* and the congruence between mitochondrial and nuclear sequences suggested the Atlantic Ocean as the origin of the *Scomber* genus. Population differentiation among eastern Atlantic *S. scombrus* stocks was also detected by Nesbø et al., 2000 using Cytb and D-loop markers.

Multiple migration routes were also proposed to explain the current geographical distribution of these species (Cheng et al., 2011).

Most population genetics studies have primarily focused on the Pacific region. A phylogeographic study on *S. japonicus* in the northwest Pacific was conducted, analyzing complete mitochondrial D-loop and Cytb sequences, revealing population isolation during the Pleistocene era (Yan et al., 2015). Genetic differentiation was observed between Chinese and Japanese populations, while no significant structure was found along the Chinese coast. The authors hypothesized that historical events, biological characteristics, and extrinsic factors such as ocean currents might contribute to the current phylogeographic pattern of *S. japonicus* in the northwest Pacific. Similar findings were also reported using microsatellites in the Northwestern Pacific and along the coast of China (Zeng et al., 2012; Cheng et al., 2015). Additionally, studies on the population structure of *S. japonicus* in the Taiwan Strait, utilizing D-loop sequences (T. D. Tzeng et al., 2007) or COI fragments (L. Zhang et al., 2019), provided further insights into the genetic makeup of this species in that specific geographic area.

Microsatellite and mitochondrial analyses conducted on blue mackerel (*S. australasicus*) in the western North Pacific concluded it as a distinct taxonomic species, suggesting it should be treated separately from *S. japonicus* in statistical studies for fisheries management (C. H. Tzeng et al., 2009). In a recent study on genetic diversity, using seven microsatellite loci, Atlantic chub mackerel populations in the

Central East Atlantic displayed significant genetic differentiation, indicative of the formation of distinct subpopulations (Stroganov et al., 2023).

2.4. Molecular authentication of Scomber products

In general, Forensically Informative Nucleotide Sequencing (FINS) is the preeminent technique for DNA analysis in discerning seafood products, with a particular focus on mackerel seafoods, which involves sequencing the PCR products and comparing the sequences with those in public databases (Bartlett and Davidson, 1992; Lago et al., 2011; Armani et al., 2013). Nonetheless, conventional Sanger sequencing proves inadequate for samples containing multiple species (Lenstra, 2003). However, alternative methods like PCR-RFLP or species-specific PCR techniques can offer efficient solutions (Applewhite et al., 2016). Markers as Cytb and COI are commonly used for seafood species differentiation, with COI serving as the DNA barcoding standard (Ward et al., 2009). However, it has been proven that both genes have limitations in identifying closely related species and some species like tuna may be better identified using other mitochondrial regions (Viñas and Tudela, 2009; Ardura et al., 2013), while the reliable identification of hybridization between species may require the use of nuclear markers (Sevilla et al., 2007; Rehbein, 2013). Additionally, other genetic regions such as 12S rRNA, 16S rRNA, or D-loop are also employed for differentiation in heavily processed seafood (Ardura et al., 2010; Armani et al., 2015; Mitchell and Hellberg, 2016; Roungchun et al., 2022; Mottola, Piredda, Catanese, Lorusso, et al., 2022).

For the identification of mackerels, the sequencing-based method has been employed to analyse mainly specific genetic mitochondrial DNA regions, which exhibit variations among different species. By comparing the DNA sequences obtained from the target region of interest with reference sequences from known species, it is possible to identify and differentiate various *Scomber* species.

The differences in DNA genes were used to develop various molecular methods as potential tools for the direct authentication and identification of *Scomber* mackerels in the seafood industry. Aranishi, 2005b proposed a rapid and reliable polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis

for discriminating between imported mackerel *S. scombrus* and domestic mackerel *S. japonicus* in Japan. This method utilized *Scomber*-specific primers to amplify the nuclear 5S ribosomal DNA non-transcribed spacer, followed by direct digestion of the PCR products using Pvu II or Hae III restriction enzymes, which generated species-specific profiles for accurate identification.

Expanding on this approach, Aranishi, 2005a developed a PCR-based method for differentiating *Scomber* mackerels from other Scombrid fish species. The researchers used *Scomber* genus-specific primers to amplify the non-transcribed spacer (NTS) of the 5S rDNA, and subsequent PCR-RFLP analysis using Sca I endonuclease resulted in unique restriction patterns specific to each *Scomber* species.

A method based on the mitochondrial NADH dehydrogenase subunit 5 (mtND5) gene, was developed for identifying *Scomber* species (Martinez et al., 2012). By sequencing specific fragments of the mt ND5 and cytochrome b genes, the researchers could accurately identify each of the four *Scomber* species, with the exception of *S. japonicus* and *S. colias*, which had the same restriction sites in the ND5 amplicon.

To authenticate Atlantic mackerel *S. scombrus* in commercial canned products, Infante and Manchado, 2006 developed a multiplex-PCR assay. This method involved amplification of a *S. scombrus*-specific fragment from the mitochondrial NADH dehydrogenase subunit 5, along with a positive amplification control from the small rRNA 12S subunit. Similarly, Infante and Manchado, 2006 devised a multiplex-PCR system for the authentication of *S. colias* in commercial canned products. The authors utilized an *S. colias*-specific fragment from the non-transcribed spacer (NTS) sequence, along with a *Scomber* genus-specific PCR product from the 5S rRNA gene, as a positive amplification control.

In another study by Catanese, Manchado, Fernández-Trujillo, and Infante, 2010, a novel multiplex-PCR assay was developed for the simultaneous authentication of *Scomber* mackerels including *S. japonicus*,*S. australasicus*, *S. colias*, and *S. scombrus* in processed food. This method involved amplifying species-specific small amplicons from the mitochondrial (D-loop) and nuclear (NTS) markers, and using a positive amplification control from the small mitochondrial 12S rRNA subunit.

In a more recent work, Armani et al., 2015 developed new primers for the amplification and sequencing of short fragments of the mitochondrial 16S ribosomal RNA (16S rRNA) gene. These primers were applied to analyze highly degraded DNA extracted from canned food containing tuna, bonito, or mackerel and the obtained

sequences were subjected to BLAST and FINS analysis.
An interesting and alternative work introduced a Real Time-PCR method based on TaqMan technology for the identification of *S. scombrus* (Velasco et al., 2011). This approach employed specific primers and a Minor Groove Binding (MGB) TaqMan probe targeting sequences from the mitochondrial Cytb region.
For simultaneous detection of horse mackerel (*Trachurus trachurus*) and mackerel (*S. scombrus*) DNA, Prado et al., 2013 also developed a real-time PCR method using a target fragment from the parvalbumin gene. Differentiation between *S. japonicus* and *S. scombrus* was then achieved by Ahn et al., 2017 through the detection of polymorphisms in the mitochondrial 16s rRNA gene using fluorescence melting curve analysis and locked nucleic acid probes. The method successfully distinguished the two species based on the difference in melting temperatures caused by the specificity of the locked nucleic acid probes. New DNA-based assays were designed for the identification of fresh mackerel species and commercial products (Frigerio et al., 2023). These researchers developed a primer pair targeting the 5S rRNA gene and non-transcribed spacer (NTS), allowing for species identification of fresh mackerel (*Scomber spp.*) and processed commercial products. They also developed a rapid and cost-effective assay using recombinase polymerase amplification (RPA) coupled with lateral flow visualization specifically for the differentiation of *S. scombrus* and *S. japonicus/S. colias*.
Finally, Mottola, Piredda, Catanese, Lorusso, et al., 2022 implemented a two-step mini barcoding approach for the identification of canned mackerel sold in Italian markets. They employed mini-barcode universal primers targeting a COI fragment for initial identification of *S. scombrus*, followed by specific primers designed in the mitochondrial D-loop to discriminate between *S. colias*, *S. japonicus*, and *S. australasicus*.

2.5. in-silico evaluation of interspecific variation for DNA metabarcoding approach

In this study we used *in-silico* simulations to evaluate the possibility of metabarcoding application, that is using a primer pair designed for other species we checked

their applicability to *Scomber* species. This approach is a barcoding of DNA using primers that allow for the identification of many taxa within the same sample, even simultaneously.

The *in-silico* simulations for *Scomber* species mitochondrial 12S rDNA gene revealed 7 primer pairs capable of amplification, generating fragments ranging from 105 bp to 435 bp (**Table 2.1**). Analysis of GenBank sequences showed conserved nucleotide positions only in *S. scombrus*, varying from 3 to 15 mutations (1.60% to 10.4% of fragment length) compared to other *Scomber* species. Two *S. colias* sequences and one *S. australasicus* sequence displayed mutations. Primers generating < 200 bp fragments, suitable for degraded DNA, from Riaz et al., 2011, Miya et al., 2015, Taberlet et al., 2018, and Valentini et al., 2016 effectively differentiate *S. scombrus*, identifying 3, 9, 9, and 11 nucleotide positions, respectively. Similarly, primers producing fragments of 280-435 bp facilitate differentiation, with 7-15 specific positions for *S. scombrus* identification among other *Scomber* species.

For the mitochondrial 16S rDNA gene, the *in-silico* simulations revealed 11 primer pairs capable of amplification in *Scomber* spp., producing fragments ranging from 112 to 392 bp (**Table 1B**). Specific nucleotide positions within this gene could differentiate *S. scombrus* from *S. australasicus*, *S. japonicus*, and *S. colias*, with mutations ranging from 3 to 8 nucleotides (1.78% to 3.57% of fragment length). One *S. colias* sequence displayed 3-6 differences, and one *S. scombrus* sequence showed indels. The primers described by Kitano et al., 2007 exhibited 1 mutation in *S. scombrus* and 2 in *S. colias*, though based on limited sequences. Primers generating < 200 bp fragments, like those from Horreo et al., 2013, allow discrimination of *S. scombrus* from other species, identifying 4 conserved nucleotide positions.

In relation to the mitochondrial COI gene, the *in-silico* simulations on revealed potential primer applicability in *Scomber* spp. samples (**Table 1C**), producing DNA fragments of 191 to 370 bp. While most primer pairs were effective across *Scomber* species, one failed in *S. scombrus* but was valid for others. Species differentiation relied on more nucleotide positions compared to ribosomal genes, with textitS. scombrus displaying 6-32 species-specific positions (3.42% to 8.64%). Conversely, *S. australasicus*, *S. japonicus*, and *S. colias* exhibited 5-21 unconserved positions and numerous dispersed mutations. Despite greater polymorphism in this coding gene than in the ribosomal genes, differences among *S. australasicus*, *S. japonicus*, and *S. colias* lacked consistency within species.

The *in-silico* simulations identified 7 primer pairs for amplifying a fragment of the mitochondrial Cytb gene in *Scomber* spp. (**Table 1D**), yielding fragments ranging from 80 to 464 bp. Conserved nucleotide variations were detected in *S. scombrus* (3-45 differences; 3.75% to 9.69%), yet no species-identifying positions were found among the other three *Scomber* species. Notably, primers producing PCR fragments smaller than 100 bp, suitable for processed products analysis, exhibited extremely low melting temperatures, posing challenges for application.

Finally, all these simulations and analyses of published sequences reinforce the assertion that, at the mitochondrial level the D-loop region emerges as the sole mitochondrial marker capable of distinguishing all *Scomber* species, including those in processed products, with fragments of approximately 200 bp suitable for multiplex-PCR (Catanese, Manchado, Fernández-Trujillo, and Infante, 2010) and mini barcoding sequencing (Mottola, Piredda, Catanese, Lorusso, et al., 2022). However, due to its variability, finding primers for amplification across different genera is challenging. Initially, primers from mitochondrial D-loop mini barcoding assays used for tuna authentication were assessed (Mitchell and Hellberg, 2016; Roungchun et al., 2022; Klapper et al., 2023) (**Table 1E**). Unfortunately, only two primer pairs (TunaCR_F/TunaCR_R2 and MH-TunaCRV2/TunaCR_R2) demonstrated amplification potential in *Scomber* species. Nonetheless, nucleotide differences compared to reference sequences could pose challenges during the hybridization step, with 20 and 11 differences for the first primer pair and 3 and 13 differences for the second.

Analyzing the D-loop fragment targeted by the TunaCR_F/TunaCR_R primers, substantial differences are evident among *Scomber* species. *S. scombrus* exhibits approximately 64 specific nucleotide positions and 8 indels compared to other species (21.9%); *S. japonicus* has 10 plus one indel (3.35%); *S. colias*, 13 (3.96%) and *S. australasicus*, 8 (2.43%). Using the MH-TunaCRV2/TunaCR_R2 primers, which may offer better amplification success due to fewer nucleotide differences on the reverse, *Scomber* sequences display significant nucleotide differences. *S. scombrus* shows 61 positions and 8 indels compared to other *Scomber* species (22.6%). Additionally, *S. colias* exhibits 10 specific conserved positions (3.28%), with 3 nucleotide differences in *S. australasicus* (0.98%) and 8 in *S. japonicus* (2.63%). However, the resulting fragment length in *Scomber* would be around 328 and 304 bp, too long for degraded DNA typical of processed products, usable only

in fresh, refrigerated, or frozen samples. Furthermore, Mottola, Piredda, Catanese, Lorusso, et al., 2022 described a more specific primer pair to differentiate *Scomber* species with a smaller size (207 bp). Unfortunately, this pair does not amplify in *S. scombrus* (due to too many nucleotide differences in the 3' end of the forward primer). Nonetheless, *S. japonicus* displays 6 different nucleotide positions (2.89%), 4 in *S. australasicus* (1.93%), and 3 in *S. colias* (1.44%), enabling identification in canned products.

As a consequence of the difficulties in identifying each of the *Scomber* species by sequencing using a single fragment of the Dloop, we simulated the possibility of applying new pairs of primers for this purpose. In one case, we considered only the *Scomber* species, simulating the PCR amplification of a 140 bp fragment (MiniScomberCR_F/MiniScomberCR_R). In two other cases, their application, in addition to *Scomber* spp., would also be extended to other species of Scombridae (*Thunnus thynnus*, *T. albacares*, *T. alalunga*, *T. orientalis*, *T. obesus*, *Auxis rochei*, *A. thazard*, *Euthynnus alletteratus*, *Katsuwonus pelamis* and *Scomberomorus cavalla*). The fragments generated could vary between 178 and 182 bp for one pair (Scombridae179CR_F/Scombridae179CR_R) and between 247 and 255 bp for the second pair (Scombridae255CR_F/Scombridae255CR_R).

The fragment which includes only *Scomber* spp. revealed that *S. scombrus* has 28 species-specific nucleotide positions, 2 *S. colias*, 6 *S. japonicus* and 3 *S. australasicus*, which allow the correct identification of each of them (20%, 1.42%, 4.28% and 2.14% respectively). The fragment for Scombridae of 179 bp as well as that of 255 bp, in addition to presenting numerous variations between the species of the genus *Thunnus*, *Auxis*, *Katsuwonus* and *Scomberomorus*, shown for *S. scombrus* between 34 and 54 species-specific nucleotide positions (from 13.3% to 30.1%), between 4 and 5 for *S. colias* (from 1.56% to 2.79%), between 6 and 8 for *S. japonicus* (from 2.35% to 4.46%) and finally between 2 and 4 for *S. australasicus* (from 0.78% to 2.23%). All these positions would allow the identification of each of the considered species, even if a high number of haplotypes must be analyzed among the *Thunnus* species to be sure we have the ability to differentiate each those species.

Table 2.1: List of primers used in the *in-silico* evaluations for **12S rRNA gene**

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
12S_p1	AcMDB07 F AcMDB07 R	GCCTATATACCGCCGTCG GTACACTTACCATGTTACGACTT	321 bp	53°C	Actinopterygii	Bylemans et al., 2018	329 bp	56°C
12S_p2	Ac12s F Ac12s R	ACTGGGATTAGATACCCCACTATG GAGAGTGACGGGCGGTGT	385-429 bp	58°-63°C	Actinopterygii	Evans et al., 2016/Bylemans et al., 2018	435 bp	58°C
12S_p3	Am12s F Am12s R	AGCCACCGCGGTTATACG CAAGTCCTTTGGGTTTTAAGC	241 bp	60°-65°C	Actinopterygii	Evans et al., 2016	282 bp	56°C
12S_p5	MiFish-U-F MiFish-U-R	GTCGGTAAAACTCGTGCCAGC CATAGTGGGGTATCTAATCCCAGTTTG	219 bp	61°C	eDNA sampled from Aquarium with known species; also sampled natural seawaters around coral reefs. total number of species detected to 232 (from 70 families and 152 genera)	Miya et al., 2015	217 bp	58°C
12S_p6	12SV5 F 12SV5 R	TAGAACAGGCTCCTCTAG TTAGATACCCCACTATGC	99 bp (73-110)	50°-52°C	Vertebrata	Riaz et al., 2011	136 bp	54°C
12S_p8	Tele02 F Tele02 R	AAACTCGTGCCAGCCACC GGGTATCTAATCCCAGTTTG	167 bp	50°C	Teleostei /eDNA water samples sequence variants assigned to 62 fish taxa	Taberlet et al., 2018	203 bp	56°C
12S_p9	Teleo F Teleo R	ACACCGCCCGTCACTCT CTTCCGGTACACTTACCATG	100 bp	55°C	Teleostei	Valentini et al., 2016	105 bp	54°C

Table 2.2: List of primers used in the *in-silico* evaluations for **16S rRNA gene**

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
16S_p1	16S1F 16S2R	GACGAKAAGACCCTA CGCTGTTATCCCTADRGTAACT	180-270 bp	54°C	Fish, cephalopods and crustaceans	Deagle et al., 2007	259 bp	53°C

Continued on next page

Table 2.2: List of primers used in the *in-silico* evaluations for **16S rRNA gene** (Continued)

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
16S_p2	16FORF-CEP3	GAGAAGACCCTDTKGAGCTT	206-145 bp	56°C	cephalopod species	modified from Armani et al., 2015	255 bp	55°C
	16REVF-CEP	GCTGTTATCCCTAKGGTAAC						
16S_p3	16S-forward	AYAAGACGAGAAGACCC	250 bp	53°C	Universal animal mini-barcode	Sarri et al., 2014	268 bp	54°C
	16S-reverse	GATTGCGCTGTTATTCC						
16S_p4	Vert-16S F	AGACGAGAAGACCCYdTGGAGCTT	264 bp	65°C	Vertebrata	Vences et al., 2016	316 bp	58°C
	Vert-16S R	GATCCAACATCGAGGTCGTAA						
16S_p5	FOR16Spc	TGCCCGTGCAGAAGCGG	327-334 bp	60°C	fish species belonging to Clupeidae, Engraulidae, Salangidae, and Scombridae	Armani et al., 2015	342 bp	58°C
	REV16Spc	CAACATCGAGGTCGTAAACCC						
16S_p7	16SF/D	GACCCTATGGAGCTTTAGAC	203 bp	54°C	Fish	DiBattista et al., 2017	252 bp	55°C
	16S2R-degenerate	CGCTGTTATCCCTADRGTAACT						
16S_p8	Ac16s F	CCTTTTGCATCATGATTTAGC	330 bp	58°-63°C	Freshwater fishes and amphibians	Evans et al., 2016	392 bp	56°C
	Ac16s R	CAGGTGGCTGCTTTTAGGC						
16S_p9	Ve16s F	CGAGAAGACCCTATGGAGCTTA	310 bp	60°-65°C	Freshwater fishes and amphibians	Evans et al., 2016	364 bp	57°C
	Ve16s R	AATCGTTGAACAAACGAACC						
16S_p10	16S-HF	ATAACACGAGAAGACCCT	80 -125 bp	61°C	allows species identification of food and seafood of animal origin over a wide range of species.Detected most of species of fish, mammalia (pig, cattle,sheep) and aves (chicken, turkey)	Horreo et al., 2013	123 bp	51°C
	16S-HR1	CCCACGGTCGCCCCAAC						
16S_p11	16S-HF	ATAACACGAGAAGACCCT	80 -125 bp	61°C	allows species identification of food and seafood of animal origin over a wide range of species.Detected most of species of fish, mammalia (pig, cattle,sheep) and aves (chicken, turkey)	Horreo et al., 2013	123 bp	51°C
	16S-HR2	CCCGCGGTCGCCCCAAC						
16S_p12	L2513	GCCTGTTTACCAAAAACATCAC	244 bp	58°C	Vertebrata	Kitano et al., 2007	239 bp	52°C
	H2714	CTCCATAGGGTCTTCTCGTCTT						

Table 2.3: List of primers used in the *in-silico* evaluations for **COI gene**

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
COI_p01	FISHCOILBC Revshort1	TCAACYAATCAYAAAGATATYGGCAC GGYATNACTATRAAGAAAATTATTAC	139 bp	51°C	fish	Armani et al., 2015	191 bp	45°C/not run in *S.scombrus*
COI_p02	mlCOIintF HCO2198	GGWACWGGWTGAACWGTWTAYCCYCC TAAACTTCAGGGTGACCAAAAAATCA	313 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	365 bp	38°C
COI_p03	mlCOIintF dgHCO2198	GGWACWGGWTGAACWGTWTAYCCYCC TAAACTTCAGGGTGACCAAARAAYCA	313 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	365 bp	48°C
COI_p04	mlCOIintF jgHCO2198	GGWACWGGWTGAACWGTWTAYCCYCC TAIACYTCIGGRTGICCRAARAAYCA	313 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	365 bp	60°C
COI_p05	LCO1490 mlCOIintR	GGTCAACAAATCATAAAGATATTGG GGRGGRTASACSGTTCASCCSGTSCC	319 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	370 bp	53°C
COI_p06	dgLCO1490 mlCOIintR	GGTCAACAAATCATAAAGAYATYGG GGRGGRTASACSGTTCASCCSGTSCC	319 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	370 bp	55°C
COI_p07	jgLCO1490 mlCOIintR	TITCIACIAAYCAYAARGAYATTGG GGRGGRTASACSGTTCASCCSGTSCC	319 bp	46°C	metazoan diversity: application for characterizing coral reef fish gut contents	Leray et al., 2013	368 bp	56°C
COI_p09	mini-COI-F (LCO1490) mini-COI-R	GGTCAACAAATCATAAAGATATTGG ACTATAAAGAAGATTATTACAAAGGC	136 bp	48°C	edible species (fishes, shrimps, birds, and mammals)	Pan et al., 2020	187 bp	50°C
COI_p10	Fish_miniA_F_t Fish_miniA_R_t	ACIAAICAIAAAGAYATYGGC AARAAAATYATAACRAAIGCRTGIGC	129 bp	46°C	fish	Shokralla et al., 2015	176 bp	52°C
COI_p12	Fish_miniC_F_t Fish_miniC_R_t	ACYAAICAYAAAGAYATIGGCAC GAARATCATAATGAAGGCATGIGC	127 bp	46°C	fish	Shokralla et al., 2015	175 bp	51°C
COI_p13	Fish_miniD_F_t Fish_miniD_R_t	GGIACIGGITGRACIGTITAYCCYCC GTRATICCIGCIGCIAGIAC	208 bp	50°C	fish	Shokralla et al., 2015	253 bp	57°C
COI_p14	Fish_miniE_F_t Fish_miniE_R_t	ACYAAICAYAAAGAYATIGGCAC CTTATRTTRTTTATICGIGGRAAIGC	226 bp	46°C	fish	Shokralla et al., 2015	275 bp	55°C

Continued on next page

Table 2.3: List of primers used in the *in-silico* evaluations for **COI gene** (Continued)

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
COI_p15	Fish_miniF_F_t	GGIACIGGITGRACIGTITAYCCYCC	314 bp	49°C	fish	Shokralla et al., 2015	361 bp	57°C
	Fish_miniF_R_t	CTTCAGGGTGICCGAARAATC						
COI_p16	Forward	ATCACAAAGACATTGGCACCCT	295 bp	59°C	fish and non-fish species	Sultana et al., 2018	295 bp	53°C
	Reverse	AATGAAGGGGGGAGGAGTCAGAA						
COI_p17	mlCOIintF-XT	GGWACWRGWTGRACWITITAYCCYCC	ca. 313 bp	46-62°C	for biodiversity characterization of structurally complex natural marine hard-bottom communities	Wangensteen et al., 2018	365 bp	57°C
	jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA						

Table 2.4: List of primers used in the *in-silico* evaluations for **Cytb gene**

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
cytb_p01	L14735	AAAAACCACCGTTGTTATTCAACTA	413 bp	50°C	fish and anfibian species	Burgener and Hübner, 1998	464 bp	55°C
	H15149	GCCCCTCAGAATGATATTTGTCCTCA						
cytb_p04	Cytb_L14841	AAAAACCACCGTTGTTATTCAACTA	460 bp	50°C	Vertebrata	Hänfling et al., 2016	368 bp	55°C
	Cytb_H15149R	GCDCCTCARAATGAYATTTGTCCTCA						
cytb_p05	BDR-L	GCMAACGGSGCNTCYTTCTTCT	131 bp	55°C	tuna tins: mixed tuna samples	Kappel et al., 2017	100 bp	30°C
	BDR-H-mod1	TGACGGTAGCHCCTCAGRADGACATTTGTCCYCA						
cytb_p07	L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	307 bp	50°C	Vertebrata	Kocher et al., 1989	376 bp	54°C
	H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA						
cytb_p08	L14912	TTCCTAGCCATACAYTAYAC	235 bp	54°C	Teleostei	Miya and Nishida, 2000	286 bp	45°C
	H15149	GGTGGCKCCTCAGAAGGACATTTGKCCYCA						
cytb_p12	Fish2bCBR	GATGGCGTAGGCAAACAAGA	80 bp	50°C	Fish	Thomsen et al., 2012	80 bp	15°C
	Fish2CBL	ACAACTTCACCCCTGCAAAC						

Continued on next page

Table 2.4: List of primers used in the *in-silico* evaluations for **Cytb gene** (Continued)

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
cytb_p13	Fish2degCBL	ACAACTTCACCCCTGCRAAY	80 bp	50°C	Fish	Thomsen et al., 2012	80 bp	15°C
	Fish2CBR	GATGGCGTAGGCAAATAGGA						

Table 2.5: List of primers used in the *in-silico* evaluations for **D-loop region**

Code	Primers Name	Primers Sequences	Fragment Length	Tm	Target Group	Reference	Fragment Length in *Scomber* spp.	Tm opt. *Scomber* spp.
Dloop_p1	TunaCR_F	CACGACGTTGTAAAACGACGCAYGTAC ATATATGTAAYTACACC	326 bp	49°C	*Thunnus* spp.	Mitchell and Hellberg, 2016	328 bp	47°C
	TunaCR_R2	GGATAACAATTTCACACAGGCTGGATG GTAGGYTCTTACTGCG						
Dloop_p2	MH-Tuna-CR-V2	GACATAYATGTATTAWAACCAT	242 bp	49°C	*Thunnus* spp.	Klapper et al., 2023	304 bp	47°C
	TunaCR_R2	GGATAACAATTTCACACAGGCTGGATG GTAGGYTCTTACTGCG						
Dloop_p3	MiniCR_F	GGAACACCCCACACAAAGTCG	207 bp	50°C	*Scomber* spp.	Mottola, Piredda, Catanese, Lorusso, et al., 2022	207 bp	not run in *S.scombrus* /50°C
	MiniCR_R	TATCAAGTTATGGCCCTGAGTTAGGAA						
New primers								
Dloop_p4	MiniScombCR_F	TACTTTCACGGATAGGCGAA			*Scomber* spp.		140 bp	50°C
	MiniscombCR_R	ACCGTTAACATTAAGTTATGAGCTTG						
Dloop_p5	Scombridae179CR_F	CCATATATATGATTAATATGACATAAACCA	178-182 bp	42°C	Scombridae		179 bp	48°C
	Scombridae179CR_R	ATGAGCTTGTTGGTGGGCTCT						
Dloop_p6	Scombridae255CR_F	GCATATTTAGATATATGTATTTACACCAT	247-254 bp	42°C	Scombridae		255 bp	48°C
	Scombridae255CR_R	TTCGACGGGATGTTGGTACTTGGTA						

2.6. New analytical perspectives

Authentication and traceability of seafood products are crucial for ensuring food safety, verifying label information, combating food fraud, and promoting transparency in marketing. They also help in enforcing regulations, protecting legitimate fishermen, ensuring compliance with international trade standards, addressing food contamination events promptly, and conserving endangered species and marine ecosystems. Overall, authentication and traceability are vital for safeguarding food security, promoting sustainability, and protecting both consumers and marine environments. Advancements in techniques are continually improving the authenticity verification of seafood products including the use of droplet digital PCR (ddPCR), High-Resolution Melting (HRM) analysis and applications of next-generation sequencing (NGS) (Chatzoglou et al., 2023).

The DNA barcoding has advanced in recent years, with improved reference databases, sequencing technologies (like NGS), and refined bioinformatic tools, leading to enhanced accuracy and efficiency in identifying species, even for closely related or processed seafood products. The advancement of NGS technologies has revolutionized SNP detection, significantly improving the speed and reliability of fish species identification and fraud detection in the commercial sector.

Metagenomics involves sequencing the entire DNA content of a sample, and may enable more accurate and comprehensive species identification by analyzing the complete genetic profile of a seafood sample, including mixed samples identifying the target and no target species (Kappel et al., 2017; Mottola, Piredda, Catanese, Giorelli, et al., 2022; Klapper et al., 2023; Giusti et al., 2023). DNA metabarcoding combines high-throughput NGS sequencing with DNA barcoding, analyzing simultaneously multiple barcode regions or mix of species. This involves extracting DNA from food sources, amplifying a specific barcode region (typically 120-600 bp), and sequencing the resulting amplicon. Particularly effective for processed foods with degraded DNA, this method enables comprehensive analysis through specialized pipelines. The DNA metabarcoding approach has proven valuable also for quantitative analysis utilizing variations in sequence read abundance to infer species abundance within a sample. Its efficacy in seafood authentication and traceability has been demonstrated across various species (Arulandhu et al., 2017; Giusti et al., 2017; Carvalho et al., 2017; Gense et al., 2021).

Furthermore, integrated with innovative sequencing methods, SNP-based approaches are increasingly vital for enhancing food safety and maintaining product quality (Nielsen et al., 2012). An intriguing technique has recently emerged for high-throughput barcoding, which could also find application in the realm of seafood. This cost-effective approach, termed microfluidic enrichment barcoding (MEBarcoding), involves the synergistic utilization of the Fluidigm Access Array and NGS technologies (Gostel et al., 2020). This innovative method enables the construction of a highly comprehensive barcode database and showcases the efficiency of the proposed approach in distinguishing a vast array of species present within a food-borne matrix concurrently.

DNA-based biosensors utilize DNA probes discerning between samples that differ by only a single nucleotide in their sequence, underscoring the promising potential of this technique in SNP-based food authenticity assessments. A particular advancement is the SNP genotyping system reliant on a nanofluidic array, utilizing integrated fluidic circuits for high-throughput real-time PCR. These protocols have successfully authenticated cultivars, identified adulterants in products and distinguished between fish populations (Narum et al., 2009; Catanese et al., 2017; Catanese et al., 2020).

Another analysis whose application is growing more is microbiome sequencing. The microbiome analysis refers to the associate community of microorganisms present in a sample. By employing advanced sequencing techniques such as 16S rRNA gene sequencing or metagenomic sequencing, researchers can identify and characterize the microbiota in seafood samples, identifying specific microbial markers associated with different species or geographic origin. In fact, although post-harvest activities can affect the microbiome in fish products, it has been demonstrated that geographic origins of seafood samples can be distinguished by analyzing the microbiome associated with the fresh, chilled or frozen mackerel (Piredda et al., 2023; Meriggi et al., 2024). Moreover, this method can be particularly useful for identifying if no processed seafood products have been defrosted during the transport and preparation handling.

We also have to consider that biostatistics in seafood authenticity analysis using Next-Generation Sequencing (NGS) ensures data accuracy and reliability by managing preprocessing, alignment, variant calling, and downstream analyses, crucial for preventing misleading interpretations of genetic variants and disease associations,

while statistical methods like PCA, AMOVA, MANOVA, and cluster analysis aid in interpreting large datasets to authenticate and detect adulteration in fish and seafood based on genetic, morphometric, proteomic, and metabolomic analyses (K. Kotsanopoulos et al., 2024).

However, the integration of molecular and non-molecular methods can improve the reliability of traceability and authentication in commercial fishery products. In fact, as well to DNA-based methods, advanced analytical techniques that are already used for plant-based food products such as spectroscopy (MIR, NIR, Raman, UV-Vis) and chromatography (HPLC, GC), could be integrated to identify species and detect adulteration in seafood products (K. V. Kotsanopoulos and Exadactylos, 2022). In addition to genomic approaches, other high-throughput technologies can characterize food matrices, such as transcriptomics (mRNA), proteomics (proteins), lipidomics (lipids content) and metabolomics (metabolites) (Mohanty et al., 2019). For instance, proteomic was successfully applied for seafood authentication by Chien et al., 2022. Similarly, lipidomic was used to species identification or to differentiate between wild and farmed fish, as well as to assess seafood freshness (K. V. Kotsanopoulos et al., 2021). Also, metabolomics has been applied for geographical origin assessmentand for shrimps or *Thunnus thynnus* species determination (Chatterjee et al., 2019; Cappello et al., 2018). Finally, multi-omics approaches could make it possible to identify unique molecular signatures specific to each species, providing a comprehensive view of the molecular composition of seafood and improving the accuracy and reliability of seafood authentication.

Artificial intelligence (AI) could play a crucial role by improving efficiency, accuracy and reliability in DNA sequencing, data management and certification processes. In fact, by constantly monitoring data, artificial intelligence could also identify trends and irregularities, optimize certification processes and learn from historical data to detect anomalies. Furthermore, it can manage large databases, update fish species information and visually identify species when molecular traceability is not available. Bioinformatics tools are leveraging machine learning to analyze DNA sequences for food authentication, particularly through high-throughput sequencing to enhance DNA barcoding for species-specific marker identification crucial in verifying food product authenticity. Moreover, the promising growth of integrating machine learning with DNA analysis techniques for food and fish authentication can ensures accuracy in species identification and food product

traceability, utilizing advanced models like EN-SAE with KDE to enhance DNA barcoding for precise species classification in food authentication. (Jin et al., 2021). In industry, the combination of DNA sequencing and machine learning aids in species identification and adulteration detection within complex food matrices, alongside recent advancements applying deep learning for automated species identification through image and video analysis or photography app for smartphones (Taheri-Garavand et al., 2020; Rossi et al., 2016; Jayasundara et al., 2023;).

In conclusion, the continuous development of new genetic authentication techniques and applications is proving increasingly beneficial, ensuring the integrity of mackerel in commercial products, bolstering consumer confidence, and promoting sustainable fishing practices.

3. Authentication of seafood species on the ASFIS list (FAO) by in-silico evaluation of primers for metabarcoding

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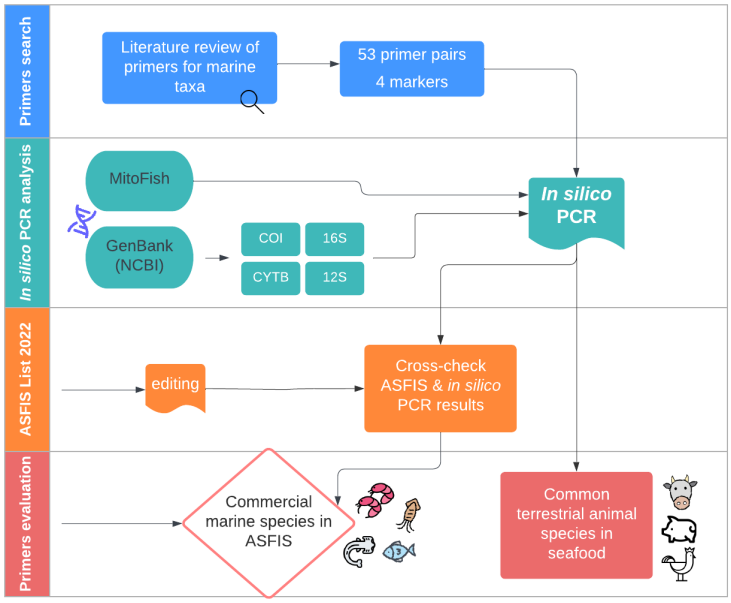
3.1. Abstract



Given the remarkable biodiversity among the seafood species entering the food supply chain, authentication is a crucial component in ensuring transparent trade. DNA metabarcoding stands out as the leading methodology for determining species composition within complex food matrices. Nevertheless, the selection of primer pairs is critical, as it may introduce biases into species identification. In this investigation, we evaluated previously described primers for their *in-silico* efficacy in identifying both edible marine species on the ASFIS (FAO) list and the main terrestrial animal species frequently incorporated as ingredients in processed seafood products. Selected primers covered the Cytochrome c Oxidase subunit I (COI), the Cytochrome b (cytb), the 16S rRNA (16S) and the 12S rRNA (12S) mitochondrial regions. Results showed that, out of 53 pairs analysed, one primer pair targeting the COI region obtained the best performance in terms of taxonomic coverage for both ASFIS and terrestrial species. Notably, each primer pair showed a unique taxonomic profile, suggesting their potential for addressing various seafood authentication issues as the detection of endangered species or novel food ingredients (e.g. jellyfish, red algae, insects). Overall, these outcomes deliver valuable insights for selecting metabarcoding primers to improve the traceability of seafood products and contribute to the development of an innovative Food Safety Management System.

3.2. Introduction

The defence of food authenticity is crucial for public health and consumer protection, but, in the complex context of the seafood supply chain, ensuring product authenticity is often a challenging task[1]. Authenticity refers to the truthfulness of the information declared on the labels which connect producers and retailers with the consumer, requiring transparency from the first two and enabling conscious choices by the latter (Robson et al., 2021). The crucial role of labelling and the importance of consistent legislation has been recognised by the European Council. Indeed, seafood labelling is currently governed by Regulation (EU) No. 1379/2013 for all unprocessed and some processed fishery and aquaculture products (e.g. dried, salted, or smoked products), and by Regulation (EU) No. 1169/2011 for processed pre-packed products. The latter requires only a generic declaration of the presence of fish, molluscs, and crustaceans probably because it used to be impossible to verify which species were being used as ingredients in the mixtures (Piredda et al., 2022). In contrast, Regulation (EU) No. 1379/2013 includes a set of mandatory information, such as scientific name and its respective commercial designation, origin, type of production and whether it is a fresh or thawed product. The global trade in seafood requires rules to improve food security and mitigate the environmental impact of production, so each European Union Member State has to publish the Official List of marketable species within its territory; similar guidelines have been established by other non-European Union countries such as the United Kingdom, United States and Canada, including the scientific name and the commercial designation in the original language of the country (FAO, 2018). Application of molecular techniques is the most powerful tool for the traceability of fishery products and the verification of compliance with the information shown on labels (Staats et al., 2016). DNA barcoding has been used for several years as a regulatory tool to combat seafood mislabelling by the Canadian Food Inspection Agency (CFIA) and the Food and Drug Administration (FDA) in the United States, as well as being reported in literature in several studies across several countries and target species (Handy et al., 2011; Shehata et al., 2018). More recently, with the advent of Next Generation Sequencing (NGS) platforms, the application of

[1]http://futureoffish.com/resources/research-reports/making-sense-wild-seafood-supply-chains

DNA barcoding for single species has evolved into a new technique, called DNA metabarcoding, which is able to identify a full list of species from complex matrices (Franco et al., 2021). This innovative approach is a powerful strategy for the traceability of processed seafood products (fish burgers, surimi-based products, breaded minced fish,...), a product category which is growing in popularity, being time-saving, convenient, and having a long shelf life (Piredda et al., 2022). Several studies have shown that metabarcoding can identify a list of ingredients in seafood products, sometimes even discovering the presence of unexpected taxa (Franco et al., 2021; Giusti et al., 2024; Mottola, Piredda, Catanese, Giorelli, et al., 2022; Toxqui Rodríguez et al., 2023).

Despite its promising future, the success of a DNA metabarcoding assessment is subject to the same weaknesses as those reported for DNA barcoding: the choice of the barcode region, universality of the primers, discrimination power at species level (barcoding gap) and availability of reference sequences in public repositories (Hestetun et al., 2020; Wiemers and Fiedler, 2007). In addition, DNA metabarcoding has constraints with regard to amplicon size imposed by the Next Generation Sequencing platforms, reducing the potential use of primer pairs already designed and applied in DNA barcoding studies, as these are usually long fragments. Indeed, in the case of Illumina platforms, the ones most commonly used in metabarcoding experiments, the maximum length allowed (including primers) is less than 600 bp, obtained by the partial overlap of 2x300 bp reads[2]. Since 2003, with the launch of the DNA barcoding project, several studies have described several universal primers located in different genomic regions (Antil et al., 2023). The mitochondrial cytochrome c oxidase 1 gene (COI) was the first region proposed and, thanks to the several primer pairs designed, is considered a standard method for the identification of metazoans, including insects, mammals, amphibians, reptiles, birds and fish (Andújar et al., 2018). Nevertheless, other barcode candidates have been evaluated and compared with COI, including segments from mitochondrial cytochrome b (cytb), 12S and 16S ribosomal RNA (12S and 16S) (Fernandes et al., 2021; Giusti et al., 2024). Alternative regions and primers have often been developed to overcome difficulties in the identification of some taxonomic groups in biodiverse contexts (Neigel et al., 2007; Rach et al., 2017), but very few studies have designed primers targeting species identification from seafood matrices (Chapela et al., 2002; Mottola,

[2]www.illumina.com/systems/miseq/performance_specifications.html

Piredda, Catanese, Lorusso, et al., 2022; Pan et al., 2020). Seafood species are the part of marine biodiversity that is involved in human nutrition. Every year since 2000, the Food and Agriculture Organization of the United Nations (FAO) has issued a catalogue of organisms included in that diversity in the Aquatic Sciences and Fisheries Information System (ASFIS) List. The ASFIS list uses several sources (e.g., scientific knowledge, fishing reports, statistical data, and industry experts) to identify a wide range of marine organisms from protists to fish, with high value for human nutrition, the economy worldwide and market demand[3].

The aim of this study was to perform a comprehensive evaluation of several primer pairs already described in the literature, and whose fragment length enables them to be used in metabarcoding applications ($< 600bp$), to assess their potential use in studies on the traceability of seafood products. To this end, the pairs collected were used to perform *in-silico* PCR against the reference sequences available in public repositories, and the results obtained were analysed and evaluated using the commercial marine species listed on the ASFIS list. Information and data from this study will help a wide range of researchers, greatly enhancing primer choices for metabarcoding studies applied to seafood products, helping to set up future genetic authentication pipelines and develop new primer pairs.

3.3. Materials and methods

3.3.1. Literature review and primer search

In order to draw up a comprehensive list of primers from literature, we searched Google Scholar (March-June 2022) using the key words "primers" AND "fish OR seafood" AND "barcoding OR metabarcoding". We filtered papers reporting primer pairs targeting marine taxa such as *Actinopteri* and more in general metazoans so as to include molluscs, crustaceans and terrestrial species commonly used in food. Since the aim of the present study was to evaluate primer performance for metabarcoding studies, primers were also filtered based on amplicon length up to

[3] www.fao.org/fishery/en/collection/asfis/en

600 bp.

3.3.2. ASFIS list

The ASFIS list is compiled by the FAO Statistics Team (NFISS) of the Fisheries and Aquaculture Division and species were selected according to their interest or relation to fisheries and aquaculture derived from several sources[4]. The ASFIS list sets out the item, the *ISCAAP code* (assigned according to the FAO International Standard Statistical Classification of Aquatic Animals and Plant), the *taxonomic code* (a twelve-digit alphanumerical code for classificatory purposes), *alpha-3A code* (Inter-agency 3-alpha code related to the scientific or English name of the listed species), the scientific name, common English name and, where possible, the French, Spanish, Arabic, Chinese and Russian name, *the author* (of the scientific name), Family, Order and *Stats data* (indication of records are those for which either capture or aquaculture statistics are available in the FAO databases). The most recent version of the ASFIS list (2022) was downloaded[5], and then edited to make it easy to cross-reference with the *in-silico* PCR results: i) any items not at species level (i.e., Order, Family, Genus), hybrid species or Subgenus names were discarded; ii) all the columns were removed and only the alpha-3A code and the scientific name kept; iii) for each species, the NCBI Taxonomy ID (taxID) was retrieved from the NCBI names.dmp file that includes scientific names for each species together with any synonyms; iv) the online WoRMS Taxon Match Tool (updated July 24, 2023 [6]) was used to solve instances of misspellings or of outdated scientific names in ASFIS with accepted species names.

[4] www.fao.org/fishery/static/ASFIS/ASFIS_biblio.pdf
[5] www.fao.org/fishery/en/collection/asfis/en
[6] www.marinespecies.org/aphia.php?p=match

3.3.3. in-silico PCR analysis

To perform *in-silico* PCR, we used the Creating Reference databases for Amplicon-Based Sequencing software (CRABS version 0.1.1) following the pipeline provided by the authors (Jeunen et al., 2023) and including complete and partial mitogenomes from the Mitochondrial Genome Database of Fish (MitoFish)[7] and all the sequences of COI, 16S, 12S and cytb gene available at the National Center for Biotechnology Information (NCBI) (June 2023)[8]. Any sequences with poor or incomplete taxonomic information (environmental, sp., cf., aff.) were removed from the outputs.

3.3.4. Data analysis

To evaluate the performance of the primer pairs in terms of commercial seafood species, the species lists from the *in-silico* PCR were cross-referenced with the list of species included in the ASFIS document. Statistical description and plots were performed using the R packages phyloseq v1.32 (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2011), tidyverse (Wickham and Wickham, 2017) and microeco (Liu et al., 2021). In addition, we also verified for each pair the ability to detect the three most important terrestrial animal species used as additional ingredients in seafood products (i.e., *Bos taurus*, *Gallus gallus* and *Sus scrofa*).

[7] mitofish.aori.u-tokyo.ac.jp/
[8] www.ncbi.nlm.nih.gov/

3.4. Results and Discussion

3.4.1. Primers list and reference sequences

The revision of the literature selected a total of 32 articles, 21 reporting primer pairs designed for biodiversity studies, and 11 designed for food authenticity purposes. Overall, articles included 53 primer pairs covering four different genomic regions all belonging to the mitochondrial genome, thus confirming the crucial role played by mitochondrial genes in studies on the identification of marine metazoan taxa (Fernandes et al., 2021). The list of the primers included 17 pairs for the Cytochrome c Oxidase subunit I (COI), 14 pairs for the Cytochrome b (cytb), 13 pairs for the 16S rRNA (16S) and 9 primer pairs for the 12S rRNA (12S) (**Table 3.1**). Careful inspection of the literature revealed that, in several cases, a new forward or reverse had been coupled with an already published forward or reverse (COIp02 - COIp07, COIp09), or else the primer pair is a new combination of forward or reverse already designed in other studies (COIp01).

Although seafood species can be considered as part of marine biodiversity, the list of target reference taxa considered by the several authors in the primer design step could play an important role in the ability to identify commercial species used in seafood products. From the 11 articles targeting seafood species, we found 19 primer pairs described none of them being in the 12S region. Distribution of pairs in the other mitochondrial regions revealed that four pairs were designed in cytb, six in 16S and nine in COI. Despite the fact that COI showed the highest number, six of these pairs were part of a single study by Shokralla et al., 2015 where the authors proposed and tested COI mini-barcode primer sets, made up of six pairs, identifying one as the most favourable and the others as alternatives in the event of failure.

In terms of reference sequences available, the download from GenBank confirmed the strong dominance of molecular data from COI genes (4,406,030 sequences) since this is the official region for the DNA Barcoding Project's effect (Hebert et al., 2003) having very good performance for species identification in metazoans. The other regions showed lower number of sequences (840,641 for cytb, 637,700 for 16S and 371,301 for 12S), whereas 802,927 partial and total mitochondrial

genomes were available from MitoFish database. Interestingly, the number of fish mitochondrial genomes was almost equal to the number of sequences of the cytb gene (the second most abundant reference gene) suggesting that decreasing the cost for High-Throughput Sequencing and the availability of online bioinformatic tools for assembly, made the generation of a mitogenome in fish easier and less expensive.

Table 3.1: List of primer pairs selected. For each pair, we report the code used in this study and the original name attributed to forward and reverse sequences, the fragment length, the taxonomic target group they were designed for and the bibliographical citation.

Code	Primer name	Primer Sequences (5'-3')	Fragment length	Target group	Reference
12S_p01	AcMDB07	For-GCCTATATACCGCCGTCG Rev-GTACACTTACCATGTTACGACTT	281 bp	Actinopteri	Bylemans et al., 2018
12S_p02	Ac12s	For-ACTGGGATTAGATACCCCACTATG Rev-GAGAGTGACGGGCGGTGT	385 bp	Actinopteri	Evans et al., 2016
12S_p03	Am12s	For-AGCCACCGCGGTTATACG Rev-CAAGTCCTTTGGGTTTTAAGC	241 bp	Actinopteri	Evans et al., 2016
12S_p04	L1085 H1259	For-CCCAAACTGGGATTAGATACCC Rev-GTTTGCTGAAGATGGCGGTA	215 bp	Vertebrata	Kitano et al., 2007
12S_p05	MiFish-U-F MiFish-U-R	For-GTCGGTAAAACTCGTGCCAGC Rev-CATAGTGGGGTATCTAATCCCAGTTTG	170 bp	Fish	Miya et al., 2015
12S_p06	12SV5	For-TAGAACAGGCTCCTCTAG Rev-TTAGATACCCCACTATGC	99 bp (73-110)	Vertebrata	Riaz et al., 2011
12S_p07	12SF1/R1	For-AGGGATAACAGCGCAATC Rev-TCGTTGAACAAACGAACC	63 - 84 bp	Vertebrata	Riaz et al., 2011
12S_p08	Tele02	For-AAACTCGTGCCAGCCACC Rev-GGGTATCTAATCCCAGTTTG	167 bp	Teleostei	Taberlet et al., 2018
12S_p09	Teleo	For-ACACCGCCCGTCACTCT Rev-CTTCCGGTACACTTACCATG	63 bp	Teleostei	Valentini et al., 2016
16S_p01	16S1F 16S2R	For-GACGAKAAGACCCTA Rev-CGCTGTTATCCCTADRGTAACT	250 bp	Fish, cephalopods and crustaceans	Deagle et al., 2007
16S_p02	16FORF-CEP3 16REVF-CEP	For-GAGAAGACCCTDTKGAGCTT Rev-GCTGTTATCCCTAKGGTAAC	206 - 145 bp	cephalopod species	Giusti et al., 2017*

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Table 3.1: List of primer pairs selected. For each pair, we report the code used in this study and the original name attributed to forward and reverse sequences, the fragment length, the taxonomic target group they were designed for and the bibliographical citation. (Continued)

Code	Primer name	Primer Sequences (5'-3')	Fragment length	Target group	Reference
16S_p03	16S-forward	For-AYAAGACGAGAAGACCC	250 bp	Universal animal mini-barcode	Sarri et al., 2014*
	16S-reverse	Rev-GATTGCGCTGTTATTCC			
16S_p04	Vert-16S	For-AGACGAGAAGACCCYdTGGAGCTT	264 bp	Vertebrata	Vences et al., 2016
		Rev-GATCCAACATCGAGGTCGTAA			
16S_p05	FOR16Spc	For-TGCCCGTGCAGAAGCGG	295 - 339 bp	Clupeidae, Engraulidae, Salangidae, Scombridae	Armani et al., 2012*
	REV16Spc	Rev-CAACATCGAGGTCGTAAACCC			
16S_p06	16sf-var	For-CAAATTACGCTGTTATCCCTATGG	148 - 209 bp	cephalopod species (Ommastrephidae, Loliginidae)	Chapela et al., 2002*
	16sr-var	Rev-GACGAGAAGACCCTAATGAGCTTT			
16S_p07	Fish16SF	For-GACCCTATGGAGCTTTAGAC	203 bp	Fish	DiBattista et al., 2017
	16S2R-degenerate	Rev-CGCTGTTATCCCTADRGTAACT			
16S_p08	Ac16s	For-CCTTTTGCATCATGATTTAGC	330 bp	Freshwater fishes and amphibians	Evans et al., 2016
		Rev-CAGGTGGCTGCTTTTAGGC			
16S_p09	Ve16s	For-CGAGAAGACCCTATGGAGCTTA	310 bp	Freshwater fishes and amphibians	Evans et al., 2016
		Rev-AATCGTTGAACAAACGAACC			
16S_p10	16S-HF	For-ATAACACGAGAAGACCCT	80 - 125 bp	Animal species in food	Horreo et al., 2013*
	16S-HR1	Rev-CCCACGGTCGCCCCAAC			
16S_p11	16S-HF	For-ATAACACGAGAAGACCCT	80 - 125 bp	Animal species in food	Horreo et al., 2013*
	16S-HR2	Rev-CCCGCGGTCGCCCCAAC			
16S_p12	L2513/H2714	For-GCCTGTTTACCAAAAACATCAC	244 bp	Vertebrata	Kitano et al., 2007
		Rev-CTCCATAGGGTCTTCTCGTCTT			
16S_p13	16S fish-specific	For-GGTCGCCCCAACCRAAG	68 bp	Fish	Shaw et al., 2016
	16S fish-specific	Rev-CGAGAAGACCCTWTGGAGCTTIAG			

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Table 3.1: List of primer pairs selected. For each pair, we report the code used in this study and the original name attributed to forward and reverse sequences, the fragment length, the taxonomic target group they were designed for and the bibliographical citation. (Continued)

Code	Primer name	Primer Sequences (5'-3')	Fragment length	Target group	Reference
COI_p01	FISHCOILBC	For-CTCAACYAATCAYAAAGATATYGGCAC	139 bp	Fish	Giusti et al., 2017*
	Revshort1	Rev-GGYATNACTATRAAGAAAATTATTAC			
COI_p02	mlCOIintF	For-GGWACWGGWTGAACWGTWTAYCCYCC	313 bp	Marine metazoa	Leray et al., 2013
	HCO2198	Rev-TAAACTTCAGGGTGACCAAAAAATCA			
COI_p03	mlCOIintF	For-GGWACWGGWTGAACWGTWTAYCCYCC	313 bp	Marine metazoa	Leray et al., 2013
	dgHCO2198	Rev-TAAACTTCAGGGTGACCAAARAAYCA			
COI_p04	mlCOIintF	For-GGWACWGGWTGAACWGTWTAYCCYCC	313 bp	Marine metazoa	Leray et al., 2013
	jgHCO2198	Rev-TAIACYTCIGGRTGICCRAARAAYCA			
COI_p05	LCO1490	For-GGTCAACAAATCATAAAGATATTGG	319 bp	Marine metazoa	Leray et al., 2013
	mlCOIintR	Rev-GGRGGRTASACSGTTCASCCSGTSCC			
COI_p06	dgLCO1490	For-GGTCAACAAATCATAAAGAYATYGG	319 bp	Marine metazoa	Leray et al., 2013
	mlCOIintR	Rev-GGRGGRTASACSGTTCASCCSGTSCC			
COI_p07	jgLCO1490	For-TITCIACIAAYCAYAARGAYATTGG	319 bp	Marine metazoa	Leray et al., 2013
	mlCOIintR	Rev-GGRGGRTASACSGTTCASCCSGTSCC			
COI_p08	Uni-MinibarF1	For-TCCACTAATCACAARGATATTGGTAC	127 bp	Universal animal mini-barcode	Meusnier et al., 2008
	Uni-MinibarR1	Rev-GAAAATCATAATGAAGGCATGAGC			
COI_p09	mini-COI-F (LCO1490)	For-GGTCAACAAATCATAAAGATATTGG	136 bp	edible species (fish, shrimps, birds, mammals)	Pan et al., 2020*
	mini-COI-R	Rev-ACTATAAAGAAGATTATTACAAAGGC			
COI_p10	Fish_miniA_F_t	For-ACIAAICAIAAAGAYATYGGC	129 bp	fish	Shokralla et al., 2015*
	Fish_miniA_R_t	Rev-AARAAAATYATAACRAAIGCRTGIGC			

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Table 3.1: List of primer pairs selected. For each pair, we report the code used in this study and the original name attributed to forward and reverse sequences, the fragment length, the taxonomic target group they were designed for and the bibliographical citation. (Continued)

Code	Primer name	Primer Sequences (5'-3')	Fragment length	Target group	Reference
COI_p11	Fish_miniB_F_t	For-GCIGGIRTYTCITCIATYYTAG	227 bp	fish	Shokralla et al., 2015*
	Fish_miniB_R_t	Rev-ACTTCAGGGTGICCGAARAATCA			
COI_p12	Fish_miniC_F_t	For-ACYAAICAYAAAGAYATIGGCAC	127 bp	fish	Shokralla et al., 2015*
	Fish_miniC_R_t	Rev-GAARATCATAATGAAGGCATGIGC			
COI_p13	Fish_miniD_F_t	For-GGIACIGGITGRACIGTITAYCCYCC	208 bp	fish	Shokralla et al., 2015*
	Fish_miniD_R_t	Rev-GTRATICCIGCIGCIAGIAC			
COI_p14	Fish_miniE_F_t	For-ACYAAICAYAAAGAYATIGGCAC	226 bp	fish	Shokralla et al., 2015*
	Fish_miniE_R_t	Rev-CTTATRTTRTTTATICGIGGRAAIGC			
COI_p15	Fish_miniF_F_t	For-GGIACIGGITGRACIGTITAYCCYCC	314 bp	fish	Shokralla et al., 2015*
	Fish_miniF_R_t	Rev-CTTCAGGGTGICCGAARAATC			
COI_p16	F	For-ATCACAAAGACATTGGCACCCT	295 bp	fish and non-fish species	Sultana et al., 2018*
	R	Rev-AATGAAGGGGGGAGGAGTCAGAA			
COI_p17	mlCOIintF-XT	For-GGWACWRGWTGRACWITITAYCCYCC	ca. 313 bp	Marine metazoa	Wangensteen et al., 2018
	jgHCO2198	Rev-TAIACYTCIGGRTGICCRAARAAYCA			
CYTB_p01	L14912/H15149c	For-AAAAACCACCGTTGTTATTCAACTA	413 bp	Fish and amphibians	Burgener and Hübner, 1998
		Rev-GCCCCTCAGAATGATATTTGTCCTCA			
CYTB_p02	Cytb1F	For-CAGCTATTCCATATGTTGGTGA	297 bp	Loliginidae and Ommastrephidae	Chapela et al., 2002*
	Cytb1R	Rev-GGTTACTAAAGGATTAGCTGGA			
CYTB_p03	CytBL1C modified	For-CCWGCWAAYATWWCAACTTTRTGAARGTTTGG	~357 bp	Crustaceans and molluscs in seafood	Dwiyitno et al., 2021*
	CytBHW modified	Rev-CYCCYCARAAWGATATTTGYCCYCA			
CYTB_p04	L14735/H15149c2	For-AAAAACCACCGTTGTTATTCAACTA	413 bp	Vertebrata	Hänfling et al., 2016
		Rev-GCDCCTCARAATGAYATTTGTCCTCA			

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Table 3.1: List of primer pairs selected. For each pair, we report the code used in this study and the original name attributed to forward and reverse sequences, the fragment length, the taxonomic target group they were designed for and the bibliographical citation. (Continued)

Code	Primer name	Primer Sequences (5'-3')	Fragment length	Target group	Reference
CYTB_p05	BDR-L BDR-H-mod1	For-GCMAACGGSGCNTCYTTCTTCT Rev-TGACGGTAGCHCCTCAGRADGACATTTGTCCYCA	131 bp	Mixed canned fish	Kappel et al., 2017*
CYTB_p06	BMID-L-mod1 BMID-H-mod1	For-ATCYCATTCCACCCATACTWCTC Rev-AATAGGAARTATCATTCRGGTTTRATG	126 bp]	Mixed canned fish	Kappel et al., 2017*
CYTB_p07	L14841/H15149	For-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA Rev-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	307 bp	Vertebrata	Kocher et al., 1989
CYTB_p08	L14912/H15149	For-TTCCTAGCCATACAYTAYAC Rev-GGTGGCKCCTCAGAAGGACATTTGKCCYCA	235 bp	Teleostei	Miya and Nishida, 2000
CYTB_p09	L14816 H15173	For-CCATCCAACATCTCAGCATGATGAAA Rev-CCCCTCGAATGATATTTGTCCTCA	357 bp	Universal vertebrate animal mini-barcode	Parson et al., 2000
CYTB_p10	CEF-H H15149AD	For-TTATGGKTGRGTRYTDCGTTAT Rev-GCICCTCARAATGAYATTTGTCCTCA	160 bp	Loliginidae, Ommastrephidae, Sepiidae, Octopodidae	Santaclara et al., 2007*
CYTB_p11	FishCBL/CBR	For-TCCTTTTGAGGCGCTACAGT Rev-GGAATGCGAAGAATCGTGTT	90 bp	Fish	Thomsen et al., 2012
CYTB_p12	Fish2bCBR/CBL	For-GATGGCGTAGGCAAACAAGA Rev-ACAACTTCACCCCTGCAAAC	40 bp	Fish	Thomsen et al., 2012
CYTB_p13	Fish2degCBL/CBR	For-ACAACTTCACCCCTGCRAAY Rev-GATGGCGTAGGCAAATAGGA	40 bp	Fish	Thomsen et al., 2012
CYTB_p14	mcb398 mcb869	For-TACCATGAGGACAAATATCATTCTG Rev-CCTCCTAGTTTGTTAGGGATTGATCG	421 bp	Fish, mammals, birds, reptiles	Verma and Singh, 2002

3.4.2. Description of ASFIS content

From the original ASFIS list, constituted by a total of 13,417 items, we initially kept 12,301 items reporting the species level. The list contained several cases of misspelling, synonyms and unaccepted names that were fixed using the WoRMS Taxon Match Tool, thus reducing the list to 12,193 taxa (Supplementary Table 1). It highlights the issue related to the fact that the taxonomy of reference sequences is constantly being revised, as species are dynamic entities (Hleap et al., 2021). An overview of the ASFIS list revealed that six taxonomic groups contained 92% of the total species on the list: *Actinopteri* included 62% of total species, followed by *Mollusca* (e.g. *Bivalvia*, *Gastropoda* and *Cephalopoda*) (13% of total species), *Malacostraca* (8%), *Chondrichthyes* (6%), *Aves* (1%) and *Mammalia* (1%). These six groups included the most common seafood taxa, while the remaining 8% was made up of taxonomic groups with less widespread or novel seafood edible species; it can be divided into a group comprising 4% of the list made up of *Cnidaria*, *Echinodermata*, *Arthropoda* (excluding *Malacostraca*) and *Rhodophyta*, each contributing 1% of species, while the other 4% included a long list of minor taxa, each contributing a total number of species of $< 1\%$. Molecular data were not available for all the species in the ASFIS list, and their presence in public repositories was inferred by the possibility of associating taxID (in GenBank) to species name in the ASFIS list. Data crossing revealed that molecular data were not available for 18% of the species on the list: the highest number of taxa without molecular data belonged to *Actinopteri* (56%), followed by *Mollusca* (21%), *Malacostraca* (8%), *Chondrichthyes* (8%). One *Aves* and two *Mammalia* species on the ASIFS list, highlighted the lack of molecular data. Finally, the remaining 7% of taxa without molecular information belonged to the less widespread or minor taxa.

3.4.3. In-silico PCR

The plots of amplicon length distributions, generated after *in-silico* PCR for each pair, showed distribution peaks in line with the size declared by the authors in the original paper describing the primer pair (**Table 3.1**; Supplementary Fig. 1).

However, in the different taxonomic groups, amplicon length can deviate from the expected size. Thus, such plots constituted an important base to guide researchers in the filtering of metabarcoding raw data (set up of minimum and maximum amplicon length) thus facilitating the filtering of spurious data. The pair COIp13 collected the highest number of species (177,320), whereas the pair CYTBp07 captured the lowest (27) (**Table 3.2**). The latter pair was designed by Kocher et al., 1989 to estimate the evolution rate among Vertebrata, and the poor performance found in our study confirmed previous results obtained *in-silico* PCR by S. Zhang et al., 2020.

Table 3.2: Summary of results obtained by *in-silico* PCR. After the code of primer pairs, the columns represent the number of sequences and the corresponding numbers of total species and ASFIS species recovered. The last column shows the percentage of ASFIS species recovered by each primer pair on the total of 12,193 species included in the ASFIS list.

Code	Number of sequences after PCR	Total species	ASFIS species	Total percentage ASFIS coverage (%)
12Sp01	43,206	19,615	3,853	31.6
12Sp02	59,850	28,283	4,101	33.6
12Sp03	43,113	20,231	4,039	33.1
12Sp04	13,767	8,421	2,137	17.5
12Sp05	25,165	14,917	3,595	29.5
12Sp06	28,063	21,078	3,680	30.2
12Sp07	25,064	18,764	4,816	39.5
12Sp08	32,901	19,543	3,810	31.2
12Sp09	21,440	14,604	3,342	27.4
16Sp01	173,193	87,750	6,533	53.6
16Sp02	156,769	80,696	6,486	53.2
16Sp03	157,220	80,667	6,182	50.7
16Sp04	140,031	69,467	6,096	49.9
16Sp05	55,274	21,659	4,763	39.1
16Sp06	55,308	35,792	988	8.1
16Sp07	92,172	44,676	5,377	44.1
16Sp08	23,537	12,888	3,073	25.2
16Sp09	104,455	40,816	5,298	43.5
16Sp10	62,515	33,614	5,129	42.1
16Sp11	61,328	32,912	5,119	42
16Sp12	92,059	43,211	5,231	42.9
16Sp13	70,445	40,973	5,517	45.3

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Table 3.2: Summary of results obtained by *in-silico* PCR. After the code of primer pairs, the columns represent the number of sequences and the corresponding numbers of total species and ASFIS species recovered. The last column shows the percentage of ASFIS species recovered by each primer pair on the total of 12,193 species included in the ASFIS list. (Continued)

Code	Number of sequences after PCR	Total species	ASFIS species	Total percentage ASFIS coverage (%)
COIp01	194,828	60,616	4,800	39.4
COIp02	287,205	58,556	3,901	32
COIp03	312,104	63,636	4,616	37.9
COIp04	319,205	65,125	4,824	39.6
COIp05	69,085	13,679	815	6.9
COIp06	93,954	18,179	1,795	14.7
COIp07	145,087	29,173	3,655	30
COIp08	105,269	37,988	1,439	11.8
COIp09	84,315	28,801	1,347	11
COIp10	82,372	36,641	5,031	41.3
COIp11	135,564	50,918	4,787	39.3
COIp12	115,198	43,198	3,616	29.7
COIp13	488,713	177,320	7,811	64.1
COIp14	127,902	37,362	4,830	39.6
COIp15	272,850	63,921	4,993	41
COIp16	65,252	17,202	3,245	26.6
COIp17	256,958	62,651	4,894	40.1
CYTBp01	76,402	8,496	2,226	18.3
CYTBp02	1,317	629	80	0.7
CYTBp03	3,633	1,538	94	0.8
CYTBp04	79,148	8,846	2,329	19.1
CYTBp05	61,702	21,566	3,525	28.9
CYTBp06	93,303	29,219	3,867	31.7
CYTBp07	84	27	6	0.05
CYTBp08	127,715	33,538	4,015	32.9
CYTBp09	37,709	6,937	919	7.5
CYTBp10	6,980	2,723	333	2.7
CYTBp11	55,528	22,364	3,206	26.3
CYTBp12	26,064	15,073	2,647	21.7
CYTBp13	39,061	21,362	3,494	28.6
CYTBp14	144,276	28,835	3,260	26.7

3.4.4. Primer performance in commercial species (ASFIS List)

Data crossing between *in-silico* results and ASFIS list showed that the primer pairs retrieved 8,919 (73%) of the species included in the ASFIS table (Supplementary Table 1). The taxonomic composition of these 8,919 species well fit the original proportion of Classes included on the ASFIS list and described above. Indeed, the species found belonged to 48 different Classes and were dominated by *Actinopteri* with 5,734 species (64%), followed by 798 species of *Malacostraca* (9%), 552 species of *Chondrichthyes* (6%), 404 species of *Bivalvia* (5%), 342 species of *Gastropoda* (4%), 208 species of *Cephalopoda* (2%), 158 species of *Aves* (2%) and 127 species of *Mammalia* (1%). Beyond these eight Classes, each of the other 40 Classes included taxa with abundances equal to or below 1%, showing the same proportions previously found on the ASFIS list (**Fig. 3.1**). All the pairs were able to retrieve at least one *Actinopteri* species, in a range between 5,219 (COIp13) and 4 (CYTBp02); *Chondrichthyes* were retrieved by 51/53 pairs, with the highest number being retrieved by the COIp13 pair (522 species); 47/53 pairs were able to capture *Malacostraca* species, with the highest number being retrieved by the 16Sp01 pair (703 species); *Bivalvia* were retrieved by 41/53 pairs, with the highest number retrieved by the 16Sp03 pair (301 species) and *Cephalopoda* were retrieved by 37/53 pairs, with the highest number by the COIp13 pair (189 species).

Focus on the most widespread seafood species, (*Actinopteri*, *Chondrichthyes*, *Malacostraca*, *Bivalvia* and *Cephalopoda*), showed different patterns of richness (number of species) captured by each primer pair within each molecular region (**Fig. 3.2**). One primer pair from the COI region (COIp13) showed the highest value of richness (7,811), but the boxplots also revealed that primer pairs located in the 16S gene generally retrieved higher and quite homogeneous numbers of ASFIS species, in contrast with the primer pairs located within the cytb gene that retrieved the lowest and the most heterogeneous numbers of species among them. Interestingly, the best performer pair, i.e. COIp13, was one of the six pairs included in the mini-barcode primer sets proposed for fish products by Shokralla et al., 2015. In the original paper, the pair SH-E (corresponding to COIp14 in our **Table 3.1**) was identified as the most favourable for use in mini-barcoding, but, in our analysis, it showed lower richness value than COIp13 (4,830 vs 7,811 species for COIp14

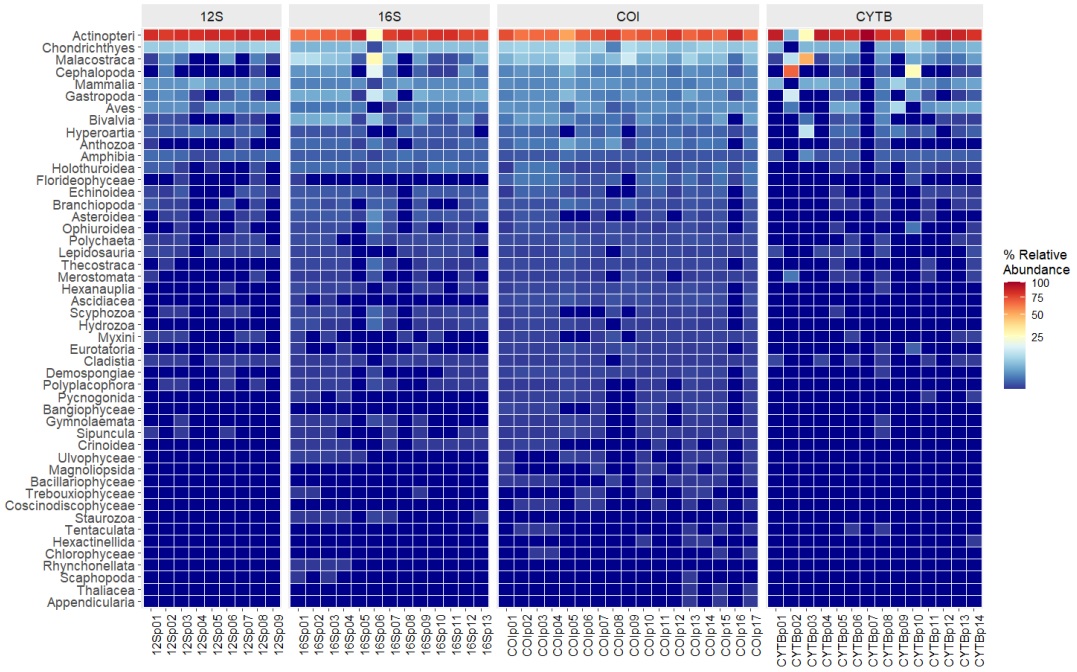

Figure 3.1.: Heatmap presenting the taxonomic coverage of all 48 Classes retrieved, expressed as relative percentage abundance.

and COIp13, respectively) and lower percentage of ASFIS species recovered (40% vs 64% ASFIS species for COIp14 and COIp13, respectively). However, such a discrepancy was not a surprise since the actual amplification may differ from the *in-silico* results and is affected by the composition of the dataset used for the test. Indeed, the original analysis was restricted to sequences from specimens representing 200 species of commercial fish, whereas in our test a wider number of fish and other seafood species have been incorporated. The taxonomic profile generated by COIp13 covered all the most important seafood species including 67% of *Actinopteri* species, 2% *Bivalvia*, 2% *Cephalopoda*, 7% *Chondrichthyes*, and 9% of *Malacrostaca* (**Fig. 3.3**).

After the best performing pair, a group of four pairs from the 16S region, 16Sp02, 16Sp03 (designed for seafood) and 16Sp01,16Sp04 (for general marine biodiversity) revealed high and similar richness values (6,533 to 6,096 number of specie). The percentages of species captured at Class level were also very similar: *Actinopteri* made up 64%-68%; *Bivalvia* (4%-5%), *Cephalopoda* (3%), *Chondrichthyes* (5%) and *Malacrostaca* (6%-11%). A second group of pairs from the COI region, COIp03,

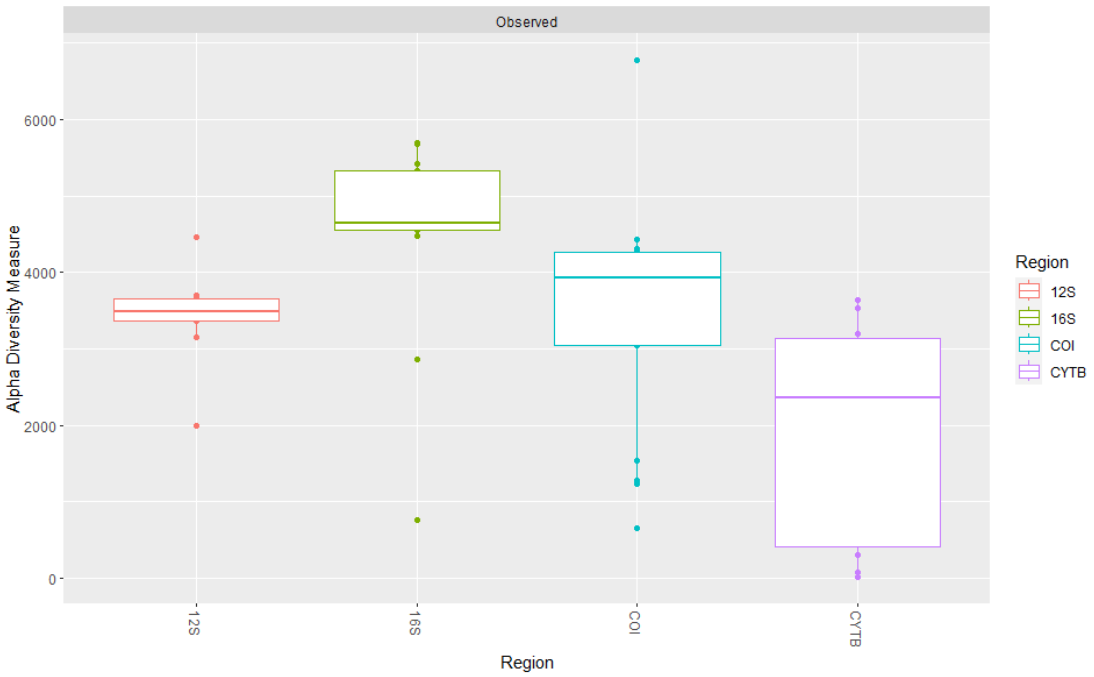

Figure 3.2.: Boxplots of richness values calculated for primer pairs in each primer region

COIp04 and COIp17 (designed for general marine biodiversity) and COIp11, COIp14 and COIp15 (designed for seafood) retrieved lower richness values (4,616 – 4,993) but the percentages of species captured at Class level were very similar to those of 16S pairs. Both 12S and CYTB pairs showed more affinity for fish taxa (*Actinopteri* and *Chondrichthyes*), and their performances deviated from the proportion of species described for the ASFIS list. Indeed, in the case of cytb, the group of pairs CYTBp05, CYTBp06 (designed for seafood) and CYTBp08, CYTBp11, CYTBp13 and CYTBp14 (designed for general marine biodiversity), revealed an average percentage of 83% of *Actinopteri*, 5% of *Chondrichthyes* and only 2% of *Malacrostaca*. In addition, a group of three primer pairs showed very peculiar taxonomic profiles: the pair CYTBp07 captured only 6 ASFIS species, all belonging to *Actinopteri* (Tetraodontidae); the pair CYTBp03 retrieved the highest percentage of *Malacrostaca* (49%) and CYTBp02 the highest percentage of *Cephalopoda* (67%) within a total of 94 and 80 ASFIS species, respectively. In general, the taxonomic profiles generated by 12S primer pairs showed a strong bias toward *Actinopteri* and *Chondrichthyes*, that made up, on average, respectively 83% and 8% of total species collected. On the contrary, worse results were reported for the other Classes: *Bivalvia* were collected by 5/9 pairs (average composition of

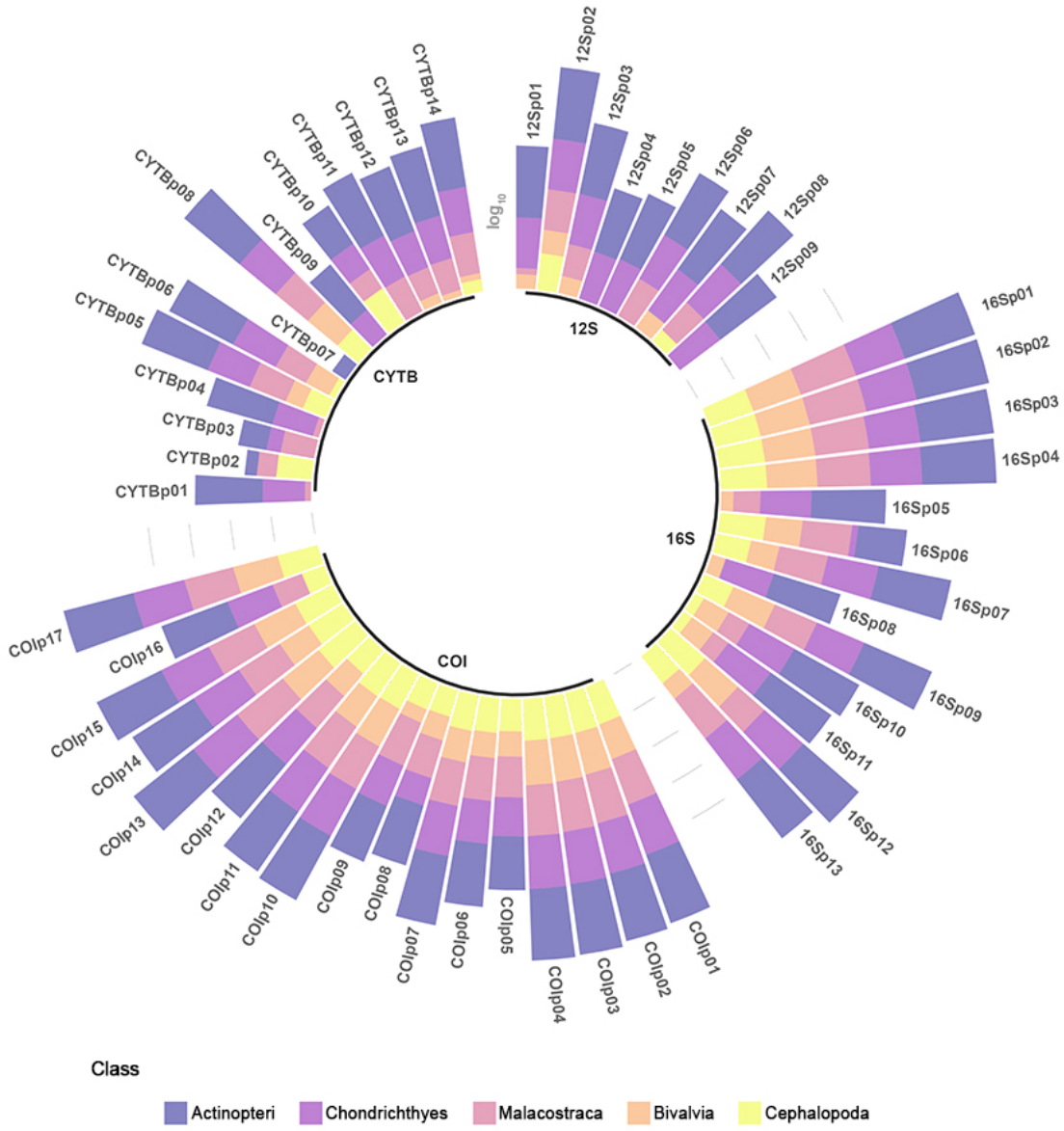

Figure 3.3.: Circular bar plots show the taxonomic composition captured by each primer pair shown in terms of abundance (absolute number of species) at Class level (Actinopteri, Cephalopoda, Bivalvia, Malacrostaca and Chondrichthyes). A log_10 scale was applied to optimize data visualization.

0.2%), *Cephalopoda* by 2/9 pairs (average of 0.8%) and *Malacostraca* by 6/9 pairs (average composition of 1%) (**Fig. 3.3**).

The described patterns confirmed, once again, the key role of molecular region and choice of primers as potential biases in the taxonomic profiles generated. Venn diagrams highlighted that the four regions (COI, 16S, 12S, cytb) were able to provide very similar taxonomic profiles at Class, Order, and Family levels (**Fig. 3.4**). Nevertheless, at Family level, each molecular region showed the ability to recover a part of the taxa that was not shared with the other regions. Such patterns were more evident at Genus or Species level, where the value of shared taxa among regions decreased (89% at genus level and 77% at species level) and the percentage of exclusive taxa of each region increased (2.4% for COI, 0.6% for 16S, 0.3% for CYTB and 0.2% for 12S) (**Fig. 3.4**). Deeper exploration, focused on primer pairs within each molecular region, highlighted that primer pairs from 12S were able to provide a quite homogeneous taxonomic profile, even at species level, with about 50% of shared species. However, as reported above, 12S was unbalanced towards *Actinopteri* and *Chondrichthyes*; thus, it cannot guarantee a full profile of seafood products. By contrast, the primer pairs from cytb showed no shared Order, Family, Genus or species. Actually, this pattern was mainly due to the group of pairs discussed above (CYTBp02, CYTBp03 and CYTBp07), which produced biased taxonomic profiles. The COI and 16S pairs showed similar results at species level (2.6% for COI, 2% for 16S); however, at Order, Family and Genus levels, the COI pairs revealed more similar profiles (Supplementary Fig. 2). Overall, these outcomes once again confirmed the potential biases introduced by the different primer pairs, suggesting the simultaneous use of two or more pairs to overcome some weaknesses as well as caution in the comparison of taxonomic profiles generated with different molecular regions and/or primer pairs.

3.4.5. Less common seafood (ASFIS List)

The 40 Classes captured by the *in-silico* PCR with abundances equal to or below 1%, included: *Holothuroidea*, *Asteroidea* and *Echinoidea* (*Echinodermata*); *Hyperoartia* and *Myxini* (*Agnatha*); *Hydrozoa*, *Scyphozoa* and *Hydrozoa* (*Cnidaria*); *Thaliacea*, *Appendicularia* and *Ascidiacea* (*Tunicata*); *Amphibia*; *Polychaeta*; *Florideophyceae*

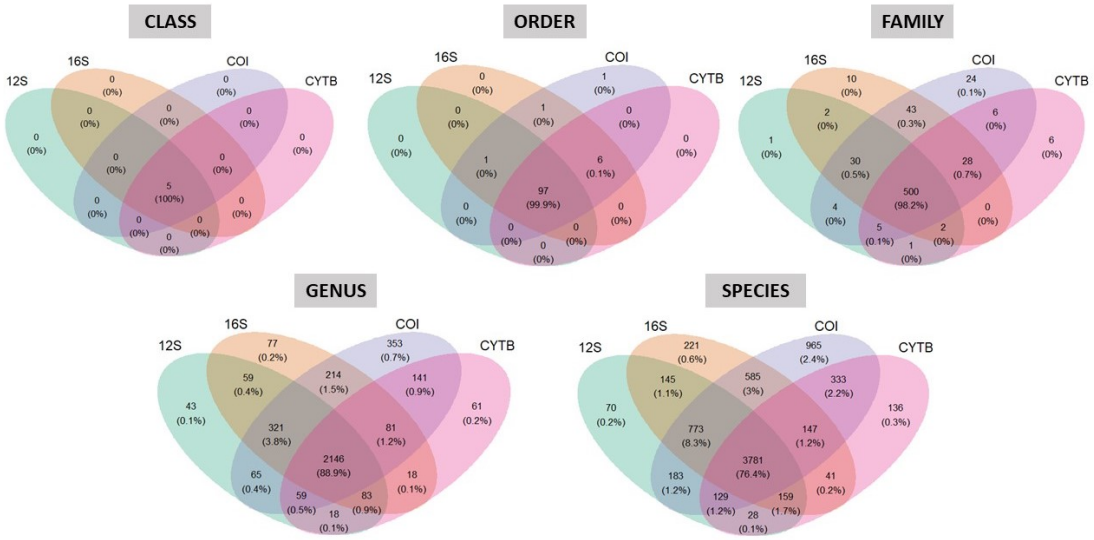

Figure 3.4.: Venn diagrams show the value of shared taxonomic profiles among the four marker regions at Class, Order, Family, Genus, and Species levels.

and *Bangiophyceae* (*Rhodophyta*), *Trebouxiophyceae*, *Chlorophyceae* and *Ulvophyceae* (*Chlorophyta*) (**Fig. 3.1**). Such taxa, in the past mainly involved in traditional foods or in the gastronomic heritage of human populations, are now moving into the lives of people in other parts of the world. Indeed, the use of such lesser known taxa is growing and spreading due to globalisation, migration, ethnic diversity, and the search for new sources of nutrients. This phenomenon is not a surprise, since changes in diets and food habits are recurrent in world history, as happened with, for example, the introduction of tomatoes, potatoes, and cocoa, which were not originally part of the European diet (Scaffardi, 2022). However, as Regulatory Agencies are responsible for establishing standards and policies governing the safety and nutritional quality of foods, a series of labelling policies related to health and nutrition and the implementation of traceability tools need to be developed. An important case in point is that of some Classes of *Cnidaria*, such as *Hydrozoa* and *Scyphozoa* (i.e., jellyfish), that have been launched as novel foods in Western countries due to their antioxidant properties and good protein profile even though they are not yet authorized in the European Union (De Domenico et al., 2019; Leone et al., 2019; Torri et al., 2020). In addition, *Anthozoa* (i.e., deep sea coral) are eaten in some areas of Asia and North America but also in Spain and Sardinia and have now been included on the IUCN Red List (Ballesteros-Contreras

et al., 2022). Overall, the ASFIS list includes 137 *Cnidaria* species and the primer pairs retrieved 84 species of *Anthozoa*, 13 species of *Hydrozoa*, 9 species of *Scyphozoa* and one of *Staurozoa*. COIp17 was the pair that alone collected the highest number of *Anthozoa* species (65), while 16Sp01 and 16Sp02 retrieved the highest number of *Hydrozoa* species (13) and COIp13 the most *Scyphozoa* species (8). Also, the ASFIS list included 190 *Echinodermata* (*Holothuroidea*, *Asteroidea* and *Echinoidea*) mainly captured by the pairs belonging to the 16S and COI regions; 46 *Agnatha* (*Hyperoartia* and *Myxini*) bound by all the molecular regions, with the highest number of species being captured by COIp13 (42). For the 46 species of *Tunicata* (*Thaliacea*, *Appendicularia* and *Ascidiacea*), included on the ASFIS list, best results were reported by COI pairs, while very poor results were found for the other molecular regions. Finally, the 34 Amphibia, particularly appreciated and traded in several countries, both in the West and Asia (Auliya et al., 2023), were collected mainly by the 16S primer pairs. The ASFIS list is also characterized by the presence of non-animal taxa. *Rhodophyta* (*Florideophyceae* and *Bangiophyceae*) are appreciated in some countries as food for their high concentration in vitamins, carotenoids, protein and in worldwide food industries for the extraction of additives (Baweja et al., 2016; Rioux and Turgeon, 2015). Among the 140 *Rhodophyta* in the ASFIS list, the primer pair COIp13 collected the highest number of *Florideophyceae* (79) and *Bangiophyceae* (7). All COI primers, except COIp09, retrieved some of these Classes, confirming the barcoding power of this region for red algae (Robba et al., 2006; Urban et al., 2022). In contrast, 12S and 16S primer pairs retrieved *Rhodophyta* only in a few cases, and in one case within the cytb region (CYTBp10).

3.4.6. Terrestrial components not included on the ASFIS list

Exploration of terrestrial taxa potentially used as additional ingredients in seafood products (*Sus scrofa*, *Gallus gallus* and *Bos taurus*) revealed that all the 12S and 16S primer pairs were able to recover them, except for the 16Sp06 pair that only recovered *Bos taurus*. Less homogenous patterns were exhibited by pairs within the COI and cytb regions, where only 10/17 COI primers and 7/14 cytb primers were potentially able to amplify the three terrestrial species. The need to trace these species within seafood products has been reported in several studies, because

the use of these taxa as undeclared additional ingredients in processed seafood products, has implications for consumers' health and religious issues. In addition, insects, recently included as food and feed by the Regulation (EU) 2015/2283, were able to be traced by the primer pairs tested. Indeed, while the traceability of insects is not strictly related to seafood ingredients, they could enter the processed seafood supply chain, as ingredients or raw materials from other preparations (Traynor et al., 2024). Some efforts to find mini barcodes among the COI and 16S regions have been conducted, while Leray (COIp02, COIp05) and Meusnier primers (COIp08) have already been tested for insects (Brandon-Mong et al., 2015; Clarke et al., 2014). In our test, the highest insect richness was shown in the 12S region by the 12Sp02 pair ($\sim$ 7,000 species) and in the 16S region by the 16Sp13 pair ($\sim$ 9,000 species). Among the COI pairs, the highest number of species was retrieved by COIp13 (more than 100,000 species) and within the cytb region by the pairs CYTBp08 and CYTBp14 ($\sim$ 7,500 species) (Supplementary Fig. 1).

3.4.7. Sustainability

Although the ASFIS list includes taxa involved in human nutrition and which are globally traded, some of them are species subject to exploitation, by-catch, illegal fishing, or are included on IUCN or Red lists. Indeed, concern about the impact of seafood production on marine biodiversity is growing, while the application of molecular methods has been shown to be useful for investigating illegal trade, assessing stocks, and collecting data on the composition of catches (Maiello et al., 2022; Stoeckle et al., 2021). In this context, information provided by our *in-silico* PCR can provide a preliminary screening for the identification of the best candidate pair for each target. As shown above, while Chondrichthyes (sharks) include important seafood products, they also comprise several endangered species (Cardeñosa et al., 2022; Dulvy et al., 2021; Prasetyo et al., 2023). Moreover, for the traceability of marine mammals, that are facing new threats (Jog et al., 2022; Valsecchi et al., 2020), several pairs (51/53) provided positive results, but the highest number was retrieved by pair CYTBp14 (122 out of the 130 species listed) designed by Verma and Singh, 2002.

3.5. Conclusions

Molecular approaches are essential tools for ensuring global food traceability, promoting seafood quality and safety and the implementation of a farm to fork strategy; our study provided information to support experimental design for metabarcoding studies focused on the traceability of seafood products. Beyond the main description on their overall ability to bind commercial marine species, the data can be explored by users to address several issues or targets within the seafood authentication field. Furthermore, given the importance of discrimination power between genetic targets for seafood authentication, further specific studies are needed so that the extracted molecular data can be used to perform dedicated analyses, at least for the most important commercial fish species, such as considered for the genus *Scomber* sp. by Lorusso, Mottola, et al., 2024. Our results highlight that most of the pairs recovered from literature were designed for biodiversity studies, but the best performer pair was designed and proposed for fish products. This confirmed the importance of the dataset used for the design of the primers, suggesting that new studies targeting marine species with high value for human nutrition, the economy worldwide and market demand are needed to improve the success of their traceability using metabarcoding techniques.

However, the limitations of an *in-silico* PCR approach are well known, since various parameters affect the amplification of target genes *in-vivo*. They include number, position, and type of mismatches between primer and template, primer properties (length, GC content, 3' end stability and influence of nearest-neighbouring nucleotides), PCR conditions (annealing temperature and template concentration). In addition, in the case of metabarcoding studies with complex matrices containing several species, the competition among different DNA templates is another source of variability affecting the result of amplification (Alberdi et al., 2018). Therefore, the success or otherwise of *in-vitro* PCR amplification is governed by complex molecular interactions; therefore, even though *in-silico* PCR makes it possible to evaluate a huge number of reference templates, it can lead to inaccurate results, including predictions of false positives. On the other hand, primer tests *in-vivo*, usually performed by the authors for the validation step of the pair, included a limited fraction of reference specimens or natural samples, leading to potentially incomplete and biased evaluation of universality or specificity of the primers.

Nevertheless, keeping in mind both the pros and cons, preliminary *in-silico* evaluation of primers is a powerful tool for primer choice in metabarcoding studies, helping implement standardized and dedicated pipelines in order to mitigate species substitution fraud and promote an innovative Food Safety Management System.

4. Decoding Seafood: Multi-Marker Metabarcoding for Authenticating Processed Seafood

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4.1. Abstract

Given the recognized nutritional value of fish and shifting consumer lifestyles, processed seafood has become increasingly prevalent, comprising a significant portion of global food production. Although current European Union labelling regulations do not require species declaration for these products, food business operators often voluntarily provide this information on ingredient lists. Next Generation Sequencing (NGS) approaches are currently the most effective methods for verifying the accuracy of species declarations on processed seafood labels. This study examined the species composition of 20 processed seafood products, each labelled as containing a single species, using two DNA metabarcoding markers targeting the mitochondrial cytochrome c oxidase I (COI) and 16S rRNA genes. The combined use of these markers revealed that the majority of the products contained multiple species. Furthermore, two products were found to be mislabelled, as the declared species were not detected. These findings underscore that NGS is a robust technique that could be adopted to support routine food industry activities and official control programs, thereby enhancing the 'From Boat to Plate' strategy and combating fraudulent practices in the complex fisheries supply chain.

4.2. Introduction

Seafood has emerged as one of the most highly traded food commodities globally, driven by its relatively low cost and recognized nutritional importance in the human diet (Kroetz et al., 2020; FAO, 2020). Technological advancements in the seafood industry have facilitated the development of a broad range of appealing ready-to-eat and ready-to-cook products, which have significantly altered consumer perceptions of seafood. These innovative product forms are attracting new consumer targets that avoid seafood due to preparation time or a general dislike for fresh fish products, particularly among younger consumers (Piredda et al., 2023; Verza et al., 2023). While some products, such as surimi or fish cakes, are clearly identified as mixtures of different species, others may disguise their composite nature under the appearance of higher-quality products like 'fillets', leading to misperceptions among consumers. The diffusion of these new seafood forms not only boosts industry profits but also increases risks to consumers and marine ecosystems. The assembly of mixed species in these products can facilitate illegal practices that compromise food safety, and promote fraudulent activities involving species substitution (Hopkins et al., 2024; Kendall et al., 2019; M. Pardo et al., 2016; Sharrad et al., 2023). Indeed, such practices may pose health risks, particularly when allergenic species or specimens contaminated with harmful compounds, such as toxins or heavy metals, are introduced into the supply chain (Acutis et al., 2019; Mottola, Piredda, Catanese, Giorelli, et al., 2022; J. Spink et al., 2017). Furthermore, Illegal, Unreported, and Unregulated (IUU) fishing activities contribute to the overexploitation of fish stocks and the degradation of marine ecosystems, compounding the ecological and economic challenges faced by global fisheries (Giagkazoglou et al., 2024; H. Zhang et al., 2023). Species substitution practices may also conflict with religious and ethical behaviors (Piredda et al., 2022).

To date, European Union (EU) food labeling legislation, specifically Regulation (EU) No. 1169/2011 and Regulation (EU) No. 1379/2013, has focused on ensuring transparency, safety, and fairness in the seafood industry (Deconinck et al., 2020). Regulation (EU) No. 1379/2013, in particular, encourages the use of molecular techniques to verify the identity of products. DNA-based methods have become popular for identifying seafood species due to the high resistance of target molecules during food processing (Paracchini et al., 2019; Wang et al., 2021). In particular,

DNA barcoding is a pivotal policy instrument for single-species identification (Clark, 2015; Handy et al., 2011). However, traditional barcoding based on Sanger sequencing is limited to identifying the dominant species in complex food matrices (Paracchini et al., 2019). High-throughput sequencing technologies (HTS), based on Next Generation Sequencing (NGS) platforms, enable the simultaneous sequencing of all DNA molecules present and, therefore, the identification of every species in a sample (Haynes et al., 2019). This approach, blending traditional DNA barcoding with NGS (DNA metabarcoding), has been primarily used in ecological studies (G. Ficetola and Taberlet, 2023; Piredda et al., 2018; Van Driessche et al., 2024) but has also proven effective in analyzing various food matrices. These include the floral composition of honey (Pathiraja et al., 2023; Prosser and Hebert, 2017), ingredients in complex food products such as candies (Muñoz-Colmenero et al., 2017), meat (Kappel et al., 2023; Mottola et al., 2023; Ribani et al., 2018; Xing et al., 2019), seafood (Piredda et al., 2022; Wang et al., 2021; Giusti et al., 2017; Toxqui Rodríguez et al., 2023), dairy products (Ribani et al., 2018), spices (Flügge et al., 2023; Raclariu-Manolică et al., 2021), herbal supplements and teas (Cottenet et al., 2022; Frigerio et al., 2021; N. Ivanova et al., 2016), and pet food (Kattoor et al., 2024; Palumbo et al., 2020). Despite its advantages, this approach has limitations that can significantly affect the results (Da Silva et al., 2019; Da Silva et al., 2019; De Barba et al., 2014), particularly concerning the choice of molecular markers and primer pairs. These choices can influence PCR amplification, taxonomic profiles, and species-level resolution. To mitigate these biases, the use of multiple primer pairs from different molecular regions has been suggested for various applications, including biodiversity surveys, animal dietary analyses (Da Silva et al., 2019; Da Silva et al., 2019; De Barba et al., 2014), mesozooplankton composition monitoring (Stefanni et al., 2018), identification of herbal teas and traditional medicines (Frigerio et al., 2021; Arulandhu et al., 2017), and the authentication of mixed seafood products and canned tuna (Toxqui Rodríguez et al., 2023; Klapper et al., 2023).

In this study, we assess the authenticity of processed single-species fish products using a multi-marker DNA metabarcoding approach. Specifically, two primer pairs targeting the mitochondrial cytochrome c oxidase I (COI) and 16S rRNA genes were sequenced using the Ion Torrent platform. The analysis aimed to (i) evaluate the universality of the primer pairs utilized, (ii) assess the discriminatory power

at the species level of the two sequenced fragments, and (iii) compare molecular identifications with the list of ingredients reported on product labels.

4.3. Materials and Methods

4.3.1. Sampling

A total of 20 processed seafood samples (intact packages) from different brands and batches were collected from Italian supermarkets, including 5 burgers, 4 nuggets, 3 breaded fillets, 3 cutlets, 3 sticks, and 2 products labeled as "fish fantasy". The labels indicated that these products were made up of one (18 samples) or two species. The samples were transported to the food safety laboratory in an insulated bag. The cooled containers were maintained at +4 °C and subsequently stored at -20 °C until the time of molecular analysis.

4.3.2. DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) in compliance with ISO [20813:2019], 2019 and following the methodology described by Piredda et al., 2022, at the Food Safety Section of the Department of Veterinary Medicine of the University of Bari. Briefly, under sterile conditions, three aliquots of 25 mg from each sample were collected and subsequently added to 180 µL of ATL lysis buffer and 20 µL Proteinase K (20 mg/mL). The aliquots were incubated at 56 °C for 1 h and 30 min. Then, 200 µL of AL buffer was added and incubated at 70 °C for 10 min. Subsequently, with the addition of 200 µL ethanol, the mixture was applied to DNeasy Mini-Columns (QIAGEN, Hilden, Germany). The DNA was adsorbed to the QIAamp silica-gel membrane by centrifugation at 6000× g for 60 s and subsequently washed with 500 µL of AW1 and 500 µL of AW2 washing buffers. Finally, the DNA was eluted using 60 µL of AE elution buffer (QIAGEN, Hilden, Germany). For each sample, the 3

aliquots were pooled and subjected to subsequent molecular analysis. To ensure the quality of the extraction process, positive controls (*Gadus chalcogrammus* Pure DNA Extract, Generon, San Prospero (MO), Italy) and negative extraction controls were included. The concentration and purity of the extracted DNA were assessed by measuring the absorbance ratios A260 nm/A280 nm and A260 nm/A230 nm using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Monza, Italy). Amplifications and sequencing were performed at the laboratory of the Central Inspectorate for Fraud Repression and Quality Protection of Agri-Food Products and Foodstuffs Ministry of Agriculture, Food Sovereignty and Forests (ICQRF-MASAF) located in Modena (Italy). The extracted DNA was amplified with the following primer pairs: 16sf-var 5′-CAAATTACGCTGTTATCCCTATGG-3′ and 16sr-var 5′-GACGAGAAGACCCTAATGAGCTTT-3′ (Chapela et al., 2002) targeting a fragment of 148–209 bp of the 16S ribosomal RNA mitochondrial (16S) gene and FishCOIlbc 5′-CTCAACYAATCAYAAAGATATYGGCAC-3′ and Revshort1 5′-GGYATNACTATRAAGAAAATTATTAC-3′ (Giusti et al., 2017), for the COI gene. These primers targeted fragments of 148–209 bp and 139 bp, respectively. After accurate assessment of dsDNA concentration using a Qubit™ fluorimeter, amplification reactions were performed for each target fragment in a final volume of 50 µL, comprising 42 µL of Platinum™ PCR SuperMix High Fidelity (Thermo Fisher Scientific), 5 µL of the primer pool 2 µM, and 3 µL of DNA template 20–50 ng. The process used a QuantStudio™ 6 PRO PCR System (Thermo Fisher Scientific) with the following thermal profile: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s (for the 16S gene) and 51 °C for 30 s (for the COI gene), and elongation at 68 °C for 2 min. Post-amplification, PCR products were purified using an Agencourt™ AMPure PCR purification kit (Beckman Coulter. Beverly, MA, USA) with a DynaMag™-96 Side Magnet (Thermo Fisher Scientific), following the manufacturer's instructions. The integrity of the PCR products was verified by 1.5% (w/v) agarose gel electrophoresis stained with Green Gel Safe 10,000 × Nucleic Acid Stain (5 µL/100 mL; Thermo Fisher Scientific, Waltham, MA, USA). The purified products were then quantified using a Qubit™ fluorimeter (Thermo Fisher Scientific, Monza, Italy) and pooled in equimolar amounts for subsequent analysis.

The DNA barcode libraries for NGS were prepared from each amplicon pool using

the Ion Plus Fragment Library Kit (Thermo Fisher, Monza, Italy), adhering to the manufacturer's guidelines. All libraries were quantified, and their quality was assessed using a Qubit™ fluorimeter (Thermo Fisher Scientific, Monza, Italy). Libraries were diluted and pooled to achieve a 100 pM equimolar concentration for the template reaction. This process involved attaching DNA fragments to Ion Sphere Particles (ISPs) for clonal amplification via emulsion PCR, conducted using the Ion Chef™ System (Thermo Fisher, Monza, Italy). Following preparation, samples were sequenced on the Ion GeneStudio S5™ Prime sequencer (Thermo Fisher Scientific, Monza, Italy) utilizing an Ion 530 chip. The sequencing reads were analyzed using Torrent Suite™ version 5.4 (Thermo Fisher Scientific. Monza, Italy).

4.3.3. Bioinformatics Analysis

After sequencing, BAM files containing nucleotide sequences in binary form were converted to FASTQ format using the SAMTOOLS Version 1.2 package (Schloss et al., 2009). For each FASTQ file, primers were removed; DNA reads were filtered by length and quality using Mothur v.1.48.0 (Schloss et al., 2009). The quality control settings included a maximum homopolymer length of 8 bp, no ambiguous bases allowed, and a read length between 80 and 250 bp. Reads were also screened for chimeras. Taxonomic assignments for representative Amplicon Sequence Variants (ASVs) were performed using the standalone BLAST+ suite (Altschul et al., 1990; Camacho et al., 2009) against a custom database including the 16S and COI mitochondrial sequences downloaded from GenBank (March 2023). Assignments with a similarity of less than 90%, indicative of potential low-quality reads or unknown taxa, were discarded. Sequences achieving 98–100% similarity were assigned at the species level (Barbuto et al., 2010), while those with less than 98% similarity were classified at the genus level. The raw sequence data were deposited in the Sequence Read Archive (SRA) under the BioProject database PRJNA 1136829.

4.3.4. Label Analysis and Mislabeling Assessment

Each product's label was examined for compliance with Regulation (EU) No. 1169/2011, checking the commercial name, ingredient list, net quantity, storage instructions, expiration date, manufacturer information, nutritional declaration, and allergens. Molecular identifications were then compared with the species listed on the labels. Cases of mislabeling were identified if the species listed, either by scientific name or commercial designation, were not detected in the molecular analysis. When labels provided only the commercial designation, Annex I of the Italian MASAF Decree dated 22 September 2017 was referenced to determine the corresponding scientific names. This Decree was also used to verify the presence of taxa unmarketable in Italy.

4.4. Results and Discussion

In this study, we employed a multi-marker DNA metabarcoding approach to assess the species composition of processed seafood products that were presumably mono-species. Interestingly, the taxonomic profiles generated indicated that none of the products tested strictly adhered to a mono-species composition, and various species were either intentionally or accidentally included as ingredients. Furthermore, the analysis highlighted several limitations in the performance of the two primer pairs used, particularly in their ability to accurately trace products predominantly composed of Gadiformes species.

4.4.1. Molecular Identifications

The molecular analysis of 20 processed seafood samples using two DNA markers revealed the presence of 18 taxa spanning 2 classes (Actinopteri, Asteroidea), 5 orders (Perciformes, Salmoniformes, Pleuronectiformes, Gadiformes, Forcipulatida), 8 families, and 11 genera. The taxonomic profiles generated by the COI and 16S

markers were only partially overlapping, which underscores the need for a multi-marker approach in metabarcoding studies for food authentication, as supported by recent research (Ribani et al., 2018; Toxqui Rodríguez et al., 2023; Frigerio et al., 2021; Klapper et al., 2023). Differences between the taxonomic profiles derived from the two primer pairs can be attributed to variations in the universality of the primers and their discrimination power at the species level. For instance, approximately 67% of sequences in the COI dataset were taxonomically assigned to the genus *Gadus* sp., followed by *Merluccius* sp. (28.3%), with other taxa such as *Sander lucioperca*, *Arctogadus glacialis*, *Pollachius* sp., *Pleuronectes platessa*, and *Sebastes* sp. detected in smaller proportions (overall <1.5%). The 16S dataset showed about 60% of sequences assigned to *Gadus sp.*, followed by Pleuronectidae (31.1%), with additional taxa including *Salmo salar*, *Pollachius virens*, *Leptasterias coei, Limanda aspera*, and *Chelidonichthys* sp. (Perciformes) detected in lesser quantities (overall <6%) (**Supplementary Table C.2**).

This analysis not only confirmed four taxa shared between markers (*Gadus* sp., *Salmo salar*, *Pollachius* sp., and Pleuronectidae) but also identified seven taxa exclusive to the COI marker (*Arctogadus glacialis*, *Merluccius* sp., *Merluccius australis*, *Merluccius hubbsi*, *Merluccius paradoxus*, *Sander lucioperca*, *Sebastes* sp.) and five exclusive to the 16S marker (*Chelidonichthys sp., Gadus chalcogrammus, Gadus morhua, Leptasterias coei*, and *Limanda aspera*) (**Fig. 4.1**).

Although both primer sets detected the presence of the genus *Gadus*, they differed in their ability to discriminate between species within the genus. The COI marker did not differentiate between *Gadus chalcogrammus* and *Gadus morhua*, whereas the 16S marker effectively discriminated between them, assigning 36% of sequences to *Gadus chalcogrammus* and 16% to *Gadus morhua*. Additionally, the genus *Merluccius* was only detected by the COI marker, which successfully identified the species *M. australis, M. hubbsi*, and *M. paradoxus*. However, four other species within this genus (i.e., *M. productus, M. gayi, M. angustimanus,* and *M. bilinearis*) were not discriminated by this marker.

These results, pertaining to two commercially significant genera (*Gadus* and *Merluccius*) in terms of economic value and global fisheries and trade, underscore the critical importance of primer selection during experimental design in metabarcoding assessments. Furthermore, although both markers detected the presence of the Pleuronectidae family, only the COI marker could identify *Pleuronectes platessa* at

the species level, as detailed in **Supplementary Table C.2**.
Despite employing two distinct primer pairs, the study encountered significant challenges in tracing the genus *Macruronus* (Order Gadiformes). The public molecular databases currently include sequences for only two species within this genus, *M. novaezelandiae* and *M. magellanicus*, but lack data for *M. capensis*. Consequently, in two samples (SM10 and SM11) where *Macruronus capensis* was declared on the label, its presence could not be verified because no reference sequences were available in public repositories. Moreover, the absence of matching sequences for the other two known species (*M. novaezelandiae, M. magellanicus*) suggests that both primer pairs were ineffective amplification for this genus. Interestingly, despite expectations based on label claim, no crustacean DNA was detected by either marker.

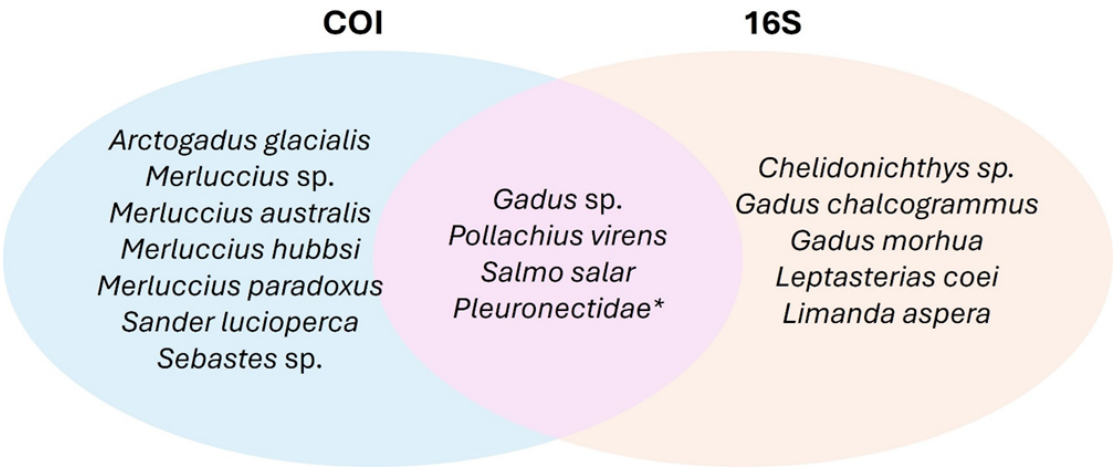

Figure 4.1.: Venn diagram illustrating the shared and exclusive taxonomic identifications between the COI and 16S gene fragments. While Pleuronectidae is identified as a shared taxon, only the COI fragment achieved species-level identification. * Family detected by both markers but only COI was able to identify *Pleuronectes platessa* to species level.

4.4.2. Label Analysis and Product Composition

The analysis of labels for all 20 processed seafood samples confirmed compliance with the mandatory requirements set by Regulation (EU) No. 1169/2011. Of these, 14 out of 20 labels (70%) voluntarily included both the commercial designation and the scientific name of the fish used. The remaining six labels (30%) provided

only the commercial designation of the fish.

Based on our mislabelling criteria, which defines misllabeling as the absence of molecular identification of the species declared on the label, the comparison of molecular identifications obtained by both markers with the information on the labels revealed that each product contained the declared species, at least at the genus level. This finding indicates a generally high level of compliance with voluntary declarations (**Table 4.1**). However, 2 out of 20 samples (10%)—SM11 and SM23—were found to be mislabelled. In SM11, the non-compliance involved the absence of crustacean species listed under the commercial name "Gambero Indopacifico". Such an outcome could be due to the actual absence of "Gambero Indopacifico" as an ingredient. Intriguingly, in sample SM23, labelled as breaded fillet of "Merluzzo d'Alaska", *Gadus chalcogrammus* was entirely substituted with species from the *Merluccius* genus.

The observed substitution does not appear to be driven by economic motives, as hakes are generally considered higher-value species compared to Pollock (Blanco-Fernandez et al., 2021). Rather, the substitution may stem from confusion arising from the common names of these species, as both are referred to as "Merluzzi" in Italian. To mitigate such potential misunderstandings, which can occur among various intermediaries within the global supply chain, the adoption of Industry 4.0 technologies—such as artificial intelligence, blockchain, and big data—could enhance traceability "From Boat to Plate". This integration promises to clarify product identities throughout the supply chain, thus supporting accurate labelling and compliance (Blanco-Fernandez et al., 2021; But et al., 2019; Mottola, Piredda, Catanese, Lorusso, et al., 2022).

Although the products analyzed were labelled as containing one or two species, molecular analyses revealed that 95% of them were multi-species (**Figure 4.2**), containing species not listed in the ingredients. These unexpected species included commercially recognized fish such as *Pleuronectes platessa, Pollachius virens, Sander lucioperca, Merluccius australis, Sebastes* sp., *Limanda aspera,* and *Chelidonichthys* sp., but also species not listed in the latest Italian MASAF Decree (2017), such as *Arctogadus glacialis* and *Leptasterias coei*. The presence of these species could be economically motivated, as the intentional addition of fish discards and low-value fish is a known industry strategy to increase product weight and boost profits (Mottola, Piredda, Catanese, Giorelli, et al., 2022; Piredda et al., 2022;

Mottola, Piredda, Catanese, Lorusso, et al., 2022; Nitsuwat et al., 2021). However, the presence of untraced seafood might also stem from illegal, unreported, and unregulated (IUU) fishing activities, raising serious ethical and ecological concerns (Hasan et al., 2024). Moreover, such practices can pose risks to human health through unintentional exposure to heavy metals, persistent organic pollutants, and microplastics (Selig et al., 2022).

On the other hand, accidental inclusion of species could occur due to contamination along the seafood supply chain, particularly when different raw materials are processed within the same facility (Piredda et al., 2022). An illustrative case was the detection of the inedible sea star species *Leptasterias coei* (Echinodermata, Asteroidea) in sample SM17, which was labelled and molecularly identified as containing *G. chalchogrammus* (Merluzzo d'Alaska). Interestingly, the geographical distribution of *Leptasterias coei* in the Northeast Pacific, specifically Alaska, provides indirect evidence of the harvesting region for *G. chalchogrammus*. Moreover, the accidental presence of this species highlights concerns about biodiversity loss associated with bottom trawling practices (Paula et al., 2022).

Table 4.1: Label details of processed seafood products alongside molecular identifications and instances of mislabelling detected using COI and 16S mitochondrial genes.

Sample ID	Type of Product	Commercial Designation	Scientific Name	Molecular Identification COI	Molecular Identification 16S	Molecular Identification COI_16S	Mislabeling COI_16S
SM10	Burger	Salmone and merluzzo sudafricano	*S. salar*[a], *Macruronus capensis or Merluccius paradoxus or Merluccius capensis*	***Salmo salar***; *Gadus sp.*[c]; ***Merluccius paradoxus***	***Salmo salar***	*Gadus sp*; ***Salmo salar***; *Merluccius paradoxus*	No
SM11	Burger	Merluzzo sudafricano and Gambero Indopacifico	*Macruronus capensis*[a] *o Merluccius paradoxus o Merluccius capensis Crustaceans*	*Merluccius sp.*[b]; *Merluccius hubbsi*; ***Merluccius paradoxus***	*Gadus chalcogrammus*; *Gadus sp.*[d]	*Merluccius hubbsi*; *Merluccius sp.*; *Gadus sp.*; *Gadus chalcogrammus*; ***Merluccius paradoxus***	Yes
SM14	Burger	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Sander lucioperca*; *Pleuronectes platessa*	*Pleuronectidae*; *Gadus sp.*[d]; *Gadus morhua*; ***Gadus chalcogrammus***	*Gadus sp.*; ***Gadus chalcogrammus***; *Gadus morhua*; *Sander lucioperca*; *Pleuronectes platessa*	No
SM15	Fish fantasy	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Pollachius virens*; *Sebastes sp.*	*Pollachius virens*; ***Gadus sp.***[d]; *Gadus morhua*	*Gadus sp.*; *Pollachius virens*; *Gadus morhua*; *Pollachius virens*; *Sebastes sp.*	No
SM16	Sticks	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Sander lucioperca*; *Pleuronectes platessa*; *Arctogadus glacialis*; *Merluccius sp.*[b]; *Sebastes sp.*	*Pleuronectidae*; *Gadus morhua*; ***Gadus sp.***[d]	*Arctogadus glacialis*; *Gadus sp.*; *Merluccius sp.*; *Sander lucioperca*; *Pleuronectes platessa*; *Gadus morhua*; *Pleuronectidae*	No

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Table 4.1: Label details of processed seafood products alongside molecular identifications and instances of mislabelling detected using COI and 16S mitochondrial genes. (Continued)

Sample ID	Type of Product	Commercial Designation	Scientific Name	Molecular Identification COI	Molecular Identification 16S	Molecular Identification COI_16S	Mislabeling COI_16S
SM17	Nuggets	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Arctogadus glacialis; Pollachius virens*	*Gadus sp.*[d]; ***Gadus chalcogrammus***; *Leptasterias coei e; Gadus morhua*	*Arctogadus glacialis; Gadus sp.; Pollachius virens; Gadus sp.; Gadus chalcogrammus; Gadus morhua; Leptasterias coei e*	No
SM18	Cutlets	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Pleuronectes platessa; Pollachius virens; Sander lucioperca; Arctogadus glacialis*	*Pleuronectidae;* ***Gadus sp.***[d]; *Gadus morhua; Gadus chalcogrammus*	*Gadus sp.; Pollachius virens; Sander lucioperca; Pleuronectes platessa; Gadus chalcogrammus; Gadus morhua; Pleuronectidae; Arctogadus glacialis*	No
SM19	Burger	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Pleuronectes platessa; Salmo salar; Sander luciorerca*	*Pleuronectidae; Chelidonichthys sp.; Limanda aspera;* ***Gadus sp.***[d]; *Gadus morhua*	***Gadus sp.***; *Pleuronectes platessa; Salmo salar; Sander luciorerca; Gadus morhua; Pleuronectidae; Chelidonichthys sp.; Limanda aspera;*	No
SM2	Braded fillet	Merluzzo Alaska	*G. chalcogrammus*[a]	***Gadus sp.***[c]; *Arctogadus glacialis*	***Gadus chalcogrammus***; *Gadus sp.*[d]; *Gadus morhua*	*Arctogadus glacialis; Gadus sp.;* ***Gadus chalcogrammus***	No
SM20	Fish fantasy	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Pollachius virens*	***Gadus chalcogrammus***; *Gadus sp.*[d]; *Gadus morhua; Pollachius virens*	*Gadus sp.; Pollachius virens;* ***Gadus chalcogrammus***; *Gadus morhua; Pollachius virens*	No

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Table 4.1: Label details of processed seafood products alongside molecular identifications and instances of mislabelling detected using COI and 16S mitochondrial genes. (Continued)

Sample ID	Type of Product	Commercial Designation	Scientific Name	Molecular Identification COI	Molecular Identification 16S	Molecular Identification COI_16S	Mislabeling COI_16S
SM21	Cutlets	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]; *Pleuronectes platessa; Sander lucioperca; Pollachius virens; Arctogadus glacialis*	*Pleuronectidae; Gadus morhua;* ***Gadus sp.***[d]	***Gadus sp.***; *Pollachius virens; Sander lucioperca; Pleuronectes platessa; Gadus morhua; Pleuronectidae*	No
SM22	Braded fillet	Merluzzo nordico	*G. morhua*	***Gadus sp.***[c]; *Arctogadus glacialis*	***Gadus morhua***; *Gadus sp.*[d];	*Arctogadus glacialis; Gadus sp.;* ***Gadus morhua***;	No
SM23	Braded fillet	Merluzzo Alaska	*G. chalcogrammus*[a]	*Merluccius sp.*[b]	*N.A.*	*Merluccius sp.*	Yes
SM25	Burger	Salmone	*S. salar*	***Salmo salar***	***Salmo salar***	***Salmo salar***	No
SM3	Nuggets	Merluzzo Alaska	*G. chalcogrammus*	***Gadus sp.***[c]	***Gadus chalcogrammus***; *Gadus sp.*[d]; *Gadus morhua*	*Gadus sp;* ***Gadus chalcogrammus***; *Gadus morhua*	No
SM4	Sticks	Pollak Alaska	*G. chalcogrammus*	***Gadus sp.***; *Arctogadus glacialis; Pollachius virens; Salmo salar*	***Gadus chalcogrammus***; *Gadus sp.*[d]	*Arctogadus glacialis; Gadus sp.; Pollachius virens; Salmo salar;* ***Gadus chalcogrammus***	No
SM5	Nuggets	Merluzzo nordico	*G. morhua*	***Gadus sp.***[c]; *Arctogadus glacialis*	***Gadus morhua***; *Gadus sp.*[d]	*Arctogadus glacialis; Gadus sp.;* ***Gadus morhua***	No
SM6	Nuggets	Merluzzo Alaska	*G. chalcogrammus* [a]	***Gadus sp.***[c]; *Arctogadus glacialis*	***Gadus chalcogrammus***; *Gadus sp.*[d]; *Gadus morhua*	*Arctogadus glacialis; Gadus sp.;* ***Gadus chalcogrammus***; *Gadus morhua*	No

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Table 4.1: Label details of processed seafood products alongside molecular identifications and instances of mislabelling detected using COI and 16S mitochondrial genes. (Continued)

Sample ID	Type of Product	Commercial Designation	Scientific Name	Molecular Identification COI	Molecular Identification 16S	Molecular Identification COI_16S	Mislabeling COI_16S
SM7	Cutlets	Merluzzo del Pacifico	*M. gayi*	***Merluccius sp.***[b]*;* *Merluccius australis*	*N.A.*	***Merluccius sp.***[b]*;* *Merluccius australis*	No
SM9	Sticks	Merluzzo Alaska	*G. chalcogrammus*[a]	***Gadus sp.***[c]	***Gadus chalcogrammus****;* *Gadus sp.*[d]*; Pollachius virens*	*Gadus sp.; **Gadus chalcogrammus**; Pollachius virens*	No

N.A.: Not available. Species expected based on label information are highlighted in **bold**.

[a] Species not declared on the label. The scientific name was extracted from Annex I of the Italian MASAF Decree dated 22 September 2017.

[b] *Merluccius* sp. (COI) refers to the undistinguished species *M. productus/M. gayi/M. angustimanus/M. bilinearis.*

[c] *Gadus* sp. (COI) refers to the undistinguished species *G. morhua/G. chalcogrammus.*

[d] *Gadus* sp. (16S) refers to the undistinguished species *G. morhua/G. chalcogrammus.*

[e] Species not listed in Annex I of the Italian MASAF Decree dated 22 September 2017.

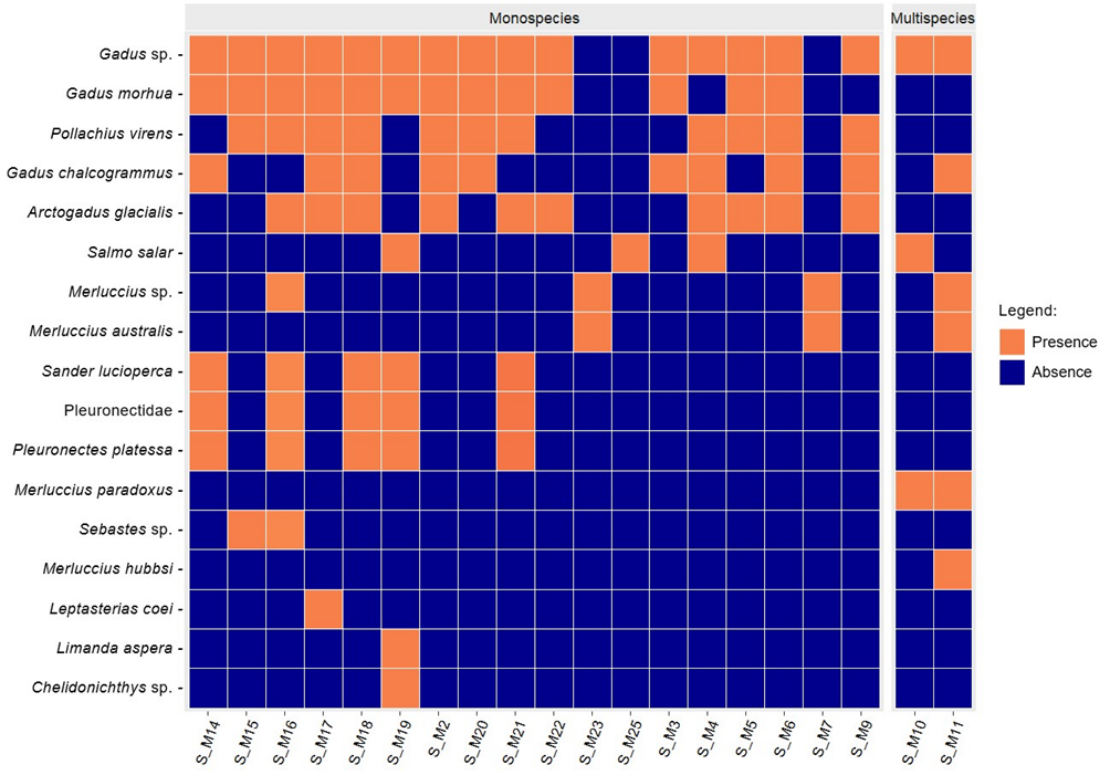

Figure 4.2.: Heatmap of presence-absence based on the integration of molecular identifications obtained with COI and 16S fragments. Taxonomic identifications are reported at the species level where possible.

4.5. Conclusions

Overall, our results confirm that the metabarcoding approach is a critical and effective tool for seafood traceability. Integrating molecular profiles obtained using multiple primer pairs has enhanced species identification success in seafood products. While both primer pairs used in this study exhibited limitations in tracing Gadiformes—the taxa most commonly used in these products—the COI fragments, considered the gold standard for metazoan assessments (Hebert et al., 2003), performed marginally better.

Given the importance of primer selection, an optimal strategy for thorough species characterization in seafood could involve pairing one primer pair designed to amplify and distinguish species listed on product labels (expected species) with another

primer pair that offers broader taxonomic coverage to detect unexpected species. Food fraud poses significant concerns for consumers, the food industry, and regulatory authorities, thus underscoring the need for innovative tools to ensure product authenticity. Although additional species detected in our study were not categorized under mislabelling criteria, our findings suggest that truly mono-species products are rare in processed seafood. Voluntary declarations on labels often present a truncated list of ingredients, potentially used by brands as a marketing tactic to appeal to consumers seeking authentic, safe, and wholesome products ostensibly made from a single species of fish (Piredda et al., 2022). The urgency for suitable verification tools is clear. For highly processed matrices, NGS approaches stand as the sole effective methods capable of tracing the full species composition of products. Therefore, further development is necessary, particularly in laboratory setups and bioinformatic pipelines, before these methodologies can be routinely employed in official controls by authorities or within the food industry (Lorusso, Piredda, et al., 2024). However, their implementation will advance the "From Boat to Plate" strategy and combat fraudulent practices along the intricate fishery supply chain, in alignment with Regulation (EU) 625/2017.

5. Mismanagement and poor transparency in the European processed seafood supply revealed by DNA metabarcoding

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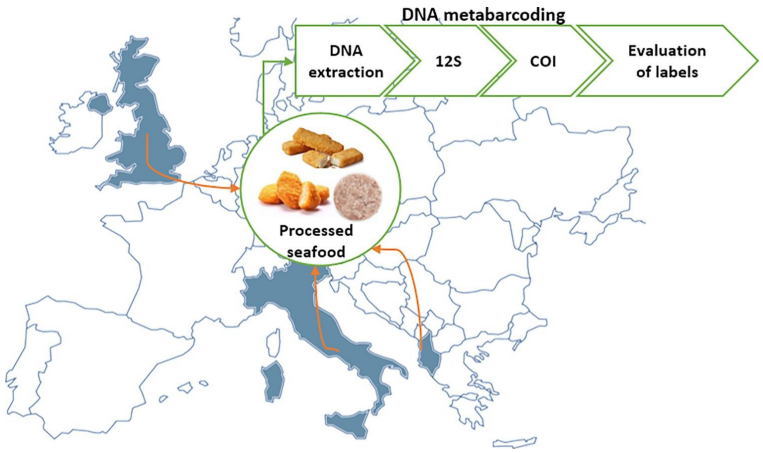
5.1. Abstract



In the global processed seafood industry, disparate actors play different roles along the supply chain, creating multiple opportunities for mistakes, malpractice, and fraud. As a consequence, consumers may be exposed to non-authentic products, which hinder informed purchasing decisions and broader efforts to improve trade transparency and sustainability. Here, we characterised the taxonomic composition of 62 processed seafood products in Italian, British and Albanian retailers, purposefully obtained from different supply routes, using multiple DNA metabarcoding markers. By combining molecular results with metadata reported on labels, we revealed patterns of mislabelling in 24 products (39%) across sampling regions, denoting lack of transparency of processed seafood products based on resources sourced from either Europe or globally. We show that the accuracy of label claims and the mis-represented and underestimated levels of traded biodiversity are largely determined by the management of raw material by global processors. Our study shows that DNA metabarcoding is a powerful and novel authentication tool that is mature for application at different stages of the seafood supply chain to protect consumers and improve the sustainable management of fish stocks.

5.2. Introduction

The seafood industry handles nearly 180 million tonnes of goods annually to meet the unprecedented demand for fish products around the world (FAO, 2024). To cater to consumers, especially in developed countries, producers, processors and retailers work synergistically to supply, transform and deliver processed "convenience seafood" to supermarket chains (Barska, 2018; Mottola, Piredda, Catanese, Giorelli, et al., 2022). Globally, at the point of sale, high quality standards are expected by regulatory frameworks and the consumers themselves. Recently, the quality aspects of seafood products have expanded to include not only sensory and safety concerns, but also traceability, authenticity, and sustainability (FAO, 2018). Indeed, in May 2020, the European Commission presented the fight against fraud along the supply chain as one of the main objectives of the 'From Farm to Fork' strategy at the core of the European Union's Green Deal (Schebesta et al., 2020). Particularly for mixed processed seafood (e.g. fish cakes, fish fingers, surimi, etc.), the length of the supply chain and the lack of morphological characteristics of the product on sale mean that there are more opportunities for fraud, such as the inclusion of species sourced from illegal, unreported, and unregulated fishing (IUU), or subject to specific management programmes (Fox et al., 2018). Also, the substitution of prized species for others of lower value is the most reported type of seafood fraud, which can deliver financial gain for the fraudulent operator, and is often a ploy to meet local and global market demands for dwindling popular species (Calosso et al., 2020; Luque and Donlan, 2019). At the same time, inaccurate labelling of mixed seafood exposes consumers to the risk of unknowingly consuming products (e.g., molluscs, crustaceans, dairy, pork, or eggs) that may be undesirable for health or personal dietary choices (Piredda et al., 2022). Labels are the tool through which consumers can make informed choices, so they must be filled in with accurate information by Food Business Operators (FBOs) purveying the products (Giusti et al., 2023, Paolacci et al., 2021). Meanwhile, consumer awareness of fishing pressure on ecosystems is moving towards the choice of eco-labels such as the Marine Stewardship Council (MSC), the leader in this sector, which has based its chain of custody management principles on maintaining target stocks at sustainable levels, and their ecosystems in a healthy status (Jardim and Currey, 2023).

Several analytical techniques have been developed to verify food authenticity, including protein-based methods (e.g., HPLC, ELISA, NMR) and DNA analysis (e.g., RAPD, LAMP, ddPCR, HRM) (Asensio et al., 2008 ; Y. Cai et al., 2017; Medina et al., 2019). However, DNA specificity, sensitivity, and thermal stability have progressively made DNA-based approaches more popular and effective for species identification especially when the species integrity is lost, or food is subjected to high technological treatments (Griffiths et al., 2014). Among them, DNA metabarcoding, based on parallel sequencing of diagnostic target fragments (i.e., 'barcodes'), is the most suitable for mixed products since it allows the simultaneous in-depth characterisation of all species present in a sample, offering throughput unmatched by traditional DNA barcoding methods or quantitative PCR (qPCR) (Clark, 2015; Staats et al., 2016; Franco et al., 2021).
In this study, we utilised a dual-marker DNA metabarcoding strategy to comprehensively characterise the taxonomic composition of pre-packaged processed seafood products in three European countries. This approach included one marker specifically designed to target fish species and another that is broadly applicable to eukaryotes, which enabled the analysis of globally sourced species processed through diverse supply routes.

5.3. Materials and methods

5.3.1. Sampling

Processed pre-packed point-of-sale seafood products (i.e., breaded, burgers, surimi-based, dumplings, and "fish steaks") were collected in three European countries spanning the range of current regulatory statuses: Italy (EU member), UK (recently exited from the EU), Albania (aiming to soon become an EU member). Sampling was carried out in the main supermarket chains of the Large-Scale Distribution (retailers) of these three countries. Specifically, we visited six different retailers in the UK, five in Italy and three in Albania, to cover the diversity of products sold by different brands (FBOs). To cast further light onto supply chain mechanisms,

we also obtained samples from an Italian supplier, ahead of their preparation for retail; and we also sampled a range of diverse processed products from two Asian retailers in the UK, aiming to increase the biological diversity of products and supply routes (**Fig. 5.1**).To group samples by product type, we pooled under the term "steaks" all those samples that came as frozen blocks of fish and appeared to mimic a unique piece of muscle, while we used the term "breaded" to simply refer to all products coated with breadcrumbs or battered crust. All samples were stored at -20 °C until DNA extraction.

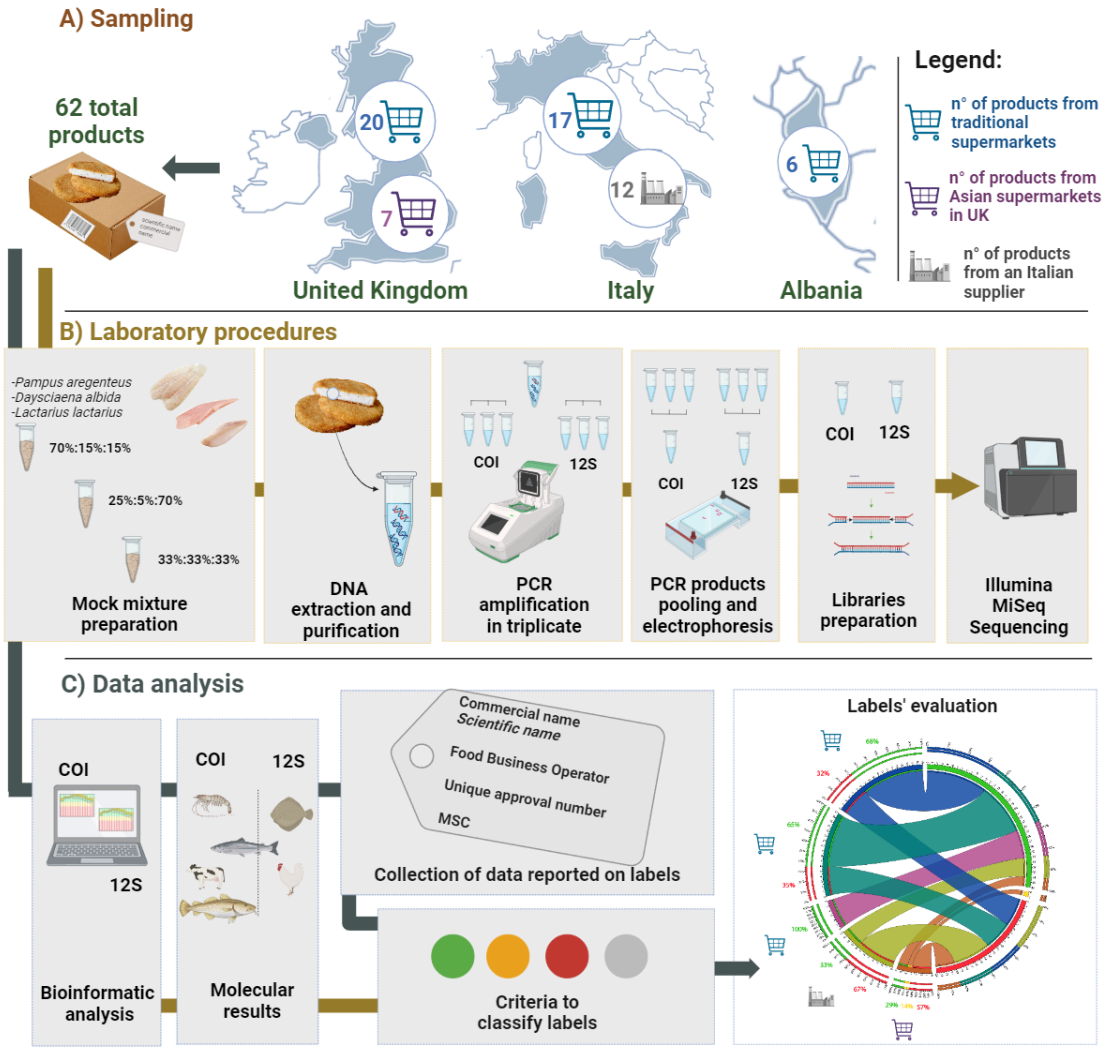


Figure 5.1.: Infographic showing the main steps of the study, from sampling, laboratory procedures and data analysis to the final cross-check against the established criteria to assess the accuracy of the labels.

Visual inspection of the labels was carried out for each purchased product to record data such as the commercial name used and the scientific name, if declared, as well as the list of ingredients, the declared presence of traces of other animal species, the name of the FBO and the unique approval number on the identification mark. Moreover, we recorded whether or not the products were certified sustainable by the MSC.

5.3.2. Laboratory procedures

Three mock mixtures were prepared blending different proportions of tissue from three fish species purchased whole and unlikely to be included in the mainstream supermarket products. These species were: silver pomfret (*Pampus argenteus*), Bengal corvina (*Daysciaena albida*) and false trevally (*Lactarius lactarius*) blended respectively in these following proportions: 70%:15%:15% (Sample 39), 25%:5%:70% (Sample 40) and 33.3%:33.3%:33.3% (Sample 41). Those mixtures were included to support our traffic light criterion for mixed market products and to determine a conservative operational threshold below which a certain percentage of reads could be disregarded as "traces" for the purpose of designating whether a sample was mislabelled. Since several packaged products contained multiple pieces (e.g., fish fingers), we endeavoured to randomly excise several pieces from each sample using a sterile scalpel and placed approximately 200 mg of each product in an 1.5 ml eppendorf tube for DNA extraction. Genomic DNA extraction and purification of the Italian and Albanian samples was performed using the DNeasy Blood and Tissue Kit (Qiagen) as reported by Piredda et al., 2022. The DNA of products sampled in England and of the three mock mixtures was isolated following the Mu-DNA extraction protocol (Sellers et al., 2018). Negative extraction controls (no added tissue) were included in both cases to verify the purity of the extraction reagents. DNA concentration and purity were evaluated by a Qubit Flex™ 4.0 fluorometer with a Qubit™ dsDNA HS Assay Kit (Invitrogen).
Two different primer sets were chosen to increase the confidence with the species identification and to compare their performance in terms of discrimination power at species level. One set of PCR amplifications were performed using the mlCOIintF 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ and jgHCO2198 5′-

TAIACYTCIGGRTGICCRAARAAYCA-3′ primers, designed to target a fragment of 313 bp of the subunit I of the Cytochrome C Oxidase (COI) in all eukaryotes (Geller et al., 2013, Leray et al., 2013). The other primer pair used was the Teleo02 (F: 5′-AAACTCGTGCCAGCCACC-3′, R: 5′-GGGTATCTAATCCCAGTTTG-3′) that amplifies ∼ 167 bp of the 12S rRNA mitochondrial gene (12S) as described by Taberlet et al., 2018 and primarily targets teleost fishes. Both primer pairs were uniquely tagged for each sample with 8 bp sequences to ensure the parsing of sample reads during demultiplexing. The PCR amplification was performed for 72 samples (62 products, 7 negatives, and 3 mocks) in triplicate, and in a final reaction volume of 20 μl containing 10 μl MyFi™ Mix (Meridian Bioscience), 0.16 μl of Bovine Serum Albumin (Thermo Scientific), 5.84 μl of UltraPure™ Distilled Water (Invitrogen), 1 μl of the forward and reverse primer (10 μM, Eurofins), and 2 μl of the extracted DNA. The thermocycling programme for the COI primer followed the initial DNA denaturation at 94 °C for 10 min, a total of 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and a final elongation at 72 °C for 5 min. For the 12S primer the following amplification conditions were applied: initial DNA denaturation for 10 min at 95 °C, 35 cycles of 30 s at 95 °C, 45 s at 60 °C and 30 s at 72 °C, and a final step of 72 °C for 5 min. PCR triplicates were then pooled and checked through gel electrophoresis on a 2% agarose gel stained with SYBRsafe (Invitrogen). The PCR product clean-up was performed using an equal volume of 45 μl of the Mag-Bind® TotalPure NGS magnetic beads (Omega Bio-tek Inc) for both primer sets. DNA quantification of all samples was used to normalize and pool samples in equimolar concentration for the two libraries' preparation. After pooling, an additional magnetic beads clean-up was performed to concentrate pooled samples in a final volume of 50 μl. The Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape (Agilent Technologies) showed the presence of a single peak of the expected size for both libraries. End repair, adapter ligation and library PCR amplification were carried out using the NEXTFLEX® Rapid DNA-Seq Kit 2.0 for Illumina® platforms (PerkinElmer) according to the manufacturer's protocol. The two libraries were quantified by a quantitative PCR (qPCR) on a Rotor-Gene Q (Qiagen) with the NEBNext® Library Quant Kit for Illumina® (New England Biolabs) and were equimolarly pooled to obtain a final concentration of 9 pM with 10% PhiX control. Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2 (500 cycles).

5.3.3. Bioinformatics

Raw data were analysed following the OBITOOLS (Boyer et al., 2016) pipeline. First, the FASTQC program was used to assess the quality of reads and exclusively the 12S raw reads were trimmed (i.e., 200 bp for forward and 180 bp for reverse sequences) to remove low-quality ends using OBICUT. Then, pair-end reads were merged through ILLUMINAPAIREDEND and retained only those with a quality score > 40. NGSFILTER allowed the demultiplexing, based on the unique tags, with a maximum mismatch threshold of 1 bp. OBIGREP was used to filter reads based on their expected lengths (i.e., 300 – 325 bp for the COI and 109 - 229 bp for the 12S), and remove reads containing ambiguous bases "N". Sequences were dereplicated through OBIUNIQUE and chimeras were removed using UCHIME (Edgar et al., 2011). The clustering into Molecular Operational Taxonomic Units (MOTUs) was performed with SWARM (Mahé et al., 2014), setting custom threshold of $d = 9$ for COI and $d = 3$ for 12S. Finally, taxonomic assignment was performed using ECOTAG against the EMBL database (release version r143) for both the COI and 12S, generated by *in-silico* PCR (ecoPCR). MOTUs with a percentage identity between 95% and 98% were manually checked on NCBI to resolve ambiguous assignments. Negative controls were used to identify the presence of contaminating MOTUs with the DECONTAM package in R, setting a threshold of 0.2 in both cases. Contaminant reads, such as *Homo sapiens* and *Pangasianodon hypophthalmus* (used as a positive control in other sequencing projects) were removed. Finally, based on the results obtained in the three mock mixtures, we set a threshold of 1% of the total percentage abundance of reads in each sample to discard all lower percentage values as representative of contamination.

5.3.4. Mislabelling criteria

Since commercial designations of seafood species vary greatly both across and within countries, we compared the ingredients provided for each product to the official list of commercial designation of the country where the product was purchased. In particular, we referred to the Commercial designations of fish of the United

Kingdom (updated to 03 February 2020)[1], the Annex I of the Decree of the Italian Ministry of Agriculture, Food Sovereignty and Forests (MASAF) of September 22nd, 2017[2], and the Appendix 3 of the Albanian Regulation No. 407 dated May, 8th 2013[3]. If a common name was declared on the label, the relevant species name was obtained searching FishBase (Froese, 2023), while if a scientific name was provided, it was contrasted directly with the molecular results. Using matches and mismatches between label information and DNA-based identification, we classified the examined products into the following categories: (i) "green" (correctly labelled product): when the proportion of reads of the declared species was at least twice as large as the second most abundant species and constitutes the majority of the bulk; (ii) "amber" (misleading product): when the proportion of the declared species was higher than any other species, but not necessarily amounting to the majority of the bulk; (iii) "red" (mislabelled product): when the declared species was either absent or not the most abundant in the mix; (iv) "grey" (undetermined product): when the declared species couldn't be genetically identified with certainty.
These categories were established after combining the "traffic light" information between the two genetic markers employed, as there were instances where one marker was unable to discriminate between two closely related species ("grey"), but the other could instead tell them apart (see Results for details).

5.3.5. Data analysis

The experimental workflow illustrated in **Fig. 5.1** was created online on Biorender.com. Label metadata, such as the unique approval number and the country where the product brand is registered, were combined with the sampling location of each product to reconstruct trade flows between and within countries of establishments and sale. Linear regressions between biomass and the Hellinger-transformed number of reads, generated with the R package vegan (Oksanen, 2018), were calculated for the mock mixtures using ggplot2 (Wickham, 2011) and ggpmisc

[1]https://www.gov.uk/government/publications/commercial-designations-of-fish-united-kingdom
[2]https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/11953
[3]https://www.mod.gov.al/images/akteligjore/qnod/09_VKM_407_08052013.pdf

(Aphalo, 2016). The number of reads of each taxon within the total number of reads in each sample were converted in proportions that were used to represent the product composition, for category and gene marker applied, generating barplots through the R package ggplot2. Visualizations of labelling results, in accordance with the labelling classification criteria, were created using the Circos online tool (Krzywinski et al., 2009). The Pearson's chi-squared test was calculated within the R environment (R Core Team, 2023) (version 4.2.3) and used to test for pair-wise differences in labelling accuracy between Italian and UK supermarkets, between them and the Italian supplier, as well as between MSC-certified and non-certified products. For the purposes of this study, documentation on the suppliers' MSC certification schemes was provided and used to assess the management of the MSC-certified species within the same company.

5.4. Results

5.4.1. Label visual inspection

The sampling expansion reflected the range of diversity and availability of processed seafood in countries of sampling. We examined 62 pre-packed seafood products obtained from Italian, Albanian, and British (UK) markets (**Fig. 5.1**), purchased from 22 distinct FBOs, distributed as follows: 10 headquartered in the UK, 10 in Italy, 1 in Spain and 1 in the Netherlands. We identified 30 different unique approval numbers, each linked to the last point of food handling, across Europe, Asia, and Africa. One sample (S20) lacked a unique approval number, but reported China as country of production, therefore a code was also assigned to that product in China. Most of the samples from the UK were supplied by British establishments, with the exception of three products from Germany and one from Poland. In the Italian supermarkets, eight products were sourced from processing plants in Italy, with the other nine coming from Spain, France, Germany, and Poland. On the other hand, none of the products purchased in Albania came from a local establishment, but were sourced from Spain, the UK, Germany, and Namibia. Twelve products

sourced from an Italian supplier (a-Italy) all came from a factory in the Netherlands. All the Asian-style cuisine products came from production sites in India, China, Thailand, and Malaysia, apart from one, and were all sold by FBOs in the UK and one in the Netherlands (**Fig. 5.2**). A total of 23 out of the 62 products held MSC certification (**Supplementary Table 1–3**). Overall, 51 of 62 products claimed to be made exclusively of a single species (**Supplementary Tables 1–3**).

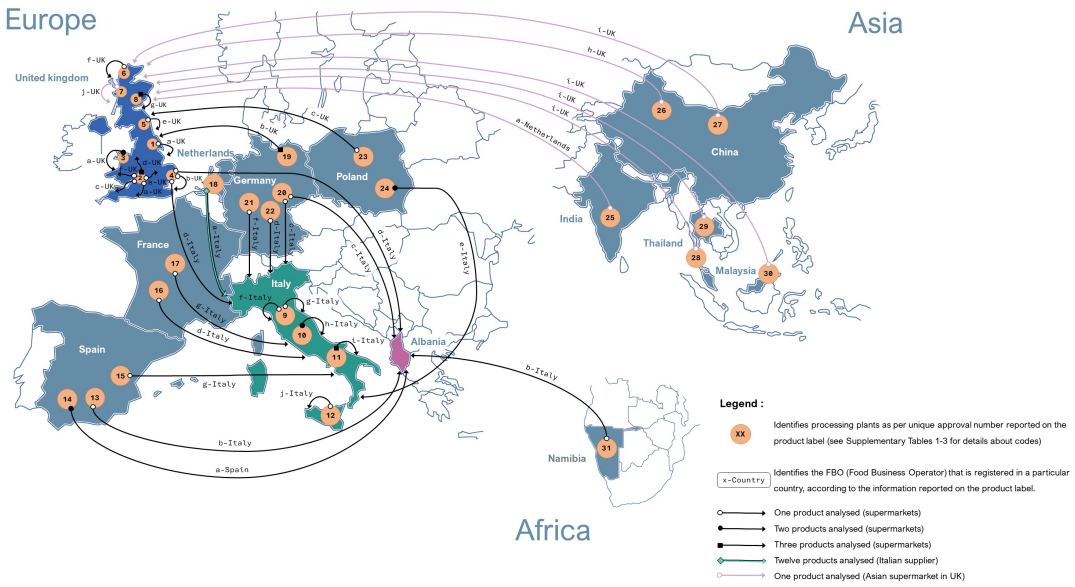


Figure 5.2.: The roadmap mapping the information provided on the labels about the unique approval number of the establishment from which the products originated and the brand selling the products, based on the country in which they are registered (Supplementary Tables 1–3). Their position in the country map does not represent the real geographical coordinates.

5.4.2. Primer performance and taxonomic characterization

A successful COI amplification was obtained for all samples, except for one (5 reads in sample S2). The total number of reads obtained with the COI primer pair was 5,077,768, which came down to 4,775,115 after applying all filters (Supplementary Material), resulting in a mean read number per sample (61 products + 3 mocks) of $\sim$ 75 k. The COI dataset consisted of 53 total taxa (49 at species level, three at genus level and one at family level), belonging to three kingdoms (Metazoa, Fungi and Stramenopiles), five phyla (Arthropoda, Chordata, Nematoda, Ascomycota

and Ochrophyta), eight classes (Arachnida, Actinopterigyii, Chromadorea, Insecta, Mammalia, Malacostraca, Saccharomycetes and Chrysophyceae), 19 orders, and 30 families.

The COI molecular identifications highlighted that in the 44 breaded products (e.g., fish cakes and fish fingers) the main component was Alaska pollock (*Gadus chalcogrammus*), reaching over $>$ 98% of the reads in 14 of the samples, and between 44% and 63% in 11 other samples (**Fig. 5.3; Supplementary Tables 1–3**). Atlantic cod (*G. morhua*) contributed $\sim$ 40% of reads in eight samples; European plaice (*Pleuronectes platessa*) accounted for at least the 75% of reads in six samples; haddock (*Melanogrammus aeglefinus*) amounted to $\sim$ 78% of reads in three samples (S14, S15, S26); Pacific hake (*Merluccius productus*) reached at least the 90% of reads in three other samples (S54, S59 and S70); saithe (*Pollachius virens*) represented $\sim$ 60% of reads in one sample (S62), and Atlantic salmon (*Salmo salar*) comprised the entirety of sample S25. The main species in the five burgers, accounting for at least the 89% of the reads, were Alaska Pollock (S54 and S60), Atlantic salmon (S57 and S72), and Pacific hake (S58). In the four "steaks", Alaska Pollock, hakes and *Macruronus* sp. (S48) were predominant, amounting to at least 80% in each sample. In one of the shrimp-based dumplings (S20), 100% of the reads belonged to Louisiana crawfish (*Procrambarus clarkii*) and in the other one (S21) the whiteleg shrimp (*Penaeus vannamei*) represented over 80% of reads. The six surimi-based products were constituted by a broad range of species from fisheries across the world, with the predominant species being: herring (*Clupea harengus*) with $>$ 93% of reads in S22, Pacific hake with 84% of reads in S42, cutlassfishes (*Trichiurus japonicus*) with 82% in S19, and threadfin breams (*Nemipterus* sp.) with over 59% of reads in S18 (**Fig. 5.3**; **Supplementary Tables 1–3**).

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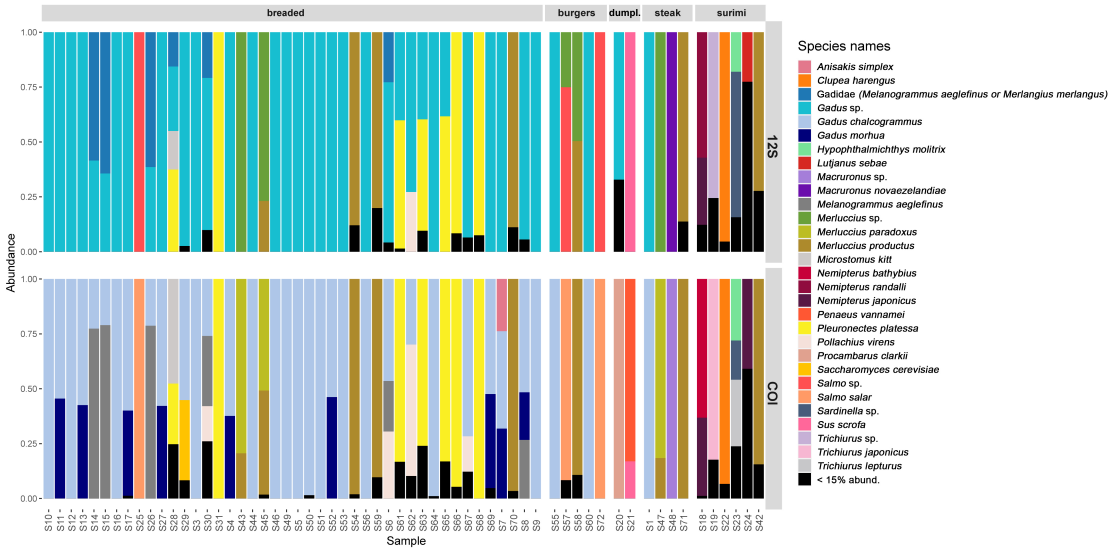

Figure 5.3.: Barplots showing the proportion of reads of each taxon detected by the 12S and COI primer pairs separately, grouped according to the type of product (i.e., breaded, burgers, dumplings, steaks and surimi).

The 12S amplification was successful for all samples. The total number of reads obtained with this primer pair was 5,643,616, which was reduced to 4,880,925 after filtering (with mean read number per sample of ∼ 75 k) (Supplementary Material). The cleaned 12S dataset consisted of 50 taxa (38 at species level, nine at genus level, and two at family level). The 12S dataset exclusively included the phylum Chordata, three classes (Actinopterigyii, Mammalia and Aves), 19 orders and 28

families. Haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) could not be distinguished by this marker and were recorded as 'Gadidae'. For the MOTU assigned to the Genus *Gadus* it was not possible to unambiguously assign a species-level identification.

The 12S molecular identifications highlighted that the genus Gadus sp. was the main component in the 44 breaded products, reaching $> 93\%$ of the reads in 26 samples (**Fig. 5.3; Supplementary Tables 1–3**). European plaice accounted for at least $\sim 50\%$ of reads in six samples. Also, reads of Gadidae (*Melanogrammus aeglefinus* and *Merlangius merlangus*) reached an average 61% of reads in three samples. Hakes (*Merluccius* sp.) reached at least the 91% of reads in five samples. Salmon (*Salmo* sp.) constituted sample S25 in its entirety. Composition in the five burgers also mirrored the findings from the COI data set, with at least 75% of the reads of *Gadus* sp. in S55 and S60, *Salmo* sp. in S57 and S72, and *Merluccius* sp. in S58. The four "steaks" were entirely composed of *Merluccius* sp. (S47 and S71), *Gadus* sp. (S1), and hoki (*Macruronus novaezelandiae*) in S48. In one shrimp-based dumpling *Sus scrofa* reached 100% of the reads (S21), while in the other one *Gadus* sp. amounted to 78% of the reads (S20), in contrast to the COI results. In the six surimi-based products, in the midst of the vast number of species detected, the results confirmed the dominance of *Clupea harengus* in S22, *Nemipterus* sp. in S18, *Merluccius* sp. in S42, and *Trichiurus* sp. in S19, with only S23 showing discrepancy with COI, as *Sardinella* sp. dominated with 66% of reads (**Fig. 5.3; Supplementary Tables 1–3**).

To add support to our criteria for the interpretation of proportional read counts, we used mock mixtures of three known fish species. These included silver pomfret (*Pampus argenteus*), Bengal corvina (*Daysciaena albida*) and false trevally (*Lactarius lactarius*), which were mixed in varying proportions. Results obtained from the three mock mixtures, from both primer sets, confirmed the presence of the three fish species included, with similar abilities of both markers to resolve the known compositions (**Supplementary Fig. 1**). Overall, we observed that both primers returned proportional read counts that fit well with the input proportions of tissues, with 12S (designed for teleosts) reflecting the actual proportions with greater accuracy. Notably, in S41 (where input proportions were equal across the three species), no species resulted dominant in the read counts for neither marker. To corroborate the choice of excluding taxa with $< 1\%$ reads, we never found anything

other than the three input species with read counts beyond the 1% threshold.

5.4.3. Mislabelling assessment

To determine labelling accuracy, we compared molecular identifications of each marker with the commercial and/or scientific names reported on the labels. The COI dataset allowed accurate assessment for 60 out of 62 products' labels (**Fig. 5.4A**), while with 12S it was not possible to unequivocally determine label conformity in 40 samples, in large part due to DNA sequence ambiguity within the genus *Gadus* (**Fig. 5.4B**). In all those cases, COI information was instrumental to resolve the ambiguity. Mislabelled or "red" samples occurred when the declared species was either absent or not the most abundant in the product, while correctly labelled products or "green" samples needed to meet a conservative abundance (see Methods). In 21 of 61 cases there was an agreement of both markers with the traffic light criterion. In all other cases, whenever one marker failed to resolve taxonomy, the other one was able to settle identification to the species level. Overall, 36/61 (59%) labels were "green", 24/61 (39.3%) were "red", and one (1.6%) was "amber" (the declared species was higher than any other species, but not the majority of the bulk). Three products were labelled with a generic name (e.g. "whitefish" and "surimi") to which no specific taxon could be attributed. Consequently, these three products were deemed "green", regardless of the identifications obtained. All six product labels form the Albanian supermarkets were "green" (100%). Of the 17 product labels from the Italian supermarkets, 11 were "green" (65%) and six "red" (35%), among the 19 samples purchased from UK supermarkets, 13 labels were "green" (68%) and six "red" (32%). In contrast, among the 12 products' labels from the Italian supplier, four were "green" (33%) and eight "red" (67%). Products sampled at Asian markets located in UK included two "green" (29%), four "red" (57%), and one "amber" (14%) labels (**Fig. 5.4C**).

The rate of mislabelling did not differ significantly between Italian and UK supermarkets ($\chi^2 = 0.056, p\text{-value} = 0.81$), but the mislabelling rate between the Italian supplier and both Italian and UK supermarkets was significantly different ($\chi^2 = 3.833, p\text{-value} = 0.05$). Of the 20 supermarket products that were MSC

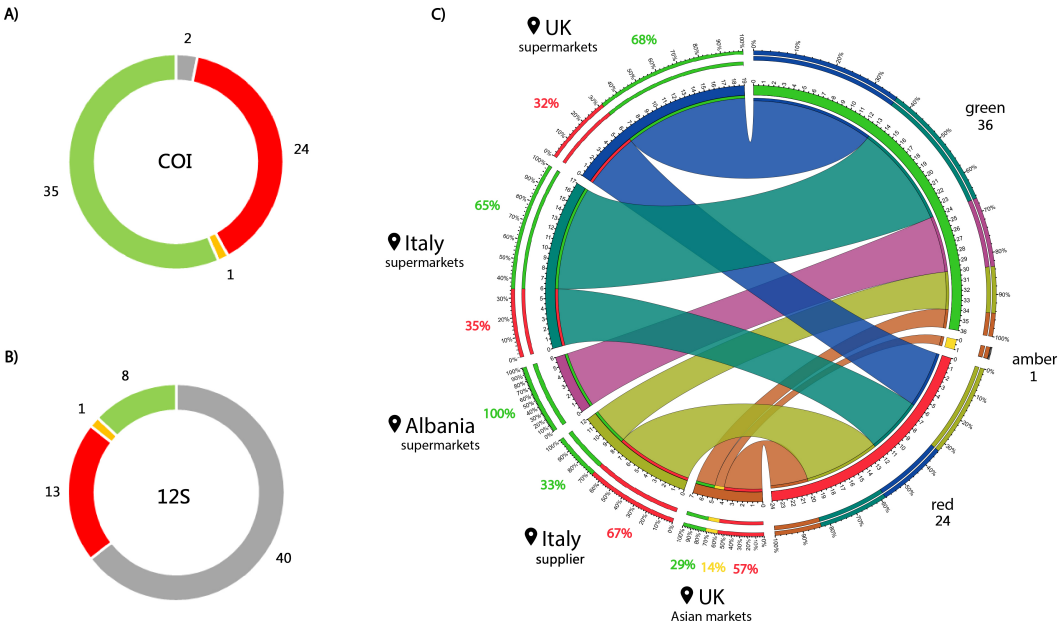

Figure 5.4.: Circos plots **A)** and **B)** number of "green" (correct), "red" (mislabel), "amber" (mislead) and "grey" (undetermined) labels highlighted following our criteria by the COI and 12S primer pairs. **C)** corresponding abundance of correct, misleading and mislabelled products for each sampling location, after collapsing the attributions.

certified, 15 (75%) were "green", and five (25%) were deemed "red". By contrast, of the 29 non-MSC certified products, 17 (58.6%) were "green", and 12 (41.4%) "red" or "amber" ($\chi^2 = 1.402, p\text{-value} = 0.24$); however, two out of three MSC-certified products handled by the Italian supplier were deemed mislabelled (**Supplementary Table 3b**). All the species found in MSC-certified products were within the MSC programme of their suppliers, except for *Microstomus kitt*, *Melanogrammus aeglefinus* and *Sander lucioperca*, which were all found in products from the same supplier.

5.5. Discussion

The advent of massively parallel sequencing is revolutionising the field of food research and monitoring (Mottola et al., 2023; Noh et al., 2021; Özkök et al., 2023).

Here we show that the biodiversity underpinning widely traded processed seafood is greater than what is usually declared on labels, frequently even in products that are retailed as being of "single-species". Comparisons among countries, and between levels along the supply chain, can help form a better understanding of the mechanisms that regulate the provision and use of aquatic species in processed foods and can identify a roadmap for improving practice towards more informed consumers, a responsible trade, and sustainable fish production.

5.5.1. Molecular identification

The DNA metabarcoding approach has proven to be a powerful tool for ingredient authentication in processed seafood products, primarily owing to its ability to resolve species identity in complex mixture (Giusti et al., 2023; Kappel et al., 2023; Klapper et al., 2023; Piredda et al., 2022; Toxqui Rodríguez et al., 2023). However, to date, there is no standardised workflow for food authentication of processed, potentially mixed, products, especially in terms of the choice of primers to generate taxonomic profiles (Lorusso, Piredda, et al., 2024). In this study, the inclusion of two different primer pairs (one, 12S, focused on fishes, the other, COI, broadly encompassing all animals Geller et al., 2013, Leray et al., 2013, Taberlet et al., 2018), provided a comprehensive assessment of species composition, and helped to verify their reciprocal performance in multispecies seafood. As part of our test, the use of mock mixtures comprising known proportions of three species, showed that the starting biomass of each species was positively correlated with the transformed number of sequence reads for both primers, consistent with previous evidence (**Supplementary Fig. C.2**) (Evans et al., 2016; Laporte et al., 2021) and supporting a semi-quantitative interpretation of our results. Several factors are known to affect the proportion of reads generated from complex matrices (i.e., primer affinity, PCR conditions or inhibitors, as well as competition among templates) (Deagle et al., 2019; Klapper et al., 2023), which means that we opted for a conservative approach when assessing label claim compliance (see "traffic light" criteria in the Methods). Most of the sequences in the two marker datasets belonged to the same taxa, at least at the genus level: *Gadus* sp., *Merluccius* sp. and *Pleuronectes platessa* were the most common taxa in both datasets, accounting

for – alongside haddock and salmon – over 80% of the total number of reads. The two primers performed in a complementary way, not only due to their different ability to discriminate species. Indeed, COI was able to efficiently discriminate *G. chalcogrammus* and *G. morhua* and reach species level in *M. aeglefinus* and *Salmo salar*, whereas 12S proved to be crucial in discriminating the genus *Macruronus* (**Fig. 5.3**). Moreover, only COI was able to detect crustaceans, necessary to assess the use of the declared species in shrimp-based dumplings (i.e., *Procrambarus clarkii* and *Penaeus vannamei*) or burgers (i.e., *Solenocera crassicornis* and *Parapenaeopsis hardwickii*). Additionally, 34 and 24 low-abundance species exclusively detected in the COI and 12S datasets respectively, contributed to the overall characterisation of the products.

The authenticity of food products does not only involve the accuracy of the information on the label concerning the main raw material used, but also other aspects such as the presence of allergens or uncommon species. For instance, even if the COI molecular result for S20 (dumpling) confirmed the use of the crustacean species declared on the label, those from 12S sequencing revealed the presence of terrestrial species, i.e., pig (10%) and chicken (∼1%), which were not declared either in the list of ingredients or as possible traces. The presence of fish species (e.g., *Gadus* sp., Gadidae and *Gadiculus argenteus*) was also detected, possibly due to the presence of "seafood flavoured sauce", reported in the list of ingredients. Irrespective of product compliance in terms of the declared crustacean species, the UK Food Labelling Regulation also requires the declaration of presence of fish and eggs (chicken) as allergens. Again, the 12S primer detected chicken (∼2%) in sample S63 (breaded), with the possible presence of egg traces being correctly declared on the label, in accordance with Regulation (EU) No. 1169/2011 (Mottola, Piredda, Catanese, Lorusso, et al., 2022). Sample S59 (breaded) contained cow DNA, as detected by both primers, which may be linked to the presence of cheese, declared in the list of ingredients. The additional power of DNA metabarcoding is revealed through the detection of uncommon species for these products. For example, the detection of freshwater species, such as zander (*Sander lucioperca*), not declared on the label and typically uncommon in processed seafood products, rises concern about the intentional sourcing and use of species of unknown origin (Nikolić et al., 2023; Selig et al., 2022). Specifically, the supplier in question publicly advertises their trade in zander products, which may accidentally lead to traces of

this species being incorporated in different end products. Other useful findings from the metabarcoding approach include the detection of potential pathogens (Poms et al., 2004). For instance, sample S7 (breaded) included the DNA of *Anisakis simplex*, a parasitic nematode that can infests fish fleshes, and can be harmful for human ingestion causing allergic reactions as urticaria, angioedema and/or anaphylactic shocks, sometimes even when consuming cooked or frozen products (Packi et al., 2023; Polimeno et al., 2021). In addition, in sample S24 (surimi) the undeclared presence of *Lutjanus sebae* was detected, which may contain toxins that are not destroyed by cooking and cause ciguatera poisoning (Abraham et al., 2012, Hamilton et al., 2002). Sample S19 (surimi) showed the presence of *Ochromonas danica*, a freshwater phytoflagellate microalga that produces chlorosulpholipids, marine toxins responsible for human poisoning from seafood consumption (Nilewski et al., 2009). This calls for the need to improve the quality control of the raw materials before including them in added-value preparation.
Also, it is interesting to note the presence of the beetle *Sitophilus oryzae*, in sample S69 (breaded), and *Acarus siro*, in sample S67 (breaded), both common pests of raw materials in the food industry, therefore probably present in the wheat flour used for breadcrumbs (Bell, 2014). The reads belonging to yeast, *Saccharomyces cerevisiae*, in some samples detected by the COI marker may also be due to the declared use of breadcrumbs. However, samples S28, S29 and S30 (breaded) - all from the same processing plant ("18", **Fig. 5.2**) showed an unexpectedly high presence (8.17% - 36.48%), which could represent an intentional addition of brewer's yeast as a flavour enhancer in the fish mixture (Ferreira et al., 2010).
Our set threshold of 1% for contamination was already used by the UK Food Safety Authority and by the Department for Environment Food and Rural Affairs (Defra), and it is also in accordance with other metabarcoding studies for food authentication (Giusti et al., 2023). Details on the, at times, curious findings from these trace reads can be found in the Supplementary Material.

5.5.2. Supply flows and mislabelling drivers

Although processed pre-packed seafood product labels do not currently require information on commercial and scientific names (Paolacci et al., 2021), several

product labels did actually report some species names: eight out of the 15 (53%) processed products sold in Italy, six out of the 20 (30%) in UK, but none in Albania. Also, six out of the seven (86%) products from Asian markets voluntarily indicated a scientific name, at least at the genus level, for the main species used. This is probably a marketing strategy of the FBOs or a way to anticipate what might become a much-needed future labelling requirement also for highly processed seafood products.

Overall, UK supermarket products primarily claimed Alaska pollock, cod, haddock and salmon. In Italy and Albania, the same types of products claimed Alaska pollock, South-Pacific hake, Cape hake, North-Pacific hake, hoki, cod and salmon (**Supplementary Fig. C.2**). Thus, a pattern of preference of hakes in Mediterranean markets was observed in contrast with the UK market, probably due to consumers' historical familiarity with certain regional products (EUMOFA, Directorate-General for Maritime Affairs and Fisheries, 2023; Penca et al., 2021). These claims were all largely corroborated by the molecular results and mirrored a similar pattern in surimi products, where different sets of species are used as raw materials to produce similar products. Surimi prepared in Europe – such as sample S42, processed in Spain and sold in Albania – tends to be based on cold temperate species, such as hakes and anchovies, which contrasts with the species used for the preparation of surimi from Asian countries, which tend to be of tropical origin, as also documented in other studies (An et al., 2020; X. Zhang, Giusti, et al., 2024). It is also worth noting that in some of the products from Asian countries, species were found that do not currently feature in the official list of the UK commercial designations of fish (**Supplementary Table S4**). Their absence within the list may stem from a variety of reasons, including their danger for consumption (such as in the case of pufferfishes) or simply because they are not known as a commercial target. This has a twofold implication for government action, as the genetic discovery of these species in seafood could either prompt more frequent updates of these approved lists or escalate prosecution for illegally marketed organisms.

By placing label metadata in a geographically explicit context, it is possible to visualise trade flows and the otherwise under-appreciated supplier-retailer relationships that may help explain mislabelling patterns (**Fig. 5.2**). Most of the UK products are supplied by UK-based processors (eight out of 10 processors being British),

while Italy mostly relied on international supply (only four out of 12 providers being based in Italy), with Albania being entirely reliant on external suppliers. Such differences likely reflect the legacy of different levels of establishment of industrial fishing and processing in the various countries (Wilcox, 2012). Interestingly, however, such different supply or retail mechanisms do not seem to affect the overall extent of mislabelling in the products, which was essentially the same in Italy and the UK (**Fig. 5.4**). This seems to indicate that major trans-national suppliers of processed seafood depend on the level of good practice enacted in individual processing plants, echoing similar inference obtained from the supply of unprocessed seafood (D. Miller et al., 2012).

In this study we could also peer into the dynamics between a supplier/distributor and the products it obtains from their manufacturing hubs. Analysis of the samples provided by the Italian supplier revealed that in 11 of the 12 products declared to be based on Alaska pollock, the DNA of several other species was also detected, including saithe (S29, S30, S62, S65 and S67), haddock (S28 and S30), lemon sole (S28 and S66), European plaice (S28, S61, S63, S65, S66 and S68), zander (S30 and S63), and Atlantic cod (S29, S30, S61, S62, S63, S64, S65 and S67), sometimes in substantial proportions (**Fig. 5.3; Supplementary Tables 1–3**). All products come from a factory in the Netherlands (**Fig. 5.2**), which appears to include these additional species in the processing of the products for reasons that require further investigation. Such biocomplexity associated with the supply chain is unlikely to be unravelled without a DNA testing programme. By far the most recurrent case of mislabelling is the existence of cod products that contain substantial amounts of Alaska pollock, in both the UK and Italy, which may be explained by the latter's greater availability, and lower price compared to Cod (M. Pardo et al., 2016) (**Supplementary Fig. C.2**). Consistently, even products deemed "green", were shown to contain minority amounts of DNA sequences from species not declared on the labels and, even if these are approximations of the real proportions, there are no doubts that other species are simultaneously present in these products (Klapper et al., 2023). This is also the case of one of the "steak" products where the unexpected presence of *M. productus*, along with the declared *M. paradoxus*, may be due to its inclusion as entire fillets or parts of them. Samples S14, S15 and S26, all labelled as haddock (*M. aeglefinus*), still contained a significant percentage of Alaska pollock. These products originate, respectively, from processors "2", "19",

and "8", which also provide traditional Alaska pollock products (such as S3, S9, S10 and S16 from establishment "2", or S5 from "19"), or where Alaska pollock was identified alongside the declared species (cod) but not explicitly stated (e.g., S27 from "8"). The addition of readily available cheaper species to bulk up haddock and cod products creates the conditions for financial gain (Carvalho et al., 2017), as well as when in two products (S59 and S70) from the same processing plant and brand, the declared Alaska pollock was substituted with *M. productus*, as found in frozen unprocessed products (Blanco-Fernandez et al., 2021). These large scale gadoid species substitution patterns may not just necessarily result from deliberate financial incentives, but also from the need to secure a steady and secure supply of raw whitefish. Since the regulations do not mandate the reporting of the exact species in this type of products, such practices would be acceptable, if the items on retail were not making inaccurate marketing claims. Indeed, products labelled as made of "whitefish", from the same processor, simultaneously contained *G. chalcogrammus*, *P. virens* and *M. aeglefinus* (S6) and *G. chalcogrammus*, *M. aeglefinus* and *G. morhua* (S8). Yet, as mentioned above, neither the retailers nor the large distribution companies would be in the position to ascertain the true composition of these products, without conducting DNA metabarcoding checks.
These intricate dynamics along the processing and supply chain have some bearing on sustainability schemes. We found that all the undeclared species in MSC-certified products sampled in supermarkets were included in the MSC certification programme. Therefore, these products could still be considered environmentally friendly, overall. On the other hand, the three MSC certified products from the Dutch-Italian supply flow, as discussed above, contained lemon sole, haddock, and zander, none of which is within the MSC scope. This raises concerns around some certified companies' commitment to comply with the MSC programme, as well as for the ability of MSC to control such compliance. Since the MSC already showed willingness to monitor labelling accuracy across the scheme by carrying out DNA barcoding tests on unprocessed fillet (Barendse et al., 2019), a case could be made for the expansion of testing to include metabarcoding analysis of processed mixed seafood.

5.6. Conclusions

Our findings highlighted the highly diverse nature of pre-packed processed fish products, even where a single species is declared. Such diversity can currently only be characterised in detail through DNA metabarcoding. By combining data on species composition and label information, we could uncover some of the mechanisms and stages of the global supply chain that are vulnerable to unintentional errors and/or deliberate malpractice. Raw material management in production plants plays a key role in determining species composition, while the long distance that exists between raw material and consumers enhances exposure to mislabelling. Our results showed that current practices expose consumers to a significant amount of poorly traceable and mislabelled products, hindering progress towards a truly sustainable seafood supply. Without more frequent and systematic DNA-based monitoring, it remains difficult to fully understand mechanisms and motives (Fox et al., 2018), as non-compliance at one of the nodes along the chain can irreversibly compromise the authenticity of the product all the way down the supply (Luque and Donlan, 2019). As a natural progression from the transformative impact that DNA barcoding had on seafood market operations globally (Mariani et al., 2015), we argue that the now established metabarcoding approach can play a major role in monitoring authenticity in complex production sectors across the agri-food arena. To further boost such a transformation, the use of low-cost, real-time and long reads sequencers, such as nanopore technology, could accelerate progress in statutory product identification in the seafood industry (Ho et al., 2020, Shum et al., 2024), although dedicated studies need to be carried out to verify its robustness (e.g., error rate) compared to traditional sequencing methods. These changes may entail voluntary monitoring within the industry and eco-labelling organisations, as well as the implementation of government-led testing programmes, which would improve the safety of products, the rights of consumers and brands, and the sustainable management of fish stocks.

6. Contrasting Nanopore Sequencing Effectiveness for Processed Seafood Authentication against the Illumina "Gold Standard"

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6.1. Abstract

Ensuring species authenticity in processed seafood is critical to combat bad behaviour and malpractice whether it is deliberate or not in the seafood industry, yet current methods face challenges in scalability and accuracy. This study evaluates the performance of Oxford Nanopore Technologies (ONT) and Illumina sequencing for species identification in 62 commercial seafood products using short (313 bp, S-COI) and long (652 bp, L-COI) COI barcodes. Nanopore sequencing generated 10.1 million (S-COI) and 2.7 million (L-COI) filtered reads, detecting 42 and 55 taxa, respectively, compared to 53 species via Illumina, and all methods shared a core of 26 taxa ($\sim$ 84% of reads). However, the nanopore short reads showed higher error rates for certain taxa (e.g., 84.8% similarity for *Gadus chalcogrammus*). Despite technical limitations, ONT long reads achieved $\geq$ 98 % similarity with Illumina for three of four focal species after trimming. These findings demonstrate ONT potential for seafood authentication but underscore the need for multi-marker approaches and improved error correction. Integrating long-read breadth with short-read precision offers a pragmatic strategy for supply chain compliance, pending advancements in ONT accuracy and standardisation.

6.2. Introduction

Global seafood supply chains are vulnerable to species substitution and undeclared ingredients, undermining consumer trust and regulatory compliance (Fox et al., 2018). Processed products, where morphological identification is impossible, require rapid, portable DNA-based tools for traceability. While Illumina sequencing remains the gold standard for DNA metabarcoding, its reliance on centralised facilities limits real-time supply chain interventions. Oxford Nanopore Technology (ONT) offers a promising alternative with real-time sequencing, portability, and cost-effectiveness (Ho et al., 2020; Franco et al., 2021). Also, the ability to generate long reads, make ONT particularly attractive for improving taxonomic resolution in complex food matrices (Ho et al., 2020; Shum et al., 2024). Despite its potential, however, one of the current challenges in applying ONT to food species authentication lies in the development of robust experimental workflows, as well as in the standardization of data processing pipelines. The majority of ONT applications have been focused to the biomedical and environmental sciences, while its application in authenticity of mixed food products remains limited (Goordial et al., 2017; Knobloch et al., 2024; Lee et al., 2024; Rheman et al., 2024). Existing ONT seafood studies fall into two categories: artificial mixtures (Voorhuijzen-Harink et al., 2019) and commercial products with unknown compositions (Detcharoen et al., 2024). Critical gaps persist in benchmarking ONT against Illumina for commercial products and optimising workflows for degraded DNA typical in processed seafood and cross-platform comparisons are lacking, particularly for long-read vs. short-read performance in complex matrices. This study bridges this gap by evaluating the feasibility of ONT for the authentication of commercial processed seafood products, by comparing results with those previously obtained using the Illumina MiSeq platform (Lorusso, Shum, et al., 2024). Specifically, an evaluation of two COI fragments were assessed on how read length influences taxonomic resolution, error rates, and concordance with Illumina. Our work advances the validation of ONT for real-world applications, addressing both technical feasibility and the challenges of standardization in food authentication.

6.3. Materials and Methods

6.3.1. Laboratory procedures

Genomic DNA extracts from Lorusso, Shum, et al., 2024 were used to assess the applicability of Nanopore sequencing. The study included 72 samples: 62 commercial processed seafood products, 7 DNA extraction negative controls, and 3 mock communities. The mock communities comprised of silver pomfret (*Pampus argenteus*), Bengal corvina (*Daysciaena albida*) and false trevally (*Lactarius lactarius*) tissues blended respectively in these following proportions: 70%:15%:15% (Sample S39), 25%:5%:70% (Sample S40) and 33.3%:33.3%:33.3% (Sample S41) for DNA extractions. The DNA was quantified using a Qubit Flex™ 4.0 fluorometer (Thermo Fisher Scientific) with the Qubit™ dsDNA HS Assay Kit (Invitrogen). Two cytochrome C oxidase I (COI) regions were targeted, the first PCR amplifications targeted a short fragment ($\sim$ 313 bp) of the COI gene (hereafter termed "S-COI") using the forward primer 5′-GGWACWGGWTGAACWGTWTAYCCYCC-3′ (Leray et al., 2013) and the reverse primer 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′ (Geller et al., 2013), which were also employed in previous Illumina-based analyses (Lorusso, Shum, et al., 2024). S-COI PCR amplifications were carried out in triplicate in a final reaction volume of 20 μl, comprising 10 μl of MyFi™ Mix (Meridian Bioscience), 0.16 μl of Bovine Serum Albumin (Thermo Scientific), 5.84 μl of UltraPure™ Distilled Water (Invitrogen), 1 μl of each primer (10 µM, Eurofins), and 2 μl of extracted DNA. The thermal cycling conditions consisted of an initial denaturation at 94°C for 10 minutes, followed by 35 cycles of 1 minute at 94°C, 1 minute at 45°C, and 1 minute at 72°C, with a final elongation step at 72°C for 5 minutes. The second region amplified a longer COI fragment ($\sim$ 652 bp, hereafter "L-COI") using the PCR primer cocktail (*C_FishF1t1–C_FishR1t1*) described by N. V. Ivanova et al., 2007. L-COI PCR amplifications were also conducted in triplicate in a total reaction volume of 20 μl, containing 10 μl of MyFi™ Mix (Meridian Bioscience), 0.16 μl of Bovine Serum Albumin (Thermo Scientific), 6.84 μl of UltraPure™ Distilled Water (Invitrogen), 1 μl of primer cocktail (10 µM, Eurofins), and 2 μl of extracted DNA. The PCR thermal profile included an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 30 seconds at 94°C, 40 seconds at 52°C, and 1 minute at

72°C, with a final elongation step at 72°C for 10 minutes. For both primer sets, PCR triplicates per sample were pooled and subjected to electrophoresis on a 2% agarose gel stained with SYBR Safe (Invitrogen). PCR residual components were removed adding 45 μl of Agencourt AMPure XP beads to an equal volume of each PCR product following the manufacturer's instructions. Library preparation followed ONT's Native barcoding kit with amplicons (SQK-NDB112-96) with modifications described below. Two distinct libraries per primer set were prepared according to the manufacturer's protocol. Briefly, DNA repair and end-prep were carried out mixing 12 μl of DNA at 200pM concentration per sample, 1.75 μl of NEBNext Ultra II End-prep reaction buffer and 0.75 μl of Ultra II End-prep enzyme mix and incubating the reaction mix at 20°C for 5 min and 65°C for 5 min. Native barcode ligations were performed adding 0.75 μl of end-prepped DNA, 1.25 μl of a specific native barcode (Native Barcoding Kit 96), 3 μl of nuclease-free water and 5 μl of Blunt/TA Ligase Master Mix, and subsequently incubating the reactions at room temperature for 20 min. Pooled barcoded samples were purified using AMPure XP beads at 1:1 ratio. After, 30 μl of pooled barcoded sample were adaptors ligated adding 10 μl of NEBNext Quick Ligation Reaction Buffer (5X), 5 μl of Adapter Mix II and 5 μl of Quick T4 DNA Ligase, then purified by magnetic beads and eluted in 15 μl of elution buffer (ONT). The flow cell priming mix was prepared mixing 30 μl of Flush Tether and 970 μl of Flush Buffer and was loaded according to the manufacturer's protocol (ONT). Finally, 12 μl of DNA library was mixed with 37.5 μl of Sequencing Buffer II and 25.5 μl of Loading Beads II. The S-COI and the L-COI libraries were loaded into two separate Nanopore FLO-MIN112 flow cells and sequenced on a MinION Mk1C device both for 44 hours.

6.3.2. Bioinformatic analysis

Basecalling was performed using Guppy v6.5.7 implemented within the MinKNOW software. First, fastq files for each barcode sample were concatenated into a single fastq file. For both S-COI and L-COI datasets, raw reads were trimmed for primers using Primerchop[1]. Reads were filtered by removing sequences with a quality score below 8 and retained only reads within an expected read length range (244-340

[1]https://gitlab.com/mcfrith/primer-chop

bp for S-COI and 600-710 for L-COI) using Chopper [2]. For each library, all reads were concatenated to generate a consensus database. Consensus building was performed starting from sequences dereplication and clustering using VSEARCH, then the consensus sequences were generated using Racon and further polishing was performed with medaka[3]. Then, each sample were aligned to the polished consensus sequence using Minimap2 (H. Li, 2018).
Taxonomic assignment was performed using a Bayesian-based Lowest Common Ancestor (BLCA) approach for Molecular Operational Taxonomic Units (MOTUs), against a custom BLCA reference database containing all non-environmental mitochondrial COI sequences available in the nt_euk database in GenBank, along with their taxonomic information. Ambiguous assignments, between 98% and 90% of percentage identity, were manually checked through nBLAST against GenBank. Then, reads with low percentage identity scores ($< 90\%$) and unclassified were filtered as well as *Homo sapiens* (contaminants) and *Pangasianodon hypophthalmus reads* (used as a positive control in other sequencing projects). In the assessment of the mock communities, a relative proportion threshold was set at 1% to discard all reads below this threshold to remove unexpected taxa.

6.3.3. Statistical analysis

Data analysis

Linear regressions between biomass and the Hellinger-transformed number of reads, generated with the R package vegan (Oksanen, 2018), were calculated for the mock mixtures using ggplot2 (Wickham, 2011) and ggpmisc (Aphalo, 2016). The number of reads of each taxon within the total number of reads in each sample were converted in proportions that were used to represent the product composition, for category and marker applied, generating bubble plots through the R package ggplot2.

[2]https://github.com/wdecoster/chopper
[3]https://github.com/nanoporetech/medaka

Rarefaction

We assessed dataset-level species richness with rarefaction curves. For each sequencing dataset – Illumina, S-COI and L-COI – we summed read counts across all samples to create a single abundance vector per dataset. These three pooled abundance vectors were combined into a matrix in which rows represented sequencing platforms and columns represented MOTUs. Using the vegan package in R (function rarecurve()), we generated one rarefaction curve per platform with a constant sampling step (e.g. step = 100). The relationship between accumulated MOTU richness and sequencing depth, and the degree to which each curve approached an asymptote, were used to judge whether overall sequencing depth was sufficient for each platform.

Species overlap

We compiled the unique set of detected species for each method (after excluding mock samples). Venn diagrams were generated to compare overlap of species detections. We plotted three-set Venn-diagram (Illumina vs S-COI vs L-COI) at the species level using VennDiagram in R (Chen and Boutros, 2011). Overlapping regions quantify shared taxa, while non-overlapping MOTUs indicate unique detections.

Error analysis

To benchmark cross-platform sequence accuracy, we extracted the consensus COI barcodes generated for four focal taxa (*Gadus morhua*, *G. chalcogrammus*, *Merluccius productus* and *Pleuronectes platessa*) by the Illumina short-read workflow ($\sim$ 313 bp), the Nanopore 313 bp assay (S-COI) and the Nanopore $\sim$ 650 bp assay (L-COI). The short-read nanopore and Illumina fragments are fully nested within the L-COI nanopore region, each pair of sequences was globally aligned using MUSCLE implemented in MEGA11 (Tamura et al., 2021). The longer L-COI

read was then trimmed to the 300-bp Illumina window to eliminate edge effects. Sequence similarity was calculated as the proportion of identical positions across the trimmed window and expressed as percent similarity, with percent error reported as 1 - similarity.

6.4. Results and Discussion

For the S-COI dataset, a total of 11,890,238 reads were obtained after pre-processing across 65 samples. Following the application of filtering, the final read count was 10,127,063, corresponding to an average of approximately 150k reads per sample (62 products and three mock mixtures). The S-COI dataset comprised 42 taxa, of which 39 identified at species level and three at genus level, distributed across four phyla (*Arthropoda*, *Ascomycota*, *Chordata*, and *Nematoda*), eight classes (*Actinopterygii*, *Arachnida*, *Chromadorea*, *Eurotiomycetes*, *Malacostraca*, *Mammalia*, *Pichiomycetes*, and *Saccharomycetes*), 16 orders, and 23 families. For the L-COI dataset, after basecalling, no reads were recovered for barcode 33, which corresponded to mock mixture S40. After pre-processing, a total of 2,716,692 reads were obtained from 64 samples. Following data filtering, 2,555,985 reads remained, resulting in a mean of approximately 40k reads per sample (62 products and two mock mixtures). The L-COI dataset comprised 55 taxa, of which 49 identified at species level and six at genus level, spanning two phyla (*Arthropoda* and *Chordata*), four classes (*Actinopterygii*, *Malacostraca*, *Mammalia*, and *Aves*), 19 orders, and 26 families. All three rarefaction curves levelled off at comparable values (42–55 MOTUs), confirming that each dataset was sequenced deeply enough to capture most recoverable diversity (**Fig.6.1** a)). Accurate species identification within the mock mixtures was achieved, with all three target species successfully detected. However, correlation analyses based on Nanopore sequencing revealed a weak relationship between input biomass (mg) and transformed read counts in both datasets (**Supplementary Figure D.1**). Notably, the discrepancy in correlation between the Illumina COI and the Nanopore S-COI datasets cannot be attributed to differences in primer affinity, as the same Leray-Geller primer pair was used in both cases. This suggests that the reduced correlation observed in Nanopore data

is more likely due to differences associated with library preparation, sequencing and/or raw data pre-processing.

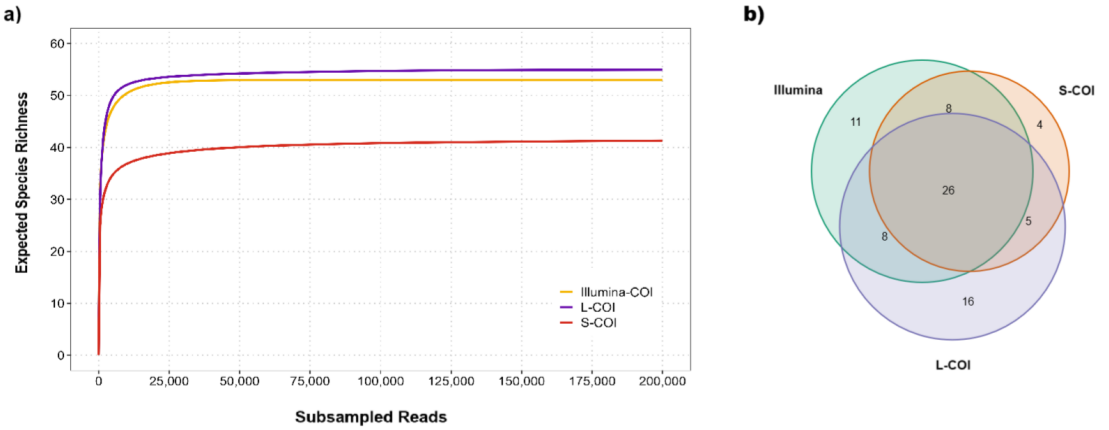

Figure 6.1.: a) Dataset-level rarefaction curves show expected species richness as a function of subsampled reads for Illumina-COI (orange), S-COI (red) and L-COI (purple); curves flatten, indicating adequate sequencing depth. **b)** Three-way Venn diagram of species detections highlights overlap (26 species common to all) and method-specific taxa after zero-count OTUs were removed.

The three methods overlapped in a core set of taxa but also detected many unique species. In total we observed 53 taxa in the Illumina dataset, 42 in the Nanopore S-COI, and 55 in Nanopore L-COI. Of these, 26 taxa were common to all three (e.g. *Clupea harengus*, *Salmo salar*, *Gadus morhua*, see **Supplementary Table D.1**). Those 26 taxa amounted to 88%, 80%, and 84% of all filtered reads for S-COI, L-COI, and Illumina data respectively. Illumina and S-COI shared 34 species (including the 26 common), Illumina and L-COI shared 34, and S-COI and L-COI shared 31 (**Fig. 6.1 b)**). Unique detections numbered 11 for Illumina-only (e.g., the microalga *Ochromonas danica* and the insect *Sitophilus oryzae*), 4 for S-COI-only (e.g., *Penicillium sclerotiorum* and *Pichia kluyveri*), and 16 for L-COI-only. Overall, the long-COI dataset recovered more species, many of which were missed by the shorter barcodes. This suggests that the extended fragment captured additional diversity (whether true ingredients or incidental DNA) beyond what the shorter reads could amplify or match. The remaining taxa not shared by all three methods amounted to trace detected ranging from 0.007%-7.8% of the total read proportion for Illumina, 0.002%-8.3% for S-COI and 0.0018%- 10.2% for L-COI. This pattern shows that relying on a single barcode can miss a non-trivial

fraction of the community, even when overall species counts appear saturated. At the same time, the 26-taxa core shared by all three workflows demonstrates that each platform robustly detects the dominant taxa. Together, these results suggest a complementary strategy: pair short-read barcodes – which tolerate degraded DNA and offer high-throughput quantification – with longer Nanopore reads that extend taxonomic breadth by capturing additional, otherwise overlooked species.

Nanopore results across most commercial products (**Fig. 6.2, 6.3, 6.4, 6.5**) showed overall consistency in both the number of species detected per sample and their relative abundance. This agreement suggests that, beyond the intrinsic differences in primer affinities and related different sequencing technologies, the analytical workflow developed in this study was effective in recovering the general taxonomic profiles of commercially processed products.

Nevertheless, notable incongruency emerged in the detection of congeneric species such as those belonging to the genus *Gadus*, *Merluccius*, and *Nemipterus*, when they co-occur within the same sample. This may be due to the introduction of sequencing errors or in raw data processing biases. Since this short region had already demonstrated effective species-level discrimination in previous analyses, the use of longer reads did not consistently lead to improved taxonomic resolution. Interestingly, identification of *Macruronus* sp. at the genus level, previously achieved with Illumina, was also found with the L-COI fragment, confirming the limited genetic variability within the COI region for the species *M. megellanicus* and *M. novaezelandiae* observed by Ward, 2009. Notably, species-level resolution was instead achieved with the 12S marker, highlighting the complementary power of alternative mitochondrial loci in discriminating closely related species (Fernandes et al., 2021; Lorusso, Shum, et al., 2024).

It is also important to consider that, in highly processed seafood products, DNA can be degraded, which may hinder the amplification of longer fragments, driving the development of mini-barcode primers specifically designed for degraded templates (Shokralla et al., 2015; Mottola, Piredda, Catanese, Lorusso, et al., 2022). Therefore, both biological and technical variables must be taken into account when interpreting species composition results. However, concordance among workflows was generally

high: nine of the twelve pairwise comparisons exceeded 98% similarity, corresponding to error rates $\leq 2\%$. The L-COI fragment produced perfect or near-perfect matches for *G. morhua*, *G. chalcogrammus* and *P. platessa* but dropped to 92.4% similarity for *M. productus*, whereas S-COI yielded the highest identity for *M. productus* (99.7%) yet the lowest for *G. chalcogrammus* (84.8%). Illumina reads agreed with S-COI at $\geq 98\%$ for all taxa except *G. chalcogrammus*, mirroring the pattern observed in the S-COI versus L-COI comparison (**Table 6.1; Supplementary Table D.2**). These results indicate that long-read Nanopore barcodes usually retain high accuracy once trimmed, although occasional species-specific errors persist, while the short-amplicon nanopore assay may underperform for certain taxa. Integrating both nanopore read lengths with Illumina data therefore provides complementary validation and maximises confidence in species assignments.

Species	Best-matching workflow	% Similarity	Worst-matching workflow	% Similarity
G. morhua	L-COI (Nanopore-650)	**100%**	S-COI (Nanopore-313)	98%
G. chalcogrammus	L-COI	**98.60%**	S-COI	84.80%
M. productus	S-COI	**99.70%**	L-COI	92.40%
P. platessa	L-COI	**99.70%**	S-COI	98.40%

Table 6.1.: Pairwise sequence similarity among Illumina, Nanopore 313 bp (S-COI) and Nanopore 650 bp (L-COI) consensus barcodes for four focal taxa after trimming all reads to the 300-bp Illumina window. Values are expressed as percent similarity; the most and least similar workflow for each species are indicated in boldface within the table.

The presence of some false positive taxa within samples was noticed. Since the same DNA extracts were used for the two experiments (Illumina and Nanopore) and a primer pair was in common, it is unlikely that these unshared taxa were effectively present, or the DNA extracts were contaminated. Since at time of analysis the available flow cell chemistry was the R.9.4, improvements in sequencing accuracy promised by the newly released chemistry (now at R.10.4.1 version) could further improve the accuracy and the taxonomic resolution [4]. Also, errors in the barcodes generated during sequencing could have led the inclusion of minor taxa as false positives into samples. Ultimately, the presence of non-target species could also be associated to the sequencing depth maybe linked to the duration of the sequencing runs (Toxqui Rodríguez et al., 2023). These results further highlight the need of accurate methodological choices for food metabarcoding analysis, comprising the

[4](https://store.nanoporetech.com/eu/flow-cells.html)

inclusion of negatives and positives samples (Giusti et al., 2024). Indeed, taxa included in our mock mixtures (*Pampus* sp. and *Lactarius lactarius*) were detected in S72 (L-COI) along with other unexpected species, according to the results of the other markers, in proportion superior of the 1% (**Fig. 6.2**). Other examples of samples containing unshared taxa, even at genus level, were S19 (surimi **Fig. 6.3**; *Merluccius* sp., *Melanogrammus aeglefinus*), S31 (breaded **Fig. 6.4**a; *Nemipterus*, *Merluccius*, and *Gadus*), S66 (breaded **Fig. 6.4**a; *Microstomus kitt*, *Merluccius paradoxus*), and S68 (breaded **Fig. 6.4**a; *Nemipterus*, *Merluccius*, and *Gadus*) in S-COI dataset, and S20 (dumpling **Fig. 6.6**; *Pleuronectes platessa* and *Gadus*) in L-COI dataset.

Terrestrial taxa *Bos taurus* and *Sus scrofa* were detected by both S-COI and L-COI markers, in agreement with the Illumina results. While the detection of *Gallus gallus* in S63 using L-COI, is not reflected in either COI Illumina and S-COI results, but is in accordance with the 12S primer (**Supplementary Table S4 in Chapter 5**), confirming the need of multi-markers strategies to improve taxonomic characterization of samples.

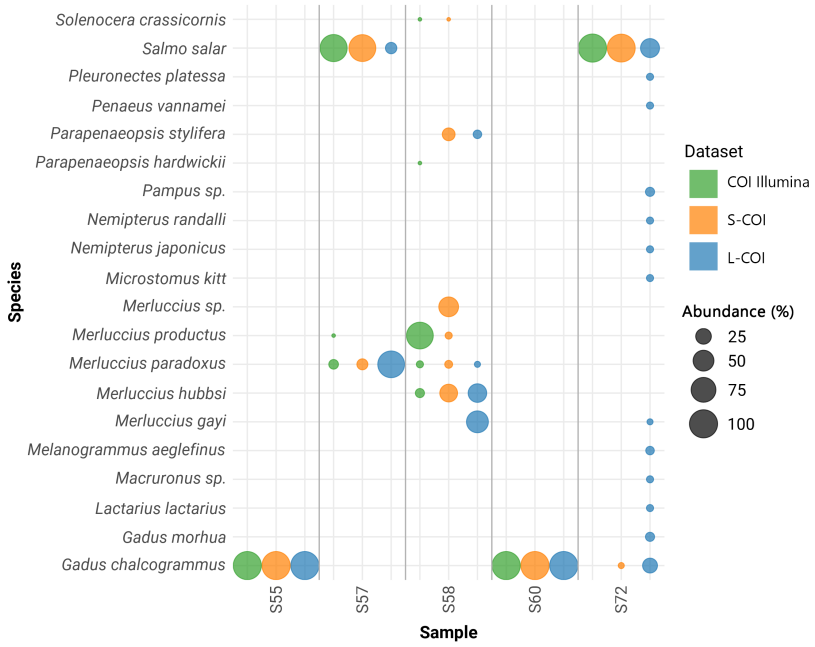

Figure 6.2.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "burgers" in Chapter 5

Figure 6.3.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "surimi" in Chapter 5

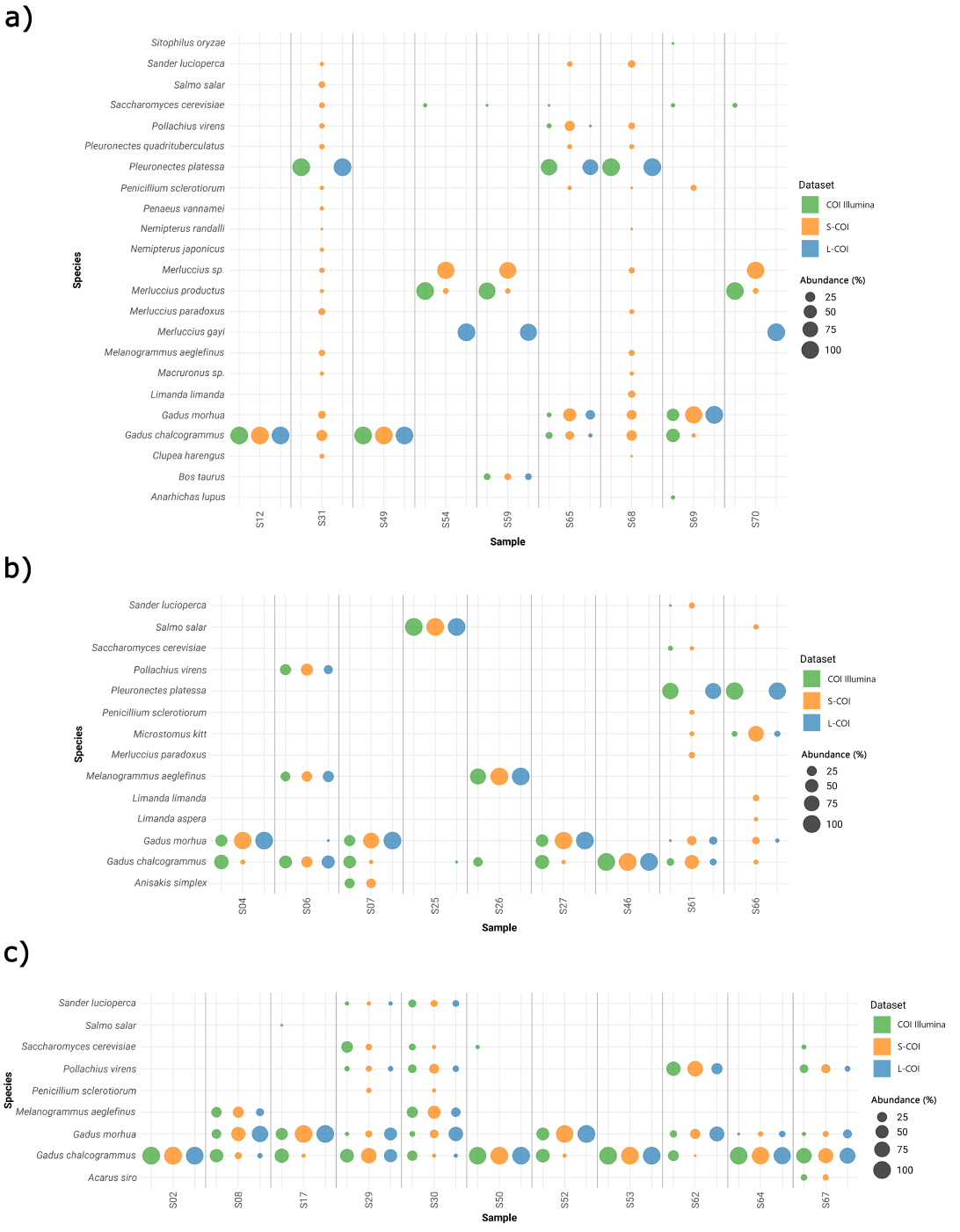

Figure 6.4.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "breaded a) Fillets, b) Fishcakes, c) Nuggets" in Chapter 5. S02 sample in COI Illumina analysis detected only 5 reads of *G. chalcogrammus*.

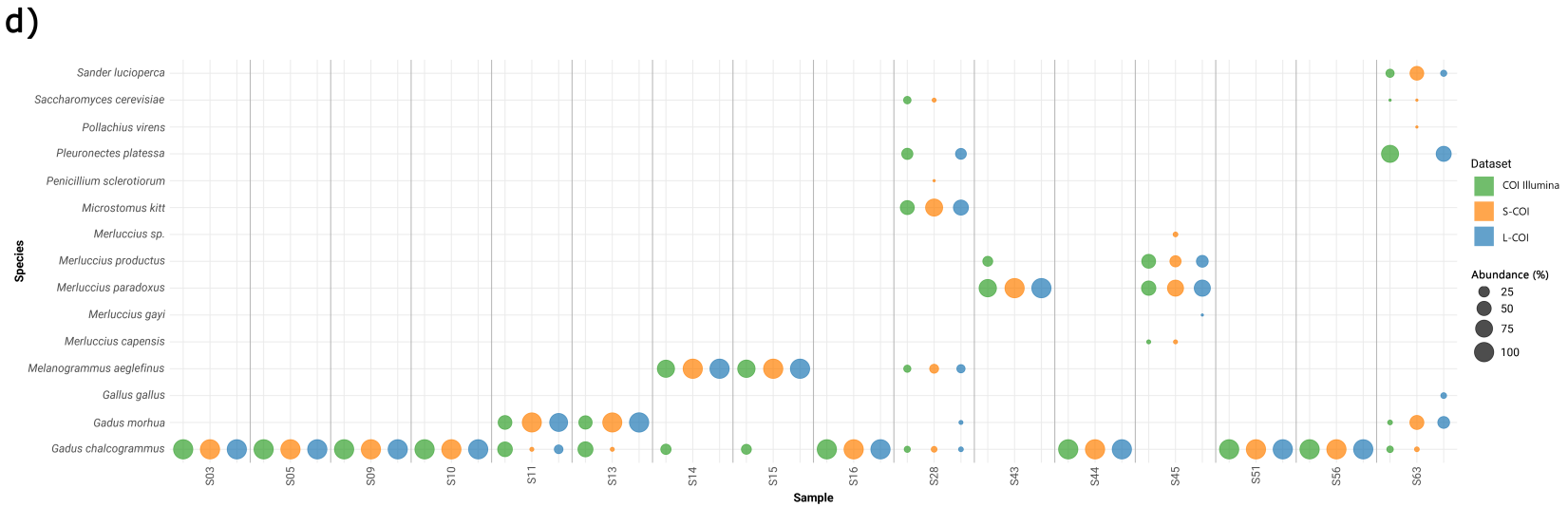

Figure 6.5.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "breaded d) fish finger" in Chapter 5

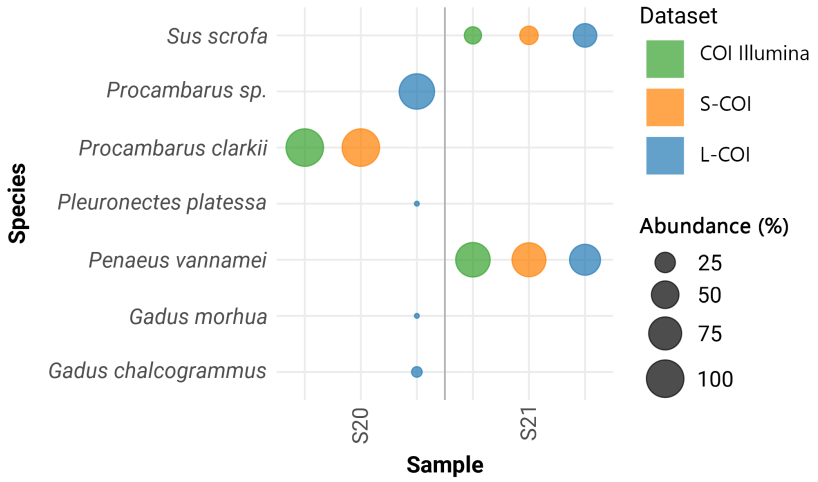

Figure 6.6.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "dumplings" in Chapter 5

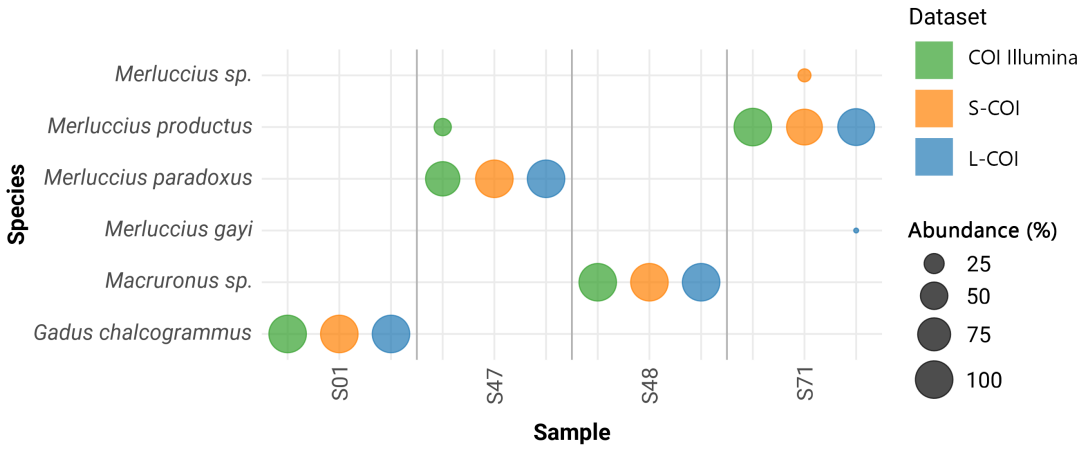

Figure 6.7.: Bubble plots showing read proportions of all taxa identified across the three methods in products categorised as "Steak" in Chapter 5

6.5. Conclusions

This study confirms that Oxford Nanopore Technologies (ONT) sequencing is a practical alternative to Illumina for species authentication in processed seafood—and, in some cases, adds complementary taxonomic coverage. Although overall MOTU richness was similar across platforms (42–55), the long-read Nanopore assay (L-COI, $\sim$ 650 bp) contributed 16 species that were not detected by either Illumina or the short-read Nanopore barcode, demonstrating its ability to pick up additional, low-abundance or difficult-to-amplify taxa in complex food matrices. After trimming to the shared 313 bp window, L-COI consensus sequences matched Illumina reads at 98–99% identity for all four focal species, confirming that extended fragments do not compromise accuracy. Conversely, the short-read ONT assay (S-COI) occasionally showed lower identity for certain closely related taxa (e.g., *Gadus chalcogrammus*), reflecting the greater impact of per-base errors on very short amplicons. Together, these results highlight ONT's portability and real-time sequencing as strong assets for on-site seafood verification, while showing that combining long- and short-read workflows can maximise taxonomic detection without sacrificing reliability. Notably, ONT's portability and affordability democratise access to sequencing, enabling in-field or local laboratory use where Illumina's infrastructure is cost-prohibitive. Current chemistry advancements (e.g., Q25 accuracy with R10.4.1 flow cells) further enhance its potential beyond the Q15 performance observed here. While false positives emphasise the need for replication and stringent controls, these are manageable through methodological refinements, such as multi-locus workflows and integrated reference databases. Ultimately, ONT's ability to deliver rapid, high-depth sequencing – combined with its taxonomic breadth – positions it as a pragmatic tool for supply chain compliance. Though not yet a full replacement for Illumina in standardised workflows, its strengths in portability, long-read resolution, and adaptability to degraded DNA make it indispensable for decentralising seafood authentication. Future efforts should prioritise protocol standardisation and hybrid approaches (e.g., coupling ONT with Illumina or qPCR) to maximise reliability while retaining ONT's unique advantages.

7. General conclusions and Future directions

Seafood products are frequently commercialised in processed forms to meet evolving consumer demands, enhancing palatability, extending shelf life, and facilitating access to global markets. However, the disparity in commercial value among fish species often motivates intentional species substitutions, with implications that extend from economic deception to consumer health risks, and the sustainable exploitation of marine resources. In the same way that morphological characteristics are essential for the taxonomic recognition of whole specimens, the nucleotide sequence of specific genes enables the molecular identification of the species included in processed food products. Therefore, molecular techniques remain the most reliable approach for verifying species authenticity, for both official control authorities and within industry. This PhD thesis confirms DNA metabarcoding as a powerful tool for food authenticity testing, offering increased sensitivity and enhanced species detection capabilities. Furthermore, by placing a strong emphasis on the evaluation of primer performance, this thesis offers valuable contributions to their effective application in the development of standardised protocols for species authentication in processed seafood.

Specifically, Chapter 2 provides a critical review of the molecular methodologies currently employed for the traceability and authentication of seafood products, with particular emphasis on DNA metabarcoding. The chapter presents a focused case study on mackerel species, which are of specific interest due to their frequent use in highly processed foods, such as canned products. The review highlights the importance of molecular identification techniques in distinguishing between mackerel species, particularly in processed products where morphological features

are no longer discernible. It is noted that many primer pairs and molecular regions commonly used in authentication studies show limited discriminatory power among mackerel species. Therefore, to ensure accurate species authentication in commercial mackerel products, it is essential to test alternative genetic regions and to carefully select the most appropriate primers. The combined use of preliminary *in-silico* analyses and subsequent *in-vitro* validation to select primers, suited to the intended application, is proving to be the most effective strategy for the detection and differentiation of closely related species. Moreover, the chapter underscores the lack of global regulatory harmonisation, which is crucial to ensure consistency, clarity, and that currently hinders interoperability between control systems.

Chapter 3 presents a comparative analysis of primer pairs previously reported in literature and potentially applicable to DNA metabarcoding studies, for the identification of commercially relevant marine species. *In-silico* PCR, based on publicly available reference sequences, were used to assess the taxonomic coverage of each primer pair. Among the primer pairs evaluated, some demonstrated superior performance, combining broad taxonomic coverage with a balanced amplification profile across detected taxa, while others exhibited limited coverage or strong taxonomic biases. The chapter emphasises the importance to increase the number of reference sequences in databases and highlights the need to design new primers specifically targeting marine species of high economic and nutritional relevance. Beyond the general assessment of these primers' universality for both marine and terrestrial animal species - whether listed in the FAO database or not - that could potentially enter the seafood supply chain, the findings confirm the necessity of adopting a multiple-marker strategy for the authentication of complex processed seafood products, ensuring broader coverage and greater reliability in species identification.

For these reasons, Chapter 4 applied DNA metabarcoding using two mitochondrial markers, targeting COI and 16S rRNA genes, for verifying species composition of processed seafood products labelled as single species. It revealed that most of the samples contained multiple species, despite label indications, and two products were mislabelled as the declared species were not detected at all. Notably, the analysis revealed the presence of species that were not reported on the labels, including both expected commercial species and others not listed in the Italian commercial designation decree. The two markers showed complementary taxonomic resolution,

reinforcing the need for multi-marker approaches in metabarcoding. However, both showed limitations in discriminating Gadiformes, a major component of processed seafood. In few cases, substitution may not have been economically motivated but rather due to confusion from commercial naming, only two samples violated labelling expectations, but the widespread undeclared multi-species content raises questions about transparency and consumer rights. The study suggests that some undeclared inclusions might result from cross-contamination during production, while others could reflect intentional addition of lower-value species.

Chapter 5 reports on the application of DNA metabarcoding using an alternative dual-marker combination, targeting the COI and 12S rRNA mitochondrial genes, to analyse pre-packaged seafood products, including fish fingers, burgers, and surimi, sourced from Italy, the United Kingdom, and Albania. The analysis revealed multiple instances of species substitution, the presence of undeclared species, and even potentially harmful taxa. In several cases, lower-value species were used in place of higher-value declared species raising concerns about supply chain integrity, even in products bearing sustainability certifications such as MSC. These findings suggest that irregularities are more likely to occur at the industrial processing stage, which is often managed by globally distributed operators located far from the final point of sale, rather than at the retail level.

As discussed in Chapters 4 and 5, the selection of primer pairs plays a critical role in the success of DNA metabarcoding analyses. While both chapters employed primers targeting regions of the COI gene, only the primer pair employed in chapter 5 proved capable of unambiguously distinguishing species within the *Gadus* genus.

As explored in Chapter 6, the development of third-generation ONT technology, with its lower cost and portability, seems promising for enabling rapid molecular testing at strategic points along the supply chain. However, the robustness and reliability of Illumina sequencing remain unmatched. To take advantages of this emerging platform, careful methodological choices are essential, including the use of multiple replicates, both positive and negative extraction controls, to support data cleaning and accurate result interpretation.

Overall, this thesis proposes robust DNA metabarcoding frameworks for species identification and authentication of processed products, allowing accurate verification of labelling information. It tests, validates and contrasts emerging technologies, providing practical advice on their use. However, results also highlight that the

full potential of this approach in the authentication of processed, multi-species seafood, remains underutilized. This thesis strongly emphasizes the urgent need for comprehensive controls throughout the supply chain. Indeed, it is unacceptable that, despite significant advancements in research and the availability of this method, it is still not implemented by regulatory authorities or the seafood industry. Can we still afford years of repeated attempts in pursuit of the perfect protocol? Can the seafood industry continue to operate without systematic monitoring? In today's context, ensuring food safety demands the integration of powerful analytical tools such as DNA metabarcoding in routine inspection practices. Meeting the global challenges of marine resource protection requires concrete, science-based actions to ensure safety, transparency and traceability across the seafood supply chain.

Others Publications

Species authentication of canned mackerel: Challenges in molecular identification and potential drivers of mislabelling

Mottola, A., Piredda, R., Catanese, G., **Lorusso, L.**, Ciccarese, G., Di Pinto, A. (2022).

Food Control

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The market for canned mackerel is growing due to their competitive price and to the excellent properties of the meat. However, the weak rules of current legislation, coupled with the loss of discrimination power due to being obliged to use short barcode regions, open up new challenges for traceability in processed products. In this study, for the first time, we applied a two-step mini-barcoding approach to identify canned mackerel sold in Italian markets and make assumptions on the potential drivers of mislabelling. Preliminary identification was performed using mini-barcode universal primers targeting a COI-fragment; then, in order to discriminate within the complex *Scomber colias/S. japonicus/S. australasicus*, we applied a second step, using new specific primers designed in the mitochondrial control region (D-loop). Comparison between labels and molecular identifications highlighted that the chance of mislabelling could be either 45% or 0%, depending on the interpretation of the generic terms used on the labels. Therefore, the study revealed that the ambiguous use, in the ingredient list, of 'umbrella' terms for naming fish species, such as 'sgombro' could be related to geographic origin of products and creates opportunities for fraudulent activities, but also misleads consumers. In light of this pattern, an urgent update of European legislation is needed to face current and future challenges for full chain traceability.

Preliminary study on species authentication in poultry meat products by next-generation sequencing

Mottola, A., Piredda, R., **Lorusso, L.**, Armani, A., Di Pinto, A. (2023).

Food Control

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The growing demand for poultry meat and the complexity of the supply chain affects traceability. Therefore, the aim of this study was to apply DNA metabarcoding to verify the labelling compliance of multi-species poultry meat commercial products. Overall, the molecular identifications conducted in this study confirm that all products contain the species declared in the label and all the non-conformities regard the presence of one or more undeclared meat species. In particular, undeclared species, such as pig and cattle, were highlighted in 8/13 of the samples. Such pattern could be due to technological purposes or accidental contamination linked to inappropriate sanitation practices during processing. However, the presence of undeclared species can affect the ability to choose for consumers with of specific needs (e.g., ethic, religious) or health risk and should be not neglected. Results of this study show that metabarcoding is a promising tool to identify meat species in mixtures. Therefore, its application by food industry and competent institutions, could help to innovate the food management system with the creation of a favourable environment for the protection and respect of the consumer needs.

Microbiome-based study in wild-caught Scomber scombrus fish products at the end of the supply chain

Piredda, R., Mottola, A., **Lorusso, L.**, Ranieri, L., Catanese, G., Cipriano, G., Carlucci, R., Anaclerio, D., & Di Pinto, A. (2023)

LWT

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Fresh fish remain the dominant seafood forms and preservation technologies have enabled them to access ever more distant markets. In this study, we used metabarcoding of the 16S rRNA gene to generate gill microbiomes from *Scomber scombrus* bought at fishmonger's stores as fresh products but whose labels showed they had been harvested in the Atlantic or Mediterranean FAO fishing areas. Microbial data were analysed with the aim of evaluating their ability to maintain signals from their different geographical origins and the presence of taxa which can potentially act as spoilers, foodborne pathogens, or histamine producers. Results revealed that microbiota, at the end of the wild fish supply chain, had differences related to the two FAO fishing areas (Atlantic vs Mediterranean). Despite the presence of microbial genera potentially associated with spoilage, histamine-production or foodborne pathogens, their patterns confirmed that low-temperature storage is a traditional but effective method of preservation. However, the ongoing spoilage processes were more evident in fresh non-local specimens, dominated by psychrophilic Gram-negative bacteria, whereas fresh local specimens contained Planctomycetes taxa. Therefore, despite the current limitations mainly related to time and cost of the method, our study highlighted that microbiome-based applications are an emergent tool for food system transformation.

Species substitution in goat yoghurt supply chain using melting-curve analysis

Mottola, A., Piredda, R., **Lorusso, L.**, Ranieri, L., & Di Pinto, A. (2024).

Journal of Food Composition and Analysis

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Mislabelling of dairy products has been widely reported worldwide but there are relatively few data available to help assess species substitution in goat yogurt. The purpose of this study was to investigate the presence of bovine and sheep milk in yogurt labelled as being made exclusively from goat milk, using qualitative real-time PCR. The results confirm that fermented products are targets for food species substitution, highlighting the undeclared presence of cow and/or sheep milk in 39.6% of samples analysed. Moreover, the study shows that the qualitative real-time PCR used here is a rapid, sensitive (LODabs of 0.015 ng/L), specific (no primer-dimers or non-specific products in the reactions), straightforward and low-cost molecular tool for discriminating adulterants in the dairy supply chain. Therefore, melt-curve analysis is a promising tool for facilitating routine safety, quality and authenticity checks in the dairy industry, and for implementing mitigation strategies against fraudulent activities. Application of DNA-based approaches is crucial for designing an innovative food safety management system and for helping to implement both a Food Fraud Vulnerability Assessment and a Food Fraud Prevention Strategy as common practices in the dairy sector.

DNA Metabarcoding Approach as a Potential Tool for Supporting Official Food Control Programs: A Case Study

Mottola, A., Intermite, C., Piredda, R., **Lorusso, L.**, Ranieri, L., Carpino, S., Celano, G.V., & Di Pinto, A.

Foods

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Food authentication significantly impacts consumer health and the credibility of Food Business Operators (FBOs). As European regulations mandate the verification of food authenticity and supply chain integrity, competent authorities require access to innovative analytical methods to identify and prevent food fraud. This study utilizes the DNA metabarcoding approach on meat preparations, sampled during an official control activity. It assesses animal and plant composition by amplifying DNA fragments of the 12S rRNA and trnL (UAA) genes, respectively. The results not only confirmed the declared species but also revealed undeclared and unexpected taxa in products labelled as containing a single animal species and various unspecified plant species. Notable findings such as the presence of Murinae, *Sus scrofa*, *Ovis aries*, and *Pisum sativum* could raise public health concerns, compromise consumer choices made for ethical or religious reasons, and reflect the hygienic conditions of the processing plant. This study demonstrates that the DNA metabarcoding approach looks to be a promising support tool for official control authorities to ensure food authenticity and safety, and to develop risk profiles along the supply chain.

Bibliography

Aakre, I., Næss, S., Kjellevold, M., Markhus, M. W., Alvheim, A. R., Dalane, J. Ø., Kielland, E., & Dahl, L. (2019). New data on nutrient composition in large selection of commercially available seafood products and its impact on micronutrient intake. *Food & Nutrition Research*, *63*, 10–29219.

Abraham, A., Jester, E. L. E., Granade, H. R., Plakas, S. M., & Dickey, R. W. (2012). Caribbean ciguatoxin profile in raw and cooked fish implicated in ciguatera. *Food Chemistry*, *131*, 192–198.

Acutis, P., Cambiotti, V., Riina, M., Meistro, S., Maurella, C., Massaro, M., Stacchini, P., Gili, S., Malandra, R., & Pezzolato, M. (2019). Detection of fish species substitution frauds in italy: A targeted national monitoring plan. *Food Control*, *101*, 151–155. https://doi.org/10.1016/j.foodcont.2019.02.020

Ahn, J. J., Kim, Y., Hong, J. Y., Kim, G. W., Kim, S. J., & Hwang, S. Y. (2017). Differentiation of Scomber japonicus from Scomber scombrus by using a single locked nucleic acid probe. *Mitochondrial DNA Part A*, *28*(3), 379–382. https://doi.org/10.3109/19401736.2015.1126831

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147. https://doi.org/10.1111/2041-210X.12849

Altschul, S., Gish, W., Miller, W., Myers, E., & Lipman, D. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Amarasinghe, S. L., Su, S., Dong, X., Zappia, L., Ritchie, M. E., & Gouil, Q. (2020). Opportunities and challenges in long-read sequencing data analysis. *Genome biology*, *21*(1), 30.

An, Y., Qian, Y. L., Alcazar Magana, A., Xiong, S., & Qian, M. C. (2020). Comparative characterization of aroma compounds in silver carp (hypophthalmichthys molitrix), pacific whiting (merluccius productus), and alaska pollock (theragra chalcogramma) surimi by aroma extract dilution analysis, odor activity value, and aroma recombination studies. *Journal of Agricultural and Food Chemistry*, *68*(38), 10403–10413. https://doi.org/10.1021/acs.jafc.9b07621

Andújar, C., Arribas, P., Yu, D. W., Vogler, A. P., & Emerson, B. C. (2018). Why the COI barcode should be the community DNA metabarcode for the Metazoa. *Molecular Ecology*, *27*(20), 3968–3975. https://doi.org/10.1111/mec.14844

Antil, S., Abraham, J. S., Sripoorna, S., Maurya, S., Dagar, J., Makhija, S., Bhagat, P., Gupta, R., Sood, U., Lal, R., & Toteja, R. (2023). Dna barcoding, an effective tool for species identification: A review. *Molecular Biology Reports*, *50*(1), 761–775. https://doi.org/10.1007/s11033-022-08015-7

Aphalo, P. J. (2016). Ggpmisc: An r package.

Applewhite, L., Larkin, P., & Naaum, A. M. (2016). Species identification using other tools. In *Seafood authenticity and traceability* (pp. 133–148). Academic Press. https://doi.org/10.1016/B978-0-12-801592-6.00007-3

Aranishi, F. (2005a). Pcr-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. *Journal of Agricultural and Food Chemistry*, *53*, 508–511. https://doi.org/10.1021/jf0484881

Aranishi, F. (2005b). Rapid PCR-RFLP method for discrimination of imported and domestic mackerel. *Marine Biotechnology*, *7*(6), 571–575. https://doi.org/10.1007/s10126-004-4102-1

Ardura, A., Planes, S., & Garcia-Vazquez, E. (2013). Applications of DNA barcoding to fish landings: Authentication and diversity assessment. *Zookeys*, *30*(365), 49–65. https://doi.org/10.3897/zookeys.365.6409

Ardura, A., Pola, I. G., Linde, A. R., & Garcia-Vazquez, E. (2010). Dna-based methods for species authentication of amazonian commercial fish. *Food Research International*, *43*, 2259–2302. https://doi.org/10.1016/j.foodres.2010.08.004

Armani, A., Castigliego, L., Tinacci, L., Gianfaldoni, D., & Guidi, A. (2012). Multiplex conventional and real-time PCR for fish species identification of Bianchetto (juvenile form of Sardina pilchardus), Rossetto (Aphia minuta),

and Icefish in fresh, marinated and cooked products. *Food Chemistry*, *133*(1), 184–192. https://doi.org/10.1016/j.foodchem.2011.12.076

Armani, A., Tinacci, L., Giusti, A., Castigliego, L., Gianfaldoni, D., & Guidi, A. (2013). What is inside the jar? forensically informative nucleotide sequencing (fins) of a short mitochondrial coi gene fragment reveals a high percentage of mislabeling in jellyfish food products. *Food Research International*, *54*(2), 1383–1393.

Armani, A., Tinacci, L., Xiong, X., Castigliego, L., Gianfaldoni, D., & Guidi, A. (2015). Fish species identification in canned pet food by blast and forensically informative nucleotide sequencing (fins) analysis of short fragments of the mitochondrial 16s ribosomal rna gene (16s rrna). *Food Control*, *50*, 821–830.

Arulandhu, A. J., Staats, M., Hagelaar, R., Voorhuijzen, M. M., Prins, T. W., Scholtens, I., Costessi, A., Duijsings, D., Rechenmann, F., Gaspar, F. B., et al. (2017). Development and validation of a multi-locus dna metabarcoding method to identify endangered species in complex samples. *Gigascience*, *6*(10), gix080.

Asensio, L., González, I., García, T., & Martín, R. (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (elisa). *Food Control*, *19*(1), 1–8.

Auliya, M., Altherr, S., Nithart, C., Hughes, A., & Bickford, D. (2023). Numerous uncertainties in the multifaceted global trade in frogs' legs with the EU as the major consumer. *Nature Conservation*, *51*, 71–135. https://doi.org/10.3897/natureconservation.51.93868

Baker, E., & Collette, B. B. (1998). Mackerel from the northern Indian Ocean and the Red Sea are Scomber australisicus, not Scomber japonicus. *Ichthyological Research*, *45*(1), 29–33. https://doi.org/10.1007/BF02678572

Ballesteros-Contreras, D. C., Barrios, L. M., & Preziosi, R. (2022). Development of microsatellite markers for the deep coral Madracis myriaster (Pocilloporidae: Anthozoa). *Scientific Reports*, *12*(1), 13193. https://doi.org/10.1038/s41598-022-14322-7

Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P., & Casiraghi, M. (2010). Dna barcoding reveals fraudulent substitutions in shark seafood products: The italian case of "palombo" (mustelus spp.) *Food Research International*, *43*, 376–381.

Barendse, J., Roel, A., Longo, C., Andriessen, L., Webster, L. M. I., Ogden, R., & Neat, F. (2019). Dna barcoding validates species labelling of certified seafood. *Current Biology*, *29*(6), R198–R199. https://doi.org/10.1016/j.cub.2019.02.014

Barska, A. (2018). Millennial consumers in the convenience food market. *Management*, *22*(1), 251–264. https://doi.org/10.2478/manment-2018-0018

Bartlett, S. E., & Davidson, W. S. (1992). FINS (forensically informative nucleotide sequencing): A procedure for identifying the animal origin of biological specimens. *Biotechniques*, *12*, 408–411.

Baweja, P., Kumar, S., Sahoo, D., & Levine, I. (2016). Biology of seaweeds. In *Seaweed in health and disease prevention* (pp. 41–106). Elsevier. https://doi.org/10.1016/B978-0-12-802772-1.00003-8

Bell, C. H. (2014). *Food safety assurance systems: Infestation management in food production premises*. https://doi.org/10.1016/B978-0-12-378612-8.00354-1

Blaha, F., & Katafono, K. (2020). Blockchain application in seafood value chains. *FAO Fisheries and Aquaculture Circular*, (C1207), I–43.

Blanco-Fernandez, C., Garcia-Vazquez, E., & Machado-Schiaffino, G. (2021). Seventeen years analyzing mislabeling from DNA barcodes: Towards hake sustainability. *Food Control*, *123*, 107723. https://doi.org/10.1016/j.foodcont.2020.107723

Blasco, G. D., Ferraro, D. M., Cottrell, R. S., Halpern, B. S., & Froehlich, H. E. (2020). Substantial gaps in the current fisheries data landscape. *Frontiers in Marine Science*, *7*, 612831.

Borit, M., & Olsen, P. (2012). Evaluation framework for regulatory requirements related to data recording and traceability designed to prevent illegal, unreported and unregulated fishing. *Marine Policy*, *36*(1), 96–102. https://doi.org/https://doi.org/10.1016/j.marpol.2011.03.012

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). Obitools: A unix -inspired software package for dna metabarcoding. *Molecular Ecology Resources*, *16*(1), 176–182. https://doi.org/10.1111/1755-0998.12428

Brandon-Mong, G.-J., Gan, H.-M., Sing, K.-W., Lee, P.-S., Lim, P.-E., & Wilson, J.-J. (2015). Dna metabarcoding of insects and allies: An evaluation of

primers and pipelines. *Bulletin of Entomological Research*, *105*(6), 717–727. https://doi.org/10.1017/S0007485315000681

Bruels, C. C., Littel, H. R., Daugherty, A. L., Stafki, S., Estrella, E. A., McGaughy, E. S., Truong, D., Badalamenti, J. P., Pais, L., Ganesh, V. S., et al. (2022). Diagnostic capabilities of nanopore long-read sequencing in muscular dystrophy. *Annals of clinical and translational neurology*, *9*(8), 1302–1309.

Bruno, A., Sandionigi, A., Agostinetto, G., Bernabovi, L., Frigerio, J., Casiraghi, M., & Labra, M. (2019). Food tracking perspective: Dna metabarcoding to identify plant composition in complex and processed food products. *Genes*, *10*(3), 248.

Burgener, M., & Hübner, P. (1998). Mitochondrial DNA enrichment for species identification and evolutionary analysis. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, *207*, 261–263. https://doi.org/10.1007/s002170050329

But, G.-C., Wu, H.-Y., & Shaw, P.-C. (2019). Identification of fish species of sushi products in hong kong. *Food Control*, *98*, 164–173.

Butler, F., Luijckx, N. L., Marvin, H. J., Bouzembrak, Y., & Mojtahed, V. (2021). Role of analytical testing for food fraud risk mitigation–a commentary on implementation of analytical fraud testing: Role of analytical testing for food fraud mitigation. *Current Research in Food Science*, *4*, 301–307.

Bylemans, J., Gleeson, D. M., Hardy, C. M., & Furlan, E. (2018). Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray–Darling Basin (Australia). *Ecology and Evolution*, *8*(17), 8697–8712. https://doi.org/10.1002/ece3.4387

Byrne, A., Beaudin, A. E., Olsen, H. E., Jain, M., Cole, C., Palmer, T., DuBois, R. M., Forsberg, E. C., Akeson, M., & Vollmers, C. (2017). Nanopore long-read rnaseq reveals widespread transcriptional variation among the surface receptors of individual b cells. *Nature communications*, *8*(1), 16027.

Cai, C., Hou, X., Huang, B., He, J., Wu, X., & He, Q. (2025). Lamp assay coupled crispr/lbcas12a system in a single-tube method for visual detection of meat adulteration. *Food Control*, *167*, 110809. https://doi.org/https://doi.org/10.1016/j.foodcont.2024.110809

Cai, Y., He, Y., Lv, R., Chen, H., Wang, Q., & Pan, L. (2017). Detection and quantification of beef and pork materials in meat products by duplex droplet

digital pcr. *PLoS One*, *12*(8), Article e0181949. https://doi.org/10.1371/journal.pone.0181949

Calosso, M. C., Claydon, J. A. B., Mariani, S., & Cawthorn, D.-M. (2020). Global footprint of mislabelled seafood on a small island nation. *Biological Conservation*, *245*, 108557. https://doi.org/10.1016/j.biocon.2020.108557

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. (2009). Blast+: Architecture and applications. *BMC Bioinformatics*, *10*, 421. https://doi.org/10.1186/1471-2105-10-421

Cappello, T., Giannetto, A., Parrino, V., De Marco, G., Mauceri, A., & Maisano, M. (2018). Food safety using nmr-based metabolomics: Assessment of the atlantic bluefin tuna, thunnus thynnus, from the mediterranean sea. *Food and Chemical Toxicology*, *115*, 391–397.

Cardeñosa, D., Shea, S. K., Zhang, H., Fischer, G. A., Simpfendorfer, C. A., & Chapman, D. D. (2022). Two thirds of species in a global shark fin trade hub are threatened with extinction: Conservation potential of international trade regulations for coastal sharks. *Conservation Letters*, *15*(5), e12910. https://doi.org/10.1111/conl.12910

Carvalho, D. C., Palhares, R. M., Drummond, M. G., & Gadanho, M. (2017). Food metagenomics: Next generation sequencing identifies species mixtures and mislabeling within highly processed cod products. *Food Control*, *80*, 183–186. https://doi.org/10.1016/j.foodcont.2017.04.049

Castro Hernandez, J., & Santana Ortega, A. (2000). Synopsis of biological data on the chub mackerel (scomber japonicus houttuyn, 1782). fao fisheries synopsis. no. 157. rome, fao. 2000. 77p.

Catanese, G., Pérez, L., & Funes, V. (2007). Diferenciación genética del estornino (scomber colias), procedente del litoral andaluz, mediante análisis de la región mitocondrial atco. xi congreso nacional de acuicultura. genética y reproducción.; 263-266; fundación oesa; cima. xunta de galicia.; vigo; españa.

Catanese, G., Di Capua, I., Iriondo, M., Bonanno, A., Estonba, A., & Procaccini, G. (2020). Application of high-throughput single nucleotide polymorphism genotyping for assessing the origin of engraulis encrasicolus eggs. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *30*(7), 1313–1324.

Catanese, G., Manchado, M., Fernández-Trujillo, A., & Infante, C. (2010). A multiplex-pcr assay for the authentication of mackerels of the genus scomber in processed fish products. *Food chemistry*, *122*(1), 319–326.

Catanese, G., Manchado, M., & Infante, C. (2010). Evolutionary relatedness of mackerels of the genus scomber based on complete mitochondrial genomes: Strong support to the recognition of atlantic scomber colias and pacific scomber japonicus as distinct species. *Gene*, *452*(1), 35–43.

Catanese, G., Watteaux, R., Montes, I., Barra, M., Rumolo, P., Borme, D., Buongiorno Nardelli, B., Botte, V., Mazzocchi, M. G., Genovese, S., et al. (2017). Insights on the drivers of genetic divergence in the european anchovy. *Scientific Reports*, *7*(1), 4180.

Cavelius, L. S., Goebelbecker, J. M., & Morlock, G. E. (2023). Legal and normative requirements for food safety culture–a consolidated overview for food companies within the eu. *Trends in Food Science & Technology*, *142*, 104222.

Cawthorn, D.-M., Murphy, T. E., Naaum, A. M., & Hanner, R. H. (2021). Vague labelling laws and outdated fish naming lists undermine seafood market transparency in canada. *Marine Policy*, *125*, 104335.

Chapela, M. J., Sotelo, C. G., Calo-Mata, P., Perez-Martin, R. I., Rehbein, H., Hold, G. L., Quinteiro, J., Rey-Mendez, M., Rosa, C., & Santos, A. T. (2002). Identification of cephalopod species (Ommastrephidae and Loliginidae) in seafood products by forensically informative nucleotide sequencing (FINS). *Journal of Food Science*, *67*(5), 1672–1676. https://doi.org/10.1111/j.1365-2621.2002.tb08703.x

Chatterjee, N. S., Chevallier, O. P., Wielogorska, E., Black, C., & Elliott, C. T. (2019). Simultaneous authentication of species identity and geographical origin of shrimps: Untargeted metabolomics to recurrent biomarker ions. *Journal of Chromatography A*, *1599*, 75–84.

Chatzoglou, E., Tsaousi, N., Apostolidis, A. P., Exadactylos, A., Sandaltzopoulos, R., Giantsis, I. A., Gkafas, G. A., Malandrakis, E. E., Sarantopoulou, J., Tokamani, M., et al. (2023). High-resolution melting (hrm) analysis for rapid molecular identification of sparidae species in the greek fish market. *Genes*, *14*(6), 1255.

Chen, H., & Boutros, P. C. (2011). Venndiagram: A package for the generation of highly-customizable venn and euler diagrams in r. *BMC bioinformatics*, *12*, 1–7.

Cheng, J., Gao, T., Miao, Z., & Yanagimoto, T. (2011). Molecular phylogeny and evolution of scomber (teleostei: Scombridae) based on mitochondrial and nuclear dna sequences. *Chinese Journal of Oceanology and Limnology*, *29*(2), 297–310.

Cheng, J., Yanagimoto, T., Song, N., & Gao, T.-x. (2015). Population genetic structure of chub mackerel scomber japonicus in the northwestern pacific inferred from microsatellite analysis. *Molecular Biology Reports*, *42*, 373–382.

Chien, H.-J., Huang, Y.-H., Zheng, Y.-F., Wang, W.-C., Kuo, C.-Y., Wei, G.-J., & Lai, C.-C. (2022). Proteomics for species authentication of cod and corresponding fishery products. *Food Chemistry*, *374*, 131631.

Clark, L. (2015). The current status of dna barcoding technology for species identification in fish value chains. *Food Policy*, *54*, 85–94. https://doi.org/10.1016/j.foodpol.2015.05.005

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160–1170. https://doi.org/10.1111/1755-0998.12265

Cohen, N. J., Deeds, J. R., Wong, E. S., Hanner, R. H., Yancy, H. F., White, K. D., Thompson, T. M., Wahl, I., Pham, T. D., Guichard, F. M., Huh, I., Austin, C., Dizikes, G., & Gerber, S. I. (2009). Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. *Journal of Food Protection*, *72*(4), 810–817. https://doi.org/https://doi.org/10.4315/0362-028X-72.4.810

Collette, B. B., & Nauen, C. E. (1983). *Fao species catalogue, vol. 2: Scombrids of the world: An annotated and illustrated catalogue of tunas, mackerels, bonitos, and related species known to date*. FAO. https://www.fao.org/3/ac478e/ac478e00.htm

Collette, B. (1999). Mackerels, molecules, and morphology. *Soc Fr Ichthyol*, 149–164.

Cottenet, G., Cavin, C., Blancpain, C., Chuah, P., Pellesi, R., Suman, M., Nogueira, S., & Gadanho, M. (2022). A dna metabarcoding workflow to identify species

in spices and herbs. *Journal of AOAC International*, *106*, 65–72. https://doi.org/10.1093/jaoacint/qsac099

Da Silva, L., Mata, V., Lopes, P., Pereira, P., Jarman, S., Lopes, R., & Beja, P. (2019). Advancing the integration of multi-marker metabarcoding data in dietary analysis of trophic generalists. *Molecular Ecology Resources*, *19*, 1420–1432. https://doi.org/10.1111/1755-0998.13060

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). Dna metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, *14*, 306–323. https://doi.org/10.1111/1755-0998.12188

De Domenico, S., De Rinaldis, G., Paulmery, M., Piraino, S., & Leone, A. (2019). Barrel jellyfish (Rhizostoma pulmo) as source of antioxidant peptides. *Marine Drugs*, *17*(2), 134. https://doi.org/10.3390/md17020134

Deagle, B. E., Gales, N. J., Evans, K., Jarman, S. N., Robinson, S., Trebilco, R., & Hindell, M. A. (2007). Studying seabird diet through genetic analysis of faeces: A case study on macaroni penguins (Eudyptes chrysolophus). *PLoS One*, *2*(9), e831. https://doi.org/10.1371/journal.pone.0000831

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R., & Eveson, J. P. (2019). Counting with dna in metabarcoding studies: How should we convert sequence reads to dietary data? *Molecular Ecology*, *28*(2), 391–406. https://doi.org/10.1111/mec.14734

Deconinck, D., Volckaert, F., Hostens, K., Panicz, R., Eljasik, P., Faria, M., Monteiro, C., Robbens, J., & Derycke, S. (2020). A high-quality genetic reference database for european commercial fishes reveals substitution fraud of processed atlantic cod (gadus morhua) and common sole (solea solea) at different steps in the belgian supply chain. *Food and Chemical Toxicology*, *141*, 111417. https://doi.org/10.1016/j.fct.2020.111417

Deng, R., Xu, L., Zhang, Y., Zhang, X., Yuan, Z., Chen, J., & Xia, X. (2024). Crispr-based nucleic acid assays for food authentication. *Trends in Food Science & Technology*, *145*, 104351. https://doi.org/https://doi.org/10.1016/j.tifs.2024.104351

Deng, Z., Wang, T., Zheng, Y., Zhang, W., & Yun, Y.-H. (2024). Deep learning in food authenticity: Recent advances and future trends. *Trends in Food*

Science & Technology, *144*, 104344. https://doi.org/https://doi.org/10.1016/j.tifs.2024.104344

Detcharoen, M., Khrueakaew, P., Sukkapat, P., Benjakul, S., & Saetang, J. (2024). Metabarcoding for authentication of fish species in surimi-based products by nanopore sequencing. *Food Bioscience*, *61*, 104628.

Di Pinto, A., Marchetti, P., Mottola, A., Bozzo, G., Bonerba, E., Ceci, E., Bottaro, M., & Tantillo, G. (2015). Species identification in fish fillet products using dna barcoding. *Fisheries Research*, *170*, 9–13. https://doi.org/https://doi.org/10.1016/j.fishres.2015.05.006

DiBattista, J. D., Coker, D. J., Sinclair-Taylor, T. H., Stat, M., Berumen, M. L., & Bunce, M. (2017). Assessing the utility of eDNA as a tool to survey reef-fish communities in the Red Sea. *Coral Reefs*, *36*(4), 1245–1252. https://doi.org/10.1007/s00338-017-1618-1

Dobrovolny, S., Blaschitz, M., Weinmaier, T., Pechatschek, J., Cichna-Markl, M., Indra, A., Hufnagl, P., & Hochegger, R. (2019). Development of a dna metabarcoding method for the identification of fifteen mammalian and six poultry species in food. *Food chemistry*, *272*, 354–361.

Domínguez-Petit, R., Navarro, M. R., Cousido-Rocha, M., Tornero, J., Ramos, F., & Jurado-Ruzafa, A. e. a. (2022). Spatial variability of life-history parameters of the Atlantic chub mackerel (Scomber colias), an expanding species in the northeast Atlantic. *Scientia Marina*, *86*, e048. https://doi.org/10.3989/scimar.05296.048

Dooley, J. J., Sage, H. D., Clarke, M.-A. L., Brown, H. M., & Garrett, S. D. (2005). Fish species identification using pcr- rflp analysis and lab-on-a-chip capillary electrophoresis: Application to detect white fish species in food products and an interlaboratory study. *Journal of agricultural and food chemistry*, *53*(9), 3348–3357.

dos Santos Fogaça, F. H., Cassol, G. Z., Rezende-de-Souza, J. H., Martin, J. G. P., & Savay-da-Silva, L. K. (2023). Chapter 10 - south american fermented fish and meat-based products. In O. A. Adebo, C. E. Chinma, A. O. Obadina, A. G. Soares, S. K. Panda, & R.-Y. Gan (Eds.), *Indigenous fermented foods for the tropics* (pp. 149–168). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-323-98341-9.00028-1

Dulvy, N. K., Pacoureau, N., Rigby, C. L., Pollom, R. A., Jabado, R. W., Ebert, D. A., Finucci, B., Pollock, C. M., Cheok, J., Derrick, D. H., Herman, K. B., Sherman, C. S., VanderWright, W. J., Lawson, J. M., Walls, R. H. L., Carlson, J. K., Charvet, P., Bineesh, K. K., Fernando, D., et al. (2021). Overfishing drives over one-third of all sharks and rays toward a global extinction crisis. *Current Biology*, *31*(21), 4773–4787.e8. https://doi.org/10.1016/j.cub.2021.08.062

Dwiyitno, D., Hoffman, S., Parmentier, K., & Keer, C. V. (2021). Universal primer design for crustacean and bivalve-mollusc authenticity based on cytochrome-b gene. *Biodiversitas Journal of Biological Diversity*, *23*(1). https://doi.org/10.13057/biodiv/d230103

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). Uchime improves sensitivity and speed of chimera detection. *Bioinformatics*, *27*(16), 2194–2200. https://doi.org/10.1093/bioinformatics/btr381

EUMOFA, Directorate-General for Maritime Affairs and Fisheries. (2023). The EU fish market – 2023 edition. https://doi.org/10.2771/716731

European Commission. (2021). *Eu consumer habits regarding fishery and aquaculture products: Special eurobarometer 515 report* (Eurobarometer Report). European Union. https://europa.eu/eurobarometer/api/deliverable/download/file?deliverableId=76466

European Commission. (2024). First report on the implementation of the multiannual plan for the fisheries exploiting demersal stocks in the western mediterranean sea: Report from the commission to the european parliament and the council [Accessed April 23, 2025]. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:52024DC0322

European Food Safety Authority (EFSA). (2018). Occurrence of residues of fipronil and other acaricides in chicken eggs and poultry muscle/fat. *EFSA Journal*, *16*(5), e05164. https://doi.org/10.2903/j.efsa.2018.5164

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., Mahon, A. R., Pfrender, M. E., Lamberti, G. A., & Lodge, D. M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular Ecology Resources*, *16*(1), 29–41. https://doi.org/10.1111/1755-0998.12433

Everstine, K. D., Chin, H. B., Lopes, F. A., & Moore, J. C. (2024). Database of food fraud records: Summary of data from 1980 to 2022. *Journal of Food Protection*, *87*(3), 100227. https://doi.org/https://doi.org/10.1016/j.jfp.2024.100227

FAO. (2018). *Overview of food fraud in the fisheries sector, by alan reilly* (tech. rep. No. 1165). FAO. Rome, Italy.

FAO. (2020). *The state of world fisheries and aquaculture 2020*.

FAO. (2022). *The state of world fisheries and aquaculture 2022 - towards blue transformation*. https://doi.org/10.4060/cc0461en

FAO. (2024). *The state of world fisheries and aquaculture 2024 - blue transformation in action*. https://doi.org/10.4060/cd0683en

FAO & WHO. (2024). *Joint fao/who expert consultation on the risks and benefits of fish consumption: Meeting report, rome, 9–13 october 2023*. Food, Agriculture Organization of the United Nations (FAO), and World Health Organization (WHO). FAO & WHO.

Fernandes, T. J. R., Amaral, J. S., & Mafra, I. (2021). Dna barcode markers applied to seafood authentication: An updated review. *Critical Reviews in Food Science and Nutrition*, *61*(22), 3904–3935. https://doi.org/10.1080/10408398.2020.1811200

Ferreira, I. M. P. L. V. O., Pinho, O., Vieira, E., & Tavarela, J. G. (2010). Brewer's saccharomyces yeast biomass: Characteristics and potential applications. *Trends in Food Science and Technology*, *21*, 77–84.

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An in silico approach for the evaluation of dna barcodes. *BMC genomics*, *11*, 1–10.

Ficetola, G., & Taberlet, P. (2023). Towards exhaustive community ecology via dna metabarcoding. *Molecular Ecology*, *32*, 6320–6329. https://doi.org/10.1111/mec.16881

Flügge, F., Kerkow, T., Kowalski, P., Bornhöft, J., Seemann, E., Creydt, M., Schütze, B., & Günther, U. (2023). Qualitative and quantitative food authentication of oregano using ngs and nmr with chemometrics. *Food Control*, *145*, 109497. https://doi.org/10.1016/j.foodcont.2022.109497

Fox, M., Mitchell, M., Dean, M., Elliott, C., & Campbell, K. (2018). The seafood supply chain from a fraudulent perspective. *Food Security*, *10*(4), 939–963. https://doi.org/10.1007/s12571-018-0826-z

Franco, C. M., Ambrosio, R. L., Cepeda, A., & Anastasio, A. (2021). Fish intended for human consumption: From dna barcoding to a next-generation sequencing (NGS)-based approach. *Current Opinion in Food Science*, *42*, 86–92. https://doi.org/10.1016/j.cofs.2021.05.005

Friedemann, M. (2019). Ciguatera fish poisoning outbreaks from 2012 to 2017 in germany caused by snappers from india, indonesia, and vietnam. *Journal of Consumer Protection and Food Safety*, *14*(1), 71–80. https://doi.org/10.1007/s00003-018-1191-8

Friedman, M. A., Fernandez, M., Backer, L. C., Dickey, R. W., Bernstein, J., Schrank, K., Kibler, S., Stephan, W., Gribble, M. O., Bienfang, P., Bowen, R. E., Degrasse, S., Flores Quintana, H. A., Loeffler, C. R., Weisman, R., Blythe, D., Berdalet, E., Ayyar, R., Clarkson-Townsend, D., … Fleming, L. E. (2017). An updated review of ciguatera fish poisoning: Clinical, epidemiological, environmental, and public health management. *Marine Drugs*, *15*(3). https://doi.org/10.3390/md15030072

Frigerio, J., Agostinetto, G., Mezzasalma, V., De Mattia, F., Labra, M., & Bruno, A. (2021). Dna-based herbal teas' authentication: An its2 and psba-trnh multi-marker dna metabarcoding approach. *Plants*, *10*, 2120. https://doi.org/10.3390/plants10102120

Frigerio, J., Gorini, T., Palumbo, C., De Mattia, F., Labra, M., & Mezzasalma, V. (2023). A fast and simple DNA mini-barcoding and RPA assay coupled with lateral flow assay for fresh and canned mackerel authentication. *Food Analytical Methods*, *16*, 426–435. https://doi.org/10.1007/s12161-022-02429-6

Froese, R. (2023). Fishbase. world wide web electronic publication. http://www.fishbase.org

Fromentin, J.-M., Emery, M. R., Donaldson, J., Balachander, G., Barron, E. S., Chaudhary, R. P., Danner, M.-C., Gasalla, M. A., Hallosserie, A., Halmy, M., et al. (2023). Status, challenges and pathways to the sustainable use of wild species. *Global Environmental Change*, *81*, 102692.

Galal-Khallaf, A., Ardura, A., Mohammed-Geba, K., Borrell, Y. J., & Garcia-Vazquez, E. (2014). Dna barcoding reveals a high level of mislabeling in egyptian fish fillets. *Food Control*, *46*, 441–445. https://doi.org/https://doi.org/10.1016/j.foodcont.2014.06.016

Garibaldi, L., & Busilacchi, S. (2002). Asfis list of species for fishery statistics purposes/liste asfis des espèces pour les besoins des statistiques des pêches/lista asfis de especies para los fines de estadísticas de pesca.

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of pcr primers for mitochondrial cytochrome c oxidase subunit i for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, *13*(5), 851–861. https://doi.org/10.1111/1755-0998.12138

Gense, K., Peterseil, V., Licina, A., Wagner, M., Cichna-Markl, M., & Dobrovolny, S. e. a. (2021). Development of a DNA metabarcoding method for the identification of bivalve species in seafood products. *Foods*, *10*, 2618. https://doi.org/10.3390/foods10112618

Gephart, J. A., & Pace, M. L. (2015). Structure and evolution of the global seafood trade network. *Environmental Research Letters*, *10*(12), 125014. https://doi.org/10.1088/1748-9326/10/12/125014

Giagkazoglou, Z., Loukovitis, D., Gubili, C., Chatziplis, D., Symeonidis, A., & Imsiridou, A. (2024). Untangling the cephalopod market: Authentication of seafood products in greece with dna-barcoding. *Food Control*, *163*, 110523. https://doi.org/10.1016/j.foodcont.2024.110523

Giusti, A., Malloggi, C., Lonzi, V., Forzano, R., Meneghetti, B., Solimeo, A., Tinacci, L., & Armani, A. (2023). Metabarcoding for the authentication of complex seafood products: The fish burger case. *Journal of Food Composition and Analysis*, *123*, 105559. https://doi.org/10.1016/j.jfca.2023.105559

Giusti, A., Malloggi, C., Magagna, G., Filipello, V., & Armani, A. (2024). Is the metabarcoding ripe enough to be applied to the authentication of foodstuff of animal origin? a systematic review. *Comprehensive Reviews in Food Science and Food Safety*, *23*(1), 1–21.

Giusti, A., Ricci, E., Guarducci, M., Gasperetti, L., Davidovich, N., Guidi, A., & Armani, A. (2018). Emerging risks in the european seafood chain: Molecular identification of toxic lagocephalus spp. in fresh and processed products.

Food Control, *91*, 311–320. https://doi.org/https://doi.org/10.1016/j.foodcont.2018.04.013

Giusti, A., Tinacci, L., Sotelo, C. G., Marchetti, M., Guidi, A., Zheng, W., & Armani, A. (2017). Seafood identification in multispecies products: Assessment of 16srrna, cytb, and COI universal primers' efficiency as a preliminary analytical step for setting up metabarcoding next-generation sequencing techniques. *Journal of Agricultural and Food Chemistry*, *65*(13), 2902–2912. https://doi.org/10.1021/acs.jafc.6b05802

Goordial, J., Altshuler, I., Hindson, K., Chan-Yam, K., Marcolefas, E., & Whyte, L. G. (2017). In situ field sequencing and life detection in remote (79 26 n) canadian high arctic permafrost ice wedge microbial communities. *Frontiers in microbiology*, *8*, 2594.

Gordoa, A., Carreras, G., Sanz, N., & Viñas, J. (2017). Tuna species substitution in the spanish commercial chain: A knock-on effect. *PLoS One*, *12*(1), e0170809. https://doi.org/10.1371/journal.pone.0170809

Gostel, M. R., Zúñiga, J. D., & Kress, W. J. e. a. (2020). Microfluidic enrichment barcoding (MEBarcoding): A new method for high throughput plant DNA barcoding. *Scientific Reports*, *10*, 8701. https://doi.org/10.1038/s41598-020-64919-z

Grainger, S. (2020). *The story of garum: Fermented fish sauce and salted fish in the ancient world*. Routledge.

Granata, L. A., Flick Jr, G. J., & Martin, R. E. (2012). *The seafood industry: Species, products, processing, and safety*. John Wiley & Sons.

Griffiths, A., Sotelo, C., Mendes, R., Pérez-Martín, R. I., Schröder, U., & Shorten, M. e. a. (2014). Current methods for seafood authenticity testing in Europe: Is there a need for harmonisation? *Food Control*, *45*, 95–100. https://doi.org/10.1016/j.foodcont.2014.04.020

Hajibabaei, M., & McKenna, C. (2012). Dna mini-barcodes. In *Methods in molecular biology* (pp. 339–353, Vol. 858). https://doi.org/10.1007/978-1-61779-591-6_15

Hamilton, B., Hurbungs, M., Jones, A., & Lewis, R. J. (2002). Multiple ciguatoxins present in indian ocean reef fish. *Toxicon*, *40*, 1347–1353.

Handy, S. M., Deeds, J. R., Ivanova, N. V., Hebert, P. D. N., Hanner, R. H., Ormos, A., Weigt, L. A., Moore, M. M., & Yancy, H. F. (2011). A single-laboratory

validated method for the generation of dna barcodes for the identification of fish for regulatory compliance. *Journal of AOAC INTERNATIONAL*, *94*(1), 201–210. https://doi.org/10.1093/jaoac/94.1.201

Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver, A., & Winfield, I. J. (2016). Environmental dna metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, *25*(13), 3101–3119. https://doi.org/10.1111/mec.13660

Hasan, Y., Nurbaiti, S., & Hamid, A. (2024). The urgency of handling illegal, unreported and unregulated fishing in the indonesian border region perspective: Marine security in the makassar strait. *Padjadjaran Journal of International Law*, *8*, 63–74. https://doi.org/10.23920/pjil.v8i1.1622

Hassoun, A., Siddiqui, S. A., Smaoui, S., Ucak, İ., Arshad, R. N., Garcia-Oliveira, P., Prieto, M. A., Aït-Kaddour, A., Perestrelo, R., Câmara, J. S., & Bono, G. (2022). Seafood processing, preservation, and analytical techniques in the age of industry 4.0. *Applied Sciences*, *12*(3). https://doi.org/10.3390/app12031703

Hawkins, J., De Vere, N., Griffith, A., Ford, C. R., Allainguillaume, J., Hegarty, M. J., Baillie, L., & Adams-Groom, B. (2015). Using dna metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. *PloS one*, *10*(8), e0134735.

Haynes, E., Jimenez, E., Pardo, M., & Helyar, S. (2019). The future of ngs (next generation sequencing) analysis in testing food authenticity. *Food Control*, *101*, 134–143. https://doi.org/10.1016/j.foodcont.2019.02.010

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through dna barcodes. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Hestetun, J. T., Bye-Ingebrigtsen, E., Nilsson, R. H., Glover, A. G., Johansen, P.-O., & Dahlgren, T. G. (2020). Significant taxon sampling gaps in dna databases limit the operational use of marine macrofauna metabarcoding. *Marine Biodiversity*, *50*(5), 70. https://doi.org/10.1007/s12526-020-01093-5

Hleap, J. S., Littlefair, J. E., Steinke, D., Hebert, P. D. N., & Cristescu, M. E. (2021). Assessment of current taxonomic assignment strategies for metabarcoding

eukaryotes. *Molecular Ecology Resources*, *21*(7), 2190–2203. https://doi.org/10.1111/1755-0998.13407

Ho, J. K., Puniamoorthy, J., Srivathsan, A., & Meier, R. (2020). Minion sequencing of seafood in singapore reveals creatively labelled flatfishes, confused roe, pig dna in squid balls, and phantom crustaceans. *Food Control*, *112*, 107144.

Hopkins, C., Roberts, S., Caveen, A., Graham, C., & Burns, N. (2024). Improved traceability in seafood supply chains is achievable by minimising vulnerable nodes in processing and distribution networks. *Marine Policy*, *159*, 105910. https://doi.org/10.1016/j.marpol.2023.105910

Horreo, J. L., Ardura, A., Pola, I. G., Martínez, J. L., & Garcia-Vazquez, E. (2013). Universal primers for species authentication of animal foodstuff in a single polymerase chain reaction. *Journal of the Science of Food and Agriculture*, *93*(2), 354–361. https://doi.org/10.1002/jsfa.5766

Hu, T., Chitnis, N., Monos, D., & Dinh, A. (2021). Next-generation sequencing technologies: An overview. *Human immunology*, *82*(11), 801–811.

ICQRF. (2024). Report attività 2023: Prospettive 2024 [Accessed May 2025].

Infante, C., & Manchado, M. (2006). Multiplex-polymerase chain reaction assay for the authentication of the mackerel Scomber colias in commercial canned products. *Journal of AOAC International*, *89*(3), 708–711. https://doi.org/10.1093/jaoac/89.3.708

Infante, C., Blanco, E., Zuasti, E., Crespo, A., & Manchado, M. (2007). Phylogenetic differentiation between atlantic scomber colias and pacific scomber japonicus based on nuclear dna sequences. *Genetica*, *130*, 1–8.

Infante, C., Catanese, G., Ponce, M., & Manchado, M. (2004). Novel method for the authentication of frigate tunas (auxis thazard and auxis rochei) in commercial canned products. *Journal of Agricultural and Food Chemistry*, *52*(25), 7435–7443.

Infante, C., Crespo, A., Zuasti, E., Ponce, M., Pérez, L., Funes, V., Catanese, G., & Manchado, M. (2006). Pcr-based methodology for the authentication of the atlantic mackerel scomber scombrus in commercial canned products. *Food Research International*, *39*(9), 1023–1028.

ISO. (2019). Iso 20813:2019 – molecular biomarker analysis—methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods)—general requirements and definitions.

Ivanova, N. V., Zemlak, T. S., Hanner, R. H., & Hebert, P. D. (2007). Universal primer cocktails for fish dna barcoding. *Molecular ecology notes*, *7*(4), 544–548.

Ivanova, N., Kuzmina, M., Braukmann, T., Borisenko, A., & Zakharov, E. (2016). Authentication of herbal supplements using next-generation sequencing. *PLoS ONE*, *11*, e0156426. https://doi.org/10.1371/journal.pone.0156426

Jardim, E., & Currey, R. J. C. (2023). The msc fisheries standard review policy development process. *Marine Policy*, *157*, 105855. https://doi.org/10.1016/j.marpol.2023.105855

Jayasundara, J. M. V. D. B., Ramanayake, R. M. L. S., Senarath, H. M. N. B., Herath, H. M. S. L., Godaliyadda, G. M. R. I., & Ekanayake, M. P. B. e. a. (2023). Deep learning for automated fish grading. *Journal of Agriculture and Food Research*, *14*, 100711.

Jeunen, G.-J., Dowle, E., Edgecombe, J., von Ammon, U., Gemmell, N. J., & Cross, H. (2023). Crabs—a software program to generate curated reference databases for metabarcoding sequencing data. *Molecular ecology resources*, *23*(3), 725–738.

Jin, L., Yu, J., Yuan, X., & Du, X. (2021). Fish classification using DNA barcode sequences through deep learning method. *Symmetry*, *13*, 1599. https://doi.org/10.3390/sym13091599

Jog, K., Sutaria, D., Diedrich, A., Grech, A., & Marsh, H. (2022). Marine mammal interactions with fisheries: Review of research and management trends across commercial and small-scale fisheries. *Frontiers in Marine Science*, *9*, 758013. https://doi.org/10.3389/fmars.2022.758013

Kappel, K., Gadelmeier, A., Denay, G., Gerdes, L., Graff, A., Hagen, M., Hassel, M., Huber, I., Näumann, G., Pavlovic, M., et al. (2023). Detection of adulterated meat products by a next-generation sequencing-based metabarcoding analysis within the framework of the operation opson x. *Journal of Consumer Protection and Food Safety*, *18*, 375–391. https://doi.org/10.1007/s00003-023-01437-w

Kappel, K., Haase, I., Käppel, C., Sotelo, C. G., & Schröder, U. (2017). Species identification in mixed tuna samples with next-generation sequencing targeting two short cytochrome b gene fragments. *Food Chemistry*, *234*, 212–219. https://doi.org/10.1016/j.foodchem.2017.04.178

Kasianowicz, J. J., Brandin, E., Branton, D., & Deamer, D. W. (1996). Characterization of individual polynucleotide molecules using a membrane channel. *Proceedings of the National Academy of Sciences*, *93*(24), 13770–13773.

Kattoor, J., Guag, J., Nemser, S., & Wilkes, R. (2024). Development of ion torrent-based targeted next-generation sequencing panel for identification of animal species in pet foods. *Research in Veterinary Science*, *167*, 105117. https://doi.org/10.1016/j.rvsc.2023.105117

Kendall, H., Clark, B., Rhymer, C., Kuznesof, S., Hajslova, J., Tomaniova, M., Brereton, P., & Frewer, L. (2019). A systematic review of consumer perceptions of food fraud and authenticity: A european perspective. *Trends in Food Science & Technology*, *94*, 79–90. https://doi.org/10.1016/j.tifs.2019.10.005

Khalili Tilami, S., & Sampels, S. (2018). Nutritional value of fish: Lipids, proteins, vitamins, and minerals. *Reviews in Fisheries Science & Aquaculture*, *26*(2), 243–253.

Kitano, T., Umetsu, K., Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine*, *121*(5), 423–427. https://doi.org/10.1007/s00414-006-0113-y

Klapper, R., Velasco, A., Döring, M., Schröder, U., Sotelo, C., Brinks, E., & Muñoz-Colmenero, M. (2023). A next-generation sequencing approach for the detection of mixed species in canned tuna. *Food Chemistry X*, *17*, 100560. https://doi.org/10.1016/j.fochx.2023.100560

Knobloch, S., Salimi, F., Buaya, A., Ploch, S., & Thines, M. (2024). Rapid: A rapid and accurate plant pathogen identification pipeline for on-site nanopore sequencing. *PeerJ*, *12*, e17893.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X., & Wilson, A. C. (1989). Dynamics of mitochondrial dna evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, *86*(16), 6196–6200. https://doi.org/10.1073/pnas.86.16.6196

Kotsanopoulos, K., Martsikalis, P. V., Gkafas, G. A., & Exadactylos, A. (2024). The use of various statistical methods for authenticity and detection of adulteration in fish and seafood. *Critical Reviews in Food Science and Nutrition*, *64*, 1553–1571.

Kotsanopoulos, K. V., & Exadactylos, A. (2022). Methods and techniques for verifying authenticity and detecting adulteration. *In: Authenticity of foods of plant origin*, 32–82.

Kotsanopoulos, K. V., Exadactylos, A., Gkafas, G. A., Martsikalis, P. V., Parlapani, F. F., Boziaris, I. S., & Arvanitoyannis, I. S. (2021). The use of molecular markers in the verification of fish and seafood authenticity and the detection of adulteration. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1584–1654.

Kroetz, K., Luque, G., Gephart, J., Jardine, S., Lee, P., Chicojay Moore, K., Cole, C., Steinkruger, A., & Donlan, C. (2020). Consequences of seafood mislabeling for marine populations and fisheries management. *Proceedings of the National Academy of Sciences of the USA*, *117*, 30318–30323. https://doi.org/10.1073/pnas.2003741117

Kron, G. (2014). Ancient fishing and fish farming. *The Oxford handbook of animals in classical thought and life*, 192–202.

Krzywinski, M., Schein, J., Birol, İ., Connors, J., Gascoyne, R., Horsman, D., Jones, S. J., & Marra, M. A. (2009). Circos: An information aesthetic for comparative genomics. *Genome Research*, *19*(9), 1639–1645. https://doi.org/10.1101/gr.092759.109

Kusche, H., & Hanel, R. (2021). Consumers of mislabeled tropical fish exhibit increased risks of ciguatera intoxication: A report on substitution patterns in fish imported at frankfurt airport, germany. *Food Control*, *121*, 107647. https://doi.org/https://doi.org/10.1016/j.foodcont.2020.107647

Kwasi Bannor, R., Arthur, K. K., Oppong, D., & Oppong-Kyeremeh, H. (2023). A comprehensive systematic review and bibliometric analysis of food fraud from a global perspective. *Journal of Agriculture and Food Research*, *14*, 100686. https://doi.org/https://doi.org/10.1016/j.jafr.2023.100686

Lago, F. C., Herrero, B., Vieites, J. M., & Espiñeira, M. (2011). Fins methodology to identification of sardines and related species in canned products and detection of mixture by means of snp analysis systems. *European Food Research and Technology*, *232*, 1077–1086. https://doi.org/10.1007/s00217-011-1481-1

Laporte, M., Reny-Nolin, E., Chouinard, V., Hernandez, C., Normandeau, E., Bougas, B., Côté, C., Behmel, S., & Bernatchez, L. (2021). Proper environ-

mental dna metabarcoding data transformation reveals temporal stability of fish communities in a dendritic river system. *Environmental DNA*, *3*(5), 1007–1022. https://doi.org/10.1002/edn3.224

Lawrence, S., Elliott, C., Huisman, W., Dean, M., & van Ruth, S. (2022). The 11 sins of seafood: Assessing a decade of food fraud reports in the global supply chain. *Comprehensive Reviews in Food Science and Food Safety*, *21*(4), 3746–3769. https://doi.org/https://doi.org/10.1111/1541-4337.12998

Lee, A. W.-T., Ng, I. C.-F., Wong, E. Y.-K., Wong, I. T.-F., Sze, R. P.-P., Chan, K.-Y., So, T.-Y., Zhang, Z., Fung, S. K.-Y., Wong, S. C.-Y., et al. (2024). Comprehensive identification of pathogenic microbes and antimicrobial resistance genes in food products using nanopore sequencing-based metagenomics. *Food Microbiology*, *121*, 104493.

Lenstra, J. (2003). Dna methods for identifying plant and animal species in food. In M. Lees (Ed.), *Food authenticity and traceability* (pp. 34–53). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9781855737181.1.34

Leone, A., Lecci, R. M., Milisenda, G., & Piraino, S. (2019). Mediterranean jellyfish as novel food: Effects of thermal processing on antioxidant, phenolic, and protein contents. *European Food Research and Technology*, *245*(8), 1611–1627. https://doi.org/10.1007/s00217-019-03248-6

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., & Ranwez, V. e. a. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: Application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 1–14. https://doi.org/10.1186/1742-9994-10-34

Li, H. (2018). Minimap2: Pairwise alignment for nucleotide sequences. *Bioinformatics*, *34*(18), 3094–3100.

Li, N., Shen, Q., Wang, J., Han, C., Ji, R., Li, F., & and, T. J. (2015). Tetrodotoxin detection and species identification of pufferfish in retail roasted fish fillet by dna barcoding in china [PMID: 26413972]. *Food Additives & Contaminants: Part A*, *32*(12), 2148–2153. https://doi.org/10.1080/19440049.2015.1087056

Li, Q., Song, P., & Wen, J. (2019). Melamine and food safety: A 10-year review [Food Toxicology • Food Safety]. *Current Opinion in Food Science*, *30*, 79–84. https://doi.org/https://doi.org/10.1016/j.cofs.2019.05.008

Liou, P., Banda, A., Isaacs, R. B., & Hellberg, R. S. (2020). Labeling compliance and species authentication of fish fillets sold at grocery stores in southern california. *Food Control*, *112*, 107137. https://doi.org/https://doi.org/10.1016/j.foodcont.2020.107137

Liu, C., Cui, Y., Li, X., & Yao, M. (2021). Microeco: An r package for data mining in microbial community ecology. *FEMS Microbiology Ecology*, *97*(2), fiaa255. https://doi.org/10.1093/femsec/fiaa255

Loeffler, C. R., & Spielmeyer, A. (2024). Faster ciguatoxin extraction methods for toxicity screening. *Scientific Reports*, *14*(1), 21715. https://doi.org/10.1038/s41598-024-72708-1

Lorusso, L., Mottola, A., Piredda, R., Di Pinto, A., Intermite, C., Ranieri, L., & Catanese, G. (2024). Revisiting molecular techniques for the authentication of mackerels in commercial products. approaches to prevent seafood fraud. *Trends in Food Science & Technology*. https://doi.org/10.1016/j.tifs.2024.104572

Lorusso, L., Piredda, R., Mottola, A., Intermite, C., Ranieri, L., Carpino, S., & Di Pinto, A. (2024). Authentication of seafood species on the asfis list (fao) by in-silico evaluation of primers for metabarcoding. *Food Control*, *165*, 110663. https://doi.org/10.1016/j.foodcont.2024.110663

Lorusso, L., Shum, P., Piredda, R., Mottola, A., Maiello, G., Cartledge, E. L., Neave, E. F., Di Pinto, A., & Mariani, S. (2024). Mismanagement and poor transparency in the european processed seafood supply revealed by dna metabarcoding. *Food Research International*, *194*, 114901.

Luque, G. M., & Donlan, C. J. (2019). The characterization of seafood mislabeling: A global meta-analysis. *Biological Conservation*, *236*, 556–570. https://doi.org/10.1016/j.biocon.2019.04.006

Mahé, F., Rognes, T., Quince, C., De Vargas, C., & Dunthorn, M. (2014). Swarm: Robust and fast clustering method for amplicon-based studies. *PeerJ*, *2*, e593. https://doi.org/10.7717/peerj.593

Maiello, G., Talarico, L., Carpentieri, P., De Angelis, F., Franceschini, S., Harper, L. R., Neave, E. F., Rickards, O., Sbrana, A., Shum, P., Veltre, V., Mariani, S., & Russo, T. (2022). Little samplers, big fleet: Edna metabarcoding from commercial trawlers enhances ocean monitoring. *Fisheries Research*, *249*, 106259. https://doi.org/10.1016/j.fishres.2022.106259

Mariani, S., Griffiths, A. M., Velasco, A., Kappel, K., Jérôme, M., Perez-Martin, R. I., Schröder, U., Verrez-Bagnis, V., Silva, H., Vandamme, S. G., Boufana, B., Mendes, R., Shorten, M., Smith, C., Hankard, E., Hook, S. A., Weymer, A. S., Gunning, D., & Sotelo, C. G. (2015). Low mislabeling rates indicate marked improvements in european seafood market operations. *Frontiers in Ecology and the Environment*, *13*(10), 536–540. https://doi.org/10.1890/150119

Martinez, I., Velasco, A., Pérez-Martín, R., Forås, E., Yamashita, M., & Sotelo, C. (2012). Evaluation of a fast method based on the presence of two restriction sites in the mitochondrial nd5 (mt ND5) gene for the identification of Scomber species. *Journal of Aquatic Food Product Technology*, *21*, 289–297. https://doi.org/10.1080/10498850.2012.664804

Marvin, H. J., Bouzembrak, Y., Janssen, E. M., van der Fels- Klerx, H., van Asselt, E. D., & Kleter, G. A. (2016). A holistic approach to food safety risks: Food fraud as an example. *Food Research International*, *89*, 463–470. https://doi.org/https://doi.org/10.1016/j.foodres.2016.08.028

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An r package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Medina, S., Perestrelo, R., Silva, P., Pereira, J. A., & Câmara, J. S. (2019). Current trends and recent advances on food authenticity technologies and chemometric approaches. *Trends in Food Science & Technology*, *85*, 163–176. https://doi.org/https://doi.org/10.1016/j.tifs.2019.01.017

Meriggi, N., Russo, A., Renzi, S., Cerasuolo, B., Nerini, M., & Ugolini, A. e. a. (2024). Enhancing seafood traceability: Tracking the origin of seabass and seabream from the tuscan coast area by the analysis of the gill bacterial communities. *Animal Microbiome*, *6*, 13. https://doi.org/10.1186/s42523-024-00300-z

Meusnier, I., Singer, G. A., Landry, J.-F., Hickey, D. A., Hebert, P. D., & Hajibabaei, M. (2008). A universal dna mini-barcode for biodiversity analysis. *BMC Genomics*, *9*(1), 214. https://doi.org/10.1186/1471-2164-9-214

Miller, D., Jessel, A., & Mariani, S. (2012). Seafood mislabelling: Comparisons of two western european case studies assist in defining influencing factors, mechanisms and motives. *Fish and Fisheries*, *13*(3), 345–358. https://doi.org/10.1111/j.1467-2979.2011.00426.x

Miller, D. D., & Mariani, S. (2010). Smoke, mirrors, and mislabeled cod: Poor transparency in the european seafood industry. *Frontiers in Ecology and the Environment*, *8*(10), 517–521. https://doi.org/https://doi.org/10.1890/090212

Mitchell, J. K., & Hellberg, R. S. (2016). Use of the mitochondrial control region as a potential dna mini-barcoding target for the identification of canned tuna species. *Food Analytical Methods*, *9*, 2711–2720. https://doi.org/10.1007/s12161-016-0460-3

Miya, M., & Nishida, M. (2000). Use of mitogenomic information in teleostean molecular phylogenetics: A tree-based exploration under the maximum-parsimony optimality criterion. *Molecular Phylogenetics and Evolution*, *17*(3), 437–455. https://doi.org/10.1006/mpev.2000.0839

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kondoh, M., & Iwasaki, W. (2015). Mifish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *Royal Society Open Science*, *2*(7), 150088. https://doi.org/10.1098/rsos.150088

Mohanty, B. P., Mohanty, S., Mitra, T., Mahanty, A., Ganguly, S., & Singh, S. (2019). Omics technology in fisheries and aquaculture. *Advances in Fish Research*, *7*, 1–30.

Mottola, A., Piredda, R., Catanese, G., Giorelli, F., Cagnazzo, G., Ciccarese, G., Dambrosio, A., Quaglia, N. C., & Di Pinto, A. (2022). Dna metabarcoding for identification of species used in fish burgers. *Italian Journal of Food Safety*, *11*(3). https://doi.org/10.4081/ijfs.2022.10412

Mottola, A., Piredda, R., Catanese, G., Lorusso, L., Ciccarese, G., & Di Pinto, A. (2022). Species authentication of canned mackerel: Challenges in molecular identification and potential drivers of mislabelling. *Food Control*, 108880. https://doi.org/10.1016/j.foodcont.2022.108880

Mottola, A., Piredda, R., Lorusso, L., Armani, A., & Di Pinto, A. (2023). Preliminary study on species authentication in poultry meat products by next-generation sequencing. *Food Control*, *145*, 109459. https://doi.org/10.1016/j.foodcont.2022.109459

Mottola, A., Intermite, C., Piredda, R., Lorusso, L., Ranieri, L., Carpino, S., Celano, G. V., & Di Pinto, A. (2024). Dna metabarcoding approach as a potential tool for supporting official food control programs: A case study. *Foods*, *13*(18), 2941.

Muñoz-Colmenero, M., Martínez, J., Roca, A., & Garcia-Vazquez, E. (2017). Ngs tools for traceability in candies as high processed food products: Ion torrent pgm versus conventional pcr-cloning. *Food Chemistry*, *214*, 631–636.

Naaum, A. M., Cusa, M., Singh, M., Bleicher, Z., Elliott, C., Goodhead, I. B., Hanner, R. H., Helyar, S. J., Mariani, S., Rice, J. E., Wangh, L. J., & Sanchez, J. A. (2021). Validation of fastfish-id: A new commercial platform for rapid fish species authentication via universal closed-tube barcoding. *Food Research International*, *141*, 110035. https://doi.org/https://doi.org/10.1016/j.foodres.2020.110035

Naaum, A. M., & Hanner, R. (2015). Community engagement in seafood identification using dna barcoding reveals market substitution in canadian seafood. *DNA Barcodes*, *3*(1), 74–79.

Narum, S., Campbell, N., & Yi, Y. (2009). High-throughput snp genotyping in salmon and steelhead. *Proceedings of Plant and Animal Genomes XVII Conference*, 10–14.

Narzary, Y., Das, S., Goyal, A. K., Lam, S. S., Sarma, H., & Sharma, D. (2021). Fermented fish products in south and southeast asian cuisine: Indigenous technology processes, nutrient composition, and cultural significance. *Journal of Ethnic Foods*, *8*, 1–19.

Naylor, R. L., Kishore, A., Sumaila, U. R., Issifu, I., Hunter, B. P., Belton, B., Bush, S. R., Cao, L., Gelcich, S., Gephart, J. A., et al. (2021). Blue food demand across geographic and temporal scales. *Nature communications*, *12*(1), 5413.

Neigel, J., Domingo, A., & Stake, J. (2007). Dna barcoding as a tool for coral reef conservation. *Coral Reefs*, *26*(3), 487–499. https://doi.org/10.1007/s00338-007-0248-4

Nesbø, C. L., Rueness, E. K., Iversen, S. A., Skagen, D. W., & Jakobsen, K. S. (2000). Phylogeography and population history of atlantic mackerel (Scomber scombrus L.): A genealogical approach reveals genetic structuring among the

eastern atlantic stocks. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*, 281–292. https://doi.org/10.1098/rspb.2000.0998

Nielsen, E. E., Cariani, A., Mac Aoidh, E., Maes, G. E., Milano, I., Ogden, R., & et al. (2012). Gene-associated markers provide tools for tackling illegal fishing and false eco-certification. *Nature Communications*, *3*, 851. https://doi.org/10.1038/ncomms1845

Nikolić, D., Poleksić, V., Tasić, A., Smederevac-Lalić, M., Djikanović, V., & Rašković, B. (2023). Two age groups of adult pikeperch (sander lucioperca) as bioindicators of aquatic pollution. *Sustainability*, *15*(14), 11321.

Nilewski, C., Geisser, R. W., & Carreira, E. M. (2009). Total synthesis of a chlorosulpholipid cytotoxin associated with seafood poisoning. *Nature*, *457*, 573–576.

Nitsuwat, S., Zhang, P., Ng, K., & Fang, Z. (2021). Fish gelatin as an alternative to mammalian gelatin for food industry: A meta-analysis. *LWT*, *141*, 110899. https://doi.org/10.1016/j.lwt.2021.110899

NOAA). (2016). *National bycatch reduction strategy* (Strategy Document). NOAA. https://www.fisheries.noaa.gov/resource/document/national-bycatch-reduction-strategy

NOAA. (2023). *Status of stocks 2023: Annual report to congress on the status of u.s. fisheries* (Annual Report). NOAA. https://www.fisheries.noaa.gov/national/sustainable-fisheries/status-stocks-2023

Noh, E. S., Lee, M.-N., Kim, E. M., Nam, B.-H., Noh, J. K., Park, J. Y., Kim, K.-H., & Kang, J.-H. (2021). Discrimination of raw material species in mixed seafood products (surimi) using the next generation sequencing method. *Food Bioscience*, *41*, 100786. https://doi.org/10.1016/j.fbio.2020.100786

Oksanen, J. (2018). Vegan: Community ecology package. ordination methods, diversity analysis and other functions for community and vegetation ecologists. http://vegan.r-forge.r-project.org/

OLAF. (2023). OLAF Report 2023 [Accessed May 2025].

Olafsdottir, A. H., Utne, K., Jacobsen, J., Jansen, T., Óskarsson, G., & Nøttestad, L. e. a. (2018). Geographical expansion of northeast atlantic mackerel (Scomber scombrus) in the nordic seas from 2007-2016 was primarily driven by stock size and constrained by low temperatures. *Deep Sea Research Part*

II: Topical Studies in Oceanography, *159*. https://doi.org/10.1016/j.dsr2.2018.05.023

Özkök, A., Akel Bilgiç, H., Kosukcu, C., Arık, G., Canlı, D., Yet, İ., & Karaaslan, C. (2023). Comparing the melissopalynological and next generation sequencing (ngs) methods for the determining of botanical origin of honey. *Food Control*, *148*, 109630. https://doi.org/10.1016/j.foodcont.2023.109630

Packi, K., Rudek, A., Matysiak, J., Klimczak, S., Matuszewska, E., Rzetecka, N., & Matysiak, J. (2023). Food allergies and parasites in children. *Foods*, *12*(13), 2465. https://doi.org/10.3390/foods12132465

Palumbo, F., Scariolo, F., Vannozzi, A., & Barcaccia, G. (2020). Ngs-based barcoding with mini-coi gene target is useful for pet food market surveys aimed at mislabelling detection. *Scientific Reports*, *10*, 17767.

Pan, Y., Qiu, D., Chen, J., & Yue, Q. (2020). Combining a COI mini-barcode with next-generation sequencing for animal origin ingredients identification in processed meat product. *Journal of Food Quality*, 1–9. https://doi.org/10.1155/2020/2907670

Pangsorn, S., Laong-manee, P., & Siriraksophon, S. (2007). Trend of surimi raw materials in the southeast asia. *Res. Pap. Ser. Train. Dep. Southeast Asian Fish. Dev. Cent*, 1–17.

Paolacci, S., Mendes, R., Klapper, R., Velasco, A., Ramilo-Fernandez, G., Muñoz-Colmenero, M., Potts, T., Martins, S., Avignon, S., Maguire, J., De Paz, E., Johnson, M., Denis, F., Pardo, M. A., McElligott, D., & Sotelo, C. G. (2021). Labels on seafood products in different european countries and their compliance to eu legislation. *Marine Policy*, *134*, 104810. https://doi.org/10.1016/j.marpol.2021.104810

Paracchini, V., Petrillo, M., Lievens, A., Kagkli, D.-M., & Angers-Loustau, A. (2019). Nuclear dna barcodes for cod identification in mildly-treated and processed food products. *Food Additives & Contaminants: Part A*, *36*, 1–14. https://doi.org/10.1080/19440049.2018.1556402

Pardo, M., Jiménez, E., & Pérez-Villarreal, B. (2016). Misdescription incidents in seafood sector. *Food Control*, *62*, 277–283. https://doi.org/10.1016/j.foodcont.2015.10.048

Pardo, M. Á., Jiménez, E., Viarsson, J. R., Ólafsson, K., Ólafsdóttir, G., Daníelsdóttir, A. K., & Pérez-Villareal, B. (2018). Dna barcoding revealing mislabeling of seafood in european mass caterings. *Food Control*, *92*, 7–16.

Parson, W., Pegoraro, K., Niederstätter, H., Föger, M., & Steinlechner, M. (2000). Species identification by means of the cytochrome b gene. *International Journal of Legal Medicine*, *114*(1–2), 23–28. https://doi.org/10.1007/s004140000134

Pathiraja, D., Cho, J., Kim, J., & Choi, I.-G. (2023). Metabarcoding of edna for tracking the floral and geographical origins of bee honey. *Food Research International*, *164*, 112413. https://doi.org/10.1016/j.foodres.2022.112413

Paula, M., Freitas, R., Ribeiro, R., Barboza, C., & Ruta, C. (2022). Bycatch of asteroidea from shrimp trawl fishery in the southwestern atlantic ocean–brazil. *Papéis Avulsos de Zoologia*, *62*, e202262007. https://doi.org/10.11606/1807-0205/2022.62.007

Pauly, D., Christensen, V., Guénette, S., Pitcher, T. J., Sumaila, U. R., Walters, C. J., Watson, R., & Zeller, D. (2002). Towards sustainability in world fisheries. *Nature*, *418*(6898), 689–695.

Penca, J., Said, A., Cavallé, M., Pita, C., & Libralato, S. (2021). Sustainable small-scale fisheries markets in the mediterranean: Weaknesses and opportunities. *Maritime Studies*, *20*(2), 141–155. https://doi.org/10.1007/s40152-021-00222-5

Piredda, R., Claverie, J.-M., Decelle, J., De Vargas, C., Dunthorn, M., Edvardsen, B., Eikrem, W., Forster, D., Kooistra, W., Logares, R., et al. (2018). Diatom diversity through hts-metabarcoding in coastal european seas. *Scientific Reports*, *8*, 18059. https://doi.org/10.1038/s41598-018-36345-9

Piredda, R., Mottola, A., Cipriano, G., Carlucci, R., Ciccarese, G., & Di Pinto, A. (2022). Next generation sequencing (NGS) approach applied to species identification in mixed processed seafood products. *Food Control*, *133*, 108590. https://doi.org/10.1016/j.foodcont.2021.108590

Piredda, R., Mottola, A., Lorusso, L., Ranieri, L., Catanese, G., Cipriano, G., & et al. (2023). Microbiome-based study in wild-caught Scomber scombrus fish products at the end of the supply chain. *Food Research International*, *186*, 115264. https://doi.org/10.1016/j.lwt.2023.115264

Polimeno, L., Lisanti, M. T., Rossini, M., Giacovazzo, E., Polimeno, L., Debellis, L., Ballini, A., Topi, S., & Santacroce, L. (2021). Anisakis allergy: Is aquacultured fish a safe and alternative food to wild-capture fisheries for anisakis simplex-sensitized patients? *Biology*, *10*(2), 106. https://doi.org/10.3390/biology10020106

Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: A review. *Food Additives and Contaminants*, *21*(1), 1–31. https://doi.org/10.1080/02652030310001620423

Prado, M., Boix, A., & von Holst, C. (2013). Development of a real-time PCR method for the simultaneous detection of mackerel and horse mackerel. *Food Control*, *34*(1), 19–23. https://doi.org/10.1016/j.foodcont.2013.04.007

Prasetyo, A. P., Murray, J. M., Kurniawan, M. F. A. K., Sales, N. G., McDevitt, A. D., & Mariani, S. (2023). Shark-dust: Application of high-throughput dna sequencing of processing residues for trade monitoring of threatened sharks and rays. *Conservation Letters*, *16*(5), e12971. https://doi.org/10.1111/conl.12971

Prosser, S., & Hebert, P. (2017). Rapid identification of the botanical and entomological sources of honey using dna metabarcoding. *Food Chemistry*, *214*, 183–191.

Quinteiro, J. (2011). *Filogenia molecular, estructura poblacional y trazabilidad genética de escómbridos (pisces, scombridae)* [Doctoral dissertation, Universidade de Santiago de Compostela].

Quinteiro, J., Sotelo, C. G., Rehbein, H., Pryde, S. E., Medina, I., Perez-Martin, R. I., Rey-Mendez, M., & Mackie, I. M. (1998). Use of mtdna direct polymerase chain reaction (PCR) sequencing and pcr-restriction fragment length polymorphism methodologies in species identification of canned tuna. *Journal of Agricultural and Food Chemistry*, *46*, 1662–1669. https://doi.org/10.1021/jf970552+

R Core Team. (2023). R: A language and environment for statistical computing [[Computer software]]. https://www.R-project.org/

Rabiepour, A., Zahmatkesh, F., & Babakhani, A. (2024). Preservation techniques to increase the shelf life of seafood products: An overview. *Journal of Food Engineering and Technology*, *13*(1), 1–24.

Rach, J., Bergmann, T., Paknia, O., DeSalle, R., Schierwater, B., & Hadrys, H. (2017). The marker choice: Unexpected resolving power of an unexplored CO1 region for layered dna barcoding approaches. *PLOS ONE*, *12*(4), e0174842. https://doi.org/10.1371/journal.pone.0174842

Raclariu-Manolică, A., Anmarkrud, J., Kierczak, M., Rafati, N., Thorbek, B., Schrøder-Nielsen, A., & De Boer, H. (2021). Dna metabarcoding for quality control of basil, oregano, and paprika. *Frontiers in Plant Science*, *12*, 665618. https://doi.org/10.3389/fpls.2021.665618

Rehbein, H. (2013). Differentiation of fish species by PCR-based DNA analysis of nuclear genes. *European Food Research and Technology*, *236*, 979–990. https://doi.org/10.1007/s00217-013-1961-6

Rheman, S., Hossain, S., Sarker, M. S., Akter, F., Khor, L., Gan, H. M., Powell, A., Card, R. M., Hounmanou, Y. M. G., Dalsgaard, A., et al. (2024). Nanopore sequencing for identification and characterization of antimicrobial-resistant escherichia coli and salmonella spp. from tilapia and shrimp sold at wet markets in dhaka, bangladesh. *Frontiers in Microbiology*, *15*, 1329620.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). Ecoprimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, *39*(21), e145. https://doi.org/10.1093/nar/gkr732

Ribani, A., Schiavo, G., Utzeri, V., Bertolini, F., Geraci, C., Bovo, S., & Fontanesi, L. (2018). Application of next generation semiconductor based sequencing for species identification in dairy products. *Food Chemistry*, *246*, 90–98. https://doi.org/10.1016/j.foodchem.2017.11.006

Rioux, L.-E., & Turgeon, S. L. (2015). Seaweed carbohydrates. In *Seaweed sustainability* (pp. 141–192). Elsevier. https://doi.org/10.1016/B978-0-12-418697-2.00007-6

Robba, L., Russell, S. J., Barker, G. L., & Brodie, J. (2006). Assessing the use of the mitochondrial cox1 marker for use in dna barcoding of red algae (Rhodophyta). *American Journal of Botany*, *93*(8), 1101–1108. https://doi.org/10.3732/ajb.93.8.1101

Robeson, M. S., O'Rourke, D. R., Kaehler, B. D., Ziemski, M., Dillon, M. R., Foster, J. T., & Bokulich, N. A. (2021). Rescript: Reproducible sequence

taxonomy reference database management. *PLoS computational biology*, *17*(11), e1009581.

Robson, K., Dean, M., Haughey, S., & Elliott, C. (2021). A comprehensive review of food fraud terminologies and food fraud mitigation guides. *Food Control*, *120*, 107516. https://doi.org/10.1016/j.foodcont.2020.107516

Rossi, F., Benso, A., Carlo, S. D., Politano, G., Savino, A., & Acutis, P. L. (2016). Fishapp: A mobile app to detect fish falsification through image processing and machine learning techniques. *2016 IEEE International Conference on Automation, Quality and Testing, Robotics (AQTR)*, 1–6.

Roungchun, J. B., Tabb, A. M., & Hellberg, R. S. (2022). Identification of tuna species in raw and processed products using DNA mini-barcoding of the mitochondrial control region. *Food Control*, *134*, 108752. https://doi.org/10.1016/j.foodcont.2021.108752

Sabatino, L., Esposito, G., & Meloni, D. (2024). Occurrence of silver-cheeked toadfish lagocephalus sceleratus (tetraodontiformes: Tetraodontidae) and tetrodotoxins in the mediterranean sea: Implications for the italian fishing sector? *Regional Studies in Marine Science*, *75*, 103562. https://doi.org/https://doi.org/10.1016/j.rsma.2024.103562

Santaclara, F. J., Espiñeira, M., & Vieites, J. M. (2007). Genetic identification of squids (families ommastrephidae and loliginidae) by pcr–rflp and fins methodologies. *Journal of Agricultural and Food Chemistry*, *55*(24), 9913–9920. https://doi.org/10.1021/jf0707177

Sarno, E., Pezzutto, D., Rossi, M., Liebana, E., & Rizzi, V. (2021). A review of significant european foodborne outbreaks in the last decade. *Journal of Food Protection*, *84*(12), 2059–2070. https://doi.org/https://doi.org/10.4315/JFP-21-096

Sarri, C., Stamatis, C., Sarafidou, T., Galara, I., Godosopoulos, V., Kolovos, M., Liakou, C., Tastsoglou, S., & Mamuris, Z. (2014). A new set of 16s rrna universal primers for identification of animal species. *Food Control*, *43*, 35–41. https://doi.org/10.1016/j.foodcont.2014.02.036

Scaffardi, L. (2022). A peculiar category of novel foods: Traditional foods coming from third countries and the regulatory issues involving sustainability, food security, food safety, and the free circulation of goods. In L. Scaffardi & G. Formici (Eds.), *Novel foods and edible insects in the european union*

(pp. 37–58). Springer International Publishing. https://doi.org/10.1007/978-3-031-13494-4_3

Schebesta, H., Bernaz, N., & Macchi, C. (2020). The european union farm to fork strategy: Sustainability and responsible business in the food supply chain. *Sustainability*, *15*, 420–427.

Schloss, P., Westcott, S., Ryabin, T., Hall, J., Hartmann, M., Hollister, E., Lesniewski, R., Oakley, B., Parks, D., & Robinson, C. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*, 7537–7541.

Scientific, Technical and Economic Committee for Fisheries (STECF). (2019). *The eu fish processing sector. economic report* (tech. rep. No. STECF-19-15) (JRC119498). Scientific, Technical and Economic Committee for Fisheries (STECF). Luxembourg, Publications Office of the European Union. https://doi.org/10.2760/30373

Scoles, D., Collette, B. B., & Graves, J. (1998). Global phylogeography of mackerels of the genus Scomber. *Fishery Bulletin*, *96*(4), 823–842.

Selig, E., Nakayama, S., Wabnitz, C., Österblom, H., Spijkers, J., Miller, N., Bebbington, J., & Decker Sparks, J. (2022). Revealing global risks of labor abuse and illegal, unreported, and unregulated fishing. *Nature Communications*, *13*, 1612. https://doi.org/10.1038/s41467-022-28916-2

Sellers, G. S., Di Muri, C., Gómez, A., & Hänfling, B. (2018). Mu-dna: A modular universal dna extraction method adaptable for a wide range of sample types. *Metabarcoding and Metagenomics*, *2*, e24556. https://doi.org/10.3897/mbmg.2.24556

Sevilla, R. G., Diez, A., Noren, M., Mouchel, O., Jerome, M., Verrez-Bagnis, V., & et al. (2007). Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. *Molecular Ecology Notes*, *7*(5), 730–734. https://doi.org/10.1111/j.1471-8286.2007.01863.x

Sharrad, A., Reis-Santos, P., Austin, J., & Gillanders, B. (2023). Umbrella terms conceal the sale of threatened shark species: A dna barcoding approach. *Food Control*, *148*, 109606. https://doi.org/10.1016/j.foodcont.2023.109606

Shaw, J. L. A., Clarke, L. J., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., & Cooper, A. (2016). Comparison of environmental dna metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, *197*, 131–138. https://doi.org/10.1016/j.biocon.2016.03.010

Shehata, H. R., Naaum, A. M., Garduño, R. A., & Hanner, R. (2018). Dna barcoding as a regulatory tool for seafood authentication in Canada. *Food Control*, *92*, 147–153. https://doi.org/10.1016/j.foodcont.2018.04.045

Shokralla, S., Hellberg, R. S., Handy, S. M., King, I., & Hajibabaei, M. (2015). A DNA mini-barcoding system for authentication of processed fish products. *Scientific Reports*, *5*, 15894. https://doi.org/10.1038/srep15894

Shum, P., Cusa, M., Prasetyo, A., & Mariani, S. (2024). Nanopore sequencing facilitates screening of diversity and provenance of seafood and marine wildlife. *Food Control*, *161*, 110382.

Smith, R., Manning, L., & McElwee, G. (2017). Critiquing the inter-disciplinary literature on food fraud. *International Journal of Rural Criminology*, *3*(2), 250–270.

Spink, J., Ortega, D., Chen, C., & Wu, F. (2017). Food fraud prevention shifts the food risk focus to vulnerability. *Trends in Food Science & Technology*, *62*, 215–220. https://doi.org/10.1016/j.tifs.2017.02.012

Spink, J., & Moyer, D. C. (2011). Defining the public health threat of food fraud. *Journal of Food Science*, *76*(9), R157–R163. https://doi.org/https://doi.org/10.1111/j.1750-3841.2011.02417.x

Spink, J. W. (2019). The current state of food fraud prevention: Overview and requirements to address 'how to start?'and 'how much is enough?' *Current Opinion in Food Science*, *27*, 130–138.

Staats, M., Arulandhu, A. J., Gravendeel, B., Holst-Jensen, A., Scholtens, I., Peelen, T., Prins, T. W., & Kok, E. (2016). Advances in dna metabarcoding for food and wildlife forensic species identification. *Analytical and Bioanalytical Chemistry*, *408*(17), 4615–4630. https://doi.org/10.1007/s00216-016-9595-8

Stefanni, S., Stanković, D., Borme, D., De Olazabal, A., Juretić, T., Pallavicini, A., & Tirelli, V. (2018). Multi-marker metabarcoding approach to study mesozooplankton at basin scale. *Scientific Reports*, *8*, 12085. https://doi.org/10.1038/s41598-018-30157-7

Stefánsson, G., Nielsen, H. H., Skåra, T., Schubring, R., Oehlenschläger, J., Luten, J., Derrick, S., & Gudmundsdóttir, G. (2000). Frozen herring as raw material for spice-salting. *Journal of the Science of Food and Agriculture*, *80*(9), 1319–1324. https://doi.org/https://doi.org/10.1002/1097-0010(200007)80:9⟨1319::AID-JSFA637⟩3.0.CO;2-N

Stoeckle, M. Y., Adolf, J., Charlop-Powers, Z., Dunton, K. J., Hinks, G., & VanMorter, S. M. (2021). Trawl and edna assessment of marine fish diversity, seasonality, and relative abundance in coastal new jersey, usa. *ICES Journal of Marine Science*, *78*(1), 293–304. https://doi.org/10.1093/icesjms/fsaa225

Stroganov, A. N., Nikitenko, A. I., Rakitskaya, T. A., & et al. (2023). Study of population genetic structure of atlantic mackerel Scomber colias Gmelin, 1789 (Scombridae) in the central east atlantic. *Russian Journal of Genetics*, *59*, 66–72. https://doi.org/10.1134/S1022795423010118

Sultana, S., Ali, M. E., Hossain, M. M., Naquiah, N., & Zaidul, I. S. M. (2018). Universal mini COI barcode for the identification of fish species in processed products. *Food Research International*, *105*, 19–28. https://doi.org/10.1016/j.foodres.2017.10.065

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: For biodiversity research and monitoring*. Oxford University Press. https://doi.org/10.1093/oso/9780198767220.001.0001

Taheri-Garavand, A., Nasiri, A., Banan, A., & Zhang, Y. D. (2020). Smart deep learning-based approach for non-destructive freshness diagnosis of common carp fish. *Journal of Food Engineering*, *278*, 109930.

Tamura, K., Stecher, G., & Kumar, S. (2021). Mega11: Molecular evolutionary genetics analysis version 11. *Molecular biology and evolution*, *38*(7), 3022–3027.

TFEU. (2010). Consolidated version of the treaty on the functioning of the european union [Published by the European Union]. *Official Journal of the European Union*, *53*(C83), 47–200.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Tigchelaar, M., Leape, J., Micheli, F., Allison, E. H., Basurto, X., Bennett, A., Bush, S. R., Cao, L., Cheung, W. W., Crona, B., et al. (2022). The vital roles of blue foods in the global food system. *Global Food Security*, *33*, 100637.

Torri, L., Tuccillo, F., Bonelli, S., Piraino, S., & Leone, A. (2020). The attitudes of italian consumers towards jellyfish as novel food. *Food Quality and Preference*, *79*, 103782. https://doi.org/10.1016/j.foodqual.2019.103782

Toxqui Rodríguez, M. d. S., Vanhollebeke, J., & Derycke, S. (2023). Evaluation of dna metabarcoding using oxford nanopore sequencing for authentication of mixed seafood products. *Food Control*, *145*, 109388. https://doi.org/10.1016/j.foodcont.2022.109388

Travaille, K. L. T., Lindley, J., Kendrick, G. A., Crowder, L. B., & Clifton, J. (2019). The market for sustainable seafood drives transformative change in fishery social-ecological systems. *Global Environmental Change*, *57*, 101919.

Traynor, A., Burns, D. T., Wu, D., Karoonuthaisiri, N., Petchkongkaew, A., & Elliott, C. T. (2024). An analysis of emerging food safety and fraud risks of novel insect proteins within complex supply chains. *npj Science of Food*, *8*(1), 7. https://doi.org/10.1038/s41538-023-00241-y

Tzeng, C. H., Chen, C. S., Tang, P. C., & Chiu, T. S. (2009). Microsatellite and mitochondrial haplotype differentiation in blue mackerel (Scomber australasicus) from the western North Pacific. *ICES Journal of Marine Science*, *66*, 816–825. https://doi.org/10.1093/icesjms/fsp120

Tzeng, T. D., Wang, D., Haung, H. L., & Yeh, S. Y. (2007). Genetic diversity and population expansion of the common mackerel (Scomber japonicus) off Taiwan. *Journal of Fishery Society of Taiwan*, *34*, 281–289.

Urban, P., Præbel, K., Bhat, S., Dierking, J., & Wangensteen, O. S. (2022). Dna metabarcoding reveals the importance of gelatinous zooplankton in the diet of Pandalus borealis, a keystone species in the Arctic. *Molecular Ecology*, *31*(5), 1562–1576. https://doi.org/10.1111/mec.16332

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., Gaboriaud, C., Jean, P., Poulet, N., Roset, N., Copp, G. H., Geniez, P., Pont, D., Argillier, C., Baudoin, J.-M., et al. (2016). Next-generation monitoring of aquatic

biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*(4), 929–942. https://doi.org/10.1111/mec.13428

Valsecchi, E., Bylemans, J., Goodman, S. J., Lombardi, R., Carr, I., Castellano, L., Galimberti, A., & Galli, P. (2020). Novel universal primers for metabarcoding environmental DNA surveys of marine mammals and other marine vertebrates. *Environmental DNA*, *2*(4), 460–476. https://doi.org/10.1002/edn3.72

Van Driessche, C., Everts, T., Neyrinck, S., Halfmaerten, D., Verschelde, P., Breine, J., Bonte, D., & Brys, R. (2024). Environmental dna metabarcoding reflects spatiotemporal fish community shifts in the scheldt estuary. *Science of The Total Environment*, *934*, 173242. https://doi.org/10.1016/j.scitotenv.2024.173242

Velasco, E. M., Del Arbol, J., Baro, J., & Sobrino, I. (2011). Age and growth of the spanish chub mackerel (Scomber colias) off southern spain: A comparison between samples from the NE Atlantic and the SW Mediterranean. *Revista de Biología Marina y Oceanografía*, *46*(1), 27–34.

Vences, M., Lyra, M. L., Perl, R. B., Bletz, M. C., Stanković, D., & Lopes, e. a. (2016). Freshwater vertebrate metabarcoding on illumina platforms using double-indexed primers of the mitochondrial 16s rrna gene. *Conservation Genetics Resources*, *8*, 323–327. https://doi.org/10.1007/s12686-016-0550-y

Verma, S. K., & Singh, L. (2002). Novel universal primers establish identity of an enormous number of animal species for forensic application: Primer note. *Molecular Ecology Notes*, *3*(1), 28–31. https://doi.org/10.1046/j.1471-8286.2003.00340.x

Verza, M., Camanzi, L., Rota, C., Cerjak, M., Mulazzani, L., & Malorgio, G. (2023). Consumer sentiments and emotions in new seafood product concept development: A co-creation approach using online discussion rooms in croatia, italy and spain. *Foods*, *12*, 1729. https://doi.org/10.3390/foods12081729

Viñas, J., & Tudela, S. (2009). A validated methodology for genetic identification of tuna species (Genus Thunnus). *PLoS ONE*, *4*, e7606. https://doi.org/10.1371/journal.pone.0007606

Voorhuijzen-Harink, M. M., Hagelaar, R., van Dijk, J. P., Prins, T. W., Kok, E. J., & Staats, M. (2019). Toward on-site food authentication using nanopore sequencing. *Food Chemistry: X*, *2*, 100035.

Wang, N., Xing, R.-R., Zhou, M.-Y., Sun, R.-X., Han, J.-X., Zhang, J.-K., Zheng, W.-J., & Chen, Y. (2021). Application of dna barcoding and metabarcoding for species identification in salmon products. *Food Additives & Contaminants: Part A*, *38*, 754–768.

Wangensteen, O. S., Palacín, C., Guardiola, M., & Turon, X. (2018). Dna metabarcoding of littoral hard-bottom communities: High diversity and database gaps revealed by two molecular markers. *PeerJ*, *6*, e4705. https://doi.org/10.7717/peerj.4705

Ward, R. D., Hanner, R., & Hebert, P. D. N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, *74*, 329–356. https://doi.org/10.1111/j.1095-8649.2008.02080.x

Ward, R. D. (2009). Dna barcode divergence among species and genera of birds and fishes. *Molecular ecology resources*, *9*(4), 1077–1085.

Westbury, M. (2018). *Unraveling evolution through next generation sequencing* [Doctoral dissertation, Universität Potsdam].

Wickham, H. (2011). Ggplot2. *WIREs Computational Statistics*, *3*(2), 180–185. https://doi.org/10.1002/wics.147

Wickham, H., & Wickham, M. H. (2017). Package tidyverse. easily install and load the tidyverse [R package].

Wiemers, M., & Fiedler, K. (2007). Does the dna barcoding gap exist? – a case study in blue butterflies (lepidoptera: Lycaenidae). *Frontiers in Zoology*, *4*(1), 8. https://doi.org/10.1186/1742-9994-4-8

Wilcox, M. (2012). Railways, roads and the british white fish industry, 1920–70. *Business History*, *54*(5), 741–764. https://doi.org/10.1080/00076791.2011.631128

WWF. (2019). Sharks in crisis: A call to action for the mediterranean.

Xing, R.-R., Wang, N., Hu, R.-R., Zhang, J.-K., Han, J.-X., & Chen, Y. (2019). Application of next generation sequencing for species identification in meat and poultry products: A dna metabarcoding approach. *Food Control*, *101*, 173–179.

Xiong, X., Yuan, F., Huang, M., Lu, L., Xiong, X., & Wen, J. (2019). Dna barcoding revealed mislabeling and potential health concerns with roasted fish products sold across china. *Journal of Food Protection*, *82*(7), 1200–1209. https://doi.org/https://doi.org/10.4315/0362-028X.JFP-18-514

Yan, S., Catanese, G., Brown, C. L., Wang, M., Yang, C., & Yang, T. (2015). Phylogeographic study on the chub mackerel (Scomber japonicus) in the Northwestern Pacific indicates the late Pleistocene population isolation. *Marine Ecology*, *36*(3), 753–765. https://doi.org/10.1111/maec.12267

Zardoya, R., Castilho, R., Grande, C., Favre-Krey, L., Caetano, S., Marcato, S., & Patarnello, T. (2004). Differential population structuring of two closely related fish species, the mackerel (Scomber scombrus) and the chub mackerel (Scomber japonicus), in the Mediterranean Sea. *Molecular Ecology*, *13*(7), 1785–1798. https://doi.org/10.1111/j.1365-294X.2004.02198.x

Zeng, L., Cheng, Q., & Chen, X. (2012). Microsatellite analysis reveals the population structure and migration patterns of scomber japonicus (scombridae) with continuous distribution in the east and south china seas. *Biochemical Systematics and Ecology*, *42*, 83–93.

Zhang, H., Li, D., Zhong, X., & Xiong, Y. (2023). Assessment of ecological damage from illegal fishing and judicial practice for damage compensation. *Ocean & Coastal Management*, *246*, 106909. https://doi.org/10.1016/j.ocecoaman.2023.106909

Zhang, J., Hua, M. Z., Chen, H., Hou, H., Hu, Y., & Lu, X. (2025). Food authentication using the loop-mediated isothermal amplification (lamp) method: Current trends and future prospects. *Trends in Food Science & Technology*, *156*, 104874. https://doi.org/https://doi.org/10.1016/j.tifs.2025.104874

Zhang, L., Zhou, Z., Li, H., Qiao, Y., & Zhang, Y. (2019). The mitochondrial COI gene reveals the genetic diversity of chub mackerel (Scomber japonicus) in the Taiwan Strait and its adjacent waters. *Pakistan Journal of Zoology*, *51*, 935–943. https://doi.org/10.17582/journal.pjz/2019.51.3.935.943

Zhang, S., Zhao, J., & Yao, M. (2020). A comprehensive and comparative evaluation of primers for metabarcoding edna from fish. *Methods in Ecology and Evolution*, *11*(12), 1609–1625. https://doi.org/10.1111/2041-210X.13485

Zhang, X., Giusti, A., Sun, Z., Li, Y., Guo, J., Deng, W., Chen, Y., He, A., Peng, H., Tinacci, L., Armani, A., & Wen, J. (2024). Molecular authentication

of surimi-based products (fish cakes,) sold on the chinese e-commerce: Traditional (dna barcoding) and innovative techniques (metabarcoding) to tackle seafood fraud. *Food Control*, *155*, 110110. https://doi.org/10.1016/j.foodcont.2023.110110

Zhang, X., Tinacci, L., Sun, Z., Li, Y., Guo, J., & Deng, W. e. a. (2024). Labeling compliance assessment and molecular authentication of grilled fish products sold on chinese e-commerce: Traceability issues related to the use of umbrella trade names. *Food Control*, *155*, 110043. https://doi.org/10.1016/j.foodcont.2023.110043

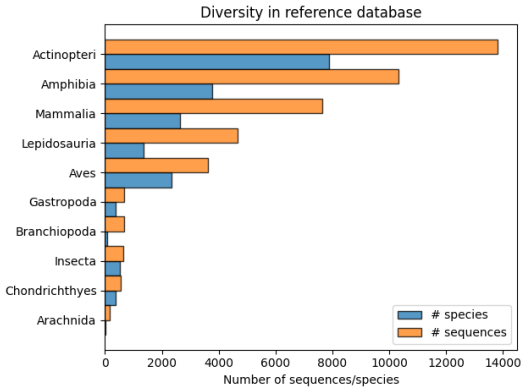
Appendix

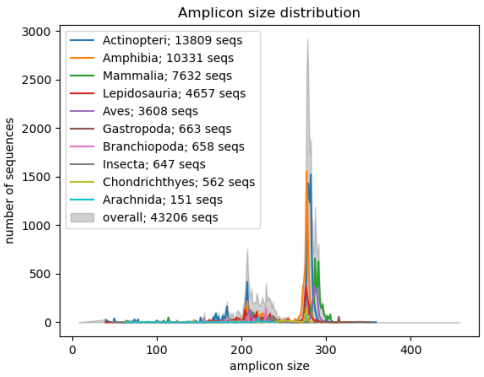
Appendix A.

Supplementary Chapter 3

Given the size of the Supplementary Table 1, it can be accessed through the associated DOI: https://doi.org/10.1016/j.foodcont.2024.110663

12Sp01





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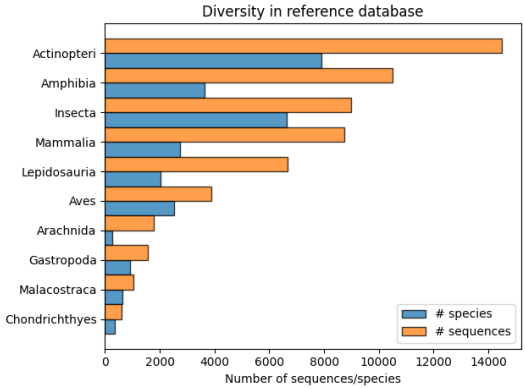

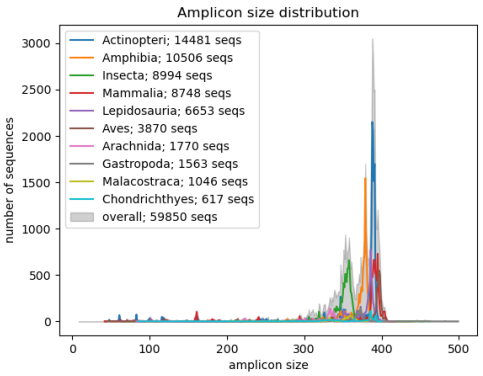

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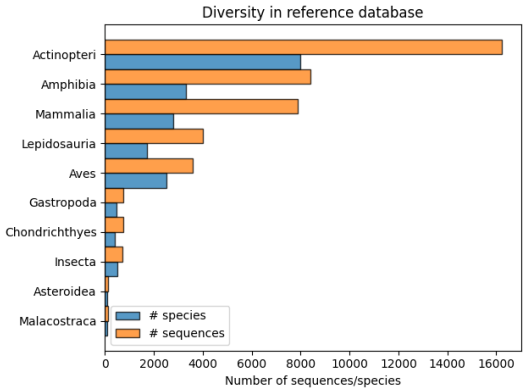

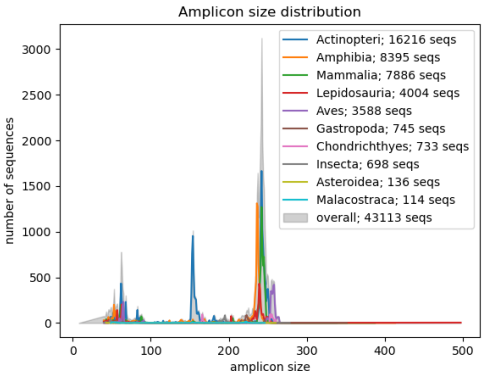

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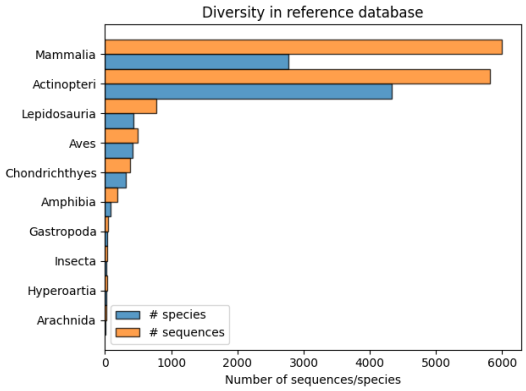
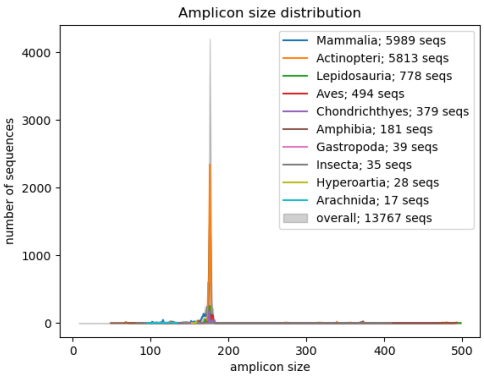
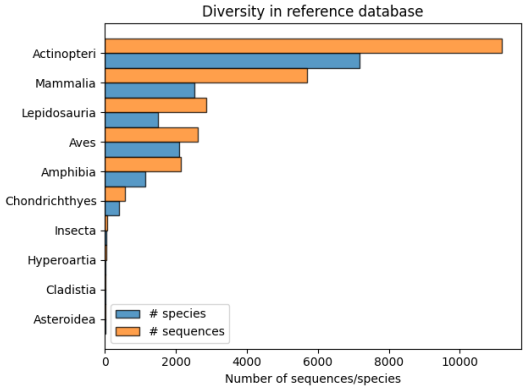
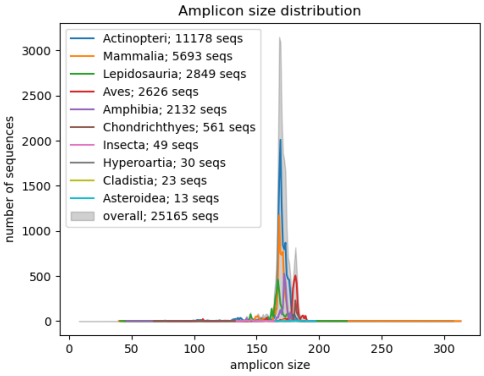


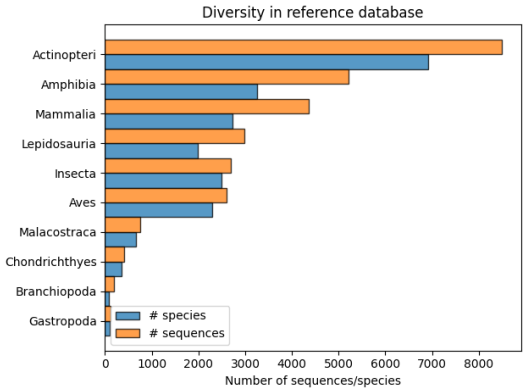


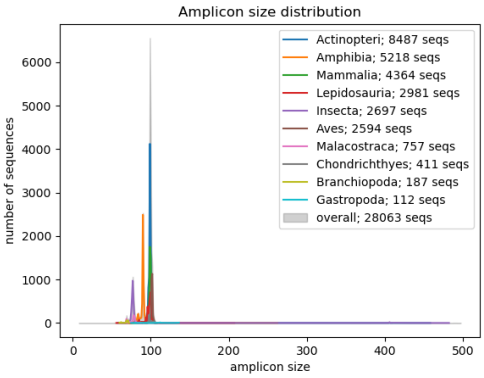
12Sp05



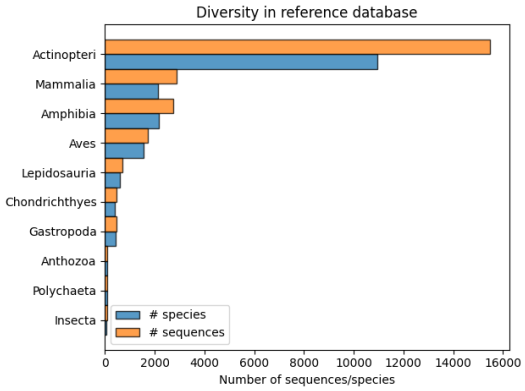


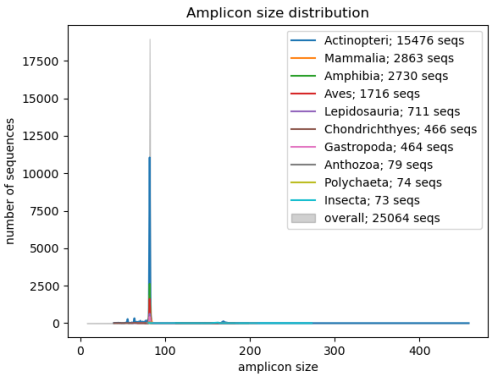
12Sp06





12Sp07





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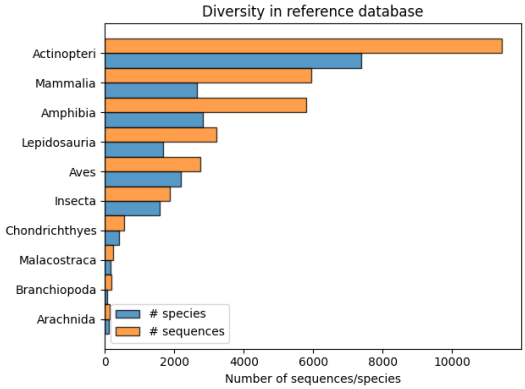

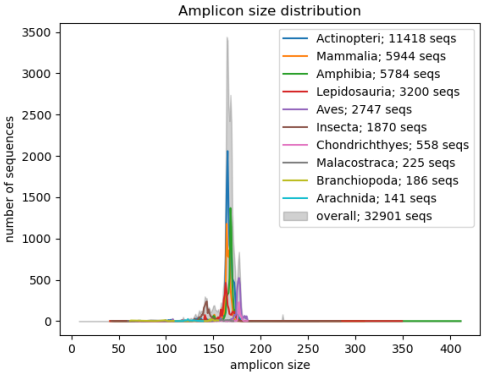

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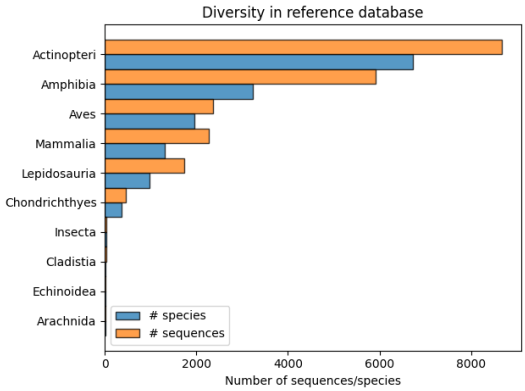
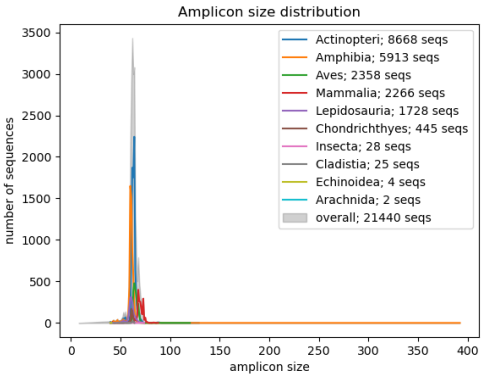
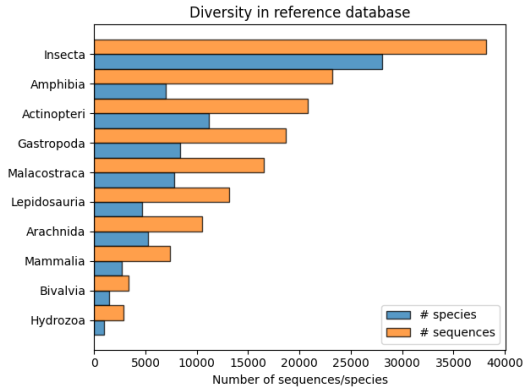
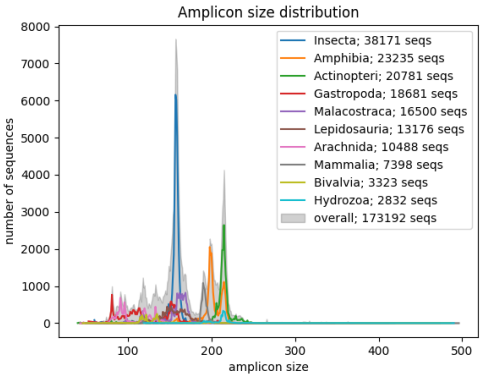




16Sp01





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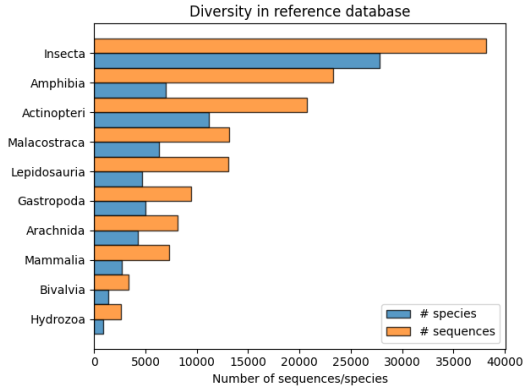
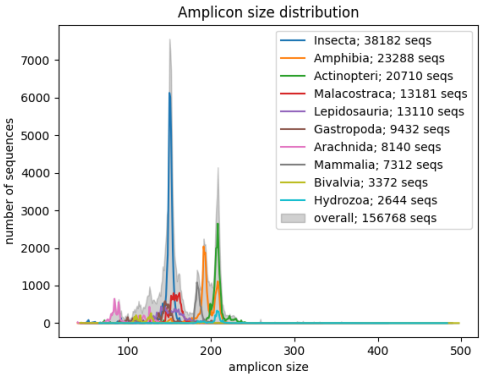
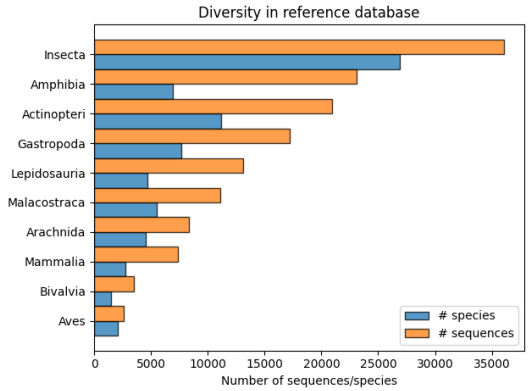
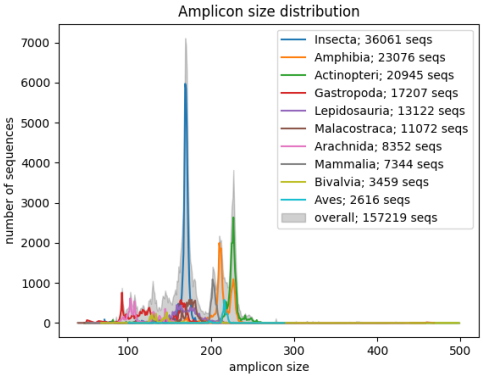




16Sp03





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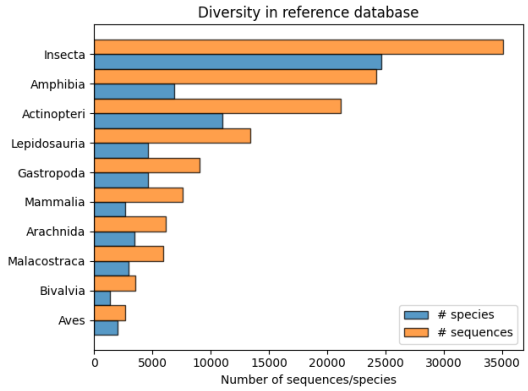

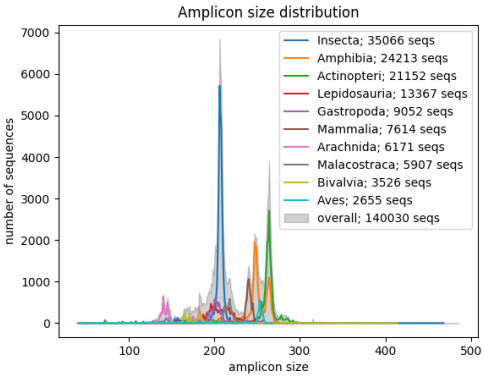

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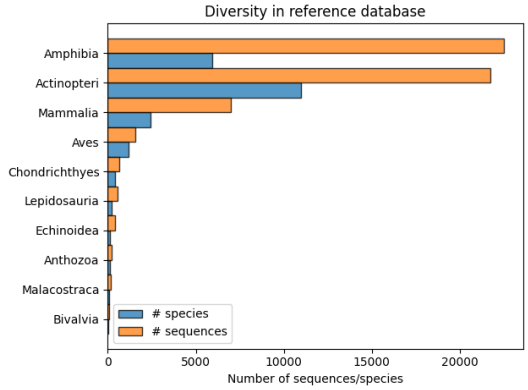

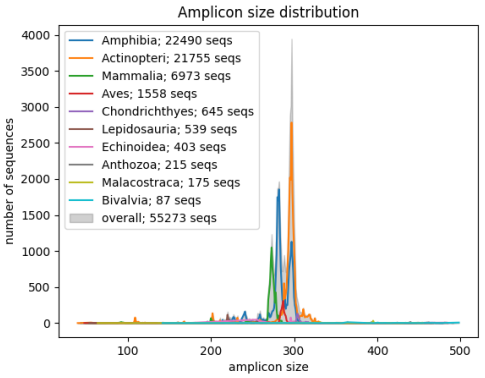

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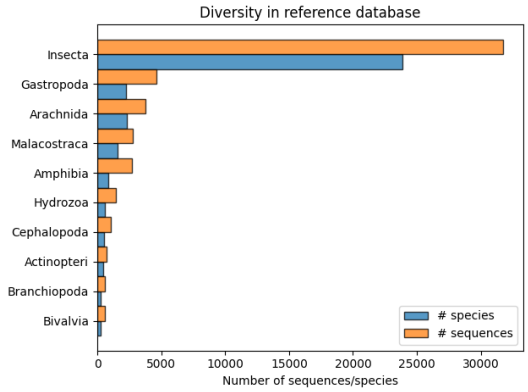

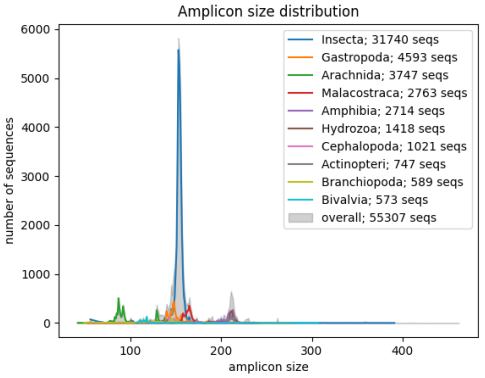

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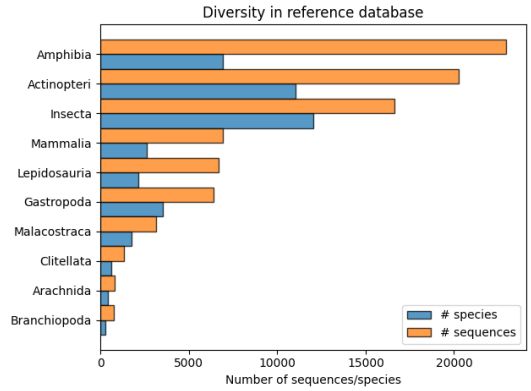

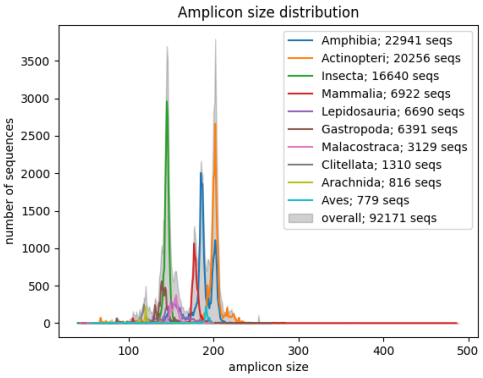

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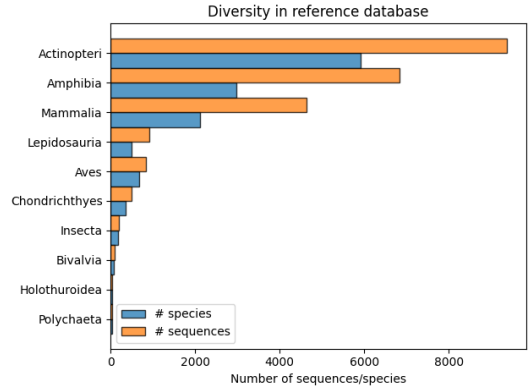

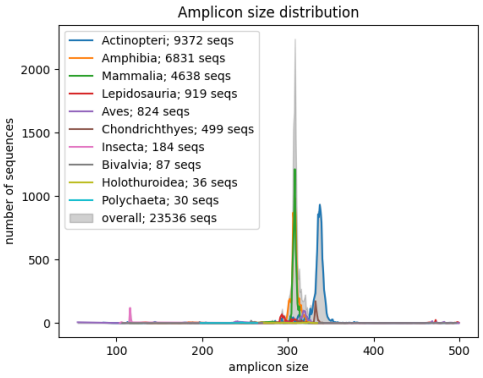

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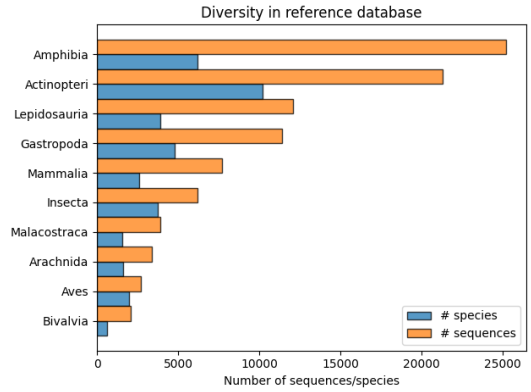

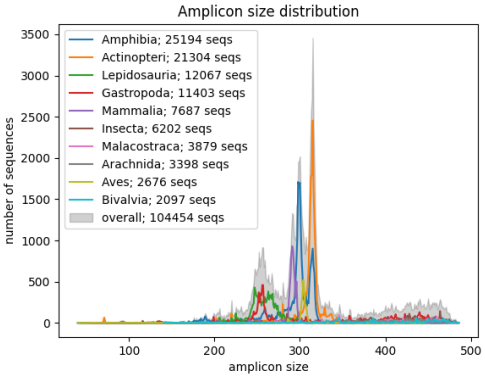

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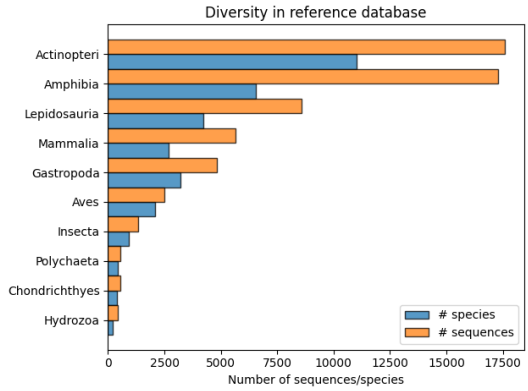
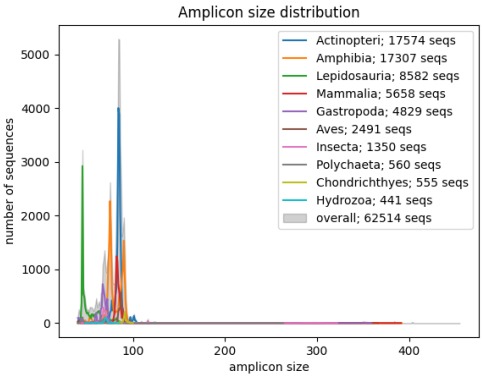
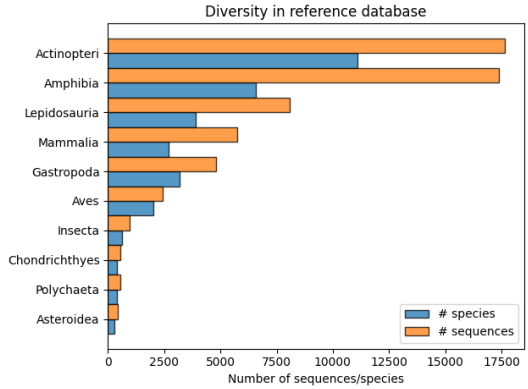
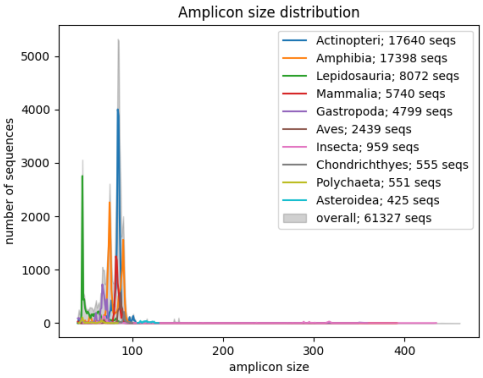


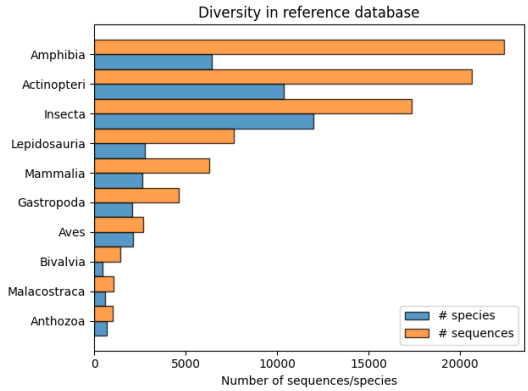


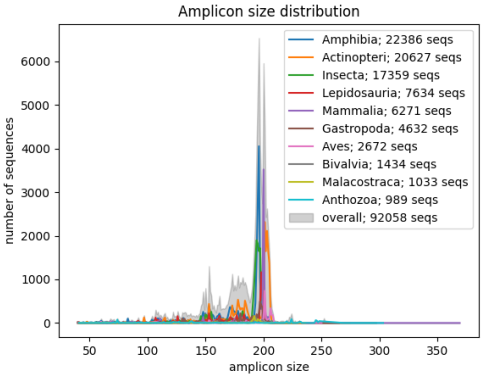
16Sp11



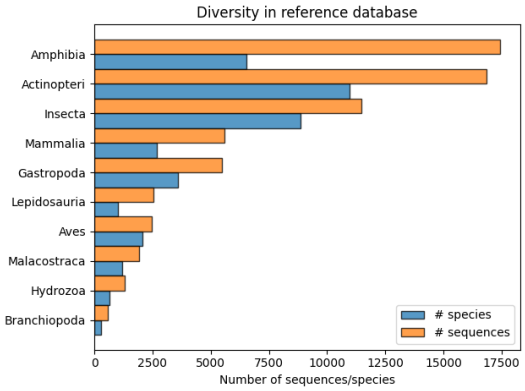


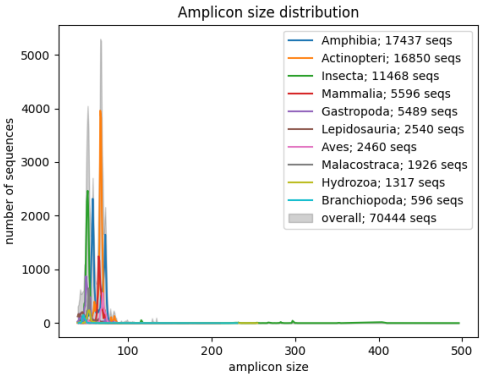
16Sp12



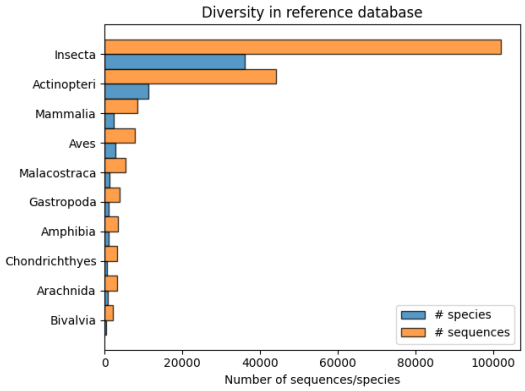


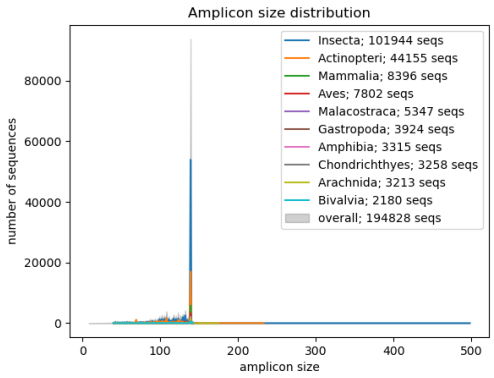
16Sp13


Diversity in reference database
Amphibia
Actinopteri
Insecta
Mammalia
Gastropoda
Lepidosauria
Aves
Malacostraca
Hydrozoa
Branchiopoda
species
sequences
0
2500
5000
7500
10000
12500
15000
17500
Number of sequences/species

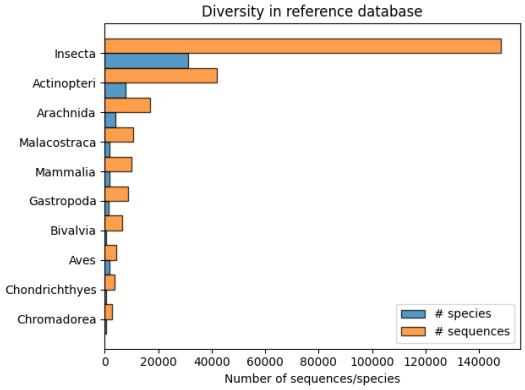

Amplicon size distribution
Amphibia; 17437 seqs
Actinopteri; 16850 seqs
Insecta; 11468 seqs
Mammalia; 5596 seqs
Gastropoda; 5489 seqs
Lepidosauria; 2540 seqs
Aves; 2460 seqs
Malacostraca; 1926 seqs
Hydrozoa; 1317 seqs
Branchiopoda; 596 seqs
overall; 70444 seqs
number of sequences
0
1000
2000
3000
4000
5000
100
200
300
400
500
amplicon size

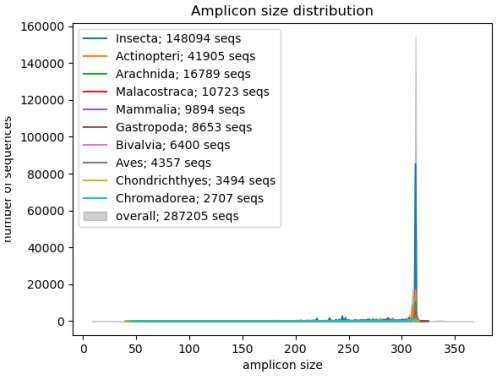
COIp01



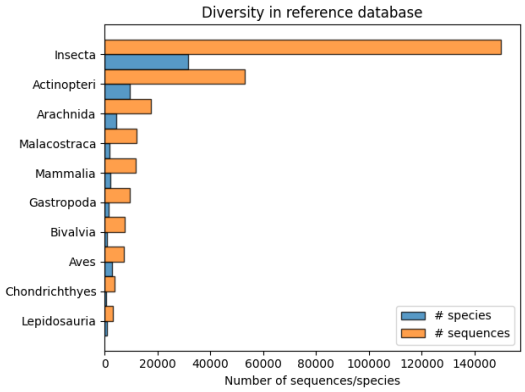


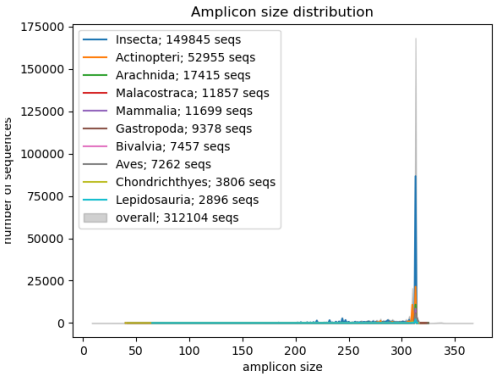
COIp02



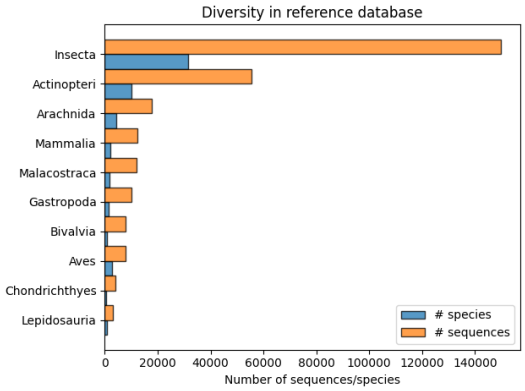


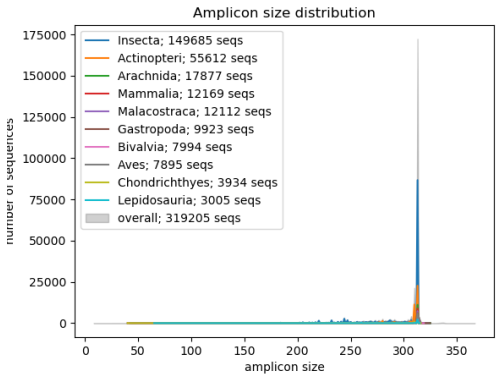
COIp03



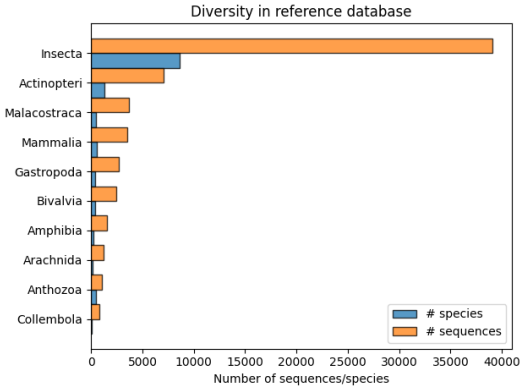


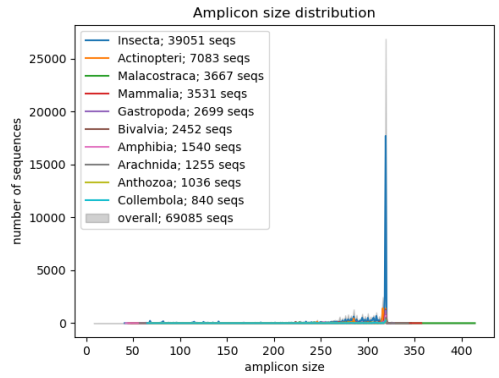
COIp04



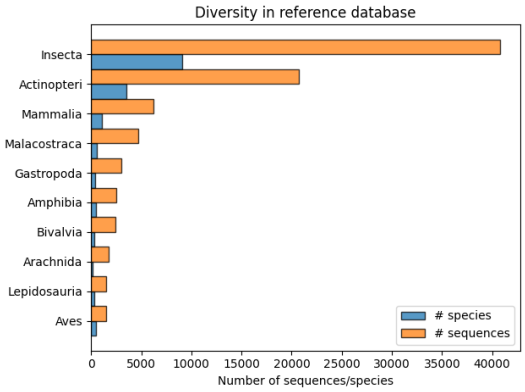


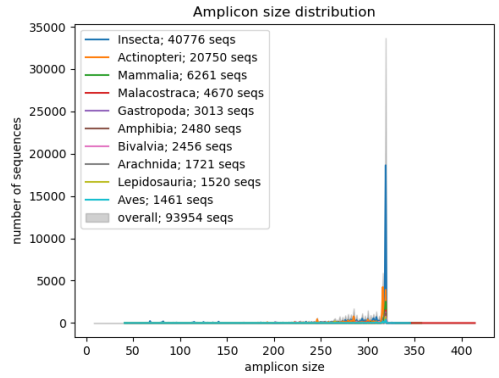
COIp05



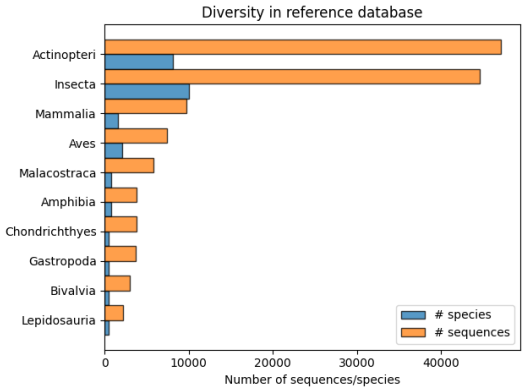


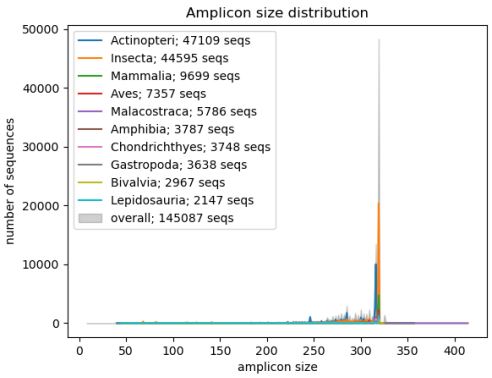
COIp06



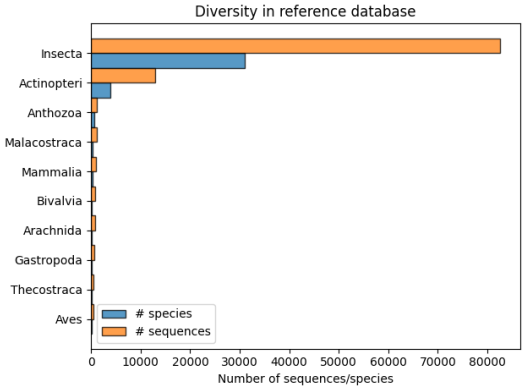


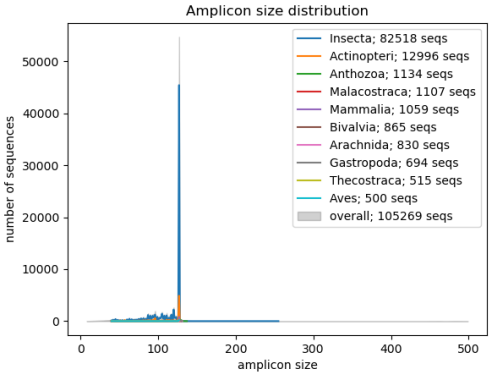
COIp07



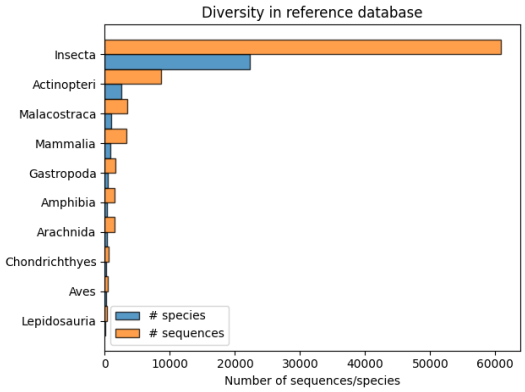


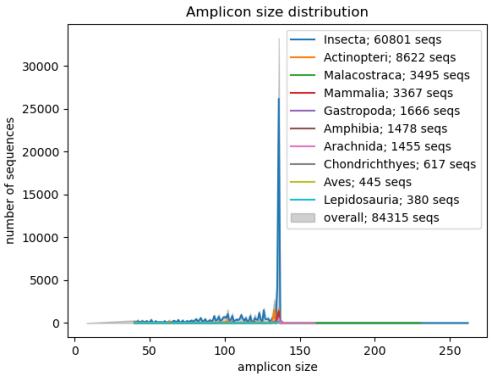
COIp08



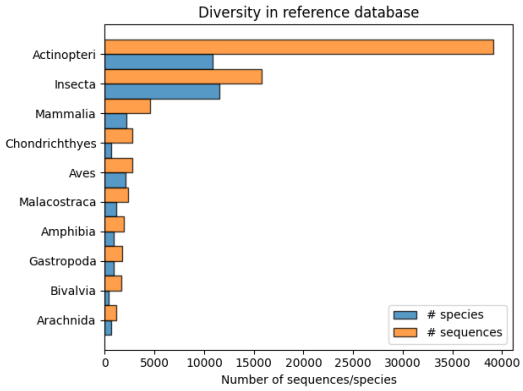


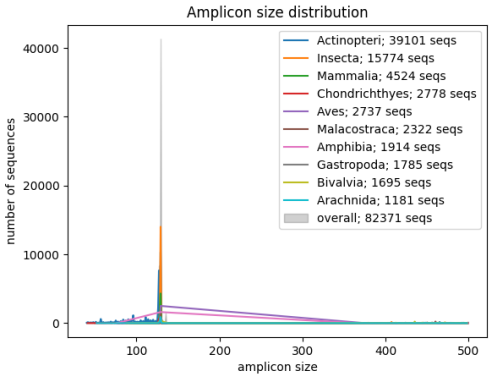
COIp09



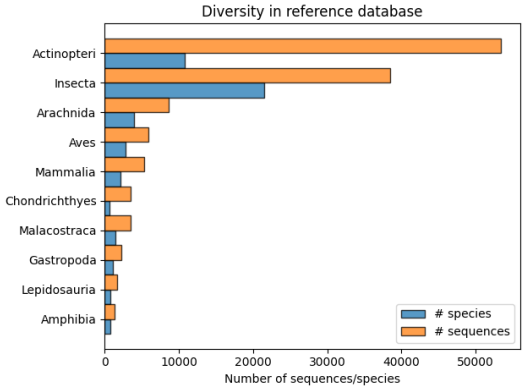


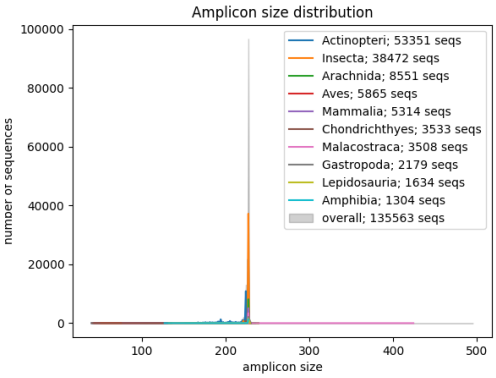
COIp10





COIp11





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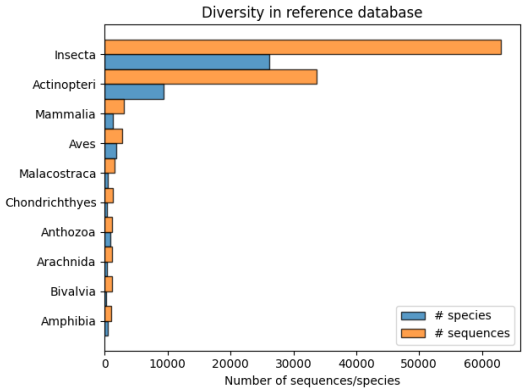

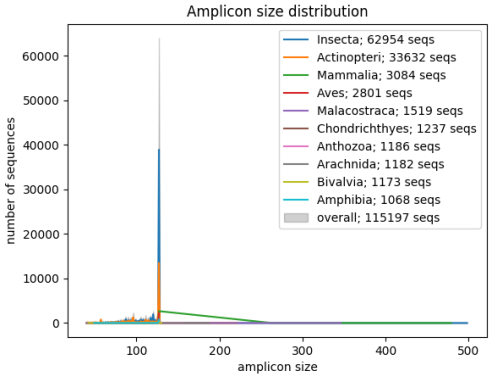

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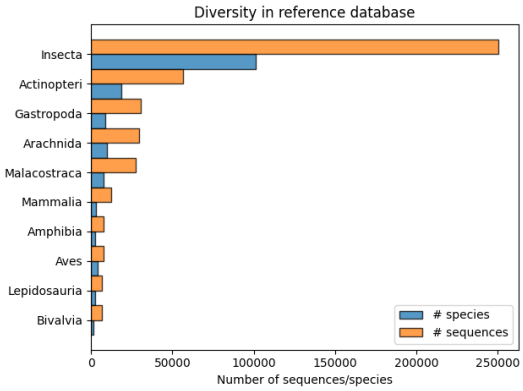

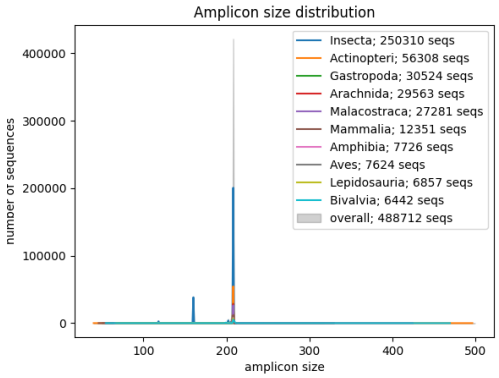

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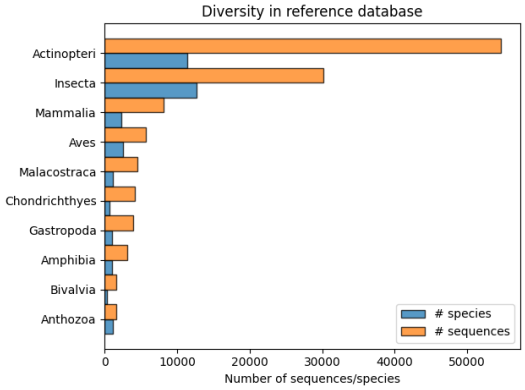

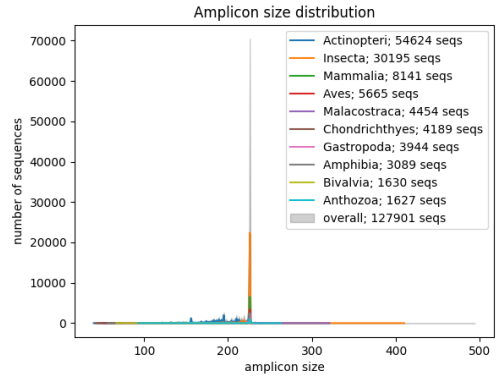

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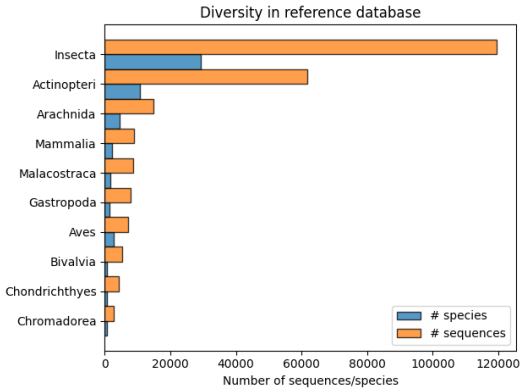

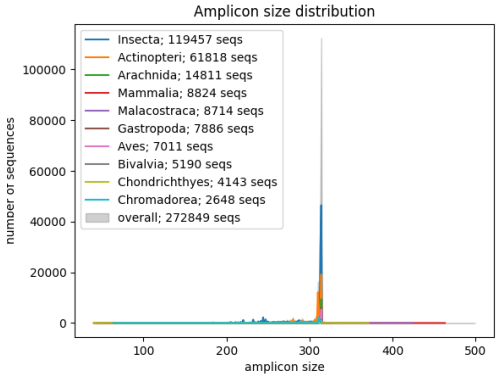

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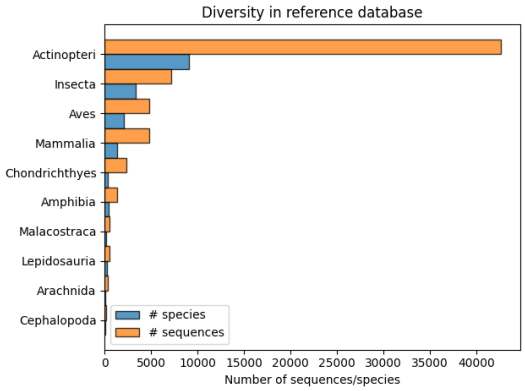
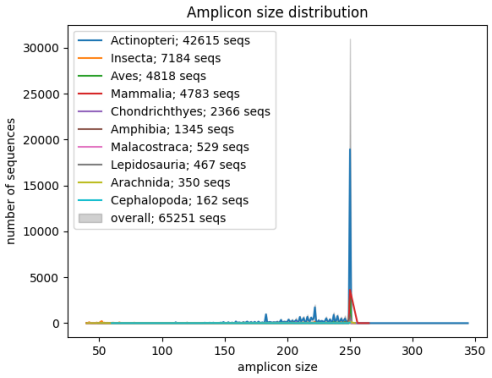
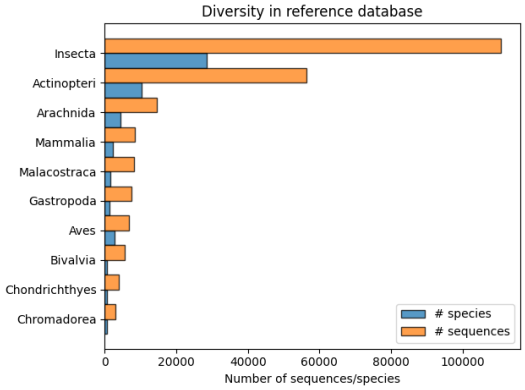
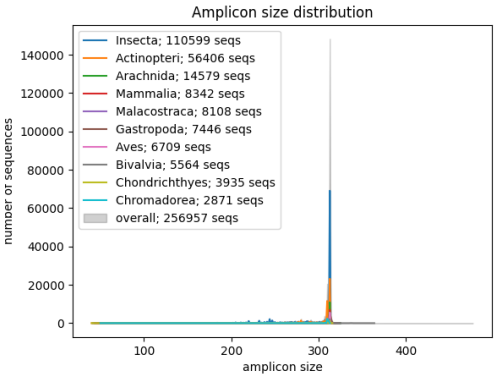




COIp17





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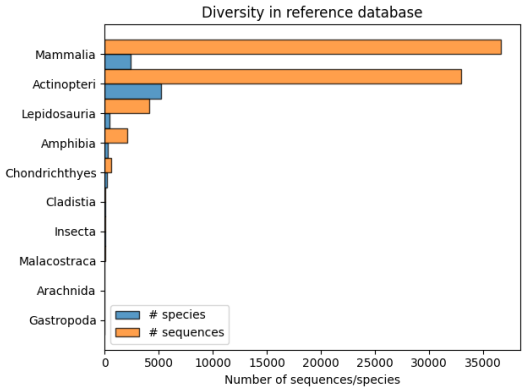

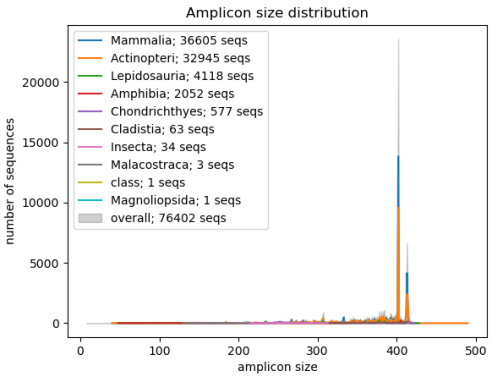

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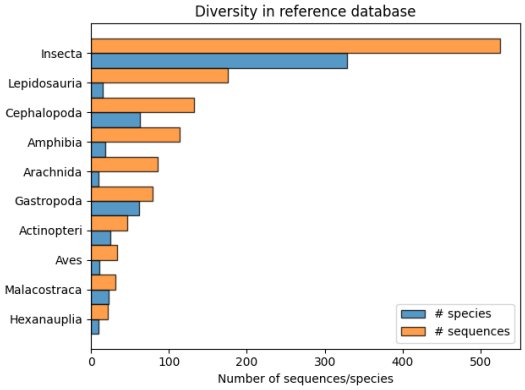

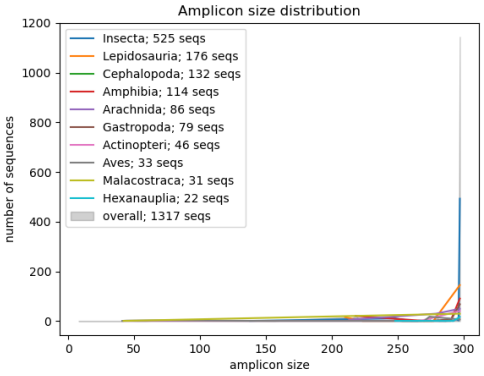

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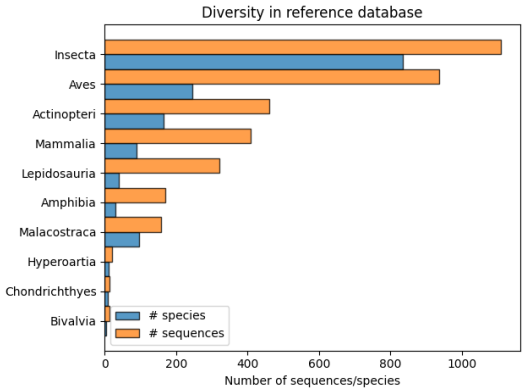

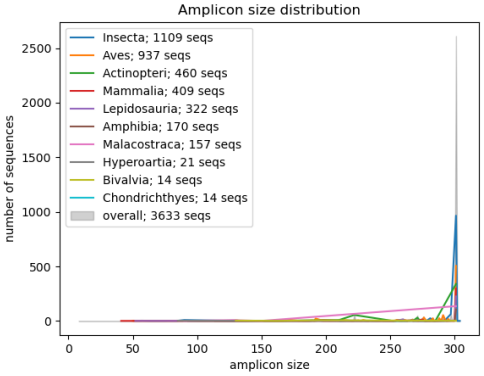

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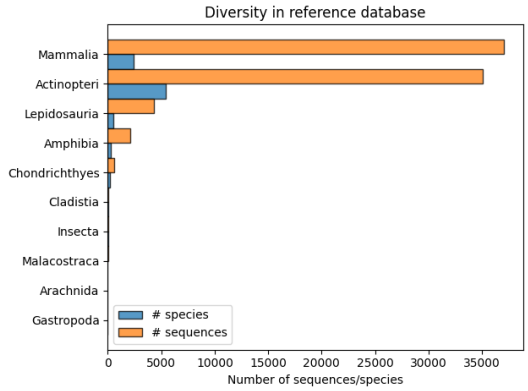

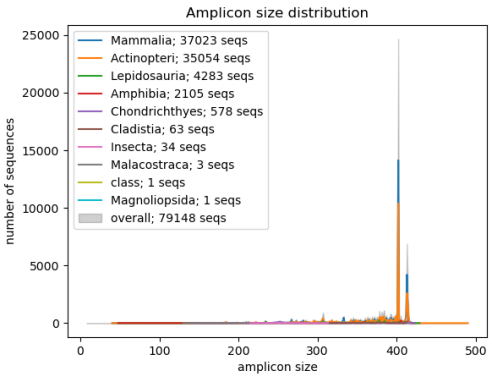

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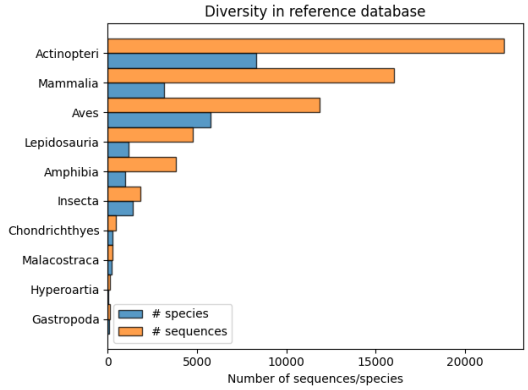

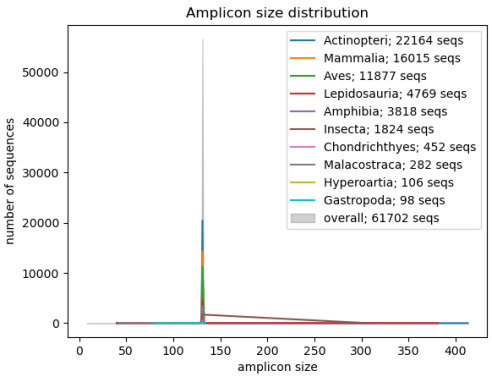

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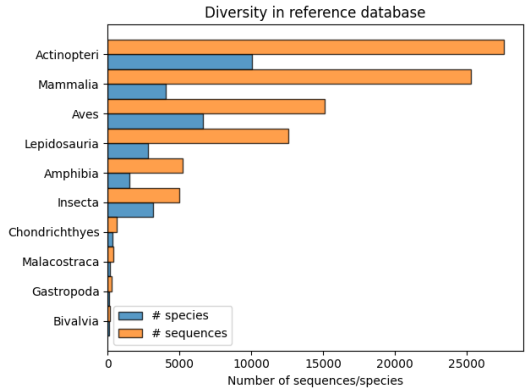

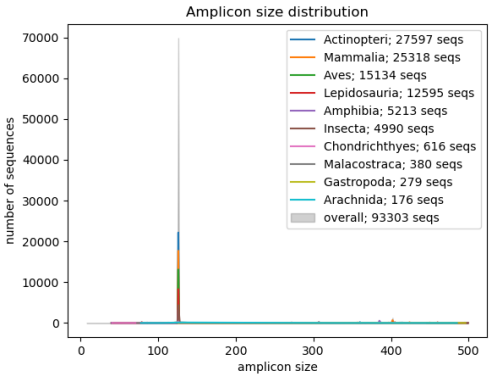

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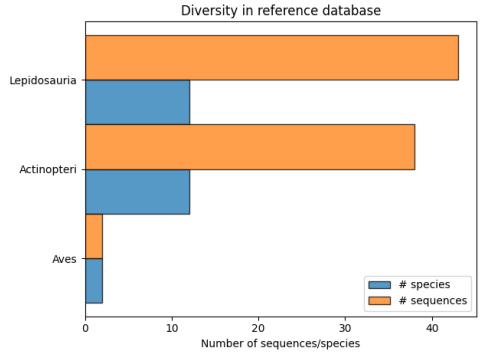

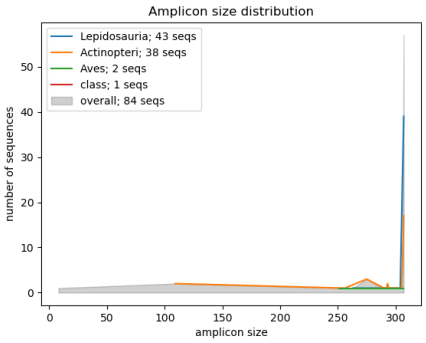

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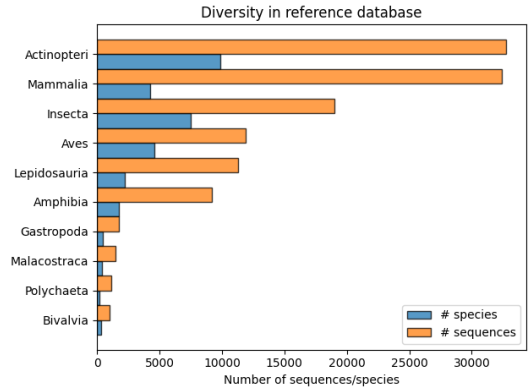

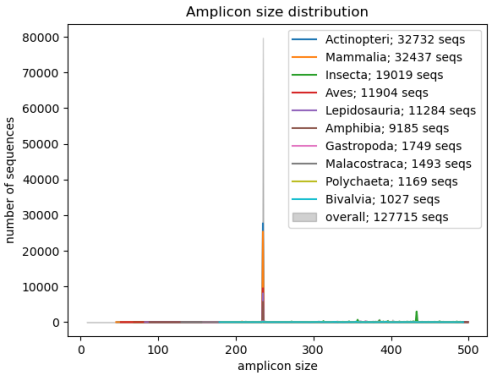

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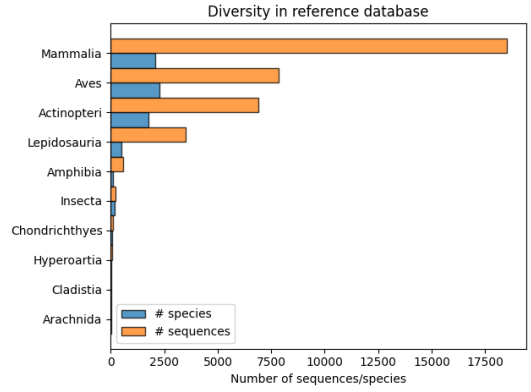

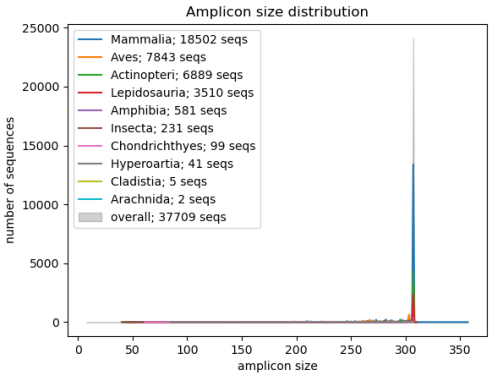

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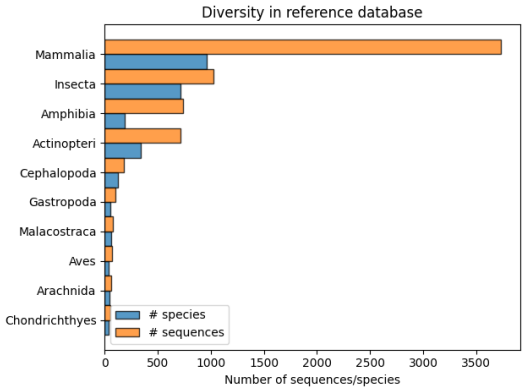

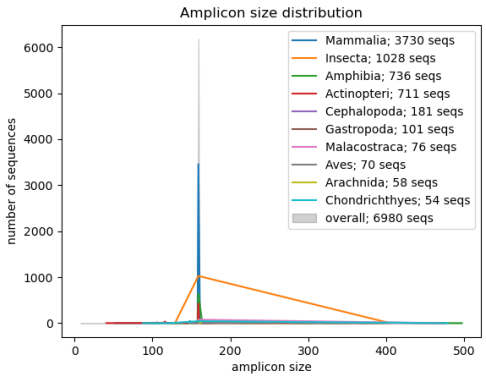

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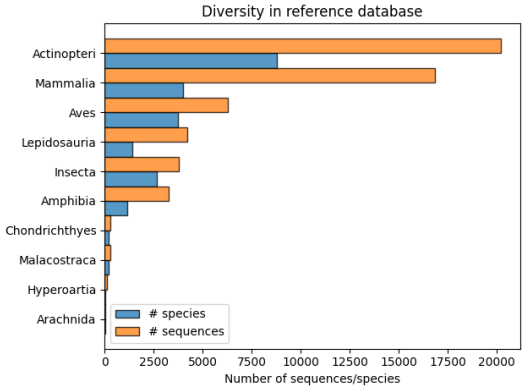

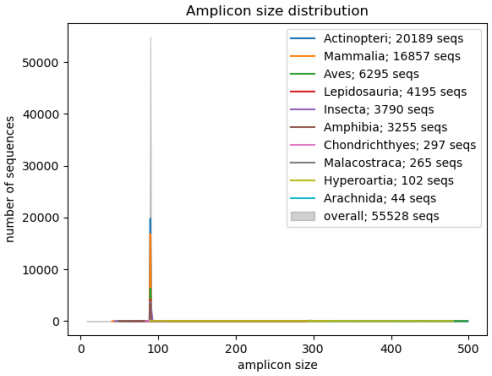

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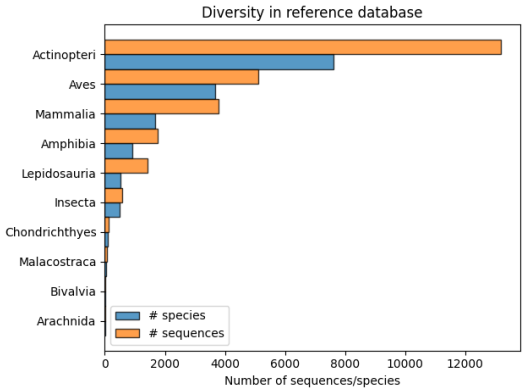

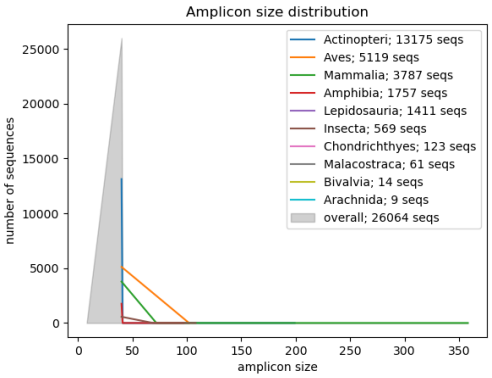

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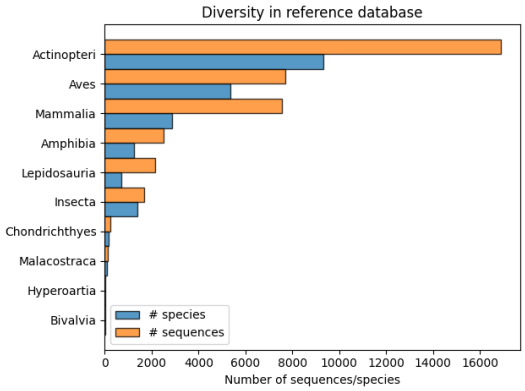

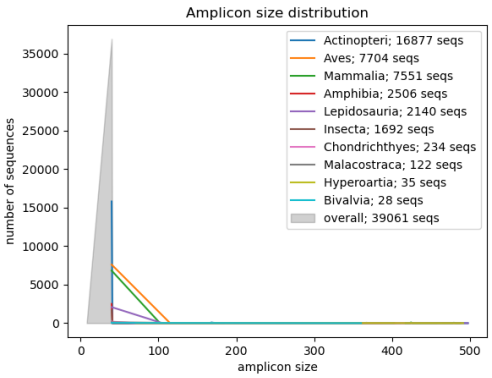

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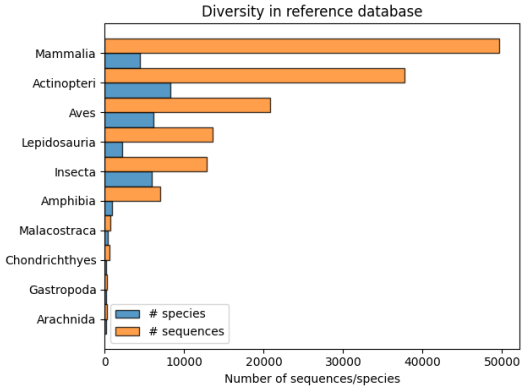
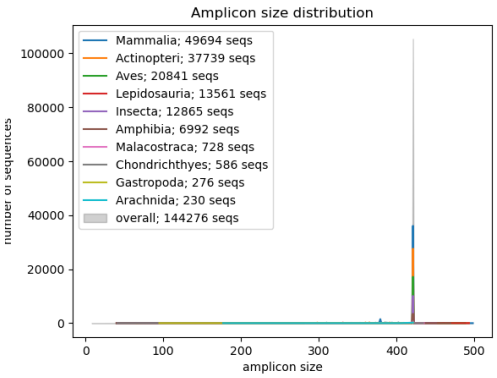
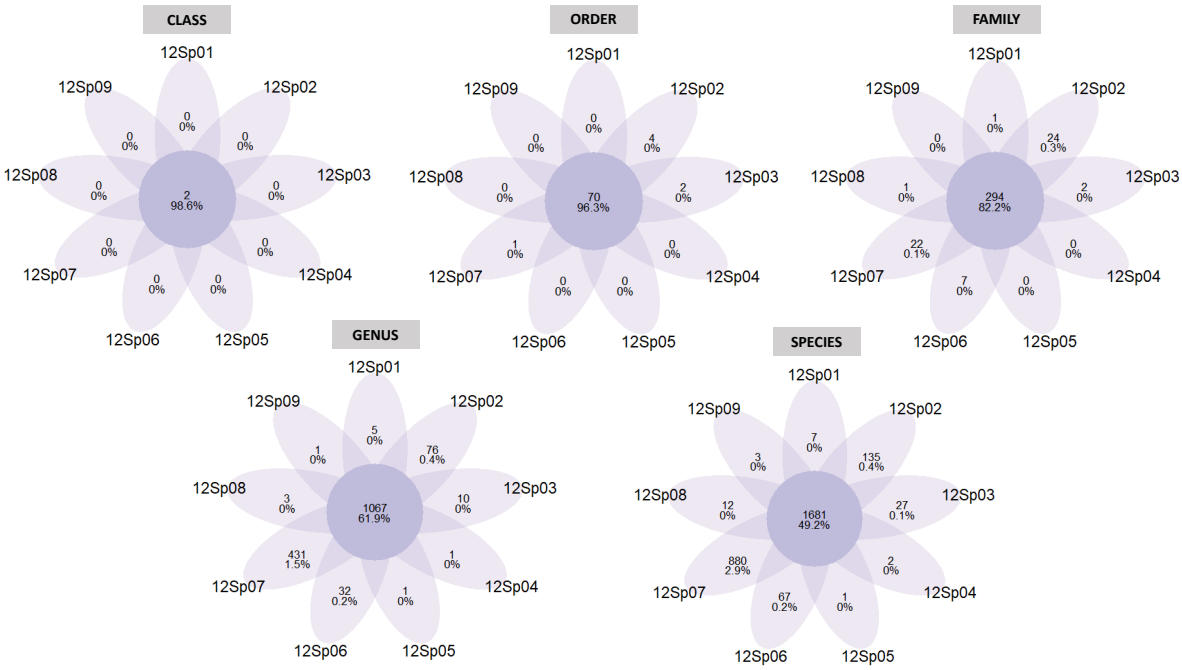
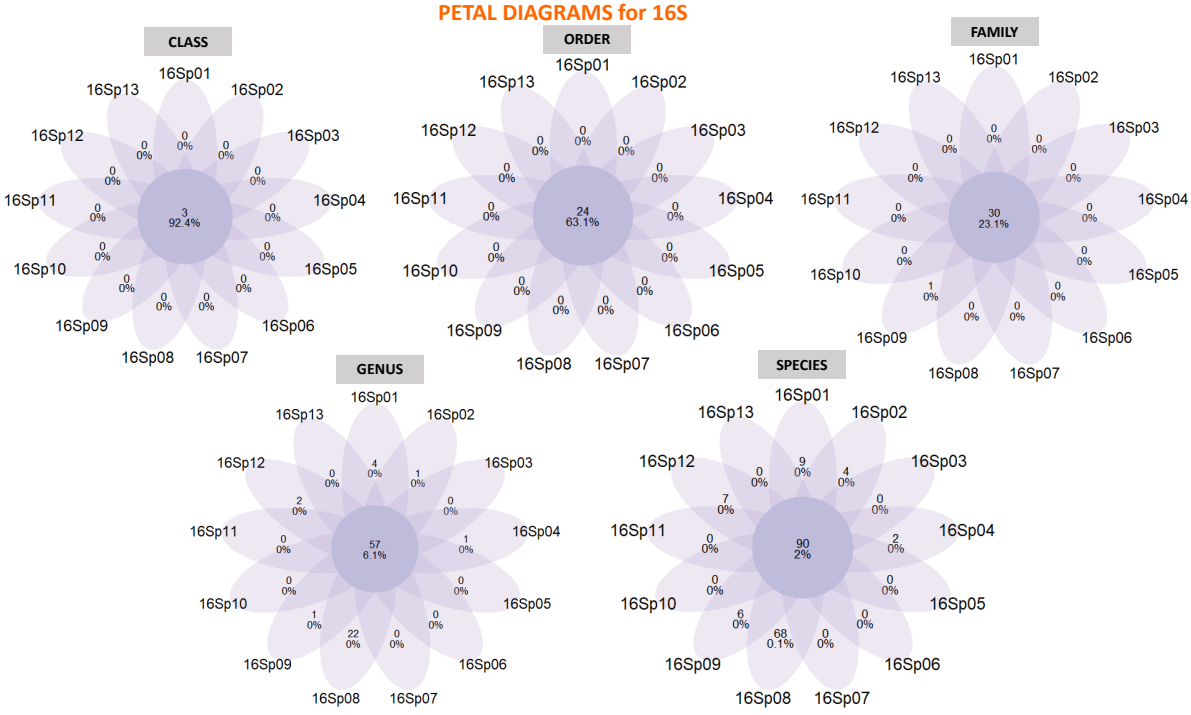




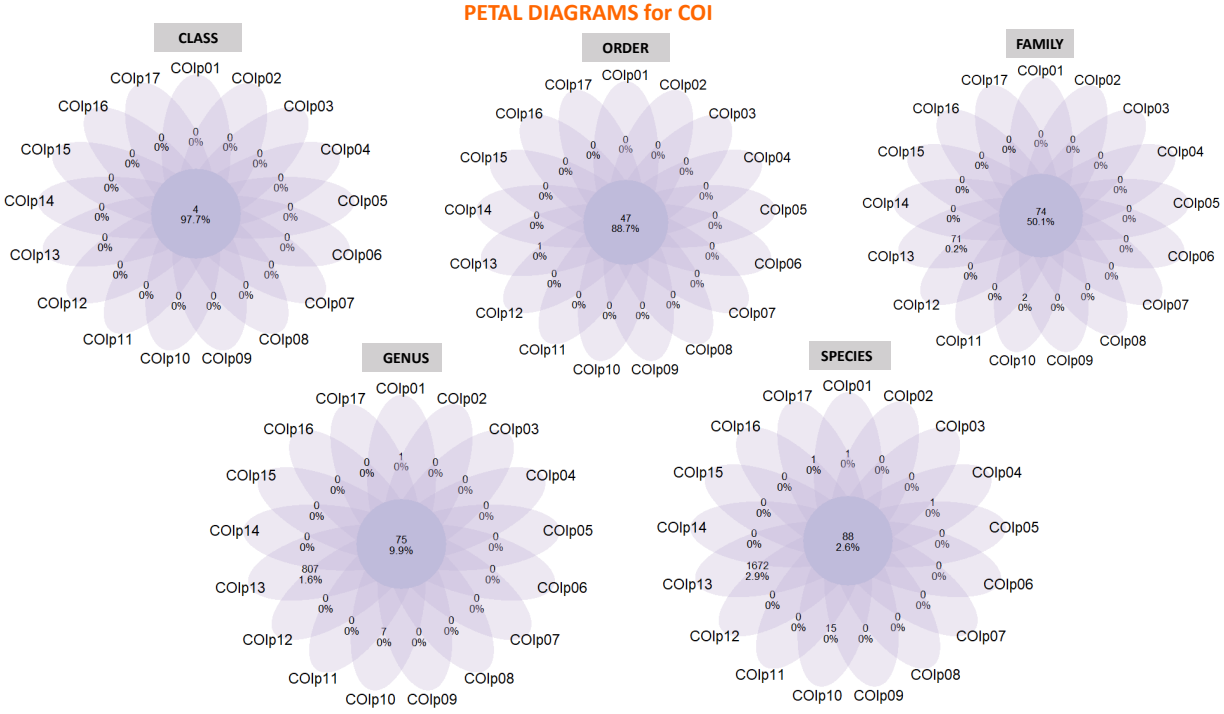
PETAL DIAGRAMS for 12S



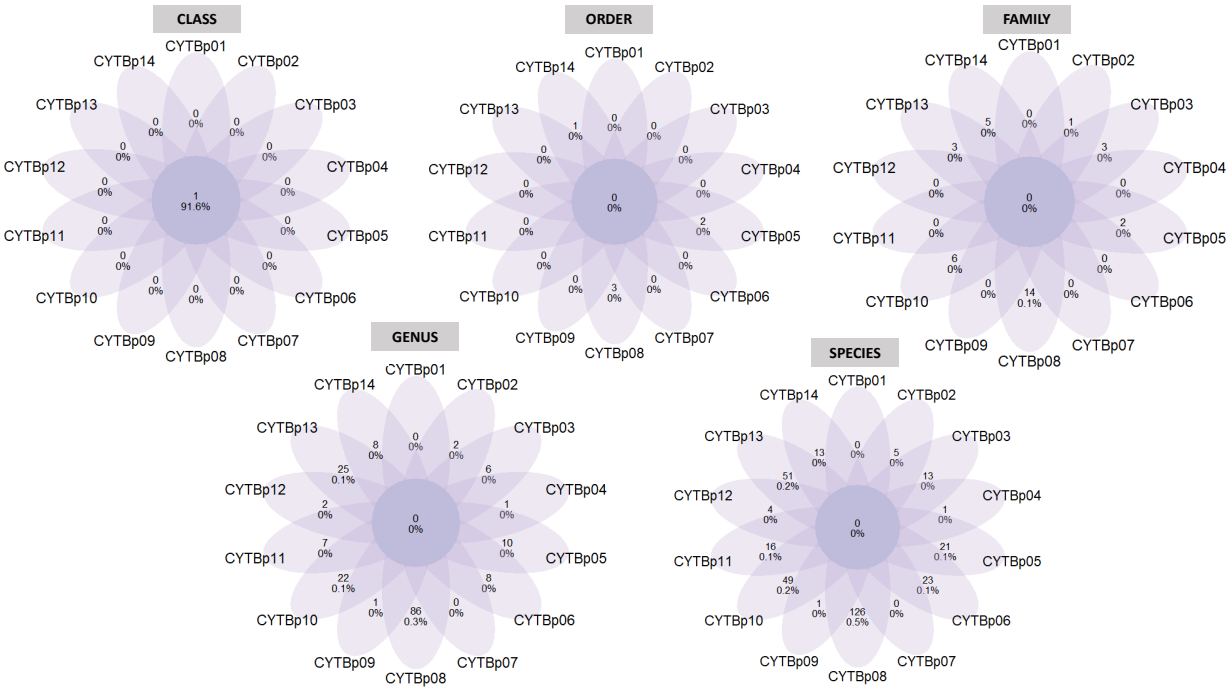
PETAL DIAGRAMS for 16S



PETAL DIAGRAMS for COI



PETAL DIAGRAMS for CYTB



Appendix B.

Supplementary Chapter 4

Table B.1:

Species	S_M14	S_M15	S_M17	S_M18	S_M19	S_M2	S_M20	S_M21	S_M23	S_M3	S_M4	S_M5	S_M6	S_M9	S_M16	S_M22	S_M10	S_M11	S_M7	S_M25
Arctogadus_glacialis_COI	0.0	0.0	0.2	0.1	0.0	0.1	0.0	0.2	0.0	0.0	0.1	0.1	0.2	0.0	0.2	0.3	0.0	0.0	0.0	0.0
Gadus_chalco_morhua_COI	95.0	75.3	99.7	96.0	80.8	99.9	98.6	66.1	0.0	100.0	99.7	99.9	99.7	100.0	82.7	99.7	1.6	0.0	0.0	0.0
Pollachius_virens_COI	0.0	24.7	0.1	1.5	0.0	0.0	1.4	0.5	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Merluccius_australis_COI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.0
Merluccius_hubbsi_COI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	42.1	0.0	0.0
Merluccius_spp_COI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	57.6	99.9	0.0
Sander_lucioperca_COI	3.8	0.0	0.0	1.0	0.6	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.0	16.0	0.0	0.0	0.0	0.0	0.0
Pleuronectes_platessa_COI	1.3	0.0	0.0	1.5	16.8	0.0	0.0	30.2	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0
Salmo_salar_COI	0.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	97.6	0.0	0.0	100.0
Sebastes_undetermined_COI	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Merluccius_paradoxus_COI	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.1	0.0	0.0

Species	S_M14	S_M15	S_M17	S_M18	S_M19	S_M2	S_M20	S_M21	S_M23	S_M3	S_M4	S_M5	S_M6	S_M9	S_M16	S_M22	S_M10	S_M11	S_M7	S_M25
Gadus_spp_16S	17.2	32.1	45.9	14.1	1.1	43.4	43.2	0.8	0.0	40.0	49.0	44.5	45.3	45.2	21.1	45.8	0.0	33.3	0.0	0.0
Gadus_chalcogrammus_16S	5.2	0.0	38.2	1.8	0.0	56.3	46.9	0.0	0.0	59.8	51.0	0.0	54.5	54.7	0.0	0.0	0.0	66.7	0.0	0.0
Gadus_morhua_16S	13.9	21.0	5.9	5.3	0.2	0.3	5.1	0.9	0.0	0.2	0.0	55.5	0.2	0.0	22.5	54.1	0.0	0.0	0.0	0.0
Pollachius_virens_16S	0.0	47.0	0.0	0.0	0.0	0.0	4.7	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Pleuronectidae_16S	63.7	0.0	0.0	78.9	96.0	0.0	0.0	98.3	0.0	0.0	0.0	0.0	0.0	0.0	56.5	0.0	0.0	0.0	0.0	0.0
Salmo_salar_16S	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	100.0
Leptasterias_coei_16S	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chelidonichthys_und_16S	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Limanda_aspera_16S	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix C.

Supplementary Chapter 5

Full metadata details of all samples are provided in the supplementary tables (1-3), which can be accessed at the following DOI: https://doi.org/10.1016/j.foodres.2024.114901

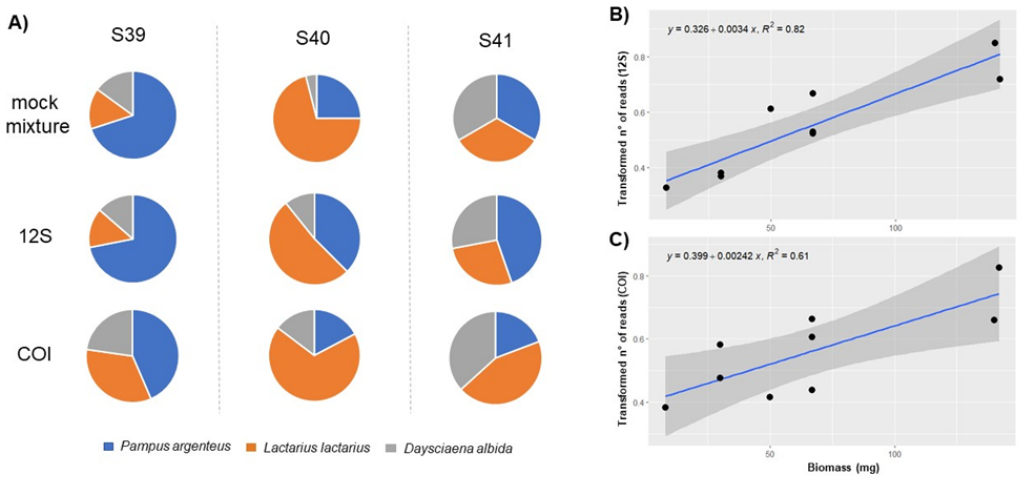


Figure C.1.: A) proportion of added biomass in the three mock mixtures and the corresponding proportion of reads found with the 12S and COI primer pairs that support our criteria for the interpretation of data. B) and C) linear regressions between the biomass and the Hellinger-transformed number of reads obtained with the two markers.

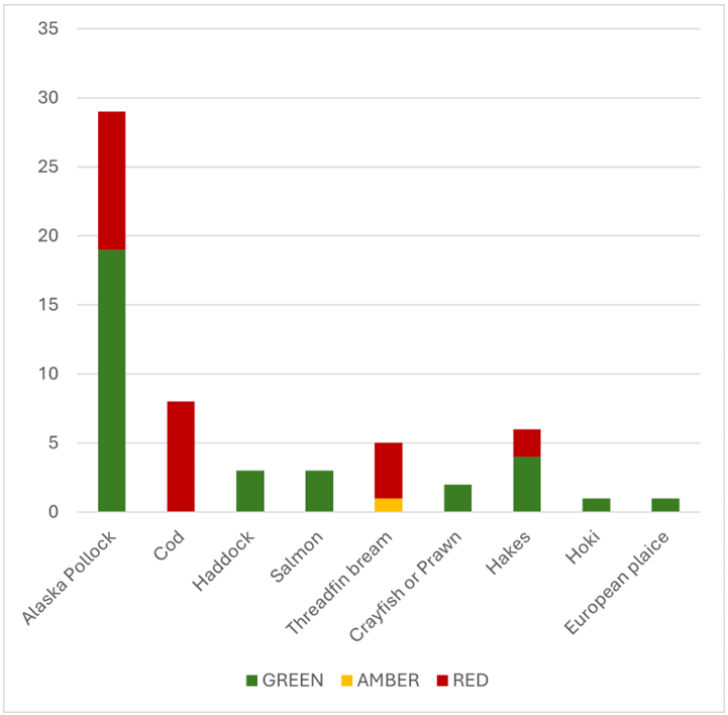


Figure C.2.: Frequency in the green, red, and amber categories for the declared species used as ingredients.

Table C.1: Supplementary Table S4: List of taxa detected by both markers in all the samples, with the exception of those that were only found in the mock mixture. Shared taxa, at least at genus level, were reported in bold.

COI	12S
Acarus siro [mite]	*Anodontostoma chacunda [fish]**
Anarhichas lupus [fish]	*Ariomma indicum [fish]**
Anchoa nasus [fish]	***Bos taurus [cow]***
Anisakis simplex [nematode]	***Clupea harengus [fish]***
Bos taurus [cow]	*Decapterus maruadsi [fish]*
Clavispora lusitaniae [yeast]	***Engraulis ringens [fish]***
Clupea harengus [fish]	*Gadiculus argenteus [fish]**
Engraulis ringens [fish]	**Gadidae (*Melanogrammus aeglefinus* or *Merlangius merlangus*) [fish]**
Gadus chalcogrammus [fish]	***Gadus sp. [fish]***
Gadus morhua [fish]	*Gallus gallus [bird]*
Hypophthalmichthys molitrix [fish]	*Hilsa kelee [fish]**
Limanda aspera [fish]	***Hypophthalmichthys molitrix [fish]***
Lutjanus ophuysenii [fish]	*Ilisha melastoma [fish]**
Lutjanus vitta [fish]	*Johnius borneensis [fish]**
Macruronus sp. [fish]	***Lutjanus sebae [fish]***
Melanogrammus aeglefinus [fish]	***Lutjanus lutjanus [fish]***
Merluccius capensis [fish]	***Macruronus novaezelandiae [fish]***
Merluccius hubbsi [fish]	***Merluccius sp. [fish]***
Merluccius paradoxus [fish]	***Merluccius productus [fish]***
Merluccius productus [fish]	***Microstomus kitt [fish]***
Microstomus kitt [fish]	***Nemipterus aurora [fish]***
Nemipterus aurora [fish]	***Nemipterus japonicus [fish]***
Nemipterus bathybius [fish]	***Nemipterus randalli [fish]***
Nemipterus japonicus [fish]	*Odonus niger [fish]**
Nemipterus nemurus [fish]	***Pleuronectes platessa [fish]***
Ochromonas danica [alga]	***Pollachius virens [fish]***
Parapenaeopsis hardwickii [shrimp]	*Pomatoschistus microps [fish]**

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Table C.1: Supplementary Table S4: List of taxa detected by both markers in all the samples, with the exception of those that were only found in the mock mixture. Shared taxa, at least at genus level, were reported in bold. (Continued)

COI	12S
Penaeus vannamei [shrimp]	***Priacanthus macracanthus [fish]***
*Pennahia anea [fish]**	***Priacanthus tayenus [fish]***
*Pentaprion longimanus [fish]**	*Pristipomoides multidens [fish]*
Pichia kudriavzevii [yeast]	***Salmo sp. [fish]***
Pleuronectes platessa [fish]	***Sander sp. [fish]***
Pollachius virens [fish]	***Sardinella sp. [fish]***
Priacanthus macracanthus [fish]	*Saurida umeyoshii [fish]**
Priacanthus tayenus [fish]	*Saurida undosquamis [fish]**
Procambarus clarkii [shrimp]	**Sciaenidae [fish]**
Saccharomyces cerevisiae [yeast]	*Scomber sp. [fish]*
Salmo salar [fish]	*Selaroides leptolepis [fish]*
Sander lucioperca [fish]	*Seriola quinqueradiata [fish]*
Sardinella sp. [fish]	*Sphyraena pinguis [fish]*
Sardinella melanura [fish]	***Sus scrofa [swine]***
Sciaenidae [fish]	*Symphodus ocellatus [fish]*
Sitophilus oryzae [beetle]	*Trachurus japonicus [fish]*
Solenocera crassicornis [shrimp]	***Trichiurus sp. [fish]***
Sus scrofa [swine]	***Upeneus sp. [fish]***
Trichiurus japonicus [fish]	***Upeneus moluccensis [fish]***
Trichiurus lepturus [fish]	***Upeneus sulphureus [fish]***
Upeneus japonicus [fish]	
Upeneus sulphureus [fish]	
Upeneus tragula [fish]	

* species not included on the UK list of commercial designations of fish (updated to 03 February 2020), used as a reference for Asian-style products sold in the UK.

Table C.2.: Supplementary material. Filtering steps and their corresponding total number of reads in each dataset.

	COI n° of reads	**12S n° of reads**
Pre-processed raw data	5,077,768	5,643,616
No contaminants and no negatives	4,831,834	4,921,563
Reads >1%	4,775,115	4,880,925

Some of the rarer species that were discarded (< 1%) offer additional insights (and concerns) around seafood production. Within the 12S dataset, we found DNA belonging to animals that may live around seafood processing plants, such as sea gulls (*Larus* sp.) in S18 (surimi) (47 out of 45,229 reads) and S19 (surimi) (12 out of 74,580 reads) and pigeon (*Columba livia*) in S18 (250 reads) and S30 (breaded) (5 out of 45,695 reads), as well as cat (*Felis catus*) DNA in sample S19 (8 reads). Moreover, we detected the presence of pufferfish (*Lagocephalus* sp.) in S23 (surimi) (8 out of 86,288 reads) and in S24 (surimi) (30 out of 109,427 reads), previously recorded in surimi-based products (Piredda et al., 2022), and responsible for several documented cases of tetrodotoxin poisoning (Alhatali et al., 2022; Al-Sulaimani et al., 2022). Meanwhile, the COI marker also detected the presence of some species of fungi, such as *Aspergillus* and *Fusarium*, in most of the breaded products, probably for the contamination of wheat, hence raising concern for the possible intrusion of mycotoxins (Gurikar et al., 2023).

Appendix D.

Supplementary Chapter 6

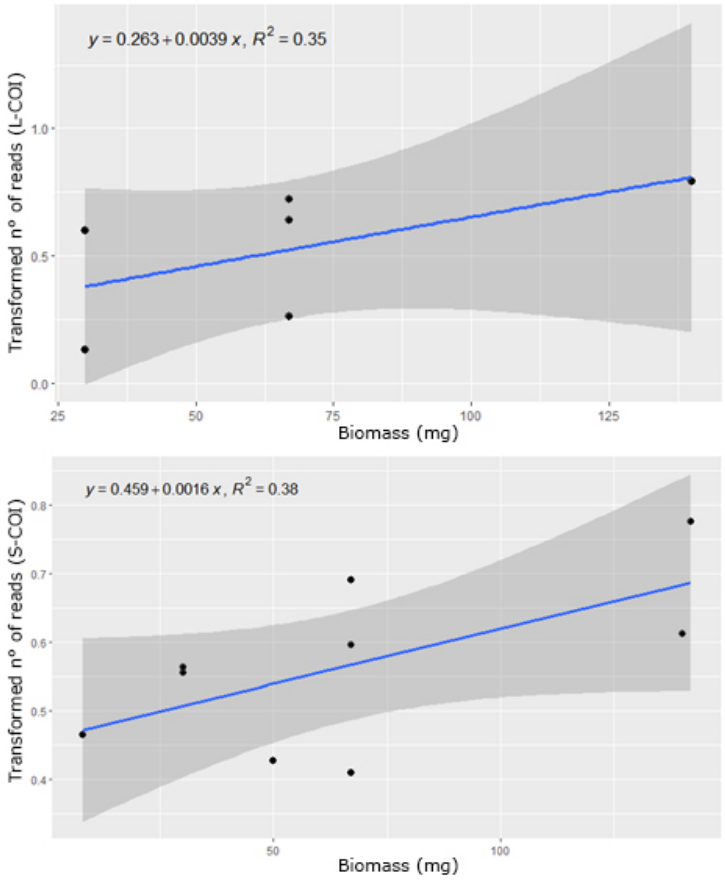

Figure D.1.: Linear correlation between the input biomass (mg) of the three fish species and the Hellinger-transformed read counts obtained through Nanopore sequencing (L-COI and S-COI dataset).

Table D.1: Supplementary table 1: sequence comparison between Illumina, S-COI, and L-COI datasets for selected species. For each comparison, the number of matching bases, reference sequence length, percentage of similarity, and associated error rate are reported.

Species	Comparison	Matching Bases	Reference Length	% Similarity	% Error
G. morhua	Illumina vs. S-COI	294/300	300	98%	0.02%
	Illumina vs. L-COI	300/300	300	100%	0%
	S-COI vs. L-COI	294/300	300	98%	0.02%
G. chalcogrammus	Illumina vs. S-COI	251/296	296	84.8%	0.152%
	Illumina vs. L-COI	292/296	296	98.6%	0.014%
	S-COI vs. L-COI	251/296	296	84.8%	0.152%
M. productus	Illumina vs. S-COI	303/304	304	99.70%	0.30%
	Illumina vs. L-COI	281/304	304	92.40%	7.60%
	S-COI vs. L-COI	281/304	304	92.40%	7.60%
P. platessa	Illumina vs. S-COI	299/304	304	98.40%	1.60%
	Illumina vs. L-COI	303/304	304	99.70%	0.30%
	S-COI vs. L-COI	299/304	304	98.40%	1.60%

Table D.2: Supplementary table 2: List of all taxa detected across the three datasets (COI Illumina, S-COI, and L-COI). An "X" indicates the detection of a given taxon in at least one sample.

All taxa	COI Illumina	S-COI	L-COI
Acarus siro	X	X	
Anarhichas lupus	X		
Anchoa nasus	X	X	X
Anisakis simplex	X	X	
Bos taurus	X	X	X
Clavispora lusitaniae	X		
Clupea harengus	X	X	X
Daysciaena albida	X	X	X
Engraulis ringens	X	X	
Gadus chalcogrammus	X	X	X
Gadus morhua	X	X	X
Gallus gallus			X

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Table D.2: Supplementary table 2: List of all taxa detected across the three datasets (COI Illumina, S-COI, and L-COI). An "X" indicates the detection of a given taxon in at least one sample. (Continued)

All taxa	COI Illumina	S-COI	L-COI
Hypophthalmichthys molitrix	X	X	X
Lactarius lactarius	X	X	X
Limanda aspera	X	X	X
Limanda limanda		X	X
Lutjanus ophuysenii	X		
Lutjanus sp.			X
Lutjanus vitta	X		
Macruronus sp.	X	X	X
Melanogrammus aeglefinus	X	X	X
Merluccius capensis	X	X	
Merluccius gayi			X
Merluccius hubbsi	X	X	X
Merluccius paradoxus	X	X	X
Merluccius productus	X	X	X
Merluccius sp.		X	
Microstomus kitt	X	X	X
Nemipterus aurora	X	X	X
Nemipterus bathybius	X	X	
Nemipterus furcosus		X	X
Nemipterus japonicus	X	X	X
Nemipterus marginatus			X
Nemipterus mesoprion			X
Nemipterus nemurus	X		
Nemipterus randalli		X	X
Ochromonas danica	X		
Odonus niger			X
Pampus sp.	X	X	X
Parapenaeopsis hardwickii	X		

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Table D.2: Supplementary table 2: List of all taxa detected across the three datasets (COI Illumina, S-COI, and L-COI). An "X" indicates the detection of a given taxon in at least one sample. (Continued)

All taxa	COI Illumina	S-COI	L-COI
Parapenaeopsis stylifera		X	X
Parascolopsis aspinosa			X
Penaeus vannamei	X	X	X
Penicillium sclerotiorum		X	
Pennahia anea	X		
Pentaprion longimanus	X		X
Pichia kluyveri		X	
Pichia kudriavzevii	X		
Pleuronectes platessa	X		X
Pleuronectes quadrituberculatus		X	
Pollachius virens	X	X	X
Priacanthus macracanthus	X		X
Priacanthus tayenus	X		X
Procambarus clarkii	X	X	
Procambarus sp.			X
Saccharomyces cerevisiae	X	X	
Salmo salar	X	X	X
Sander lucioperca	X	X	X
Sardinella fijiensis			X
Sardinella gibbosa			X
Sardinella melanura	X	X	X
Sardinella sp.	X		X
Saurida undosquamis			X
Sciaenidae	X		
Selaroides leptolepis			X
Seriola sp.			X
Sitophilus oryzae	X		
Solenocera crassicornis	X	X	

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Table D.2: Supplementary table 2: List of all taxa detected across the three datasets (COI Illumina, S-COI, and L-COI). An "X" indicates the detection of a given taxon in at least one sample. (Continued)

All taxa	**COI Illumina**	**S-COI**	**L-COI**
Sphyraena pinguis			X
Sus scrofa	X	X	X
Trachinocephalus myops			X
Trichiurus japonicus	X	X	X
Trichiurus lepturus	X	X	X
Upeneus guttatus			X
Upeneus japonicus	X		X
Upeneus moluccensis			X
Upeneus sulphureus	X		X
Upeneus tragula	X		X

DATI DOTTORANDO

Cognome Nome: **Lorusso Lucilia**

Codice borsa: **DOT1302911**

CUP: **H99j21010120001**

Asse IV "Istruzione e ricerca per il recupero – REACT-EU"
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