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

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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 novel food ingredients (e.g., jellyfish, red algae, insects) or endangered species. 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.

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

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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 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 & 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 (www.illumina.com/systems/miseq/performance_specifications.html). Since 2003, with its 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, 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 (www.fao.org/fishery/en/collection/asfis/en).

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.

2. Materials and methods

2.1. 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 600 bp.

2.2. 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 (www.fao.org/fishery/static/ASFIS/ASFIS_biblio.pdf). 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 (www.fao.org/fishery/en/collection/asfis/en), 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, www.marinespecies.org/aphia.php?p=match) was used to solve instances of misspellings or of outdated scientific names in ASFIS with accepted species names.

2.3. 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) (mitofish.aori.u-tokyo.ac.jp/) and all the sequences of COI, 16S, 12S and cytb gene available at the National Center for Biotechnology Information (NCBI) (June 2023) (www.ncbi.nlm.nih.gov/). Any sequences with poor or incomplete taxonomic information (environmental, sp., cf., aff.) were removed from the outputs.

2.4. 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 & Holmes, 2013), ggplot2 (Wickham, 2011), tidyverse (Wickham & 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*).

3. Results and discussion

3.1. 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 COI, 14 pairs for the cytb, 13 pairs for the 16S and 9 primer pairs for the 12S (Table 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 (e.g., COIp02 - COIp07, COIp09), or else the primer pair is a new combination of forward or reverse already designed in other studies (e.g., 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.

3.2. 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.

3.3. 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 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 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 Zhang et al. (2020).

3.4. 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. 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. 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 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 and COIp13, respectively) and lower percentage of ASFIS species recovered (40% vs 64% ASFIS species for COIp14 and COIp13, respectively) (Table 2). However, such a discrepancy was not a surprise since the actual amplification may differ from the *in-silico* results and is

Table 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					
12Sp01	AcMDB07	For-GCCTATATACCGCCGTCG Rev-GTACACTTACCATGTTACGACTT	281 bp	Actinopteri	Bylemans et al. (2018)
12Sp02	Ac12s	For-ACTGGGATTAGATACCCCACTATG Rev-GAGAGTGACGGGCGGTGT	385 bp	Actinopteri	Evans et al. (2016)
12Sp03	Am12s	For-AGCCACCGCGGTTATACG Rev-CAAGTCCTTTGGGTTTTAAGC	241 bp	Actinopteri	Evans et al. (2016)
12Sp04	L1085 H1259	For-CCCAAACTGGGATTAGATACCC Rev-GTTTGCTGAAGATGGCGGTA	215 bp	Vertebrata	Kitano et al. (2007)
12Sp05	MiFish-U-F MiFish-U-R	For-GTCGGTAAAACTCGTGCCAGC Rev-CATAGTGGGGTATCTAATCCCAGTTTG	170 bp	Fish	Miya et al. (2015)
12Sp06	12SV5	For-TAGAACAGGCTCCTCTAG Rev-TTAGATACCCCACTATGC	99 bp (73–110)	Vertebrata	Riaz et al. (2011)
12Sp07	12SF1/R1	For-AGGGATAACAGCGCAATC Rev-TCGTTGAACAAACGAACC	63-84 bp	Vertebrata	Riaz et al. (2011)
12Sp08	Tele02	For-AAACTCGTGCCAGCCACC Rev-GGGTATCTAATCCCAGTTTG	167 bp	Teleostei	Taberlet et al. (2018)
12Sp09	Teleo	For-ACACCGCCCGTCACTCT Rev- CTTCCGGTACACTTACCATG	63 bp	Teleostei	Valentini et al. (2016)
16S					
16Sp01	16S1F 16S2R	For-GACGAKAAGACCCTA Rev-CGCTGTTATCCCTADRGTAACT	250 bp	Fish, cephalopods and crustaceans	Deagle et al. (2007)
16Sp02	16FORF-CEP3 16REVF-CEP	For-GAGAAGACCCTDTKGAGCTT Rev-GCTGTTATCCCTAKGGTAAC	206-145 bp	cephalopod species	Giusti et al. (2017) [a]
16Sp03	16S-forward 16S-reverse	For-AYAAGACGAGAAGACCC Rev-GATTGCGCTGTTATTCC	250 bp	Universal animal mini-barcode	(Sarri et al., 2014) [a]
16Sp04	Vert-16S	For-AGACGAGAAGACCCYdTGGAGCTT Rev-GATCCAACATCGAGGTCGTAA	264 bp	Vertebrata	Vences et al. (2016)
16Sp05	FOR16Spc REV16Spc	For-TGCCCGTGCAGAAGCGG Rev-CAACATCGAGGTCGTAAACCC	295-339 bp	Clupeidae, Engraulidae, Salangidae, Scombridae	Armani et al. (2012) [a]
16Sp06	16sf-var 16sr-var	For-CAAATTACGCTGTTATCCCTATGG Rev-GACGAGAAGACCCTAATGAGCTTT	148-209 bp	cephalopod species belonging to Ommastrephidae and Loliginidae	(Chapela et al., 2002) [a]
16Sp07	Fish16SF 16S2R-degenerate	For-GACCCTATGGAGCTTTAGAC Rev-CGCTGTTATCCCTADRGTAACT	203 bp	Fish	DiBattista et al. (2017)
16Sp08	Ac16s	For-CCTTTTGCATCATGATTTAGC Rev-CAGGTGGCTGCTTTTAGGC	330 bp	Freshwater fishes and amphibians	Evans et al. (2016)
16Sp09	Ve16s	For-CGAGAAGACCCTATGGAGCTTA Rev-AATCGTTGAACAAACGAACC	310 bp	Freshwater fishes and amphibians	Evans et al. (2016)
16Sp10	16S-HF 16S-HR1	For-ATAACACGAGAAGACCCT Rev-CCCACGGTCGCCCCAAC	80-125 bp	Animal species in food	Horreo et al. (2013) [a]
16Sp11	16S-HF 16S-HR2	For-ATAACACGAGAAGACCCT Rev-CCCGCGGTCGCCCCAAC	80-125 bp	Animal species in food	Horreo et al. (2013) [a]
16Sp12	L2513/H2714	For-GCCTGTTTACCAAAAACATCAC Rev-CTCCATAGGGTCTTCTCGTCTT	244 bp	Vertebrata	Kitano et al. (2007)
16Sp13	16S fish-specific 16S fish-specific	For-GGTCGCCCCAACCRAAG Rev-CGAGAAGACCCTWTGGAGCTTIAG	68 bp	Fish	Shaw et al. (2016)
COI					
COIp01	FISHCOILBC Revshort1	For-CTCAACYAATCAYAAAGATATYGGCAC Rev-GGYATNACTATRAAGAAAATTATTAC	139 bp	Fish	Giusti et al. (2017) [a]
COIp02	mlCOIintF HCO2198	For-GGWACWGGWTGAACWGTWTAYCCYCC Rev-TAAACTTCAGGGTGACCAAAAAATCA	313 bp	Marine metazoa	Leray et al. (2013)
COIp03	mlCOIintF dgHCO2198	For-GGWACWGGWTGAACWGTWTAYCCYCC Rev-TAAACTTCAGGGTGACCAAARAAYCA	313 bp	Marine metazoa	Leray et al. (2013)
COIp04	mlCOIintF jgHCO2198	For-GGWACWGGWTGAACWGTWTAYCCYCC Rev-TAIACYTCIGGRTGICCRAARAAYCA	313 bp	Marine metazoa	Leray et al. (2013)
COIp05	LCO1490 mlCOIintR	For-GGTCAACAAATCATAAAGATATTGG Rev-GGRGGRTASACSGTTCASCCSGTSCC	319 bp	Marine metazoa	Leray et al. (2013)
COIp06	dgLCO1490 mlCOIintR	For-GGTCAACAAATCATAAAGAYATYGG Rev-GGRGGRTASACSGTTCASCCSGTSCC	319 bp	Marine metazoa	Leray et al. (2013)
COIp07	jgLCO1490 mlCOIintR	For-TITCIACIAAYCAYAARGAYATTGG Rev-GGRGGRTASACSGTTCASCCSGTSCC	319 bp	Marine metazoa	Leray et al. (2013)
COIp08	Uni-MinibarF1 Uni-MinibarR1	For-TCCACTAATCACAARGATATTGGTAC Rev-GAAAATCATAATGAAGGCATGAGC	127 bp	Universal animal mini-barcode	Meusnier et al. (2008)

(*continued on next page*)

Table 1 (*continued*)

Code	Primer name	Primer Sequences (5′-3′)	Fragment length	Target group	Reference
COIp09	mini-COI-F (LCO1490)	For-GGTCAACAAATCATAAAGATATTGG	136 bp	edible species (fish, shrimps, birds, and mammals)	(Pan et al., 2020) [a]
	mini-COI-R	Rev-ACTATAAAGAAGATTATTACAAAGGC			
COIp10	Fish_miniA_F_t	For-ACIAAICAIAAAGAYATYGGC	129 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniA_R_t	Rev-AARAAAATYATAACRAAIGCRTGIGC			
COIp11	Fish_miniB_F_t	For-GCIGGIRTYTCITCIATYYTAG	227 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniB_R_t	Rev-ACTTCAGGGTGICCGAARAATCA			
COIp12	Fish_miniC_F_t	For-ACYAAICAYAAAGAYATIGGCAC	127 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniC_R_t	Rev-GAARATCATAATGAAGGCATGIGC			
COIp13	Fish_miniD_F_t	For-GGIACIGGITGRACIGTITAYCCYCC	208 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniD_R_t	Rev-GTRATICCIGCIGCIAGIAC			
COIp14	Fish_miniE_F_t	For-ACYAAICAYAAAGAYATIGGCAC	226 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniE_R_t	Rev-CTTATRTTRTTTATICGIGGRAAIGC			
COIp15	Fish_miniF_F_t	For-GGIACIGGITGRACIGTITAYCCYCC	314 bp	fish	Shokralla et al. (2015) [a]
	Fish_miniF_R_t	Rev-CTTCAGGGTGICCGAARAATC			
COIp16	F	For-ATCACAAAGACATTGGCACCCT	295 bp	fish and non-fish species	Sultana et al. (2018) [a]
	R	Rev-AATGAAGGGGGGAGGAGTCAGAA			
COIp17	mlCOIintF-XT	For-GGWACWRGWTGRACWITITAYCCYCC	ca. 313 bp	Marine metazoa	Wangensteen et al. (2018)
	jgHCO2198	Rev-TAIACYTCIGGRTGICCRAARAAYCA			
CYTB					
CYTBp01	L14912/H15149c	For-AAAAACCACCGTTGTTATTCAACTA	413 bp	Fish and amphibians	Burgener and Hübner (1998)
		Rev-GCCCCTCAGAATGATATTTGTCCTCA			
CYTBp02	Cytb1F	For-CAGCTATTCCATATGTTGGTGA	297 bp	Loliginidae and Ommastrephidae	Chapela et al. (2002) [a]
	Cytb1R	Rev-GGTTACTAAAGGATTAGCTGGA			
CYTBp03	CytBL1C modified	For-CCWGCWAAYATWWCAACTTTRTGAARGTTTGG	~357 bp	Crustaceans and molluscs in seafood	Dwiyitno et al. (2021) [a]
	CytBHW modified	Rev-CYCCYCARAAWGATATTTGYCCYCA			
CYTBp04	L14735/H15149c2	For-AAAAACCACCGTTGTTATTCAACTA	413 bp	Vertebrata	Hänfling et al. (2016)
		Rev-GCDCCTCARAATGAYATTTGTCCTCA			
CYTBp05	BDR-L	For-GCMAACGGSGCNTCYTTCTTCT	131 bp	Mixed canned fish	Kappel et al. (2017) [a]
	BDR-H-mod1	Rev-TGACGGTAGCHCCTCAGRADGACATTTGTCCYCA			
CYTBp06	BMID-L-mod1	For-ATCYCATTCCACCCATACTWCTC	126 bp	Mixed canned fish	Kappel et al. (2017) [a]
	BMID-H-mod1	Rev-AATAGGAARTATCATTCRGGTTTRATG			
CYTBp07	L14841/H15149	For-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	307 bp	Vertebrata	Kocher et al. (1989)
		Rev-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA			
CYTBp08	L14912/H15149	For-TTCCTAGCCATACAYTAYAC	235 bp	Teleostei	Miya and Nishida (2000)
		Rev-GGTGGCKCCTCAGAAGGACATTTGKCCYCA			
CYTBp09	L14816	For-CCATCCAACATCTCAGCATGATGAAA	357 bp	Universal vertebrate animal mini-barcode	Parson et al. (2000)
	H15173	Rev-CCCCTCGAATGATATTTGTCCTCA			
CYTBp10	CEF-H	For-TTATGGKTGRGTRYTDCGTTAT	160 bp	Loliginidae, Ommastrephidae, Sepiidae, and Octopodidae	Santaclara et al. (2007) [a]
	H15149AD	Rev-GCICCTCARAATGAYATTTGTCCTCA			
CYTBp11	FishCBL/CBR	For-TCCTTTTGAGGCGCTACAGT	90 bp	Fish	Thomsen et al. (2012)
		Rev-GGAATGCGAAGAATCGTGTT			
CYTBp12	Fish2bCBR/CBL	For-GATGGCGTAGGCAAACAAGA	40 bp	Fish	Thomsen et al. (2012)
		Rev-ACAACTTCACCCCTGCAAAC			
CYTBp13	Fish2degCBL/CBR	For-ACAACTTCACCCCTGCRAAY	40 bp	Fish	Thomsen et al. (2012)
		Rev-GATGGCGTAGGCAAATAGGA			
CYTBp14	mcb398	For-TACCATGAGGACAAATATCATTCTG	421 bp	Fish, mammals, birds, reptiles	Verma and Singh (2002)
	mcb869	Rev-CCTCCTAGTTTGTTAGGGATTGATCG			

[a] articles described for food authenticity.

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).

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, 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,

Table 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 (%)
12S				
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
16S				
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
COI				
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
CYTB				
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

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

3.5. *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* and *Bangiophyceae* (*Rhodophyta*), *Trebouxiophyceae, Chlorophyceae* and *Ulvophyceae* (*Chlorophyta*) (Fig. 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,*

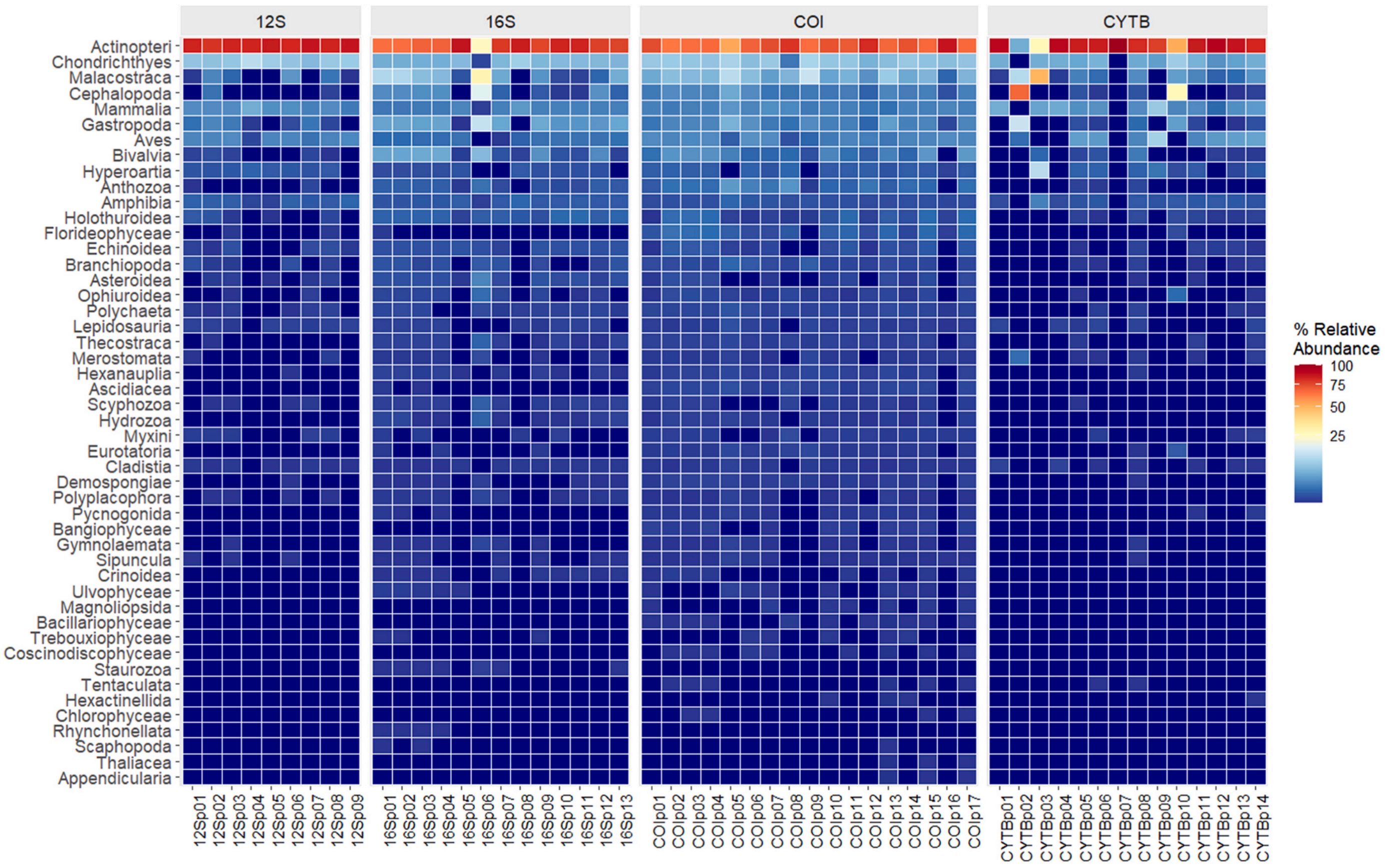

Fig. 1. Heatmap presenting the taxonomic coverage of all 48 Classes retrieved, expressed as relative percentage abundance.

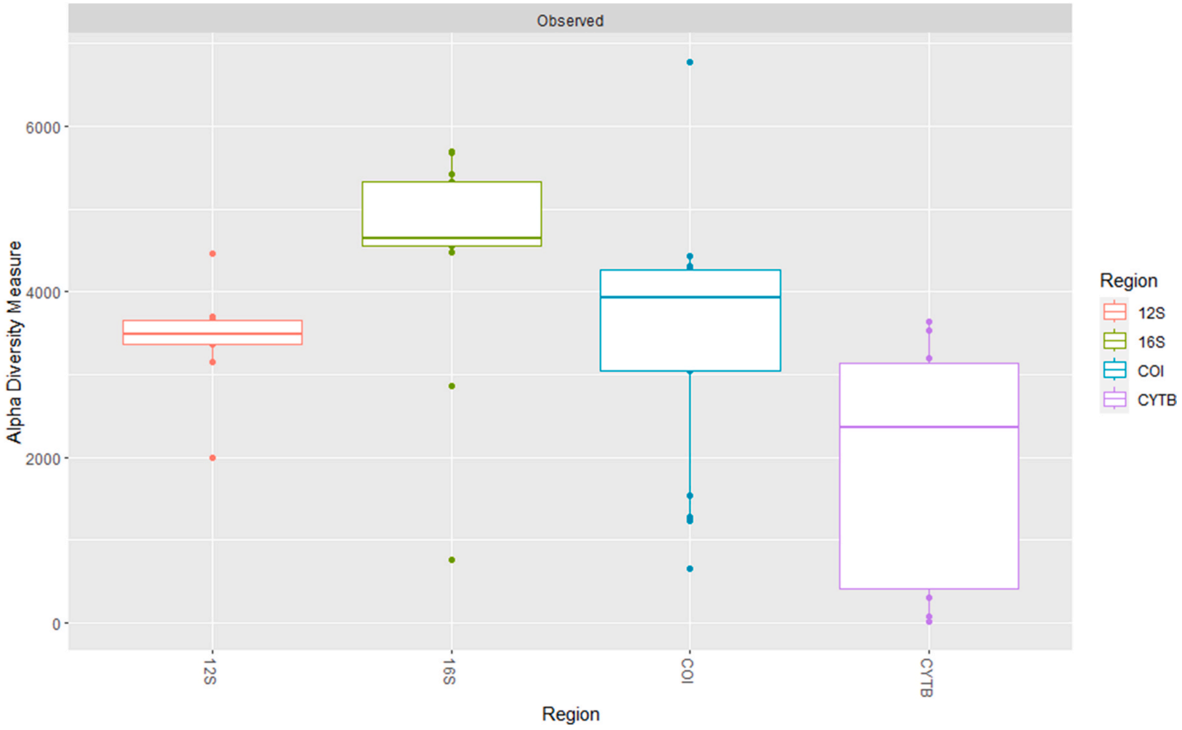

Fig. 2. Boxplots of richness values calculated for primer pairs in each primer region.

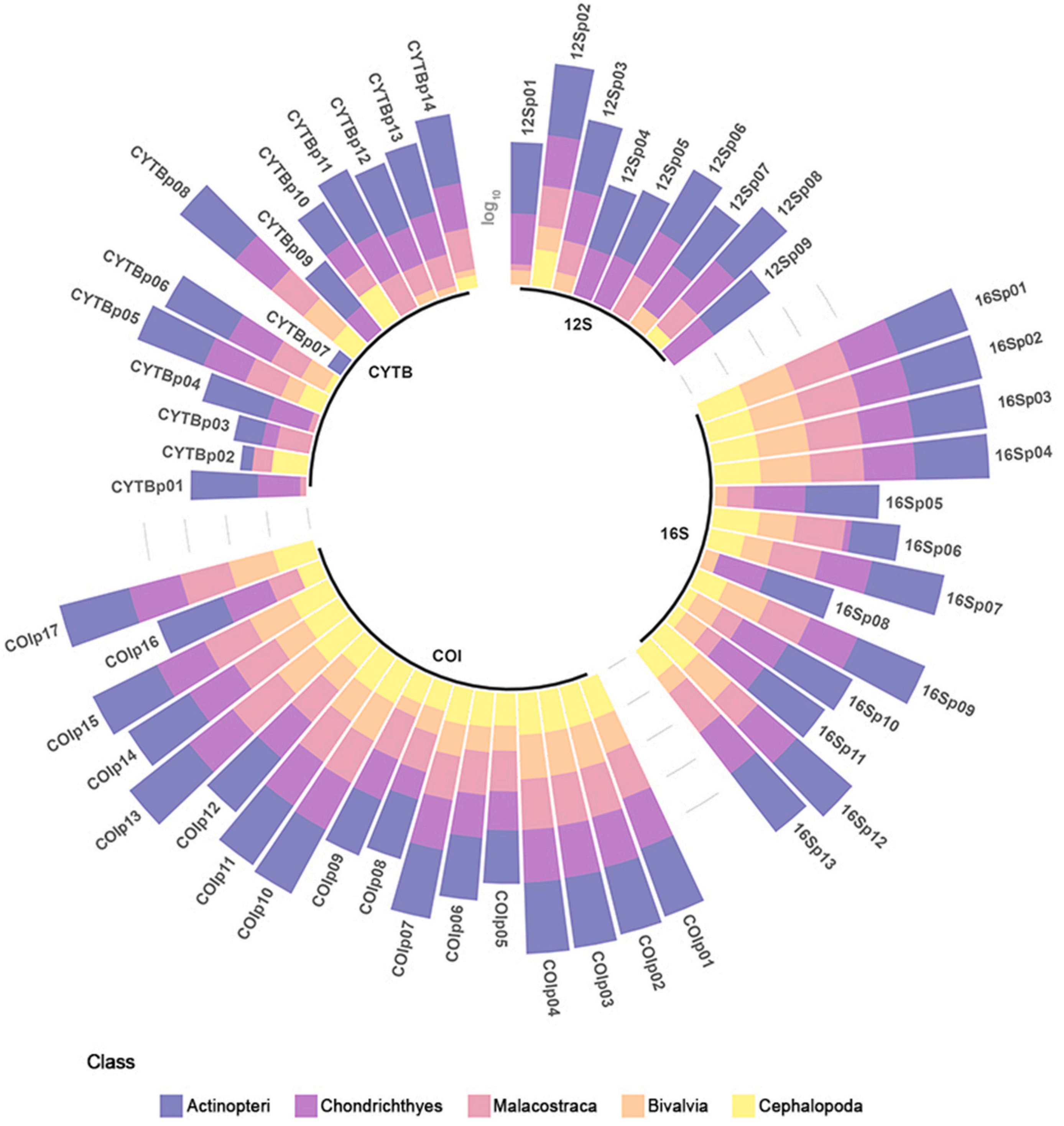

Fig. 3. Circular bar plots show the taxonomic composition captured by each primer pair shown in terms of abundance (absolute number of species) at Class level (Actinopteri, Cephalopoda, Bivalvia, Malacrostaca and Chondrichthyes). A $\log_{10}$ scale was applied to optimize data visualization.

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 & 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).

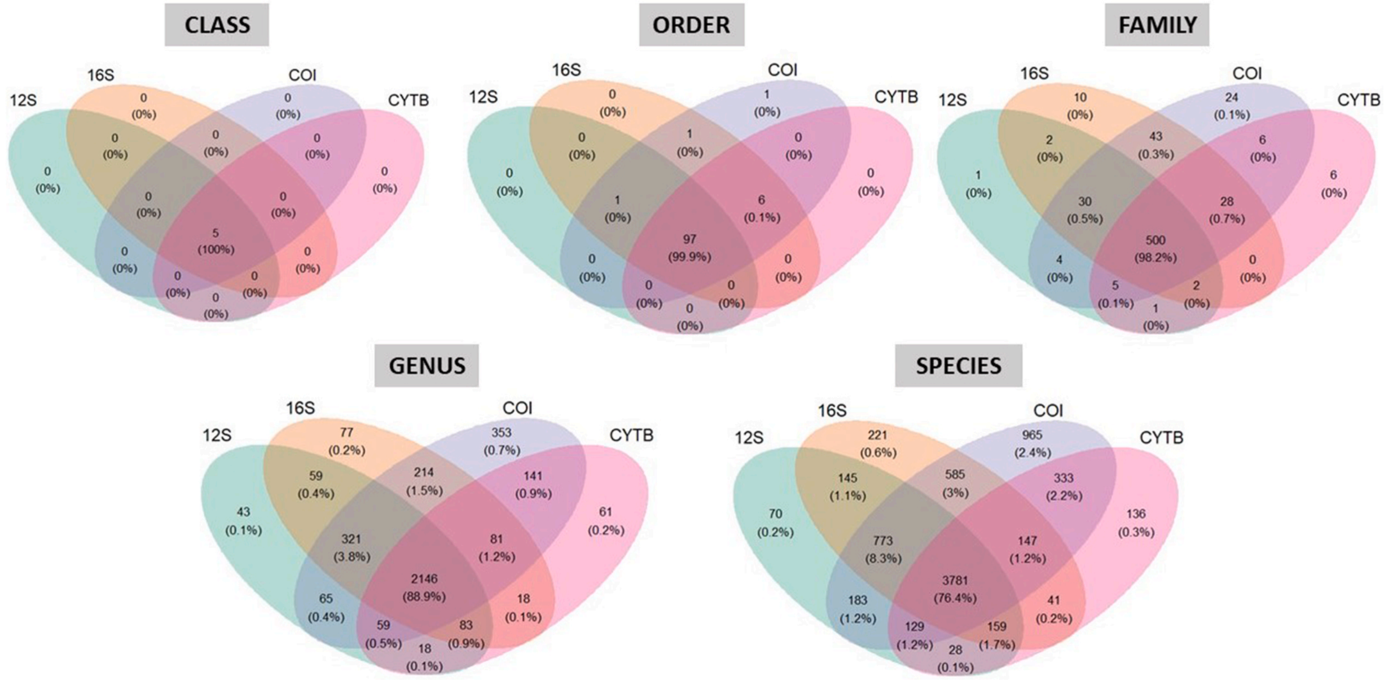

Fig. 4. Venn diagrams show the value of shared taxonomic profiles among the four marker regions at Class, Order, Family, Genus, and Species levels.

3.6. 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 (~7,000 species) and in the 16S region by the 16Sp13 pair (~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 (~7,500 species) (Supplementary Fig. 1).

3.7. 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 activities, 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).

4. 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 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 vivo* 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.

CRediT authorship contribution statement

Lucilia Lorusso: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Conceptualization. **Roberta Piredda:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Conceptualization. **Anna Mottola:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Chiara Intermite:** Writing – review & editing, Formal analysis. **Lucia Ranieri:** Writing – review & editing, Formal analysis. **Stefania Carpino:** Writing – review & editing. **Angela Di Pinto:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The manuscript entitled 'Authentication of seafood species on the ASFIS list (FAO) by in-silico evaluation of primers for metabarcoding' has not been submitted to, nor is under review at, another journal or other publishing venue.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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