DNA metabarcoding for identification of species used in fish burgers

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Abstract

The absence of morphological identification characters, together with the complexity of the fish supply chain make processed seafood vulnerable to cases of species substitution. Therefore, the authentication and the traceability of such products play a strategic role in ensuring quality and safety. The aim of the present study was to detect species used in the production of multi-species fish burgers and to evaluate mislabelling rates, using a DNA metabarcoding approach by sequencing a fragment of the 16S rRNA mitochondrial gene. The study highlighted the presence of 16 marine and 2 mammalian taxa with an overall mislabelling rate of 80%, including cases of species substitution, the undeclared presence of molluscs and of taxa whose use is not permitted by current Italian legislation. The presence of swine DNA as well as the inclusion of undeclared taxa potentially causing allergies raise concerns regarding consumer safety and protection regarding ethical or religious issues. Overall, the study shows that the application of DNA metabarcoding is a promising approach for successfully enforcing traceability systems targeting multi-species processed food and for supporting control activities, as a guarantee of an innovative food safety management system.

Introduction

In the last decades, changes in people's lifestyles and the growing awareness of health issues and the importance of healthy eating have shifted consumer eating habits towards 'time-saving' foods that also provide health benefits. In particular, the consumption of fish and fish products, essential sources of animal proteins, micronutrients and omega-3 fatty acids, has grown (FAO, 2020a). Behind only fillets, fish burgers have been recognised as the second-healthiest such products, being popular among consumers worldwide, owing to their ease of use, rich nutritional value and attractive appearance (Zhou *et al.*, 2021).

Fish burgers are seafood products with a circular form, either breaded and pre-fried or unbreaded, mainly obtained from fish fillets or fish pulp mixed with sunflower oil, wheat flour, potato flakes, egg white, salt, cheese, lemon juice and natural flavours. The main fish species used in their production are European sea bass (*Dicentrarchus labrax*), codfish (*Gadus morhua*), Argentine hake (*Merluccius hubbsi*), Atlantic salmon (*Salmo salar*) and yellowfin tuna (*Thunnus albacares*) (Husein, 2019).

As widely reported by several authors (D'Amico *et al.*, 2016; Walker *et al.*, 2017; Marchetti *et al.*, 2020; Piredda *et al.*, 2022), the absence of morphological identification characters, together with the complexity of the fish supply chain, as well as the price differential between lookalike species, and the multiplicity of species and their corresponding values, make processed fishery products very vulnerable to food fraud and mislabelling (Pardo et al, 2016; FAO, 2020b, Mottola *et al.*, 2022). To date, food labelling is the most important instrument for safeguarding consumer safety and should prevent fraud and provide consumers with sufficient information to perform conscious food choices in terms of ethical and sustainable production (Varunjikar *et al.*, 2022). Specifically, regarding processed seafood, the current EU law on food labelling, Reg. (EU) 1169/2011, the instrument aimed to improve transparency and conscious food choices, establishes that it is not mandatory to provide the commercial and/or scientific name or the catching area of the seafood species used, whereas the presence of allergens must be reported. Therefore, the enforcement of food labelling legislation and effective control systems are crucial for the authentication of processed seafood products.

To this aim, DNA-barcoding is well established and has been widely used for species identification (Barbuto *et al.*, 2010; Marchetti *et al.*, 2020). However, this approach is not suitable for species identification of multi-species food matrices, given that Sanger sequencing is able to detect only dominant species, thus failing to identify other species used as ingredients (Paracchini *et al.*, 2019). Recent advances

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in analysis tools came up with Next Generation Sequencing, an assay that simultaneously sequences many molecules of DNA of different targets contained in composite foods, as a promising tool for the authentication of mixed food matrices. In this sense, different studies have applied this technology to identifying ingredients in various types of food, including meat (Xing *et al.*, 2019), honey (Milla *et al.*, 2021), food supplements (Raclariu *et al.*, 2018), vegetable products and herbal medicinals (Bruno *et al.*, 2019; Seethapathy *et al.*, 2019), processed seafood (Giusti *et al.*, 2017; Noh *et al.*, 2021; Piredda *et al.*, 2022), and petfood (Preckel *et al.*, 2021).

The importance of the trade and the authentication of mixed fish products is an important issue concerning food safety and environmental sustainability, given that processing (*e.g.* mincing or mixing) make difficult the identification of species used, as well as such products could be produced by including unexpected rare, endangered or protected species from illegal fishing or toxic species with impacts on marine ecosystems and consumer health (Piredda *et al*., 2022). Considering this, the aim of this study was to apply DNA metabarcoding to track fish species used in the production of multi-species fish burgers sold in Italian markets and verify the compliance with the current food labelling law Reg. (UE) 1169/11.

Materials and methods

Sampling

A total of 5 fish burger samples were purchased from different markets and supermarkets in the Apulia region (Italy). Samples were stored at -20°C until processed.

DNA extraction, purification and sequencing

The extraction and purification of genomic DNA were performed starting from aliquots of 25 mg of each sample, using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), as reported by Piredda *et al.* (2022). In order to verify the purity of the extraction reagents, negative extraction control (no added tissue) (ENC) was included. DNA concentration and purity were established by evaluating the ratio A260 nm/A280 nm using a BioPhotometer D30 filter (Eppendorf, Milan, Italy). Then, the extracted DNA was amplified using the primer pairs 16sf-var 5 '-CAAATTACGCTGTTATCCCTATGG-3 ' and 16sr-var 5 '-GACGAGAAGACCCTAATGAGCTTT-3 ' designed by Chapela *et al.* (2002), amplifying a fragment of 148–209 bp of the 16S ribosomal RNA mitochondrial gene, and previously tested by Giusti *et al.* (2017). The sequencing was carried out on the Illumina NextSeq platform by LGC Genomics GmbH (Berlin, Germany), with a paired-end approach (2×150 bp). PCR negative controls (no template) (PNC) were introduced during the amplification step of library preparation. Raw sequences were deposited in the Sequence Read Archive (SRA) with the following BioSample codes: SRX12375038, SRX12375039, SRX12375040, SRX12375041, SRX12375042 (Table 1).

Data processing and taxonomic assignment

Paired-end reads were processed using the DADA2 R package, and raw sequences were merged into amplicon sequence variants (ASVs) (Callahan *et al.*, 2016). Taxonomic assignment was performed using standalone blast in the blast + suite (Camacho *et al.*, 2009) against the 16S mitochondrial custom database (16S_DB). Assignments with a similarity of <90% were discarded and reads assigned to the same species in the range 100-98% similarity were merged and considered at species level (Barbuto *et al.*, 2010), and values lower than 98% as genus. In the case of species sharing the same sequences, the Lowest Common Ancestor (LCA) (Huson *et al.*, 2007) approach was applied.

Analysis of labels and mislabelling assessment

The label for each sample was checked regarding mandatory information required by Regulation (EU) No. 1169/2011 (*i.e.* commercial name, ingredient list, net quantity, conservation instructions, best before date, company name or code and nutrition declaration, allergens). Then, molecular identifications of each sample were compared with the species declared in the ingredient list. Mislabelling was established if: a) labels did not report all mandatory information reported above; b) the scientific name and/or the commercial designation did not correspond to that detected by the molecular analysis (Piredda *et al*., 2022; Giusti *et al.,* 2017). Furthermore, molecular identifications were cross-checked against the Annex I, Italian MiPAAF Decree dated 22 September 2017 in order to detect the presence of taxa unmarketable in Italy.

Results

DNA metabarcoding analysis and identification

All 5 burger samples passed our quality

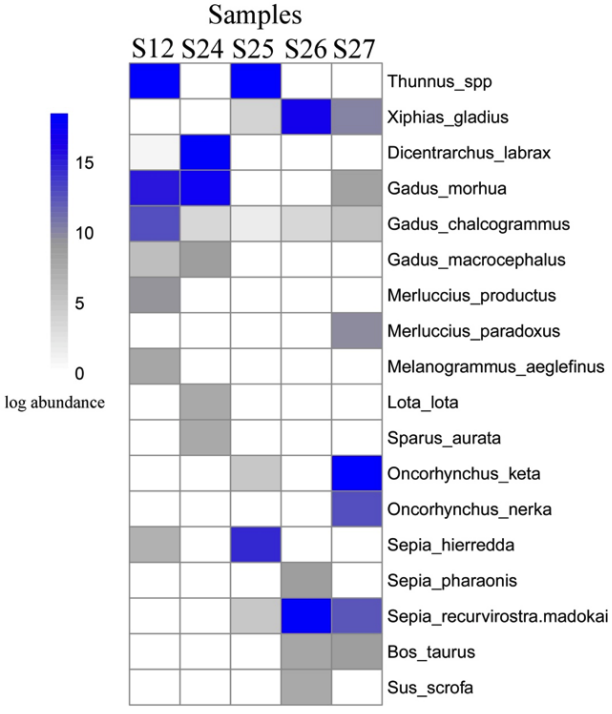

Figure 1. Heat map of taxa in fish burger. Colour gradients correspond to log of relative abundance of sequences.

trimming criteria. PCR negative controls (PNC) did not yield any taxonomic identification. Illumina sequencing generated a total of 1,536,027 raw reads, and quality filtering reduced the dataset to 1,234,869 reads (Supplementary Table 1). After the removal of ASVs with total abundance ≤50 and taxonomic assignment, the final curated dataset included 936,254 reads corresponding to 16 different taxa, including marine and mammalian species. The length of fragment varied in the different taxonomic groups with an average of 214 bp in Actinopterygii and 144 bp in Cephalopoda.

Sixteen out of 18 taxa were unambiguously assigned at species level, with a level of similarity of between 99 and 100%, whereas in two cases different sister species shared the same sequences (*Thunnus tonggol/Thunnus maccoyii/Thunnus thynnus thynnus/Thunnus albacares* and *Sepia recurvirostra/Sepia madokai*) (Supplementary Table 2).

Overall, the dataset included 2 Classes (Actinopterygii and Cephalopoda), 4 Orders (Gadiformes, Perciformes, Salmoniformes and Sepiida), 8 Families and 10 Genera (Supplementary Table 2). At species level, sample S12 was dominated by *Thunnus* spp. (86.2%), *Gadus morhua* (11.8%), *Gadus chalcogrammus* (1.7%), *Melanogrammus aeglefinus* (0.7%), *Merluccius productus* (0.2%), *Sepia hierredda* (0.03%), *Gadus macrocephalus* (0.01%) and *Dicentrarchus labrax* (0.001%). In sample S24, we found two main components *i.e. Dicentrarchus labrax* (61.6%) and *Gadus morhua* (38.2%), with other species found only in traces, *i.e. Lota lota* (0.5%), *Sparus aurata* (0.5%), *Gadus macrocephalus* (0.1%), and *Gadus chalcogrammus* (0.003%). Sample S25 was dominated again by *Thunnus* spp. (94.2%), in combination with *Sepia hierredda* (5.83%) and traces of *Oncorhynchus keta* (0.01%), *Sepia* spp. (0.01%), *Xiphias gladius* (0.004%), and *Gadus chalcogrammus* (0.001%). In sample S26, we found *Sepia* spp. (65.5%) *Xiphias gladius* (34.4%) as dominant and traces of *Sepia pharaonic* (0.12%) and *Gadus chalcogrammus* (0.003%). Finally sample S27 revealed the dominance of *Oncorhynchus keta* (95.9%), low presence of *Oncorhynchus nerka* (1.9%) and *Sepia* spp. (1,4%), and traces of *Xiphias gladius* (0.3%), *Merluccius paradoxus* (0.2%), *Gadus morhua* (0.1%) and *Gadus chalcogrammus* (0.01%) (Table 1, Supplementary Table 3 and Figure 1).

In two samples (S26 and S27), the presence of non-marine taxa was detected. Specifically, *Bos taurus* was detected in samples S26 (0.07%) and S27 (0.012%) and *Sus scrofa* was traced in sample S26 (0.05%) (Table 1, Supplementary Table 3 and Figure 1).

Analysis of labels and mislabelling assessment

Label analysis revealed that all 5 samples were correctly labelled according to the mandatory requirements of Regulation (EU) No. 1169/2011 and that all labels voluntarily reported the commercial designation and the scientific name of the fish used (Table 1).

Considering the mislabelling criterion defined, molecular analysis revealed 4/5 (80%) cases of mislabelling. In particular, in 1/5 sample (S12), the unreported presence of molluscs (allergens) was detected in traces of *Sepia hierredda*, and in 3/5 cases labels failed to match the voluntarily declared scientific names in the ingredient list. In detail, species substitution cases regarded sample S25 where *Sepiella japonicus* was substituted with *Sepia hierredda*; sample S26, where *Sepiella japonicus* was substituted with *Sepia* spp., and sample S27, where *Salmo salar* was substituted with *Oncorhynchus keta* and *Oncorhynchus nerka.* Furthermore, molecular identification also highlighted the presence of one taxa (*Sepia hierredda* in samples S12 and S25) not included in Annex I of the MiPAAF 2017 Decree (Table 1).

Discussion

In this study, a DNA metabarcoding approach was applied for the authentication of commercial multi-species fish burgers. In particular, our comparison between the list of molecular species obtained and the list of labelled species revealed several discrepancies and the presence of other taxa not expressly stated on the label. According to other studies obtained by DNA metabarcoding approaches on multi-species processed seafoods purchased in Italy, Spain and Korea (Giusti *et al.*, 2017; Noh *et al.*, 2021 and Piredda *et al.*, 2022), our research confirmed that DNA metabarcoding is a powerful approach able to detect the raw materials

Table 1. Description of sample labels, molecular identification and mislabelling assessment.

Id sample	Country of production	Packaging	Commercial designation	(Main) ingredients	Declared commercial and scientific name	Molecular identification	Mislabelling	BioSample code
S12	Italy	Frozen	Frozen tuna burger	Yellow fin tuna (40%), Atlantic cod, egg whites	Yellow fin tuna -*Thunnus albacares* and Atlantic cod - *Gadus morhua*	*Thunnus* spp. 86.20%, *Gadus morhua* 11.78%, *Gadus chalcogrammus* 1.73%, *Melanogrammus aeglefinus* 0.7%, *Merluccius productus* 0.2%, ***Sepia hierredda* 0.03%** (*), *Gadus macrocephalus* 0.01% *Dicentrarchus labrax* 0.001%	Yes (a) (*)	SRX12375038
S24	Italy	Frozen	Frozen sea bass burger	Sea bass (45%), Atlantic cod, egg whites, potato flakes	Sea bass - *Dicentrarchus labrax* and Atlantic cod - *Gadus morhua*	*Dicentrarchus labrax* 61.6%, *Gadus morhua* 38.18%, *Lota lota* 0.5%, *Sparus aurata* 0.5%, *Gadus macrocephalus* 0.1%, *Gadus chalcogrammus* 0.003%	No	SRX12375039
S25	Italy	Defrosted, modified atmosphere	Tuna burger	Yellow fin tuna (63%), Japanese spineless cuttlefish, cheese. May contain traces of molluscs, eggs	Yellow fin tuna - *Thunnus albacares* and Japanese spineless cuttlefish - *Sepiella japonica*	*Thunnus* spp. 94.2%, ***Sepia hierredda* 5.8%** (*), *Sepia* spp. 0.01%, *Oncorhynchus keta* 0.01%, *Xiphias gladius* 0.004%, *Gadus chalcogrammus* 0.001%	Yes (b) (*)	SRX12375040
S26	Italy	Defrosted, modified atmosphere	Swordfish burger	Swordfish 58%, Japanese spineless cuttlefish, cheese. May contain traces of molluscs, eggs	Swordfish - *Xiphias gladius* and Japanese spineless cuttlefish - *Sepiella japonica*	*Sepia* spp. 65.5%, *Xiphias gladius* 34.4%, *Sepia pharaonis* 0.12%, ***Bos taurus* 0.07%** (**), ***Sus scrofa* 0.05%** (**) *Gadus chalcogrammus* 0.003%	Yes (b) (**)	SRX12375041
S27	Italy	Defrosted, modified atmosphere	Salmon burger	Salmon 58%, South African hake, cheese. May contain traces of molluscs, eggs	Salmon - *Salmo salar* and South African hake - *Merluccius capensis* or *Merluccius paradoxus*	*Oncorhynchus keta* 96%, *Oncorhynchus nerka* 1.95%, *Sepia* spp. 1.42%, *Merluccius paradoxus* 0.22%, ***Bos taurus* 0.12%** (*), *Gadus morhua* 0.1%, *Gadus chalcogrammus* 0.01%	Yes (b) (**)	SRX12375042

(a) labels did not report all mandatory information required by EU Reg. 1169/2011
(b) the scientific name and/or the commercial designation did not correspond to that detected by the molecular analysis.
*Presence of taxa not included in Annex I of the Italian MiPAAF Decree dated 22 September 2017
**Presence of mammalian DNA

from processed seafood, also identifying unexpected species. These outcomes raise concerns for consumers and suggest the need for urgent measures to enforce food traceability systems.

Overall, the non-conformities are mainly linked to inconsistencies between molecular identification of species and the commercial or scientific name voluntarily declared on the label (60%), as well as to the unreported presence of molluscs (20%). In particular, these cases of incorrect labelling evidently produce not only the violation of the transparent food labelling systems implemented both by the general European food legislation (Reg. (EC) 178/02), but also by the specific law on food labelling (EU Reg. 1169/2011). Furthermore, mislabelling observed also violates the code of conduct on the management of food allergens established by the recent Commission Regulation (EU) 2021/382, as the omission of an allergen declaration also entails important risks for allergic consumers.

In addition to cases of mislabelling, the metabarcoding approach detected the presence of taxa not included in ingredient lists and/or marine species unmarketable in Italy, a phenomenon that may be explained by various factors, including economical or technological ones. The presence of *Sepia hierredda*, a species not listed in Annex I of the Italian MiPAAF Decree of 2017, could be linked either with economic gain or to the intentional mixing of fish gelatine to improve the texture (*i.e.*, viscosity, texture and stability of fish products) of burger products. Indeed, as previously reported by other authors, the use of fish gelatines is an alternative to mammalian gelatine and is, at the same time, a promising solution to enhance the economic value of the fish by-products, thus also reducing the waste generated by the seafood industry (Nitsuwat *et al.*, 2021). On the other hand, the presence of traces of non-listed taxa could be unintentional and linked to the adventitious contamination along the complex fishery supply chain of processed seafood and thus associated with the fact that different fish species are processed within the same processing plants (Di Pinto *et al.*, 2019).

Furthermore, we detected traces of swine DNA, confirming that its presence is probably related to the addition of gelatine extracted from porcine skin, as previously observed in other multi-species processed seafoods (Noh *et al.*, 2021, Piredda *et al.*, 2022). This poses important safety and ethical implications for consumers. Indeed, the addition of swine gelatine could not only infringe cultural and religious dietary laws of the Jewish (Kashrut) and Islamic (Halal) communities, who forbid the consumption of porcine material, but could also entail safety implications, especially as it might be associated with allergic reactions in gelatine-allergic patients (Caponetto *et al.*, 2013; Zin *et al.*, 2021).

From a legislative point of view, the study underlines the need to strengthen European food legislation regarding labelling, as well as to update monitoring systems to ensure the integrity and authenticity of multi-species foods. Indeed, the mislabeling cases observed are mainly due to a mismatch between the species as voluntarily declared on the label and those actually detected by us. On the other hand, producers may voluntarily provide consumers with additional information not so much out of a desire to increase the transparency of their fish product, but rather as a marketing strategy to attract consumers. Indeed, the presence of more detail on labels improves consumer perception of safer and healthier food (Piredda *et al.*, 2022). Therefore, given that advanced molecular approaches can successfully trace species in complex matrices, an update to the current food labelling legislation by extending Reg. (EU) No 1379/2013 to processed seafood could be required. Furthermore, there is a need to improve dedicated official food control programs targeting food fraud to assess the degree of compliance with fish labelling regulations and to minimize health risks for consumers (FAO, 2018). In this sense, even though the recent Regulation (EU) 2017/625 authorizes official control authorities to react to fraudulent practices, European legislation must provide an internationally agreed regulatory definition of "food fraud" for the authentication of food, thus clarifying which infringements the official food control authorities are required to control, and how such controls should be carried out. Also, given that in some cases the presence of undeclared species could be unintentional, the food legislation must clearly define of "accidental and unintentional presence" by defining how to identify and consider unintentional presence and thereby establish a threshold limit for discriminating accidental contamination from intentional practices. Furthermore, food control authorities will have to authenticate and validate traceability in order to implement effective food control programs.

Conclusions

This study shows that, unlike other DNA-based methodologies, innovations in molecular methods make it possible to trace ingredients in mixed fishery products. Though the application of metabarcoding in seafood is still in its infancy, our study helps to highlight the potential use of this approach for food authentication, as well its cost-related limitations and the need for standardization of dedicated pipelines. Advances in these new technologies will soon make it possible to revise current food legislation, so as to introduce an innovative food safety management system for complex global supply chains. The resulting Food Safety Management System (FSMS) should be based on scientific information, historical results, and specific prevention, intervention, and monitoring systems enabling global food safety assessments to be carried out.

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