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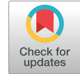
Fish intended for human consumption: from DNA barcoding to a next-generation sequencing (NGS)-based approach



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In food authentication research, species identification was first developed by comparing the sequences of several mitochondrial genes isolated from food with NCBI or Barcode of Life Database (BOLD) data. These methods usually use Sanger methodology for sequencing although they have difficulty in identifying mixed species in processed foods. The first research proposing the use of next-generation sequencing (NGS) for identification of fish for food consumption appeared in 2012. Recently, several platforms used for NGS have shown their capacity to identify up to 15 different fish species or more in a single highly processed fish product.

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Introduction

According to the Food and Agriculture Organization (FAO) [1], fish production and consumption have increased intensely during recent decades, registering global food fish consumption of 156 million tonnes (about 88% of all production) in 2018. Fish demand has grown drastically and in 2017 these foods accounted for 17% of the whole human intake of animal protein. As a consequence of the expansion in the global marketing of fish and fish products, that accounts for a huge number of different species, a significant development in food quality and safety standards was needed. After several food safety crises, global regulations have promoted better supervision of the food production chain, supporting control and traceability systems to ensure the healthiness of products and correct labelling, and to avoid commercial frauds. Nevertheless, there are several safety issues that still need to be controlled, such as inedible and toxic fish [2]. Indeed, chemical hazards such as toxic metal compounds [3] and plastics [4] and/or biological ones pathogenic bacteria, derived compounds such as histamine [5] or parasites [6] can be widely found in some fish species because of their specific habitat or behaviour which make them particularly exposed and susceptible.

In this context, taxonomic identification of commercially valuable fish plays an important control role, despite its complexity that depends on the species of several genera. This approach is useful for controlling and reducing economic fraud, as seafood has been involved in a large number of mislabelling episodes [7]. Furthermore, consumers are increasingly demanding information and understandable labels, preferring otherwise not to buy [8].

Molecular control of fish species using DNA-based methods

Since the beginning of this century, the development of different molecular methods has resulted in the application of DNA-based technologies for species identification. These methods were initially applied for identifying land animals in food products and also in feed, mainly derived from controls related to the bovine spongiform encephalopathy crisis [9]. The first methods to be developed target specific regions such as the mitochondrial cytochrome *b* (*cytb*) gene. Mitochondrial DNA lends itself well to species identification as it has a high number of copies of gene targets, ideal for highly processed products or products with a small amount of tissue. In 2003, Hebert *et al.* [10] launched a global barcoding system based on sequences of the mitochondrial Cytochrome Oxidase 1 (COI) gene, known as the Barcode of Life Database (BOLD). Recent researches on the comparison of the mitochondrial genome of fish species have shown that sequences analyses allows to identify novel gene markers, different from the conventional ones, but effective for unambiguous species identification. This method allows a sequencing-free recognition, through the design of species-specific primers. The technique has been applied to study the mitochondrial genome of 13 Sparidae

[11••,12,13••], recognizing the NAD5 and the NAD2 genes, better than the *COI* or *cytb* genes for species identification, and developing a new method without PCR to extract fish mitochondrial genome for NGS analysis [14]. Despite the high number (273 426) of entries [15] registered as fish in the BOLD database, it would currently be possible to identify only 19 863 fish at species level using DNA barcoding with both GenBank and BOLD ID [15]. Currently, the most common DNA based identification process usually consists of a) extraction of DNA from fish or fish parts, maybe without the preserved morphological aspects needed for taxonomic identification, b) PCR amplification of a fragment of ≈650 bp from the *COI* mitochondrial gene, c) fragment sequencing using Sanger methodology and d) alignment of the sequenced amplicon against the BOLD Systems or GenBank reference databases to identify fish species. This methodology has been widely applied to identify fish fraud worldwide and also for research with pure taxonomic purposes. Overall, the use of DNA barcoding for fish identification follows temporal sequences with a continuous use along the last decade, in Europe [7,16,17], America [18–20], Africa [21–23] and Asia [24,25]. Additionally, in an attempt to identify fish and other species in highly processed products, a version of the DNA barcoding system, called mini-barcoding, has been developed which is capable of amplifying shorter regions (295 bp) of the COI gene [26] or a 198 bp region of the mitochondrial 16S rRNA gene [27]. In any case, this mini-barcoding system is usually less accurate at species identification level. Interestingly, DNA barcoding has recently been applied in combination with the PCR-RFLP (PCR restriction fragment length polymorphism) method to investigate labelling accuracy in processed anchovy [28], in convenience seafood (*Gadus chalcogrammus*, *Merluccius merluccius*, *Merluccius productus* and *Merluccius paradoxus*) [29] and in 'caviar' species [30]. The development of COIBar-RFLP aimed to validate the efficacy of a new method that reduces the costs and the time of fish identifications. It is worth noting that the authors successfully demonstrated its applicability, underlining the ease of the method.

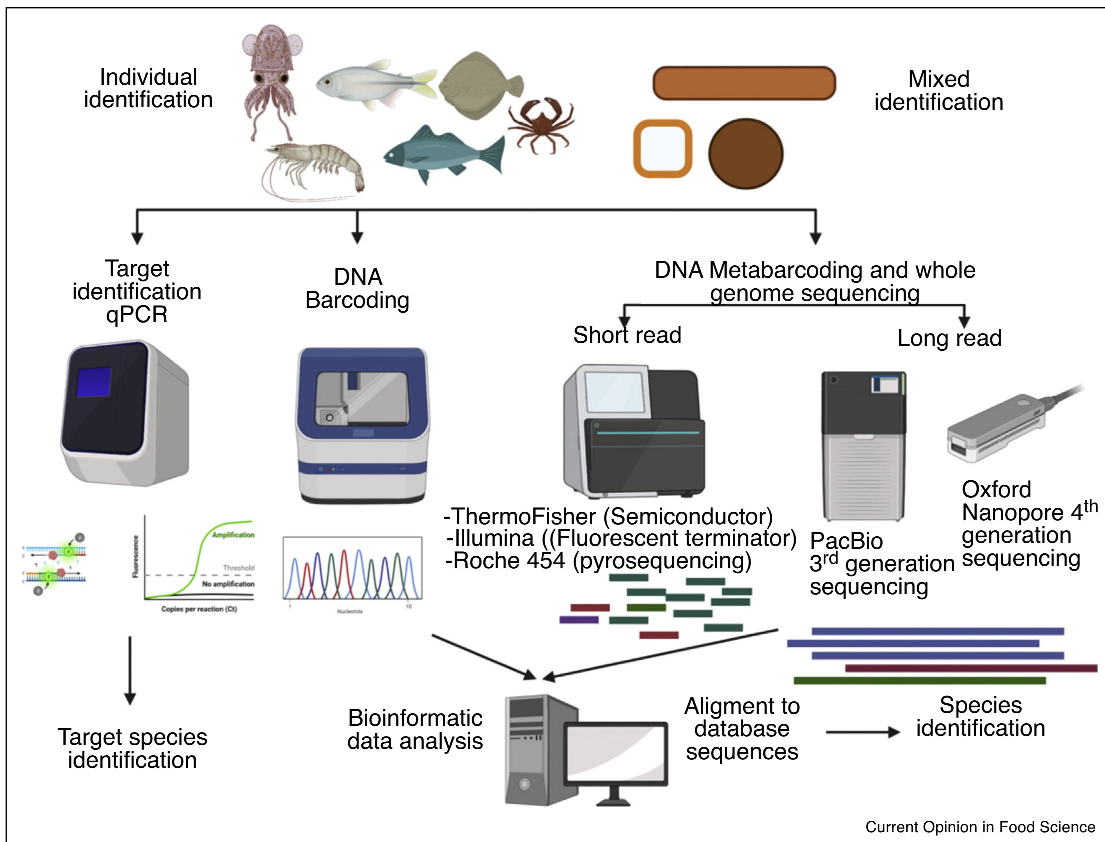
The rise of NGS for fish identification in foods and other related purposes

Despite the widespread use of DNA barcoding, this methodology has several drawbacks. First of all, the number of sequence entries is limited to some fish species. FishBase indicates that there are 33 932 living fish species [31] but only 19 863 species can be identified by the COI gene; the most important flaw is that the Sanger methodology for DNA fragment sequencing, that has limitations for the identification of mixed fish species, especially in highly processed foods [14]. So-called next-generation sequencing (NGS) allows complete sequencing in a sample containing multiple different fragments corresponding to the same gene after PCR amplification, and the identification of different species by combining this technology with bioinformatic tools; in other words, all interesting DNA extracted from a sample can be sequenced at one time. There are several second-generation NGS platforms; according to the scientific literature, the most important and most used are those characterized by 'sequencing by synthesis' where the system detects some aspects produced after each event of fragment synthesis. Examples are pyrosequencing, commercialized by Biotage®, and later Qiagen® and Roche® (discontinued in 2013), which determines the fluorescence of the pyrophosphate released; the Illumina® platform, with fluorescent labelling of a reversible terminator nucleotide; and Thermo Fisher Scientific®'s Ion Torrent™ sequencer, based on detection of the H^+ released during nucleotide incorporation for DNA synthesis using a semiconductor. These technologies have in common the characteristic of preparing the DNA by fragmenting the target DNA into smaller fragments, known as 'library preparation', before sequencing. This is an important feature because this sequencing method uses fragments of 150–800 bp, depending on the platform. Furthermore, third- and fourth-generation NGS sequencing allow direct reading (single molecule in real time) of the genetic sequence without the use of an amplicon template used in the 'sequencing by synthesis' described above. Third-generation NGS is capable of reading fragments of up to 40 kb using platforms such as PacBio RS from Pacific Biosciences®, CGA from Complete Genomics® and HeliScope from Helicos®. Fourth-generation NGS platforms, in addition to the ability to read large DNA fragments, differ in that they are highly portable. The systems developed by Oxford Nanopore Technologies® are called MinION and GridION (high-throughput version) (Figure 1).

Second-generation NGS platforms and fish for human consumption

Park *et al.* [32] made the first approach to using NGS sequencing for fish traceability in the food chain, showing the possibilities of NGS technology. They compared NGS with the use of microarray to detect fish species in fish cakes, using the GS Junior Titanium Sequencing machine from 454 Life Sciences, a technology now discontinued. A couple of years later, De Battisti *et al.* [33] proposed the use of pyrosequencing for the detection of commercial fish fraud. In this work, three different amplicons targeting 16S rRNA, NADH dehydrogenase subunit II and the *cytb* gene were used to control two groups of fish frequently involved in fraudulent practices: the Clupeidae and potential substitutes, and the Pleuronectidae as well as other flatfish, evaluating a total of 116 fish species. The authors proposed a two-step protocol with 16S rRNA amplicon sequencing as the main target for species identification, using the two other targets only in the case of ambiguous identification with 16S RNA. In any case, De Battisti *et al.* [33] indicated that complete analysis could

Figure 1



Main DNA based technologies used to control fish species intended for human consumption.

be carried out in a single run, for a faster procedure. However, they did not evaluate this NGS procedure in highly processed samples containing a mix of several species. The same Italian research group also used pyrosequencing for identification of bivalves intended for human consumption. In this case, amplicons of the 16S rRNA gene (203–288 bp) and COI gene (444 bp) with the use of different primers and the identification nucleotide section ranging from 30 to 40 bp allowed discrimination of the 15 species included in the study [34]. The method employed could detect the simultaneous presence of different species in processed products. Although pyrosequencing can currently be considered an old and no competitive method, it is still useful for identifying fish species in complex fish products. With this technology, it has been possible to distinguish more than 15 species in surimi [35•]. To date, pyrosequencing has been widely used for research on environmental metabarcoding of fish [36], the gut microbiota of fish species [37,38] and antibiotic resistance [39] and also for fish traceability in aquafeed formulations.

Galal-Khallaf *et al.* [21] detected a total of 13 fish species in samples of fish feed for which no fish species were declared among ingredients on the label, using metabarcoding and targeting a short fragment (150 $\approx$ 200 bp) of the COI gene, as recommended for degraded DNA samples. Their important discovery was that most of the fish species detected are overexploited. In spite of the use of pyrosequencing for bacteria, environmental studies or otherwise, this platform is not commonly used for identification of fish in food intended for human consumption, aside it was one of the first to appear. It may be that the rapid development of other second-generation sequencers by Illumina® and Thermo Fisher® caused the displacement of pyrosequencing for this purpose. A group of Italian and Spanish researchers evaluated the use of the NGS semiconductor platform from Thermo Fisher® to detect fish species as well as cephalopods in 16 samples of complex food matrices such as surimi, produced in EU and extra EU countries [40]. This study used primers previously assessed to be used in NGS sequencing [41] for library construction targeting

16S rRNA, *cytb* and *COI* genes capable of amplifying the DNA of over 80 fish and cephalopod species. These researchers found in the 16 samples a total of 13 families, 19 genera and 16 species of fish, and three families with three genera of cephalopods. They found DNA belonging to Gadidae in each sample and also performed relative quantification based on the number of readings. Likewise, they detected up to 15 different species (12 fish samples + 3 cephalopods) in one of the samples, with the majority of the samples consisting of more than three different species. Brazilian and Portuguese researchers [42] also used the Thermo Fisher® platform to detect species mixtures within highly processed cod products. This group sampled a total of 22 cod products consisting of pieces, fish cakes and vacuum-packaged cooked dishes from 17 brands. They found nine samples illegally labelled as containing more than one species or other genera in addition to *Gadus*, therefore proving the possibility of using NGS for the identification of raw or highly processed cooked cod. Second-generation NGS was also used to identify some Scombridae such as mixed tuna samples [43]. The researchers used nine samples, six containing muscle mixtures prepared by grinding and mixing different proportions of five different tuna species (*Thunnus alalunga*, *Thunnus albacares*, *Thunnus obesus*, *Katsuwonus pelamis* and *Sarda sarda*), and three purchased tins of canned tuna. They used two *cytb* fragments amplified in two different PCRs, with product lengths of 131 and 126 bp. Sequencing was carried out using the Illumina® MiSeq platform and using two runs; the researchers concluded that this NGS approach allows the detection of admixtures of as low as 1%. In an imaginative study, the Illumina® platform was also used for a more complex purpose: tracing seafood at high spatial resolution with NGS data and machine learning [44]. The objective was to benefit from the large amount of data generated by NGS to obtain a signature of seafood origin, investigating the SNPs of clams, and the related microbiome in order to study the connection between fish food safety and its specific production place. The authors concluded that the use of machine learning can be useful for processing such a large amount of data generated by NGS, and that microbiome data are better for traceability than typing by SNPs.

Third-generation and fourth-generation NGS platforms and fish for human consumption

Regarding the third generation of NGS machines, little information is available on their use in control of fish species specifically intended for human consumption. However, in the last two or three years, several works have sequenced the whole genomes of different fish species such as *Pelteobagrus fulvidraco* [45], *Larimichthys crocea* [46], *Liza haematocheila* [47] and *Takifugu bimaculatus* [48], among others, mainly using the PacBio® platform from Pacific Systems. In all these cases, PacBio was used for sequencing in combination with the Illumina platform. PacBio carried out long reads, facilitating de novo assembly, and Illumina short reads helped polish the final assembly, increasing accuracy. Compared to the scarce use of third-generation NGS platforms for fish-derived food control, the use of the relatively new fourth-generation platforms has been evaluated for this purpose, specifically Oxford Nanopore Technologies®'s

Table 1

Platform, genes and fragments used for NGS for research on authentication of fish or processed mixed fish intended for human consumption

Platform (method)	Target genes	Length	Reference
Roche 454 (pyrosequencing)	n.d.	300–400 bp	Park *et al.* [32]
PiroMark (Biotage®) (pyrosequencing)	16S rRNA NADH dehydrogenase sub II *cytb*	289 bp 291 bp 520 bp	De Battisti *et al.* [33]
PiroMark (Biotage®) (pyrosequencing)	16S rRNA COI	203–288 bp 444 bp	Abbadi *et al.* [34]
Roche 454 (pyrosequencing)	COI	703 and 737 bp	Noh *et al.* [35•]
Thermo Fisher (semiconductor)	16S rRNA *cytb* COI	<500 bp	Giusti *et al.* [40]
Thermo Fisher (semiconductor)	16S rRNA	250–260 bp 190–200 bp	Giusti *et al.* [41]
Thermo Fisher (semiconductor)	*cytb* COI	100–150 bp	Carvalho *et al.* [18]
Illumina MiSeq (fluorescent terminator)	*cytb*	126 and 131 bp	Kappel *et al.* [43]
Illumina MiSeq (fluorescent terminator)	*NAD5*	265 and 505 bp	Ceruso *et al.* [11••]
Illumina MiSeq (fluorescent terminator)	Complete mitogenome	≈ 16 000 bp	Mascolo *et al.* [14]
MinION Oxford Nanopore Technologies (single molecule real time)	COI *cytb*	500–800 bp	Voorhuijzen-Harink *et al.* [49]
MinION Oxford Nanopore Technologies (single molecule real time)	COI	313 bp ≈ 650 bp	Ho *et al.* [50•]

MinION. For example, Voorhuijzen-Harink *et al.* [49] used two synthetic fish mixtures with known rates of each species in the mixture (one with six different fish species and another with 11 species), adopting PCR enrichment of the *cytb* or *COI* mitochondrial genes and comparing the MinION sequencing with that by MiSeq from Illumina®. Both MinION and MiSeq were able to detect all the species by combining the use of the two sequences, but both platforms failed to identify *Limanda aspera* from *cytb* sequences using R9.4 MinION flow cell chemistry but not with R9.5 MinION flow cell chemistry, and also failed to identify *Scophthalmus rhombus* and *Scophthalmus maximus* from the *COI* sequence. The systems always allowed the detection of weight quantities of at least 5%. Working with real supermarket samples in Singapore, instead of synthetic mixtures, Ho *et al.* [50•] studied a total of 105 samples of fresh and frozen seafood by sequencing of fragments corresponding to the full-length *COI* barcode and a shorter mini-barcode (313 bp). These researchers used MinION with a large number of samples, sequencing a total of 105 samples in one flow cell using a tagged amplicon system, after obtaining a consensus barcode for each genus-specific read set. They reported that clean barcodes, using a single-species approach, failed in some cases due to interference with the bacteria present in the product. However, from our point of view, the high read error rate for MinION, reaching 15% [51] if compared, for example, to Illumina MiSeq's 0.1% [52], could be at the base of these failures. In any case, the portability and pricing benefits of MinION likely compensate this flaw. After modifying the bioinformatic procedures, the most interesting results obtained by Ho *et al.* [50•] concerned product samples containing fish, cephalopods, crustaceans or similar species mixed, purchased from supermarkets and labelled specifically as prawn balls or cuttlefish balls. They were able to identify up to 15 different species in a single sample. Likewise, the identification of pork (*Sus scrofa*) in a significant number of these processed fish balls with obvious labelling errors should be noted. Identity accuracy was above 99% in most cases. The same research team also suggested a 'reasonable capacity would be closer to 1000 samples' in one flow cell, which is a good indication of the vast sequencing capacity of this platform (Table 1).

Conclusions and perspectives

Compared to the employment of conventional Sanger sequencing methods, the use of novel NGS platforms for determining fish authenticity has been poorly proven. Although these systems have been widely used for sequencing whole genomes or for microbiome control, only a dozen works involving fish products for human consumption have benefited from the use of these high-power platforms. However, the ability of NGS systems to detect mixtures of more than 15 different species has been proven. The increase in the number of processed foods consumed by the population indicates using these sequencing methods to simultaneously detect several product characteristics, although this has not currently been done. This may include the detection in surimi, fish balls or any type of highly processed mixture of fish of not only fish and/or cephalopod species but also bacterial pathogens [53,54], antibiotic resistance genes [55,56] or parasitic contaminants [6,57]. All these features can be simultaneously accessible with NGS systems. Although the search for new barcoding regions for fish identification has continued over the past decade, that is, using nuclear targets [58,59•], the future will likely show much more work on employing NGS to control several combined aspects of fish intended for human consumption.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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The authors adopted *NAD2* genes as a DNA barcoding marker for direct identification analysis. The analytic approach, adopted and proposed by researchers, proved to be fast and effective. Indeed, they proposed DNA-sequencing-free method which was able to identify and differentiate the *D. dentex* species from their most substituted species.

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