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COI barcode-HRM as a novel approach for the discrimination of hake species

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ABSTRACT

Hake species of *Merluccius* genus represent an important group of fish commonly sold all over the world. Therefore, they are highly prone to be adulterated, particularly the substitution of *M. merluccius* by other species with lower market value. The present work intended the development of a highly sensitive methodology for the rapid detection and differentiation of hake species based on mini-barcoding of cytochrome c oxidase subunit I (COI) gene combined with high resolution melting (HRM) analysis. The method allowed the full discrimination of *M. merluccius*, *M. productus*, *M. hubbsi*, *M. capensis* and *M. paradoxus* with high levels of confidence. Real-time PCR assay targeting COI mini-barcode provided a high sensitive tool to detect hake species down to 0.2–20 pg of DNA with adequate performance parameters. The application of the COI-HRM approach to 45 fish-containing foods showed that two samples did not comply with the declared species, suggesting mislabelling or species substitution. These findings highlight the need of controlling processed fish-containing foods and the feasibility of the proposed tool for their authentication at trace levels.

1. Introduction

The fish sector is significantly growing over the years due to the global increase of fish consumption worldwide. Merluccidae family encompasses some of the most commercially relevant fish species that are heavily captured nowadays. *Merluccius merluccius* (European hake) is the most appreciated species and its market value is often higher than other hakes within the *Merluccius* genus. Nonetheless, there are important and commonly sold Merluccidae species from other geographical locations, such as *M. productus* (North Pacific hake), *M. hubbsi* (Argentine hake), *M. capensis* (whiting) and *M. paradoxus* (deepwater hake) (Hubalkova et al., 2009; Machado-Schiaffino et al., 2008; Muñoz-Colmenero et al., 2015; Sánchez et al., 2009). Similarly to other fish families, the identification based on visual analysis is very difficult or even impossible due to the phenotypic resemblances of *Merluccius* spp. or when the morphological characteristics are lost (e.g. fillets, tails, loins, compressed blocks, fish sticks), making them prone to mislabelling and fraudulent practices. Species substitution has been widely reported both in the media and in the scientific community, being usually through the deliberate replacement of high-valued species by lower cost ones without the respective labelling correction (Abdullah and Rehbein, 2016; Castigliego et al., 2015; Di Pinto et al., 2013; Hubalkova et al., 2008). According to the European Union (EU) legislation, unprocessed and some processed seafood products (e.g. salted, smoked products) must be labelled with the commercial and the scientific name of the species, while in other products, such as, canned, composite products and breaded products, the inclusion of this type of information is voluntary (Regulation, 2013). Additionally, Regulation (EU) No 1169/2011 (Regulation, 2011) demands the obligatory labelling of a list of allergenic foods, from which fish is included, that should be highlighted from the listed ingredients, regardless of their quantity. Therefore, clear and reliable mechanisms for species certification constitute a benefit for consumers and producers, not only for authentication purposes, but also for assessing the presence of potentially allergenic foods that might be inadvertently undeclared (Hubalkova et al., 2009; Sánchez et al., 2009).

DNA-based methods have proved to be suitable alternatives to the classical protein-based methods and became the methods of preference for the analysis of a wide range of seafood products. Species-specific polymerase chain reaction (PCR), multiplex PCR, PCR followed by analysis of restriction fragment length polymorphisms (PCR-RFLP), forensically informative nucleotide sequencing (PCR-FINS) and single-stranded conformational polymorphism (PCR-SSCP), targeting mostly mitochondrial (mt) DNA markers, are some examples of methods developed for the identification and discrimination of fish species, such as flatfish, gadoids, scombroids, salmonids and percoids (Mafra et al., 2008; Rasmussen and Morrissey, 2008, 2009). More recently, real-time PCR methods have been widely used for fish species identification in foods due to the advantages of high specificity and sensitivity, combined with simplicity and rapidity (Armani et al., 2012; Hird et al., 2012; Sánchez et al., 2009; Sánchez et al., 2013; Taboada et al., 2017). Another recent approach applied to fish species discrimination is based

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on DNA barcoding, for which the cytochrome c oxidase subunit I (COI) gene is the target most commonly used (Armani et al., 2015; Deli Antoni et al., 2015; Di Pinto et al., 2013; Fernandes et al., 2017; Ferrito et al., 2016). DNA barcoding relies on the sequence variation within a short and standardised region of the genome, designated as a "barcode", to enable species identification. In particular, COI fragments of ~650 base pairs have shown to discriminate animal species reliably, including fish (Carvalho et al., 2017; Costa and Carvalho, 2007; Handy et al., 2011; Hebert et al., 2003; Pappalardo and Ferrito, 2015; Pappalardo et al., 2015; Ward et al., 2005). The use of even smaller DNA barcodes (mini-barcodes) has also been used and further recommended when analysing highly processed foods containing degraded DNA (Fernandes et al., 2017; Fields et al., 2015; Little, 2014; Mitchell and Hellberg, 2016; Villa et al., 2016). Besides, mini-barcodes can be combined with high resolution melting (HRM) analysis, which can be used as a cost-effective, specific and high-throughput tool for the discrimination of fragments with small nucleotide differences, therefore avoiding the need of sequencing (Fernandes et al., 2017; Villa et al., 2016; Xanthopoulou et al., 2016). However, only few studies report the approach of mini-barcoding combined with HRM analysis for fish species differentiation (Fernandes et al., 2017; Fitzcharles, 2012).

The main goal of the present work was to develop a highly sensitive methodology for the rapid detection and differentiation of hake species based on mini-barcoding combined with HRM analysis. A COI mini-barcode was *in silico* analysed regarding five *Merluccius* species (*M. merluccius*, *M. products*, *M. paradoxus*, *M. hubbsi* and *M. capensis*), taking into account their genetic variability for primer design and HRM analysis. The method was validated through sequencing and its applicability was further demonstrated by analysing processed fish-containing foods to identify Merluccidae species and to verify labelling compliance.

2. Materials and methods

2.1. Sampling and preparation

Twenty specimens of five *Merluccius* species were acquired at different local markets and further authenticated by sequencing: European hake − *M. merluccius* (n = 4); North Pacific hake − *M. productus* (n = 4); Argentine hake − *M. hubsi* (n = 4); whiting − *M. capensis* (n = 4) and deepwater hake − *M. paradoxus* (n = 4). A total of 49 specimens, one of each species, comprising other fish, crustaceans and molluscs (n = 21) (Atlantic cod − *Gadus morhua*; Alaska pollock − *Theragra chalcogramma;* saithe − *Pollachius virens*; Atlantic salmon − *Salmo salar*; gilt-head bream − *Sparus aurata*; ray − *Raja* spp.; common sole − *Solea solea*; European pilchard − *Sardina pilchardus*; yellowfin tuna − *Thunnus albacares*; squid − *Loligo* spp.; mussel − *Mytilus spp.*; undulated surf clam − *Paphia undulata*; crab − *Portunus validus*; edible crab − *Cancer pagurus*; Caribbean spiny lobster − *Panulirus argus*; Norway lobster − *Nephrops norvegicus*; Whiteleg shrimp − *Litopenaeus vannamei*; Giant tiger prawn − *Penaeus monodon*; Indian white prawn − *Fenneropenaeus indicus*; Jinga shrimp − *Metapenaeus affinis* and Striped shrimp, *Melicertus kerathurus*), meat species (n = 9) (cow, chicken, rabbit, deer, turkey, pork, lamb, goat, ostrich) and plant species (n = 19) (soybean, oat, rye, mint, wheat, lupine, maize, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, nectarine, apricot, strawberry, raspberry, honey, cashew), commonly used as food, were tested to assess the assay specificity. Several processed seafood products (fish sticks, hake/fish fillets, breaded hake/fish fillets, hake patties, hake medallion, fish pies, surimi, pre-cooked dishes, among others, n = 45) were also acquired at local markets and analysed to verify assay applicability.

All the specimens and samples were individually ground and homogenised in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) and stored at −20 °C until additional analysis. All containers and material used during this procedure were previously treated with DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany).

2.2. In silico DNA barcode analysis and primer design

COI sequences of *Merluccius* spp. were obtained from NCBI database with the respective accession numbers (*M. merluccius* − KJ679916.1*; M. productus* − KM528138.1; *M. hubbsi* − KF604889.1; *M. campensis* − KJ879298.1*; M. paradoxus* − KC789253.1). The sequences were aligned with BioEdit v.7.2.5 software (Ibis Biosciences, Canada) and further analysed to check the regions with high sequence homology for primer design, but also including several nucleotide variations to allow amplicon differentiation. Accordingly, two sets of primers were designed, namely MER1COI-F (TCACGGCACACGCCTTCGTAA)/MER1COI-R (TGTCGGGGGCTCCGATCATTA) to produce a fragment of 102 bp for the development of a real-time PCR assay combined with HRM analysis and MER2COI-F (GCATAGTCGGAACAGCCCTAA)/MER2COI-R (CCCAGAATTGATGAAACGCC) targeting a region of 400 bp for sequencing purposes. To check the primer properties, namely GC content, estimated annealing temperature, the absence of hairpins and self-hybridisation, as well as to evaluate their *in silico* specificity towards the available database nucleotide sequences (GenBank), the OligoCalc (http://www.basic.northwestern.edu./biotools/oligocalc.html) and the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) softwares, respectively, were used. Primers were synthesised by STABVIDA (Lisbon, Portugal).

2.3. DNA extraction and its quality assessment

For DNA extraction, the Nucleospin® Food kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's instructions with minor alterations. Briefly, 200 mg of each sample were used and 2 µL of RNAse (2 mg/mL) were added for 5 min at room temperature immediately after the lysis step (1 h incubation at 65 °C). The extractions were performed in duplicate for each sample and all the extracts were kept at −20 °C until further analysis.

For DNA yield and purity determinations, the UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA) and a Take3 micro-volume plate accessory were used, following the defined nucleic acid quantification protocol (double-strand DNA) in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA). Electrophoresis in a 1.0% agarose gel containing Gel Red 1 x (Biotium, Hayward, CA, USA) for staining and carried out in STGB 1 x (GRISP, Porto, Portugal) for 25 min at 200 V was performed to check DNA integrity of the extracts. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. PCR amplification

Reactional mixtures of 25 µL of total volume containing 2 µL of DNA extract (100 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of $MgCl_2$ and 200 nM of each primer (MER1COI-F/MER1COI-R or MER2COI-F/MER2COI-R). The amplifications were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following temperature program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 60 °C for 30 s (or 45 s with primers MER2COI-F/MER2COI-R) and 72 °C for 30 s (or 60 s with primers MER2COI-F/MER2COI-R); and a final extension at 72 °C for 5 min.

PCR amplicons were run in a 1.5% agarose gel containing 1 × Gel Red (Biotium, CA, USA) for staining and carried out in 1 × SGTB buffer (GRISP, Porto, Portugal) for about 20–25 min at 200 V. A digital image