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DNA barcoding coupled to HRM analysis as a new and simple tool for the authentication of Gadidae fish species

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ABSTRACT

This work aimed to exploit the use of DNA mini-barcodes combined with high resolution melting (HRM) for the authentication of gadoid species: Atlantic cod (*Gadus morhua*), Pacific cod (*Gadus macrocephalus*), Alaska pollock (*Theragra chalcogramma*) and saithe (*Pollachius virens*). Two DNA barcode regions, namely cytochrome c oxidase subunit I (COI) and cytochrome b (*cytb*), were analysed *in silico* to identify genetic variability among the four species and used, subsequently, to develop a real-time PCR method coupled with HRM analysis. The *cytb* mini-barcode enabled best discrimination of the target species with a high level of confidence (99.3%). The approach was applied successfully to identify gadoid species in 30 fish-containing foods, 30% of which were not as declared on the label. Herein, a novel approach for rapid, simple and cost-effective discrimination/clustering, as a tool to authenticate Gadidae fish species, according to their genetic relationship, is proposed.

1. Introduction

The nutritional value of seafood, as part of a healthy diet, has resulted in a significant increase in demand for fish over several decades. The collapse of some fish stocks, allied with price and the pursuit for increased profit, has led to high levels of deliberate substitution and mislabelling of fish products (Hellberg, Kawalek, Van, Shen, & Williams-Hill, 2014; Mueller et al., 2015; Sampels, 2015; Wetten, Wilson, & Andersen, 2012). In the European Union (EU), to control and reduce economic fraud, through substitution and/or mislabelling, fish products must be labelled with the commercial and scientific names, fishing and production methods, catch area and the fishing gear. For other processed foods, such as canned, composite products and breaded products, this information is voluntary (Armani et al., 2015; Di Pinto et al., 2013; Regulation (EC) No 104/2000; Regulation (EC) No 206/2009; Regulation (EU) No 1379/2013). Approximately 18% of the world's total catch is fish from the Gadiform order, which corresponded to almost 5.9 million tonnes in 2011 (FAO, 2014). The Gadidae family, in particular, represents an important marine resource and includes commercially important species that are common traditional dietary components for populations around the world. Atlantic cod (*Gadus morhua*), Pacific cod (*Gadus macrocephalus*), Alaska pollock (*Theragra chalcogramma*) and saithe (*Pollachius virens*) belong to this family, and are usually referred to as cod-like species or gadoids. Most of these species are similar in appearance, which makes their morphological identification very difficult or almost impossible. This can contribute to the misbranding of processed codfish products (Calo-Mata et al., 2003; Di Pinto et al., 2013; Hellberg et al., 2014; Wetten et al., 2012).

Food authenticity can be assessed using a broad variety of methods, such as those based on protein or DNA analysis. Within the sphere of DNA-based approaches, much attention has been devoted to DNA barcoding, which relies on sequence variation within a short and standardised region of the genome, designated as "barcode", which provides accurate species identification (Hebert, Cywinska, Ball, & de Waard, 2003). Currently, the mitochondrial genes coding for cytochrome c oxidase subunit I (COI) and cytochrome b (*cytb*) are considered reliable DNA barcodes for the discrimination of animal species (Hebert et al., 2003; Hellberg et al., 2014; Mueller et al., 2015). For the identification of fish species, mitochondrial *loci* have been preferred to nuclear genes because fish genomes are haploid; they are present in high copy numbers (particularly in fish tissues) and their mutation rate is greater than that of nuclear genes (Cline, 2012; Rehbein, 2013). The number of DNA barcodes deposited in databases is growing continuously (http://www.fishbol.org/). So far, several studies have used COI or *cytb* mitochondrial DNA barcoding to identify seafood products and investigate broad patterns in fish mislabelling (Cline, 2012; Di Pinto et al., 2013; Miller & Mariani, 2010; Rasmussen & Morrissey, 2008; Wong & Hanner, 2008; Yancy et al., 2008).

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Despite being a good basis for species differentiation, DNA barcoding cannot be considered cost-effective since it depends on DNA sequencing. To overcome this, real-time PCR coupled to high resolution melting (HRM) analysis offers a rapid, reliable and more economic alternative to exploit DNA barcoding.

HRM analysis is regarded as an excellent tool for the identification and differentiation of closely related species or cultivars, identification of pathogens, screening of genetically modified organisms and detection of food allergens (Druml & Cichna-Markl, 2014; Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014). This analysis has emerged from recent advances in high-resolution instrumentation using new generation fluorescent DNA-binding dyes (e.g. EvaGreen, LC Green and SYTO9). Owing to their ability to specifically bind double stranded DNA, these new dyes can be used at high concentrations and are less likely to cause non-specific amplification or PCR inhibition. This enables detection of subtle fluorescent changes during gradual melting of PCR fragments. HRM consists of a closed-tube post-PCR analysis based on the shape of melting transitions for real-time PCR products, allowing identification of small variations in DNA sequences, such as a single base change (deletion or addition). Recently, real-time PCR coupled to HRM analysis has provided simple, rapid, cost-effective and high-throughput approaches for food testing (Costa, 2013; Costa, Mafra, & Oliveira, 2012). So far, few works have reported the application of HRM analysis to differentiate fish species. McGlauflin et al. (2010) described the use of HRM analysis for the identification of 11 single-nucleotide polymorphisms (SNP) to distinguish rainbow trout (*Oncorhynchus mykiss*) and cutthroat trout (*Oncorhynchus clarkii*). Fitzcharles (2012) also reinforced the potential of HRM analysis as a rapid, robust and reliable technique for the discrimination of species among four Antarctic fish, even when analysing samples with poor DNA quality and quantity. However, none concerned the identification of codfish or cod-like species.

The aim of this work was to develop a robust and highly sensitive methodology for the rapid discrimination of four closely-related fish species from the Gadidae family. For this purpose, two DNA barcode regions, namely COI and the *cytb* genes of the selected species (*G. morhua*, *G. macrocephalus*, *T. chalcogramma* and *P. virens*) were analysed *in silico* to search for genetic variability among them. This variability was exploited using HRM analysis, targeting DNA mini-barcode regions to develop a specific, rapid and cost-effective approach for the identification of gadoid fish species. The proposed method was applied to processed fish-containing foods for species identification and verification of labelling compliance.

2. Materials and methods

2.1. Sample collection and preparation

Authentic samples of Atlantic cod (*G. morhua*) ($n = 4$), Pacific cod (*G. macrocephalus*) ($n = 3$), Alaska pollock (*T. chalcogramma*) ($n = 2$) and saithe (*P. virens*) ($n = 2$) were kindly provided by Pascoal & Filhos SA. In order to evaluate the specificity of the proposed approach, a total of 34 samples, including different fish, crustacean and mollusc species ($n = 8$) (Atlantic salmon – *Salmo salar*, gilthead bream – *Sparus aurata*, ray – *Raja* spp., common sole – *Solea solea*, European pilchard – *Sardina pilchardus*, common shrimp – *Crangon crangon*, yellowfin tuna – *Thunus albacares*, squid – *Loligo* spp.), as well as other animal ($n = 9$) (rabbit, deer, cow, chicken, turkey, pork, lamb, goat, ostrich) and plant species ($n = 17$) (soybean, oat, rye, mint, wheat, lupine, maize, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, nectarine, apricot, strawberry, raspberry) acquired at local markets, were tested. A total of 42 fish-containing foods were also purchased at local markets, comprising a variety of frequently consumed traditional Portuguese products (codfish with cream, codfish cakes, "brás" style codfish, codfish "patanisca"), fish sticks, fish fillets, and patties, among others. The samples were milled and homogenised separately using a laboratory knife mill, Grindomix GM200 (Retsch, Haan, Germany), before being stored at −20 °C for analysis. All containers and material used during this procedure were treated previously with a DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany).

2.2. In silico DNA barcode analysis and primer design

Sequences from *G. morhua*, *G. macrocephalus*, *T. chalcogramma* and *P. virens* were obtained from the NCBI database using the respective accession numbers for COI (KC015380.1, JQ354097.1, JQ354518.1 and KF930285.1, respectively) and *cytb* (EU492141.1, AB078152.1, AB078151.1 and EU492147.1, respectively) regions. Sequence alignment was performed with BioEdit v.7.2.5 (Ibis Biosciences, Carlsbad, USA) to examine variation in both COI (Fig. 1) and *cytb* (Fig. 2) genes among the selected species and their suitability for primer design. Accordingly, two sets of primers were designed for each DNA barcode region (COI and *cytb*), with the aim of using these sequences (Gad1COI-F/Gad2COI-R and Gad2CytB-F/Gad1Cytb-R) in the development of new real-time PCR system (Gad1COI-F/Gad1COI-R and Gad1CytB-F/Gad1Cytb-R) (Table 1). The nucleotide sequences were submitted to a basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which identifies regions of local similarity among homologue sequences from different species and calculates the statistical significance of the matches. Primer specificity was assessed using the Primer-BLAST tool that allows homologies in relation to all sequences available in the NCBI database to be revealed. Primer properties, and the absence of hairpins and self-hybridisation, were assessed using OligoCalc (http://www.basic.northwestern.edu./biotools/oligocalc.html). Primers were synthesised by STABVIDA (Lisbon, Portugal).

2.3. DNA extraction

DNA from all samples was extracted with a Nucleospin® Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions, using 200 mg of each sample. The extractions were performed in duplicate. All extracts were kept at −20 °C until further analysis.

2.4. DNA quality

Yield and purity of extracts were assessed using UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA) fitted with a Take3 micro-volume plate accessory. The DNA content was determined using the nucleic acid quantification protocol with sample type defined as double-strand DNA in Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA). The quality of extracted DNA was also analysed by electrophoresis in a 1% agarose gel containing 1 × Gel Red (Biotium, Hayward, CA, USA) for staining and carried out in 1 × STGB (GRISP, Porto, Portugal) for 25 min at 200 V. The agarose gel was visualised under an 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).