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# White fish authentication by COIBar-RFLP: Toward a common strategy for the rapid identification of species in convenience seafood

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ABSTRACT

The global trade and the increased demand for seafood products have encouraged the common practice of replacement of valuable species with species of lower value worldwide. The species of the genus *Merluccius* are often subject to fraudulent substitution due to their high commercial interest. The present investigation of labeling accuracy on 54 samples taken from 20 convenience seafood products collected from Southern Italy markets, allowed the identification of four species through DNA barcoding: *Gadus chalcogrammus*, *Merluccius merluccius, Merluccius productus* and *Merluccius paradoxus*. Mislabeling was observed in seven of 20 (35%) products (frozen breaded steaks and fish fingers), six of which (30%) were labeled as hake (*M. merluccius*). To reduce analysis time of fish species authentication, a COIBar-RFLP, using DNA barcoding in combination with PCR-RFLP methods, was performed for species discrimination. The restriction enzyme *Hinf*I yielded differential digestion patterns suitable for unveiling inconsistencies between product labels and genetic species identification. The COIBar-RFLP represents an effective tool for fish authentication in convenience seafood and responds to the emerging interest in molecular identification technologies that reduce processing time and eliminate the need for lab-based DNA sequencing.



## 1. Introduction

The growing scientific literature dealing with seafood product traceability has demonstrated that seafood mislabeling has reached alarming levels worldwide (Armani et al., 2015; Benard-Capelle et al., 2015; Changizi, Farahmand, Soltani, Darvish, & Elmdoost, 2013; Garcia-Vazquez et al., 2011; Helyar et al., 2014; Huang et al., 2014; Lamendin, Miller, & Ward, 2015; Pappalardo & Ferrito, 2015a). Contributing factors include trade globalization, an increased demand for fishery products and, at the same time, the depletion of fishery resources in some areas where the consumers demand is growing (Cawthorn, Duncan, Kastern, Francis, & Hoffman, 2015). As a result, commercial fraud based on the intentional substitution of species of high economic value with species of lower value, is greatly increasing. In this context, molecular biology tools have been identified to effectively respond to the growing demand of consumers to know exactly what they eating (what species is it and where was it caught?). This is particularly true for transformed seafood products, because, due to processing, they lose those morphological characters useful in species identification. To address this issue, multiple marker types (mitochondrial genes, microsatellites, SNPs) have been submitted to analytical methods such as nucleotide sequencing, fragment analysis and genotyping (Armani, Castigliego, Tinacci, Gianfaldoni, & Guidi, 2011; Huang et al., 2014; Jerome et al., 2008; Li, Li, Zhang, He, & Pan, 2013; Martinsohn & Ogden, 2009; Rasmussen Hellberg & Morrissey, 2011; Scarano & Rao, 2014). Among these molecular markers, two mitochondrial genes have been widely used for fish species identification in transformed fishery products, namely cytochrome oxidase I (COI) and cytochrome *b* (cyt *b*) (Ogden, 2008). In particular, a partial sequence of the COI gene, referred to as a barcode sequence, enables discrimination of more than 98% of animal species and is currently being used to differentiate between animal taxa, including fish (e.g., Dawnay, Ogden, McEwing, Carvalho, & Thorpe, 2007; Hebert, Cywinska, Ball, & deWaard, 2003, Hebert, Ratnasingham, & deWaard, 2003; Hebert, Stoeckle, Zemlak, & Francis, 2004; Hajibabaei et al., 2006; Lefebure, Douady, Gouy, & Gibert, 2006; Pappalardo, Guarino, Reina, Messina, & De Pinto,

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2011, Pappalardo, Cuttitta et al., 2015, Pappalardo & Ferrito, 2015a, b; Ward, Zemlak, Innes, Last, & Hebert, 2005). Thus, large databases of COI barcode reference sequences generated from voucher specimens worldwide, have been built (Barcode of Life Database- BOLD, Fish Barcode of Life- FishBOL) (Ward, Hanner, & Hebert, 2009), allowing the identification of samples through DNA sequence matching. The socio-economically important applications of DNA barcoding, such as wildlife forensics investigations and marketplace surveys of seafoods and medicinal plants, were recently highlighted by the international scientific community at the 6th International Barcode of Life Conference (Adamowicz, 2015).

The species of the genus *Merluccius* are often subject to fraudulent substitution due to their high commercial interest (Muñoz-Colmenero et al., 2015). The genus comprises twelve morphologically well characterized species of which only *Merluccius merluccius*, the European hake, is distributed along the north east Atlantic coasts of Europe and in the Mediterranean Sea (Campo, Machado-Schiaffino, Perez, & Garcia-Vazquez, 2007). The European hake is one of the most valuable and heavily exploited demersal species whose flesh quality and organoleptic properties are highly valued. The population size of northern European hake precipitously declined during the late 1990s and it was considered at risk of being harvested unsustainably (Murua, Lucio, Santurtun, & Motos, 2006). Although over-fishing remains the major threat to European hake, it should be noted that over the last five years the spawning stock biomass of the species seems to have increased, allowing a shift in status from Vulnerable to Least Concern on the IUCN red list (http://www.iucnredlist.org/details/198562/1). However, the European hake falls within the European Community (EC) priority species for enforcement and/or conservation. Indeed, it was included among the target species of the EC project on the traceability of fish populations and fish products carried out by the consortium FishPopTrace (https://fishpoptrace.jrc.ec.europa.eu/). Several investigations have demonstrated that, on the international market, *M. merluccius* is often substituted in transformed products by less valuable species, which calls for strategies to ensure product authenticity of this high value meat (Quinteiro et al., 2001; Tantillo et al., 2015). The more recent literature on the molecular identification of fish species of commercial interest, advocates COI barcoding as a reliable tool for fish authentication in transformed products (Armani et al., 2015; Benard-Capelle et al., 2015; Cutarelli et al., 2014; Di Pinto et al., 2015; Ferri et al., 2015; Galal-Kallaf, Ardura, Mohammed-Jeba, Borrel, & Garcia-Vazquez, 2014; Helyar et al., 2014; Huxley-Jones, Shaw, Fletcher, Parnell, & Watts, 2012; Lamendin et al., 2015; Mueller et al., 2015; Pappalardo et al., 2015, Pappalardo, Federico, Sabella, Saccone, & Ferrito, 2015). This methodology has also been validated for forensic species identification (Dawnay et al., 2007). Interestingly, the aim of some of these investigations was to search for standardized, rapid and low cost methodologies more suitable for routine species identification screening by authorities for food control. In this regard, PCR restriction fragment length polymorphism (PCR-RFLP) has often been proposed as a practical, simple and rapid technique (Partis et al., 2000) which does not require a high level of expertise in molecular genetics for interpreting results obtained on agarose gels (Akasaki, Yanagimoto, Yamakami, Tomonaga, & Sato, 2006; Besbes, Fattouch, & Sadok, 2012; Hsieh et al., 2010; Rea, Storani, Mascaro, Stocchi, & Loschi, 2009; Sebastio, Zanelli, & Neri, 2001; Zhang, Huang, Cai, & Huang, 2006). Accordingly, DNA barcoding was successfully used in combination with the PCR-RFLP method, the COIBar-RFLP, to investigate labeling accuracy in processed anchovy products by Pappalardo and Ferrito (2015b).

Based on the considerations above and considering that only PCR-RFLP analysis of cytochrome *b* (Akasaki et al., 2006; Hold et al., 2001; Pepe et al., 2005) and mitochondrial DNA control region sequences (Quinteiro et al., 2001) have been used to identify Merlucciidae and Gadidae species in processed fish products, in this study we investigated fish mislabeling in convenience seafood labeled as hake, cod and surimi, using COIBar-RFLP. The aim is to extend the application of this methodology coupling two consolidated methods, that could be considered a good candidate for a common strategy for the rapid identification of species in convenience seafood.

## 2. Materials and methods

### 2.1. Samples and DNA extraction

A total of 54 samples were obtained from 20 different convenience seafood products (such as fish fingers and fillets, seafood and frozen breaded sticks) purchased from several local markets in Southern Italy in 2015. Four brands of fish fingers were labeled as Alaska Pollock and two as “fish”; two brands of frozen breaded steaks were labeled as cod and Pacific hake respectively; two brands of seafood sticks were labeled as surimi, whereas the remaining fish fingers and fillets were labeled as hake. For each brand containing multiple samples, such as fish and hake fingers and seafood sticks, three units or samples were chosen randomly and processed to investigate the presence of multiple species in the product; hake fillets and frozen breaded steaks were instead, purchased and processed as single sample (Table 1). Total genomic DNA was extracted from muscle tissue (25–30 mg) using the DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

### 2.2. COI barcode amplification, sequencing and data analysis

COI sequences were obtained using the primer combination of universal primers VF2_t1- 5′ TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC-3′ and FishR2_t1- 5′CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA-3′ (described in Ward et al., 2005) appended with M13 tails to aid in sequencing (Messing, 1983). All PCR amplifications were carried out in 25 μl using approximately 50 ng of the isolated DNA as a template. In addition, each PCR contained 1X Taq DNA polymerase buffer (supplied by the respective Taq DNA polymerase manufacturer), 1.5–2 mM of $MgCl_2$, 200 mM of each dNTP, 10 pmol of each primer and 0.5 U of Taq DNA polymerase (Platinum Taq DNA polymerase, Invitrogen). An initial denaturation at 94 °C for 7 min was followed by 35 cycles (denaturation at 94 °C for 30 s, annealing at 52 °C for 45 s, and extension at 72 °C for 1 min) and a final extension at 72 °C for 7 min. Negative controls were included in all PCR runs to confirm that no cross-contamination occurred. Double-stranded products were checked by agarose gel electrophoresis and purified with the QIAquick PCR purification kit (Qiagen) and subsequently sequenced in the forward and reverse direction by Genechron (http://www.genechron.it/index.php/sanger-sequencing) using an ABI Prism 3100 automated sequencer (Applied Biosystems) and M13 forward and M13 reverse primers. Sequences were carefully checked and deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 1). The chromatograms obtained were assembled and checked by eye. Edited sequences were aligned using the default settings in ClustalX software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and the alignment was manually revised in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A COI reference library of fifteen *Merluccius* and *Gadus* species sequences from GenBank and fifty-four sequences from processed samples were used to build a dendrogram of K2P distance Neighbor-Joining (NJ) in MEGA v 6.0 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).