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MONOGRAPH

Seafood species identification by DNA barcoding, a molecular tool for food traceability

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ABSTRACT Traceability contributes to improve food safety giving information on animal species, origin, authenticity, composition and production system. Species identification is an important step of seafood traceability and molecular tools have been proved far superior to all other diagnostic methods previously used. The seafood products are particularly affected by commercial frauds based on unintentional or deliberate species substitutions of low value fish species for high value fish. In this review, we summarize the data concerning the level of fish species misidentification in processed products in the Italian fish markets and strengthen that DNA barcoding is an effective molecular tool to track down mislabeling and food frauds. Furthermore, we highlight the COIBar-RFLP (Cytochrome Oxidase I Barcode-Restriction Fragment Lengh Polymorphism), combining two consolidated techniques (COI barcoding and PCR-RFLP) in a new molecular strategy as a rapid method for routine screening to detect the mislabeling of seafood products.

KEY WORDS COIBar-RFLP; DNA Barcoding; Frauds; Seafood products.

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INTRODUCTION

The fish trade globalization and the increased demand for fishery products, have raised important concerns about the food authentication due to the alarming levels of seafood mislabeling worldwide detected (Garcia-Vazquez et al., 2011; Changizi et al., 2013; Helyar et al., 2014; Huang et al., 2014; Armani et al., 2015; Benard-Capelle et al., 2015; Lamendin et al., 2015). As a result, a high and growing interest in the origin of seafood products has been triggered in consumers who demand for food quality and safety assurance. In this context, seafood traceability has become very important to respond to the consumers demand to know what they eating. According to the European Union (EU) regulation 178/2002, traceability is the ability to track any food through all stages of production, processing and distribution (including importation and at retail). More specifically, product tracking is the process that follows the product from upstream to downstream (from beginning to the end) so that, at every stage of the process, appropriate traces or informations can be supplied. Product tracing is the reverse process of the food supply chain, or a method in gathering the informations previously released (Fig. 1). Therefore, traceability contributes to improve food safety giving information on animal species, origin, authenticity, composition and production system.

Focusing on species identification, that is an important step of seafood traceability, advances in molecular biology technologies opened new avenues in the field of food-safety, offering new analytical controls suitable both to enhance the food-safety and food-authenticity of foodstuff for humans and to detect frauds. The reliability and sensitivity of species authentication through molecular biology techniques is far superior to all other diagnostic methods previously used, since it is based both on the study of genes, from which the uniqueness that characterizes all living things, and on stability of DNA to every kind of treatment that is used in the food processing industry. In particular, molecular biology tools allowed to exceed the limits of the morphological approach in species identification. The morphological identification of gross anatomical features of the whole fish according to dichotomous key proposals by the Food and Agriculture Organization (FAO), has represented, for example in Italy, the only method used in identification of fish species as legal standard of value. However, a growing scientific literature dealing with seafood products authentication has demonstrated that the highly automated biomolecular techniques can greatly improve species identification in processed seafood products, especially when due to the industrial processing, species lose those morphological characters useful to recognize them. Multiple marker types (mitochondrial genes, microsatellites, SNPs) have been submitted to analytical methods such as nucleotide sequencing, fragment analysis and genotyping for species identification in processed products. Among these molecular markers, a partial sequence of the mitochondrial gene cytochrome oxidase I (COI) referred to as a barcode sequence, has been widely used for fish species identification in transformed fishery products (Ogden, 2008). The COI DNA barcode has been validated for forensic species identification (Dawnay et al., 2007) and is currently being used to differentiate between animal taxa enabling discrimination for more than 98% of animal species (e.g., Hebert et al., 2003a, b; 2004; Paquin & Hedin, 2004; Ward et al., 2005; Hajibabaei et al., 2006; Lefebure et al., 2006). Based on considerations above, and considering that the new food habits have led to an increased consumption of fresh or frozen cuts, processed and ready to eat food, making species identification very difficult, the aims of the present review are:

1) to summarize the data concerning the level of fish species misidentification in processed products in the Italian fish markets;

2), to strengthen that DNA barcoding is an effective molecular tool to track down mislabeling and food frauds;

3) to recommend the formal adoption of DNA-based procedures for the establishment of effective standardized traceability systems by policy government.

For these purposes, we will describe first the DNA barcoding methodology and then we will report on several cases of fish species substitutions. Finally, we will deal with analytical approaches allowing to improve the rapid identification of species in convenience seafood useful for routine species identification by local authorities.

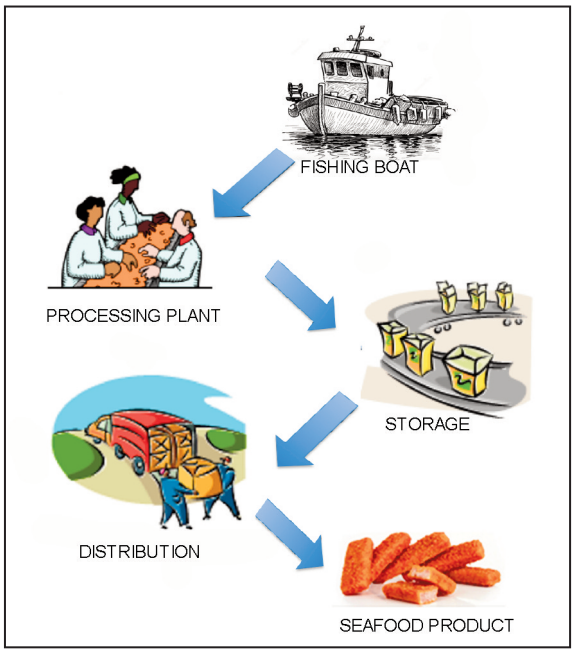

Figure 1. Flowchart of traceability in seafood industry. Arrows indicate product tracking or the process that follows the product from upstream to downstream (from beginning to the end).

DISCUSSION

DNA barcoding as a prime tool of species authentication

Over the last decade, DNA barcoding has

emerged as a universal method to identify living organism. It is based on the sequencing of a short and standardized gene region for the recognition and identification of animal species. However, DNA barcoding does not seek to throw away the morphological studies in support of a narrow and entirely molecular identification system. The overall purpose is to build an alliance between molecular and morphological taxonomists for rapid and unequivocally species identification (Bhattacharya et al., 2015). The quest for a genetic marker useful to determine unambiguously the species is still a matter of debate. Such a genetic marker should have several features. It should show high interspecific but low intraspecific variation to avoid ambiguities in the authentication of species. From the technical point of view it should be characterized by well-preserved PCR-primer sequences at the borders, to guarantee PCR amplification reliable, reproducible, productive and without the risk of producing false negatives, especially in a cluster analysis. Tipically, mitochondrial genes are used for DNA barcoding in animal: the mtDNA has a higher rate of mutation compared to the nuclear genome, is maternally inherited, has a high copy number, which promotes PCR amplification (Hebert et al., 2004). The best candidate to this role has been proposed to be, at least for animals, an approximately 648 bp region, near the 5' end of the mitochondrial Cytochrome Oxidase I (COI) gene, a highly conserved, bioenergetic gene encoding for protein subunits of the respiratory chain and is referred as a "barcode sequence" (e.g. Hebert et al. 2003a, b, 2004; Paquin & Hedin, 2004; Ward et al., 2005; Pappalardo et al., 2011; Pappalardo & Ferrito, 2015a, b; Pappalardo et al., 2015). This gene region generally shows little variation within species but substantial divergence between species, allowing for taxa differentiation (e.g. "barcoding gap") (Mayer & Paulay, 2005). The Consortium of Barcode of Life (CBOL) has indicated this sequence, also know as the "Folmer region", to be the reference barcode for animal organisms. Until now, the adoption of COI as a DNA barcode has been successful in the species identification and in the discovery of cryptic species among amphibians (Vences et al., 2005), ants (Smith et al. 2005), birds (Hebert et al., 2004), collemboles (Hogg & Hebert, 2004), fishes (Ward et al., 2005), mots and butterflies (Ball & Armstrong, 2006; Hajibabaei et al., 2006) and spiders (Barret & Hebert, 2005). Most of this studied species (>94%) showed well separated barcodes, suitable for identification purpose (Ward et al. 2005; Hajibabaei et al., 2006). Generally, two approaches have been employed to analyze DNA barcode sequences and to verify the identity of unknown samples: a similarity search which is conducted with the DNA Identification Engine at BOLD (Barcode of Life Database), based on the Hidden Markov Model (HMM) algorithm (Eddy, 1998), and BLAST algorithm of GenBank (Altschul et al., 1990); and the Neighbour-Joining (NJ) trees built with a distance-based approach to illustrate sequence identity based on tree topology. However, conventional DNA barcoding encounters a problem: DNA degradation in processed biological material often prevents the recovery of PCR fragments longer than 200 bp, impeding full barcode recovery (Hajibabaei et al., 2006). Some authors have proposed the use of a "mini-barcode" sequence to overcome this problem. The mini-barcode system dramatically broadens the applications of DNA barcoding and several authors as Meusnier et al. (2008) have demonstrated that shorter barcode sequences (< 150 bp) represent efficient tags for species identification. According to Ferri et al. (2015) the power of the DNA barcoding is to merge in a single approach the molecularization of identification process, the standardization of molecular markers and analytical procedures and the data computerization of identification results. Information gathered from DNA barcodes can be used across many fields of biology, where species identification play a central role, including ecology, conservation biology, biosecurity, medicine and pharmacology (Pečnikar & Buzan, 2014). Furthermore, a relatively recent and important application aspect of DNA barcoding method concerns the food safety, since the rapid and accurate species identification through this promising tool has proved very useful to detect potentially frauds particularly in transformed seafood products.

Fish market frauds

In the last ten years, a large number of scientific reports have highlighted that fraudulent fish species substitution based on willful or unintentional substitution of low value fish species for high value fish, is common in processed products, such as

fillets and transformed products, due that the morphological identification of the processed species is very difficult or impossible. More specifically, the recent literature deals with the proper identification of species contained in food through the DNA barcoding methodology (Barcaccia et al., 2015) and several investigations have been carried out on seafood products from various marketed brands and on samples purchased in fish marketplaces.

The Italian markets have been investigated to verify the label information of several seafood products. For example, 69 samples of fresh and frozen fish fillets obtained from department stores and fishmongers of four different regions of Northern and Central Italy (Emilia-Romagna, Liguria, Tuscany and Latium) were investigated for label information trough COI DNA barcoding (Filonzi et al., 2010). It was shown that the identified species did not matched with the ones declared on label in 22 samples (32%). The amount of commercial frauds in the trading of shark slices labeled as ‘‘palombo” in Italian markets, was evaluated by Barbuto et al. (2010), which highlighted a relevant economical impact for consumers. Indeed, the recognition of commercialized shark species through the DNA barcoding approach showed a high amount of commercial frauds rising the 80% of analysed ‘‘palombo” slices. Studies by Nicolè et al. (2012) used a multi-locus DNA barcoding strategy for genetic identification of the marine species present in 37 seafood products (30 fish, 3 crustacean and 4 mollusk samples) some of which were fresh or frozen skinned fillets, or heat treated or canned samples. The results of this study showed that the identified species of five samples (13.5 %) did not matched the label information and supported the use of CO1-based identification of fish sample as an efficient tool for food authentication.

More recently, Cutarelli et al. (2014) ascertained possible labeling frauds, made substituting value species with less precious ones, in 58 Italian commercial seafood products from Southern Italy markets (40 samples were whole fish caught in the Mediterranean Sea and 18 samples were commercial fish products). No mislabeling was found for the whole fish sample, while two important frauds were detected in transformed products (11.1%): in a sample sold as cod fillets in butter, the species *Gadus macrocephalus* Tilesius, 1810 (Gadiformes Gadidae) and *G. morhua* Linnaeus, 1758 were substituted by the less valuable species *Pollachius virens* (Linnaeus, 1758), and in a sample sold as frozen grouper fillets that were made of halibut, *Reinhardtius hippoglossoides* (Walbaum, 1792) (Pleuronectiformes Pleuronectidae), instead of grouper, *Epinephelus marginatus* (Lowe, 1834) (Perciformes Serranidae). A 56.6% of mislabeling (17 products out of 30) was reported by Tantillo et al. (2015) for *Merluccius merluccius* (Linnaeus, 1758) (Gadiformes Merlucciidae) or European hake fillet in Southern Italy (Apulia), while only 5% of mislabeling (6 sample on 120) was detected by Di Pinto et al. (2016) in the same region (Apulia) in packaged frozen fishery products sold as breaded hake cutlets, croquettes and sticks, and breaded plaice fillets in market, supermarket and hypermarket chains. However, it would be noted that none of the products analyzed by Di Pinto et al. (2016) declared the presence of *M. merluccius* on the label, suggesting that the substitution of the European hake, when it occurs, is deliberate (Ferrito et al. 2016). The screening of forty fresh and frozen fillet samples labeled as European plaice, *Pleuronectes platessa* Linnaeus, 1758 (Pleuronectiformes Pleuronectidae) and common sole, *Solea solea* (Linnaeus, 1758) (Pleuronectiformes Soleidae) randomly purchased at several supermarkets in Sicily and Calabria, allowed to detect mislabeled products both for European plaice (35 % of the cases) and common sole (41 % of the cases). *Pleuronectes platessa* was replaced by *Platichthys flesus* (Linnaeus, 1758) (Pleuronectiformes Pleuronectidae), *Limanda limanda* (Linnaeus, 1758) and the river fish *Pangasius hypophtalmus* (Sauvage, 1878) (Siluriformes Pangasiidae); *Solea solea* was replaced by *Arnoglossus laterna* (Walbaum, 1792) (Pleuronectiformes Bothidae) (Pappalardo & Ferrito, 2015a).

Toward a common strategy for a rapid identification of fish species: the COIBar-RFLP

Recently, two consolidated methods including COI barcoding and PCR-RFLP were combined in a new molecular strategy (COIBar-RFLP, Cytochrome Oxidase I Barcode-Restriction Fragment Lengh Polymorphism) for fish species identification in processed seafood products (Pappalardo & Ferrito, 2015b; Ferrito et al., 2016) (Fig. 2). The aim was to perform a rapid and easy molecular approach

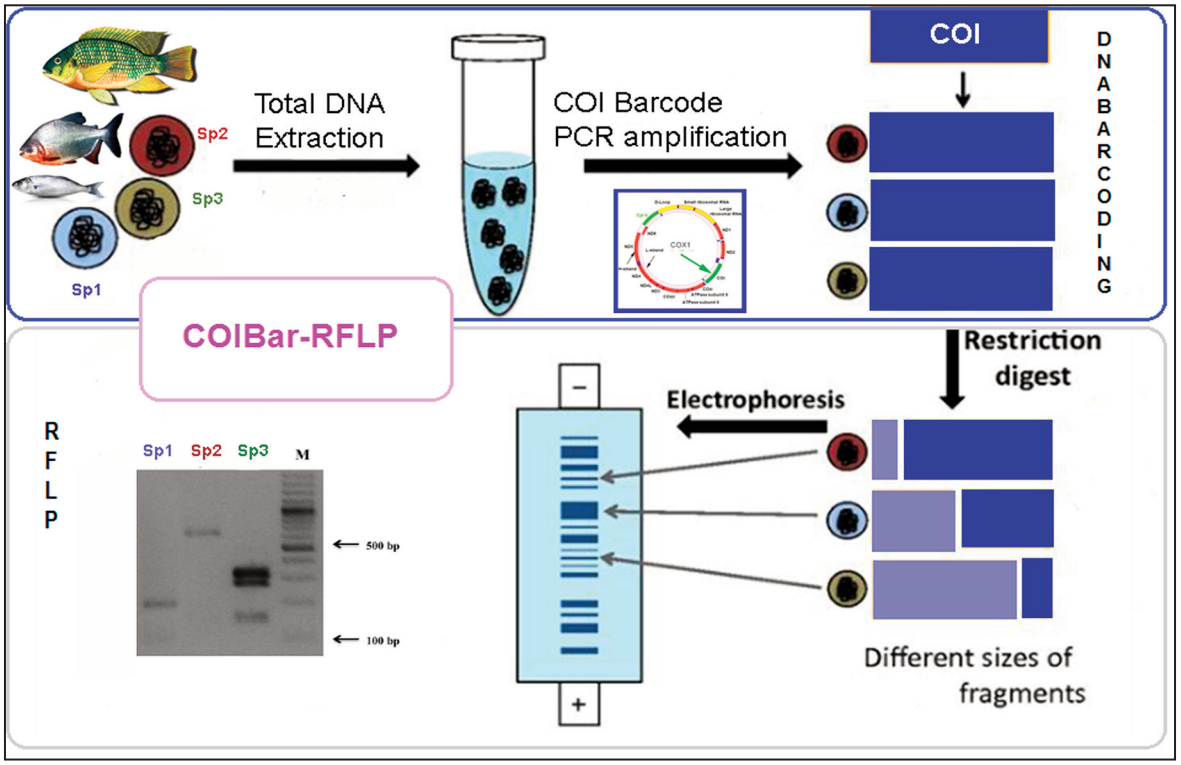

Figure 2. Diagram summarizing the steps of the DNA barcoding method (above) and of the RFLP (Random fragment Length Polymorphism) method (below) combined in the COIBar-RFLP strategy.

by using the conventional DNA barcoding and a traditional PCR-restriction fragment length polymorphism method to unveil potential mislabeling commercial frauds. Emerging molecular techniques have recently been used for seafood fish species identification, but most of them are currently only available for use by specialists in specially-equipped laboratories and they include very expensive methods such as real-time PCR, microarray technology, and next-generation sequencing (NGS) (e.g. Balitzki-Korte et al., 2005; Kochzius et al., 2008; Teletchea et al., 2008; Helberg & Morrissey, 2011; Pascoal et al., 2012; Chuang et al., 2012; Li et al. 2013; Prado et al., 2013). On the other hand, PCR-restriction fragment length polymorphism (PCR-RFLP) has proven to be a practical, simple and rapid technique (Partis et al., 2000) and a high level of expertise in molecular genetics is not necessary for interpreting results obtained on agarose gels. In RFLP analysis, the DNA is cutted into fragments by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. Therefore, PCR-RFLP may be considered a suitable technique for routine species identification in processed fishery products, showing excellent potential even in the case of mixtures of species (Rea et al., 2009).

The COIBar-RFLP analysis was applied to investigate labeling accuracy in processed anchovy products to unveil putative fish fraud involving the replacement of the European anchovy, *Engraulis encrasicolus* (Linnaeus, 1758), with less valuable Engraulidae and Clupeidae species (Pappalardo & Ferrito, 2015b). Four different species, *E. encrasicolus*, *E. japonicus* (Temminck et Schlegel, 1846), *Sardinella aurita* Valenciennes, 1847 and *Sardina pilchardus* (Walbaum, 1792),were found in the processed products labeled as European anchovy and the COIBar-RFLP yielded differential patterns of MboI restriction sites allowing the unambiguous discrimination of European anchovy from the other species. The COIBar-RFLP was also performed for white fish authentication in convenience seafood (Ferrito et al., 2016). In conflict with the Italian Ministerial Decree (MD) of January, 31, 2008 stating that fish products labeled as hake must

contain only the species *M. merluccius*, four species, *Gadus chalcogrammus* Pallas, 1814, *M. merluccius*, *M. productus* (Ayres, 1855) and *M. paradoxus* Franca, 1960, were found in 30% of products (frozen breaded steaks and fish fingers) collected from Southern Italy markets and labeled as hake. The restriction enzyme HinfI yielded differential digestion patterns suitable to discriminate the four species and to unveil inconsistencies between product labels and genetic species identification.

CONCLUSIONS

Mislabeling detected through molecular tools has been reported for seafood products worldwide (e.g. Garcia-Vasquez et al., 2011, Chanzigi et al., 2013, Galal-Kallaf et al., 2014, Benard-Capelle et al., 2015, Carvalho et al., 2015, Cawthorn et al. 2015, Lamendin et al., 2015). In particular, COI DNA barcoding has been adopted by the United States Food and Drug Administration (FDA) as the primary method of regulatory control of seafood products in the United States (Handy et al., 2011); by the governmental Brazilian Consumers Protection Agency for application of financial penalties, due to detection of mislabeling and species substitution in seafood products (Carvalho et al., 2015); and in Canada, which is in the process of incorporating DNA barcoding into its regulatory framework for fish species authentication (Clark, 2015). The incorporation of DNA barcoding methods of identification for law enforcement in the Italian food control system, although inevitable in the future, today remains a challenge (Ferrito et al., 2016). We hope for the formal adoption of DNA-based procedures for the establishment of effective standardized traceability systems in Italy, and in this context we encourage local authorities to carry out pilot projects on the effectiveness of traceability molecular tools such as COIBar-RFLP for routine screening to detect the mislabeling of seafood products.

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