I.A.M.C.-CNR di Capo Granitola

Development of a simple and fast “DNA extraction kit” for sea food identification and marine species.

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Introduction

Seafood products fraud, the misrepresentation of them, have been discovered all around the world in different forms as false labeling, species substitution, short-weighting or over glazing in order to hide the correct identity, origin or weight of the seafood products.

Due to the value of seafood products such as canned tuna, swordfish or grouper, these species are the subject of the commercial fraud is mainly there placement of valuable species with other little or no value species. A similar situation occurs with the shelled shrimp or shellfish that are reduced into pieces for the commercialization.

Food fraud by species substitution is an emerging risk given the increasingly global food supply chain and the potential food safety issues. Economic food fraud is committed when food is deliberately placed on the market, for financial gain deceiving consumers (Woolfe, M. & Primrose, S. 2004). As a result of the increased demand and the globalization of the seafood supply, more fish species are encountered in the market. In this scenary, it becomes essential to unequivocally identify the species.

The traditional taxonomy, based primarily on identification keys of species, has shown a number of limitations in the use of the distinctive features in many animal taxa, amplified when fish, crustacean or shellfish are commercially transformed. Many fish species show a similar texture, thus the certification of fish products is particularly important when fishes have undergone procedures which affect the overall anatomical structure, such as heading, slicing or filleting (Marko et al., 2004). The absence of morphological traits, a main characteristic usually used to identify animal species, represents a challenge and molecular identification methods are required. Among them, DNA-based methods are more frequently employed for food authentication (Lockley & Bardsley, 2000).

In addition to food authentication and traceability, studies of taxonomy, population and conservation genetics as well as analysis of dietary habits and prey selection, also rely on genetic analyses including the DNA barcoding technology (Arroyave & Stiassny, 2014; Galimberti et al., 2013; Mafra, Ferreira, & Oliveira, 2008; Nicolé et al., 2012; Rasmussen & Morrissey, 2008), consisting in PCR amplification and sequencing of a COI mitochondrial gene specific region.

The system proposed by P. Hebert et al. (2003) locates inside the mitochondrial COI gene (cytochrome oxidase subunit I) the bioidentification system useful in taxonomic identification of species (Lo Brutto et al., 2007).

The COI region, used for genetic identification - DNA barcode - is short enough to allow, with the current technology, to decode sequence (the pairs of nucleotide bases) in a single

step. Despite, this region only represents a tiny fraction of the mitochondrial DNA content in each cell, the COI region has sufficient variability to distinguish the majority of species among them (Biondo et al. 2016).

This technique has been already employed to address the demand of assessing the actual identity and/or provenance of marketed products, as well as to unmask mislabelling and fraudulent substitutions, difficult to detect especially in manufactured seafood (Barbuto et al., 2010; Galimberti et al., 2013; Filonzi, Chiesa, Vaghi, & Nonnis Marzano, 2010). Nowadays,the research concerns the use of genetic markers to identify not only the species and/or varieties of fish, but also to identify molecular characters able to trace the origin and to provide an effective control tool forproducers and consumers as a supply chain in agreementwith local regulations.

Materials and Methods

Ten species of high economic interest were selected after doing a survey on local species. Market survey was conducted through a questionnaire at the fish shops and fish markets, school students and local restaurants. Ten species greatest economic impact were selected, these include five fish species (including 1 also achieved through mariculture industry); 3 molluscs and 2 crustaceans were the following:

- sardine (*Sardina pilchardus*);
- mackerel (*Scomber scombrus*);
- sea bream (*Sparus aurata*);
- swordfish (*Xiphias gladius*);
- bass (*Dicentrarchus labrax*);
- red shrimp (*Aristaeomorpha foliacea*);
- lobster (*Palinurus elephas*);
- cuttlefish (*Sepia officinalis*);
- squid (*Loligo vulgaris*);
- tattler(*Todarodes sagittatus*).

DNA extraction protocol

DNA is extracted using a fast DNA extraction method for sea food and marine species identification described by Tagliavia et al (2016). A little piece of muscle tissue (size approximately 2-20 mg), is cut with the help of scalpels and tweezers. The sample is positioned into 1.5 ml tubes; 100 mL of solution of Sol 1 (200 mM KOH, 2 mM Na 2 EDTA, 0.2% Triton X-100) is added to the sample into the tube and this is incubated at 60°C in a thermal bath for 15minutes or until the sample appears not dissolved.

When the sample is digested 3 volumes of Sol 2 (Tris-HCl 100 mM) are added into the tube. At this point, total DNA is extracted and represent the template to use for PCR reaction.

Sequences and primers selection

In order to investigate the fish diversity through a molecular approach, a bibliographic survey was carried out using different queries on different digital platforms, including PubMed database at NCBI and Europe PMC database in EBI. After evaluation and alignment of available sequences from the database, it was selected regions that contained elements of unique nucleotide sequence. On this basis, for each species were identified sub-species-specific region, that permits the discrimination of species of commercial interest by others (kindred). In all species analyzed it was identified a highly specific region such as to allow its use for the unique identification of the species. Oligonucleotides pairs were designed (by software Primer3Plus) to find perfect complementarity, and then allow the amplification of each region identified exclusively in the species of interest. Amplification of species-specific region in each sample is assured due to the creation of highly specific primers complementary to conserved regions at the level cyt b and COI. In the construction of species-specific primers, it has paid particular attention to possible similar sequences present in related species, in order to exclude possible false positive in the case where the sample is made from related species.

Each sample was tested for the amplification of universal DNA sequences in vitro (*Barcoding approach*), with primers common to all fish (Ward et al., 2005) and universal primers for invertebrates (Folmer etal. 1994). The correct amplification of these regions is a "necessary quality control" of the biological sample and its suitability for carrying out molecular tests and it is used as a internal control. Contemporaneously to the control region for each sample it was amplified a species-specific sequence that allow the correct identification of the species.. Each test involves in a PCR reaction (Polymerase Chain Reaction) in wich the use of three or four oligonucleotides (Folmar’s or Ward’s couple primers) and specie-specific primer are used. Whose combination allows the simultaneous amplification of an amplicon that serves as an internal control reaction, and

a second amplicon species-specific, whose presence confirms the species of belonging of the sample.

PCR amplification

The PCR reactions are set up using 1 µl of total DNA as template in a total volume of 25 µl according to the following reaction scheme.

Component	Reaction of 25 µl	Final concentration
dH_2O	until 25 µl	
5x Reaction buffer (not supplied in the kit)	5 µl	1x
10 mM dNTPs	X µl	200 µM
Primer mix (ID_n o U_n)	X µl	0,5 µM
DNA	1 µl	
Phyre Hot Start DNA Polymerase (not supplied in the kit)	0,5 µl	

Each sample is subjected to two PCR, a reaction with specific primers (IDn) and a second one with the universal primers (Un). The U1 primers (Ward et al. 2005) represents the internal control for the fish samples. The U2 primers (Folmer et al. 1994) represent the internal control for the specific reactions of molluscs and crustaceans. The U3 primers represent the internal control for *Sardina pilchardus* samples (Tab. 1). Each species has its own primers.

Table 1: List of control primers used in the amplification reactions by PCR.

Controls	oligonucleotide sequenze (5'-3')
U1 **Fish**	TCAACCAACCACAAAGACATTGGCACA[a] TAGACTTCTGGGTGGCCAAAGAATCA[b]
U2 **Shellfish-Crustaceans**	GGTCAACAAATCATAAAGATATTGG[a] TAAACTTCAGGGTGACCAAAAAATCA[b]
U3 ***Sardina pilchardus***	GGCACCCTCTACCTAGTGTTTG[a] CCCACACCATTCCTATGTAGC[b]

[a]Forward primer [b]Reverse primer

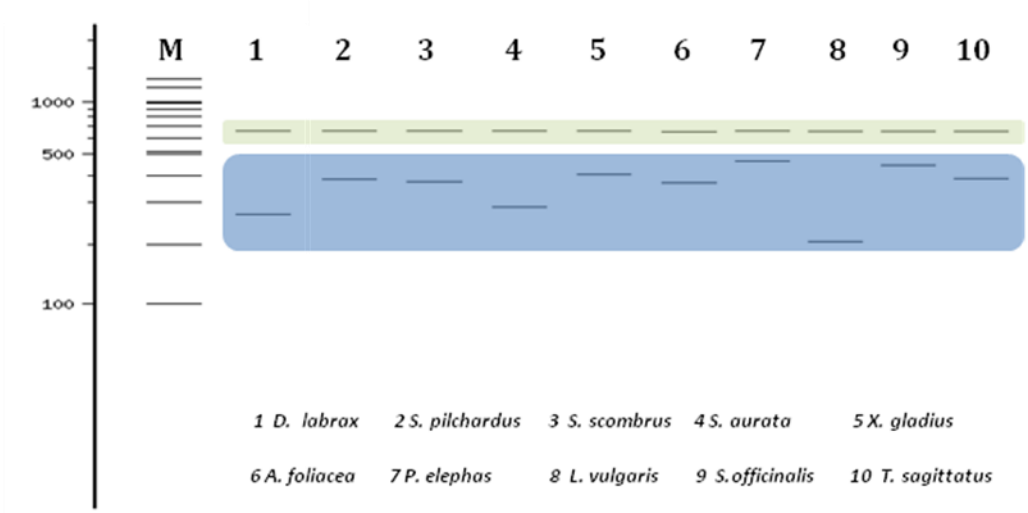
Reaction profile

The PCR reaction profiles to be used for the samples to be analyzed are described in Table 1 below:

Sample	Reaction profile
Sparus aurata	*Initial denaturation*
Xiphias gladius	15 seconds at 98° C
Sardina pilchardus	*40 cycles of the following profile*
Scomber scombrus	10 seconds at 98° C
Dicentrarchus labrax	10 seconds at 55 ° C
	15 seconds at 72 ° C
	Final extension
	1 minute at 72C°
Palinurus elephas	*Initial denaturation*
Aristeomorpha foliacea	15 seconds at 98° C
Loligo vulgaris	*40 cycles of the following profile*
Sepia officinalis	10 seconds at 98° C
Todarodes sagittatus	15 seconds at 48 ° C
	20 seconds at 72 ° C
	Final extension
	1 minute at 72C°
Sardina pilchardus	*Initial denaturation*
	15 seconds at 98° C
	20 cycles of the following profile
	10 seconds at 98° C
	15 seconds at 54 ° C
	20 seconds at 72 ° C
	20 cycles of the following profile
	10 seconds at 98° C
	15 seconds at 56° C
	20 seconds at 72 ° C
	Final extension
	1 minute at 72C°

Results

Graphic representation of the expected results for individual species on agarose gel 2% after electrophoretic run; the band corresponding to 650 bp represents the internal control, the other bands are the species-specific control for each different species.



References

Armstrong KF, Ball SL (2005) DNA barcodes for biosecurity: invasive species identification. Philos T Roy Soc B 360: 1813-1823.

Arroyave, J., & Stiassny, M. L. J. (2014). DNA barcoding reveals novel insights into pterygophagy and prey selection in distichodontid fishes (Characiformes: Distichodontidae). Ecology and Evolution, 4(23), 4534–4542.

Azzurro E, Moschella P, Maynou F (2011) Tracking Signals of Change in Mediterranean Fish Diversity Based on Local Ecological Knowledge. PLoS ONE 6: e24885.

Barbuto, M., Galimberti, A., Ferri, E., Labra, M., Malandra, R., Galli, P., et al. (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products: The Italian case of "palombo" (Mustelus spp.). Food Research International, 43. 376-381.

Bergsten J, Bilton DT, Fujisawa T, Elliott M, Monaghan MT, et al. (2012) The effect of Geographical Scale of Sampling on DNA Barcoding. SystBiol 61: 1-19.

Bianchi CN, Morri C (2000) Marine biodiversity of the Mediterranean Sea: situation, problems and prospects for future research. Mar Pollut Bull 40: 367-376.

Biondo, G., Armeri, G.M., Bennici, C. D., Nicosia, A., Tagliavia, M., Maneiro, I., Torri M., Masullo ,T., Monastero, C., Di Natale, M.V., Mangiaracina, F., Giaramita, N., Maggio, T., Lo Brutto, S., Arculeo, M., Mazzola, S. and Cuttitta ,A..

Cerutti-Pereyra F, Meekan MG, Wei N-WV, O'Shea O, Bradshaw CJA, et al. (2012) Identification of Rays through DNA Barcoding: An Application for Ecologists. PLoS ONE 7: e36479. doi:10.1371/journal.pone.0036479.

Coll M, Piroddi C, Steenbeek J, Kaschner K, Ben RaisLasram F, et al. (2010) The biodiversity of the Mediterranean Sea: Estimates, Patterns and Threats. PLoS ONE 5: e11842.

Costa FO, Antunes PM (2012) The contribution of the Barcode of Life initiative to the discovery and monitoring of Biodiversity. In: Mendonc¸a, A., et al. (Ed.).Natural.

Costa FO, Carvalho GR (2007) The Barcode of Life Initiative: synopsis and prospective societal impacts of DNA barcoding of fish. Genomics, Society and Policy 3: 29-40.

Costa FO, Carvalho GR (2010) New insights into molecular evolution: prospects from the Barcode of Life Initiative (BOLI). TheorBiosci 129: 149-157.

Costa FO, Landi M, Martins R, Costa MH, Costa ME, et al. (2012) A ranking system for reference libraries of DNA barcodes: application to marine fish species from Portugal. PLoS ONE 7: e35858.

FAO (2003a) Trends in oceanic captures and clustering of large marine ecosystems-2 studies based on the FAO capture database. FAO fisheries technical paper 435. 71 p.

FAO (2003b) Fisheries Management-2. The Ecosystem Approach to Fisheries. FAO Technical Guidelines for Responsible Fisheries 4. 112 p.

Filonzi, L., Chiesa, S., Vaghi, M., & Nonnis Marzano, F. (2010). Molecular barcoding reveals mislabelling of commercial fish products in Italy. Food Research International, 43, 1383-1388.

Folmer O, Black M, Hoeh W, Lutz R. et Vrijenhoek (1994). DNA primers for amplification of mitochondrial

cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294-299.

Galimberti, A., De Mattia, F., Losa, A., Bruni, I., Federici, S., et al. (2013). DNA barcoding as a new tool for food traceability. Food Research International., 50, 55–63.

Hajibabaei M, Smith MA, Janzen DH, Rodriguez JJ, Whitfield JB, et al. (2006) A minimalist barcode can identify a specimen whose DNA is degraded. MolEcol Notes 6: 959-964.

Handy S M et al. (2011). A single laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. J AOAC Int 94, 201-210.

Handy SM, Deeds JR, Ivanova NV, Hebert PD, Hanner RH, et al. (2011) A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. J AOAC Int 94: 201-210.

Hanner R, Becker S, Ivanova NV, Steinke D (2011) FISH-BOL and seafood identification: Geographically dispersed case studies reveal systemic market substitution across Canada. Mitochondrial DNA 22: 106-122.
Hebert PDN, Cywinska A, Ball SL, de Waard JR (2003) Biological identification through DNA barcodes. Proc R SocLond B 270: 313-322.

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, et al. (2008) Identifying Canadian Freshwater Fishes through DNA Barcodes. PLoS ONE 3(6): e2490.doi:10.1371/journal.pone.0002490.

Joly S, Davies TJ, Archambault A, Bruneau A, Derry A, et al. (2014) Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. MolEcol Res 14: 221-232.

Knibb W, Whatmore P, Lamont R, Quinn J, Powell D, Elizur A, Anderson T, Remilton C, Hong Nguyen N. Can genetic diversity be maintained in long term mass selected populations without pedigree information? - A case study using banana shrimp Fenneropenaeus merguiensis. Aquaculture, 2014,428-429,7.

Landi M, Dimech M, Arculeo M, Biondo G, Martins R, et al. (2014) DNA Barcoding for Species Assignment: The Case of Mediterranean Marine Fishes. PLoSONE 9(9): e106135. doi:10.1371/journal.pone.0106135
Lo Brutto S, Arculeo M, Grant WS (2011) Climate change and population genetic structure of marine species. ChemEcol 27: 107-119.

Lockley, A., & Bardsley, R. (2000). DNA-based methods for food authentication. Trends in Food Science and Technology, 11, 67–77.

Loukovitis, D., Sarropoulou, E., Vogiatzi, E., Tsigenopoulos, C. S., Kotoulas, G., Magoulas, A. and Chatziplis, D. (2012). Genetic variation in farmed populations of the gilthead sea bream Sparusaurata in Greece using microsatellite DNA markers. Aquaculture Research, 43: 239-246. doi: 10.1111/j.1365-2109.2011.02821.x.

Mafra, I., Ferreira, I. M. P. V. O., & Oliveira, M. B. P. P. O. (2008). Food authentication by PCR-based methods. European Food Research and Technology, 227, 649-665.

Marko, P. B. et al. Fisheries: mislabelling of a depleted reef fish. Nature ***430,*** *309–310 (2004).*

Matzen da Silva J, Creer S, dos Santos A, Costa AC, Cunha MR, et al. (2011) Systematic and Evolutionary Insights Derived from mtDNA COI Barcode Diversity in the Decapoda (Crustacea: Malacostraca). PLoS ONE 6(5): e19449. doi:10.1371/journal.pone.0019449.

Nicolé, S., Negrisolo, E., Eccher, G., Mantovani, R., Patarnello, T., Erickson, D. L., et al. (2012). DNA barcoding as a reliable method for the authentication of commercial seafood products. Food Technology and Biotechnology, 50(4), 387.

Pèrez-Barros P, d'Amato ME, Guzmàn NV, Lovrich GA (2008) Taxonomic status of two South American sympatric squat lobsters, Munidagregaria and Munidasubrugosa (Crustacea: Decapoda: Galatheidae), challenged by DNA sequence information. Biological Journal of the Linnean Society 94: 421-434.

Rasmussen RS, Morrissey MT (2008) DNA-Based Methods for the Identification of Commercial Fish and Seafood Species. Compr Rev Food Sci Food Saf 7: 280-295.

Rasmussen, R. S., & Morrissey, M. T. (2008). DNA-based methods for the identification of commercial fish and seafood species. Comprehensive Reviews in Food Science and Food Safety, 7, 280-295.

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). MolEcol Notes 7: 355-364.

Sawayama E & Takagi M. Genetic diversity and structure of domesticated strains of red sea bream, Pagrus major, inferred from microsatellite DNA markers. Aquaculture Research, 2015, 46, 9.

Steinke D, Hanner R (2010). The FISH-BOL collaborators' protocol. Mitochondrial DNA. 21: 1-5.
Steinke D, Zemlak T S, Gavin H, Hebert (2009). DNA barcoding of Pacific Canada's fishes. Mar Biol 156, 2641-2647.

Tagliavia, M., Nicosia, A., Salamone, M., Biondo, G., Bennici, C. D., Mazzola, S., Cuttitta A. Development of a fast DNA extraction method for sea food and marine species identification. Food Chemistry 203 (2016) 375–378.

Tamura K, Dudley J, Nei M, Kumar (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes. FISH-BOL. J Fish Biol 74: 329-356.

Woolfe, M. & Primrose, S. Food forensics: using DNA technology to combat misdescription and fraud. Trends Biotechnol ***22,*** *222–226 (2004).*

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- Project Bandiera RITMARESP2_WP4_AZ2_UO04_D06. A. Cuttitta.

- Project: “Tecnologie e processi per il miglioramento della shelf-life dei prodotti del comparto agroalimentare attraverso l’uso di film edibili innovativi a base pectinica” ("PON FILMEDIBILI", Cod. PON01_02286 - CUP: B68F12000360007.

- Project: " Sistema di Comunicazione Informazione e Diffusione dell' Osservatorio della Biodiversità della Sicilia". PO FESR 2007/2013 linea di intervento 3.2.1.2 Regione Siciliana - Assesorato Regionale Territorio e Ambiente - Dipartimento Regionale dell' Ambiente Progetto.