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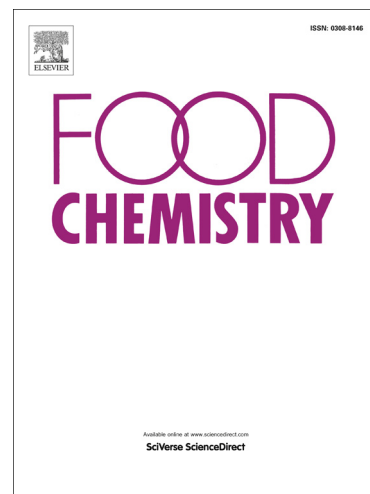
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**DEVELOPMENT OF AN IN-HOUSE FAST REAL-TIME PCR METHOD FOR
DETECTION OF FISH ALLERGEN IN FOODS AND COMPARISON WITH A
COMMERCIAL KIT**

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Abstract

Food allergy is recognized as an important human health problem. Fish represent one of the most important causes of food hypersensitivity reaction. Small amounts of the allergen can cause severe reactions in sensitive individuals, so correct labelling is essential to ensure the protection of consumers. The objective of the present work was to develop a reliable, sensitive and specific real-time PCR method for the detection of fish and traces of fish in all kind of products included those that have undergone aggressive treatments such as high temperature or pressure. This methodology was validated simulating products likely to contain this allergen and spiking them with fish cooking water. In addition, a comparison between the performance of in-house methodology and a commercial kit, both of them based on real-time PCR, was carried out.

This work is relevant because it is the first, rapid real-time PCR method developed to date for the detection of fish in processed food products.

The results obtained confirm the present assay is a useful tool in detecting fish and, therefore, minimizing exposure and reducing incidences of allergic reaction to fish in contaminated products.

KEYWORDS: Fish, allergy, fast real-time PCR, detection.

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30 **1. INTRODUCTION**

31 Food allergies have become an important human health concern due to increasing prevalence
32 (Li, Zhang, Pawar, Wang & Lin, 2011). They are an adverse immune response to food proteins
33 due to ingestion, contact, or inhalation. Any food can cause an allergic reaction in a person
34 sensitive to particular allergen, but the most common foods causing allergic reactions are: milk,
35 egg, peanuts, tree nuts, fish, and shellfish (Venter & Arshad, 2011). The amount of allergen
36 required to produce an allergic reaction depends on the sensitivity of each person to a particular
37 allergen (CIAA, 2010). Thus, removing the allergen from the diet and, therefore, all products
38 likely to contain it is the option.

39 In order to achieve a high level of health protection for consumers and to guarantee their right to
40 information, consumers should be informed about the content of the foods they consume. In this
41 sense, the ingredient statement on the label of food products is essential. The European Union
42 (EU) has implemented regulations that require the presence of some potential allergens, which
43 are present as ingredients, to be declared by manufacturers (EU Directive 2003/89/EC and EU
44 Directive 2007/68/EC, both amending 2000/13/EC and EU Regulation 1169/2011 which will
45 come into force in 2014) (EU., 2000, 2003, 2007, 2011). However, whilst known allergens can
46 be included in the label, potentially hazardous residues/contaminants can be present in some
47 products as result of common practices in the food industry. This is due to cross-contamination
48 between raw materials, production lines, or equipment. On the other hand, sometime the
49 substitution of non-allergenic ingredients with allergenic ingredients of less commercial value
50 occurs (e.g. meat or fish may be substituted for soy since they increase the weight of the final
51 product reducing the production costs) and not declared on the label (Herrero, Vieites &
52 Espiñeira, 2012).

53 Fish is among the most prevalent causes of allergic reactions mediated by IgE in food. The
54 consumption or contact with fish or the inhalation of vapors can produce in sensitized

55 individuals different symptoms, which affect the skin, gastrointestinal tract and the respiratory
56 system, and sometimes are cause of severe anaphylactic reactions (Swoboda, 2011). The main
57 fish allergen is parvalbumin, a small calcium-binding muscle protein (Swoboda, 2011).

58 Polysensitization to different fish species linked to cross-recognition of different parvalbumins
59 has been reported (Griesmeier et al., 2010; Hilger et al., 2004) and, therefore, those with fish
60 allergy are advised to avoid eating any fish. For all these reasons, the development of detection
61 methods to ensure consumer protection and compliance with food labelling regulations is
62 necessary. The most commonly used techniques are protein-based methods such as the enzyme-
63 linked immunosorbent assay (ELISA) (Fæste & Plassen, 2008; Gajewski, 2009) and DNA-
64 based methods such as PCR technologies (Aranishi & Okimoto, 2004; van Hengel, 2007). The
65 main advantage of the latter is that they are not sensitivity to factors such as pressure and heat,
66 which can produce changes in the protein conformation and can inhibit its detection
67 (Hildebrandt S. & Garber EAE., 2010). In this sense, the real-time PCR provides a useful,
68 sensitive and specific tool for the detection of DNA fish in adulterated food and products where
69 there may be cross-contamination. Moreover, the results are obtained in a short time avoiding
70 economic losses arising from delays in analysis. Some work in the scientific literature has used
71 real-time PCR for detection of fish (Benedetto, Abete, & Squadrone, 2011; Martin et al., 2010;
72 Sun, Liang, Gao, Lin, & Deng, 2009), but specificity was tested with only a small number of
73 species or non-food matrices meaning they were not subjected to the typical preparation
74 processes of commercial food products. There are some commercial kits that detect different
75 allergens using real-time PCR, but their validation need to be extended to verify specificity and
76 possible crossing-reactions with closely related species.

77 In this work, a real-time PCR method was developed for the detection of fish in all kind of
78 products, independent of the degree of transformation to which they have been subjected (as
79 frozen or processed products) and compared with a commercial kit. Due to the high sensitivity
80 and specificity of the method developed, it was thought to be appropriate for the analysis of
81 cross-contamination in production chains, which would make it ideal to ensure food quality and
82 safety, and protect the rights of the consumers to know what they are eating. In addition, it

constitutes the first fast real-time PCR developed up to date for the detection of fish in all kinds of food including those that have been subjected to treatments (e.g. high temperatures and pressure) during processing.

2. MATERIALS AND METHODS

2.1. Sample Collection, Storage and DNA Extraction

Specimens of fish obtained for the development of this methodology were authenticated by genetic analyses (sequencing). Also, different species of bivalves, cephalopods, crustaceans, meats, vegetables and spices were obtained to establish specificity of the method (Table 1).

DNA extraction was performed for both methods (in-house method and commercial kit) using the *SureFood PREP Allergen kit (R-Biopharm)* according to the protocol described by the manufacturer. The quality and concentration of extracted DNA was determined by measuring the absorbance at 260 nm and the 260/280 nm and 234/260 ratios using a NanoDrop ND-1000 spectrophotometer (*ThermoScientific*) (Winfrey, Rott & Wortman, 1997). Subsequently, DNA extractions were labeled and preserved at -80 °C.

2.2. Design of a Specific Fast Real-time PCR Method to Detect Fish.

18S RNA gene sequences were downloaded from the *National Center for Biotechnology Information (NCBI)* (Table 1). From the resulting alignment with Clustal W (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997) integrated into BioEdit 7.0 (Hall, 1999), a specific primers/probe set was designed by using *Primer Express* software (*Applied Biosystems*): FISH ALLERGEN F (5'-GGC TCA TTA AAT CAG TTA TG-3'), FISH ALLERGEN R (5'-CCG AGT TAT CTA GAG TCA-3') and FISH ALLERGEN PROBE (5'-6-FAM-CCG TAC TTG GAT AAC TGT GGC AAT TC-BHQ1-3').

The PCR reactions were carried out in a total volume of 20 µL containing 100 ng of DNA template, 10 µL of *TaqMan Fast Advanced Master Mix (Applied Biosystems)*, primers and probe and molecular biology grade water (*Eppendorf*) to adjust to the final volume. The optimal

111 concentrations of primers and probe were evaluated by preparing dilution series. To determine
 112 the minimum primer concentrations giving the maximum ΔR_n (the maximum fluorescence
 113 emission intensity and the lowest threshold cycle, C_t), forward and reverse primer
 114 concentrations of 50, 300, 500, and 900 nmol/L were used. All were evaluated at a constant
 115 probe concentration of 50 nmol/L. Subsequently, the probe concentration was optimized with
 116 concentrations of 50-250 nmol/LM (Murray & Curran, 2005).

117 The reactions were performed in triplicate on DNA samples in *MicroAmp Optical 96-well*
 118 *reaction plates (Applied Biosystems)* with *MicroAmp optical caps (Applied Biosystems)* using
 119 the *ViiA 7 Real-Time PCR System (Applied Biosystems)*. Amplification was carried out with the
 120 following conditions: 95 °C for 20 s and 40 cycles each of 95 °C for 1 s and 60 °C for 20s.

121 The specificity of the assay was tested with different species of bivalves, cephalopods,
 122 crustaceans, meats, vegetables and spices among which are several potential cross-reacting
 123 species; the species tested are shown in Table 1.

124 A serial dilution of fish DNA was performed with DNA from other seafood species at levels
 125 ranging from 500 ng to 5 pg, and the fluorescence signal was used to calculate the limit of
 126 detection (LOD). The samples were prepared by adding fish DNA and DNA from different
 127 species until the final concentration (500 ng) was achieved. All measurements were performed
 128 in triplicate from five samples independently.

129

130 **2.3. Methodological Validation**

131 Species of fish obtained for the development of the present method were used to spike different
 132 products. The fish were cooked at 99 °C in water for 20 min and the water used to spike
 133 different food matrices and processed products, which simulated canned and precooked food.

134 The amount of DNA present in the cooking water was the lowest concentration detected during
 135 development of the method. The presence of additives such as spices or sauces used by the food
 136 industry can affect DNA amplification. Moreover, due to the sensitive of DNA to acid and
 137 alkaline agents in different kinds of sauces, the quantity and quality of the DNA extracted can
 138 be affected as in the case of pickled products, for example, where that low pH produces higher

DNA degradation (Herrero, Vieites & Espiñeira, 2012). All treatments applied to samples were performed in the pilot plant of ANFACO-CECOPESCA. The products were analyzed according to the same method described for the standard samples. The objective of this method validation was to prove treatments applied to the processed products do not affect real-time PCR detection of fish contamination/adulteration.

2.4. Comparison of In-house and Commercial Real-time PCR Systems

The method proposed was compared in terms of sensitivity, specificity, diagnostic cost and time required with a commercial real-time PCR test kit for fish allergen, *SureFood® Allergen Fish* (R-Biopharm). This kit was used according to the manufacturer's protocol.

2.5. Application of the Methodology Developed to the Food Industry

The method developed was applied to samples from different points in the production chain, from raw materials to finished products, taking account of surfaces control (Table 3). These samples were mainly from companies involved in the production of fish products. Often these industries use the same processing line for manufacturing products containing different ingredients, such as crustaceans, bivalves, cephalopods or fish. This can cause cross contamination of a product by another including allergens.

3. RESULTS AND DISCUSSION

3.1. Design of a Specific Fast Real-time PCR Method to Detect Fish.

In the present work, a specific fast real-time PCR method for fish detection was designed. A successful real-time PCR assay requires amplification and detection under optimal conditions and each component of the reaction can affect the result. The quality and integrity of DNA is essential to conducting a successful experiment. One way to measure these characteristics in the DNA is by absorbance ratio A_{260}/A_{280} , and in all samples this ratio was between 1.7 and 2.

166 Values outside this range may indicate the presence of impurities or contaminants in the
167 solution such as proteins or phenol, which may affect the results.

168 On the other hand, optimal performance is achieved by selecting the primer and probe
169 concentrations that provide the lowest quantification cycle (C_q) and highest ΔRn (normalized
170 reporter, defined as emission intensity of reporter/emission intensity of passive reference)
171 (Bustin, 2004).

172 The optimal conditions in terms of specificity and sensitivity for detecting fish were established.
173 Finally, the optimal concentrations were 900 nmol/L for both primers and 250 nmol/L for the
174 probe. The specificity of the primer/probe set was confirmed using pure genomic DNA from
175 different species of bivalves, cephalopods, crustaceans, meats, vegetables and spices (normally
176 used as a condiment in prepared food). Processed food products, especially pre-cooked plates,
177 contain a large number of ingredients. For this reason, it is necessary to verify the specificity of
178 the method against with these ingredients although they are not related genetically to the fish.
179 No cross-reactivity was detected with any of the samples tested (Table 1; Figure 1). The optimal
180 annealing temperature that allowed correct detection of the fish was 60 °C. The mean values of
181 C_q obtained were 20 ± 2 in fresh and frozen fish with a concentration of 100 ng of DNA.

182 On the other hand, robust and precise assays are usually correlated with high PCR efficiency.
183 The amplification plot of the experiment using the primer/probe fish set generated a slope of -
184 3.3009 or 100.885% of efficiency, with a correlation coefficient of 0.99.

185 The C_q and efficiency values obtained demonstrate the applicability of the rapid real-time PCR
186 method developed to detect fish in food. LOD is defined as the lowest concentration of fish
187 DNA at which 95% of positive samples are detected (Bustin SA. et al., 2009). In the present
188 assay, a minimum of 0.05 ng of fish was necessary to obtain a positive result.

189

190 **3.2. Methodological Validation**

191 The aim of the validation step was to evaluate the correct operation of the proposed method, and
192 check if treatment of the processed foods had any influence on detection of fish. The products

193 elaborated in the pilot plant of ANFACO-CECOPESCA with the cooking water of fish were
194 analysed using the method developed.

195 The conditions of technological treatment such as mechanical stress, high temperature, pH
196 variations, enzymatic activities, fermentations or pressure may lead the DNA degradation or
197 reduce the efficiency of the amplification especially in processed food (Gryson, 2010).

198 Moreover, the presence of sauces, fats or additives can affect the reaction by inhibiting PCR or
199 increasing the DNA degradation. As a result, some authors recommend only fragments with
200 fewer than 200 bp should be amplified to ensure the correct amplification (Herrero, Vieites &
201 Espiñeira, 2012). In the present work, fish was detected in all contaminated samples using the
202 real-time PCR proposed.

203 In the processed products C_q was higher. However, the presence of fish was always detected
204 without any reservation. In the cross-reactivity analysis, no false-positive results were observed,
205 even under the most severe conditions, as shown by C_q values 'undetected' (UND).

206 These results demonstrate the method developed herein could be applied in all kind of foods
207 including processed products.

208

209 **3.3. Comparison of In-house and Commercial Real-time PCR Systems**

210 Two real-time PCR based method for the detection of fish in food, commercially available kit
211 and an in-house method, were evaluated in this study (Table 2).

212 The specificity of both methods was compared with the same samples. In the present study, the
213 specificity of the method was verified in-house against the commercial kit. The commercial kit
214 experienced cross-reactivity with some species of bivalves, cephalopods, crustacean, meats and
215 vegetables (Table 1). This demonstrates the importance of realizing validation more widely
216 when a commercial kit is chosen since the analysis of the manufacturer is not enough.

217 Sensitivity and specificity are the main criteria when a kit is evaluated, but other considerations
218 important in this task are handling, time-consuming, cost, applicability or simplicity. The in-
219 house real-time PCR required more handling, but was cheaper than the commercial kit. Data

from the in-house method were obtained in 43 minutes while the commercial kit took 75 minutes (both used the *ViiA 7 Real-Time PCR System*).

In summary, the results suggest that the in-house assay was more sensitive and specific, and was cheaper and less time-consuming than the commercial kit tested.

3.4. Application of the Methodology Developed to the Food Industry

Food security must be considered in parallel with physical, chemical and microbiological risks.

Thus, management of allergen in the food industry should be seen as an integral part of the management of existing food safety, and should take into account all operations from raw material to manufacturing and packaging of the finished product.

Many manufacturers produce different products in the same factory, using the same production lines. Single manufacture lines for multiple products can cause cross-contamination of production lines, particularly by food allergens. In addition, raw materials may be composites products that contain undeclared ingredients. Hence, management of allergens consists must address two points: (1) identification of allergens or derivatives in raw materials received from the supplier that were added intentionally and (2) presence of any allergen arising from cross-contamination in the suppliers' premises by (a) assessing risk of cross-contamination at installations level (e.g. production lines, machinery...) and (b) environmental pollution using swabs to keep track of cleaning and detection of allergens on surfaces and equipment.

Thus, detection of fish material using the proposed method was developed with samples encompassing the entire production chain (Table 3).

Overall, our study demonstrated the presence of fish contamination in different parts of the production chain. Moreover, application of the proposed method to highly processed samples, such as those subjected to high pressure and heat treatments (e.g. canned products) was successful because genomic material is more stable than proteins.

4. CONCLUSIONS

248 Food allergies are recognized as a food safety issue. Fish are one of the main causes of food
249 hypersensitive reactions. Sensitized individuals can react to a wide range of allergenic foods and
250 different quantities, and must avoid the consumption of foods containing them meaning it is
251 essential labelling of products is accurate. In this sense, the first fast real-time PCR to detect fish
252 in all kind of products including processed products was developed successfully. This method
253 provides advantages with respect to accuracy, sensitivity, specificity, dynamic range, high-
254 throughput capacity, and lower risk of contamination, and reduces the time for analysis to 43
255 minutes. Furthermore, the method was compared with a commercial kit, which highlighted
256 several advantages of the in-house real-time PCR. The method is a useful tool to address
257 questions regarding food quality and security, and in the verification the implementation of
258 labelling regulations to ensure the consumer protection.

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ACCEPTED MANUSCRIPT

347 Tables

348

349

350 Table 1. Species included in the present work.

351

352

	Scientific name	Access n° NCBI	Common name ^a	Detection with in-house method	Detection with commercial kit
Fishes	<i>Anguilla anguilla</i>		European eel	+	+
	<i>Anguilla australis</i>	FJ710814	Australian shortfinned eel	+	+
	<i>Auxis rochei</i>		Bullet tuna	+	+
	<i>Brama australis</i>		South rays bream	+	+
	<i>Clupea harengus</i>	X98845	Atlantic herring	+	+
	<i>Cynoglossus spp.</i>		Tonguesole	+	+
	<i>Cynoglossus zanzibarensis</i>		Zanzibar tonguesole	+	+
	<i>Decapterus macarellus</i>		Mackerel scad	+	+
	<i>Dicentrarchus labrax</i>	AM419038	Capemouth	+	+
	<i>Dissostichus eleginoides</i>		Chilean seabass	+	+
	<i>Engraulis encrasicolus</i>		European anchovy	+	+
	<i>Engraulis japonicus</i>		Southern African anchovy	+	+
	<i>Epinephelus bruneus</i>	JN603833	Kelp grouper	+	+
	<i>Gadus macrocephalus</i>		Atlantic cod	+	+
	<i>Gadus morhua</i>	AF518205	Atlantic cod	+	+
	<i>Genypterus blacodes</i>		Pink cusk-eel	+	+
	<i>Glyptocephalus cynoglossus</i>		Witch	+	+
	<i>Helicolenus dactylopterus</i>		Bluemouth rockfish	+	+
	<i>Hoplostethus atlanticus</i>		Atlantic slimehead	+	+
	<i>Istiophorus spp.</i>		Sailfish	+	+
	<i>Isurus oxyrinchus</i>		Shortfin mako	+	+
	<i>Katsuwonus pelamis</i>		Skipjack tuna	+	+
	<i>Lepidocybium flavobrunneum</i>		Escolar	+	+
	<i>Lophius budegassa</i>		Black-bellied angler	+	+
	<i>Lophius litulon</i>		Anglerfish	+	+
	<i>Lophius piscatorius</i>		Anglerfish	+	+
	<i>Merluccius australis</i>		Patagonian hake	+	+
	<i>Merluccius merluccius</i>	HQ189426	European hake	+	+
	<i>Molva dypterygia</i>		Blue ling	+	+
	<i>Molva molva</i>		Ling	+	+
	<i>Oreochromis aureus</i>		Blue tilapia	+	+
	<i>Platichthys flesus</i>	AJ715485	Flounder	+	+
	<i>Pollachius virens</i>		Saithe	+	+
	<i>Raja binoculata</i>		Big skate	+	+
	<i>Raja miraletus</i>		Brown ray	+	+
	<i>Rastrellinger kanagurta</i>		Indian mackerel	+	+
	<i>Ruvettus pretiosus</i>		Castor oil fish	+	+
	<i>Salmo trutta</i>	FJ710887	Brown trout	+	+
	<i>Sardina pilchardus</i>		European pilchard	+	+
	<i>Sardinella aurita</i>		Round sardinella	+	+
	<i>Scomber coilis</i>		Atlantic chub mackerel	+	+
	<i>Scorpaena scorpa</i>		Largescalled scorpionfish	+	+
	<i>Sebastes mentella</i>		Beaked redfish	+	+
	<i>Sebastes oculatus</i>	AF518200	Patagonian redfish	+	+
	<i>Seriola spp.</i>		Amberjacks	+	+
	<i>Solea lascaris</i>		Sand sole	+	+
	<i>Solea senegalensis</i>	EF126042	Senegalese sole	+	+
	<i>Sparus aurata</i>	AY993930	Gilthead seabream	+	+
	<i>Sprattus fuergensis</i>		Falkland sprat	+	+
	<i>Thunnus orientalis</i>	AY695887	Pacificbluefin tuna	+	+
	<i>Thunnus thynnus</i>		Bluefin tuna	+	+
	<i>Trachurus mediterraneus</i>		Mediterranean horse mackerel	+	+
	<i>Trachurus trachurus</i>		Atlantic horse mackerel	+	+
	<i>Xiphias gladius</i>		Swordfish	+	+

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	Scientific name	Access n° NCBI	Common name ^a	Detection with in-house method ^b	Detection with commercial kit ^b
Fishes	<i>Anguilla anguilla</i>		European eel	+	+
	<i>Anguilla australis</i>	FJ710814	Bullet tuna	+	+
	<i>Auxis rochei</i>		South rays bream	+	+
	<i>Brama australis</i>		Atlantic herring	+	+
	<i>Clupea harengus</i>	X98845	Tonguesole	+	+
	<i>Cynoglossus spp.</i>		Tonguesole	+	+
	<i>Cynoglossus zanzibarensis</i>		Zanzibar tonguesole	+	+
	<i>Decapterus macarellus</i>		Mackerel scad	+	+
	<i>Dicentrarchus labrax</i>	AM419038	Capemouth	+	+
	<i>Dissostichus eleginoides</i>		Chilean seabass	+	+
	<i>Engraulis encrasicolus</i>		European anchovy	+	+
	<i>Engraulis japonicus</i>		Southern African anchovy	+	+
	<i>Epinephelus bruneus</i>	JN603833	Kelp grouper	+	+
	<i>Gadus macrocephalus</i>		Atlantic cod	+	+
	<i>Gadus morhua</i>	AF518205	Atlantic cod	+	+
	<i>Genypterus blacodes</i>		Pink cusk-eel	+	+
	<i>Glyptocephalus cynoglossus</i>		Witch	+	+
	<i>Helicolenus dactylopterus</i>		Bluemouth rockfish	+	+
	<i>Hoplostethus atlanticus</i>		Atlantic slimehead	+	+
	<i>Istiophorus spp.</i>		Sailfish	+	+
	<i>Isurus oxyrinchus</i>		Shortfin mako	+	+
	<i>Katsuwonus pelamis</i>		Skipjack tuna	+	+
	<i>Lepidocybium flavobrunneum</i>		Escolar	+	+
	<i>Lophius budegassa</i>		Black-bellied angler	+	+
	<i>Lophius litulon</i>		Anglerfish	+	+
	<i>Lophius piscatorius</i>		Anglerfish	+	+
	<i>Merluccius australis</i>		Patagonian hake	+	+
	<i>Merluccius merluccius</i>	HQ189426	European hake	+	+
	<i>Molva dypterygia</i>		Blue ling	+	+
	<i>Molva molva</i>		Ling	+	+
	<i>Oreochromis aureus</i>		Blue tilapia	+	+
	<i>Platichthys flesus</i>	AJ715485	Flounder	+	+
	<i>Pollachius virens</i>		Saithe	+	+
	<i>Raja binoculata</i>		Big skate	+	+
	<i>Raja miraletus</i>		Brown ray	+	+
	<i>Rastrellinger kanagurta</i>		Indian mackerel	+	+
	<i>Ruvettus pretiosus</i>		Castor oil fish	+	+
	<i>Salmo trutta</i>	FJ710887	Brown trout	+	+
	<i>Sardina pilchardus</i>		European pilchard	+	+
	<i>Sardinella aurita</i>		Round sardinella	+	+
	<i>Scomber coilis</i>		Atlantic chub mackerel	+	+
	<i>Scorpaena scorpa</i>		Largescalled scorpionfish	+	+
	<i>Sebastes mentella</i>		Beaked redfish	+	+
	<i>Sebastes oculatus</i>	AF518200	Patagonian redfish	+	+
	<i>Seriola spp.</i>		Amberjacks	+	+
	<i>Solea lascaris</i>		Sand sole	+	+
	<i>Solea senegalensis</i>	EF126042	Senegalese sole	+	+
	<i>Sparus aurata</i>	AY993930	Gilthead seabream	+	+
	<i>Sprattus fuergensis</i>		Falkland sprat	+	+
	<i>Thunnus orientalis</i>	AY695887	Pacificbluefin tuna	+	+
	<i>Thunnus thynnus</i>		Bluefin tuna	+	+
	<i>Trachurus mediterraneus</i>		Mediterranean horse mackerel	+	+
	<i>Trachurus trachurus</i>		Atlantic horse mackerel	+	+
	<i>Xiphias gladius</i>		Swordfish	+	+

OUTGROUPS	Scientific name	Access n° NCBI	Common name ^a	Detection with in-house method	Detection with commercial kit
Cephalopods	<i>Illex argentinus</i>		Argentine shortfin squid	-	-
	<i>Loligo vulgaris</i>		European squid	-	+
	<i>Octopus vulgaris</i>		Common octopus	-	+
	<i>Sepia officinalis</i>		Common cuttlefish	-	+
Bivalves	<i>Cerastoderma edule</i>		Common cockle	-	-
	<i>Cerastoderma glaucum</i>		Olive green cockle	-	-
	<i>Crassostrea gigas</i>	AM182263	Giant Pacific oyster	-	-
	<i>Ensis ensis</i>	AF120555	Pod razor shell	-	-
	<i>Ensis macha</i>		Macha jackknife clam	-	-
	<i>Ensis siliqua</i>		Sword razor shell	-	-
	<i>Mytilus galloprovincialis</i>		Mediterranean mussel	-	+
	<i>Mytilus edulis</i>	AY527062	Blue mussel	-	-
	<i>Pecten jacobaeus</i>	AY070112	Great Mediterranean scallop	-	-
	<i>Spisula solid</i>	AM774552	Solid surf clam	-	-
	<i>Tapes decussata</i>		Grooved carpet shell	-	+
	<i>Venerupis philippinarum</i>	AM774568	Japanese carpet shell	-	-
	<i>Bathynectes maravigna</i>		Deep-sea crab	-	-
	<i>Cancer pagurus</i>	DQ079743	Edible crab	-	-
Crustacean	<i>Homarus gammarus</i>	DQ079749	European lobster	-	-
	<i>Macropus tuberculatus</i>		Knobby swimcrab	-	+
	<i>Maja squinado</i>	DQ079758	Spider crab	-	-
	<i>Necora puber</i>	DQ079759	Velvet swimming crab	-	-
	<i>Penaeus spp.</i>	EU920969	Prawn	-	-
	<i>Pollicipes pollicipes</i>	EU370427	Goose neck barnacle	-	-
	<i>Anas platyrhynchos</i>		Duck	-	-
	<i>Bos taurus</i>	NR_036642	Cow	-	-
	<i>Bubalus bubalis</i>		Water buffalo	-	+
	<i>Capra hircus</i>		Goat	-	-
Meats	<i>Equus caballus</i>		Horse	-	-
	<i>Gallus gallus</i>	HQ873432	Chicken	-	+
	<i>Meleagris gallopavo</i>	AJ419877	Turkey	-	+
	<i>Ovis aries</i>		Sheep	-	-
	<i>Sus scrofa</i>	NR_046261	Wild boar	-	-
	<i>Apium graveolens</i>		Celery	-	+
	<i>Avena sterilis</i>		Animated oat	-	-
	<i>Glycine max</i>		Edamame	-	-
	<i>Hordeum vulgare</i>		Barley	-	-
	<i>Lupinus luteus</i>		European yellow lupine	-	-
Vegetables	<i>Oryza sativa</i>		Burgundy rice	-	-
	<i>Pennisetum glaucum</i>		American fourtaingrass	-	-
	<i>Secale cereale</i>		Cereal rye	-	-
	<i>Triticum aestivum</i>		Bread wheat	-	-
	<i>Triticum burgidum</i>		Rivet wheat	-	-
	<i>Origanum vulgare</i>		Oregano	-	-
	<i>Laurus nobilis</i>		Bay Leaf	-	-
	<i>Petroselinum sativum</i>		Parsley	-	-
	<i>Mentha sativa</i>		Mint	-	-
	<i>Mentha sativa</i>		Mint	-	-

OUTGROUPS	Scientific name	Access n° NCBI	Common name ^a	Detection with in-house method ^b	Detection with commercial kit ^b
Cephalopods	<i>Illex argentinus</i>		Argentine shortfin squid	-	-
	<i>Loligo vulgaris</i>		European squid	-	+
	<i>Octopus vulgaris</i>		Common octopus	-	+
	<i>Sepia officinalis</i>		Common cuttlefish	-	+
Bivalves	<i>Cerastoderma edule</i>		Common cockle	-	-
	<i>Cerastoderma glaucum</i>		Olive green cockle	-	-
	<i>Crassostrea gigas</i>	AM182263	Giant Pacific oyster	-	-
	<i>Ensis ensis</i>	AF120555	Pod razor shell	-	-
	<i>Ensis macha</i>		Macha jackknife clam	-	-
	<i>Ensis siliqua</i>		Sword razor shell	-	-
	<i>Mytilus galloprovincialis</i>		Mediterranean mussel	-	+
	<i>Mytilus edulis</i>	AY527062	Blue mussel	-	-
	<i>Pecten jacobaeus</i>	AY070112	Great Mediterranean scallop	-	-
	<i>Spisula solid</i>	AM774552	Solid surf clam	-	-
	<i>Tapes decussata</i>		Grooved carpet shell	-	+
	<i>Venerupis philippinarum</i>	AM774568	Japanese carpet shell	-	-
	<i>Bathynectes maravigna</i>		Deep-sea crab	-	-
	<i>Cancer pagurus</i>	DQ079743	Edible crab	-	-
Crustacean	<i>Homarus gammarus</i>	DQ079749	European lobster	-	-
	<i>Macropus tuberculatus</i>		Knobby swimcrab	-	+
	<i>Maja squinado</i>	DQ079758	Spider crab	-	-
	<i>Necora puber</i>	DQ079759	Velvet swimming crab	-	-
	<i>Penaeus spp.</i>	EU920969	Prawn	-	-
	<i>Pollicipes pollicipes</i>	EU370427	Goose neck barnacle	-	-
	<i>Anas platyrhynchos</i>		Duck	-	-
	<i>Bos taurus</i>	NR_036642	Cow	-	-
	<i>Bubalus bubalis</i>		Water buffalo	-	+
	<i>Capra hircus</i>		Goat	-	-
Meats	<i>Equus caballus</i>		Horse	-	-
	<i>Gallus gallus</i>	HQ873432	Chicken	-	+
	<i>Meleagris gallopavo</i>	AJ419877	Turkey	-	+
	<i>Ovis aries</i>		Sheep	-	-
	<i>Sus scrofa</i>	NR_046261	Wild boar	-	-
	<i>Apium graveolens</i>		Celery	-	+
	<i>Avena sterilis</i>		Animated oat	-	-
	<i>Glycine max</i>		Edamame	-	-
	<i>Hordeum vulgare</i>		Barley	-	-
	<i>Lupinus luteus</i>		European yellow lupine	-	-
Vegetables	<i>Oryza sativa</i>		Burgundy rice	-	-
	<i>Pennisetum glaucum</i>		American fourtaingrass	-	-
	<i>Secale cereale</i>		Cereal rye	-	-
	<i>Triticum aestivum</i>		Bread wheat	-	-
	<i>Triticum burgidum</i>		Rivet wheat	-	-
	<i>Origanum vulgare</i>		Oregano	-	-
	<i>Laurus nobilis</i>		Bay Leaf	-	-
	<i>Petroselinum sativum</i>		Parsley	-	-
	<i>Mentha sativa</i>		Mint	-	-
	<i>Mentha sativa</i>		Mint	-	-

^a Only it is shown one of the possible common names for each species.

^b Three individuals of each species were used.

Table 2. Comparison between commercial kit and in-house real-time PCR for fish detection.

	In-house method	Commercial kit
No of samples	500	100
Reaction volume (μl)	20	25
Maximum volume of DNA (μl)	9,59	5
Chemestry	Taqman probes	Taqman probes
Rapid thermocycling	Yes	No
Time of the analysis	43 min	75 min

Table 3. Commercial samples analyzed with real-time PCR herein developed.

Product	Production Line	Samples	Result
Julienne clams	Raw material	2	Detected
		2	Not detected
	FinnaI product	2	Detected
	(Frozen meat)	2	Not detected
Mussels	Raw material	9	Detected
		6	Not detected
	FinnaI product	11	Detected
	(Frozen meat)	8	Not detected
Roman squid rings	FinnaI product	2	Detected
	(Prepared foods)	2	Not detected
Scallops	Raw material	1	Detected
		8	Not detected
	FinnaI product	2	Detected
	(Frozen meat)	9	Not detected
Tuberculate cockles	FinnaI product	2	Detected
	(Canned)	7	Not detected
Vegetable shrimps	FinnaI product (Prepared foods)	1	Detected
Surface Controls	Format	Samples	Result
Surface controls	Swabs	8	Detected
		3	Not detected

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515 **Highlights**

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- Fast real-time PCR to detect fish in food.
- Detection of fish as ingredient or as contamination in raw and processed food.
- Comparison between the performance of in-house methodology and a commercial kit.
- Results more specific, sensitive, rapidity and cheaper with in-house method.

ACCEPTED MANUSCRIPT