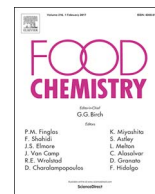




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Exploiting 16S rRNA gene for the detection and quantification of fish as a potential allergenic food: A comparison of two real-time PCR approaches

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ABSTRACT

Fish is one of the most common allergenic foods that should be accurately labelled to protect the health of allergic consumers. In this work, two real-time PCR systems based on the EvaGreen dye and a TaqMan probe are proposed and compared. New primers were designed to target the 16S rRNA gene, as a universal maker for fish detection, with fully demonstrated specificity for a wide range of fish species. Both systems showed similar absolute sensitivities, down to 0.01 pg of fish DNA, and adequate real-time PCR performance parameters. The probe system showed higher relative sensitivity and dynamic range (0.0001–50%) than the EvaGreen (0.05–50%). They were both precise, but trueness was compromised at the highest tested level with the EvaGreen assay. Therefore, both systems were successful, although the probe one exhibited the best performance. Its application to verify labelling compliance of foodstuffs suggested a high level of mislabelling and/or fraudulent practices.

1. Introduction

In geographical areas where fish has an important role in socioeconomic and cultural behaviours, such as Portugal, Spain and the Scandinavian countries, the prevalence of fish allergy also tends to be higher than in countries without a traditional strong fish industry (Calderon-Rodriguez, Pineda, Perez, & Muñoz, 2016; Mourad & Bahna, 2015). Fish allergy is typically IgE-mediated with clinical features that can range from mild to potentially life-threatening (Mourad & Bahna, 2015; Sharp & Lopata, 2014; Thalayasingam & Lee, 2015). Common clinical symptoms of fish allergy are gastrointestinal (vomiting, diarrhoea and abdominal pain), cutaneous (urticaria or angioedema) and respiratory (rhinitis, conjunctivitis, cough and wheezing), although more severe symptoms, such as laryngeal oedema or anaphylaxis, might also occur (Mourad & Bahna, 2015; Sharp & Lopata, 2014; Thalayasingam & Lee, 2015). The main fish allergen is parvalbumin, a small calcium-binding muscle protein, although other proteins, such as enolases and aldolases are also responsible for fish allergy (Fernandes, Costa, Oliveira, & Mafra, 2015; Mourad & Bahna, 2015). The content in parvalbumin differs considerably among fish species. Cartilaginous fish muscle (dark tissues) contains mainly alpha-parvalbumin (non-allergenic protein), whereas bony fish muscle (white tissues) expresses its beta-homolog (allergenic protein), which explains their distinct potential of allergenicity (Kobayashi et al., 2006; Kuehn, Scheuermann, Hilger, & Hentges, 2010; Mourad & Bahna, 2015). Owing to differences

in the amount of expressed allergenic parvalbumin in white or dark fish muscle, some sensitised/allergic patients tolerate one or more species of fish with less parvalbumin. Amongst the white fish species that are globally consumed, hakes (Merlucciidae family) are one group of the most allergenic, representing a high proportion of food allergies, namely in children (Calderon-Rodriguez et al., 2016; Pascual, Esteban, & Crespo, 1992; Triantafyllidis et al., 2010). Due to cross-reactivity phenomena among different species, parvalbumins are responsible for the polysensitisation of allergic individuals to several fish species, who are advised avoiding their consumption (Fernandes et al., 2015; Triantafyllidis et al., 2010).

Despite the compulsory labelling of food products that may contain allergenic ingredients (Regulation (EU) No. 1169/2011), the allergic individuals can inadvertently consume the offending foods due to mislabelling or cross-contamination during processing. This circumstance emphasises the need of verifying labelling compliance, which should rely on highly sensitive, accurate, specific and fast methods to enable the detection of trace levels of allergens in foods. Currently, several methods targeting protein and DNA markers have been suggested to detect fish allergens. The most widely used approaches are the enzyme linked immunosorbent assay (ELISA) (Chen & Hsieh, 2014; Faeste & Plassen, 2008; Weber, Steinhart, & Paschke, 2009) and the DNA amplification by real-time polymerase chain reaction (PCR) (Herrero, Vieites, & Espiñeira, 2014; Houhoula, Dimitriou, Mengjezi, Kyrana, & Lougovois, 2015; Sun, Liang, Gao, Lin, & Deng, 2009; Tetzlaff

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& Mäde, 2017). ELISA allows the direct detection and quantification of allergens, mainly parvalbumins in the case of fish, usually providing a rapid and simple method. PCR-based methods are able to detect marker sequences of the allergenic species, which can be allergen encoding genes, providing an indirect detection of the target (Fernandes, Costa, Carrapatoso, Oliveira, & Mafra, 2017). However, immunochemical methods, such as ELISA, are particularly prone to cross-reactivity phenomena and to be affected by structural changes in proteins after food processing, often conducting to false positive or negative results. On the other hand, PCR methods take advantage of the higher stability of DNA markers compared to proteins, being adequate to detect allergens in processed and complex food matrices with high specificity and sensitivity (Fernandes, Costa, Carrapatoso et al., 2017; Prado et al., 2016). A real-time PCR assay for the specific detection of parvalbumin gene in fish was developed by Sun et al. (2009), allowing a sensitivity of 5 pg. Herrero et al. (2014) proposed a real-time PCR method targeting a universal DNA marker of fish (18S rRNA gene), which detected fish DNA down to 50 pg and was successfully applied to commercial food samples. More recently, Tetzlaff and Mäde (2017) reported a real-time PCR assay targeting the *Hoxc13* gene to detect the Teleostei subclass of fish, reaching sensitivities of 10–100 mg/kg, depending on the matrix, and 2.5 DNA copies. Those real-time PCR reports are all based on the use of TaqMan probes targeting mostly nuclear genes. Mitochondrial DNA markers, such as the encoding genes of 16S rRNA, 12S rRNA, cytochrome c oxidase subunit I (COI) and cytochrome b (cyt b) have been frequently targeted in fish species differentiation for authentication purposes due to their high copy numbers, allowing for the universal primer design (Abdullah & Rehbein, 2016; Feng, Wu, Xie, Dai, & Liu, 2017; Fernandes, Costa, Oliveira, & Mafra, 2017; Giusti et al., 2017; Pegels, González, López-Calleja, García, & Martín, 2013).

In the present work, the development of two real-time PCR approaches is proposed targeting the 16S rRNA mitochondrial region, as a universal marker for fish detection in foods at trace levels. The two approaches based of the universal EvaGreen dye and TaqMan probes are compared in terms of assay performance, validation and applicability to model mixtures with spiked fish. Finally, several commercial samples are analysed and the results compared with the label statements.

2. Materials and methods

2.1. Sample collection and preparation

Several specimens of commercially relevant fish species available on the Portuguese market ($n = 26$) were acquired: Atlantic cod (*Gadus morhua*), Pacific cod (*Gadus macrocephalus*), Alaska pollock (*Theragra chalcogramma*), saithe (*Pollachius virens*) and ling (*Molva molva*) were kindly provided by Pascoal & Filhos S.A; European hake (*Merluccius merluccius*), North Pacific hake (*Merluccius productus*), Argentine hake (*Merluccius hubsi*), whiting (*Merluccius capensis*), deepwater hake (*Merluccius paradoxus*) were acquired at local markets and authenticated by sequencing (Fernandes, Costa, Oliveira, & Mafra, 2018); European pilchard (*Sardina pilchardus*), ray (*Raja* spp.), Atlantic horse mackerel (*Trachurus trachurus*), gilt-head bream (*Sparus aurata*), common sole (*Solea solea*) and Pacific mackerel (*Scomber japonicus*) were acquired at local markets as fresh whole specimens, allowing morphological identification; Atlantic salmon (*Salmo salar*), yellowfin tuna (*Thunnus albacares*), European seabass (*Dicentrarchus labrax*), whiting-pout (*Trisopterus luscus*), tadpole codling (*Saliota australis*), rose fish (*Sebastes marinus*), rock ling (*Genypterus blacodes*), black scabbardfish (*Aphanopus carbo*), Nile perch (*Lates niloticus*) and sutchi catfish (*Pangasius hypophthalmus*) were acquired at local markets as frozen specimens. Other non-fish species were also acquired for reactivity testing of the assay ($n = 40$), namely crustaceans and molluscs (squid, mussel, undulated surf clam, crab, edible crab, Caribbean spiny lobster, Norway lobster, whiteleg shrimp, giant tiger prawn, Indian white

prawn, jinga shrimp and striped shrimp), meat species (cow, chicken, rabbit, deer, turkey, pork, lamb, goat, ostrich) and plant species (soybean, potato, oat, rye, wheat, lupine, maize, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, nectarine, cassava, strawberry, raspberry, honey, cashew).

Several processed seafood products (fish sticks ($n = 12$), breaded fish fillets ($n = 2$), powder soups ($n = 3$), fish patties/pies ($n = 6$), surimi ($n = 2$), pre-cooked dishes ($n = 9$), among others) were also acquired at local markets and analysed to verify assay applicability.

All the specimens and samples were individually ground and homogenised in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) and stored at -20°C until additional analysis. All containers and material used during this procedure were previously treated with DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany).

2.2. Preparation of model mixtures and samples

The preparation of binary model mixtures was attempted to simulate the stuffing/filling of processed foods, such as patties and pies. Hake fillets were boiled in water during 15 min, drained and ground in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). The first mixture containing 50% (w/w) of ground-cooked fish in béchamel sauce was prepared by the addition of 200 g of fish to the same amount of sauce. Successive additions of béchamel sauce to the 50% homogenised mixture enabled the preparation of the following binary mixtures: 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005 and 0.0001% (w/w) of fish in béchamel. For method validation, blind mixtures were similarly prepared, in order to contain 8.0, 4.0, 2.5 and 0.25% (w/w) of fish in béchamel.

All the binary mixtures were also ground and homogenised in a laboratory knife mill and stored at -20°C until further analysis. All containers and material were previously treated with DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany).

2.3. Oligonucleotide design

Sequences of the 16S rRNA gene were retrieved from NCBI database from a set of 24 different fish species (Fig. S1, supplementary material). The majority of these species match with the ones most frequently consumed as food. However, it was not always possible to find available sequences on the web for all fish species commonly marketed, and vice-versa. Sequence alignments were performed with BioEdit v.7.2.5 software (Ibis Biosciences, Canada) and examined for suitable regions for primer and probe design. Accordingly, primers (16SFish1-F – 5'-AAC GGCCGCGGTATTTTAAC-3'/16SFish1-R – 5'-TGCACGGGGAGGTCAA TTT-3') and probe (16SFish-P – FAM – 5'-CTTTTAAATGAAGACCTGTA TGAATGGCAT-3'-BHQ1) were designed and submitted to a basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for the *in silico* analysis of primer specificity. Primer properties, self-hybridisation and the absence of hairpins were evaluated using the software OligoCalc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) and, finally, the oligonucleotides were synthesised by STABVIDA (Lisbon, Portugal).

2.4. DNA extraction

The DNA was extracted using the Nucleospin® Food kit (Macherey-Nagel, Düren, Germany) with minor modifications. All DNA extractions were done in duplicate assays using 200 mg of each sample and 2 μL of RNase (2 mg/mL) that were added for 5 min at room temperature immediately after the lysis step. All the extracts were kept at -20°C until further analysis.

The concentration and purity of the extracts were assessed by UV spectrophotometric DNA quantification on a Synergy HT multi-mode

Table 1

Qualitative PCR results for eukaryotic and fish testing and cycle threshold values for several fish species with real-time PCR amplification with a TaqMan probe.

Species	Scientific name	Qualitative PCR ^a		Real-time PCR Ct $\pm$ SD ^b
		Eukaryotic (18S rRNA gene)	Fish (16S rRNA gene)	
Atlantic cod	<i>Gadus morhua</i>	+	+	18.81 $\pm$ 0.09
Pacific cod	<i>Gadus macrocephalus</i>	+	+	20.59 $\pm$ 0.26
Alaska pollock	<i>Theragra chalcogramma</i>	+	+	20.67 $\pm$ 0.58
Saithe	<i>Pollachius virens</i>	+	+	18.94 $\pm$ 0.08
Ling	<i>Molva molva</i>	+	+	18.86 $\pm$ 0.16
European hake	<i>Merluccius merluccius</i>	+	+	18.31 $\pm$ 0.03
Argentine hake	<i>Merluccius hubbsi</i>	+	+	23.63 $\pm$ 0.09
North Pacific hake	<i>Merluccius productus</i>	+	+	23.75 $\pm$ 0.27
Deepwater hake	<i>Merluccius paradoxus</i>	+	+	22.30 $\pm$ 0.01
Whiting	<i>Merluccius capensis</i>	+	+	18.62 $\pm$ 0.13
Atlantic salmon	<i>Salmo Salar</i>	+	+	21.19 $\pm$ 0.12
European pilchard	<i>Sardina pilchardus</i>	+	+	29.43 $\pm$ 0.86
Yellowfin tuna	<i>Thunnus albacares</i>	+	+	18.72 $\pm$ 0.21
Gilt-head bream	<i>Sparus aurata</i>	+	+	21.04 $\pm$ 0.03
European seabass	<i>Dicentrarchus labrax</i>	+	+	19.56 $\pm$ 0.16
Whiting-pout	<i>Trisopterus luscus</i>	+	+	21.30 $\pm$ 0.14
Pacific mackerel	<i>Scomber japonicus</i>	+	+	20.89 $\pm$ 0.37
Atlantic horse mackerel	<i>Trachurus trachurus</i>	+	+	22.44 $\pm$ 0.12
Rose fish	<i>Sebastes marinus</i>	+	+	19.79 $\pm$ 0.10
Black scabbardfish	<i>Aphanopus carbo</i>	+	+	23.32 $\pm$ 0.45
Nile perch	<i>Lates niloticus</i>	+	+	20.30 $\pm$ 0.11
Common sole	<i>Solea solea</i>	+	+	21.78 $\pm$ 0.11
Ray	<i>Raja spp.</i>	+	+	21.87 $\pm$ 0.42 ^c
Tadpole codling	<i>Salilota australis</i>	+	+	19.28 $\pm$ 0.19 ^c
Rock ling	<i>Genypterus blacodes</i>	+	+	22.46 $\pm$ 0.30 ^c
Sutchi catfish	<i>Pangasius hypophthalmus</i>	+	+	19.24 $\pm$ 0.08 ^c

^a (+) positive qualitative PCR amplification.^b Mean values of cycle threshold (Ct) $\pm$ standard deviation (SD) of replicate assays ($n = 4$).^c These values were obtained by real-time PCR with EvaGreen dye.

microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA). The quality of extracted DNA was also analysed by electrophoresis in a 1.0% agarose gel containing Gel Red 1 $\times$ (Biotium, Hayward, CA, USA) for staining and carried out in STGB 1 $\times$ (GRISP, Porto, Portugal) for 25 min at 200 V. The agarose gel was visualised under a 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).

2.5. Qualitative PCR

Amplifications by qualitative PCR were carried out in 25 μ L of total reaction volume containing 2 μ L of DNA extract (100 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.01% of Tween 20, 3.0 mM of MgCl₂, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 200 μ M of each dNTP (Bioron, Ludwigshafen, Germany) and 200 nM of each primer (16SFish1-F/16SFish1-R). The PCR runs were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following cycling program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The amplicons were then analysed by electrophoresis in a 1.5% agarose gel containing 1 $\times$ Gel Red (Biotium, Hayward, CA, USA) for staining and carried out in 1 $\times$ SGTB buffer (GRISP, Porto, Portugal) for about 20–25 min at 200 V. The agarose gel was visualised under a 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).

2.6. Real-time PCR

Two different real-time PCR approaches were assayed using EvaGreen dye and melting curve analysis and a TaqMan probe. Real-time PCR assays with Evagreen were carried out in 20 μ L of total reaction volume containing 2 μ L of DNA, 1 $\times$ SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 300 nM of each primer (16SFish1-F/16SFish1-R). The real-time PCR runs were performed on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95 °C for 5 min; 45 cycles at 95 °C for 10 s, 57 °C for 15 s and 72 °C for 20 s, with collection of fluorescence signal at the end of each cycle. Data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). For melting curve analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 60 °C for 3 min, for the correct formation of DNA duplexes. These two steps were followed by melting curve ranging from 60 °C up to 90 °C with temperature increments of 0.2 °C per 10 s. The fluorescence data were acquired at the end of each melting temperature.

For the real-time PCR amplifications with a TaqMan probe, the 20 μ L of the reaction mixture included 2 μ L of DNA extract, 1 $\times$ SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer (16SFish1-F/16SFish1-R) and 200 nM of probe (16SFish1-P). The following temperature conditions were used: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 60 °C for 45 s, with collection of fluorescence signal at the end of each cycle. Bio-Rad CFX Manager 3.1 software was used to process and analyse the fluorescence data. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at automatic threshold settings.

3. Results and discussion

3.1. Specificity

In the present work, a sequence of the 16S rRNA gene was selected as a marker for the detection of a wide range of fish species, including those most commonly consumed. For that purpose, an exhaustive *in silico* study was performed to identify homologue regions and enable the design of universal primers for fish (Fig. S1, supplementary material). To confirm, experimentally, the specificity of the primers, several relevant fish species were tested by qualitative PCR with the newly designed primers (16SFish1-F/16SFish1-R). Table 1 shows that all the 26 fish species were amplified with the proposed set of primers, producing the expected fragment of 131 bp (Fig. S2A, supplementary material). The amplification of DNA from the binary mixtures showed that the optimised fish-specific PCR assay allow reaching a sensitivity down to 0.0001% (w/w) of cooked fish in béchamel (Fig. S3, supplementary material). The specificity of primers was further demonstrated using the DNA from several non-target species of crustaceans, molluscs, mammals, avian and plants, all relevant as foods. No cross-reactivity was obtained with the non-target species, thus confirming the specificity of the designed primers for fish detection (Fig. S2B, supplementary material). Prior to the specific amplification, all DNA extracts were tested for their amplifiability with universal eukaryotic primers (18SRG-F/18SRG-R), as described by Costa, Oliveira, and Mafra (2013). All the fish (Table 1), the other animal and the plant species produced the expected PCR fragment (113 bp) with the universal primers, confirming the absence of any false negative result.

3.2. Real-time PCR with EvaGreen dye

DNA binding dyes allow the acquisition of high fluorescence upon intercalation into double-stranded DNA (dsDNA) or by binding to the minor grooves of dsDNA. This type of binding chemistry enables the quantitative estimation of the DNA target due to the increase of the total fluorescence signal in direct proportion to the DNA quantity in the sample (Salihah, Hossain, Lubis, & Ahmed, 2016). In this work, the enhanced fluorescent EvaGreen dye was used for method development and further compared with a TaqMan probe system. The performance of the real-time PCR system with EvaGreen dye was analysed using a serially diluted fish-DNA extract and the binary model mixtures with fish to estimate both absolute and relative sensitivities, respectively. The guidelines of “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (Bustin et al., 2009) and the requirements set by the European Network of GMO Laboratories (ENGL) document “Definition of minimum performance requirements for analytical methods of GMO testing” (ENGL, 2015) were carefully considered for real-time PCR method development.

Table 2 presents the average results of two independent assays for each set of standards, while Fig. 1 shows an example assay with the amplification, calibration and melting curves. For the determination of the absolute sensitivity, a 10-fold serially diluted fish (hake) DNA extract (100,000 pg to 0.001 pg) was used to establish the dynamic range, which allowed covering the amplification of 8 orders of magnitude of the target analyte (Table 2, Fig. 1A, C). The limit of detection (LOD) was considered as the lowest concentration level with amplification at least 95% of the times. Accordingly, the LOD was established as 0.01 pg of fish DNA, which was also considered as the limit of quantification (LOQ) since it was within the linear range of the calibration curve. The average parameters of PCR efficiency (110.6%), slope (−3.092) and R^2 (0.998) were all within the acceptance criteria, suggesting a high performance of the assay (Table 2) (Bustin et al., 2009; ENGL, 2015). The presence of melt peaks with the same temperature (81.0 °C) is indicative of one single group of PCR products and the absence of primer dimer or other non-specific fragments (Fig. 1C).

The use of binary mixtures as reference standards with known

Table 2

Real-time PCR results with EvaGreen dye and TaqMan probe for the absolute and relative detection of fish targeting the 16S rRNA gene.

	Cycle threshold $\pm$ SD ^a	
	EvaGreen dye	TaqMan probe
<i>Absolute amount (pg fish DNA)</i>		
100 000	12.93 $\pm$ 0.31 (16/16)	14.78 $\pm$ 0.19 (16/16)
10 000	15.79 $\pm$ 0.35 (16/16)	18.08 $\pm$ 0.12 (16/16)
1 000	18.83 $\pm$ 0.32 (16/16)	21.09 $\pm$ 0.14 (16/16)
100	22.06 $\pm$ 0.28 (16/16)	24.33 $\pm$ 0.27 (16/16)
10	25.17 $\pm$ 0.28 (16/16)	27.36 $\pm$ 0.17 (16/16)
1	28.36 $\pm$ 0.29 (16/16)	30.73 $\pm$ 0.22 (16/16)
0.1	31.56 $\pm$ 0.35 (16/16)	33.56 $\pm$ 0.36 (16/16)
0.01	34.15 $\pm$ 0.81 (16/16)	37.23 $\pm$ 0.91 (16/16)
0.001	43.25 $\pm$ 0.29 (2/16)	nd ^b
PCR efficiency (%)	110.6 $\pm$ 1.9	107.4 $\pm$ 1.3
Correlation coefficient (R^2)	0.998 $\pm$ 0.002	0.999 $\pm$ 0.001
Slope	−3.092 $\pm$ 0.04	−3.156 $\pm$ 0.028
<i>Relative amount (% w/w of fish in béchamel)</i>		
50	16.84 $\pm$ 0.11 (8/8)	17.84 $\pm$ 0.40 (8/8)
10	19.01 $\pm$ 0.07 (8/8)	19.53 $\pm$ 0.77 (8/8)
5	20.21 $\pm$ 0.09 (8/8)	21.21 $\pm$ 0.13 (8/8)
1	22.64 $\pm$ 0.11 (8/8)	23.57 $\pm$ 0.17 (8/8)
0.5	23.81 $\pm$ 0.90 (8/8)	24.22 $\pm$ 0.17 (8/8)
0.1	26.02 $\pm$ 0.37 (8/8)	26.53 $\pm$ 0.64 (8/8)
0.05	27.11 $\pm$ 0.10 (8/8)	27.80 $\pm$ 0.18 (8/8)
0.01	ns ^c	31.29 $\pm$ 0.54 (8/8)
0.005	ns	32.26 $\pm$ 0.34 (8/8)
0.001	ns	34.61 $\pm$ 0.55 (8/8)
0.0005	ns	35.88 $\pm$ 0.57 (8/8)
0.0001	ns	36.67 $\pm$ 0.99 (8/8)
0	ns	nd
PCR efficiency (%)	96.3 $\pm$ 1.6	90.3 $\pm$ 0.6
Correlation coefficient (R^2)	0.999 $\pm$ 0.001	0.987 $\pm$ 0.004
Slope	−3.415 $\pm$ 0.042	−3.578 $\pm$ 0.018

^a Mean values $\pm$ standard deviation (SD) of replicates (in brackets are the positive amplifications/total replicates) obtained in two independent assays.

^b nd, not detected.

^c ns, non-specific amplification.

amounts of spiked fish enabled to establish a distinct dynamic range (0.05–50%) and the relative LOD. In this case, a calibration curve with 4 orders of magnitude was obtained, with LOD and LOQ down to 0.05% (500 mg/kg) (Table 2, Fig. 1B, D) and adequate analytical performance parameters (PCR efficiency of 96.3%, slope of −3.415 and R^2 of 0.999). According to the melting curve analysis, the amplification of fragments with similar melt peaks (81.0 °C) was obtained until the level of 0.05%. After which, other groups of melt peaks at 79.8–80.0 °C appeared, suggesting the presence of non-specific products (Fig. 1F, blue and pink lines).

3.3. Real-time PCR with TaqMan probe

In real-time PCR, the use of specific fluorescent probes, such as the hydrolysis TaqMan probes, has the advantage of conferring increased specificity to the assay, without needing melting curve analysis. For comparative purposes, the performance of the real-time PCR system with a TaqMan probe was analysed using a serially diluted fish-DNA extract and the binary model mixtures with fish to estimate both absolute and relative sensitivities, respectively, as made in the previous system. The performance results of the probe system are presented in Table 2 and Fig. 2. Regarding the amplification of the serially diluted DNA, the results are very similar to the obtained in the previous EvaGreen system. Despite a delayed amplification of about 2 cycles in the probe system, the parameters of dynamic range (8 orders of magnitude) and sensitivity were identical (LOD = LOQ = 0.01 pg) (Fig. 2A, C). The other parameters suggested again a high performance of the assay (PCR efficiency of 107.4%, slope of −3.156 and R^2 of 0.999) (Table 2).

In opposition, the results for the relative sensitivity were quite

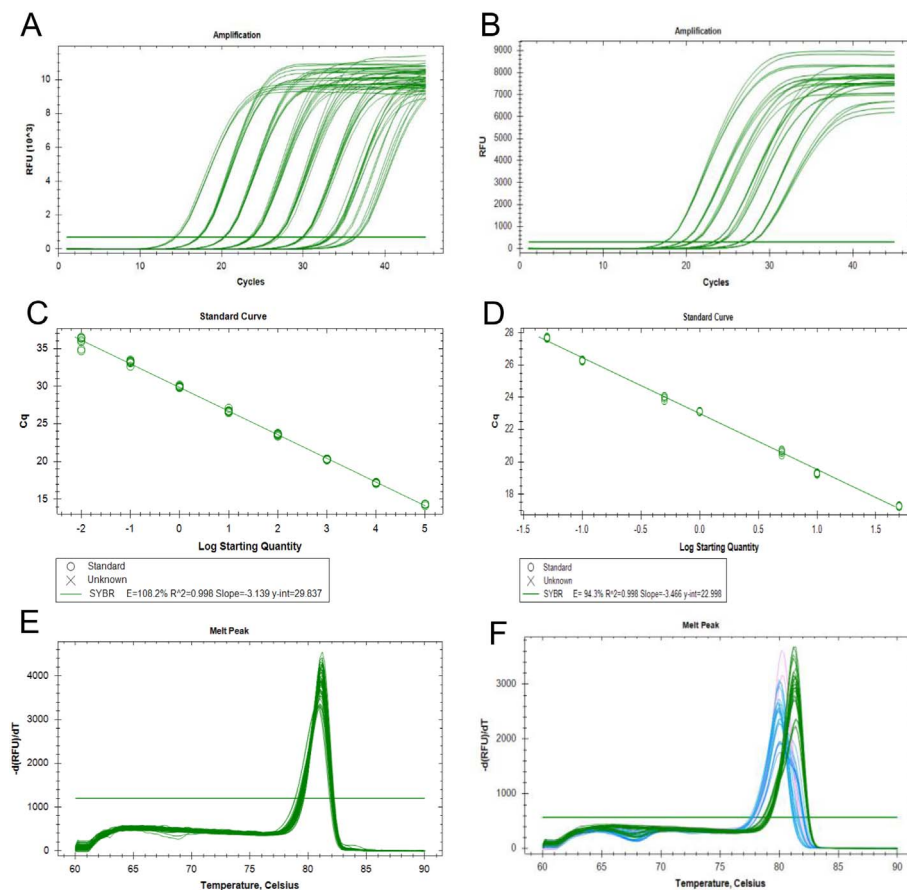


Fig. 1. Real-time PCR amplification (A, B), calibration (C, D) and melting (E, F) curves with EvaGreen dye targeting the 16S rRNA of fish using a serially diluted fish-DNA extract (100,000–0.001 pg) (A, C, E) and DNA extracts from binary model mixtures of cooked fish in béchamel sauce (50–0.001% (w/w)) (B, D, F). Non-specific melt curves are presented in blue and pink lines (F) from 0.01% to 0.0001% and 0%, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

improved with the probe system since both LOD and LOQ were reduced to 0.0001% (w/w) of fish in béchamel sauce and the dynamic range was increased to 6 orders of magnitude with high performance assay parameters (PCR efficiency of 90.3%, slope of -3.578 and R^2 of 0.987) (Table 2, Fig. 2B, D). This sensitivity (1 mg of fish per kg of food) can be considered an adequate level regarding the threshold dose (5–6000 mg of fish) for eliciting an allergic response to fish (Taylor et al., 2002).

In summary, the proposed real-time PCR with the probe enabled absolute and relative LOD of 0.01 pg of DNA and 0.0001% (1 mg/kg) of fish in béchamel sauce, which were considerably lower than the values

reported in literature. Benedetto, Abete, and Squadrone (2011) and Pegels et al. (2013), who also targeted mitochondrial DNA using real-time PCR with probe systems, reached sensitivities of 0.2 pg and 0.1 pg of fish DNA, respectively. Comparing the performance of both proposed systems, it can be inferred that the probe system was the best. Despite the same absolute sensitivities (0.01 pg) obtained by both approaches with adequate real-time PCR performance parameters, the probe system showed higher relative sensitivity and dynamic range (0.0001–50%) than the EvaGreen (0.05–50%).

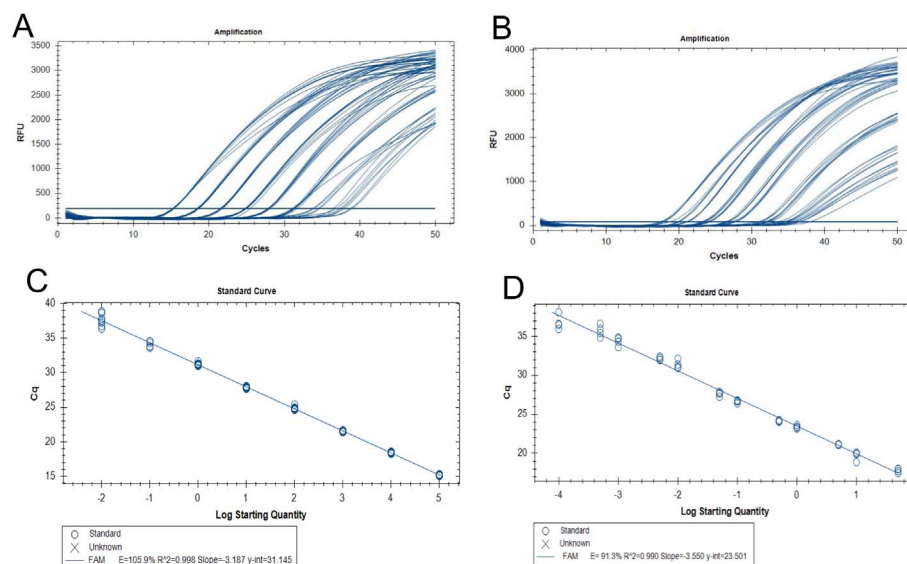


Fig. 2. Real-time PCR amplification (A, B) and calibration (C, D) curves with a TaqMan probe targeting the 16S rRNA of fish using a serially diluted fish-DNA extract (100,000–0.001 pg) (A, C) and DNA extracts from binary model mixtures of cooked fish in béchamel sauce (50–0.001% (w/w)) (B, D).

Table 3

Results for the validation of the quantitative real-time PCR assays with EvaGreen dye and TaqMan probe using blind samples.

Real-time PCR assay	Fish content (% w/w)		CV (%) ^b	Bias (%) ^c
	Actual	Estimated (mean $\pm$ SD ^a)		
EvaGreen dye	8.0	11.64 $\pm$ 1.43	12.3	45.5
	4.0	3.27 $\pm$ 0.24	7.28	−9.14
	2.5	2.80 $\pm$ 0.09	3.31	3.75
	0.25	0.23 $\pm$ 0.01	6.31	−0.29
TaqMan probe	8.0	9.52 $\pm$ 1.45	15.2	19.0
	4.0	3.60 $\pm$ 0.46	12.7	−5.02
	2.5	2.64 $\pm$ 0.20	7.52	1.80
	0.25	0.26 $\pm$ 0.02	8.33	0.10

^a Mean values $\pm$ standard deviation (SD) of replicates ($n = 8$) obtained in two independent real-time PCR assays.

^b CV, coefficient of variation.

^c Bias = ((mean estimated value – true value)/true value $\times$ 100).

3.4. Method validation

Considering that the specificity of the primers was previously demonstrated by qualitative PCR, it is also important to verify the specificity of the probe. Accordingly, all fish species were amplified by real-time PCR with the TaqMan probe, confirming most results with Ct values ranging from 18 to 23 (Table 1). The amplification with TaqMan probe was negative in four fish species (*Raja* spp., *Salilota australis*, *Genypterus blacodes*, *Pangasius hypophthalmus*). This might be due to nucleotide differences in the probe region, which can be observed in the cases of *G. blacodes* and *P. hypophthalmus* sequences (Fig. S1, supplementary material). To overcome this finding, the four species were further successfully amplified using the EvaGreen assay (Table 1).

For the validation of the quantitative assays, both EvaGreen and probe systems were tested for their aptitude to estimate the fish content using blind mixtures with fish contents of 8%, 4%, 2.5% and 0.25% (w/w) (Table 3). The comparison between the estimations and the actual values suggests that both real-time PCR systems demonstrate precision over the tested dynamic range ($\leq 25\%$) since the coefficients of variation varied between 3.31% and 15.2% (ENGL, 2015). The measured trueness, expressed as bias, was generally higher for the estimates with the EvaGreen than for those with the probe system, but still within $\pm 25\%$ of the actual value (ENGL, 2015) for all the tested levels, except for the highest one (8%) determined by the EvaGreen system that exhibited 45.5% of bias. This means that the probe system can provide accurate determinations in all tested range (0.25–8%), while in the EvaGreen system the range is lower (0.25–4%). Therefore, it can be inferred that both real-time PCR approaches were successfully developed and validated, although the TaqMan probe exhibited better performance than the EvaGreen in terms of sensitivity, dynamic range and trueness.

3.5. Analysis of processed food samples

Based on the validation results and on the previous data of assay performance, the real-time PCR system with the probe was considered the most adequate for fish allergen detection and quantification due to its higher sensitivity and dynamic range for quantification compared with the EvaGreen dye assay. For that reason, the real-time PCR assay with probe was selected to be further applied in the analysis of processed foods.

Concerning the application of the method to processed foods, the results of the 34 analysed samples are presented in Table 4. It can be noticed that most foods indicated the percentage of fish as a food component on the label. With the exception of sample #6 (Paella, “it contains fish”), all the samples were positive with the 16S rRNA primers in qualitative PCR and real-time PCR, which supports the fact that the

primers are universal markers for the presence of most fish species found in processed foods. The Ct values ranged from 20.72 to 32.66, which allowed estimating the fish content based on the developed calibration curve using the binary mixtures (Fig. 2D). The estimated contents varied from 0.02 to 48.5%, but they were, in general, much lower than the labelled values, which questions the veracity of the claimed information to consumers. In samples #9 (Surimi), #19, #20 (soups) and #28, #29 (fish sticks), the estimated fish contents are in close relation with the labelled information. In the samples #6 and #17, fish was not detected or found at trace levels, which is in good agreement with the type of foods that are not supposed to contain fish, whose labels should state the precautionary labelling “May contain fish” instead of “It contains fish”. In opposition, in sample #23, with an estimated fish content of 7.07% and labelled as “May contain fish”, fish should only be present at trace levels and not as an ingredient. These findings highlight inaccurate labelling of samples #6 and #17, and adulteration practices by partial or total replacement of shrimp by fish in sample #23. The remaining samples, with estimated contents much lower than the labelled, also suggest adulteration practices by partial substitution of fish by other ingredients.

The application of other real-time PCR methods in the detection of fish in food samples has been reported. The method of Herrero et al. (2014) was applied to processed food analysis, but only at the level of detection without quantification. The methods of Benedetto et al. (2011) and Pegels et al. (2013), although not focusing on fish as a potential allergen, but on feed authentication, were effectively applied to quantitative analysis. Benedetto et al. (2011) attempted a normalised method with binary mixtures of fish in plant material, but they did not reach an accurate quantification. The authors highlighted the need for a reliable endogenous reference for the accurate quantitative determination of fish. Pegels et al. (2013) successfully applied the assay to the authentication of industrial farm animal feeds and commercial pet foods, and exploited the quantitative potential with reproducible results. However, they were not able to obtain accurate estimates. The matrix, the DNA extraction method and the level of processing can greatly affect the DNA yield, integrity and purity, which are crucial factors that influence the quantitative real-time PCR performance.

In the present work, the proposed method was effectively applied to verify labelling compliance of processed fish-containing foods, being able to detect fish at trace levels, required as a food allergen, and to obtain estimates that could be successfully validated with blind samples. To our knowledge, this was performed for the first time.

4. Conclusion

Fish-allergic individuals may react to the ingestion of a wide range of fish species at trace levels, thus relying on the labelled information of processed foods. Therefore, the development of reliable and sensitive methods is of utmost importance to verify labelling compliance and protect the health of allergic consumers. In view of that, after an exhaustive DNA sequence search of the 16S rRNA gene, new primers for the universal detection of fish were designed whose specificity was fully demonstrated, both *in silico* and experimentally. Two real-time PCR systems based on the universal EvaGreen dye and on a TaqMan probe were successfully developed and further evaluated. Both systems were able to reach a high absolute sensitivity (0.01 pg) with adequate real-time PCR performance parameters. However, the probe system enabled a relative LOD down to 0.0001% (1 mg/kg) of cooked fish in béchamel sauce, while the EvaGreen one revealed specific amplification only until a level of 0.05%, with consequent distinct dynamic ranges of 0.0001–50% and 0.05–50%, respectively. The validation results with blind samples showed that both systems were precise in the tested range, but trueness, expressed as bias, was within $\pm 25\%$ for all tested levels (0.25–8%) with the probe system, while with EvaGreen the error was too high for the highest tested level (8%). Therefore, the real-time PCR system with the probe exhibited the best performance in terms of

Table 4

Analysed processed food samples with relevant labelled information and results of qualitative PCR and real-time PCR targeting the 16S rRNA gene of fish species.

Sample		Relevant label information	PCR ^a	Real-time PCR ^b	
				Ct ± SD	Estimated fish content (% w/w)
1	Codfish “patanísca”	Codfish 18%	+	22.94 ± 0.13	6.74 ± 0.57
2	“Brás style” codfish	Codfish 18%	+	22.56 ± 0.16	8.64 ± 0.91
3	“Gomes de Sá” style codfish	Codfish 20%	+	22.44 ± 0.02	13.43 ± 0.18
4	Codfish “alheira”	Codfish 34%	+	24.67 ± 0.29	3.47 ± 0.63
5	Codfish lasagne	Codfish 47%	+	24.64 ± 0.04	3.83 ± 0.09
6	Paella	It contains fish	–	–	
7	Codfish with cream	Codfish 11%	+	24.42 ± 0.17	4.38 ± 0.44
8	Codfish with cream	Codfish 18%	+	24.72 ± 0.06	2.14 ± 0.08
9	Surimi	Alaska pollock 49.5%	+	20.36 ± 0.02	48.51 ± 1.56
10	Surimi	Alaska pollock 49%	+	22.00 ± 0.14	19.21 ± 1.66
11	Seafood risotto	Hake 8%. Fish sauce 1%	+	26.02 ± 0.13	0.93 ± 0.08
12	Codfish pies	Codfish 23%	+	24.13 ± 0.07	5.21 ± 0.21
13	Hake/shrimp patties	Filling 42.5% (containing hake)	+	25.21 ± 0.11	2.71 ± 0.18
14	Hake patties	Hake 11%	+	23.95 ± 0.03	3.51 ± 0.07
15	Codfish patties	Codfish 26%	+	24.47 ± 0.02	2.51 ± 0.03
16	Codfish patties	Codfish 30%	+	25.04 ± 0.19	2.75 ± 0.33
17	Shrimp patties	It contains fish	+	32.66 ± 0.04	0.02 ± 0.00
18	Seafood soup	Fish 4.1%	+	30.07 ± 0.11	0.14 ± 0.01
19	Seafood soup	Fish 4.1%	+	23.91 ± 0.05	5.46 ± 0.14
20	Seafood soup	Fish 5.2%	+	24.09 ± 0.05	4.90 ± 0.14
21	Breaded fish	Alaska pollock 56%	+	21.40 ± 0.06	18.23 ± 0.69
22	Breaded fish	Alaska pollock 70%	+	20.81 ± 0.04	29.35 ± 0.79
23	Shrimp stick	May contain fish	+	23.63 ± 0.05	7.07 ± 0.21
24	Fish sticks	Fish 65%	+	22.01 ± 0.09	19.09 ± 1.05
25	Fish sticks	White fish 65%	+	21.76 ± 0.13	22.27 ± 1.80
26	Fish sticks	White fish 48%	+	24.57 ± 0.07	2.35 ± 0.11
27	Fish sticks	Hake 50%	+	21.39 ± 0.06	18.39 ± 0.61
28	Fish sticks	Hake 55%	+	20.72 ± 0.13	31.11 ± 2.41
29	Fish sticks	Hake 55%	+	20.94 ± 1.08	39.66 ± 0.87
30	Fish sticks	Hake 54%	+	22.15 ± 0.05	13.07 ± 0.43
31	Fish sticks	Hake 60%	+	21.20 ± 0.02	23.24 ± 0.31
32	Codfish sticks	Codfish 58%	+	22.33 ± 0.10	11.72 ± 0.68
33	Fish sticks	Alaska pollock 65%	+	25.34 ± 0.03	1.86 ± 0.04
34	Fish sticks	Hake 55%	+	22.20 ± 0.06	14.08 ± 0.50

^a (+) positive qualitative PCR amplification, (–) non-detected amplification.^b Mean values ± standard deviation (SD) of replicates (n = 8) obtained in two independent real-time PCR assays.

relative sensitivity, dynamic range and trueness.

The application of the real-time PCR system with the probe proved to be useful to detect trace amounts of fish in processed foods. On the other hand, the application of real-time PCR system with EvaGreen dye extended the number of fish species detected that, due to nucleotide probe mismatches, could not be amplified with the probe system. Moreover, the verification of labelling compliance of foodstuffs suggested a high level of mislabelling and/or fraudulent practices. Therefore, the proposed approaches can provide useful tools to contribute for the safety assessment of foods, particularly protecting the health of fish allergic consumers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.11.068>.

References

- Abdullah, A., & Rehbein, H. (2016). The differentiation of tuna (family: Scombridae) products through the PCR-based analysis of the cytochrome b gene and parvalbumin introns. *Journal of the Science of Food and Agriculture*, 96, 456–464.
- Benedetto, A., Abete, M. C., & Squadrone, S. (2011). Towards a quantitative application of real-time PCR technique for fish DNA detection in feedstuffs. *Food Chemistry*, 126(3), 1436–1442.
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, 611–622.
- Calderon-Rodriguez, S., Pineda, F., Perez, R., & Muñoz, C. (2016). Tolerability to dogfish in children with fish allergy. *Allergologia et Immunopathologia*, 44, 167–169.
- Chen, Y. T., & Hsieh, Y. H. P. (2014). A sandwich ELISA for the detection of fish and fish products. *Food Control*, 40, 265–273.
- Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Research International*, 54, 1722–1729.
- ENGL (2015). Definition of minimum performance requirements for analytical methods of GMO testing. European network of GMO laboratories, Joint Research Center, EURL. Available online at < http://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%202010_2015.pdf > . Last accessed on March 3, 2017.
- Faeste, C. K., & Plassen, C. (2008). Quantitative sandwich ELISA for the determination of fish in foods. *Journal of Immunology Methodologies*, 329, 45–55.
- Feng, J., Wu, Z., Xie, X., Dai, Z., & Liu, S. (2017). A real-time polymerase chain reaction method for the identification of four commercially important salmon and trout species. *Mitochondrial DNA Part A*, 28, 104–111.
- Fernandes, T. J. R., Costa, J., Carrapatoso, I., Oliveira, M. B. P. P., & Mafra, I. (2017). Advances on the molecular characterisation, clinical relevance and detection methods of Gadiform parvalbumin allergens. *Critical Reviews in Food Science and Nutrition*, 57, 3281–3296.
- Fernandes, T. J. R., Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2015). An overview on fish and shellfish allergens and current methods of detection. *Food and Agricultural Immunology*, 26, 848–869.
- Fernandes, T. J. R., Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2017). DNA barcoding coupled to HRM analysis as a new and simple tool for the authentication of Gadidae

- fish species. *Food Chemistry*, 230, 49–57.
- Fernandes, T. J. R., Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2018). COI barcode-HRM as a novel approach for the discrimination of hake species. *Fisheries Research*, 197, 50–59.
- Giusti, A., Tinacci, L., Sotelo, C. G., Marchetti, M., Guidi, A., Zheng, W., et al. (2017). Seafood identification in multispecies products: Assessment of 16S rRNA, cytb, and COI universal primers' efficiency as a preliminary analytical step for setting up metabarcoding next-generation sequencing techniques. *Journal of Agricultural and Food Chemistry*, 65, 2902–2912.
- Herrero, B., Vieites, J. M., & Espiñeira, M. (2014). Development of an in-house fast real-time PCR method for detection of fish allergen in foods and comparison with a commercial kit. *Food Chemistry*, 151, 415–420.
- Houhoula, D., Dimitriou, P., Mengjezi, G., Kyrana, V., & Lougovois, V. (2015). Quantification of parvalbumin in commercially important mediterranean seafood species using real time PCR. *Czech Journal of Food Science*, 33, 143–147.
- Kobayashi, A., Tanaka, H., Hamada, Y., Ishizaki, S., Nagashima, Y., & Shiomi, K. (2006). Comparison of allergenicity and allergens between fish white and dark muscles. *Allergy*, 61, 357–363.
- Kuehn, A., Scheuermann, T., Hilger, C., & Hentges, F. (2010). Important variations in parvalbumin content in common fish species: A factor possibly contributing to variable allergenicity. *International Archives of Allergy and Immunology*, 153, 359–366.
- Mourad, A. A., & Bahna, S. L. (2015). Fish-allergic patients may be able to eat fish. *Expert Review of Clinical Immunology*, 11, 419–430.
- Pascual, C., Esteban, M. M., & Crespo, J. F. (1992). Fish allergy: Evaluation of the importance of cross-reactivity. *The Journal of Pediatrics*, 121, S29–S34.
- Pegels, N., González, I., López-Calleja, I., García, T., & Martín, R. (2013). Detection of fish-derived ingredients in animal feeds by a TaqMan real-time PCR assay. *Food Analytical Methods*, 6, 1040–1048.
- Prado, M., Ortea, I., Vial, S., Rivas, J., Calo-Mata, P., & Barros-Velazquez, J. (2016). Advanced DNA- and protein-based methods for the detection and investigation of food allergens. *Critical Reviews in Food Science and Nutrition*, 56, 2511–2542.
- Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No. 1924/2006 and (EC) No. 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. Official Journal of the European Union, L304, 18–63.
- Salihah, N. T., Hossain, M. M., Lubis, H., & Ahmed, M. U. (2016). Trends and advances in food analysis by real-time polymerase chain reaction. *Journal of Food Science and Technology*, 53, 2196–2209.
- Sharp, M. F., & Lopata, A. L. (2014). Fish allergy: In review. *Clinical Reviews in Allergy and Immunology*, 46, 258–271.
- Sun, M., Liang, C., Gao, H., Lin, C., & Deng, M. (2009). Detection of parvalbumin, a common fish allergen gene in food, by real-time polymerase chain reaction. *Journal of AOAC International*, 92, 234–240.
- Taylor, S. L., Hefle, S. L., Bindslev-Jensen, C., Bock, S. A., Burks, A. W., Christie, L., et al. (2002). Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *Journal of Allergy and Clinical Immunology*, 109, 24–30.
- Tetzlaff, C., & Mäde, D. (2017). Development of a real-time PCR system for the detection of the potential allergen fish in food. *European Food Research and Technology*, 243, 849–857.
- Thalayasingam, M., & Lee, B. -W. (2015). Fish and shellfish allergy. M. Ebisawa, B. K. Ballmer-Weber, S. Vieths, R. A. Wood (Eds.), *Food allergy: Molecular basis and clinical practice*. Chem Immunol Allergy (pp. 152–161). Basel: Karger.
- Triantafyllidis, A., Karaiskou, N., Perez, J., Martinez, J. L., Roca, A., Lopez, B., et al. (2010). Fish allergy risk derived from ambiguous vernacular fish names: Forensic DNA-based detection in Greek markets. *Food Research International*, 43, 2214–2216.
- Weber, P., Steinhart, H., & Paschke, A. (2009). Competitive indirect ELISA for the determination of parvalbumins from various fish species in food grade fish gelatins and isinglass with PARV-19 anti-parvalbumin antibodies. *Journal of Agricultural and Food Chemistry*, 57, 11328–11334.

Supplementary material

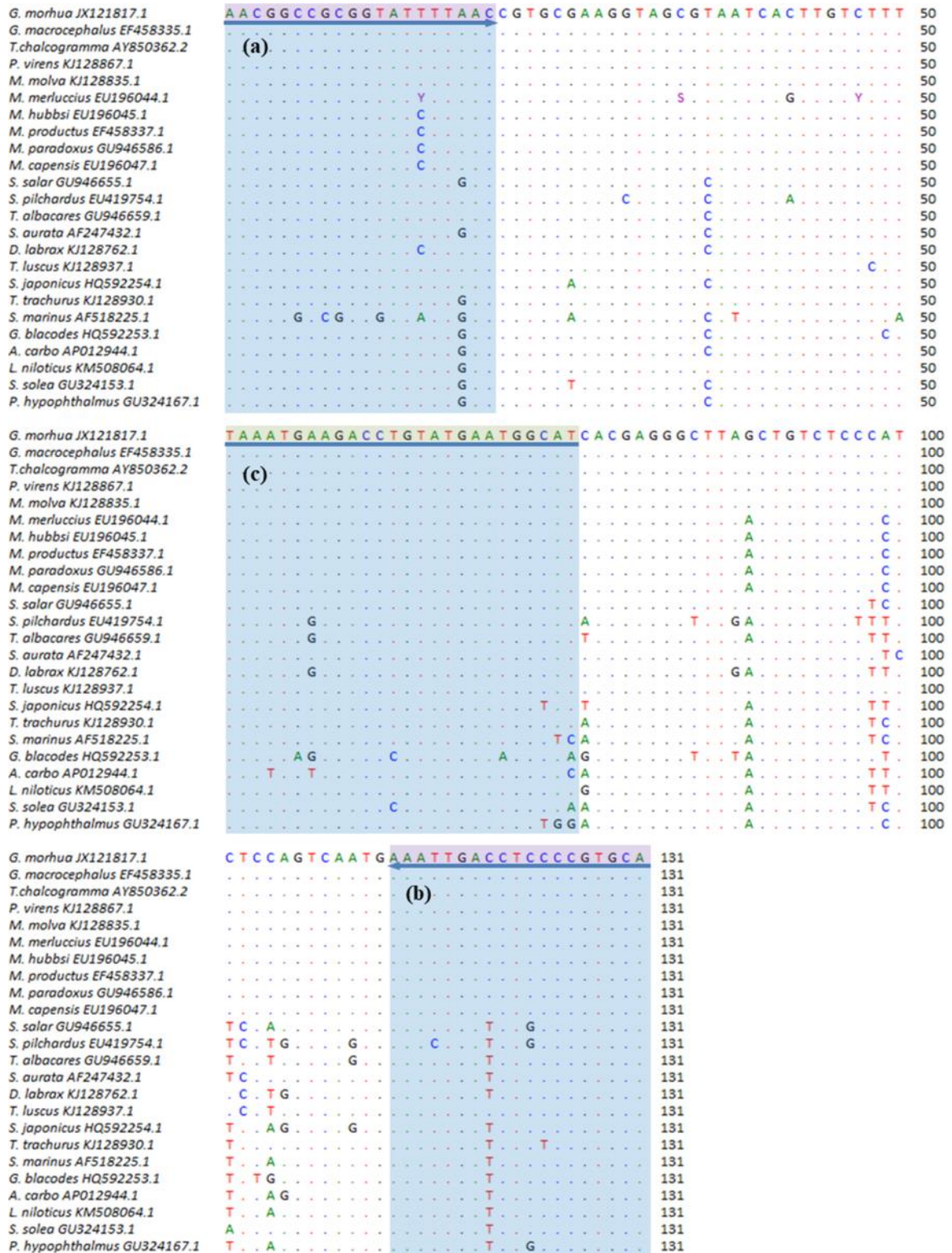


Figure S1. Sequence alignment of NCBI 16S rRNA entries showing primer and probe design regions. (a) 16SFish1-F; (b) 16SFish1-R; (c) 16SFish1-P.

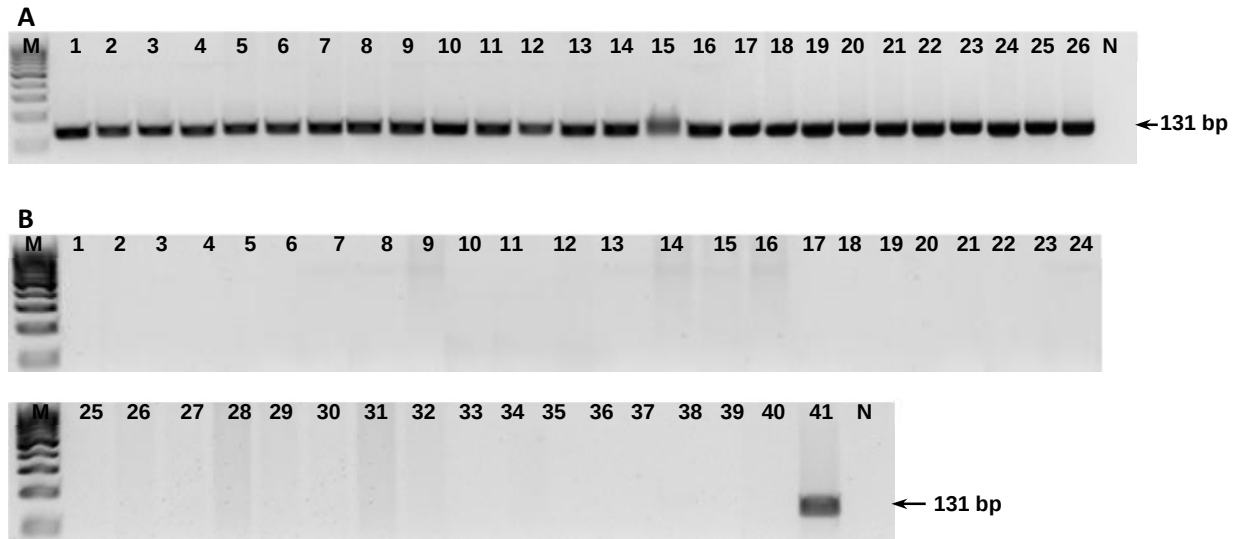


Figure S2. Agarose gel electrophoresis of PCR products targeting the 16S rRNA gene of several fish species (A) and non-target food related species (B). Legend A: Lane 1, *G. morhua*; 2, *G. macrocephalus*; 3, *T. chalcogramma*; 4, *P. virens*; 5, *M. molva*; 6, *M. merluccius*; 7, *M. productus*; 8, *M. hubsi*; 9, *M. capensis*; 10, *M. paradoxus*; 11, *S. pilchardus*; 12, *Raja* spp.; 13, *T. trachurus*; 14, *Sparus aurata*; 15, *S. solea*; 16, *S. japonicus*; 17, *S. salar*; 18, *T. albacares*; 19, *D. labrax*; 20, *T. luscus*; 21, *Saliota australis*; 22, *S. marinus*; 23, *G. blacodes*; 24, *A. carbo*; 25, *L. niloticus*; 26, *P. hypophthalmus*. Legend B: Lanes 1-12, crustacean and molluscs; 13-21, meat species; 22-40, plant species; 41, Positive control (*G. morhua*). M, 100 bp DNA ladder (Bioron, Ludwigshafen, Germany); N, negative control.

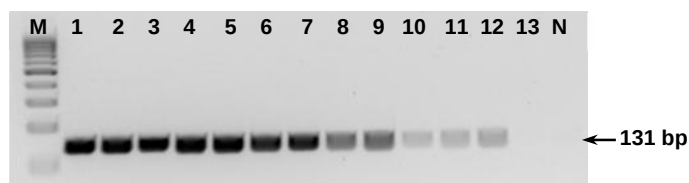


Figure S3. Agarose gel electrophoresis of PCR products targeting the 16S rRNA gene of fish species using binary model mixtures with known amounts of cooked fish in béchamel sauce. Legend: Lanes 1, 50%; 2, 10%; 3, 5%; 4, 1%; 5, 0.5%; 6, 0.1%; 7, 0.05%; 8, 0.01%; 9, 0.005%; 10, 0.001%; 11, 0.0005%; 12, 0.0001%; 13, 0% (w/w); M, 100 bp DNA ladder (Bioron, Ludwigshafen, Germany); N, Negative control.