Food Chemistry 228 (2017) 469–475

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A new real-time PCR method for rapid and specific detection of ling (*Molva molva*)

Ledicia Taboada, Ana Sánchez, Carmen G. Sotelo *

Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain

ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form 19 January 2017
Accepted 24 January 2017
Available online 8 February 2017

Keywords:
Seafood authentication
Fraud
Real-time PCR
Ling
Molva molva

ABSTRACT

Seafood fraud – often involving substitution of one species by another – has attracted much attention as it is prevalent worldwide. Whilst DNA analysis has helped to combat this type of fraud some of the methods currently in use are time-consuming and require sophisticated equipment or highly-trained personnel. This work describes the development of a new, real-time PCR TaqMan assay for the detection of ling (*Molva molva*) in seafood products. For this purpose, specific primers and a minor groove binding (MGB) TaqMan probe were designed to amplify the 81 bp region on the *cyt b* gene. Efficiency, specificity and cross-reactivity assays showed statistically significant differences between the average Ct value obtained for *Molva molva* DNA (19.45 ± 0.65) and the average Ct for non-target species DNA (38.3 ± 2.8), even with closely related species such as *Molva dypterygia* (34.9 ± 0.09). The proposed methodology has been validated with 31 commercial samples.

1. Introduction

European Union labelling regulations specify that the commercial and scientific names should be included on the labels of seafood products in order to assure their traceability and the correct identification throughout the value chain (EC 104/2000; EC 2065/2001; EU 1379/2013). The recent EU Regulation 1379/2013 on Common Market Organization requires that consumers receive precise information about the seafood they purchase, such as scientific and commercial names, fishing ground, date of durability and fishing gear. The idea behind this new regulation is to provide consumers with sufficient information to select certain products which are obtained via sustainable methods or which do not represent a threat to marine ecosystems. It is therefore clear that methods to monitor the correct labelling of seafood products on the market are necessary to ensure compliance with this and previous labelling regulations (Dalmasso et al., 2007; Mackie et al., 1999; Primrose, Woolfe, & Rollinson, 2010).

In 2012, the portion of processed fish was 74 Mt (54% of global production) and in the case of frozen fish an increase of 55% in the share was observed in developed countries (FAO, 2014), indicating that consumer demand for processed fishery products is growing. However, this trend might be accompanied by a parallel increase in fraudulent or accidental species substitution. (D'Amico et al., 2014).

Mislabelling can be done for economic benefits: undeclared cheaper or lower quality fish species are sold under the name of species with a higher price and quality. However, there are other reasons for this illegal activity such as with species that have a defined fishing quota: once this quota has been reached the fish are still caught illegally and this illegal catch can be concealed by using the name of other species with no quota or with non-filled quotas (Helyar et al., 2014).

Some of the issues arising as a consequence of fish mislabelling include consumer health risks (i.e. poisonous species and allergic reactions) (Sakaguchi et al., 2000; Triantafyllidis et al., 2010); environmental problems such as overfishing and high economic value species depletion (Marko et al., 2004; Miller & Mariani, 2010) and economic losses (Miller & Mariani, 2010).

The ling (*Molva molva*) belongs to the family Lotidae. Due to its morphological and organoleptic characteristics it is susceptible to illegal substitution with species of lower commercial value. In fact, ling could be subjected to a variety of different transformations including heading, gutting, filleting, salting, etc., which makes it easy to substitute other species for it. This may happen because in some European countries, such as France or Spain, ling is a highly prized species (Le Francois, Jobling, Carter, & Blier, 2010, chap. 6 and 13).

* Corresponding author at: Instituto de Investigaciones Marinas (IIM-CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain.
E-mail addresses: ledicia.t@gmail.com (L. Taboada), asanchez@iim.csic.es (A. Sánchez), carmen@iim.csic.es (C.G. Sotelo).

http://dx.doi.org/10.1016/j.foodchem.2017.01.117
0308-8146/

However, the opposite also happens: ling can be used as a substitute for higher-value species such as Atlantic cod (*Gadus morhua*) (Taboada et al., 2014). Furthermore, there is also the practice of labelling illegally captured species that have a defined fishing quota, no quota or a larger quota, as ling (Di Pinto et al., 2013; Miller & Mariani, 2010).

Identification of fish species in highly processed products is not possible unless DNA markers are employed, since the morphological characteristics are lost and proteins can be denatured during processing, resulting in the subsequent loss of suitable analytes for species determination.

Different techniques based on DNA have been proposed during recent decades for gadoids identification (Akasaki, Yanagimoto, Yamakami, Tomonaga, & Sato, 2006; Chapela, Sánchez, Suárez, Perez-Martin, & Sotelo, 2007; Hird et al., 2011; Maretto, Reffo, Dalvit, Barcaccia, & Mantovani, 2007; Sánchez, Quinteiro, Rey-Mendez, Perez-Martin, & Sotelo, 2009; Xiong et al., 2016). To date, there are very few publications concerning the identification of *Molva molva* and none of these include the development of a RT-PCR approach.

Some of the advantages of the RT-PCR methodology for identification of fish species are that it is a fast, easy and fully-monitorable process. Furthermore, it allows rapid analysis of a large number of samples simultaneously and no post-PCR sample treatment is required, reducing the chances of contamination. In addition, this technique may be applied to both fresh and processed products (Rasmussen & Morrissey, 2011).

With Real-time PCR it is possible to monitor amplicon accumulation due to the fact that the primers, the probes or the amplicon itself are labelled with fluorescence-emitting molecules, producing a signal change after the interaction or hybridization with the amplicon. The signal increases as the amount of amplicon increases after each amplification cycle. TaqMan technology is based on the 5′-3′exonuclease activity of the polymerase to release the two fluorochromes: a reporter placed at the 5′ head and a quencher at the 3′ edge of a specific probe. Since this probe hybridizes specifically with template DNA, the technique shows a higher specificity compared to techniques based on non-specific fluorochrome, such as the SYBR green method. Real time PCR technology has been used in the detection of species in different meat products (Hird, Chisholm, & Brown, 2005; Laube et al., 2003), vegetables (Hernandez & Esteve, 2005; Hird, Lloyd, Goodier, Brown, & Reece, 2003), bacteria (Panicker, Myers, & Bej, 2004; Weller et al., 2002) and fish (Castigliego, Armani, Tinacci, Gianfaldoni, & Guidi, 2015; Dalmasso et al., 2007; Hird et al., 2011; Sánchez et al., 2009).

This work describes the development of a rapid and precise method for identifying ling (*Molva molva*) based on TaqMan real-time PCR technology, a very fast and simple test that can be applied to fresh, frozen, and processed products to detect the fraudulent or unintentional mislabelling of this species.

Table 1
Reference species used for the study.

Species	N	Common name	Source
Orden gadiformes			
Family Lotidae			
Brosme brosme	1	Tusk	Dept. of Fish Quality, BFEL (Germany)
Molva dypterigia	1	Blue ling	Dept. of Fish Quality, BFEL (Germany)
Molva molva	15	Ling	Dept. of Fish Quality, BFEL (Germany)
Gaidropsarus ensis	1	Threadfin rockling	Fisheries and Oceans Canada
Family Gadidae			
Gadus macrocephalus	1	Pacific Cod	Instituto de Investigaciones Marinas, CSIC (Spain
Gadus morhua	1	Atlantic Cod	Instituto de Investigaciones Marinas, CSIC (Spain)
Gadus ogac	1	Greenland Cod	Instituto de Investigaciones Marinas, CSIC (Spain
Gadus chalcogrammus	1	Alaska pollock	Dept. of Fish Quality, BFEL (Germany)
Melanogrammus aeglefinus	1	Haddock	Dept. of Fish Quality, BFEL (Germany)
Merlangius merlangus	1	Whiting	Dept. of Fish Quality, BFEL (Germany)
Micromesistimus poutassous	1	Blue whiting	Instituto de Investigaciones Marinas, CSIC (Spain)
Pollachius pollachius	1	Pollack	Dept. of Fish Quality, BFEL (Germany)
Pollachius virens	1	Saithe	Dept. of Fish Quality, BFEL (Germany)
Trisopterus luscus	1	Pouting	Instituto de Investigaciones Marinas, CSIC (Spain)
Trisopterus esmarkii	1	Norway pout	Dept. of Fish Quality, BFEL (Germany)
Trisopterus minutus	1	Poor cod	Dept. of Fish Quality, BFEL (Germany)
Family Merlucidae			
Macruronus magellanicus	1	Patagonian grenadier	Isla Mar (fishing company)
Macruronus novaezelandiae	1	Blue grenadier	Europacífico (fishing company)
Merluccius bilinearis	1	Silver hake	Fisheries and Oceans Canada
Merluccius capensis	1	Shallow-water Cape hake	Marine and Coastal Management (South Africa)
Merluccius polli	1	Benguela hake	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius hubbsi	1	Argentina hake	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius merluccius	1	European hake	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius paradoxus	1	Deep-water Cape hake	Marine and Coastal Management (South Africa)
Merluccius senegalensis	1	Senegalese hake	Oviedo University (Spain)
Family Moridae			
Salilota australis	1	Tadpole codling	Instituto de Investigaciones Marinas, CSIC (Spain)
Family Phycidae			
Phycis blennoides	1	Greater forkbeard	Instituto de Investigaciones Marinas, CSIC (Spain)
Order Lophiiformes			
Family Lophiidae			
Lophius piscatorius	1	Angler	Instituto de Investigaciones Marinas, CSIC (Spain)
Order Perciformes			
Family Scombridae			
Scomber japonicus	1	Pacific mackerel	Instituto de Investigaciones Marinas, CSIC (Spain)
Family Coryphaenidae			
Coryphaena hippurus	1	Common dolphinfish	Conxemar (Spanish trade association for the Fish and Aquaculture)
Family Carangidae			
Trachurus picturatus	1	Atlantic horse mackerel	Instituto de Investigaciones Marinas, CSIC (Spain)

Table 2
Ct values resulting from target species (*Molva molva*) and non-target species for the real time PCR system designed. Average Ct values were obtained from triplicates measurements of 100 ng of DNA samples of the specimens (N = 15 for *Molva molva*, N = 1 the non-target species).

Species	Fish family	Average Ct value ± SD
Molva molva	**Lotidae**	**19,5 ± 0,65**
Brosme brosme	Lotidae	36,3 ± 0,47
Molva dypterigia	Lotidae	34,9 ± 0,09
Gaidropsarus ensis	Lotidae	40,0 ± 0,00
Gadus macrocephalus	Gadidae	40,0 ± 0,00
Gadus morhua	Gadidae	37,4 ± 0,37
Gadus ogac	Gadidae	38,3 ± 0,43
Gadus chalcogrammus	Gadidae	40,0 ± 0,00
Melanogrammus aeglefinus	Gadidae	34,5 ± 0,19
Merlangius merlangius	Gadidae	40,0 ± 0,00
Micromesistimus poutassou	Gadidae	40,0 ± 0,00
Pollachius pollachius	Gadidae	34,0 ± 0,13
Pollachius virens	Gadidae	31,7 ± 0,06
Trisopterus luscus	Gadidae	40,0 ± 0,00
Trisopterus esmarkii	Gadidae	38,7 ± 0,16
Trisopterus minutus	Gadidae	33,0 ± 0,16
Macruronus magellanicus	Merlucidae	40,0 ± 0,00
Macruronus novaezelandiae	Merlucidae	40,0 ± 0,00
Merluccius bilinearis	Merlucidae	40,0 ± 0,00
Merluccius capensis	Merlucidae	40,0 ± 0,00
Merluccius polli	Merlucidae	40,0 ± 0,00
Merluccius hubbsi	Merlucidae	40,0 ± 0,00
Merluccius merluccius	Merlucidae	40,0 ± 0,00
Merluccius paradoxus	Merlucidae	40,0 ± 0,00
Merluccius senegalensis	Merlucidae	40,0 ± 0,00
Salilota australis	Moridae	40,0 ± 0,00
Phycis blennoides	Phycidae	31,2 ± 0,34
Lophius piscatorius	Lophiidae	40,0 ± 0,00
Scomber japonicus	Scombridae	40,0 ± 0,00
Coryphaena hippurus	Coryphaenidae	40,0 ± 0,00
Trachurus picturatus	Carangidae	40,0 ± 0,00

2. Materials and methods

2.1. Fish samples

Forty-five specimens were used as reference samples, fifteen of them belonging to the species *Molva molva*, and another thirty non-target-species specimens belonging to Lophiiformes, Perciformes and Gadoids orders (see Table 1).

Thirty-one commercial seafood products, purchased from different Spanish and French markets, were used (see Table 3) and analysed in triplicate for the validation of the methodology. These samples were analysed using the optimal conditions described in Section 3.2.

2.2. DNA extraction

DNA was extracted with prior digestion in a thermo shaker at 56 °C, 0.3 g of thawed muscle was mixed with 100 μL of 5 M guanidium thiocyanate (Sigma-Aldrich), 40 μL of proteinase K (20 Unit/mg) (Gibco Invitrogen, LifeTechnologies), and 860 μL of lysis buffer. The lysis buffer was composed of 1% SDS, 150 mM NaCl, 2 mM EDTA, and 10 mM Tris-HCl at pH 8. An additional 40 μL of proteinase K was added after 3 h and the digestion was left overnight. In order to isolate DNA a Wizard DNA Clean-Up System kit (Promega) was used following the manufacturer's instructions. Extracted DNA concentration and purity were quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific) at 260 nm and the ratio 260/280, respectively. The 260/280 ratio was between 1.8 and 2.0. The DNA concentration was adjusted to 50 ng/μL for subsequent RT-PCR reactions. The purified DNA was stored at −20 °C.

Table 3
Results of the analysis of commercial products: results of FINS identification, Ct values obtained with the newly developed RT-qPCR assay and indication of presence (+) or absence (−) of *Molva molva*. Grey highlight indicate an inconsistence between commercial name and species found.

	Commercial name	Scientific name	Process type	FINS result	Average Ct value ± SD	RT-PCR result
1	Ling loins	*Molva molva*	Ling loins	*Molva molva*	19,9 ± 0,05	+
2	Ling roe	*Molva molva*	Dry-salted	*Molva molva*	16,1 ± 0,01	+
3	Ling loins	*Molva molva*	Salted	*Molva dypterigia*	32,1 ± 0,18	–
4	Ling roe	*Molva molva*	Dry-salted	*Molva molva*	16,4 ± 0,25	+
5	Ling	*Molva molva*	Salted	*Brosme brosme*	31,2 ± 0,02	–
6	Ling loins	*Molva molva*	Salted	*Molva molva*	20,1 ± 0,09	+
7	Ling	*Molva molva*	Salted	*Molva molva*	20,8 ± 0,08	+
8	Ling loins	*Molva molva*	Salted	*Gadus morhua*	35,6 ± 0,09	–
9	Ling	*Molva molva*	Fresh	*Molva molva*	19,7 ± 0,01	+
10	Ling	*Molva molva*	Fresh	*Molva molva*	20,4 ± 0,09	+
11	Ling	*Molva molva*	Fresh	*Molva molva*	17,6 ± 0,13	+
12	Ling	*Molva molva*	Fresh	*Molva molva*	19,6 ± 0,14	+
13	Ling	*Molva molva*	Fresh	*Molva molva*	17,6 ± 0,13	+
14	Cod omelette	Not avaliable	Precooked	*Inconclusive*	24,5 ± 0,22	?
15	Cod liver	Not available	Canned	*Gadus morhua*	35,1 ± 0,22	–
16	Cod	*Gadus morhua*	Salted	*Gadus morhua*	32,9 ± 0,15	–
17	Nordic cod	*Gadus morhua*	Smoked	*Gadus morhua*	34,5 ± 0,40	–
18	Minced cod	*G. morhua*	Salted	*Molva molva*	18,85 ± 0,0	+
19	Iceland cod	*Gadus morhua*	Salted	*Gadus morhua*	33,9 ± 0,22	–
20	Cod cheeks	*Gadus morhua*	Desalted	*Gadus morhua*	31,5 ± 0,08	–
21	Cod fritter	Not available	Frozen	*Gadus morhua*	35,0 ± 0,22	–
22	Cod filet	*Gadus morhua*	Frozen	*Gadus morhua*	30,0 ± 0,05	–
23	Iceland cod	*Gadus morhua*	Frozen	*Gadus morhua*	33,9 ± 0,10	–
24	Cod	*Gadus spp*	Salted	*Gadus morhua*	33,0 ± 0,38	–
25	Cod liver	Not available	Canned	*Gadus morhua*	35,7 ± 0,23	–
26	Cod	Not available	Frozen	*Molva dypterigia*	33,9 ± 0,16	–
27	Cod loins	*Gadus morhua*	Salted	*Molva molva*	20,6 ± 0,08	+
28	Minced cod	*Gadus morhua*	Salted	*G. macrocephalus*	32,8 ± 0,18	–
29	Alaska pollock	*G. chalcogrammus*	Salted	*G. chalcogrammus*	33,8 ± 0,08	–
30	Alaska Pollock loins	*G. chalcogrammus*	Frozen	*G. chalcogrammus*	33,7 ± 0,25	–
31	Alaska Pollock	*G. chalcogrammus*	Salted	*G. chalcogrammus*	31,9 ± 0,08	–

2.3. Real-time PCR system design

An extensive number of partial nuclear and mitochondrial DNA sequences (5S rRNA, rhodopsin, pantophysin, 12S rRNA, 16S rRNA, Cytochrome *b* and Cytochrome Oxidase I genes) belonging to a wide range of fish species were analysed and aligned during the study to design a specific *Molva molva* real time PCR system.

A set of primers was designed to amplify a small fragment (81 bp) of cytochrome *b* gene, surrounding the previously detected specific polymorphisms, using Oligo Analyzer v. 1.0.3 (Freeware, Teemu Kuulasmaa, Finland). The internal probe used in Real-time PCR assays was chosen in the same cytochrome *b* gene region.

2.4. Real time PCR setup

In order to determine the optimal reaction conditions (combination that gave the lowest Ct value and the highest final fluorescence), preliminary tests with concentrations of 50, 300 and 900 nM of each primer and 25, 50, 75, 100, 125, 150, 175, 200 and 225 nM of probe were carried out.

Seven 10-fold serial dilutions, from 100 ng to 10^{-4} ng were used to calculate the efficiency of the system, from Ct values versus the log DNA amount of these dilutions, and according to the equation $E = [10(-1/\text{slope}) - 1] \times 100$.

All PCR reactions were performed on a total volume of 20 μL with the following composition: 100 ng of DNA, 10 μL of TaqMan Fast Universal PCR Master Mix, no UNG Amperase (2X) (Applied Biosystems) and the optimal concentration of forward and reverse primers and probe were determined experimentally (see Section 3.2). The PCR reactions were performed in a MicroAmp fast optical 96-well reaction plate (Applied Biosystems) and these plates were covered with MicroAmp optical adhesive film (Applied Biosystems). TaqMan reactions were run on ABI 7500 Fast (Applied Biosystems) with the following thermal cycling protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The average Ct value obtained for all non-*M. molva* species was compared with that of *Molva molva* using a *t*-test, with normal distributions and different variances.

2.5. FINS identification

In order to check the reliability of the RT-PCR analysis and test the functionality of the DNA extracted, a 464 bp fragment of *cytb* gene from all reference and commercial samples was amplified and sequenced using the primers

H15149: 5′-GCICCTCARAATGAYATTTGTCCTCA-3′ and
L14735: 5′-AAAAACCACCGTTGTTATTCAACTA-3′ (Burgener, 1997).

PCR reactions were carried out in a total volume of 25 μL with 100 ng of DNA template, using PCR Ready-to-Go beads (GE Healthcare) with a final concentrations of 640 nM for forward and reverse primers on a Applied Biosystems 2720 thermocylcer. The thermal cycling protocol used was: 94 °C for 5 min; followed by 35 cycles of 94 °C for 40 s, 55 °C for 80 s, 72 °C for 80 s; and a final extension step of 72 °C for 7 min. PCR amplicons were visualized on a 2% agarose gel, using the Gel Documentation System Gel Doc XR System and the software Quantity One® v 4.5.2 (Bio-Rad).

These PCR products were sequenced on an ABI PRISM 310 genetic analyser (Applied Biosystems) using the primers described above and reactions the BigDye Terminator 1.1 (Applied Biosystems), following the supplier's instructions and sequencing. The nucleotidic sequences were analysed using BioEdit (Hall, 1999) and MEGA (Kumar, Dudley, Nei, & Tamura, 2008) software and the results were authenticated with a BLAST analysis (Altschul et al., 1997).

3. Results and discussion

The differentiation of *Molva molva* from other gadoid species is important since all of these species are very closely related and morphologically very similar. This usually means that they can easily be substituted in commercial products since some have higher commercial value than others or are subject to limited fishing quotas whereas others are not. This may encourage the substitution of one species of fish whose quota has already been reached with another fish that is not subject to a quota.

The purpose of this study was the development and evaluation of a Real-time PCR technique for the detection of ling (*Molva molva*) in elaborated products.

3.1. Real-time PCR system design assay

TaqMan real time PCR (RT-qPCR) is designed to detect sequence-specific amplicons during the course of the PCR reaction, at the beginning of the exponential phase, without a subsequent step of electrophoretic migration of the PCR product.

To prevent false negatives, the design of the system (primers and probes) requires that target regions are characterized by very low intraspecific variability.

However, to ensure that the primers and probe bind specifically to the target species, the system should be designed in a region with high interspecific variability, preferably locating the midsection of the probe in these regions. The specificity of hybridization is dependent on a number of factors including the length and GC content of the capture probe and the hybridization temperature (Pryor & Wittwer, 2006). Short probes and elevated hybridization temperatures can allow the identification of a single base mismatch in the probe target sequence (Kutyavin et al., 2000).

These factors were considered in the search for a system (primers and TaqMan probe) as short as possible, which could be useful for unequivocal identification of *Molva molva* with high sensitivity, even for thermally processed samples.

After analysing a large number of sequences of different fragments of nuclear (5S rRNA, rhodopsin and pantophysin genes) and mitochondrial (12S rRNA, 16S rRNA, Cytochrome *b* and Cytochrome Oxidase I genes) DNA genes, a fragment of mitochondrial cytochrome *b* gene was selected as a molecular marker. This selection is based on the ideal characteristic mentioned above. Also, mitochondrial DNA presents a number of advantages compared with nuclear markers such as its haploid nature and the fact that muscle tissue exhibits a large number of copies per cell, evolves faster than nuclear and is mainly maternally inherited, usually without recombination (Rastogi et al., 2007; Wilson & Turner, 2009).

Furthermore, short target DNA fragments, as we have designed in this work (81 bp, Fig. 1), turn out to be more suitable for species identification in commercial products, in which the DNA may be degraded (Teletchea 2009). Although Real-time systems aimed at gadoid species identification, particularly for Atlantic cod (*Gadus morhua*), already exist (Hird et al., 2011; Sánchez et al., 2009), this is the first such system is developed for the identification of *Molva molva*.

The sequences of the selected primers and probe were as follows:

Mmol_Cytb-F (forward) 5′-GGGTTCTCGCACTTCTATTTTCA-3′;

Mmol_Cytb-R (reverse) 5′-ATGTTAGTCCTCGTTGTTTAGAGG TATG-3′;

Mmol_Cytb-P (probe) 5′-CTAGTTCTCATAGTAGTCCCCT-3′.

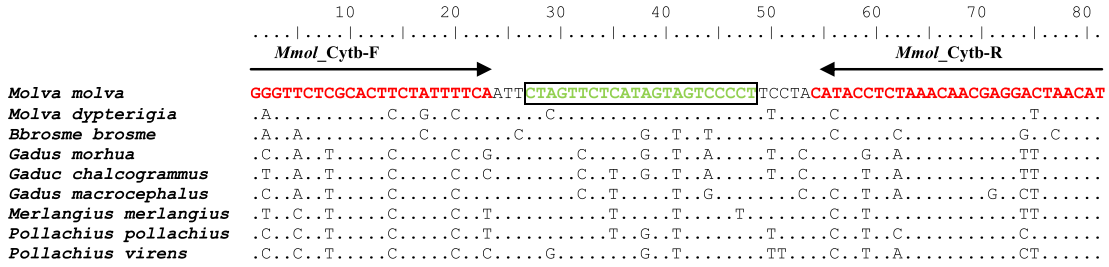

Fig. 1. Cytochrome *b* region alignment of gadoid species showing the position of the designed *Molva molva* real time PCR system.

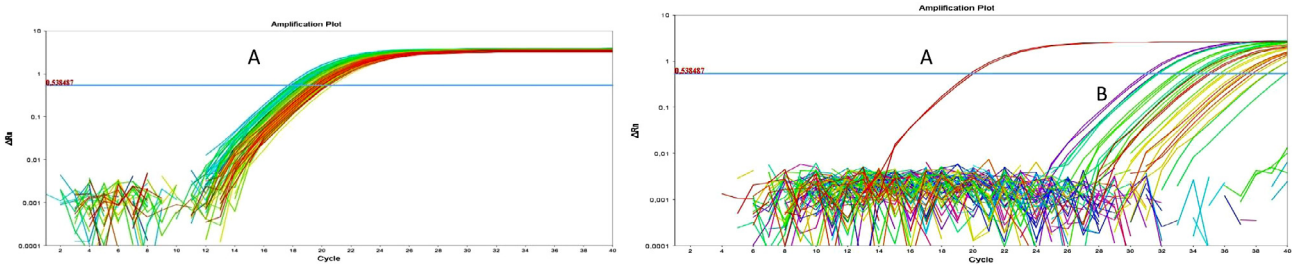

Fig. 2. Real Time PCR amplification plot. (A) Amplification patterns showed by ling (*Molva molva*) specimens. (B) Amplification patterns showed by non-ling fish species.

The 5′ end of the probe was labelled with the fluorescent reporter dye VIC, and the minor groove binding (MGB) was located at the 3′ end.

3.2. Real-Time PCR setup

Optimization tests were performed in order to find the optimal concentrations of primers and probe to be used for identifying *Molva molva* by TaqMan Real-Time PCR. The results were: 900 nM for the forward primer, 300 nM for reverse primer and 175 nM for the probe.

The limit of detection of the Mmol_Cytb primer and probe set was assessed using dilutions of *Molva molva* template DNA in water (Fig. 3). The limit of detection was determined to be a dilution of 10^{-4} ng (average Ct value 36.37).

The efficiency of the system was 102%. The response over this range of dilutions was linear with a slope of -3.25.

A linear correlation between fluorescence and DNA concentration was observed, indicating the potential of this approach for the development of quantitative estimations.

3.3. Specificity

The reference fish species (Table 1) were used to test the cross-reactivity and specificity of the system. The results are shown in Table 2. A statistically significant difference ($P < 0{,}001$) was obtained between *Molva molva* DNA samples average Ct value (19,5 ± 0,65) and the average Ct value obtained for the non-target species (38,3 ± 2,8) (to calculate this average value, the undetected samples were assigned a Ct = 40). No false positives were observed (Fig. 2). The strong signal and low Ct value obtained in *Molva molva* (Fig. 2, Table 2) were sufficient to significantly ($P < 0{,}001$) distinguish it from the other three Lotidae species, even with *Molva dypterigia*, the closest related species (Ct value 34,90 ± 0,9). The

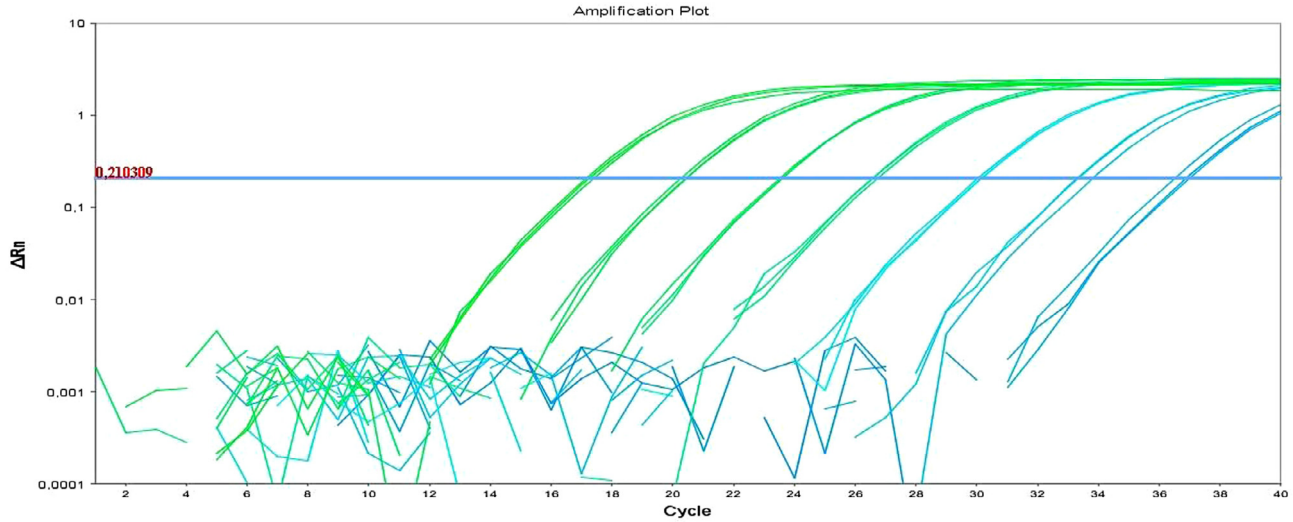

Fig. 3. Limit of detection of *Molva molva* real time PCR system on dilutions of ling template DNA in water (A:100 ng, B:10 ng, C:1 ng, D:10^{-1} ng, E: 10^{-2} ng, F: 10^{-3} ng, G: 10^{-4} ng,)

identification of all samples by RT-PCR was in line with the results found using DNA sequencing (FINS).

3.4. Validation with commercial seafood products

The method developed in this work was tested on 31 commercial samples in order to both validate the system and perform a preliminary assessment the state of labelling of these products.

As can be observed in Table 3, results obtained with RT-PCR method and FINS were in agreement. These results indicate that 19.4% of these samples were mislabelled; this percentage increased to 23% if just those products labelled as ling are taken into consideration.

It was found that three of the samples labelled as ling (*Molva molva*) turned out to be other species: two of them (sample 3 and 5) were species with lower commercial values (*Brosme brosme* and *Molva dypterigia*) and another (sample 8) was a higher value species subject to a fishing quota (*Gadus morhua*).

The first type of mislabelling – *Molva molva* replaced by *Brosme brosme* or *Molva dypterigia* – may influence efforts to sustainably manage ling stocks. Moreover, these results may reflect the existence of illegal commercial practices with economic implications and therefore the existence of fraud. As well as being a case of mislabelling, the second type - represented by replacement of ling by cod – also has obvious implications for the management of Atlantic cod stocks.

Furthermore, two samples labelled as *Gadus morhua* (sample 18 and 27) gave a positive result with the *Molva molva* species specific Real Time PCR assay and another cod sample (sample 26) was substituted with *Molva dypterigia,* giving a negative result on the RT-PCR test.

Similarly, another sample labelled as *Gadus morhua* (sample 28) gave a negative result in the RT-PCR assay, and it was effectively identified as *Gadus macrocephalus* by FINS analysis.

Only one highly processed sample (sample 14, a precooked cod omelette labelled as cod) showed inconclusive results both with FINS and RT-PCR, with a Ct value of 24,5. This result suggests the presence of a mixture of ling with other species. Mixed species have to be analysed with alternative methods such as PCR-ELISA (Taboada et al., 2014) or NGS sequencing. Using the first approach, PCR-ELISA method, we have concluded that this sample contained a mixture of *Molva molva* and *Gadus morhua*.

These results showed that this system is a good method for routine analysis aimed at species identification in commercial products.

4. Conclusions

PCR assay based on real-time TaqMan technology for ling detection was developed and optimized. The assay was successfully tested on commercial samples and, by detecting the presence or absence of ling in highly processed commercial products, was shown to be specific, sensitive and useful for the identification of products incorrectly labelled as ling.

The methodology used is a one-step protocol that does not require post-PCR sample manipulation since detection is simultaneous to amplification. This avoids contamination and reduces analysis time and cost as many samples can be analysed simultaneously.

Conflict of interest statement

Authors declare the non-existence of conflict of interest for the development of the research work presented in this paper.

Acknowledgements

The work was supported by the projects "GENTRASEA: Genetic Traceability of Fish Products. Rapid methods with DNA hybridization probes" funded by the Spanish Ministry of Science and Innovation AGL2009-13711-C03-01/ALI, and "LABELFISH: Atlantic Network on Genetic Control of Fish and Seafood Labelling and Traceability" (2011-1/163) funded by the Atlantic Area Program. The Spanish Ministry of Science and Innovation is gratefully acknowledged for the doctoral fellowship to L.T. We also acknowledge Anxela Aldrey for her technical assistance. Ledicia Taboada is a PhD student at the University of Santiago de Compostela.

References

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H., & Sato, S. (2006). Species identification and PCR-RFLP analysis of Cytochrome b gene in cod fish (order Gadiformes) products. *Journal of Food Science, 71*(3), C190–C195.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSIBLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402.

Burgener, M. (1997). *Molecular species differentiation of fish and mammals* Ph. D. Thesis. Switzerland: University of Bern.

Castigliego, L., Armani, A., Tinacci, L., Gianfaldoni, D., & Guidi, A. (2015). Two alternative multiplex PCRs for the identification of the seven species of anglerfish (Lophius spp.) using an end-point or a melting curve analysis real-time protocol. *Food Chemistry, 166*, 1–9.

Chapela, M., Sánchez, A., Suárez, M., Perez-Martin, R., & Sotelo, G. C. (2007). A rapid methodology for screening hake species (*Merluccius* spp.) by single-stranded conformasakation polymorphism analysis. *Journal of Agriculture and Food Chemistry, 55*(17), 6903–6909.

Dalmasso, A., Fontanella, E., Piatti, P., Civera, T., Secchi, C., & Bottero, M. T. (2007). Identification of four tuna species by means of real-time PCR and melting curve analysis. *Veterinary Research Communications, 31*, 355–357.

D'Amico, P., Armani, A., Castigliego, L., Sheng, G., Gianfaldoni, D., & Guidi, A. (2014). Seafood traceability issues in Chinese food business activities in the light of the European provisions. *Food Control, 35*(1), 7–13.

Di Pinto, A., Di Pinto, P., Terio, V., Bozzo, G., Bonerba, E., Ceci, E., et al. (2013). DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks. *Food Chemistry, 141*, 1757–1762.

EC (European Commission) (2013). Commission Regulation (EC) No 1379/2013 of 11 December 2013 on the common organization of the markets in fishery and aquaculture products, amending Council Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No 104/2000 (2013).

EC (European Commission) (2000). European Council Regulation No 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aqua-culture products. *Official Journal of the European Communities, L17*, 22–52.

EC (European Commission) (2001). Commission Regulation (EC) No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. *Official Journal of the European Communities, L278*, 6–8.

FAO (2014). *Fishery and aquaculture statistics*. www.fao.org/3/a-i5716t.pdf.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nuclic Acids Symposium Series, 41*, 95–98.

Helyar, S. J., Lloyd, H. A. D., de Bruyn, M., Leake, J., Bennett, N., & Carvalho, G. R. (2014). Fish product mislabelling: Failings of traceability in the production chain and implications for illegal, unreported and unregulated (IUU) fishing. *PLoS ONE, 9*(6), e98691. http://dx.doi.org/10.1371/journal.pone.0098691.

Hernandez, M., & Esteve, T. (2005). Real-time polymerase chain reaction based assays for quantitative detection of barley, rice, sunflower, and wheat.. *Journal of Agriculture and Food Chemistry, 53*, 7003–7009.

Hird, H., Chisholm, J., & Brown, J. (2005). The detection of commercial duck species in food using a single probe-multiple species-specific primer real-time PCR assay.. *European Food Research and Technology, 221*, 559–563.

Hird, H., Chisholm, J., Kaye, J., Colyer, A., Hold, G., Conyers, C. M., et al. (2011). Development of real-time PCR assays for the detection of Atlantic cod (Gadus morhua), Atlantic salmon (Salmo salar) and European plaice (Pleuronectes platessa) in complex food samples. *European Food Research Technology, 234*(1).

Hird, H., Lloyd, J., Goodier, R., Brown, J., & Reece, P. (2003). Detection of peanut using real-time polymerase chain reaction. *European Food Research and Technology, 217*, 265–268.

Kumar, S., Dudley, J., Nei, M., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform, 9*, 299–306.

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., et al. (2000). 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research, 28*(2), 655–661.

Laube, I., Spiegelberg, A., Butschke, A., Zagon, J., Schauzu, M., Kroh, L., et al. (2003). Methods for the detection of beef and pork in foods using real-time polymerase chain reaction. *International Journal of Food Science & Technology, 38*, 111–118.

Le Francois, N. R., Jobling, M., Carter, C., & Blier, P. U. (2010). *Finfish Aquaculture Diversification*. Wallingford: CABI.
Mackie, I. M., Pryde, S. E., Gonzalez-Sotelo, C., Medina, I., Peréz-Martın, R., Quinteiro, J., et al. (1999). Challenges in the identification of species of canned fish. *Trends in Food Science & Technology, 10*(1), 9–14.
Maretto, F., Reffo, E., Dalvit, C., Barcaccia, G., & Mantovani, R. (2007). Finding 16S rRNA gene-based SNPs for the genetic traceability of commercial species belonging to Gadiformes. *Italian Journal Animal Science, 6*, 161–163.
Marko, P. B., Lee, S. C., Rice, A. M., Gramling, J. M., Fitzhenry, T. M., McAlister, J. S., et al. (2004). Fisheries: Mislabelling of a depleted reef fish. *Nature, 430*(6997), 309–310.
Miller, D. D., & Mariani, S. (2010). Smoke, mirrors, and mislabeled cod: Poor transparency in the European seafood industry. *Frontiers in Ecology and the Environment, 8*(10), 517–521.
Panicker, G., Myers, M. L., & Bej, A. K. (2004). Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Applied and Environment Microbiology, 70*, 498–507.
Primrose, S., Woolfe, M., & Rollinson, S. (2010). Food forensics: Methods for determining the authenticity of foodstuffs. *Trends in Food Science & Technology, 21*(12), 582–590.
Pryor, R. J., & Wittwer, C. T. (2006). Real-time polymerase chain reaction and melting curve analysis. *Methods in Molecular Biology, 336*, 19–32.
Rasmussen, R. S., & Morrissey, M. T. (2011). Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. *Journal of Laboratory Automation, 16*(4), 308–321.
Rastogi, G., Dharne, M. S., Walujkar, S., Kumar, A., Milind, S. P., & Yogesh, S. S. (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Science, 76*, 666–674.
Sakaguchi, M., Toda, M., Ebihara, T., Irie, S., Hori, H., Imai, A., et al. (2000). IgE antibody to fish gelatin (type I collagen) in patients with fish allergy. *Journal of Allergy and Clinical Immunology, 106*(3), 579–584.
Sánchez, A., Quinteiro, J., Rey-Mendez, M., Perez-Martin, R., & Sotelo, G. C. (2009). Identification of European hake species (*Merluccius merluccius*) using real-time PCR. *Journal of Agriculture and Food Chemistry, 57*(9), 3397–3403.
Taboada, L., Sánchez, A., Velasco, A., Santaclara, F. J., Perez-Martin, R. I., & Sotelo, G. C. (2014). Identification of atlantic cod (*Gadus morhua*), ling (*Molva molva*), and alaska pollock (*Gadus chalcogrammus*) by PCR-ELISA using duplex PCR. *Journal of Agriculture and Food Chemistry, 62*, 5699–5706.
Teletchea, F. (2009). Molecular identification methods of fish species: reassessment and possible applications. *Reviews in Fish Biology and Fisheries, 19*(3), 265–293.
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*(8), 2214–2216.
Weller, S. A., Simpkins, S. A., Stead, D. E., Kurdziel, A., Hird, H., & Weekes, R. J. (2002). Identification of *Agrobacterium* spp. present within Brassica napus seed by TaqMan PCR. Implications for GM screening procedures. *Archives of Microbiology, 2002*(178), 338–343.
Wilson, W. D., & Turner, T. F. (2009). Phylogenetic analysis of the Pacific cutthroat trout (*Oncorhynchus clarki ssp.*: Salmonidae) based on partial mtDNA ND4 sequences: A closer look at the highly fragmented inland species Mol. *Phylogenet. Evol., 52*, 406–415.
Xiong, X., Guardone, L., Giusti, A., Castigliego, L., Gianfaldoni, D., Guidi, A., et al. (2016). DNA barcoding reveals chaotic labeling and misrepresentation of cod (鳕, Xue) products sold on the Chinese market. *Food Control, 60*, 519–532.