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

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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).

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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) |