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Identification of European Hake Species (*Merluccius merluccius*) Using Real-Time PCR

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A rapid and precise method for identifying European hake (*Merluccius merluccius*) based on TaqMan technology is presented. The method can be applied to fresh, frozen, and processed fish products to detect the fraudulent or unintentional mislabeling of this species. Specific primers and a minor groove binding (MGB) TaqMan probe were designed for this purpose based on partial sequences of the mitochondrial DNA control region. Combinations of primers and probe concentrations that gave the lowest *Ct* value and the highest final fluorescence value were selected to carry out efficiency, specificity, and cross-reactivity assays. The method was successfully tested on 31 commercial hake samples. A *Ct* value of about 16 was obtained when *Merluccius merluccius* was present; however, the fluorescence signal was not detected most of the time (*Ct* value 40) or presented significantly higher *Ct* values (38.2 ± 0.96) for the nonhake species.

KEYWORDS: European hake; species identification; real-time PCR; *Merluccius merluccius*; control region

INTRODUCTION

European markets, especially those in Spain, have traditionally sold *Merluccius merluccius* (European hake) mostly as a fresh whole fish, while other species belonging to the same genus (*Merluccius*, Family Merluccinae) but from other geographical locations are often marketed as frozen commercial products with different presentations (such as tails, loins, fillets, etc). Some of these species, such as the South African hake and silver hake, can also be easily found nowadays as wet fish. Since *Merluccius merluccius* is the most appreciated species within the *Merluccius* genus, and since its market value is higher than that of the other species, it is possible that some mislabeling or fraud might occur. Although European legislation establishes that seafood products should be labeled to indicate the commercial designation of species (often linked to a specific biological/ scientific name), the production method, and the catch area (Directive 2000/13/EC, EU Commission Regulation No 2065/2001), the labeling of such products is sometimes found to be incorrect. Therefore, in order to enforce these labeling rules, there arises a need for faster and cheaper analytical methods to verify seafood species and to speed up the inspection process of imported seafood at customs.

There are a number of methods published such as 2-D electrophoresis, SDS−PAGE, and MALDI-TOF MS (*1*, *2*) for detecting and identifying fish and seafood products using proteins. However, protein analysis has two major drawbacks: proteins are denatured during heat and pressure processing, and its resolution power is not enough when closely related species need to be differentiated. Under such circumstances, the use of DNA as an analytical tool is a good alternative to protein analysis because of the stability of the DNA molecules and its presence in all tissues. DNA-based technologies, such as PCR with specific primers, followed by fragment detection using gel electrophoresis, microsatellite analysis, RFLP, SSCP, and sequencing, among others, have been used as tools for species identification in foods (*2*−*4*). Moreover, several studies have already been published (*5*−*14*) on the identification of hake species.

One of the current emerging techniques, in fish species identification, is real-time PCR (RT-PCR). This technique visualizes the amount of PCR product formed during the amplification process by monitoring the fluorescence signal emitted by dyes or probes introduced into the reaction. There are two general types of RT-PCR analysis. The simplest type involves the use of intercalating dyes such as SYBR Green. These compounds bind to double-stranded DNA, giving an increase in fluorescence which correlates with the amount of dsDNA present. The major drawback is that any double-stranded product, including primer−dimers or unspecific products, will be detected, and therefore, false positives can occur. The other type involves the use of fluorescent probes. This is a more specific method for detecting the accumulation of amplicon because probes are designed to be complementary to a target sequence in the amplicon. There are several types of probes, such as TaqMan probes (hydrolysis probes), molecular beacons, and scorpions. The most commonly used TaqMan probes are labeled with a reporter and quencher

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fluorophores in the 5′ and 3′ ends, respectively. Reporter fluorescence is reduced by the quencher as long as the probe is intact regardless of whether it is attached to its target. When *Taq* polymerase, with a 5′ nuclease activity, begins to add nucleotides and hydrolyzes the probe attached to the template DNA, the quencher is separated from the reporter, thereby enabling the emission of fluorescence.

Many works have been published using this technique since Higuchi et al. (*15*) introduced it, mainly in the field of gene expression analysis, pathogen detection (*16*, *17*), and quantification of GMOs (genetically modified organisms) in food (*18*, *19*). Furthermore, RT-PCR has also been used for the identification and detection of species in different meat products (*20*, *21*), vegetables and nuts (*22*−*24*), bacteria (*25*, *26*), and fish (*27*−*32*).

The aim of this study was to find a rapid and precise method based on TaqMan technology that would be able to differentiate the European hake species from their relatives elsewhere, which can eventually appear as substitutes in fish markets. The method should be able to detect the fraudulent or at times unintentional mislabeling of European hake in fresh, frozen, and processed products.

MATERIAL AND METHODS

Collection of Authentic Hake Species and Commercial Samples. Twenty-nine specimens of *Merluccius merluccius* species and one specimen of each of the other 10 *Merluccius* species were used as reference hake species in the study. Most such species are used in marketing fish products that are commonly labeled as hake. Further 20 representative specimens of Gadiformes, Lophiiformes, Perciformes, and Pleuronectiformes orders were included in this study. Scientific and common names of these species are listed in **Table 1**. The validation step of the methodology used 31 commercial hake samples purchased from the different local markets (see **Table 2**). All samples were analyzed in triplicate.

DNA Extraction. DNA was extracted from 0.2 g of thawed muscle that was digested overnight in a thermo shaker at 56 °C with 860 μL of lysis buffer (1% SDS, 150 mM NaCl, 2 mM EDTA, and 10 mM Tris-HCl at pH 8), 100 μL of 5 M guanidium thiocyanate, and 40 μL of proteinase K (≥20 Unit/mg). After 3 h, extra proteinase K (40 μL) was added to the solution, and it was left overnight. After digestion, DNA was isolated employing the Wizard DNA Clean-Up System kit (Promega) by following the manufacturer's instructions. The purified DNA was stored at −20 °C. DNA was quantified by UV-spectrometry

Table 1. List of the Reference Species Used for the Study

species	*N*	source
Order Gadiformes		
family Gadidae		
Brosme brosme	1	Dept. of Fish Quality, BFEL (Germany)
Gadus morhua	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Melanogrammus aeglefinus	1	Dept. of Fish Quality, BFEL (Germany)
Merlangius merlangus	1	Dept. of Fish Quality, BFEL (Germany)
Micromesistius poutassou	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Molva dypterygia	1	Dept. of Fish Quality, BFEL (Germany)
Molva molva	1	Dept. of Fish Quality, BFEL (Germany)
Pollachius pollachius	1	Dept. of Fish Quality, BFEL (Germany)
Pollachius virens	1	Dept. of Fish Quality, BFEL (Germany)
Theragra chalcogramma	1	Dept. of Fish Quality, BFEL (Germany)
Trisopterus luscus	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Trisopterus minutus	1	Dept. of Fish Quality, BFEL (Germany)
family Merlucciidae		
Macruronus magellanicus	1	Isla Mar (fishing company)
Macruronus novaezelandiae	1	Europacfico (fishing company)
Merluccius albidus	1	Natural History Museum and Biodiversity Research Center, University of Kansas (USA)
Merluccius australis	1	Pescanova (fishing company)
Merluccius bilinearis	1	Fisheries and Oceans Canada
Merluccius capensis	1	Marine and Coastal Management (South Africa)
Merluccius gayi	1	Fish Market (Valparaso-Chile)
Merluccius hubbsi	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius merluccius	29	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius paradoxus	1	Marine and Coastal Management (South Africa)
Merluccius polli	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Merluccius productus	1	Northwest Fisheries Science Center (USA)
Merluccius senegalensis	1	Oviedo University (Spain)
Order Lophiiformes		
family Lophiidae		
Lophius piscatorius	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Order Perciformes		
family Sparidae		
Sparus aurata	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Order Pleuronectiformes		
family Scophthalmidae		
Psetta maximus	1	Instituto de Investigaciones Marinas, CSIC (Spain)
family Soleidae		
Solea solea	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Order Salmoniformes		
family Salmonidae		
Oncorhynchus mykyss	1	Instituto de Investigaciones Marinas, CSIC (Spain)
Salmo salar	1	Instituto de Investigaciones Marinas, CSIC (Spain)

Table 2. List of the Commercial Hake Products Tested

sample	hake product	declared species	capture zone	presentation
1	raw extruded	*Merluccius* spp.	Atl. Southwest/FAO N° 41	frozen
2	precooked hake with sauce	unidentified	unidentified	frozen
3	precooked sticks	unidentified	unidentified	frozen
4	precooked sticks	unidentified	unidentified	frozen
5	battered precooked	unidentified	unidentified	frozen
6	battered precooked	unidentified	unidentified	frozen
7	battered precooked	unidentified	unidentified	frozen
8	raw loins	*M. capensis*/*M. paradoxus*	Atl. Southeast	frozen
9	raw fillets	*Merluccius hubbsi*	Atl. Southwest/FAO N° 41	frozen
10	raw fillets	*M. capensis*/*M. paradoxus*	Atl. Southeast	frozen
11	raw fillets	*Merluccius hubbsi*	Atl. Southwest	frozen
12	raw center cuts	*M. australis*	Pacific Ocean	frozen
13	raw center cuts	*M. capensis*	Atl. Southeast	frozen
14	raw center cuts	*M. australis*	Pacific Ocean	frozen
15	raw tails	*Macruronus* spp.	FAO N° 47	frozen
16	raw center cuts	*Merluccius hubbsi*	Atl. Southwest/FAO N° 41	frozen
17	raw tails	unidentified	Malvinas	frozen
18	raw tails	unidentified	Malvinas	frozen
19	hake roes	unidentified	unidentified	canned
20	raw center cuts	unidentified	Atl. Southeast/Namibia	refrigerated
21	raw fillets	unidentified	Atl. Southeast/Namibia	refrigerated
22	raw young hake	unidentified	Atl. Northwest/EEUU-Canada	refrigerated
23	raw young hake	unidentified	Atl. Northwest/EEUU-Canada	refrigerated
24	raw center cuts	*Merluccius merluccius*	South Africa	refrigerated
25	raw young hake	unidentified	Atl. Northeast	refrigerated
26	raw center cuts	*Merluccius merluccius*	FAO 27	refrigerated
27	raw young hake	*Merluccius merluccius*	Atl. Southwest	refrigerated
28	raw young hake	*Merluccius merluccius*	Atl. Southwest	refrigerated
29	raw center cuts	unidentified	Atl. NE	refrigerated
30	raw center cuts	unidentified	Atl. SE	refrigerated
31	raw young hake	unidentified	At. Southwest	refrigerated

at 260 nm and by Quant-iT DNA Assay Kit (Molecular PROBES) for dsDNA quantification with a Perkin-Elmer LS 55 luminescence Spectrometer (excitation 510 nm, emission 527 nm). DNA concentration was adjusted to 12.5 ng/μL for subsequent RT-PCR reactions.

Design of Primers. Sequences of mitochondrial control region DNA from different hake species obtained in a previous study (*33*) with GenBank accession numbers AF112245−AF112255 were aligned to design specific primers and a MGB (minor groove binding) TaqMan probe for *M. merluccius* species by using Primer Express software (Applied Biosystems). The theoretical primers and probe specificity was evaluated by searching GenBank/EMBL/DDBJ for 100% homologous sequences using BLASTN (*34*).

Real-Time PCR Conditions. PCR reactions were performed in a total volume of 20 μL in a MicroAmp fast optical 96-well reaction plate (Applied Biosystems), covered with MicroAmp optical adhesive film (Applied Biosystems). Each reaction contained 25 ng of DNA, 10 μL of TaqMan Fast Universal PCR Master Mix no UNG Amperase (2X) and a final concentration of 900 nM for each primer and 225 nM for the probe. 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-European hake species was compared with that of *M. merluccius* using a *t*-test, with normal distributions and different variances (*35*).

FINS Identification of Hake Commercial Products. DNA from commercial samples were amplified and sequenced using Kocher et al. primers (*36*), as described in Chapela et al. (*11*), to obtain sequences of 465 bp of the cytochrome *b* gene. Sequences were analyzed using the software MEGA to construct the Neighbor-joining tree using Tamura−Nei distances as described in Chapela et al. (*11*).

RESULTS AND DISCUSSION

The differentiation of *Merluccius merluccius* species from other hake and nonhake species is challenging since they are very closely related species, and therefore, it is desirable to prevent any possible commercial fraud. Thus, the aim of this study was to design a system comprising primers and a TaqMan probe for the specific identification of *Merluccius merluccius* species and to test such a system on commercial products.

Real-Time PCR System Design. An extensive number of partial DNA sequences (nuclear and mitochondrial) and a wide number of fish species were analyzed (data not shown) during the study. The aim was to find as short a system (primers and probe) as possible, which could be useful for the unequivocal identification of European hake (*Merluccius merluccius*) with good sensitivity for processed samples. One essential condition was that no intraspecific variability should be found in the system (primers and probe) in order to avoid false negative identifications. The primers and probe must specifically bind to the target species. However, the system should be designed in a sequence region where the number of nucleotide interspecific differences is highest, preferably in the middle region of the probe sequence. The analysis of the above-mentioned sequences showed that only the interspecific nucleotide variability present in the left domain of the control region permitted the location of a potential target site for designing a real-time PCR system for the specific detection of the European hake. The designed system consisted of a 98 bp fragment (**Figure 1**). As far as it is known, it is the smallest PCR diagnostic fragment for the identification of European hake (*5*−*14*). This fact, allows the identification of European hake in commercial products that

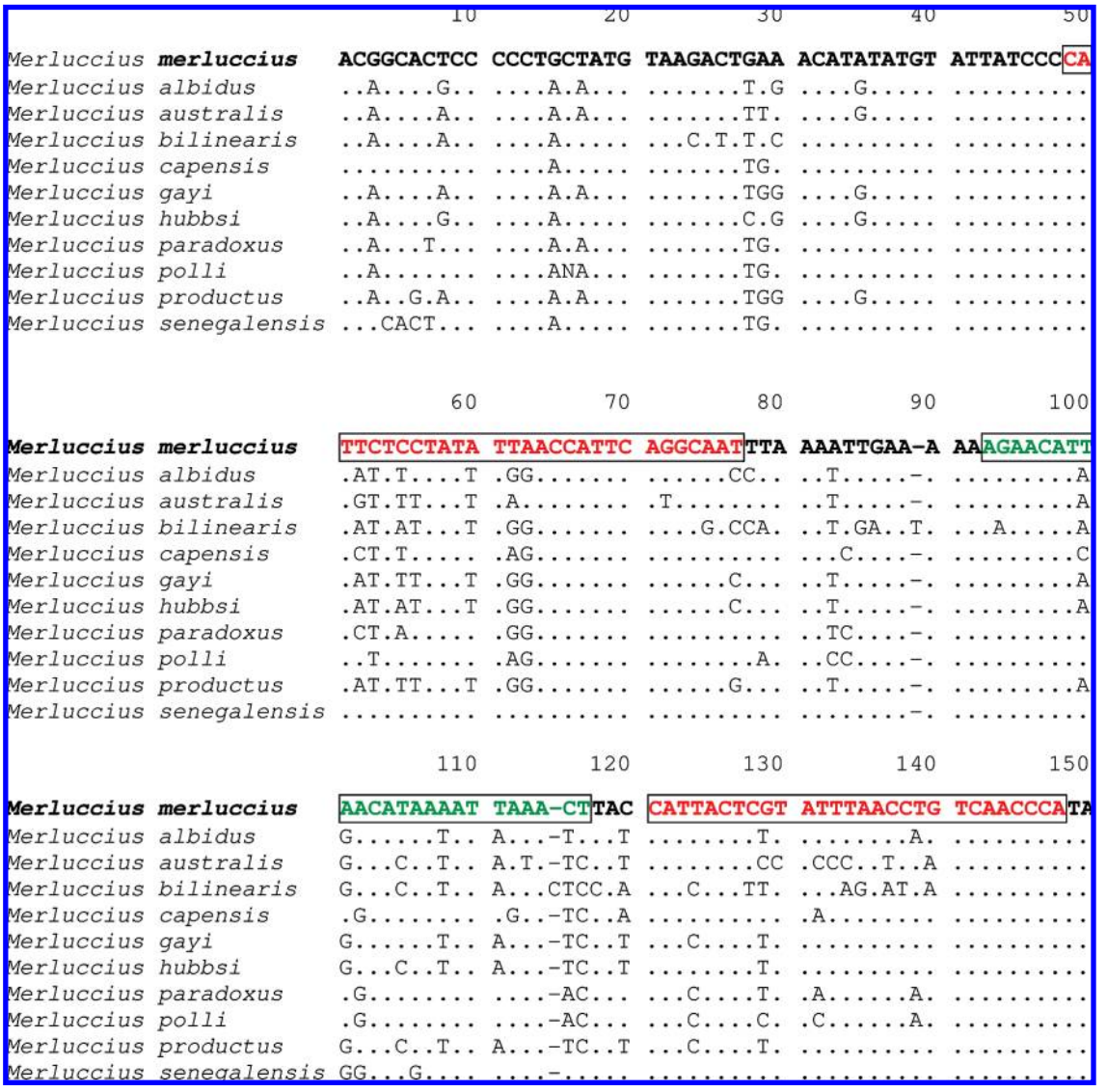

Figure 1. Alignment of mitochondrial control region sequences from hake species, showing the position of the European hake system.

have been submitted to exhaustive thermal treatment or other elaboration processes that might affect DNA integrity.

The sequences of the selected primers and probe were as follows: MMERCR4F (forward); 5′-CATTYTCYTATATTAA CCATTCAGGCAAT-3′; MMERCR5R (reverse) 5′-TGGG TTGACAGGTTAAATACGAGTAA-3′ and MMERCR 6TP 5′-AGAACATTAACATAAAATTAAACT-3′. The 5′ end of the probe was labeled with the fluorescent reporter dye VIC, and the minor groove binding (MGB) was located at the 3′ end.

Real-Time PCR Setup. Preliminary tests were performed using different concentrations of primers (50, 300, and 900 nM) and probe (25 to 225 nM) to determine the optimal concentrations of primers and the TaqMan probe. The combinations of primers and probe concentrations that gave the lowest *Ct* value and the highest final fluorescence value were selected to carry out the efficiency, specificity, and cross-reactivity assays. Detection of *Merluccius merluccius* species was performed with a final concentration of 900 nM for each primer and 225 nM for the probe.

The efficiency of the *Merluccius merluccius* system was calculated from *Ct* values versus the log DNA amount, using seven 10-fold serial dilutions, starting from 100 ng. Over this range of dilutions, the response was linear with a slope of −3.87. The efficiency of the system was of 81.43% according to the equation $E = [10^{(-1/\text{slope})} - 1] \times 100$ and allows the detection of very smalll DNA quantities (1×10^{-4}).

Specificity. The European hake TaqMan system was tested for its specificity and cross-reactivity with the reference fish species listed in **Table 1**. The European hake system amplified a 98 bp fragment from *Merluccius merluccius* mitochondrial control region DNA with an average *Ct* value of 16.7 ± 0.43, as can be seen in **Table 3**, where no amplification or amplification that was not significant was obtained from other fish species in the cross-reactivity analysis (*Ct* average of 33.7 ± 3.31 from 30 nonhake specimens). There is a statistically significant difference between the *Ct* value obtained for *M. Merluccius* and that for the rest of the analyzed species ($P < 0.001$). Although a slight signal was detected with the other species, this fact was negligible compared with the strong signals measured for European hake (**Figure 2**). This is the first european hake identification study using the real-time PCR technique.

Identification of Commercial Samples of European Hake Using RT-PCR Assays. Thirty-one commercial food products containing hake with different processing treatments were analyzed in order to detect the presence of *Merluccius*

Table 3. Specificity and Cross-Reaction of *Merluccius merluccius* Specific Real-Time PCR System (*Ct* Values Obtained from 25 ng of DNA)[a]

species	fish family	average *Ct* value ± SD
Merluccius merluccius	F. Merlucciidae	16.7 ± 0.43
Macruronus magellanicus	F. Merlucciidae	31.2 ± 0.45
Macruronus novaezelandiae	F. Merlucciidae	40
Merluccius albidus	F. Merlucciidae	35.7 ± 0.34
Merluccius australis	F. Merlucciidae	34.2 ± 0.10
Merluccius bilinearis	F. Merlucciidae	36.5 ± 0.53
Merluccius capensis	F. Merlucciidae	40
Merluccius gayi	F. Merlucciidae	39.2 ± 1.43
Merluccius hubbsi	F. Merlucciidae	37.2 ± 1.53
Merluccius paradoxus	F. Merlucciidae	35.0 ± 0.12
Merluccius polli	F. Merlucciidae	36.6 ± 0.80
Merluccius productus	F. Merlucciidae	29.3 ± 0.06
Merluccius senegalensis	F. Merlucciidae	40
Brosme brosme	F. Gadidae	33.0 ± 0.15
Gadus morhua	F. Gadidae	30.0 ± 0.06
Melanogrammus aeglefinus	F. Gadidae	32.2 ± 0.01
Merlangius merlangus	F. Gadidae	34.8 ± 0.22
Micromesistius poutassou	F. Gadidae	32.4 ± 0.05
Molva dypterygia	F. Gadidae	30.7 ± 0.08
Molva molva	F. Gadidae	32.7 ± 0.07
Pollachius pollachius	F. Gadidae	32.6 ± 0.13
Pollachius virens	F. Gadidae	31.4 ± 0.20
Theragra chalcogramma	F. Gadidae	29.2 ± 0.09
Trisopterus luscus	F. Gadidae	32.5 ± 0.13
Trisopterus minutus	F. Gadidae	34.0 ± 0.04
Lophius piscatorius	F. Lophiidae	30.7 ± 0.22
Sparus aurata	F. Sparidae	31.8 ± 0.23
Psetta maximus	F. Scophthalmidae	30.4 ± 0.09
Solea solea	F. Soleidae	35.2 ± 0.19
Oncorhynchus mykyss	F. Salmonidae	28.7 ± 0.15
Salmo salar	F. Salmonidae	33.4 ± 0.37

[a] All species with an average *Ct* value of 40 present SD = 0.

Table 4. Results of the Real-Time PCR Assay of Commercial Hake Products[a]

sample	declared species	species identified by fins	average *Ct* value ± SD
1	*Merluccius* spp.	*Merluccius paradoxus*	40
2	unidentified	*Merluccius hubbsi*	39.2 ± 0.86
3	unidentified	*Merluccius gayi*	40
4	unidentified	*Merluccius hubbsi*	40
5	unidentified	*Merluccius paradoxus*	40
6	unidentified	*Merluccius hubbsi*	40
7	unidentified	*Merluccius paradoxus*	40
8	*M. capensis*/*M. paradoxus*	*Merluccius paradoxus*	40
9	*Merluccius hubbsi*	*Merluccius hubbsi*	40
10	*M. capensis*/*M. paradoxus*	*Merluccius paradoxus*	40
11	*Merluccius hubbsi*	*Merluccius hubbsi*	40
12	*Merluccius australis*	*M. australis*	40
13	*Merluccius capensis*	*M. paradoxus*	40
14	*Merluccius australis*	*M. australis*	40
15	*Macruronus* spp.	*Macruronus* spp.	40
16	*Merluccius hubbsi*	*Merluccius hubbsi*	40
17	unidentified	*Macruronus* spp.	38.5 ± 1.58
18	unidentified	*Macruronus* spp.	36.6 ± 0.66
19	unidentified	*Merlucccius merluccius*	17.0 ± 0.04
20	unidentified	*Merluccius paradoxus*	40
21	unidentified	*Merluccius paradoxus*	40
22	unidentified	*Merluccius bilinearis*	40
23	unidentified	*Merluccius bilinearis*	40
24	*Merluccius merluccius*	*Merluccius merluccius*	16.2 ± 0.07
25	unidentified	*Merluccius merluccius*	15.5 ± 0.04
26	*Merluccius merluccius*	*Merluccius merluccius*	16.3 ± 0.11
27	*Merluccius merluccius*	*Merluccius bilinearis*	38.3 ± 0.73
28	*Merluccius merluccius*	*Merluccius bilinearis*	38.1 ± 1.61
29	unidentified	*Merluccius merluccius*	16.6 ± 0.09
30	unidentified	*Merluccius capensis*	40
31	unidentified	Merluccius merluccius	16.3 ± 0.03

[a] All samples with an average *Ct* value of 40 present SD = 0.

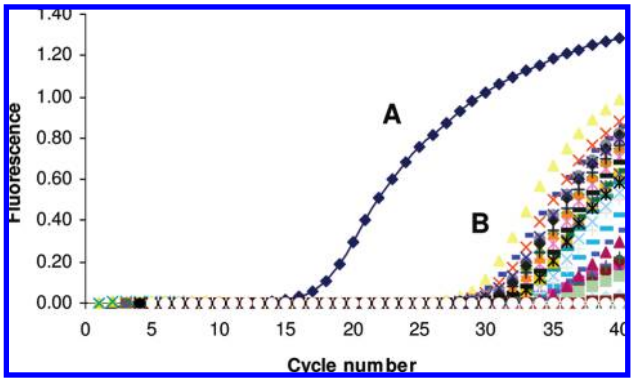

Figure 2. Cross-reactivity amplification plot. (**A**) Amplification pattern showed by the European hake. (**B**) Amplification pattern showed by non-European hake species.

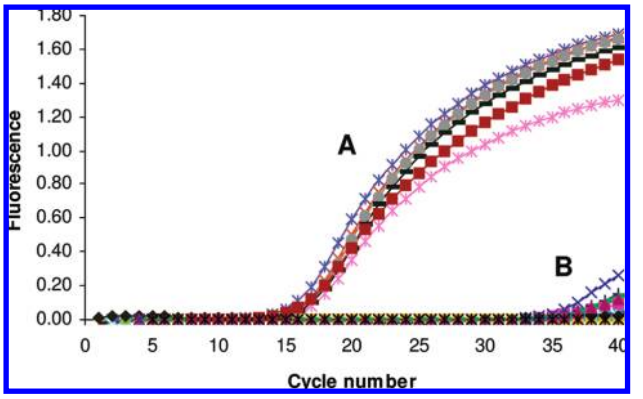

Figure 3. Commercial sample amplification plot. (**A**) Amplification pattern showed by positive samples. (**B**) Amplification pattern showed by negative samples.

merluccius. These included frozen, precooked, fresh, and canned products as shown in **Table 2**. This table also shows the labels found in each of the products analyzed, where two main types of labels were observed: labels with species declared (13 samples) and labels with no scientific name for the species, only a general commercial name (i.e., hake). Most of the samples were frozen, while others were fresh samples obtained at local markets. The total number of samples declared as *M. merluccius* were 4 out of 31, all of which were fresh hake.

Results of samples identification using the designed real-time PCR system are shown in **Table 4**, which also shows the identification of each sample by FINS.

Samples labeled with the name of the species were found to be mostly correct (10 out of 13), but only 2 samples out of 4 were correctly labeled (50%) as *M. merluccius*. RT-PCR results matched with those obtained by FINS: The *Ct* value obtained when *M. merluccius* was present was about 16 (16.3 ± 0.46); however, when *M. merluccius* was not present the fluorescence signal was mostly undetected (*Ct* value = 40) or presented *Ct* values that were significantly higher (38.1 ± 0.96) (**Figure 3**).

These results show that the RT-PCR system designed could be a very useful tool for the detection of fraud or mislabeling practices for European hake. Also, RT-PCR requires a significantly shorter analysis time than other

established methods. For instance, FINS involves one PCR amplification, sequencing reaction of the DNA strands, and the separation of sequencig fragments in a sequencer (*7*), whereas RT-PCR requires only one PCR amplification step since detection is simultaneous to amplification. Besides, DNA reference sequences and molecular phylogenetics software to carry out the identification analysis are needed for FINS. Other identification methodologies, such as PCR-RFLP and PCR-SSCP, also involve at least two separate steps, amplification and detection (*8*, *11*).

Conclusions. A real-time PCR assay for the detection of European hake species has been developed. The optimized assay is specific, highly sensitive, and applicable to complex food matrices, and it has been successfully tested on commercial samples. The designed system has been useful to detect the incorrect labeling of hake products since 50% of *M. merluccius* products were incorrectly labeled.

Real-time PCR does not require post-PCR sample handling. It thus prevents contamination and produces much faster and higher throughput assays with a wide dynamic range (more than 7 orders of magnitude). The methodology described here is a one-step protocol because detection is carried out during the amplification reaction. The method is also suitable to detect the presence or absence of European hake in highly processed food samples. Analysis costs are considerably reduced since many samples can be analyzed at the same time.

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