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ARTICLE

Authentication of Atlantic Cod (*Gadus morhua*) Using Real Time PCR

Beatriz Herrero, María Madriñán, Juan M. Vieites, and Montserrat Espiñeira*

Area of Molecular Biology and Biotechnology, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain

This work describes the development of a real-time polymerase chain reaction (RT-PCR) system for the detection and identification of Atlantic cod (*Gadus morhua*). Among the advantages of this technique, it is worth highlighting that this is reliable in terms of specificity and sensitivity. The TaqMan real-time PCR is the simplest, fastest testing process and has the highest potential for automation, therefore representing the currently most suitable method for screening, allowing the detection of fraudulent or unintentional mislabeling of this species. The method can be applied to all kinds of products, fresh, frozen, and processed products, including those undergoing intensive processes of transformation. The developed methodology using specific primer−probe set was validated and further applied to 40 commercial samples labeled as cod in order to determinate if the species used for their manufacturing corresponded to *G. morhua*, detecting 20% that were incorrectly labeled. A C_t value of about 19 was obtained when *G. morhua* was present. In samples with a species mixture, all samples that had a fluorescence signal were positive (C_t < 30) for the presence of *G. morhua* by conventional end-point RT-PCR, and the estimated limit of detection for these type of samples was of 20 pg of DNA. The methodology herein developed is useful to check the fulfilment of labeling regulations for seafood products and verify the correct traceability in commercial trade and for fisheries control.

KEYWORDS: *Gadus morhua*; cod; Taqman; real-time PCR; PCR; species identification

INTRODUCTION

Atlantic cod (*Gadus morhua*, Linneo 1758), is one of the most consumed white fish species throughout Europe. High exploitation levels and increased worldwide consumption have provoked a stock decrease and catch limitations. Overfishing data exists for this species from the 1960s, and catastrophic decrease in the volume of its stocks has been detected between 1970 and 2000 (*1*). For this reason, Atlantic cod has been included in the Red List of Threatened Species (also known as the IUCN Red List or Red Data List).

The economic importance of this fishing resource and its status of conservation forced the European Union (EU) authorities to establish Total Allowable Catches (TAC) from 1970. In 2009, the European Commission according to Norway saw an increase of 11% of the TACs for Atlantic cod in the North Sea. However, illegal, unregulated, and unreported fishing (IUU) could make difficult to check the fulfilment of European regulations.

This entire situation has caused the search and capture of alternative species, increasing their number in the market and thus attending to consumer demand. Different fishes closer to Atlantic cod are commercialized as if they were this species because they present similar organoleptic and morphological characteristics. For instance, Pacific cod (*Gadus macrocephalus*), Greeland cod (*G. ogac*), Ling (*Molva molva*), Blue ling (*M. dypterygia*), Pollock (*Pollachius pollachius*), Saithe (*P. virens*), Haddock (*Melanogrammus aeglefinus*), Alaska pollock (*Theragra chalcogramma*), Blue whiting (*Micromesistius poutassou*), Hake (*Merluccius* spp), and Whiting (*Merlangius merlangius*) can be found in the markets in various commercial formats, for example, whole, filleted, cubed, shredded, tails, etc. including different methods for conservation such as fresh, frozen, smoked, surimi, canned, and ready-to-serve dishes.

The increase in processed products and the existence of a global market may lead to substitutions of species deliberately or unintentionally. The identification of fish species is an important issue to have account of, regarding their correct labeling. The labeling regulations for products derived from fisheries are each time more demanding (*2*, *3*) as this legislation indicates the necessity of labeling fish products with both commercial and scientific denominations in order to ensure the traceability all along the chain and thus avoid possible fraud.

In the fish products commercialized with almost no manipulation, it is relatively easy to identify the original species using different morphological or meristic traits. However, in the case of organisms with morphological traits eliminated during processing such identification is not possible. Because of the above reasons, it is necessary to develop methods that permit the accurate identification of the species present in all kinds of processed products. In this sense, the most recent and innovative

*To whom correspondence should be addressed. Tel: (34) 986 469 301. Fax: 31 (34) 986 469 269. E-mail: montse@anfaco.es.

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Table 1. Samples Included in This Work and Collection Locations[a]

Order	Family	Species	Common name[a]	Samples[b]	Location	Sequences
Gadiformes	Gadidae	*Gadus morhua*	Atlantic cod	30	UK, SW, NO, Bay of Biscay, IC, FR, CA, AT	DQ173997, EU877731
		Gadus macrocephalus	Pacific cod	15	JP, PN	DQ173995
		Gadus ogac	Greeland cod	15	CA, AT	DQ356940
		Merlangius merlangus	Whiting	10	UK, ES, PT, PO, NO, ANE	AF081702
		Pollachius pollachius	Pollack	10	UK, NO, Cantabrian Sea	DQ174025
		Pollachius virens	Saithe	10	NO, AE	AF081703
		Melanogrammus aeglefinus	Haddock	7	ES, North Sea	AF081701
		Theragra chalcogramma	Alaska pollack	11	PN, CN, CA	AB182308
		Gadiculus argenteus	Silvery cod	2	IC	DQ173994
		Micromesistius poutassou	Blue whiting	12	UK, ES, M, Cantabrian Sea, CA	DQ174019
		Boreogadus saida	Arctic cod	1	PN	DQ356936
		Trisopterus esmarkii	Norway pout	3	IC, UK, NO,	AF081709
	Merlucciidae	*Merluccius* spp	Hake	15	ES, PT, FR, UK, AR, ZA	GQ503196
	Lotidae	*Brosme brosme*	Brismak	6	IC, UK, AT	EU752065
		Molva molva	Ling	14	IC, ES, PT	DQ174021
		Molva dypterygia	Blue ling	8	IC, ES, PT, ANE	
		Molva macrophthalma	Blue ling	2	UK, FR	DQ174020
		Enchelyopus cimbrius	Fourbeard rockling	2	IC	DQ173092
		Lota lota	Burbot	9	FI, NO, ANE	
		Lota lota maculosa	Burbot	1	US	
	Phycidae	*Phycis blennoides*	Greater forkbeard	2	IC, PT,	DQ174022

[a] Location abbreviations: AE, Atlantic Eastern; ANE, Atlantic Northeast; AR, Argentina; AT, Atlantic; CA, Canada; CN, China; ES, Spain; FI, Finland; FR, France; IC, Iceland; JP, Japan; NO, Norway; PN, Pacific North; PO, Poland; PT, Portugal; SW, Sweden; UK, United Kingdom; US, United States; ZA, South Africa. [b] Only one of the possible common names for each species is shown. [c] Each sample included between 3 and 10 individuals.

are the genetic techniques, which permit seafood species identification, thus granting the reliability of the commercial transactions and avoiding fraud. The genetics techniques are based on two molecules: proteins and DNA. The problem of the analysis based on proteins is that these molecules are easily denatured; thus, they do not leave possibilities for application of such methods in products subjected to thermal or pressure treatments.

For the cited reasons, the molecular methods based on DNA analysis have proven to be most suitable for species identification in all types of products independently of the processes to which these have been subjected. Among all DNA based techniques, the ones most frequently used for the study of gadoids are PCR-RFLP (*4*−*8*) and amplification of microsatellite marker, applied to population genetics (*9*, *10*) and species identification of gadoids (*11*).

A novel genetic technique for species identification is the application of specific DNA probes with the method of real time PCR. This methodology is based on the specific hybridization of a probe designed for a certain species with the DNA in the samples to be analyzed. Only the DNA complementary to the specific probe will hybridize to it. This technique is acquiring more importance because of its rapidity and sensitivity and has passed from being an almost exclusive method in microbiology to be always applied for identification of fish species, both for eggs (*12*, *13*) and adult organisms (*14*−*16*). An emphasis should be placed on the study of Taylor et al. (*17*), which identified three gadoid species of commercial importance, has several drawbacks, did not study any of the possible substitution species, and did not apply the methodology developed to processed seafood, and the verification of the method has been only realized with the three gadoid species identified.

In the present work, one methodology based on a species-specific real-time PCR assay using specific primer sets and probe was developed. The proposed technique allows the authentication of Atlantic cod, *Gadus morhua*, from other closely related species that are used as substitutes in some seafood products including those that have undergone intensive thermal treatment.

MATERIALS AND METHODS

1. Sample Collection, Storage, and DNA Extraction. Authentic gadoid samples were collected from different marine locations around the world. The species, number of specimens, location of samples, and other data are shown in **Table 1**. Samples were labeled after arriving at the laboratory and preserved at −80 °C. The specimens were identified on the basis of morphological traits according to different bibliographic references (*18*). Moreover, 40 seafood products labeled as cod were provided by import industries or purchased in supermarkets and shops from European countries in order to apply the developed methodology.

DNA was extracted from 30 mg of muscle in fresh and frozen samples according to the method described by Roger and Bendich with slight modifications (*19*). The essential modifications were as follows: DNA fraction is treated with proteinase K for the same time as the CTAB buffer and followed by phenol/chloroform, chloroform, and isopropanol washes. The second CTAB wash and the first rehydratation with TE buffer are deleted. Only one ethanol wash is carried out. In processed products, DNA was extracted from 150 mg using the NucleoSpin Tissue kit (Macherey-Nagel), following the manufacturer's instructions.

The purity and concentration of the extracted DNA was determined by measuring the absorbance at 260 nm and the purity using the absorbance ratio of 260 and 280 nm (A_{260}/A_{280}). These measures were carried out using a NanoDrop 1000 spectrophotometer (Thermo Scientific). DNA extracts were appropriately labeled and stored at −20 °C.

2. Design of a Specific RT-PCR Method to Detect and Identify *Gadus morhua*. Different sequences of the *cytochrome oxidase* subunit I gene (COI) belonging gadoid species were downloaded from the National Center for Biotechnology Information (NCBI) (accession numbers in **Table 1**). These were aligned with Clustal W integrated in the BioEdit 7.0 program pack (*20*), and from them, a specific primer set (MORHUA F 5′ TTA CAG TCG GAA TGG ACG TA 3′ and MORHUA R 5′ GAG TAG GGG TGT CTC TCA TT 3′) and a minor groove binding (MGB) TaqMan probe (MORHUA probe 5′ (FAM) CCC ATG TAA AGT TGC TAA T (MGB) 3′) for *G. morhua* were designed by using Primer Express software (Applied Biosystems).

The theoretical specificity of the primers and probe was checked with a BLASTN tool (Basic Local Alignment Search Tool-N) against the GenBank database (*21*), both available in the National Center for Biotechnology Information (NCBI) database.

The PCR reactions were carried out in a total volume of 25 μL containing 50 ng of DNA template, 12.5 μL of Taqman Master Mix (Applied Biosystems), the amount of primers and probe that were optimized, and molecular biology grade water (Eppendorf) to adjust to the final volume. Optimal amount of primers and probe were evaluated by preparing a dilution series. A common range of working stock concentrations of 50, 300, and 900 nM of each primer and 50, 100, and 250 nM of the Taqman MGB probe were used to determine the optimal concentrations.

The reactions were performed in iQ 96-well PCR plates (BIO- RAD) covered with iCycler iQ Optical Tape (BIO- RAD), and reactions were run in triplicate on a Bio-Rad iCycler iQ Real Time PCR instrument. The annealing temperature of the real-time PCR assay is one of the most critical parameters for reaction specificity. To find the optimal annealing

Table 2. Commercial Samples Analyzed[a]

products	species labeled	species identified by BLAST	presence *G. morhua* RT-PCR	average C_t value
salted cod tails	*Gadus morhua*	*Gadus macrocephalus*	negative	30.2
	Gadus morhua	*Gadus morhua*	positive	19.2
	Gadus macrocephalus	*Gadus macrocephalus*	negative	30.3
frozen cod fillet	*Gadus morhua*	*Gadus morhua*	positive	19.3
	Gadus morhua	*Gadus morhua*	positive	18.9
	Molva molva	*Molva molva*	negative	40
frozen cod fish	*Gadus morhua*	*Gadus morhua*	positive	18.7
	Gadus morhua	*Gadus morhua*	positive	19.5
	Theragra chalcogramma	*Theragra chalcogramma*	negative	40
salted cod loins	*Gadus morhua*	*Theragra chalcogramma*	negative	40
	Gadus morhua	*Gadus morhua*	positive	19.1
	Gadus macrocephalus	*Gadus macrocephalus*	negative	30.5
battered cod loins	*Gadus morhua*	*Gadus morhua*	positive	19.3
	Gadus morhua	*Gadus morhua*	positive	19.3
	Molva molva	*Molva molva*	negative	40
cod fillets	*Gadus morhua*	*Gadus morhua*	positive	18.5
	Theragra chalcogramma	*Molva molva*	negative	40
	Gadus macrocephalus	*Gadus macrocephalus*	negative	30.2
cod sticks	*Gadus* spp.	*Gadus macrocephalus*	negative	30.4
	Gadus morhua	*Gadus morhua*	positive	18.9
cod croquettes	unidentified	*Molva molva*	negative	40
	unidentified	*Theragra chalcogramma*	negative	40
salt cod balls	unidentified	*Gadus macrocephalus*	negative	30.2
	unidentified	*Molva molva*	negative	40
smoked cod roe	*Gadus* spp.	*Gadus morhua*	positive	19.3
	Gadus morhua	*Gadus morhua*	positive	19.4
small bits of cod	*Gadus morhua*	*Melanogrammus aeglefinus*	negative	40
	Gadus morhua	*Gadus macrocephalus*	negative	30.5
	Gadus macrocephalus	*Gadus macrocephalus*	negative	30.2
	Gadus macrocephalus	*Gadus macrocephalus*	negative	30.2
canned cod roe	*Gadus* spp.	*Molva dipterygia*	negative	40
	Gadus spp.	*Gadus macrocephalus*	negative	30.1
baby food	unidentified	*Molva molva*	negative	40
	unidentified	*Gadus macrocephalus*	negative	30.5
cod head	*Gadus* spp.	*Gadus morhua*	positive	19.5
cod cheeks	*Gadus* spp.	*Gadus morhua*	positive	18.8
smoked cod rissotto	unidentified	*Molva molva*	negative	40
peppers stuffed with cod	unidentified	*Theragra chalcogramma*	negative	40
canned cod fish	unidentified	*Gadus macrocephalus*	negative	30.5
canned salted cod	unidentified	*Gadus morhua*	positive	18.7

[a] Positive result: amplification pattern showed by Atlantic cod species, with C_t values about 19 ± 0.5. Negative result: amplification pattern showed by other species with C_t values >30.

temperature of the reaction, a range of temperatures was tested with the following thermal cycling protocol: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C−64 °C for 1 min. The specificity of the assay was evaluated by testing the amplification of DNA from different fish species (**Table 1**).

A serial dilution of DNA of *G. morhua* was performed with DNA of other species (**Table 1**) in levels ranging from 50 ng to 10 pg, and the fluorescence signal was determined. The dilutions were prepared by adding *G. morhua* DNA and DNA from different species until completing the final amount of 50 ng. All measurements were independently performed in triplicate from three processed samples. The relative uncertainty (relative error) was calculated as the standard deviation divided by the mean. The limit of detection (LOD) was established as the lowest concentration of DNA of *G. morhua* which yields a fluorescent signal significantly different from the negative control. LOD was calculated as indicated by the International Conference on Harmonization (ICH) guidelines by using: $\mathrm{LOD} = t \cdot s_B/m$, where s_B is the standard deviation of the nontarget control, m is the slope of the calibration curve, and $t = 3.3$ is the Student's t for a 95% confidence level.

3. Methodological Validation. Individuals from different species were authenticated on the basis of their morphological traits. Then the main treatments applied to commercial products were applied to them. These were canning, salting, smoking, marinating, and precooking (two processed products for each one of the kind processed for every species included in **Table 1**), for each of which different kinds of sauces and condiments were used. Also, the same treatments were applied in mixtures of Atlantic cod with other species to check the sensitivity and specificity of the developed method.

The treatment applied to canned samples involved 121 °C of temperature and 1.2 bar of overpressure, and the time varied depending on the size of the can. The smoking process combines two effects: on the one hand salting and drying steps and on the other the effect of temperature. The temperature corresponding to smoking of the fillets was raised to 121 °C until 60 °C was reached inside the product. The cooking time depended on the thickness of the fillets. All these treatments were carried out in the CECOPESCA (Spanish National Centre of Fish Processing Technology) pilot plant. The products were analyzed with the methodology developed in the present work. The coincidence percentage between the species identified on the basis of morphological traits and the genetic methodology developed was calculated to establish the specificity of the method.

4. Application of RT-PCR to Commercial Products. After the validation of the developed method in the present work, this was applied to 40 products labeled as cod in order to determine if the species used for their manufacturing corresponded to *Gadus morhua* (**Table 2**). These products were acquired from Spanish supermarkets, and the purpose of these analyses was to evaluate the situation regarding the labeling of these products on the market.

In order to ensure the proper working of RT-PCR, PCR products obtained using the primer set L14735/H15149AD (*22*) were sequenced. Subsequently, the identity of products was confirmed by Basic Local Alignment Search Tool (BLAST), National Center for Biotechnology

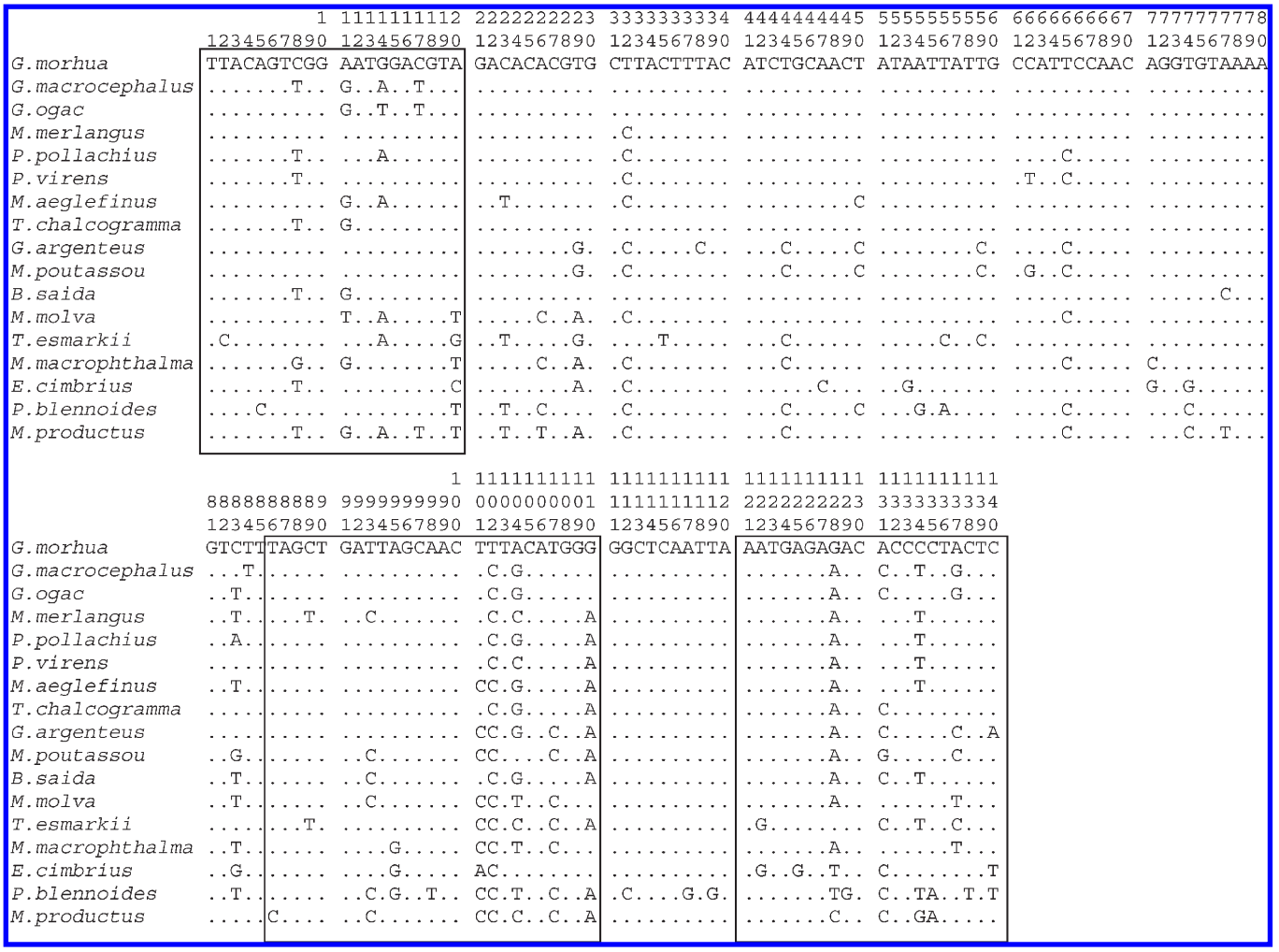

Figure 1. Sequence of the 140 bp fragment of the studied species. Shown are the positions of the primer−probe set used to amplify this region.

Information (NCBI) database (data not shown). The sequencing and identification by BLAST were carried out as described by Espiñeira et al. (*23*).

RESULTS AND DISCUSSION

1. Design of the RT-PCR Assay Specific to Identifying Atlantic Cod. Mitochondrial genes such as *cytochrome b* (*cyt b*), *cytochrome oxidase* subunits I and II (COI and COII), have been targeted in multiple PCR systems because they are present in multiple copies and are highly conserved allowing design specific primers and, in a parallel way, increase the sensitivity of the PCR. In this sense, the COI gene has been used successfully in marine species identification by RT-PCR (*24*). For all of these reasons, COI sequences of *G. morhua* were utilized to design specific primers to amplify DNA fragments from this particular species. Specific primers and the probe set were designed in a genomic region with potentially high interspecific variability but highly conserved in *G. morhua* (**Figure 1**).

The RT-PCR has several advantages with regard to end-point PCR; among them is the fact that end-point PCR requires post-PCR product separation by gel electrophoresis, or if the methodology used involves sequencing, a second thermal cycler reaction is necessary to produce sequenceable products and subsequent purification of sequences, and it is in addition time-consuming and is only semiquantitative. In this sense, among the advantages of the real-time PCR technique, it is worth highlighting its specificity, sensitivity, reproducibility, and rapidity. This technique allows one to verify the functioning of the PCR while it is running, saving time from secondary visualization or identification techniques of the PCR products. The optimization of the PCR conditions allows the highest level of sensitivity to be granted while maintaining the specificity of the technique. The TaqMan RT-PCR technology has recently gained wide acceptance in the identification of species. This system has been applied to the identification of microorganisms (*25*), plants (*26*−*28*), and animals (*14*−*16*, *29*−*32*). In this study, the primers and probe *MORHUA* herein designed amplified a 140 bp fragment, allowing the detection of *G. morhua*. The conditions that allow for obtaining the best results were established by means of a primer and probe matrix. The concentrations of 900 nM for both primers and 250 nM for the probe yielded the best results in terms of specificity and sensitivity.

2. Specificity and Sensitivity of the Method. The specificity of the primer set *MORHUA F*/*R* and *MORHUA* probe of *G. morhua* was confirmed using genomic DNA from other Gadoidae species from different geographical areas (**Table 1**). No cross-reactivity was detected with any of the ested samples. In this way, the optimal annealing conditions were established at 62 °C in order to ensure the higher specificity of the developed methodology (**Figure 2**).

In the case of RT-PCR, an important parameter reflecting the specificity and sensitivity of this methodology is the threshold cycle (C_t). The C_t is defined as the number of cycles required for the fluorescent signal to cross the threshold. C_t levels are inversely

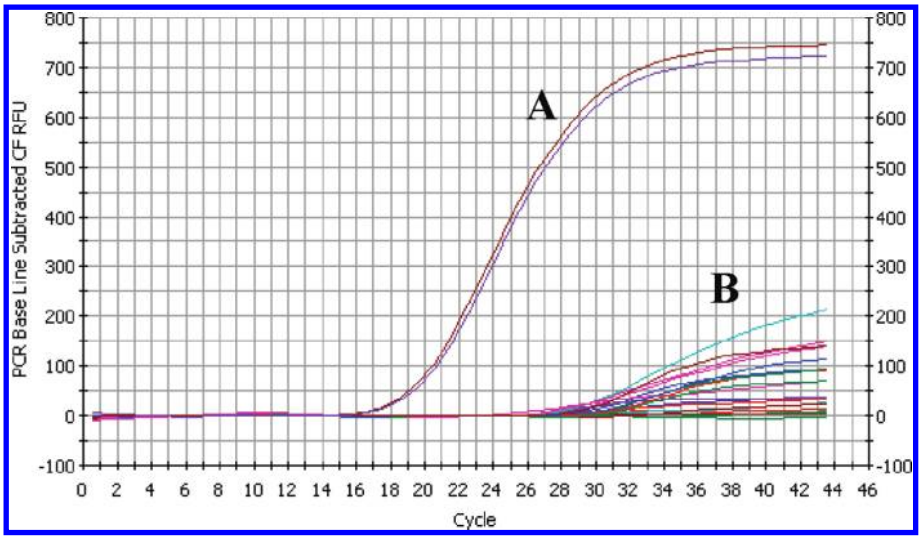

Figure 2. Specificity of the RT-PCR assay. A: amplification pattern showed by the Atlantic cod species, with C_t values about 19 ± 0.5. B: amplification pattern showed by other species with C_t values >30.

proportional to the amount of target nucleic acid in the sample (i.e., the lower the C_t level, the greater the amount of target nucleic acid in the sample). It is necessary to find the lowest C_t value and the highest final fluorescence value by means of appropriate concentrations of the primers and probe.

In all samples that contained *G. morhua*, the C_t values obtained were 19 ± 0.5. However, in the cross-reactivity analysis no false positive results were observed, under the stringent assay conditions used, as documented by C_t values > 30.

The efficiency of the developed method was calculated on the basis of the slope of the standard curve obtained using DNA serial dilutions (10-fold dilutions from 10 ng to 10 pg) as templates for RT-PCR (*33*). The mean value of the slope obtained was −3.37. From this slope, the amplification efficiency was calculated using the equation $E = (10^{(-1/\text{slope})} - 1) \times 100$, obtaining an efficiency of 98%. These values of C_t and efficiency demonstrated the utility of the RT-PCR system to identify *G. morhua*.

In samples with species mixture, the C_t values depend on the amount of template DNA of *G. morhua* in 50 ng of DNA total present in the reaction. All of these samples have a positive fluorescence signal for the presence of *G. morhua* (C_t < 30) by conventional end-point RT-PCR. The results (not shown) displayed a linear correlation of fluorescence to the presence of the DNA of *G. morhua*. The limit of detection of the developed RT-PCR assay revealed that at least 20 pg of the target DNA is necessary for positive results.

3. Methodological Validation. The aim of the methodological validation was to check whether the manufacturing process which the food underwent had no influence on the detection of *G. morhua* species. The elaborated products in the pilot plant of CECOPESCA were analyzed by the proposed methodology. The different treatments of transformation applied, allow one to evaluate the correct PCR amplification when these processes are used in these products. *G. morhua* was detected in all of these samples that contained this species independently of the transformation process. However, the high temperature and pressure to which the autoclaved products, such as canned fish, are subjected produce DNA fragmentation and will make the increment of the C_t value in this products. Despite this, it was checked that in cases in which *G. morhua* was present, it was detected because the C_t was always less than 30 and did not produce any case false negatives. Therefore, the RT-PCR system might be applied to fresh, frozen, precooked and canned fish.

4. Application to Commercial Products. Forty fresh, frozen, precooked, and canned fish were tested for the identification of *G. morhua* by using the method proposed in the present work (**Table 2**). To evaluate the degree of incorrect labeling found in the market study carried out, it is worth highlighting that, of all the samples tested, the scientific name of the species did not appear in the label of 10 of them (and only appeared under the commercial denomination cod). These samples belong to products with a high degree of transformation.

In 20% of the products analyzed, the name of the species displayed in the label was not in agreement with the species contained, as determined by genetic analysis using the RT-PCR methodology herein developed (**Table 2**) and confirmed by sequencing of the *cyt b* fragment and BLAST analysis showing concordant results for both methods.

In the commercial products tested, mixtures of different species were detected because all products where *G. morhua* was present had a C_t value of about 19, and the products where *G. morhua* was not detected had C_t > 30.

Altogether, this work describes the development of a RT-PCR method for the detection of *G. morhua* in fish products. RT-PCR has become an important technique in many fields of the food industry. Among the advantages of this technique it is worth highlighting its reliability, sensitivity, and specificity. Moreover, it is quick and cost-saving since the entire procedure can be completed within 4 h. These characteristics are turning it into a very appropriate tool for the authentication of *G. morhua* in all kinds of seafood products. The possible applications of this method are the following: normative control of raw and processed products, particularly the authenticity of imported species; the verification of the traceability of different fishing batches along the commercial chain; correct labeling and protection of the consumer's rights; fair competence among fishing operators; and the fisheries' control.

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