TITLE: Development of a Real-Time PCR method for the identification of Atlantic mackerel (*Scomber scombrus*)

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ABSTRACT: A Real Time-PCR method based on TaqMan technology for the identification of *Scomber Scombrus* has been developed. A system of specific primers and a Minor Groove Binding (MGB) TaqMan probe based on sequences of the mitochondrial cytochrome *b* region was designed. The method was successfully tested in 81 specimens of *Scomber scombrus* and related species and validated in 26 different commercial samples. An average Threshold cycle (Ct) value of 15.3 was obtained with *Scomber scombrus* DNA. With the other species tested fluorescence signal was not detected or Ct was significantly higher ($P<0.001$). The efficiency of the assay was estimated to be 92.41%, with 100% specificity, and no cross reactivity was detected with any other species. These results reveal that the developed method is a rapid and efficient tool to unequivocally identify *S. scombrus* and may aid in the prevention of fraud or mislabeling in mackerel products.

KEYWORDS: Atlantic mackerel; Scomber; species identification; pelagic fish; real-time PCR; cytochrome b.

INTRODUCTION

Scombroid fish are one of the most economically important fishery resources worldwide (FAO 2010). The family Scombridae includes 15 genera and 51 species of epipelagic marine fish (Collette, 2003) which are usually commercialized after transformation processes, which makes it difficult the species identification based on morphological characters (Sotelo and Pérez-Martín, 2007).

Atlantic mackerel *Scomber scombrus* (Linnaeus 1758), is usually marketed fresh, frozen, salted, smoked and canned. Nevertheless, since other species of *Scomber*, *Scomberomorus*, *Auxis*, and *Rastrelliger*, are commonly named mackerel in many parts of the world (Collete, 2003), and legally acceptable market names for mackerel products in many countries are nonspecific (BOE 2011), this denomination may lead to potential confusions. Also, *Scomber scombrus* can be an imported substitute of other *Scomber* species in mackerel products, and mislabeling is known to take place (Aranishi, 2005). However, the Atlantic mackerel is highly appreciated by consumers due to the excellent properties of the meat (Infante, Blanco, Zuasti, Crespo and Manchado, 2007). Furthermore, several species and/or stocks of Scombroids are endangered or overexploited, including some stocks of *Scomber scombrus* (IUCN, 2011).

To manage this situation, international authorities have developed regulations concerning labeling and worldwide fishing (EC 2001, EC 2002). Also, public health and economic concerns have increased the importance of species identification (Jacquet and Pauly 2008). In this field, DNA based techniques are faster, cheaper and more specific than protein based methods (Bottero and Dalmasso, 2011). Furthermore, protein expression in fish species is affected by

55 factors as tissue type (Martínez, Christiansen, Ofstad and Olsen, 1991), and
56 processing (Martínez, Slizyte and Dauksas, 2007). PCR based techniques have
57 been widely used for Scombroid species identification in food products, such as
58 DNA barcoding (Rasmussen and Morrissey, 2011), PCR with specific primers
59 (Aranishi and Okimoto, 2004), PCR-RFLP (Aranishi, 2005), FINS (Forensically
60 Informative Nucleotide Sequencing) (Infante et al. 2007; Espiñeira, González-
61 Lavín, Vieites and Santaclara, 2009), and others (Michelini et al. 2007;
62 Catanese, Manchado, Fernández-Trujillo and Infante, 2006; Infante et al. 2006).
63 These methods have drawbacks such as the need for post-PCR steps, costly
64 nucleotide sequencing, expensive equipment or low effectiveness in heavily
65 processed food products.

66 One of the emerging methods in species identification is Real-Time PCR (RT-
67 PCR), which allows the detection and quantification of target DNA fragments in
68 one step. It is also more sensitive and specific with standard laboratory
69 requirements, and it is suitable for samples treated under conditions that
70 endanger DNA integrity (Reischl, Wittwer and Cockerill, 2002). One of the most
71 commonly used RT-PCR analysis is based on the use of TaqMan probes,
72 designed to bind at diagnostic sites of the target DNA and release fluorescence
73 during amplification. In this context, Minor Groove Binder-DNA probes (MGB
74 probes) increase sequence specificity (Kutyavin et al. 2000). RT-PCR systems
75 have been developed for the identification of fish (Sánchez, Quinteiro, Rey-
76 Méndez, Pérez-Martín and Sotelo, 2009, López and Pardo, 2005), and in the
77 present work, this technique have been used with the aim of authenticating and
78 differentiating the Atlantic mackerel from its relatives and probable substitutes in
79 the market, developing a rapid and comparatively inexpensive method.

MATERIALS AND METHODS

Collection of samples and DNA extraction

36 specimens of *Scomber scombrus* with diverse geographic provenances and different year of capture were used as reference. Other 45 specimens of 30 related species were included. Scientific and common names are listed in table1. Also, 26 commercial samples with different types of processing and presentations (salted, dried, flavored, peppered, peppered in oil, hot smoked, cold smoked, pickled, and in tomato sauce) were collected in markets of Japan and Norway.

In addition to the above listed 107 samples, five fresh specimens of *Scomber scombrus* captured in Spain were processed in the laboratory for the validation of the method. For this, a piece of 5 g of muscle was autoclaved in a Mediclave (Selecta) at 121ºC and 1.2 atm during 20 minutes.

DNA was extracted as follows: about 0.2 to 0.3 g of muscle tissue from each sample was digested in a thermo shaker at 56ºC with 860 µL of lysis buffer (1% SDS, 150 mM NaCl, 2 mM EDTA, and 10mM Tris-HCl at pH 8), 100 µL of guanidium thiocyanate, and 40 µL of proteinase K. After 3 h, 40 µL of extra proteinase K was added and left overnight. DNA was isolated after digestion employing the Wizard DNA Clean-up System kit (Promega) by following the manufacturer's protocol. DNA was quantified by UV-spectrometry at 260 nm with Nanodrop 2000 (Thermo-scientific). DNA concentrations were adjusted to 50 ng/µL for subsequent RT-PCR reactions.

FINS identification of reference samples

All samples were authenticated by FINS 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 was amplified by using the primers described by Burguener (1997) (H15149: GCICCTCARAATGAYATTTGTCCTCA and L14735: AAAAACCACCGTTGTTATTCAACTA). The thermal cycling protocol used was: 94ºC for 5 minutes, followed by 35 cycles of amplification (94ºC for 40 seconds, 55ºC for 80 seconds and 72ºC for 80 seconds) and a final extension step of 72ºC for 7 minutes. These PCR reactions were carried out in an Applied Biosystems 2720 thermocycler, with a total reaction volume of 25 µL with 100 ng of DNA template, using PCR Ready-to-Go beads (GE Healthcare) with final concentrations of 1.5 mM of $MgCl_2$, 0.8 µM of each primer, 0.2 mM of dNTPs and 0.1 units of Taq polymerase. PCR amplicons were visualized on a 2% agarose gel, using UV transilumination.

Sequencing reactions were performed with BigDye Terminator 1.1 (Applied biosystems), following the manufacturer's instructions and sequencing was carried out in an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequences were analyzed with BioEdit (Hall, 1999) and MEGA (Kumar, Dudley, Nei and Tamura, 2008) software and the results were authenticated with BLAST (Altschul et al. 1997).

Design of primers and probe

A large number of mitochondrial regions sequences (control region, 16S rRNA, ND5, COI, 12S rRNA, and cytochrome *b*) from own databases and GenBank were analyzed to find a suitable region for the design. Sequences of

mitochondrial cytochrome *b* region from a previous study (Table 2) were selected and aligned to design a specific MGB Taq-Man primer-probe set for *Scomber scombrus* species. Primer Express software (Applied Biosystems) was used for selecting the sequence of the primers and probe set.

The sequences of the primers-probe set designed in this study are the following:

SSCO-F (forward): 5'-CTTCGGTTCCCTGCTTGGT-3'

SSCO-R (reverse): 5'-GCAAGGAATAGTCCTGTGAGGAT-3'

SSCO-P (probe): 5'-TCTGTTTAGCTTCCC-3'

The probe was labeled with the fluorochrome FAM in the 5' end and the Minor Groove Binding (MGB) in the 3' end.

Real-Time PCR conditions

Preliminary test with different concentrations of primers and probe were carried out in order to select the optimal reaction conditions. Concentrations of 50, 300 and 900 nM of each primer and 25, 50, 75, 100, 125, 150, 175, 200 and 225 nM of the probe were tested. The combination that gave the lowest Ct value and the highest final fluorescence was selected for the subsequent assays.

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 100 ng of DNA, 10 µL of TaqMan Fast Universal PCR Master Mix no UNG Amperase (2x) (Applied Biosystems) and a final concentration of 50 µM for forward primer, 300

mM for reverse primer and 150 µM for the probe. Reactions were run on ABI 7500 Fast (Applied Biosystems) with the standard thermal cycling protocol: 40 cycles of 95ºC for 15 s and 60ºC for 1 minute.

Real-Time data treatment

Each Ct value was obtained by the average of three replicates. The average Ct value obtained with *Scomber scombrus* samples was compared with the average Threshold Cycle (Ct) value obtained with 30 related species. The average Ct value found with *Scomber scombrus* was also compared with the average Ct value obtained with the other three *Scomber* species. In both cases, a One-way ANOVA F-test was used to compare variances, and a t-test with normal distributions and different variances (Yuan, Reed, Chen and Stewart, 2006) was used to compare means ($P<0.001$).

RESULTS AND DISCUSSION

Real Time PCR system design

In order to find a suitable fragment to design a specific MGB Taq-Man primer-probe system, a large number of nuclear and mitochondrial sequences were analyzed.

The aim was to find a short system, specific for the identification of the Atlantic mackerel (*Scomber scombrus*), with good sensitivity for all types of products, including heavily processed samples. Such a system (primers and probe) should not show intraspecific variability, and should be designed in a sequence region where the number of nucleotide interspecific differences is highest.

These two conditions were found in mitochondrial cytochrome *b* region. The sequence alignment of *Scomber scombrus* and related species is shown in Figure 1.

The designed system consisted of a 60 bp fragment. This size is small enough to allow identification in commercial products that have been processed with treatments that might affect DNA integrity, such as low pH, high temperatures, high pressure and hydrolysis (Bottero and Dalmasso, 2011).

Real Time PCR setup

The optimal concentrations of primers and probe used in RT-PCR reactions obtained from the results of preliminary tests were: 50 nM for the forward primer, 300 nM for reverse primer and 150 nM for the probe.

Different quantities of DNA, from 10^{-5}ng to 100ng, were used for the efficiency assay. Over this range of dilutions the response was linear with a slope of -3.52 and an efficiency of 92.41 %, following the equation: $E = [10^{(-1/\text{slope})}-1] \times 100$, which proves the utility of the system for the identification of Atlantic mackerel. It also shows a detection limit of quantities of 1×10^{-4}ng of DNA and a linear correlation between fluorescence and concentration of DNA, thus suggesting the potential of this approach for the development of quantitative estimations.

Specificity and cross-reactivity of the system was tested with the species of table 1. The average Ct value obtained with *Scomber scombrus* samples in specificity test was 15.3±0.5. There was a statistically significant difference ($P<0.001$) between this Ct value and the Ct value obtained for the rest of

samples, which showed a Ct average of 38.5±2.2 (to obtain this average value, the undetected samples were given a Ct = 40) (figure 2-I). No false positives were observed. Although some of the species in the *Scomber* genus (*Scomber australasicus* and *Scomber japonicus*) had a slight signal (Ct values of 29.5 and 33.2 respectively, table 1), the strong signal and low Ct value obtained in *Scomber scombrus* was sufficient to significantly ($P<0.001$) discriminate it from the other three *Scomber* species.

Validation in processed samples

As sterilization procedures have severe effects in DNA integrity, a laboratory assay was carried out in order to check if the manufacturing process had any effects in Ct values. The Ct values of 5 autoclaved *Scomber scombrus* did not differ significantly ($P<0.001$) from those of unprocessed specimens (figure 2-II). These results reveal that thermal and pressure treatments do not affect Ct values using the method designed in this work.

Identification of commercial samples

A total of 19 commercial samples containing *Scomber scombrus* with different degrees of processing were used for the validation of the method. The correct labeling had been previously evaluated by FINS.

Ct values obtained in all *Scomber scombrus* commercial products were within the interval 14 to 20 (table 3), including fresh, processed and commercial samples. The different transformation processes of the samples did not affect

218 the detection of the target species, as all samples tested containing *Scomber*
219 *scombrus* were positive.

220

221 The Ct values obtained in this study for *Scomber scombrus* products (Ct
222 average=16.5±1.5) are as distinctive as the values found in previous works for
223 fish species identification using Real Time-PCR (Sanchez et al. 2009; Herrero
224 et al. 2010), where positive average Ct values were within the range of 16 to 20
225 and negative values above 29. The method is also highly specific as it can
226 differentiate among the very closely related species, within the *Scomber* genus.

227

228 This work describes a very useful tool for the identification of *Scomber*
229 *scombrus* samples, both unprocessed and manufactured. Some of the
230 advantages are its reliability, sensitivity, specificity, and the relatively reduced
231 costs compared to other authentication methods for *Scomber scombus* that
232 require post-PCR steps (Aranishi, 2005; Aranishi and Okimoto, 2004; Espiñeira
233 et al. 2009; Catanese et al. 2010; Infante et al. 2006). It is also quicker, since up
234 to 96 DNA samples may be tested within a 3 to 5 hours span. Some of the
235 applications of the method developed are the fulfillment of labeling regulations
236 for commercial fish products for human consumption, and human health
237 considerations. In fish inspections it can be taken into consideration the
238 possibility of implementing a DNA extraction method that can be carried out on
239 board, eliminating tissue preservation issues. Although mitochondrial genes are
240 not the best option for quantification aims (López and Pardo, 2005), in the near
241 future this system may be also assayed for the application in this purpose in

tissue mixtures, for the efficiency results revealed a high correlation between fluorescence and DNA concentration.

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256 **FIGURE CAPTIONS**

257

258 Figure 1: Alignment of mitochondrial cytochrome *b* region sequences from
259 scombroids, showing the positions of the primer-probe set designed for this
260 study.

261

262 Figure 2: I) A: Amplification pattern shown by *Scomber scombrus* (Ct =
263 15.3±0.5). B: Amplification pattern shown by other species (Ct = 38.5±2.2). II)
264 Amplification pattern shown by fresh (Ct = 15.3±0.6) and autoclaved (Ct =
265 15.7±0.5) samples of *Scomber scombrus*.

TABLES

ɔecies name and authority	Common name	Family	Geographical origin of samples	Number of specimens
ɪbrus (Linnaeus, 1758)	Atlantic mackerel	Scombridae	Atlantic, Northeast	36
ıs (Gmelin,1789)	Atlantic chub mackerel	Scombridae	Atlantic Northeast and Mediterranean	6
ralasicus (Cuvier, 1832)	Spotted mackerel	Scombridae	Pacific, Norwest	6
nicus (Houttuyn, 1782)	Pacific mackerel	Scombridae	Pacific, Norwest	6
Risso, 1810)	Bullet tuna	Scombridae	Atlantic, Northeast	1
(Lacepède, 1800)	Frigate tuna	Scombridae	Atlantic, Northeast	1
ıelamis (Linnaeus, 1758)	Skipjack tuna	Scombridae	Atlantic, Northeast	1
ɛtteratus (Rafinesque, 1810)	Little tunny	Scombridae	Pacific, Eastern Central	1
ıticus (Lesson, 1831)	Blackfin tuna	Scombridae	Atlantic, Western Central	1
cares (Bonnaterre, 1788)	Yellowfin tuna	Scombridae	Pacific, Eastern Central	1
us (Lowe, 1839)	Bigeye tuna	Scombridae	Atlantic, Northeast	1
ıtalis (Temminck & Schlegel, 1844)	Pacific bluefin tuna	Scombridae	Pacific, Eastern Central	1
ınga (Bonnaterre, 1788)	Albacore	Scombridae	Atlantic, Southeast	1
ɔyii (Castelnau, 1872)	Southern bluefin tuna	Scombridae	Pacific, Western Central	1
gol (Bleeker, 1851)	Longtail tuna	Scombridae	Indian Ocean, East	1
nus (Linnaeus, 1758)	Bluefin tuna	Scombridae	Atlantic, Northeast	1
is (Cuvier, 1831)	Pacific bonito	Scombridae	Pacific, Eastern Central	1
Bloch, 1793)	Atlantic bonito	Scombridae	Atlantic, Northeast	1
·us cavalla (Cuvier, 1829)	King mackerel	Scombridae	Atlantic, Southwest	1
*·us sierra (*Jordan & Starks, 1895	Pacific sierra	Scombridae	Pacific, Northeast	1
·us maculatus (Mitchill, 1815)	Atlantic Spanish mackerel	Scombridae	Atlantic, Western Central	1
ɜnagurta (Cuvier, 1817)	Indian mackerel	Scombridae	Pacific, Northwest	1
ɛlovinus (Cuvier, 1830)	Patagonian blennie	Eleginopidae	Pacific, Southeast	1
*ɛellatus (*Cuvier, 1829)	Butterfish	Stromateidae	Pacific, Southeast	1
ıippurus (Linnaeus, 1758)	Common dolphinfish	Coryphaenidae	Atlantic, Northeast	1
ɬleginoides (Smitt, 1898)	Patagonian toothfish	Nototheniidae	Atlantic, Southwest	1
ɬturatus (Bowdich, 1825)	Blue jack mackerel	Carangidae	Atlantic, Northeast	1
ɛchurus (Linnaeus, 1758)	Atlantic horse mackerel	Carangidae	Atlantic, Northeast	1
ɾasicolus (Linnaeus, 1758)	European anchovy	Clupeidae	Atlantic, Northeast	1
ɪrdus (Walbaum, 1792)	European pilchard	Clupeidae	Atlantic, Northeast	1
ɾita (Valenciennes, 1847)	Round sardinella	Clupeidae	Atlantic, Eastern Central	1

Table 1: Species tested in this work and Ct values obtained from 100 ng of DNA in specificity and cross-reactivity tests.

266

species	nº haplotypes	GenBank ID
Scomber scombrus	25	JQ409567 to JQ409578, JQ409594 to JQ409606
Scomber japonicus	1	JQ409579
Scomber colias	9	JQ409580 to JQ409588
Scomber australasicus	5	JQ409589 to JQ409593
Auxis rochei	10	JQ434014 to JQ434023
Auxis thazard	3	JQ434024 to JQ434026
Katsuwonus pelamis	10	JQ434027 to JQ434036
Euthynnus alletteratus	2	JQ434037, JQ434038
Thunnus albacares	3	JQ434039 to JQ434041
Thunnus atlanticus	4	JQ434042 to JQ434045
Thunnus thynnus	2	JQ434046, JQ434047
Thunnus obesus	5	JQ434048 to JQ434052
Thunnus orientalis	3	JQ434053 to JQ434055
Thunnus alalunga	8	JQ434056 to JQ434063
Sarda chiliensis	2	JQ434070, JQ434071
Sarda sarda	5	JQ434066 to JQ434069
Scomberomorus cavalla	2	JQ434070, JQ434071
Scomberomorus sierra	4	JQ434072 to JQ434074

Table 2: List of sequences selected and compared for the design of the primers and probe.

Sample	Process	Origin of samples	Cт Mean±SD
1	Salted	Japanese market	15.2±0.1
2	Salted	Japanese market	15.9±0.1
3	Dried	Japanese market	16.4±0.1
4	Salted	Japanese market	15.3±0.1
5	Salted	Japanese market	15.8±0.0
6	Dried	Japanese market	15.6±0.0
7	Flavored	Japanese market	19.3±0.1
8	Flavored	Japanese market	17±0.0
9	Pickled	Japanese market	15±0.0
10	Peppered in oil	Norwegian market	18.5±0.1
11	In tomato sauce	Norwegian market	16.6±0.1
12	In tomato sauce	Norwegian market	15.8±0.1
13	In tomato sauce	Norwegian market	16.4±0.2
14	Cold smoked	Norwegian market	16.3±0.1
15	Hot smoked	Norwegian market	17.2±0.3
16	Hot smoked	Norwegian market	15.9±0.0
17	Hot smoked	Norwegian market	16.7±0.1
18	Peppered	Norwegian market	19.1±0.2
19	Peppered	Norwegian market	17.3±0.0
20	salted	Japanese market	14.5±0.1
21	salted	Japanese market	14.5±0.0
22	salted	Japanese market	17.3±0.1
23	salted	Japanese market	14.6±0.2
24	minced with tomato	Norwegian market	20.0±0.1
25	minced with tomato	Norwegian market	18.2±0.1
26	hot smoked	Norwegian market	15.5±0.1

Table 3: Ct values obtained from commercial samples containing *Scomber scombrus*.

267

Figure 1

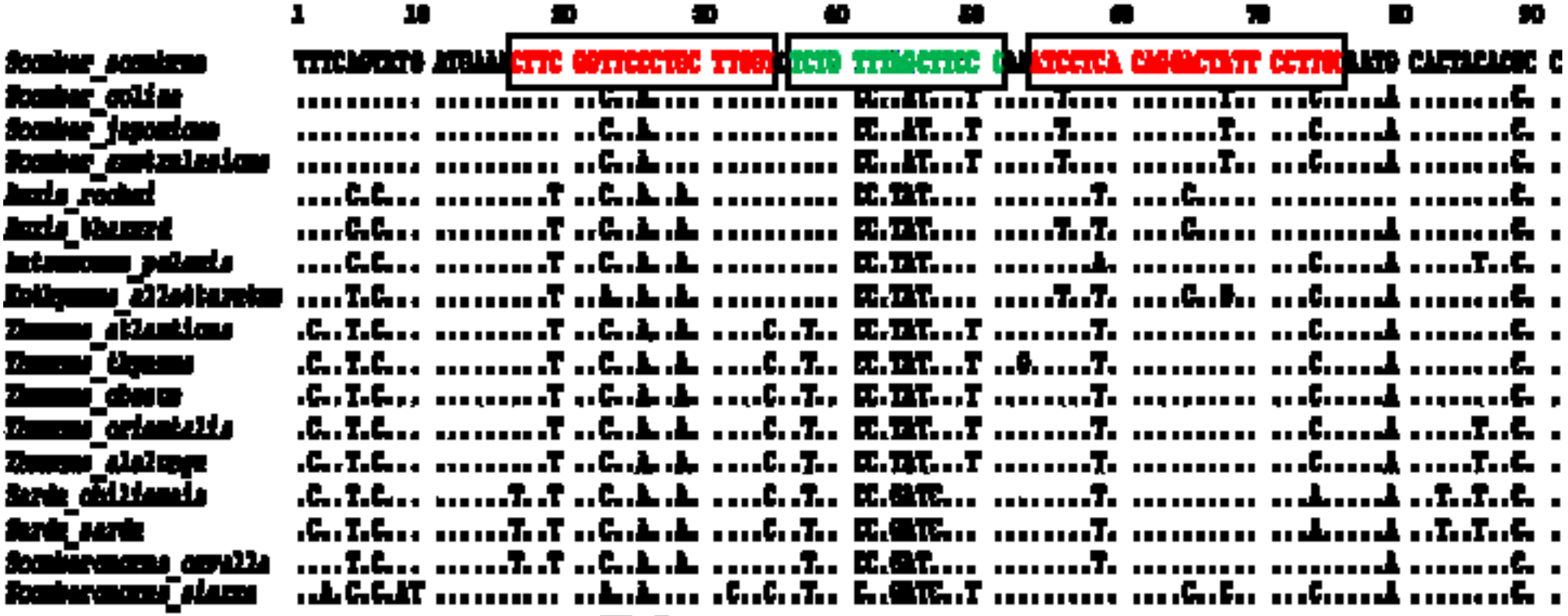
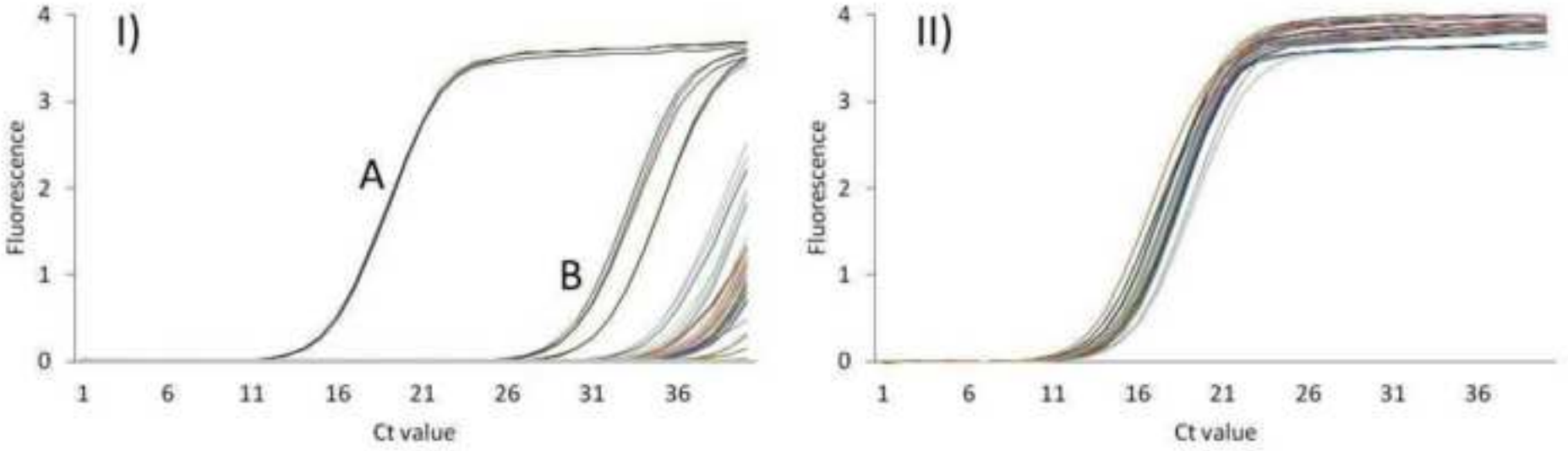
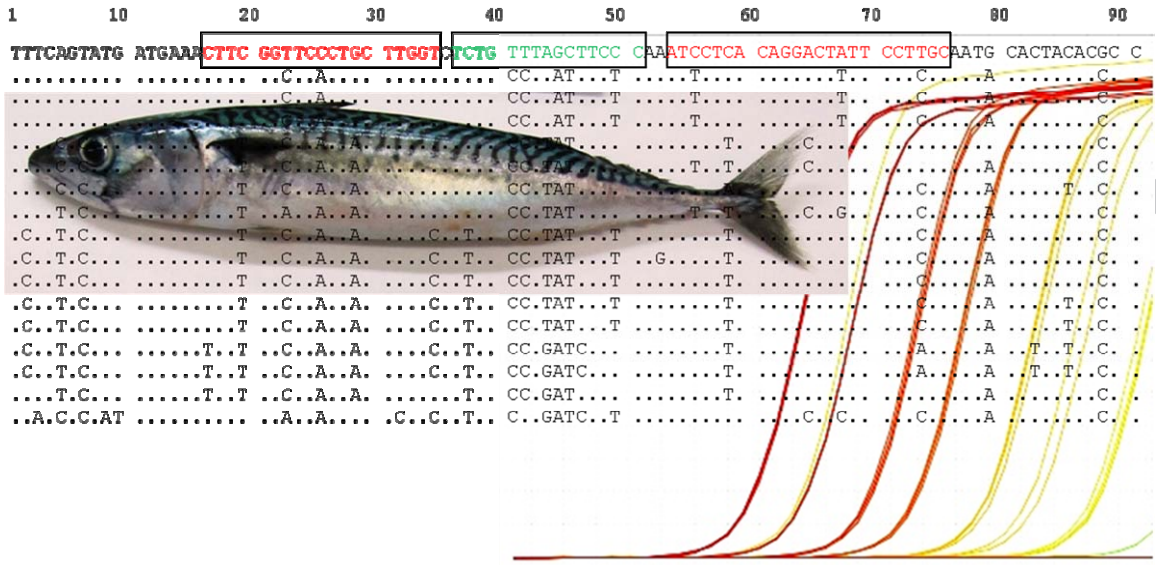


Figure 2





Higlights

- The method unequivocally identifies Atlantic mackerel
- No false positives were detected with related species
- The method was successful in heavily processed samples
- It is efficient and can be developed for quantification aims