

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

3

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14 **ABSTRACT:** A Real Time-PCR method based on TaqMan technology for the  
15 identification of *Scomber Scombrus* has been developed. A system of specific  
16 primers and a Minor Groove Binding (MGB) TaqMan probe based on  
17 sequences of the mitochondrial cytochrome *b* region was designed. The  
18 method was successfully tested in 81 specimens of *Scomber scombrus* and  
19 related species and validated in 26 different commercial samples. An average  
20 Threshold cycle (Ct) value of 15.3 was obtained with *Scomber scombrus* DNA.  
21 With the other species tested fluorescence signal was not detected or Ct was  
22 significantly higher ( $P < 0.001$ ). The efficiency of the assay was estimated to be  
23 92.41%, with 100% specificity, and no cross reactivity was detected with any  
24 other species. These results reveal that the developed method is a rapid and  
25 efficient tool to unequivocally identify *S. scombrus* and may aid in the  
26 prevention of fraud or mislabeling in mackerel products.

27

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

30 **INTRODUCTION**

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

37 Atlantic mackerel *Scomber scombrus* (Linnaeus 1758), is usually marketed  
38 fresh, frozen, salted, smoked and canned. Nevertheless, since other species of  
39 *Scomber*, *Scomberomorus*, *Auxis*, and *Rastrelliger*, are commonly named  
40 mackerel in many parts of the world (Collete, 2003), and legally acceptable  
41 market names for mackerel products in many countries are nonspecific (BOE  
42 2011), this denomination may lead to potential confusions. Also, *Scomber*  
43 *scombrus* can be an imported substitute of other *Scomber* species in mackerel  
44 products, and mislabeling is known to take place (Aranishi, 2005). However, the  
45 Atlantic mackerel is highly appreciated by consumers due to the excellent  
46 properties of the meat (Infante, Blanco, Zuasti, Crespo and Manchado, 2007).  
47 Furthermore, several species and/or stocks of Scombroids are endangered or  
48 overexploited, including some stocks of *Scomber scombrus* (IUCN, 2011).

49 To manage this situation, international authorities have developed regulations  
50 concerning labeling and worldwide fishing (EC 2001, EC 2002). Also, public  
51 health and economic concerns have increased the importance of species  
52 identification (Jacquet and Pauly 2008). In this field, DNA based techniques are  
53 faster, cheaper and more specific than protein based methods (Bottero and  
54 Dalmaso, 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 Scombrotoxic 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.

## 80 MATERIALS AND METHODS

### 81 Collection of samples and DNA extraction

82 36 specimens of *Scomber scombrus* with diverse geographic provenances and  
83 different year of capture were used as reference. Other 45 specimens of 30  
84 related species were included. Scientific and common names are listed in  
85 table1. Also, 26 commercial samples with different types of processing and  
86 presentations (salted, dried, flavored, peppered, peppered in oil, hot smoked,  
87 cold smoked, pickled, and in tomato sauce) were collected in markets of Japan  
88 and Norway.

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

93 DNA was extracted as follows: about 0.2 to 0.3 g of muscle tissue from each  
94 sample was digested in a thermo shaker at 56°C with 860 µL of lysis buffer (1%  
95 SDS, 150 mM NaCl, 2 mM EDTA, and 10mM Tris-HCl at pH 8), 100 µL of  
96 guanidium thiocyanate, and 40 µL of proteinase K. After 3 h, 40 µL of extra  
97 proteinase K was added and left overnight. DNA was isolated after digestion  
98 employing the Wizard DNA Clean-up System kit (Promega) by following the  
99 manufacturer's protocol. DNA was quantified by UV-spectrometry at 260 nm  
100 with Nanodrop 2000 (Thermo-scientific). DNA concentrations were adjusted to  
101 50 ng/µL for subsequent RT-PCR reactions.

102

**103 FINS identification of reference samples**

104 All samples were authenticated by FINS in order to check the reliability of the  
105 RT-PCR analysis and test the functionality of the DNA extracted. A 464 bp  
106 fragment of *cytb* gene was amplified by using the primers described by  
107 Burguener (1997) (H15149: GCICCTCARAATGAYATTTGTCCTCA and  
108 L14735: AAAAACCACCGTTGTTATTCAACTA). The thermal cycling protocol  
109 used was: 94°C for 5 minutes, followed by 35 cycles of amplification (94°C for  
110 40 seconds, 55°C for 80 seconds and 72°C for 80 seconds) and a final  
111 extension step of 72°C for 7 minutes. These PCR reactions were carried out in  
112 an Applied Biosystems 2720 thermocycler, with a total reaction volume of 25 µL  
113 with 100 ng of DNA template, using PCR Ready-to-Go beads (GE Healthcare)  
114 with final concentrations of 1.5 mM of MgCl<sub>2</sub>, 0.8 µM of each primer, 0.2 mM of  
115 dNTPs and 0.1 units of Taq polymerase. PCR amplicons were visualized on a  
116 2% agarose gel, using UV transillumination.

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

**123 Design of primers and probe**

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

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

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

133 SSCO-F (forward): 5'-CTTCGGTTCCTGCTTGGT-3'

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

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

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

#### 138 **Real-Time PCR conditions**

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

144 PCR reactions were performed in a total volume of 20  $\mu$ L in a MicroAmp fast  
145 optical 96-well reaction plate (Applied Biosystems), covered with MicroAmp  
146 optical adhesive film (Applied Biosystems). Each reaction contained 100 ng of  
147 DNA, 10  $\mu$ L of TaqMan Fast Universal PCR Master Mix no UNG Amperase (2x)  
148 (Applied Biosystems) and a final concentration of 50  $\mu$ M for forward primer, 300

149 mM for reverse primer and 150  $\mu$ M for the probe. Reactions were run on ABI  
150 7500 Fast (Applied Biosystems) with the standard thermal cycling protocol: 40  
151 cycles of 95°C for 15 s and 60°C for 1 minute.

## 152 **Real-Time data treatment**

153 Each Ct value was obtained by the average of three replicates. The average Ct  
154 value obtained with *Scomber scombrus* samples was compared with the  
155 average Threshold Cycle (Ct) value obtained with 30 related species. The  
156 average Ct value found with *Scomber scombrus* was also compared with the  
157 average Ct value obtained with the other three *Scomber* species. In both cases,  
158 a One-way ANOVA F-test was used to compare variances, and a t-test with  
159 normal distributions and different variances (Yuan, Reed, Chen and Stewart,  
160 2006) was used to compare means ( $P < 0.001$ ).

161

## 162 **RESULTS AND DISCUSSION**

### 163 **Real Time PCR system design**

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

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



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

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

179

#### 180 **Real Time PCR setup**

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

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

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

195 samples, which showed a Ct average of  $38.5 \pm 2.2$  (to obtain this average value,  
196 the undetected samples were given a Ct = 40) (figure 2-I). No false positives  
197 were observed. Although some of the species in the *Scomber* genus (*Scomber*  
198 *australasicus* and *Scomber japonicus*) had a slight signal (Ct values of 29.5 and  
199 33.2 respectively, table 1), the strong signal and low Ct value obtained in  
200 *Scomber scombrus* was sufficient to significantly ( $P < 0.001$ ) discriminate it from  
201 the other three *Scomber* species.

202

### 203 **Validation in processed samples**

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

210

### 211 **Identification of commercial samples**

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

215 Ct values obtained in all *Scomber scombrus* commercial products were within  
216 the interval 14 to 20 (table 3), including fresh, processed and commercial  
217 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 scombrus* 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

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

244

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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  $15.3 \pm 0.5$ ). B: Amplification pattern shown by other species (Ct =  $38.5 \pm 2.2$ ). II)  
264 Amplification pattern shown by fresh (Ct =  $15.3 \pm 0.6$ ) and autoclaved (Ct =  
265  $15.7 \pm 0.5$ ) samples of *Scomber scombrus*.

## TABLES

Species name and authority	Common name	Family	Geographical origin of samples	Number of specimens
<i>Merluccius</i> (Linnaeus, 1758)	Atlantic mackerel	Scombridae	Atlantic, Northeast	36
<i>Scomber</i> (Gmelin, 1789)	Atlantic chub mackerel	Scombridae	Atlantic Northeast and Mediterranean	6
<i>Scomber</i> (Cuvier, 1832)	Spotted mackerel	Scombridae	Pacific, Northwest	6
<i>Scomber</i> (Houttuyn, 1782)	Pacific mackerel	Scombridae	Pacific, Northwest	6
Risso, 1810)	Bullet tuna	Scombridae	Atlantic, Northeast	1
(Lacepède, 1800)	Frigate tuna	Scombridae	Atlantic, Northeast	1
<i>Thunnus</i> (Linnaeus, 1758)	Skipjack tuna	Scombridae	Atlantic, Northeast	1
<i>Thunnus</i> (Rafinesque, 1810)	Little tunny	Scombridae	Pacific, Eastern Central	1
<i>Thunnus</i> (Lesson, 1831)	Blackfin tuna	Scombridae	Atlantic, Western Central	1
<i>Thunnus</i> (Bonnaterre, 1788)	Yellowfin tuna	Scombridae	Pacific, Eastern Central	1
<i>Thunnus</i> (Lowe, 1839)	Bigeye tuna	Scombridae	Atlantic, Northeast	1
<i>Thunnus</i> (Temminck & Schlegel, 1844)	Pacific bluefin tuna	Scombridae	Pacific, Eastern Central	1
<i>Thunnus</i> (Bonnaterre, 1788)	Albacore	Scombridae	Atlantic, Southeast	1
<i>Thunnus</i> (Castelnau, 1872)	Southern bluefin tuna	Scombridae	Pacific, Western Central	1
<i>Thunnus</i> (Bleeker, 1851)	Longtail tuna	Scombridae	Indian Ocean, East	1
<i>Thunnus</i> (Linnaeus, 1758)	Bluefin tuna	Scombridae	Atlantic, Northeast	1
<i>Scomber</i> (Cuvier, 1831)	Pacific bonito	Scombridae	Pacific, Eastern Central	1
Bloch, 1793)	Atlantic bonito	Scombridae	Atlantic, Northeast	1
<i>Scomber</i> (Cuvier, 1829)	King mackerel	Scombridae	Atlantic, Southwest	1
<i>Scomber</i> (Jordan & Starks, 1895)	Pacific sierra	Scombridae	Pacific, Northeast	1
<i>Scomber</i> (Mitchill, 1815)	Atlantic Spanish mackerel	Scombridae	Atlantic, Western Central	1
<i>Scomber</i> (Cuvier, 1817)	Indian mackerel	Scombridae	Pacific, Northwest	1
<i>Scomber</i> (Cuvier, 1830)	Patagonian blennie	Eleginopidae	Pacific, Southeast	1
<i>Scomber</i> (Cuvier, 1829)	Butterfish	Stromateidae	Pacific, Southeast	1
<i>Scomber</i> (Linnaeus, 1758)	Common dolphinfish	Coryphaenidae	Atlantic, Northeast	1
<i>Scomber</i> (Smitt, 1898)	Patagonian toothfish	Nototheniidae	Atlantic, Southwest	1
<i>Scomber</i> (Bowdich, 1825)	Blue jack mackerel	Carangidae	Atlantic, Northeast	1
<i>Scomber</i> (Linnaeus, 1758)	Atlantic horse mackerel	Carangidae	Atlantic, Northeast	1
<i>Scomber</i> (Linnaeus, 1758)	European anchovy	Clupeidae	Atlantic, Northeast	1
<i>Scomber</i> (Walbaum, 1792)	European pilchard	Clupeidae	Atlantic, Northeast	1
<i>Scomber</i> (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.

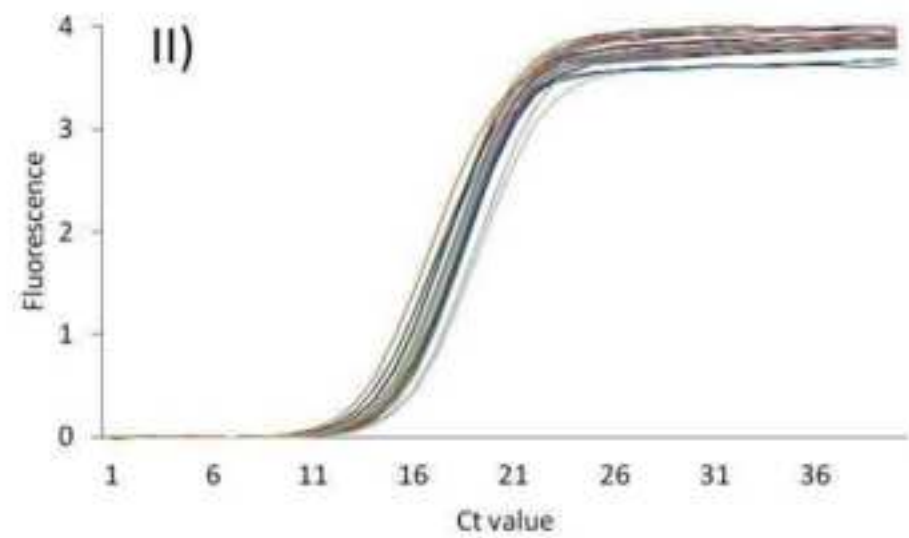
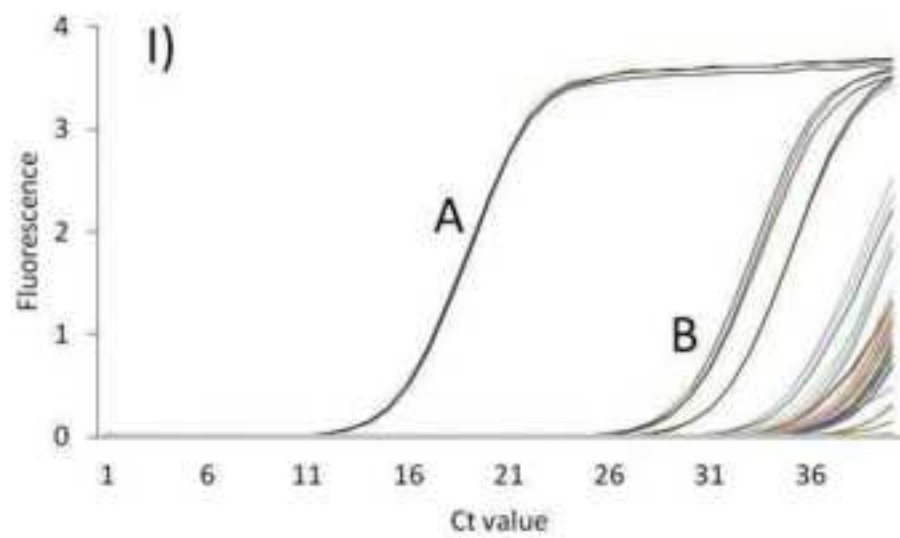
species	n° haplotypes	GenBank ID
<i>Scomber scombrus</i>	25	JQ409567 to JQ409578, JQ409594 to JQ409606
<i>Scomber japonicus</i>	1	JQ409579
<i>Scomber colias</i>	9	JQ409580 to JQ409588
<i>Scomber australasicus</i>	5	JQ409589 to JQ409593
<i>Auxis rochei</i>	10	JQ434014 to JQ434023
<i>Auxis thazard</i>	3	JQ434024 to JQ434026
<i>Katsuwonus pelamis</i>	10	JQ434027 to JQ434036
<i>Euthynnus alletteratus</i>	2	JQ434037, JQ434038
<i>Thunnus albacares</i>	3	JQ434039 to JQ434041
<i>Thunnus atlanticus</i>	4	JQ434042 to JQ434045
<i>Thunnus thynnus</i>	2	JQ434046, JQ434047
<i>Thunnus obesus</i>	5	JQ434048 to JQ434052
<i>Thunnus orientalis</i>	3	JQ434053 to JQ434055
<i>Thunnus alalunga</i>	8	JQ434056 to JQ434063
<i>Sarda chiliensis</i>	2	JQ434070, JQ434071
<i>Sarda sarda</i>	5	JQ434066 to JQ434069
<i>Scomberomorus cavalla</i>	2	JQ434070, JQ434071
<i>Scomberomorus sierra</i>	4	JQ434072 to JQ434074

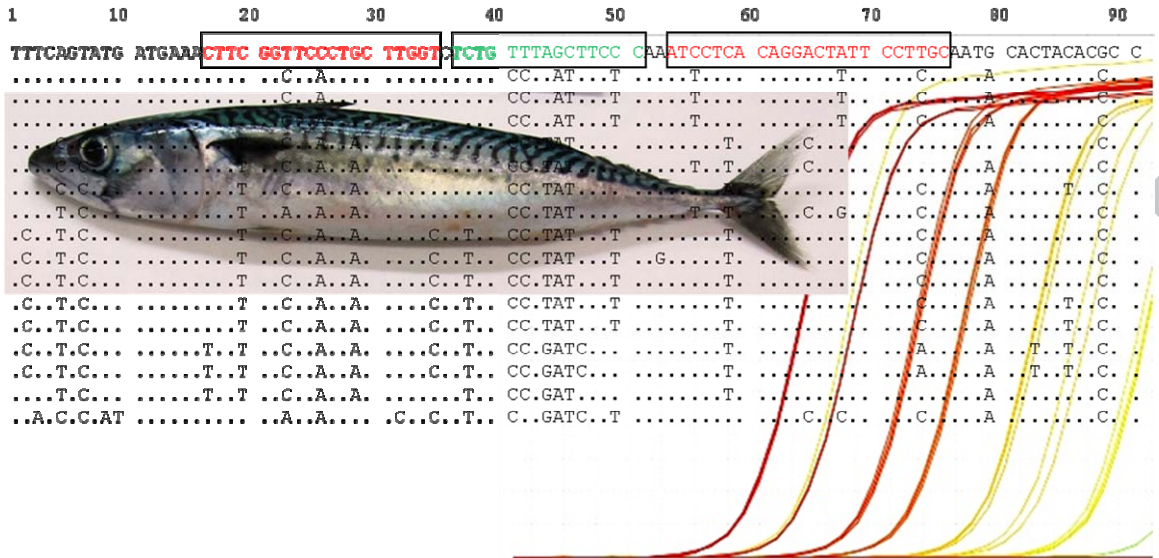
**Table 2:** List of sequences selected and compared for the design of the primers and probe.

Sample	Process	Origin of samples	Ct 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*.









268 **Highlights**

- 269
- 270
- 271
- 272
- 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

ACCEPTED MANUSCRIPT