Short communication

Development of a real-time PCR method for the simultaneous detection of mackerel and horse mackerel

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\textbf{A B S T R A C T}

We have developed a real-time PCR method for the simultaneous detection of horse mackerel (\textit{Trachurus trachurus}) and mackerel (\textit{Scomber scombrus}) DNA. This method has been designed to be used as an extension to our previously published method for the simultaneous detection of several fish species. A fragment of the parvalbumin gene was used as target for the development of the reported method. The results showed the required specificity, efficiency of the real-time PCR reaction and comparable selectivity with the previously reported method, therefore their possible complementarity for food and feed analysis is discussed. Additionally, the developed method combined with melt curve analysis allows the differentiation between both fish species which can have interesting applications for authenticity studies.

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1. Introduction

The development of analytical methods for the simultaneous detection and quantification of different fish species in food and feed is an urgent need in control laboratories. Those methods would allow on one side to effectively test food products in order to assess the compliance with the Labeling Directive (\textit{OJEU}, 2000) and its amendments (\textit{OJEU}, 2003, 2007\textit{a}) in EU, and with similar legislative initiatives in other countries such as the Food Allergy Labeling and Consumer Protection Act (FALCPA) (\textit{FDA}, 2004) in the USA which require manufacturers to declare all potentially allergenic ingredients present in pre-packaged foods, since fish is considered as one of the most common causes of food hypersensitivity (Sun, Liang, Gao, Lin, & Deng, 2009).

On the other side, it would facilitate the legal use of fishmeal in pig and poultry feed, and it would contribute to the implementation of Regulation (EC) No 1923/2006 (\textit{OJEU}, 2006) in EU. This legislation introduces the possibility of a tolerance level for fish-meals, which is nowadays not authorized on ruminant feeds.

Therefore, requires for official control purposes the availability of a specific quantitative method.

Polymerase Chain Reaction (PCR) based methods are considered as a good solution for the detection of minute amounts of one species or a group of species, since they are based on the exponential amplification of a sequence of target DNA. In addition to these advantages, real-time or quantitative PCR (qPCR) has a quantitative potential, taking into account some previously described limitations (von Holst, Boix, Marien, & Prado, 2012; Prado et al., 2009) that should be investigated for this aim.

Commonly used DNA targets include single copy, or multiple copy targets such as mitochondrial or other highly repetitive sequences. Although the latter ones have the advantage of allowing a higher sensitivity due to its higher number of copies in samples, single copy nuclear DNA is considered as more feasible for quantification purposes due to its more constant copy number in different cells from different tissues. However, public databases do not contain sequences for many of the fish species of interest, particularly from single copy nuclear DNA targets, which makes it especially cumbersome to find an appropriate target. Previously published articles about the development of real-time PCR methods in the field of fish DNA detection are focused on the use of mitochondrial DNA (mtDNA) as target, and highlight both the difficulties concerning the identification of a nuclear target and the quantification using such mtDNA targets (Benedetto, Abete, &
Squadrone, 2011; Martín et al., 2010; Pegels, González, López-Calleja, García, & Martín, 2013).

Previously, our group developed a qPCR method based on the use of a nuclear gene as target, for the simultaneous detection of fish DNA from different species and evaluated its quantification potential (Prado, Boix, & von Holst, 2012). This method was tested with 22 different fish species including those most commonly used by the food and feed industry, and with 24 negative controls reporting satisfactory results with most of them, however no positive signal was obtained from horse mackerel, while for mackerel inconsistent results were obtained for some of the replicates.

Both mackerel and horse mackerel, as well as other related species (Trachurus spp. and Scomber spp.) are important species on the fish market, captures reached 887,314 and 206,785 tons for mackerel and horse mackerel respectively in 2010 according to FAO (FAO, 2012). Both of them are key species in food products and as ingredients for fishmeals, therefore a modification of the previously described method or the development of a complementary method is required in order to include them.

### 2. Materials and methods

#### 2.1. Method development

##### 2.1.1. Target selection

Since one of the main objectives was the evaluation of the quantification potential of the developed qPCR method, single copy nuclear DNA sequences were chosen among all possible DNA targets. Single copy nuclear DNA is considered as more feasible for quantification purposes than other DNA targets, such as mitochondrial DNA, due to its more constant copy number in different cells from different tissues. The availability of DNA sequences in public databases is one of the critical factors when developing a PCR method. Our previously developed method was based on a fragment of the rhodopsin gene, due to the availability of DNA sequences for a variety of fish species, the designed primers and probes included degeneracies in order to be suitable for the amplification of such a large number of species.

Rhodopsin gene sequences from mackerel and horse mackerel were studied in order to evaluate the possibility to introduce further degeneracies in the designed primers and/or probe, or the possibility of choosing another region for amplification. However alignments performed with Geneious Pro 4.6.1. (Drummond et al., 2009) showed that further degeneracies would most likely give false positives against certain animal species.

Parvalbumin gene was chosen among available sequences for Trachurus and Scomber spp. Parvalbumins have been identified as the major allergy-eliciting proteins in fish that are responsible for over 95% of atopic responses (Sun et al., 2009). DNA sequences from parvalbumin gene from mackerel and horse mackerel were not available at the time of the development of the present method, since these sequences were aligned and a consensus sequence was obtained from them with Geneious software as seen in Fig. 1. This consensus sequence allowed the development of a qPCR method for the quantification of DNA from the fish species of interest.

#### 2.1.2. Sequence comparison and primer and probe design

Available DNA sequences from both Trachurus spp. and Scomber spp. were aligned with this consensus sequence in order to identify the most appropriate region for our aim. This region had to have a low or non-variability between the consensus sequence and both fish species, and high variability with other species, especially those most frequently used as ingredients in food and feed products.

Primers and probes for the qPCR assay were designed by using Primer Express software (Applied Biosystems), taking into account the following parameters to ensure the success of the method: (i) The product of amplification should be between 50 and 120 bp, since this would allow a positive signal and ensure the reliability of the quantification even if the heat treatment would affect the integrity of the DNA, (ii) the PCR product should be in a relatively low or non-variability between the consensus sequence and both fish species to avoid cross amplification that would lead to false positives and errors on quantification. From all the primers and probes suggested by the Primer Express software, different combinations were tested for the best results. The chosen primers/probe combination (Table 1) would give a product of amplification of 87 bp. The consensus sequence together with the selected primers and probe were aligned with sequences from public databases from a broad variety of animal species such as cattle, human or rat and also other fish species such as cod, common carp, herring or black bass to ensure that the developed method would not give a positive response with any other species apart from those species closely related with mackerel and horse mackerel (Trachurus spp. and Scomber spp.).

#### 2.2. QPCR method

##### 2.2.1. Optimization of primer and probe concentration

To ensure the most sensitive and efficient assay, the reaction conditions were carefully optimized for the primers/probe combination. The optimization included the determination of the best

### Table

<table>
<thead>
<tr>
<th>Method</th>
<th>Primers/probes optimized concentrations.</th>
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<tr>
<td>Pval1Fw</td>
<td>CTGAACGTCTCCCTCGAGAACCTT</td>
</tr>
<tr>
<td>Pval1Rev</td>
<td>GGTGACAGCTTCGTTCGAG</td>
</tr>
<tr>
<td>Pval1probe</td>
<td>[TAMRA] TCCGACGCCGAGACCAAGGC</td>
</tr>
</tbody>
</table>

**Fig. 1.** Consensus sequence from available sequences from Scomber and Trachurus genus.
primers and probe concentration and their annealing temperature in order to obtain the lowest quantification cycle (Cq), the highest ΔR and a difference in the Cq values between two tenfold dilutions of a positive control near the expected limit of detection of approximately three (Raymaekers, Smets, Maes, & Cartuyvels, 2009). To determine the best conditions, concentrations of 50, 300 and 900 nM of each primer, were tested in triplicates. Once optimized for the forward and reverse primer concentration, the probe concentration was also optimized by testing concentrations from 50 nM to 300 nM with two different concentrations (2–20 ng) of DNA from the same fish species. The annealing temperature for the best results was also established. Table 1 indicates the optimal concentration for the primers/probe combination. All reactions were performed in a total volume of 35 μl containing 17.5 μl of TaqMan Universal Mastermix (Applied Biosystems) and appropriate volumes of primers, probe (Sigma–Aldrich Co), template DNA and PCR-grade water (Sigma–Aldrich Co). All reactions were performed on an ABI7900HT thermocycler (Applied Biosystems) in groups of four replicates.

The optimized cycling conditions were as follows: 50 °C for 2 min, 94 °C for 10 min, followed by 50 cycles 94 °C for 20 s, and 60 °C for 1 min. Once optimized the reaction, the method was tested with both fish species and negative controls to check whether it was fit for the intended purpose concerning specificity. The absence of primer–dimer formation was checked by dissociation curve analysis, since the presence of primer dimers produces a lower PCR efficiency in probe-based assays (Bustin et al., 2009). All reactions were performed on an ABI7900HT thermocycler (Applied Biosystems) in triplicates. For this analysis, optimal primers concentration were used together with 17.5 μl SYBR Green PCR MasterMix (Applied Biosystems) and appropriate volumes of template DNA and PCR-grade water (Sigma–Aldrich Co). A dissociation stage was added to the thermal profile consisting of 1 single cycle of 95 °C, 58 °C and 95 °C with a 2% ramp rate in the latter case. Primers were tested with both mackerel and horse mackerel and no unspecific products were observed with any of them.

2.2.2. Data analysis

In order to evaluate the applicability of the method for quantitative purposes, it was important to determine: (i) the quantitative potential of the developed method for each individual species, (ii) if the same standard curve could be used for both fish species of interest, as part of an absolute quantification approach and (iii) if the results could be comparable with the previously developed method. When applied to food and feed samples it is very likely that the fish species of origin are unknown, and it is possible that different fish species are mixed in the sample. A reliable quantitative result should be obtained by comparison with a standard curve with comparable efficiency.

Therefore an amplification curve was created for both fish species with 10 fold dilutions of each DNA extract ranging from 0.005 ng to 50 ng. DNA extracts were amplified in triplicates for each dilution and the amplification curve was created in Excel by plotting the logarithm of the DNA concentration against the Cq values.

Efficiency of amplification was estimated for each fish species by means of its calibration curve and determined from the slope of the log-linear portion of the calibration curve as $E = 10^{\frac{1}{\text{slope}}}-1$ (Bustin et al., 2009) and compared among both fish species.

Finally we also checked whether both mackerel and horse mackerel can be quantified by using a single calibration curve obtained from pooling the calibration data from both species. Analysis of variance (ANOVA) was then applied to assess the calibration error when using a single calibration curve for both species. In addition the statistical assessment allowed for partitioning the total calibration error into the within species and between species variation.

### Table 2

<table>
<thead>
<tr>
<th>Characteristics of qPCR calibration curves. Results from 2 days calibration curve for each of the fish species.</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Intercept</td>
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<tr>
<td>Slope</td>
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<td>Efficiency</td>
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<td>R²</td>
</tr>
</tbody>
</table>

### 3. Results and discussion

#### 3.1. QPCR method

Conditions for the developed method (PvAl1) were optimized as previously described. Primers and probe optimized concentrations are shown in Table 1. Once optimized, the method was tested with different animal species and feed ingredients.

#### 3.1.1. Specificity

The specificity of the developed assay was tested using DNA extracted from 15 animal species acquired from a local supermarket including turkey, ostrich, duck, wild boar, crocodile, chicken, beef, lamb, deer, kangaroo, pork, hare, pheasant, partridge and bison, and 9 vegetal matrices commonly used by the feed industry from our sample bank of feed material including manioc, maize, soybean meal, barley, wheat gluten feed, corn gluten feed, rapeseed feed, citrus pulp and beet pulp. The method gave no signal with any of those samples. The method was also tested with other 10 different well characterized fish species acquired from the Department of Fish Quality of the Max Rubner Institute (Hamburg, Germany) including Norway pout, European pilchard, European sprat, haddock, hake, Atlantic herring, Greenland cod, blue whiting, Atlantic cod and poor cod. In all cases the Cq values were above 35, being in many cases much later and even no signal was observed in most of the cases after 40 cycles. Total DNA concentrations from negative samples tested were of 10 ng or more. As previously described, the absence of primer–dimer formation for method PvAl1 was checked by dissociation curve analysis. The results showed one clear single peak in each case corresponding to the mackerel and horse mackerel DNA amplification. A clear difference on the melting temperature was observed between both species when comparing their dissociation curve.

#### 3.1.2. Linearity, sensitivity and efficiency

As mentioned before, a calibration curve was constructed for each of the fish species. 10 fold dilutions of DNA extracts from 0.005 ng to 50 ng from both mackerel and horse mackerel were amplified in triplicates. Intercept, slope, efficiency and coefficient of correlation were calculated in each case as shown in Table 2.

Differences were observed among both fish species in terms of Cq values, and melt curve analysis. Calibration curves from *Trachurus trachurus* presented slightly lower efficiency than *Scomber scombrus* which it is most likely related with internal DNA sequence differences between both fish species.

### Table 3

Results from analysis of variance when a single calibration curve for quantifying the two different fish species.

<table>
<thead>
<tr>
<th>Concentration level (ng)</th>
<th>Within species calculation error</th>
<th>Between species calculation error</th>
<th>Total calibration error</th>
</tr>
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<tbody>
<tr>
<td>0.005</td>
<td>16</td>
<td>42</td>
<td>45</td>
</tr>
<tr>
<td>0.05</td>
<td>6.2</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>0.1</td>
<td>9.9</td>
<td>64</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>50</td>
<td>11</td>
<td>72</td>
<td>73</td>
</tr>
</tbody>
</table>
Table 3 shows the results of the statistical analysis by ANOVA when using a single calibration curve for the quantification of the target DNA from both species. Small values for the relative percentage standard deviation ranging from 6 to 16% were obtained for the intra-species variation, whereas the inter-species variation was higher since the corresponding values varied between 42 and 72%. This trend corresponded well with the results from the former study (Prado et al., 2012) thus confirming the previous conclusion that the use of a single calibration curve for quantification of DNA from different fish species requires further investigations.

Moreover, a sequences search in public databases after the development of the present method allowed us to find a newly added sequence for S. scombrus (FM949426). This sequence was compared with the previously obtained consensus sequence and the developed method as seen in Fig. 2 using Geneious Pro 4.6.1. This alignment shows that there are some small differences on the DNA sequences between Scomber and Trachurus, however the region recognized by the designed primers is identical and just the DNA sequence from S. scombrus presents a single mismatch when compared with the probe. These differences can explain the different efficiency obtained when applying the method to both species and the difference on the melt curve analysis.

These results indicate that the developed method can be used to detect and quantify both mackerel and horse mackerel DNA, with high sensitivity (up to 0.005 ng of DNA). The quantification potential for individual species of the developed method has been demonstrated; however simultaneous quantification of both groups of fish species remains challenging due to differences on PCR efficiencies. Such differences are most likely related with differences on DNA sequences, however the intrinsic variations of the complete process of analysis (sample conditions, DNA extraction/purification, DNA quantification, and qPCR reaction itself) are also an important source of uncertainty that must be taken into account in order to evaluate the final quantitative result (Burns & Valdivia, 2007).

A way to minimize the sources of uncertainty of measurement in the future could be the use of a degenerate base on the designed probe where a sequence difference between both fish species is observed, and/or a further adjustment of the melting temperature to minimize the sequence differences between both groups of fish species to obtain comparable efficiencies of amplification. Concerning other steps of the analysis process, the use of internal positive controls to be added to the sample before the DNA extraction step would contribute to minimize differences on the extraction efficiency.

At the same time, melt curve analysis results show that both species can be differentiated by a simply and costless method. Melt curve analysis is available in many qPCR instrumentation, and its use for authentication of food ingredients has been increasing in the last years, especially with the developments on the use of High Resolution Melting (HRM) analysis by different research groups (Ganopoulos, Argiriou, & Tsafaridis, 2011). However HRM usually requires specific software and reagents and it is still not available for many qPCR instruments, while melt curve is simpler and cheaper, since it can be performed using non saturating dyes such as SYBR Green I commonly used in molecular biology laboratories. This qPCR method can be applied as well to the control of fisheries overexploitation in EU, since both mackerel and horse mackerel are subjected to catch limitation in certain European waters (OJEU, 2007b, 2010).

4. Conclusions

A qPCR method has been developed for the amplification of a fragment of the parvalbumin gene from both mackerel and horse mackerel. The optimized assay shows good values of efficiency, specificity and sensitivity for both fish species. An additional and simple melt curve analysis allows differentiating between both species for authenticity studies. As previously reported, the simultaneous quantification of different groups of fish species by an absolute quantification approach, remains challenging due to differences on PCR efficiencies and the difficulties to use a universal standard calibration curve.

The approach presented here can serve to other research groups working on the development of qPCR methods for multiple species detection, and can be used as an extension of our previously developed method in order to detect a higher number of fish species by combining both methods.

References


M. Prado et al. / Food Control 34 (2013) 19–23

Fig. 2. Alignment of 1) S. scombrus (EMBL Accession number: FM949426); 2) S. japonicus (GenBank Accession number: AB091470.1); 3) T. japonicus (GenBank Accession number: AB211365.1); 4) T. japonicus (GenBank Accession number: AB211366.1); 5) S. japonicus (GenBank Accession number: AB211366.1); 6) consensus sequences from available sequences from Scomber and Trachurus genus; 7) Pval1Fw; 8) Pval1Probe; 9) Pval1Rev (reverse-complement).


