



# Interlaboratory study on real-time PCR detection and quantification of the European anglerfish, pike, and seabream parvalbumin gene

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## Abstract

This study presents a large-scale interlaboratory comparison (ILC) aimed at detecting and quantifying DNA from two European anglerfish (*Lophius budegassa*, *Lophius piscatorius*), pike (*Esox lucius*) and sea bream (*Spondyliosoma cantharus*) using real-time qPCR. To detect amplification of the parvalbumin genetic marker, single and multiplex qPCR assays using EvaGreen® dye or TaqMan™ probes were used. Genomic DNA isolated from target fish species and an advanced DNA calibrator, gBlocks® gene fragments, were used as standards. The DNA of anglerfish, pike and sea bream as well as their mixtures were analysed together with 14 other non-target fish species. All target fish samples were correctly identified by the participating laboratories. Qualitative assessment of anglerfish and seabream DNA showed an accuracy rate of 100%, while pike DNA achieved a match rate of 99%. Validation of quantitative protocols in four different laboratories consistently achieved z-scores below 2, indicating satisfactory performance and confirming the high degree of similarity of laboratory results. Furthermore, high accuracy and efficiency were demonstrated for the quantification of anglerfish and seabream DNA by triplex qPCR using TaqMan™ probes. Regarding the selected gene marker, the major fish allergenic protein parvalbumin enables indirect detection and quantification of the allergen in the sample. Therefore, the use of proposed protocols can significantly contribute to protecting the health of consumers and to controlling the food market.

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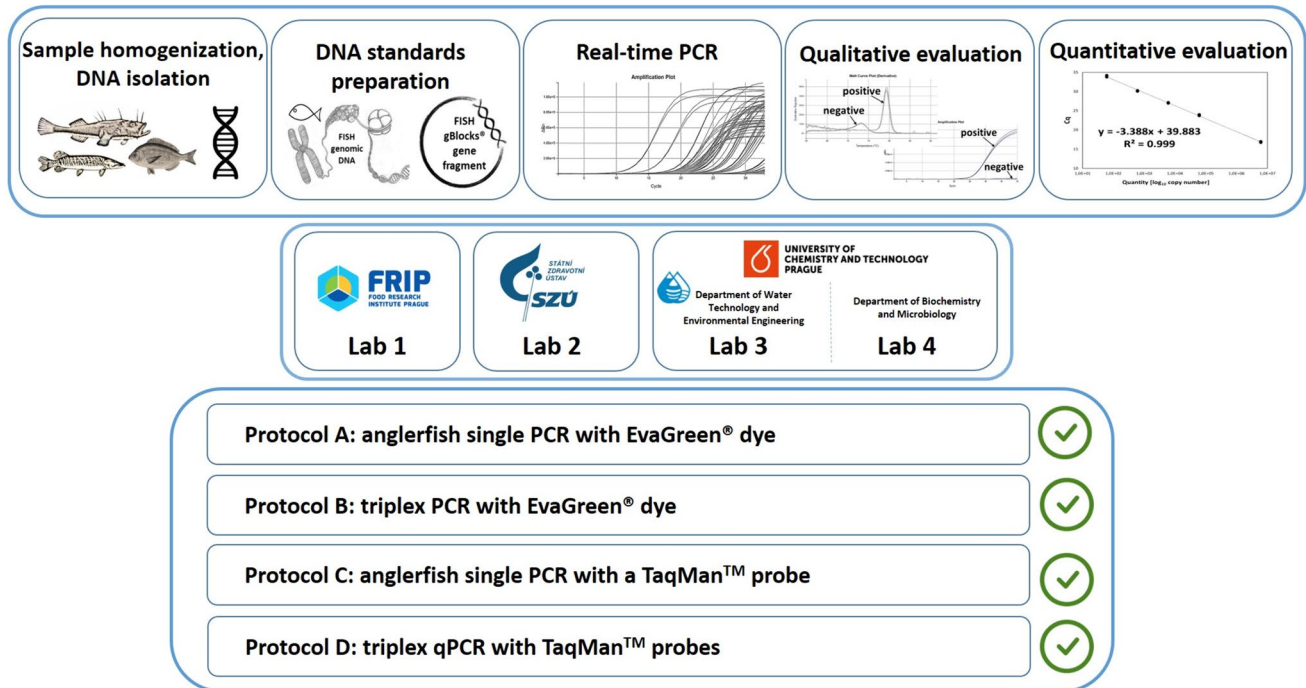
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## Graphical abstract



**Keywords** Parvalbumin · qPCR · *Lophius* · *Esox* · *Spondyliosoma* · DNA calibrator

## Introduction

DNA analyses are frequently used for the identification of fish species due to their ability to enable highly accurate and reliable control of species substitution, thus preventing fraudulent practices in commercial fisheries and consumer health protection. The accuracy and precision of DNA determination are therefore integral to verifying the quality of food control. When species-specific DNA, protocols involving amplification of chosen nucleotide sequences using PCR-based methods are widely utilised. PCR-based species identification typically relies on the analysis of specific identification markers that have unique primary DNA sequences in particular animal species. Among the most commonly used markers are in particular mitochondrial cytochrome oxidase subunit I gene (*cox1*), cytochrome b (*cytb*), or 16S rDNA markers, as well as genomic markers such as beta-actin and parvalbumin [1–3]. The advantage of mtDNA analysis is its high sensitivity due to the large number of mitochondria in cells and its higher stability due to its circular shape, which increases its resistance to fragmentation during food processing and DNA analysis. Also, due to maternal inheritance, there is less variation in mtDNA between populations. The disadvantage of mtDNA is that, unlike nuclear DNA, it cannot be used to quantify

cells, because the number of mitochondria in the cell varies [4–6].

For the identification of fish species, the nuclear coding parvalbumin gene can be advantageously used as a marker for reliable and accurate species identification and quantification. The protein-coding region of the parvalbumin gene contains three introns. In particular, the sufficiently large and species-specific second intron allows efficient identification of fish species [7], despite the immense diversity of fish. On the contrary, the second exon is almost identical in all fish species [7–9], allowing the detection and quantification of fish using universal primers. Additionally, analysis of the parvalbumin gene can be used for food control, as it provides important information on the presence of a specific fish allergen, which is the most serious health risk for fish consumers due to its high allergenicity [2, 11, 12].

The amplification of selected target nucleotide sequences using PCR is considered the gold standard for molecular biology techniques. However, even 40 years after its discovery, PCR amplification continues to improve. This improvement occurs on both the technical side, with the availability of end-point PCR, real-time PCR, or digital PCR, and on enabling a better selection of target nucleotide sequences for the species chosen due to

advances in sequencing methods. Additionally, there has been an increase in the availability and standardisation of DNA/RNA controls, facilitated by the development of nucleotide synthesis methods. The use of an appropriate DNA standard is one of the most important prerequisites for accurate analysis. Currently, three types of standards are most commonly used: (i) genomic DNA, (ii) plasmids with a known target sequence inserted, and (iii) synthetic DNA with a known primary sequence and abundance, such as gBlocks® gene fragments. In our work, we proposed methods for the amplification of the parvalbumin gene using two types of standards, namely genomic DNA and here the synthetic DNA fragment produced by Integrated DNA Technologies (IDT®). Within the ILC, four laboratories were involved in the evaluation of the accuracy of the four methods for the identification and quantification of three commercially important fish species. These were two species of European anglerfish, *Lophius budegassa* and *Lophius piscatorius*, the common pike (*Esox lucius*) and the anglerfish (*Spondyliosoma cantharus*), representative of commercial freshwater and marine fish species. 14 nontarget fish species were used for comparison.

## Material and methods

### Design of the study

First, the protocols for the detection and quantification of anglerfish, pike and seabream DNA were verified through qPCR protocols for ILC described below in detail in the organising laboratory (Lab 4, Testing Laboratory of Department of Biochemistry and Microbiology, TL DBM). Samples of DNA isolates were sent to participating laboratories and analysed as unknown, blind samples labelled with a numerical code.

The ILC included four different qPCR protocols tested, specifically:

A) The detection and quantification of anglerfish DNA using a mastermix with an intercalation dye;

- B) Triplex qPCR for the amplification of anglerfish, pike, and seabream DNA using a mastermix with an intercalation dye;
- C) qPCR with a fluorescently labelled probe complementary to anglerfish DNA;
- D) Triplex qPCR with probes for anglerfish, pike, and seabream DNA amplification.

In this study two types of known standard DNA amount were used: i) genomic DNA isolated from *L. budegassa* and ii) the 637 bp long FISH gBlocks® gene fragment (FISH\_GF) containing the sequence of the amplicons of the primers used in this study (Fig. 1; Table 1). For ILC, a uniform design of a 96-well plate was chosen: a calibration curve prepared by a ten-fold dilution of FISH\_GF was in the first row, a calibration curve prepared by diluting DNA isolated from *L. budegassa* was in the second row, and in the next lines, 23 samples were analysed in triplicate. The qualitative and quantitative parameters of the protocols were evaluated (Table 2). Nineteen samples were prepared by DNA isolation from different fish species, five of them from individuals of the target fish species and 14 from species of nontarget fish species, the remaining four samples were mixtures with different representations of the DNA of the target fish species (Table 3). Standards were shipped together with unknown samples. Guidelines unifying the critical steps of the analytical procedure are attached (Supplementary data 1).

### Participants in the interlaboratory study

Four laboratories from the Czech Republic participated in this study: (i) Food Research Institute Prague, (FRIP, Lab 1); (ii) National Institute of Public Health, Centre for Health, Nutrition and Food, (NIPH, Lab 2); (iii) Laboratory of Department of Water Technology and Environmental Engineering, University of Chemistry and Technology Prague, (UCT, Lab 3) and iv) Testing Laboratory of the Department of Biochemistry and Microbiology, University of Chemistry and Technology (TL DBM, UCT, Lab 4, operator other than the one who performed the ILC preparation).

5'tgcatgatctacgtgctgcacatgcagttacTTTTGGATCCTTGTAACGACGGCCAGT**ACAAC**TTCCCGAGAAGCTTGGTTACCTTGCTCTCTATGA**CAGCTG**CTCGTCTAATACTTACATGATCCAGATACTATAAGACCAATATAATGAATAGAATACTGTGAGAATTTTGAGATTTCTTTAAAGAACTGC**TTTCAA**AGCTCAGAAATCAAATGATATGTT**GCAAA**CTTAACTGTGATGTTGTTTCCGGTTT**GGAATCTAACTCTACTATTG**CGGTTTGAC**TTTCTCC**CTCAAGCCTCTCCAGCTCACAGTTCTAATGGAATGCTTAGAGTGAAGCACTCTAAATAGAGCAAACTACTTTTAAATGTTG**GGGCA**AAAAAGAGCAGAACTTAAAGTCCAAATAGAACAGGAACTGGTGCGTAGTTTGATTCACTCTGCTTGCTCTGTACCCATCCAGGCT**GT**TTTGTGCACTTT**CTAA**ATCTTGCTCGGTGCCTTAGAGGCCATTGCTTGCTCAAGTGTGCATTTCAGAGTGTCTACTTGAGCTCACAGGAAACAGCTATGACCTTGGATCCTTTTcactagctcagattcagtagaccgtgtg 3'

**Fig. 1** Sequence of the FISH gBlocks® gene fragment (FISH\_GF) with marked positions of the target amplicons. Adapters are marked in small font; anglerfish target amplicon is marked in blue font, pike target amplicon in green, and seabream in purple

**Table 1** Sequences of the used oligonucleotide primers and probes target to parvalbumin gene

| Fish species  | Name | Sequence of primer [5'–3']                     | Amplicon size [bp] | References |
|---|------|--|--------------------|------------|
| <i>Lophius budegassa</i> /<br><i>L. piscatorius</i> | L_F  | ACAACCTTTCCCCGAGAAGC                           | 196 bp             | [9]        |
|   | L_R  | ACAACATCACAGTTTAAGTTTTC                        |                    |            |
|   | L_P  | Cy5/FAM: TGCTCTCTATGACAGCTGTCTCGTC: BHQ        |                    |            |
| <i>Esox lucius</i>                                  | E_F  | GGAATCTAACTCCTACTATTGC                         | 223 bp             | This work  |
|   | E_R  | AACAGCCTGGATGGGTAC                             |                    |            |
|   | E_P  | FAM: A[+G]A[+G]C[+A][+G]AA[+C][+T]T[+T]AA: BHQ |                    |            |
| <i>Spondyliosoma cantharus</i>                      | S_F  | TGAGCTGAAGTAAGACACTCAGGA                       | 77 bp              | [13]       |
|   | S_R  | TCTAAAATGTTGTCTTGGTGCCTTAG                     |                    |            |
|   | S_P  | VIC: TGCACACTTGAGCAAGCAATGGCC: BHQ             |                    |            |

**Table 2** Comparison of calibration curve parameters prepared using FISH gBlocks® gene fragment, a synthetic DNA standard designed in this work, or target fish DNAs

|  | Detector channel | Specific amplification of target fish  | Parameters of calibration curves |                |       |                     |                |       |
|--|------------------|--|----------------------------------|----------------|-------|---------------------|----------------|-------|
|  |                  |  | FISH gBlocks® Gene Fragment **   |                |       | Target fish DNA *** |                |       |
|  |                  |  | Slope                            | R <sup>2</sup> | E     | Slope               | R <sup>2</sup> | E     |
| A) Anglerfish single qPCR with EvaGreen® dye | SYBR green       | <i>Lophius budegassa</i> / <i>Lophius piscatorius</i>  | − 3.214                          | 0.997          | 104.7 | − 3.119*            | 0.995          | 109.3 |
| B) Triplex qPCR with EvaGreen® dye           | SYBR green       | <i>Lophius budegassa</i> / <i>Lophius piscatorius</i> ; <i>Esox</i> ; <i>Spondyliosoma cantharus</i> | − 3.313                          | 0.998          | 100.4 | − 3.171*            | 0.997          | 106.7 |
| C) Anglerfish single qPCR with TaqMan™ probe | FAM              | <i>Lophius budegassa</i> / <i>Lophius piscatorius</i>  | − 3.134                          | 0.997          | 108.2 | − 3.271             | 0.995          | 102.2 |
|  | Cy5              | <i>Lophius budegassa</i> / <i>Lophius piscatorius</i>  | − 3.205                          | 0.996          | 105.1 | − 3.342             | 0.997          | 99.2  |
| D) Triplex qPCR with TaqMan™ probes          | Cy5              | <i>Lophius budegassa</i> / <i>Lophius piscatorius</i>  | − 3.388                          | 0.999          | 97.3  | − 3.256*            | 0.994          | 102.8 |
|  | HEX/VIC          | <i>Spondyliosoma cantharus</i>   | − 3.378                          | 0.999          | 97.7  | − 3.476             | 0.998          | 94.0  |
|  | FAM              | <i>Esox lucius</i>   | − 3.381                          | 0.998          | 97.6  | − 3.562             | 0.997          | 91.8  |

R<sup>2</sup> coefficient of determination; E efficiency; \*made by *Lophius budegassa* DNA; \*\*calibration curve was made from at least 5 subsequent decimal dilutions, 2 technical replicates of each dilution were included; \*\*\*calibration curve was made from minimally 4 subsequent 4×dilutions, 3 technical replicates of each dilution were included

## Samples

Seven individuals of the target fish species and 14 other fish species were analysed. The samples were supplied by Bidfood Czech Republic ([www.bidfood.com](http://www.bidfood.com); Table 3). Particular species were previously identified on the label and confirmed by morphological traits by experienced ichthyologists. The species of fish that comprise the panel of negative controls were selected in a way that represents the entire range of the phylogenetic system of fish in an unbiased manner. For sample analysis, 100 g of fish tissue were homogenised using an IKA A10 electric grinder (IKA-Werke, Staufen im Breisgau, Germany), weighed and stored at −20° C until DNA isolation. In addition to DNA isolated from individual fish, mixed samples with various representations

of target fish species were also prepared. These samples were prepared from *L. budegassa*, *E. lucius*, and *S. cantharus* with a DNA concentration of the samples adjusted to 40 ng/μL (fluorometrically, diluted with nuclease-free water) and the appropriate volume of DNA was mixed as shown in Table 3.

## DNA isolation

DNA was isolated from 200 mg of homogenised fish tissue samples using a cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich, MilliporeSigma, USA) method according to EN ISO 21571: 2005 with 650 μl CTAB extraction buffer added to the 200 mg homogenised sample at the beginning of the extraction process.

**Table 3** Summary of results obtained from participating laboratories

| Sample code | Fish species  | Reaction mixture                   |                          |                                  |                               | Agreement between laboratories [%] |
|-------------|---|------------------------------------|--------------------------|----------------------------------|-------------------------------|------------------------------------|
|             |   | A                                  | B                        | C                                | D                             |                                    |
|             |   | Single anglerfish qPCR (EvaGreen®) | Triplex qPCR (EvaGreen®) | Single anglerfish qPCR (TaqMan™) | Triplex qPCR (TaqMan™ probes) |                                    |
| Sa1         | Atlantic cod ( <i>Gadus morhua</i> )                          | No                                 | No                       | No                               | No                            | 100                                |
| Sa2         | European carp ( <i>Cyprinus carpio</i> )                      | No                                 | No                       | No                               | No                            | 100                                |
| Sa3         | Pink salmon/humpback salmon ( <i>Oncorhynchus gorbuscha</i> ) | No                                 | No                       | No                               | No                            | 100                                |
| Sa4         | yellowfin tuna ( <i>Thunnus albacares</i> )                   | No                                 | No                       | No                               | No                            | 100                                |
| Sa5         | Atlantic bluefin tuna ( <i>Thunnus thynnus</i> )              | No                                 | No                       | No                               | No                            | 100                                |
| Sa6         | Chinook salmon ( <i>Oncorhynchus tshawytscha</i> )            | No                                 | No                       | No                               | No                            | 100                                |
| Sa7         | Angler ( <i>Lophius piscatorius</i> ) P1                      | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa8         | Mahi-mahi ( <i>Coryphaena hippurus</i> )                      | No                                 | No                       | No                               | No                            | 100                                |
| Sa9         | Greenland halibut ( <i>Reinhardtius hippoglossoides</i> )     | No                                 | No                       | No                               | No                            | 100                                |
| Sa10        | Atlantic herring ( <i>Clupea harengus</i> )                   | No                                 | No                       | No                               | No                            | 100                                |
| Sa11        | Atlantic wolffish ( <i>Anarhichas lupus</i> )                 | No                                 | No                       | No                               | No                            | 100                                |
| Sa12        | Angler ( <i>Lophius budegassa</i> ) B1                        | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa13        | Black seabream ( <i>Spondyliosoma cantharus</i> )             | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa14        | Northern pike ( <i>Esox lucius</i> ) E1                       | No                                 | Quantified               | No                               | Quantified                    | 100                                |
| Sa15        | Atlantic mackerel ( <i>Scomber scombrus</i> )                 | No                                 | No                       | No                               | No                            | 100                                |
| Sa16        | mixture I: 2:1:7 (Angler B2/ Seabream/Pike E2)                | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa17        | mixture II: 3:1:3 (Angler B2/ Seabream/Pike E2)               | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa18        | mixture III: 1:1:5 (Angler B2/ Seabream/Pike E2)              | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa19        | mixture IV: 1:9:1 (Angler B2/ Seabream/Pike E2)               | Quantified                         | Quantified               | Quantified                       | Quantified*                   | 99                                 |
| Sa20        | Atlantic salmon ( <i>Salmo salar</i> )                        | No                                 | No                       | No                               | No                            | 100                                |
| Sa21        | Angler ( <i>Lophius piscatorius</i> ) P2                      | Quantified                         | Quantified               | Quantified                       | Quantified                    | 100                                |
| Sa22        | Rainbow trout ( <i>Oncorhynchus mykiss</i> )                  | No                                 | No                       | No                               | No                            | 100                                |
| Sa23        | Brook trout ( <i>Salvelinus fontinalis</i> )                  | No                                 | No                       | No                               | No                            | 100                                |

No not detected; \* one laboratories did not detected/quantifies pike DNA in the sample

DNA quality and quantity were checked by electrophoresis on 1% agarose gel (Serva, Heidelberg, Germany) with Midori Green Advance staining (Elisabeth Pharmakon, Croydon, United Kingdom). DNA quality and quantity were determined photometrically using NanoDrop™ One (ThermoFisher Scientific, Waltham, MA, USA) and

fluorometrically by Quantus™ Fluorimetr (Promega, Madison, WI, USA). Isolated DNA was diluted at chosen concentrations with nuclease-free water (Promega; 25 ng/μL and 5 ng/μL) based on the values obtained by the fluorimeter. All DNA samples were coded by numbers (Sa1–Sa23).



## Primers and probes

All primers and probes (FAM and Cy5 labelled, Table 1) were obtained from East Port Prague (Prague, Czech Republic), LNA probe was obtained from GeneriBiotech (Hradec Kralove, Czech Republic). One set of primers/probes was newly designed and others have been previously reported [9, 13]

## Real-time PCR

Two protocols were measured with EvaGreen® dye (reaction mixtures A and B), others with fluorescently labelled probes (C and D). Single and triplex PCRs were performed; each plate has its calibration curves. Real-time PCR was carried out on four different platforms, two machines did not have channel for Cy<sup>TM</sup>5/5.5 fluoro for (StepOne Plus<sup>TM</sup> and ABI 7900HT Fast, Lab 1 and Lab 2, respectively), while other two had (Bio-Rad CFX96 Touch and QuantStudio<sup>TM</sup> 5; Lab 3 and Lab 4, respectively). Therefore, for ILC three plates were run in laboratories 1 and 2 and four were measured in laboratories 3 and 4.

Protocols A and B were as follows: 4 µl 5×HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia), which ROX Reference Dye as a passive reference, primers at a final concentration of 0.2 mmol/L, 4 µl of template DNA and nuclease-free water, the total reaction volume 20 µl. The initial denaturation and activation of polymerase at 95 °C for 12 min, 35 cycles with denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and polymerisation at 72 °C for 30 s, followed by measurement of melting curve measurement;

Protocols C and D were as follows: 10 µl of 2×GoTaq® Probe qPCR Master Mix (Promega, Madison, Wisconsin, USA), primers at a final concentration of 0.4 mmol/L, 0.25 mmol/L probe(s), 4 µl of template DNA and nuclease-free water, the total reaction volume 20 µl. For initial denaturation and polymerase activation of the probe at 95 °C for 2 min, 35 cycles with denaturation at 95 °C for 15 s, and annealing with polymerisation at 60 °C for 60 s. Fluorescence was measured as relative fluorescence units (RFU) and plotted as a baseline-corrected normalised reporter ( $\Delta R_n$ ), i.e., the magnitude of the normalised fluorescence signal from which the normalised signal of the baseline was subtracted.

In the experiments, two calibration curves were used. The first was FISH\_GF (Fig. 1) prepared from IDT® (Integrated DNA Technologies®, Coralville, Iowa, USA) and the second was DNA isolated from *L. budegassa*. The calibration curves of FISH\_GF were measured in technical duplicates and included six concentration points prepared as serial dilutions with 1.531E + 06, 1.531E + 05, 1.531E + 04, 1.531E + 03, 1.531E + 02 and 1.53E + 01 copies in 1 µl for a single target. *L. budegassa*

DNA calibration curves were constructed from four concentration points prepared as serial dilutions of target DNA with 2.06E + 4, 5.16E + 3, 1.29E + 3 and 3.22E + 2 copies in 1 µL, technical triplicates and fish species samples were used. For the quantification of unknown samples, the values obtained by subtraction from the calibration curves of the synthetic dsDNA standard were used and statistically evaluated. Instructions, samples, and chemicals sent to laboratories are listed in the supplementary file.

The determination of the limit of quantification (LOQ) and the limit of detection (LOD) was performed by FISH\_GF at Lab 4. The LOQ of the qPCR was 18 copies, the LOD was estimated by LOD<sub>6</sub> [14]; 6 copies of the target sequence in single PCR and 10 copies in triplex.

## Data analysis

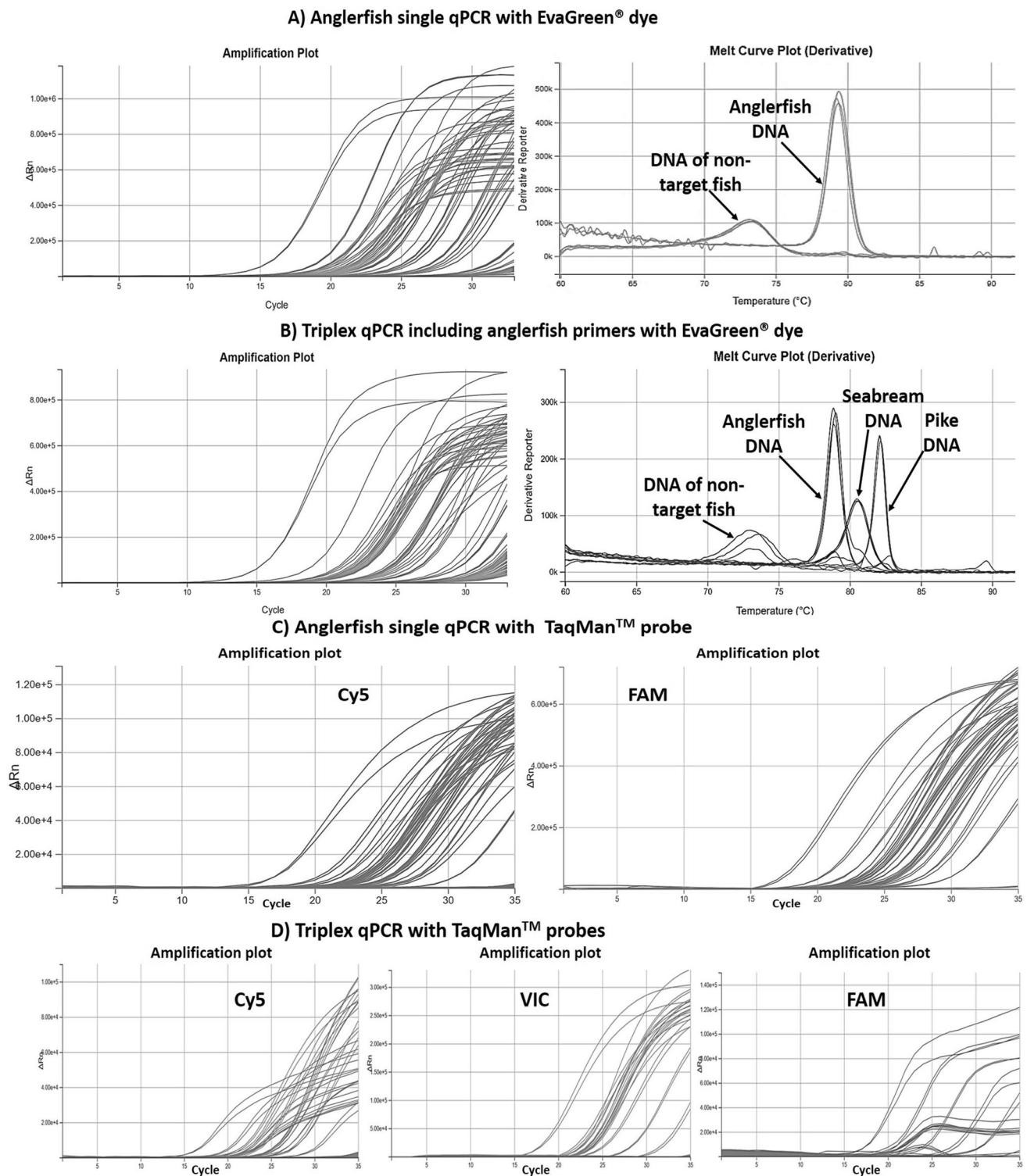
Real-time qPCR runs were analysed by Design & Analysis 2.6.0 except plates run on the Bio-Rad machine, these runs were analysed by CFX Manager<sup>TM</sup> Software 3.1. The product-moment correlation after Pearson was used to determine the degree of variation between the quantifications performed for each laboratory. This analysis was done in R using the function cor() from the package “stats” (R Core Team 2020).

The calculation of the z-score was performed according to Eq. 1, where  $x_i$  is the measured value;  $x^*$  is the median of the measured values and  $\sigma$  is the standard deviation of the measured values. The Z-score was calculated within each tested protocol itself, as well as for quantification, obtained by all tested protocols. The evaluation of z-score was as follows:  $|z| \leq 2$  satisfactory,  $2 < |z| < 3$  questionable and  $|z| \geq 3$  unsatisfactory.

$$z = |x_i - x^*| / \sigma \quad (1)$$

## Results

In our ILC study, the qPCR protocols for the detection and quantification of fish, namely European anglerfish, pike and seabream, were systematically compared. The protocols included single- and multiplexed configurations, using analysis of fluorescence emitted during qPCR by an intercalating dye (EvaGreen®) or fluorescently labelled TaqMan<sup>TM</sup> hybridisation probes. The qPCRs were verified in Lab 4 before DNA samples and reagents were sent for ILC. Examples of amplification and melting curves are shown in Fig. 2, while a summary of the quantitative parameters derived from the calibration curves is given in Table 2. Methodology for



**Fig. 2** Amplification and melting curves for qPCR targeting the second intron of *β-pvalb* gene obtained during the preparation of the interlaboratory study in the QuantStudio™ 5 cycler

verification procedures, including quantification, amplification efficiency (E), coefficient of determination ( $R^2$ ), repeatability and specificity, followed the criteria outlined in the

JRC Technical Report on Verification of Analytical Methods for GMO Testing [15].

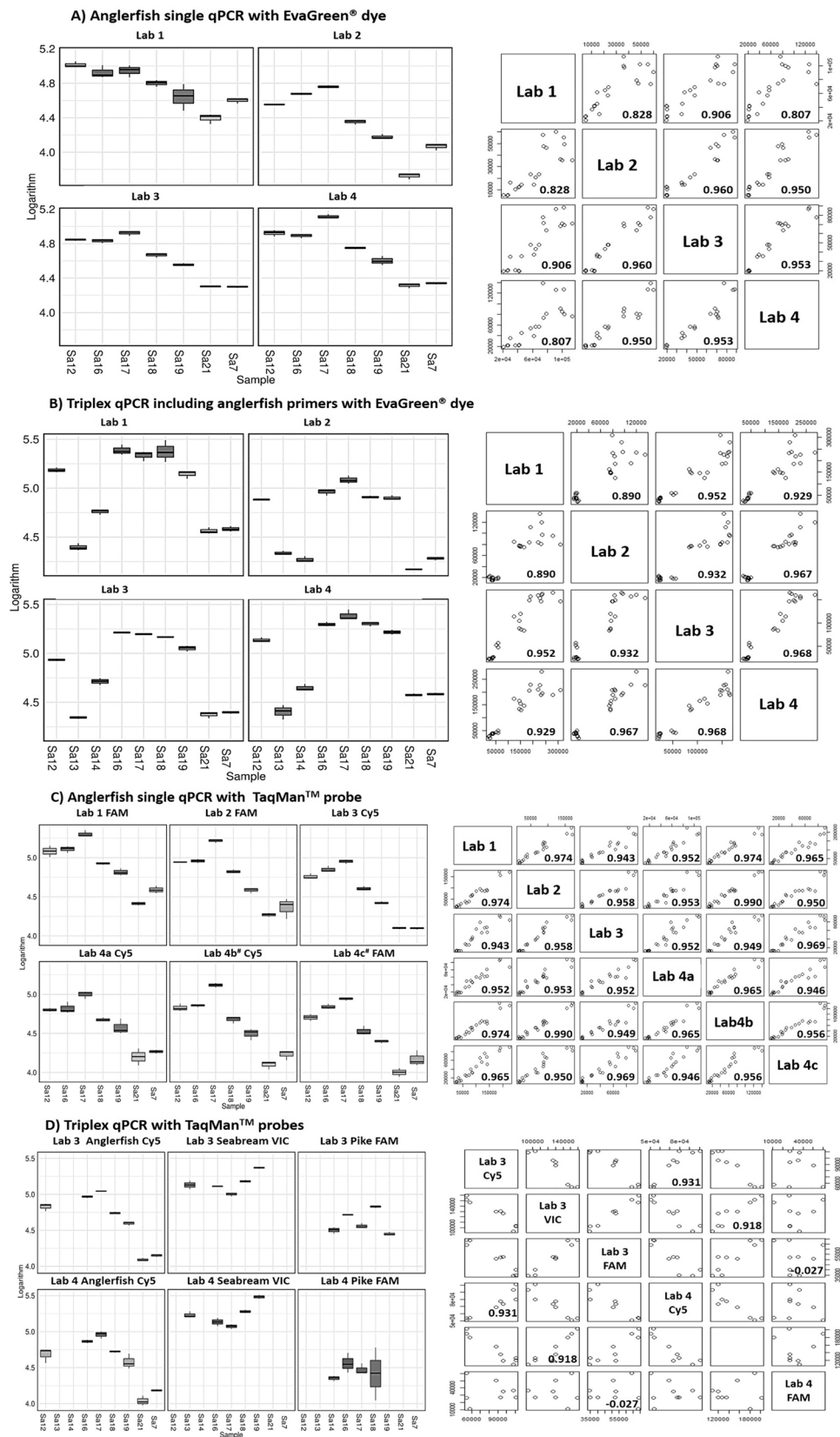
**Table 4** Comparison of the parameters of the calibrations curve obtained from the participants' laboratories

| Reaction mixture                          | Lab number         | Detector channel | Specific amplification of target fish                           | Tm of anglerfish | Tm of seabream | Tm of pike | Quantification of target DNA | Parameters of calibration curves |                |       |                    |                |       |       |
|---|--------------------|------------------|---|------------------|----------------|------------|------------------------------|----------------------------------|----------------|-------|--------------------|----------------|-------|-------|
|   |                    |                  |   |                  |                |            |                              | FISH                             |                |       | Target fish DNA*** |                |       |       |
|   |                    |                  |   |                  |                |            |                              | Slope                            | R <sup>2</sup> | E     | Slope              | R <sup>2</sup> | E     |       |
| A) Anglerfish single qPCR (EvaGreen®)     | Lab 1              | SYBR green       | <i>L. budegassa/L. piscatorius</i>                              | 78.3–78.6        | N              | N          | 0.9±0.6                      | 0.6±0.6                          | –3.339         | 0.999 | 99.3               | –3.216         | 0.994 | 104.6 |
|   | Lab 2              | SYBR green       |   | 79.0–79.6        | N              | N          | 1.5±0.3                      | 0.9±0.3                          | –3.107         | 0.999 | 109.8              | –3.127         | 0.997 | 108.9 |
|   | Lab 3              | SYBR green       |   | 78               | N              | N          | 0.1±0.1                      | 0.2±0.1                          | –3.418         | 1.000 | 96.2               | –3.198         | 0.997 | 105.5 |
|   | Lab 4              | SYBR green       |   | 79.1–79.3        | N              | N          | 0.5±0.4                      | 0.2±0.1                          | –3.337         | 0.996 | 99.4               | –3.381         | 0.997 | 97.6  |
| B) Triplex qPCR (EvaGreen®)               | Lab 1              | SYBR green       | <i>L. budegassa/ L. piscatorius; Exox lucius; Sp. cantharus</i> | 78.4–78.6        | 79.9–80.1      | 81.6–81.7  | 0.9±0.2                      | 2.4±0.4                          | –3.380         | 0.999 | 97.6               | –3.439         | 0.998 | 95.6  |
|   | Lab 2              | SYBR green       |   | 78.6–78.8        | 80.6–80.7      | 82.1–82.2  | 1.3±0.2                      | 0.3±0.3                          | –3.159         | 0.999 | 107.3              | –3.244         | 0.997 | 103.3 |
|   | Lab 3              | SYBR green       |   | 78               | 79.5           | 81         | 0.4±0.2                      | 1.0±0.5                          | –3.436         | 0.999 | 95.5               | –3.205         | 0.999 | 105.1 |
|   | Lab 4              | SYBR green       |   | 78.7–79.0        | 80.4–80.5      | 81.9–82.1  | 0.8±0.3                      | 2.2±0.3                          | –3.313         | 0.998 | 100.4              | –3.171         | 0.998 | 106.7 |
| C) Anglerfish single qPCR (TaqMan™ probe) | Lab 1              | FAM              | <i>L. budegassa/ L. piscatorius</i>                             | N                | N              | N          | 2.0±0.1                      | 1.1±0.5                          | –3.531         | 0.999 | 92.2               | –3.560         | 0.998 | 91.0  |
|   | Lab 2              | FAM              |   | N                | N              | N          | 0.9±0.2                      | 0.4±0.4                          | –3.174         | 0.998 | 106.6              | –3.122         | 0.988 | 109.1 |
|   | Lab 4 <sup>#</sup> | FAM              |   | N                | N              | N          | 0.6±0.2                      | 0.5±0.2                          | –3.247         | 0.999 | 103.2              | –3.271         | 0.995 | 102.2 |
|   | Lab 3              | Cy5              |   | N                | N              | N          | 0.4±0.2                      | 0.4±0.2                          | –3.389         | 0.999 | 97.4               | –3.375         | 0.999 | 97.8  |
| D) Triplex qPCR (TaqMan™ probes)          | Lab 4              | Cy5              |   | N                | N              | N          | 0.2±0.2                      | 0.2±0.1                          | –3.415         | 0.998 | 96.3               | –3.283         | 0.986 | 101.6 |
|   | Lab 4 <sup>#</sup> | Cy5              |   | N                | N              | N          | 0.1±0.1                      | 0.2±0.2                          | –3.305         | 0.997 | 100.7              | –3.374         | 0.993 | 97.9  |
|   | Lab 3              | Cy5              | <i>L. budegassa/ L. piscatorius</i>                             | N                | N              | N          | 0.5±0.4                      | 0.2±0.3                          | –3.346         | 0.999 | 99.0               | –3.454         | 0.997 | 94.8  |
|   | Lab 3              | HEX              | <i>Sp. cantharus</i>  | N                | N              | N          | 0.6±0.4                      | N                                | –3.366         | 0.999 | 98.2               | N              | N     | N     |
|   | Lab 3              | FAM              | <i>Exox lucius</i>  | N                | N              | N          | 0.4±0.4                      | N                                | –3.378         | 0.999 | 97.7               | N              | N     | N     |
|   | Lab 4              | Cy5              | <i>L. budegassa/ L. piscatorius</i>                             | N                | N              | N          | 0.6±0.3                      | 0.4±0.3                          | –3.321         | 0.991 | 100.0              | –3.263         | 0.995 | 102.5 |
|   | Lab 4              | HEX              | <i>Sp. cantharus</i>  | N                | N              | N          | 0.8±0.4                      | N                                | –3.242         | 0.995 | 103.5              | –3.476         | 0.998 | 94.0  |
|   | Lab 4              | FAM              | <i>Exox lucius</i>  | N                | N              | N          | 1.3±0.4                      | N                                | –3.374         | 0.990 | 97.9               | –3.559         | 0.997 | 91.0  |

Tm melting temperature; \*HRM software was not used; The mean z-score values and their standard deviation obtained from quantification of positive samples containing target DNA are shown; 1: calculation from the quantification with a specific protocol A, B, C or D; 2: calculation from the quantification by all protocols; R<sup>2</sup> coefficient of determination; E: efficiency; \*\* calibration curve was made from at least 5 subsequent decimal dilutions, 2 technical replicates of each dilution were included; \*\*\*: calibration curve was made from minimally 4 subsequent 4 × dilutions, 3 technical replicates of each dilution were included; # experiment performed by another worker in Lab 4; N not determined



**Fig. 3** Overview of the quantification of samples containing angelfish, pike, seabream, and their mixture DNA. On the left, the results of the quantification of positive samples are summarised, and on the right the correlation of the quantification carried out by the Pearson product-moment correlation



The qPCR results of the participating laboratories are summarised in Tables 3 and 4 and Fig. 3. Sample values within the concentration range of the calibration curve points were considered positive. Samples containing non-target DNA were negative in all laboratories for all protocols tested (Table 3). Samples containing anglerfish DNA (7 samples: Sa7, Sa12, Sa16, Sa17, Sa18, Sa19 and Sa21) were detected and quantified, as were samples containing seabream DNA (5 samples: Sa13, Sa16, Sa17, Sa18 and Sa19). The results of the samples containing pike DNA (5 samples: Sa14, Sa16, Sa17, Sa18 and Sa19) were analysed by two laboratories equipped with instruments capable of detecting three channels at the same time (FAM, VIC, and Cy5) using a triplex qPCR protocol with TaqMan™ probes. In the case of these samples, identical results were obtained for 4 of them. The fifth Sa19 was positive only in Lab 3 and negative in Lab 4. This sample contained a mixture of anglerfish, seabream, and pike, with most of the DNA from seabream compared to a much lower abundance of anglerfish and target pike DNA.

First, the quantitative data from the calibration curves were compared (Table 4). The amplification efficiencies of all tested qPCR protocols in all laboratories reached values of 90–110%, i.e., the slopes of the calibration curves were in the range of  $-3.6 \leq \text{slope} \leq -3.1$ . The  $R^2$  coefficients varied between 0.986 and 1.000 and were found to be highly linear. Only DNA isolated from the target fish (anglerfish, seabream, and pike) showed a positive signal. Quantification results were statistically evaluated by z-score (Table 4), and Pearson's product-moment correlation was used to assess the degree of variability in quantifications performed by each laboratory (Fig. 3). The results show that the calibration curves obtained from all laboratories participating in this interlaboratory comparison met the criteria of the JRC Technical report [15].

Protocol A, anglerfish single qPCR with EvaGreen® dye, was qualitatively evaluated according to the melting curve values of the amplicons, while the amplicons obtained by multiplying anglerfish DNA had melting curve values of  $78.8 \pm 0.8$  °C (Table 4, depending on the instrument used). In our study, 35 cycles were used in each qPCR. The non-target fish also showed fluorescence emission, and  $T_m$  values of such unspecific product/double primers were in the range of 72–73 °C, i.e., easily distinguishable from the  $T_m$  values of the target anglerfish amplicons. This distinction was achieved in samples isolated from the tissues of individual fish, as well as from DNA mixtures containing the representation of several species of fish. Furthermore, the differences between the  $C_t$  values of positive and negative samples of the same DNA concentration (fluorometric measurement) were higher than 6.5 cycles for all laboratories. Theoretically a difference in  $C_t$  between samples with a value of 6.6

corresponds to two orders lower amount of target DNA [15]. The efficiencies of the calibration curves were in the range of 96.2 to 109.8 percent, the linearity was higher than 0.99 and the z-score of samples containing anglerfish DNA was lower than 2 for all laboratories. Pearson's product-moment correlation exceeded 0.81, indicating a high correlation among the results obtained by applying this protocol in various participating laboratories (Fig. 3).

Protocol B, triplex qPCR with EvaGreen® dye, was qualitatively evaluated according to melting curve values; amplicons had melting curve values of  $78.5 \pm 0.5$  °C, seabream DNA  $80.1 \pm 0.6$  °C and pike DNA  $81.6 \pm 0.6$  °C (Table 4, depending on the instrument used) after analysing samples that have been taken out of individual fish tissues. As in protocol A, using nonspecific fluorescence emission due to intercalation of EvaGreen® dye into all dsDNA in the sample, protocol B also showed fluorescence emission of non-target fish of various  $T_m$  values. The  $T_m$  values of non-target fish were higher than 83 °C, that is, different from the  $T_m$  values of the target fish species (Table 4). Furthermore, the  $C_q$  values of non-target fish were higher than 30 except for DNA isolated from Atlantic salmon (*Salmo salar*), when the  $C_q$  values were lower, but always reached values higher than 27.5. Therefore, the difference between the  $C_q$  of positive and negative samples was greater than 5 cycles in all laboratories. Unfortunately, the analyses of fish DNA mixtures did not allow the differentiation of individual target species based on the melting curve. In mixtures with different representations of target fish species, a wider  $T_m$  peak prevailed, and it was not possible to determine the representation of individual species. In such cases, we recommend using the more expensive triplex qPCR with fluorescently labelled probes that enable the identification of species in a mixture. The efficiencies of the calibration curves ranged from 95.5 to 107.3, the linearity was greater than 0.99. The z-score based on the quantification of positive samples containing anglerfish DNA performed according to protocol B was less than 2 for all laboratories, the z-score based on quantification by all tested protocols was less than 2 for two laboratories and between 2 and 3 for other two. The Pearson product-moment correlation exceeded 0.89, indicating a strong correlation in the results obtained in various participating laboratories (Fig. 3).

Protocol C, anglerfish single qPCR with a TaqMan™ probe, was evaluated using the software based on the presence or absence of an amplification curve. Only those samples containing anglerfish DNA were detected and quantified by all laboratories using this protocol. The efficiencies of the calibration curves were in the range of 91.0 to 109.1 percent, the linearity was higher than 0.99 and the z-score of the samples containing anglerfish DNA was equal to or lower than

2 for all laboratories. Three laboratories performed analyses with a probe labelled with FAM fluorophore and two with a probe labelled with Cy5 at their 5' end; in both cases, there was a quencher at the 3' end of the probes; the results obtained with various fluorophores were similar. Pearson's product-moment correlation exceeded 0.94, the highest of the different protocols tested in this work, indicating a strong correlation among the results obtained by applying this protocol to various participating laboratories (Fig. 3).

Protocol D, triplex qPCR with TaqMan<sup>TM</sup> probes, was evaluated using the software used based on the presence or absence of an amplification curve in two laboratories. By this protocol, samples containing anglerfish and seabream DNA were detected and quantified in both laboratories. The five samples containing pike DNA were detected and quantified only in Lab 3, while one sample of pike DNA was not detected in Lab 4. The efficiencies of the calibration curves were in the range of 91.0 to 103.5%, the linearity was higher than 0.99 and the z-score of positive samples was lower than 2. The Pearson product-moment correlation for both anglerfish and seabream quantification exceeded 0.92, highlighting a robust correlation. However, for pike quantification, the correlation was near zero at  $-0.03$ , indicating a lack of association between the quantitative results obtained from the two laboratories (Fig. 3).

In summary, the ILC of the protocols confirmed their suitability for the detection and precise quantification of anglerfish and seabream DNA, including transferability between laboratories. On the contrary, the detection of pike DNA was not 100% successful, showing a low correlation of quantification between the laboratories (Fig. 3), and should be further optimised.

## Discussion

Since its discovery, PCR has been used for a variety of analyses, including verifying food authenticity and revealing adulteration, because it allows qualitative and quantitative approaches [2, 16, 17]. Qualitative evaluation enables proof of the presence of the selected target DNA sequence and its evaluation according to the length of the amplicon after electrophoresis, by the analysis of melting curves when using fluorescent intercalation dyes such as SYBR<sup>TM</sup> Green, EvaGreen®, etc. or amplicon sequencing. Quantification is then based on the correlation between the target number of copies and the threshold cycle number, and it can be either absolute or relative. Absolute quantification identifies the amount of input gene based on a standard curve. On the contrary, relative quantification determines changes relative to a reference [18–20]. One of the most commonly used techniques for quantifying DNA in the fields of environmental and food control is absolute quantification

using the qPCR standard curve approach [2, 9, 21]. If multiplex reactions are used, several target species can be identified or quantified in a single amplification reaction, allowing expenses and time reduction [17, 22, 23].

Different types of DNA are used as PCR standards for quantification, whereas DNA isolated directly from the target organism is the most commonly used [13, 24, 25]. PCR products and plasmids containing target DNA fragments are also used [9, 26–28]. Recently, techniques facilitating the precise synthesis of longer dsDNA sequences have also led to the use of standards such as the gBlocks® gene fragment [29, 30].

In the case of using DNA isolated from the target organism, a less precise quantification may be a drawback, particularly if spectrophotometric DNA concentration is the only method employed for nucleic acid quantification. However, isolated DNA may contain inhibitors of subsequent amplification reactions, which can negatively affect qPCR parameters (efficiency, LOD, or LOQ).

When using PCR products, either with or without purification, as standards in molecular biology methods, their utility can be limited by degradation during storage, resulting in a change in their copy number. To ensure more accurate quantitative results, it is recommended to clone PCR products in plasmids [28]. Plasmid calibrators (pDNA) are typically prepared through cloning, often into production strains of *E. coli* bacteria, resulting in a genetically modified organism (GMO) that may require special authorisation in certain countries. On the contrary, the use of gBlocks® standards is straightforward, user-friendly, and facilitates the development of qPCR, particularly when the analytical technique involves analysing multiple targets [30]. Additionally, both plasmid calibrators and gBlocks® standards have demonstrated high storage stability [9, 23, 31].

In our investigation, FISH\_GF and genomic DNA extracted from each of the target fish species were used. Due to its substantial popularity and high consumer demand, *L. budegassa* was chosen as the reference species [25, 32]. The calibration curves generated from both genomic DNA from *L. budegassa* and the synthetic DNA standard FISH\_GF met the criteria of the JRC technical report on the validation of analytical methods for GMO testing [15]. However, working with the FISH-GF standard versus genomic DNA was simpler and its utilisation also brings an advantage in saving financial and time costs.

Moreover, use of synthetic DNA fragments has several other advantages. They are designed to have specified sequences and lengths and hence offer a consistent reference for quantification. Second, they are not variable. Due to many factors such as the source of the sample, nucleic acid degradation that occurs during food preparation technologies, or the nucleic acid isolation technique

itself, the quality and purity of genomic DNA recovered from biological materials might vary. Third, there are no impurities present in synthetic DNA fragments, such as RNA, proteins, or other cellular remains that could obstruct measurement techniques. In our study, the synthetic DNA fragments assembled with multiple target amplicons for different PCRs were used. Hence it was easily used as a single standard for several single or multiplex methods. Essentially, reference material in the form of DNA or RNA can be designed and synthesised on the second day following the sequencing of the target organism. The artificial synthetic RNA positive control used in the detection and quantification of SARS-CoV-2 (EURM-019, JRC, 2020) is an example of one such standard.

On the other hand, genomic DNA allows the quantification of any genes or specific genetic regions of the target organism. This broader scope enables comparative analyses between different species of fish, a feature particularly valuable for biodiversity research and species conservation efforts.

### Target sequences for fish PCR detection – parvalbumin beta gene

In this investigation, we focused on identifying and measuring the  $\beta$ -parvalbumin gene in particular fish species. Parvalbumin is a significant fish allergen that is encoded in genomic DNA. It is composed of a small acidic protein with a molecular weight of approximately 12 kDa that is made up of 108–109 amino acid residues [3, 7, 33, 34]. The use of qPCR protocols to amplify genes associated with allergens is of considerable importance in the context of monitoring allergenic proteins within food products. It plays a pivotal role in ensuring accurate food labelling and thus protecting fish-allergic consumers. Detecting fish genes that encode parvalbumin can be used to demonstrate the presence of fish in the sample [8, 12, 35, 36]. However, for more precise identification of genera or fish species, after PCR amplification of the sequencing in DNA barcoding, a restrictive analysis in single-strand conformation polymorphism (SSCP) or capillary electrophoresis is needed. These analyses are most often performed for mitochondrial genes. Because of its faster rate of evolution, which guarantees a higher degree of interspecific variability that is highly helpful for differentiating between phylogenetically close species, mtDNA is frequently selected as a DNA marker for identification/species discrimination of chosen species. Additionally, because mtDNA is more abundant in cells than nuclear DNA, techniques involving it can detect it in more technologically processed products and are more sensitive. However, it is an inappropriate DNA marker for target DNA measurement due to an unknown number of copies in the cell. For example, multiplex end-point PCR

and real-time PCR with melting curve post-amplification analysis for the identification of the anglerfish (*Lophius* spp.) targeted mitochondrial *cytb* gene were tested in the work of Castigliego et al. [37]. They analysed all 7 species belonging to *Lophius* genus, i.e., *L. budegassa*, *L. piscatorius*, *L. vomerinus*, *L. vaillanti*, *L. americanus*, *L. gastrophysus* and *L. litulon*, and other fish species collected directly from the market, which are frequently sold as fillets (e.g. *Sparus aurata*, *Reinhardtius hippoglossoides* or *Oncorhynchus mykiss*). These mitochondrial-targeted multiplexes did not perform quantification.

Due to its relatively high mutation rate, mtDNA analyses can provide assays that distinguish closely related species with a low detection limit. Unfortunately, mtDNA can be present in up to thousands of copies in a cell, while genomic DNA is present in two copies in most animal somatic cells. Furthermore, the quantity of it varies substantially among tissues [2, 11, 38]. On the other hand, genomic DNA serves as a suitable target for both detection and quantification. Specifically, direct use of the parvalbumin gene amplification for the detection of *Clupea harengus* and *Clupea pallasii* [39], *Spondyliosoma cantharus* [13], *Scomber japonicus* [40] has been used.

Species belonging to the Sparidae family were identified by DNA and protein analysis at the Schiefenhövel and Rehbein work [41], where the distinguishing between various species including *Spondyliosoma cantharus*, *Sparus aurata*, *Acanthopagrus bifasciatus*, *Boops boops*, *Argyrops spinifer*, *Lithognathus mormyrus* and *Pagellus bogaraveo* of the family Sparidae were done by sequencing of amplicons after PCR of the mitochondrial *cytb* gene, PCR followed by single-strand conformation polymorphism analysis (SSCP) and isoelectric focussing (IEF) of water-soluble proteins of fish fillet. A total of 263 *Spondyliosoma cantharus* sequences, both mitochondrial DNA (*cytb*) and nuclear DNA (S7), were qualitatively analysed in the work of Neves et al. [42] for analysis of population structure in the East Atlantic and Mediterranean Sea. Quantification of *Spondyliosoma cantharus* DNA was carried out in our previous work, where an interlaboratory study of five laboratories tested real-time PCR with the TaqMan™ probe [13].

There are a limited number of published works on the identification of pike between species. On the other hand, previous research has used mitochondrial genes to analyse intraspecific variations and monitor pike migration [43, 44]. The use of the parvalbumin gene for the identification of fish species, in addition to its potential to indirectly detect allergens in the sample, represents a highly usable methodology.

Quantification of parvalbumin in selected fish species was previously used to quantify anglerfish and seabream DNA content Mukherjee et al. [10]. For European anglerfish, the intercalation dye assay was employed [25] which was



enhanced by incorporating a fluorescently labelled probe and plasmid standard as calibrators in subsequent research. In the work conducted by Akhatova et al. [13], a TaqMan™ probe detection and quantification of black seabream was implemented and widely accepted as an approach to controlling inhibition, which involved spiking negative samples with DNA from the target species. In this study, the interlaboratory transferability of qPCR protocols that amplified the nuclear marker parvalbumin was verified. In addition, synthetic fragments were successfully used to extend the DNA standards commonly used in published PCR protocols for fish detection (i.e., genomic DNA and plasmids).

## Conclusions

This work verified the effectiveness of the proposed real-time PCR assays and demonstrated their reliability in accurately identifying European anglerfish, seabream and pike, among several other fish species. The performance of the single and multiplexed qPCR protocols was thoroughly evaluated. The use of fluorescence-labelled probes, compared to protocols using an intercalation dye for fluorescence emission, demonstrated better protocol transferability between laboratories using different qPCR cyclers for the quantification of European anglerfish and seabream.

Two standards were successfully used for calibration curves, target species genomic DNA and artificial synthetic DNA consisting of three different fish genomic DNA sequences, proposed in this study. The use of synthetic DNA is proving to be a simple and cost-effective alternative for both qualitative and quantitative analysis of fish. Therefore, our results support the growing recognition of synthetic DNA fragments as a valuable tool to facilitate molecular biology analysis.

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**Data availability** Data will be made available on request.

## Declarations

**Conflict of interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Compliance with ethics requirements** All applicable international, national, and institutional guidelines for the use of animals were followed. The study was conducted following the local legislation and institutional requirements.

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