International interlaboratory study on TaqMan real-time polymerase chain reaction authentication of black seabream (*Spondyliosoma cantharus*)

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Summary

An interlaboratory study of five laboratories testing real-time polymerase chain reaction (PCR) with TaqMan probe detection of black seabream (*Spondyliosoma cantharus*) fish is presented in this work. The method is oriented to the intron of the nuclear gene encoding the main protein of the musculature of the fish, parvalbumin. This feature distinguishes the employed approach from those based on mitochondrial genes. Here, the intron is flanked by exon stretches highly conserved among species. This provides a unique advantage when a new fish species emerges as a commodity on the market: Species-versatile degenerate primers can be easily designed on these conserved exon stretches. Therefore, the initial uncertainty of the species-specific sequence of the intron can, in such case, be bypassed during the adoption of the method to this particular new species, because the amplicon obtained at this pilot stage provides the sequence of the intron itself. DNA isolates from eight exemplars of *S. cantharus* and from nineteen other fish species, the latter being used as negative controls, were tested in this study by participating laboratories. The readouts in qualitative assessment were 100% accurate. The quantitative results provided an average value and variation among samples representing particular exemplars of *S. cantharus*.

Keywords

real-time polymerase chain reaction; TaqMan; *Spondyliosoma cantharus*; fish; species identification; interlaboratory study

Fish represents an important component of the diet in highly developed countries, although it is not a staple in these societies except for some coastal areas. However, mainly due to the health benefits of the consumption of fish compared to other meat, its role as a component of the diet is highly important and a tendency towards the increased consumption of fish meat can be viewed as positive.

In this context, it is becoming critical to control the fish market of developed countries, also regarding the proper labelling according to the zoological origin of the traded fish. This is important economically, as mislabelling can result from the fraudulent substitution of species of high value with some less expensive fish. Proper labelling is also important in terms of the impact on health as fish parvalbumin can trigger allergic reactions

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in sensitive consumers [1–4]. The severity of the reaction varies, according to some reports, for each individual patient based on the particular species of fish [1, 5–7]. Therefore, it is critical to have tools available for determination of fish species, which would be versatile enough to respond, in a relatively short time, to the emergence of new fish species as a commodity on the market.

In the first instance, the species can be determined based on morphological traits [8]. Basically, this approach is the simplest, and also the easiest as well as most straightforward. Nevertheless, for routine and large-scale testing of species identity of traded fish within the worldwide market, it is basically useless for a number of reasons: 1. It provides, in fact, no possibility for automation, 2. there is a minimum chance for harmonization, 3. fish traded and imported, for instance, to landlocked countries are often compressed and frozen into large blocks suitable for handling, 4. to be able to fully cope with the issue of species determination, detection of processed and/or heat treated fish meat in complex food or served meals is required to be feasible.

To address these issues, establishment of a laboratory detection method, based on the previous morphological approach employed in the development of such a tool, seems to ideally fulfill this need.

For example, there exists a variety of protein-based analytical methods for the determination of fish species, such as protein electrophoresis [9, 10], two-dimensional electrophoresis (2-DE) [11, 12], isoelectric focusing [13, 14], urea isoelectric focusing [10] or capillary electrophoresis [15]. Immunological methods complement them [16, 17]. Other approaches for determination of the species origin of fish meat are based on chemometry [18–20]. Mass spectrometry can also be successfully employed to reach this goal [11, 21, 22].

Another approach to determination of biological characteristics of analysed food is represented by methods from the molecular biology domain. These include methods based on polymerase chain reaction (PCR) applied to determination of the taxonomic identity of plant [23–29] or animal food sources [7, 8, 30]. Various combinations of all of the aforementioned approaches also appear as detection tools in use. Restriction fragment length polymorphism (RFLP) is also widely used as the further development of PCR-based methods [31–36]. Single strand conformation polymorphism (SSCP) can also be used as the final stage of these PCR-based methods [33, 37].

Another concept relies on a real-time PCR format, which allows for obtaining quantitative results at a higher specificity provided by the employment of a probe. Some such assays were constructed based on the available DNA sequences of the fish parvalbumin gene [3, 4, 38, 39].

Among end-point PCR-based detection tools, those targeting mitochondrial genes used to be held in high esteem [31, 38, 40, 41]. The high copy number of mitochondrial genes in each cell is one of the reasons for their popularity. On the other hand, the intron regions of nuclear genes can fully serve as an equivalent alternative [14, 42–45]. While the coding parts of nuclear genes are under selection pressure and, therefore, their sequences are quite uniform among species, hence not suitable for species determination, intron regions are free of this unification, and are diverse enough to serve as a platform for species identification by PCR. Moreover, adjacent exon regions, due to their high level of unification, allow for the design of degenerate primers, fish-versatile, usable for species-specific intron sequence mining by PCR derived from these neighbouring exon regions. Such a system provides a useful tool for a relatively quick development of particular species-specific detection variants of PCR for tracing fish species that are newly emerging on the market [42].

An approach based on the nuclear intron sequence of the parvalbumin gene was designed for black seabream (*Spondyliosoma cantharus*) as a model species and later tested in the end-point PCR format as an interlaboratory study as well, proving data on its robustness for routine employment. In this fish species, the gene is composed of four exons, separated by three introns in a unique way. The length of the second intron seems to make it the most suitable for designing PCR for the discrimination of fish species [46]. To provide a higher specificity, it is possible to upgrade the format of PCR to the level of real-time PCR, which employs a labelled probe as an additional selection tool based on sequence complementarity in addition to the pairing of specific primers.

In this work, interlaboratory assessment of such real-time PCR with TaqMan probe detection of model fish species *S. cantharus* was accomplished. Encoded samples of black seabream (*S. cantharus*) were detected by the developed real-time PCR system in five participating laboratories among a panel of 19 other fish species. In parallel, absence of PCR inhibition by the matrix was tested in parallels to all samples with no specific amplification, or by spiking these parallels with *S. cantharus* DNA up to the level of 10 %, or by an internal positive control providing an amplification signal at a different wavelength than that of the *S. cantharus* probe.

MATERIALS AND METHODS

Design of the study

Samples of DNA isolates were shipped in a parcel to the participating laboratories, each sample marked by a numerical code, without any information on identity of the fish species. At the same time, standard DNA of black seabream, isolated from another exemplar, was shipped in five concentrations (50; 25; 6.25; 1.56 and 0.78 ng·μl^{-1}) together with unknown samples. Guidelines unifying the critical steps of the analytical procedure were attached.

Interlaboratory study participants

Five laboratories participated in this study: 1. Food Research Institute Prague, Czech Republic (FRIP); 2. University of Chemistry and Technology, Prague, Czech Republic (UCT); 3. Matís Ltd., Reykjavík, Iceland (MATIS); 4. Food Research Institute, National Agricultural and Food Centre, Bratislava, Slovakia (FRI-NAFC); 5. National Institute of Public Health, Centre for Health, Nutrition and Food, Brno, Czech Republic (NIPH)

Sample material

Eight black seabreams and 19 other fish species were bought from the commercial network in the Czech Republic. An overview of the tested fish species is provided in Tab. 1. The identity of the purchased fish species was taken from the label and confirmed by morphological traits. The fish species composing the panel of negative controls were selected in a way to represent, in an unbiased manner, the entire range of the phylogenetic system of fish.

DNA isolation

DNA was isolated from mechanically homogenized fish material using a method based on the use of an ionic detergent, cetyltrimethylammonium bromide (CTAB; Carl Roth, Karlsruhe, Germany). The extraction was done according to EN ISO 21571:2005 [47] with modifications, when phenol-chlorophorm-isoamyl alcohol mixture was substituted by chlorophorm-isoamyl alcohol, liquid nitrogen freezing was omitted and isopropanol precipitation was implemented. DNA quality and quantity was checked by electrophoresis in 1% Serva Premium agarose gel (Serva, Heidelberg, Germany) and determined photometrically using NanoDrop (Implen, München, Germany). The isolated DNA was adjusted to a concentration of 25 ng·μl^{-1}. All DNA samples were coded by numbers (101–146).

Design of the probe and primers

The primers and the probe were designed based on previous knowledge of the sequence (accession number JN671445.1, National Center for Biotechnology Information, Bethesda, Maryland, USA) of the second intron of the protein-coding section of the parvalbumin gene, obtained in previous work performed in an end-point format [42]. Therefore, the starting stretch of the real-time PCR amplified sequence of the intron was pre-defined by the known position of the end-point PCR primers [46]. Further placement of primers and the dual-labelled probe were designed using PrimerExpress 3.0 software (Applied Biosystems, Foster City, California, USA) to fulfill the required criteria. From the sets of primers and probe suggested by the software, the forward primer (1189B6: TGA GCT GAA GTA AGA CAC TCA GGA A), reverse primer (1189B7: TCT AAA ATG TTG TCT TGG TGC CTT AG) and dual-labelled probe (1273H9: TGC ACA CTT GAG CAA GCA ATG GCC) were selected. The probe was used as a TaqMan-type probe labelled with 6-carboxyfluorescein (FAM) as a reporter dye and Black Hole Quencher 1 (BHQ1) as a quencher. All oligonucleotides were synthetized by Generi Biotech (Hradec Králové, Czech Republic).

Real-time PCR

Real-time PCR was conducted on five different platforms, as presented in Tab. 2. All reactions contained 10 μl of 2× TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, USA), which contained Rox Reference Dye (ROX; glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester) as a passive reference dye, primers at a final concentration of 0.4 mmol·l^{-1}, 0.1 mmol·l^{-1} probe, 50 ng of template DNA and nuclease-free water, the total reaction volume being 20 μl. The following thermal cycle profile was used: initial denaturation and polymerase activation at 95 °C for 10 min, 35 cycles with denaturation at 95 °C for 15 s and annealing with polymerization at 60 °C for 60 s. Fluorescence was measured as relative fluorescence units (RFU) and plotted as baseline-corrected normalized reporter (ΔRn), i.e. the magnitude of normalized fluorescence signal from which normalized signal of baseline was subtracted.

In one extra experiment to test robustness in this respect, a pre-mixed reaction mixture qPCR ProbesMaster with lowROX (Jena Bioscience, Jena, Germany) was used as an alternative, under otherwise same conditions.

In the experiments, all samples were measured

Tab. 1. Summary of qualitative results.

Sample code	Fish species	Reaction mixture						Agreement [%]
		A					B	
		FRIP	UCT	MATIS	FRI-NAFC	NIPH	FRIP	
101	Salmon (*Salmo salar*)	–	–	–	–	–	–	100
102	European carp *(Cyprinus carpio)*	–	–	–	–	–	–	100
103	**Black seabream (*Spondyliosoma cantharus*) 1**	+	+	+	+	+	+	100
104	Atlantic bluefin tuna *(Thunnus thynnus)*	–	–	–	–	–	–	100
105	**Black seabream *(Spondyliosoma cantharus)* 2**	+	+	+	+	+	+	100
106	**Black seabream *(Spondyliosoma cantharus)* 3**	+	+	+	+	+	+	100
107	Brook trout *(Salvelinus fontinalis)*	–	–	–	–	–	–	100
108	Mahi-mahi *(Coryphaena hippurus)*	–	–	–	–	–	–	100
109	European seabass *(Dicentrarchus labrax)*	–	–	–	–	–	–	100
110	Tilapia *(Oreochromis niloticus)*	–	–	–	–	–	–	100
111	**Black seabream *(Spondyliosoma cantharus)* 4**	+	+	+	+	+	+	100
112	Atlantic herring *(Clupea harengus)*	–	–	–	–	–	–	100
113	**Black seabream *(Spondyliosoma cantharus)* 5**	+	+	+	+	+	+	100
114	European hake *(Merluccius gayi)*	–	–	–	–	–	–	100
115	Northern pike *(Esox lucius)*	–	–	–	–	–	–	100
116	Pangasius *(Pangasius hypophthalmus)*	–	–	–	–	–	–	100
117	Carassius *(Carassius carassius)*	–	–	–	–	–	–	100
118	Tench *(Tinca tinca)*	–	–	–	–	–	–	100
119	Garfish *(Belone belone)*	–	–	–	–	–	–	100
120	**Black seabream *(Spondyliosoma cantharus)* 6**	+	+	+	+	+	+	100
121	European eel *(Anguilla anguilla)*	–	–	–	–	–	–	100
122	**Black seabream *(Spondyliosoma cantharus)* 7**	+	+	+	+	+	+	100
123	Silver carp (*Hypophtalmichthys molitrix)*	–	–	–	–	–	–	100
124	Common sole *(Solea solea)*	–	–	–	–	–	–	100
125	Red scorpionfish *(Scorpena scrofa)*	–	–	–	–	–	–	100
126	**Black seabream *(Spondyliosoma cantharus)* 8**	+	+	+	+	+	+	100
127	Angler *(Lophius piscatorius)*	–	–	–	–	–	–	100

A – TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, USA), B – qPCR Probes Master with lowROX (Jena Bioscience, Jena, Germany).
FRIP – Food Research Institute Prague, Czech Republic; UCT – University of Chemistry and Technology, Prague, Czech Republic; MATIS – Matís Ltd., Reykjavík, Iceland; FRI-NAFC – Food Research Institute, National Agricultural and Food Centre, Bratislava, Slovakia; NIPH – National Institute of Public Health, Centre for Health, Nutrition and Food, Brno, Czech Republic.

Tab. 2. List of participating laboratories and instruments used.

Laboratory	Real-time instrument
FRIP	StepOnePlus (Applied Biosystems, Foster City. California, USA)
UCT	ABI 7500 (Applied Biosystems, Foster City. California, USA)
MATIS	Stratagene MX3005P (Agilent, Santa Clara, California, USA)
FRI-NAFC	Opticon 2 (MJ Research, Waltham, Massachusetts, USA)
NIPH	ABI 7900HT Fast ((Applied Biosystems, Foster City. California, USA)

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in technical triplicates. Each calibration curve was constructed from five concentration points prepared as serial dilutions of the target DNA with concentrations 50, 25, 6.25, 1.56 and 0.78 ng·μl^{-1}. Two plates were used in each laboratory, each plate with its own calibration curve.

Inhibition control

Preparation of spiked sample material

DNA of black seabream (*S. cantharus)* in a total amount of 5 ng was added to DNA samples isolated from nineteen fish species (excluding *S. cantharus*). As a result, DNA solutions with 10 % DNA of *S. cantharus* and 90 % of DNA from other fish species were obtained. In this way, the mixed samples of the aforementioned parameters of total DNA concentration 25 ng·μl^{-1} were prepared. These samples were used as parallels to samples of DNA isolates prepared from fish species from the negative panel.

Internal positive control

TaqMan exogenous internal positive control (IPC) reagents (Applied Biosystems), emitting in channel of the fluorescent dye VIC (554 nm), were used as an alternative to spiking. Reactions contained 10 μl of 2× TaqMan Environmental Master Mix 2.0, 2 μl 10× Exo IPC Mix, 0.4 μl Exo IPC DNA, 5.6 μl of target primers, probe, nuclease-free water and 2 μl of DNA solution of samples. IPC results were considered positive when the threshold cycle (*Ct*) value was lower than 30, which was considered as the positivity threshold [48].

RESULTS AND DISCUSSION

Qualitative data

Data obtained from all participating laboratories are summarized in Tab. 1. The values of unknown samples falling into the concentration range of points of the calibration curve (Fig. 1, 2) were assessed as positive *S. cantharus* results. Statistical analysis of this summary provides 100% sensitivity, 100% specificity, zero false negativity, zero false positivity and 100% accuracy. The extra experiment done with qPCR ProbesMaster with lowROX Master Mix (Fig. 1) provided the same outcome as obtained from TaqMan Environmental Master Mix in terms of sensitivity, specificity, false negativity, false positivity and accuracy.

The results of tests for the exclusion of matrix inhibition were based on two different approaches. In the first one, DNA isolated from fish species from the negative panel was spiked up to a level of 10 % with DNA from *S. cantharus* (Fig. 3). In all of these samples, the signal of amplification was detected as positive, thus excluding inhibition by matrix (Tab. 3). Another approach, based on IPC emitting at the wavelength of VIC, provided a concordant result, i.e. no inhibition by matrix in negative samples was present, as the amplification

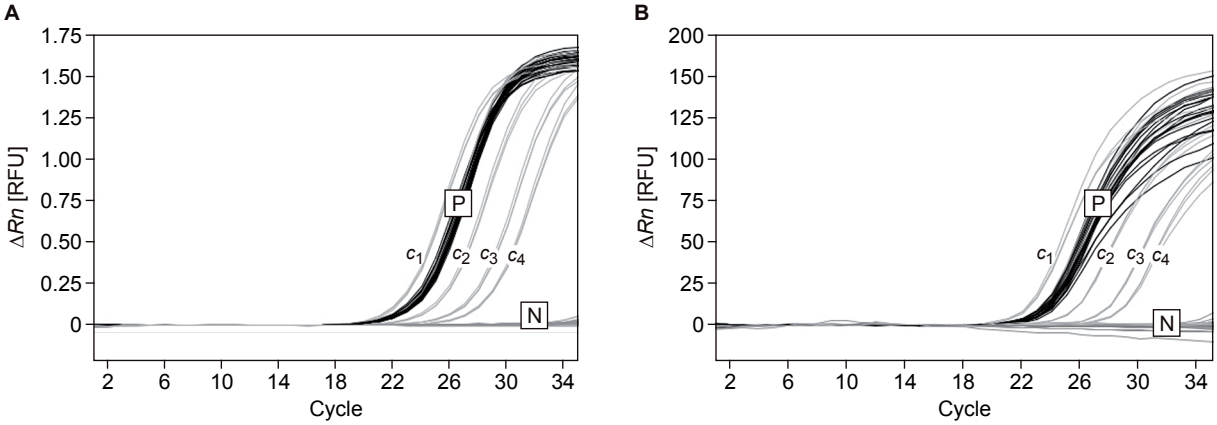

Fig. 1. Comparison of *S. cantharus* samples vs samples of various other fish species used as a panel of negative species.

A – Reaction accomplished in TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, California, USA), B – Reaction accomplished in qPCR ProbesMaster with lowROX (Jena Bioscience, Jena, Germany).

Δ*Rn* – baseline-corrected normalized reporter in relative fluorescence units (RFU); P (bunch of black curves) – *S. cantharus* samples; N (bunch of dark grey curves) – fish samples of the panel of negative species; *c* – distinct levels of concentration points of calibration, each bunch corresponding to one concentration level (c_1 = 50 ng·μl^{-1}, c_2 = 6.25 ng·μl^{-1}, c_3 = 1.56 ng·μl^{-1}, c_4 = 0.78 ng·μl^{-1}).

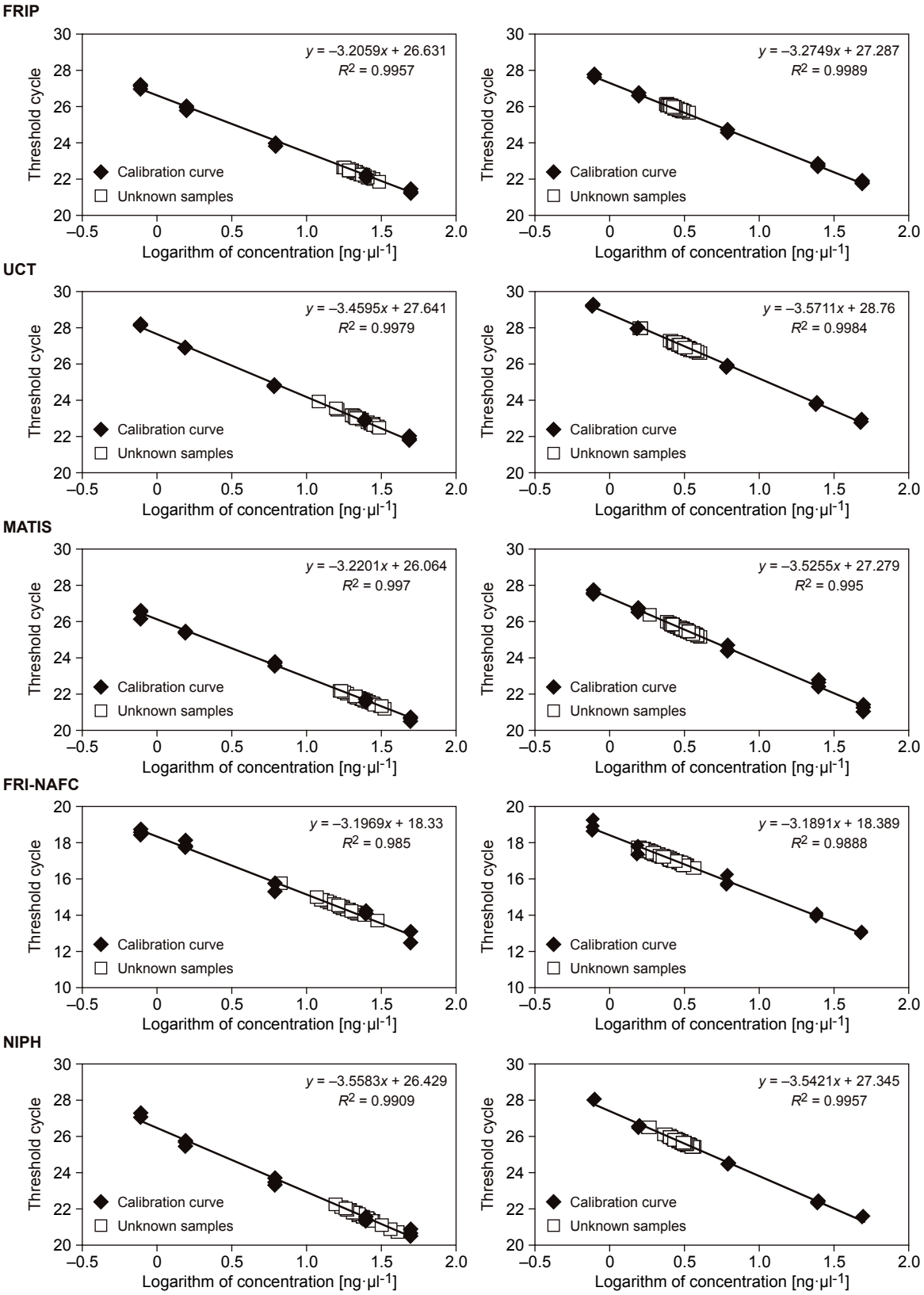

Fig. 2. Overview of calibration curves.

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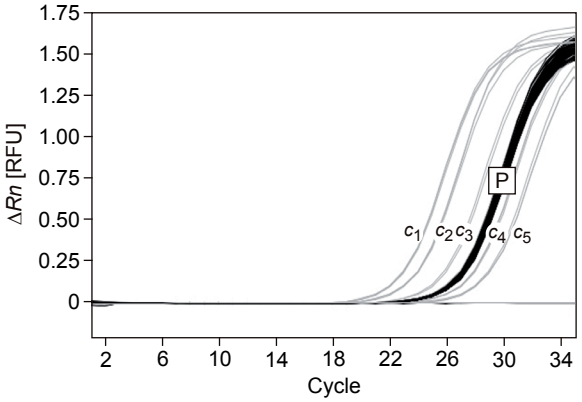

Fig. 3. Amplification plot of *S. cantharus*-specific real-time PCR obtained from spiked samples.

Δ*Rn* – baseline-corrected normalized reporter in relative fluorescence units (RFU); P (bunch of black curves) – *S. cantharus*-specific signal from spiked samples; *c* – distinct levels of concentration points of calibration, each bunch corresponding to one concentration level (c_1 = 50 ng·μl^{-1}, c_2 = 25 ng·μl^{-1}, c_3 = 6.25 ng·μl^{-1}, c_4 = 1.56 ng·μl^{-1}, c_5 = 0.78 ng·μl^{-1});

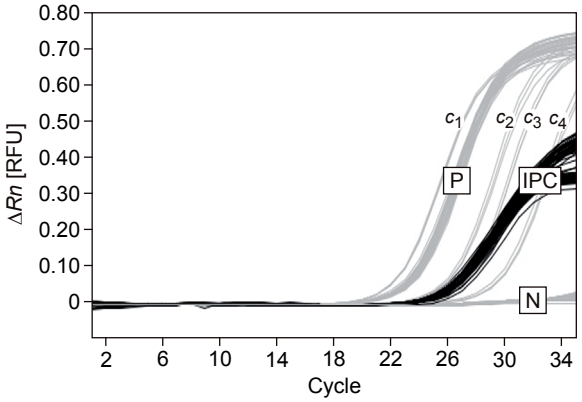

Fig. 4. Amplification plot of *S. cantharus*-specific real-time PCR together with internal positive control.

Δ*Rn* – baseline-corrected normalized reporter in relative fluorescence units (RFU); P (bunch of grey curves) – *S. cantharus* samples; N (bunch of grey curves) – fish samples of the panel of negative species; IPC (bunch of black curves) – internal positive control signal; *c* – distinct levels of concentration points of calibration, each bunch corresponding to one concentration level (c_1 = 50 ng·μl^{-1}, c_2 = 6.25 ng·μl^{-1}, c_3 = 1.56 ng·μl^{-1}, c_4 = 0.78 ng·μl^{-1});

Tab. 3. Overview of inhibition controls in spiked samples and internal positive control.

Sample code	Fish species	Spike					Agreement [%]	IPC	Agreement between spike and IPC [%]
		FRIP	UCT	MATIS	FRI-NAFC	NIPH		FRIP	
128	Salmon (*Salmo salar*)	+	+	+	+	+	100	+	100
129	European carp *(Cyprinus carpio)*	+	+	+	+	+	100	+	100
130	Atlantic bluefin tuna *(Thunnus thymus)*	+	+	+	+	+	100	+	100
131	Brook trout *(Salvelinus fontinalis)*	+	+	+	+	+	100	+	100
132	Mahi-mahi *(Coryphaena hippurus)*	+	+	+	+	+	100	+	100
133	European seabass *(Dicentrarchus labrax)*	+	+	+	+	+	100	+	100
134	Tilapia *(Oreochromis niloticus)*	+	+	+	+	+	100	+	100
135	Atlantic herring *(Clupea harengus)*	+	+	+	+	+	100	+	100
136	European hake *(Merluccius gayi)*	+	+	+	+	+	100	+	100
137	Northern pike *(Esox lucius)*	+	+	+	+	+	100	+	100
138	Pangasius *(Pangasius hypothalamus)*	+	+	+	+	+	100	+	100
139	Carassius *(Carassius carassius)*	+	+	+	+	+	100	+	100
140	Tench *(Tinca tinca)*	+	+	+	+	+	100	+	100
141	Garfish *(Belone belone)*	+	+	+	+	+	100	+	100
142	European eel *(Anguilla anguilla)*	+	+	+	+	+	100	+	100
143	Silver carp (*Hypophtalmichthys molitrix)*	+	+	+	+	+	100	+	100
144	Common sole *(Solea solea)*	+	+	+	+	+	100	+	100
145	Red scorpionfish *(Scorpena scrofa)*	+	+	+	+	+	100	+	100
146	Angler *(Lophius piscatorius)*	+	+	+	+	+	100	+	100

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IPC – exogenous internal positive control.

of IPC took place in these samples (Fig. 4). Therefore, there was 100% agreement between these two approaches (Tab. 3).

The variance of *R*-squared (R^2), efficiency and slope fell into the range recommended for real-time PCR detection of genetically modified organisms (GMO). Among calibration curves obtained from participating laboratories, the value of R^2 fell into the interval 0.983–0.999, efficiency fell into the interval from 90 % to 110 %, and the slope fell into the interval from –3.1 to –3.6. These values were within the range recommended for PCR-based detection of GMO [49, 50].

Quantitative data

For quantitative assessment of positive samples, absolute quantification was used.

For each exemplar of *S. cantharus*, the average DNA concentration among the participating laboratories was calculated, as well as the variation and standard deviation (Tab. 4). The average value of *S. cantharus* DNA concentration in true *S. cantharus* samples, among all participating laboratories, was 22.8 ng·μl^{-1} (range 19.0–28.7 ng·μl^{-1}). This might have represented the interspecies variability of the chosen trait, where the lowest and highest obtained values would be the margins of the range. In this way, the highest obtained value roughly represented 150 % of the lowest value.

Compared to the end point setup [46], the TaqMan real-time PCR provides better specificity, as another selection instance, the dually labeled probe, in addition to the primers, also pairs with the template sequence. This could successfully be used with some other fish species in which there is a slight unspecific pairing under the end-point setup [46].

Within the context of testing for the potential influence of matrix by IPC, a multiplexed setup of real-time PCR was performed. This was found to be quite promising in terms of the quality of the obtained results. The multiplexed setup can also be envisaged as a useful improvement at the detection of further fish species. The alternative approach, in which an additional dose of *S. cantharus* DNA was added to DNA of fish species from the negative panel, was shown to be equally capable of proving the inertness of the matrix. The level of 10 % was chosen based on the assumption that it should not interfere with DNA of the negative species itself but, at the same time, would be high enough to enable PCR which, in the case of inhibition by matrix, would not run. As the results of both approaches came to the same conclusion (no inhibition by matrix in any of the samples), it suggests that inhibition by matrix is not a major concern under common conditions in PCR-based species determination in fish. By comparing these two approaches, the one based on spiking by *S. cantharus* DNA is simpler and cheaper, though dependent on availability of the biological material of the tested species (*S. cantharus* meat in this case). The approach based on commercial ICP is much more expensive but is also better defined and standardized, which is more suitable for broad-range comparisons among a number of laboratories.

At the level of previous testing in end-point format, the concern of possible intraspecies variability was addressed by testing a set of exemplars of *S. cantharus* to exclude possible intraspecies variability, which might have resulted in some exemplars giving false negative outcomes of the test. This concern was sufficiently refuted

Tab. 4. Summary of quantitative results.

Sample	Absolute DNA concentration [ng·μl^{-1}]					Average	Standard deviation	Coefficient of variation
	FRIP	UCT	MATIS	FRI-NAFC	NIPH			
103	21.83	22.83	24.63	15.13	23.25	21.535	3.326	0.154
105	18.37	19.15	23.04	15.26	19.30	19.023	2.483	0.130
106	20.09	18.56	20.68	14.71	21.52	19.111	2.404	0.126
111	22.72	22.42	23.55	19.83	25.97	22.898	1.977	0.086
113	28.38	29.15	27.44	22.03	28.46	27.091	2.588	0.096
120	19.88	22.27	22.20	18.16	20.50	20.601	1.537	0.075
122	19.82	21.39	18.99	13.47	19.39	23.673	2.697	0.114
126	24.74	29.69	31.76	25.01	32.47	28.734	3.282	0.114

FRIP – Food Research Institute Prague, Czech Republic; UCT – University of Chemistry and Technology, Prague, Czech Republic; MATIS – Matís Ltd., Reykjavík, Iceland; FRI-NAFC – Food Research Institute, National Agricultural and Food Centre, Bratislava, Slovakia; NIPH – National Institute of Public Health, Centre for Health, Nutrition and Food, Brno, Czech Republic.

at that level. However, in the present real-time PCR based study with a dual-labelled TaqMan probe, eight distinct exemplars of *S. cantharus* were used and no intraspecies variability causing false negative results was observed. Therefore, the knowledge from the previous study, that intraspecies variability does not hamper PCR-based detection in the case *S. cantharus*, was confirmed in the more sophisticated format of the assay.

The data and results obtained in this study positively evaluated a method for routine precise species determination of black seabream (*S. cantharus)* among other fish species based on small pieces of meat devoid of morphological traits. The presented method was found reproducible and robust, including the use of various real-time instruments as well as various buffers. In parallel, two different methods of control of inhibition by matrix were successfully tested, when the widely used approach based on spiking negative samples with DNA from target species was supplemented with an alternative approach using IPC. Both approaches proved to be equally useful.

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