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ORIGINAL PAPER

Development of a real-time PCR system for the detection of the potential allergen fish in food

Carina Tetzlaff[1] · **Dietrich Mäde**[1]

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Abstract Fish is one of the most important triggers of food-allergic reactions. Regulation (EU) No 1169/2011 governs the labelling of allergenic ingredients including fish. A real-time PCR assay, using TaqMan® probes, was applied to detect fish in food. For generating primer and probes, the *Hox*c13 gene was selected. Based on the alignment of available DNA sequences from this gene from different fish species in public nucleic acid database, specific oligonucleotides were generated. To cover all relevant species of the phylogenetic class fish, the CTfish-system consists of two forward primers, three reverse primers and one TaqMan® probe. The real-time PCR method is able to detect specifically fish species belonging to the subclass Teleostei. The sensitivity is in an animal food product 100 mg/kg and in a vegetable food matrix 10 mg/kg relating to the fresh weight. The calculated limit of detection ($LOD_{95\ \%}$) of fish DNA in background DNA is 2.5 copies. The method developed is robust against small, deliberate changes in the reaction conditions. The CTfish-system can be used to sensitively and selectively identify the presence of fish DNA in food.

Keywords Real-time PCR · Molecular detection · Fish · Allergy

Abbreviations

BHQ1	BlackHole™ Dark Quencher
ddPCR	Droplet digital polymerase chain reaction
FAM	6-Carboxyfluorescein
PCR	Polymerase chain reaction
R	IUB code for adenine or guanine
dsDNA	Double-stranded DNA
Y	IUB code for cytosine or thymin

Introduction

From the nutritional point of view, fish is a valuable food supplying the human with essential amino acids, lipid-soluble vitamins and ω-3 fatty acids [1, 2]. Besides its nutritional benefits, fish is as well one of the most important triggers of food-allergic reactions. The consumption of fish by affected persons may cause severe anaphylactic shocks with probably fatal consequences. Currently, about 0.1 % of the population suffers from fish allergy and because of the increasing consumption of fishery products, the prevalence is rising [3]. Even small amounts of a few milligrams of protein can lead to allergic reactions immediately [4]. For this reason, the only possibility for affected persons is to avoid the intake of food containing allergens completely. To protect allergic persons, European legislation demands the obligatory labelling of 14 allergenic ingredients, including fish, by Annex II of Regulation (EU) No 1169/2011. The labelling obligations are required for consciously added ingredients only. Labelling thresholds are not defined yet in the European Union. On the other hand, hidden allergens can cause life-threatening reactions as well. Especially by "cross-contacts", resulting from contamination during production, storage or transport, allergenic substances can unintentionally get into products. Producers often use the optional labelling "may contain traces of…", which can cause uncertainty for allergic consumers. For this reasons, the development of specific and sensitive

✉ Dietrich Mäde
dietrich.maede@lav.ms.sachsen-anhalt.de

[1] Landesamt für Verbraucherschutz Sachsen-Anhalt, Fachbereich 3 - Lebensmittelsicherheit, Freiimfelder Str. 68, 06112 Halle (Saale), Germany

methods to detect the presence of fish in food is considered necessary. The German Federal Institute for Risk Assessment (BfR) specifies a limit of detection of 0.01–0.001 % for an allergenic ingredient in the final product for detection methods [5]. To define harmonised threshold values, the European Voluntary Incidental Trace Allergen Labelling (EU-VITAL) was developed. It establishes so-called action levels for the allergenic ingredients at which labelling is required [6].

For detection of fish allergens in food, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) technology are used most widely [7–11]. The majority of ELISA systems are based on the detection of the major fish allergenic protein, parvalbumin [8]. Compared to proteins, DNA is more stable against manufacturing processes. Besides this, molecular methods possess a better specificity than immunological methods [14].

The term "fish" is defined in EU legislation by the Regulation (EU) No 1379/2013. It refers to the Combined Nomenclature listing bony and cartilaginous fish. Most of the detection systems for fish available at present are specific for only a limited number of fish species, for specific allergens or use broadly reactive mitochondrial genes as target [9–13]. In this work, a real-time PCR system for the detection of all relevant species of fish which may be present in food products was developed. Single copy genes were used as target sequence to keep the possibility for molecular quantification.

Materials and methods

Fish samples and food products

The fish samples were obtained from fish farms, purchased on fish markets and supermarkets or were official samples. Food products used for spiking experiments were either purchased in local supermarkets or were sent in by local authorities as official samples for other diagnostic purposes. A summary of fish and food samples is shown in Table 1.

Preparation of spiked samples

To prepare mass fraction mixtures with different fish content, a serial dilution of fresh muscle meat (10,000, 1000, 100, 10, 1, 0.1, 0.01 mg/kg) from sample 260601 (*Abramis brama*) was done in the following food matrices: proteinshake powder and shrimp salad consisting of 245 g shrimps, 365 g pineapple and 477 g mayonnaise. The resulting spiked samples were homogenised in a mixer (Grindomix, Retsch, Haan, Germany). In addition, a serial dilution of fish DNA (20, 10, 5, 2, 1, 0.1 copies) from sample 260601 (*Abramis brama*) in background DNA of calves liver sausage (c dsDNA = 20 ng/µL) was made. The DNA copy number per microlitre of sample 260601 (*Abramis brama*) was determined by ddPCR (QX200 Droplet Generator and ddPCR Supermix for Probes, Bio-Rad, München, Germany).

DNA extraction

The DNA was extracted using a modified CTAB method [14, 15]. All DNA extractions were done in duplicates, using two portions of 200 mg for each of the samples for extraction. To prevent cross-contamination, clean instruments were used for each sample. 1 mL CTAB-extraction buffer [c (CTAB) = 20 g/L, c (NaCl) = 1.4 mol/L, c (TRIS) = 0.1 mol/L, c (Na_2EDTA) = 0.02 mol/L, pH 8.0] and 10 µL Proteinase K solution (c = 20 mg/L) were added to the sample. The samples were incubated at 60 °C under permanent agitation overnight and centrifuged for 10 min at 12,000×g afterwards. The supernatant was transferred into a new vial. 0.7 mL of chloroform was added; the sample was shaken vigorously and centrifuged at 19,000×g for 10 min. The upper phase was transferred into a new vial, and the volume was determined. Two volumes of CTAB precipitation buffer [c (CTAB) = 5 g/L, c (NaCl) = 0.04 mol/L] were added and incubated 60 min at room temperature without agitation. The samples were centrifuged for 5 min at 12,000×g, the supernatant was discarded, and the pellet was resuspended in 350 µL NaCl solution [c (NaCl) = 1.2 mol/L]. 350 µL of chloroform was added; the samples were vigorously shaken and centrifuged for 10 min at 19,000×g. The upper phase was transferred into a new vial; 0.6 vol of isopropanol was added for nucleic acid precipitation. After 20-min incubation at room temperature, the samples were centrifuged for 10 min at 12,000×g. The supernatant was discarded, the pellet was washed with 500 µL ethanol solution (c = 70 %), resolved in 100 µL 0.1× TE buffer [c (TRIS) = 1 mmol/L, c (Na_2EDTA) = 0.1 mmol/L, pH 8.0] and stored as stock solution. 5 µL of a tenfold dilution of the DNA stock solution was used as template for PCR.

The concentration and purity of the extracted nucleic acids were determined by measuring the optical density at 260 and 280 nm using a photometer (ScanDrop hotometer, Analytik Jena, Jena, Germany).

DNA sequencing

To verify the labelled species of the collected samples, the cytochrome b gene was sequenced and species identification was done by comparing with the sequences in a public database (GenBank® [16]). Additionally, the *Hox*c13 genes of three samples 030915 (*Raja brachyuran*), 201001

Table 1 Fish samples and food products used for method development. All fish samples were adjusted to a DNA concentration of about 1.8 ng/µL based on photometric determination

Trade name	Identified species	Sample number	*Cq* (quantification cycle)
Bream	*Abramis brama*	260601	28.2/28.2
Salmon	*Salmo salar*	030901	28.7/28.6
Rose fish	*Sebastes norvegicus*	030902	29.1/29.3
Saithe	*Pollachius virens*	030904	30.5/31.2
Red porgy	*Pagrus spp.*	030905	30.2/30.6
Nile perch	*Lates niloticus*	030906	29.9/29.8
Atlantic cod	*Gadus morhua*	030907	27.4/27.4
European seabass	*Dicentrarchus labrax*	030908	29.9/29.5
Atlantic halibut	*Hippoglossus hippoglossus*	030909	32.4/32.4
Brown trout	*Salmo trutta*	030910	29.3/29.0
European plaice	*Pleuronectes platessa*	030911	29.2/28.9
Tuna	*Thunnus obesus*	030912	29.6/31.4
Brook trout	*Salvelinus fontinalis*	030913	24.6/24.5
Monkfish	*Lophius piscatorius*	030914	31.8/32.6
Ray wings	*Raja brachyura*	030915	37.3/40.2
Wolffish	*Anarhichas lupus*	030916	30.7/30.5
Yellowtail amberjack	*Seriola lalandi*	030918	28.1/28.5
Atlantic herring	*Clupea harengus*	080901	24.9/25.2
Gilt-head bram	*Sparus auratus*	080902	29.7/30.0
Carp	*Cyprinus carpio*	300901	28.2/28.4
European perch	*Perca fluviatilis*	300902	29.2/28.9
Rainbow trout	*Oncorhynchus mykiss*	300903	26.2/27.6
Roach	*Rutilus rutilus*	300904	29.3/30.3
Sole	*Solea solea*	041001	26.4/26.3
Atlantic salmon	*Salmo trutta fario*	041002	27.2/26.6
Carp	*Cyprinus carpio*	041003	30.6/29.6
Lemon sole	*Pleuronectes platessa*	041005	30.0/30.5
Haddock	*Melanogrammus aeglefinus*	041006	31.9/31.1
European flounder	*Platichthys flesus*	041007	29.5/29.6
Turbot	*Scophthalmus maximus*	041008	31.4/31.6
European hake	*Merluccius merluccius*	041009	30.3/29.8
Ling	*Molva molva*	041010	31.0/30.0
Mullet	*Liza ramada*	041011	31.6/32.1
Wolffish	*Anarhichas lupus*	041012	26.8/27.8
Witch	*Scophthalmus maximus*	041013	30.7/30.1
Sea robin	*Eutrigla gurnardus*	041015	30.2/31.1
Weever	*Trachinus draco*	041016	29.2/30.1
Iridescent shark	*Pangasianodon hypophthalmus*	091001	29.4/30.3
Mackerel	*Scomber scombrus*	161001	25.2/25.7
Sprat	*Sprattus sprattus*	161002	24.9/25.7
Monkfish	*Lophius piscatorius*	161004	29.2/28.9
European pilchard	*Sardina pilchardus*	161005	27.6/27.3
Tilapia	*Oreochromis niloticus*	161006	28.3/28.3
Lump fish	*Cyclopterus lumpus*	161007	32.5/31.9
Dogfish	*Squalus acanthias*	201001	n.a./n.a.
Meagre	*Argyrosomus regius*	201002	28.0/28.1
Catfish	*Clarias gariepinus*	201003	28.8/28.7
Zander	*Sander lucioperca*	201004	31.2/31.9

Table 1 continued

Trade name	Identified species	Sample number	*Cq* (quantification cycle)
Haddock	*Melanogrammus aeglefinus*	201005	30.9/30.7
Northern pike	*Esox lucius*	201006	31.0/31.2
Lake char	*Salvelinus umbla*	201007	30.1/30.8
Eel	*Anguilla anguilla*	231001	34.2/34.6
Tench	*Tinca tinca*	231002	27.2/27.8
Blueblotch butterflyfish	*Selaroides leptolepis*	261001	28.9/28.2
Sturgeon	*Acipenser baeri*	250101	n.a./n.a.

Food	Producer
Liver sausage of calves	Halberstädter GmbH, Halberstadt, Germany
Proteinshake powder	WellMix Sport, Dirk Rossmann GmbH, Burgwedel, Germany
Shrimptails	SeaGold, Netto Marken-Discount AG and Co. KG, Marxhütte-Haidhof, Germany
Pineapple canned	Tip real-Handels GmbH, Düsseldorf, Germany
Mayonnaise	Tip real-Handels GmbH, Düsseldorf, Germany

n.a. no amplification

(*Squalus acanthias*) and 250101 (*Acipenser baeri*) were sequenced, using the sequencing-primers of the developed CTfish-system (see Table 3). In the following, differing reaction conditions for the *Hox*c13 sequencing are located in brackets. PCR products of the cytochrome b gene were generated using the primers *L14735* and *H15149ad* resulting in a PCR product of 464 bp [17]. All oligonucleotides were synthesised by TIB MOLBIOL (Berlin, Germany). PCR products, which were applied in the subsequent sequencing reaction, were produced by using 1 µL template in a total reaction volume of 25 µL. The reaction conditions were as follows: 2.5 µL 10× PCR buffer containing c ($MgCl_2$) = 15 mmol/L (Qiagen, Hilden, Germany), 3.5 µL $MgCl_2$ [c ($MgCl_2$) = 25 mmol/L] (Qiagen, Hilden, Germany) resulting in a final concentration of c ($MgCl_2$) = 5 mmol/L, 0.5 µL dNTP-Mix [c (dATP, dCTP, dGTP, dTTP) = 200 µmol/L each] (Roche Diagnostics, Mannheim, Germany), 0.5 µmol/L of each primer and 0.025 U thermostable *Taq* DNA polymerase (Hot Star Taq Polymerase, Qiagen, Hilden, Germany). Reactions were carried out on GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Darmstadt, Germany) using an initial denaturation step for 15 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 58 °C (*Hox*c13: 50 °C), 60 s at 72 °C and a final elongation step of 7 min at 72 °C. 5 µL of the PCR products was analysed on a 2 % agarose gel in TAE buffer using a 100-bp fragment length marker (Gene Ruler™, Thermo Scientific, Waltham, USA). The remaining amount of the reaction volume was purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced with the BigDye™ Terminator V 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) using the same primers as mentioned above. The reaction condition was as follows: 2 µL Big Dye® Terminator V 1.1 V 3.1 5× Sequencing Puffer (Applied Biosystems, Darmstadt, Germany), 1 µL Big Dye® Terminator V 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany), 0.5 µL primer and 1–4 µL template, depending on the intensity of the DNA band on the agarose gel. The samples were filled up with water to a total volume of 10 µL. Temperature and time conditions were as described above but using an annealing temperature of 55 °C (*Hox*c13: 48 °C). The PCR products were purified by ethanol precipitation. 1.3 µL bromophenol blue, 10 µL 3 M sodium acetate [40.83 g sodium acetate per 100 mL water, pH 4.6], 90 µL water, 250 µL 10 % (v/v) ethanol and the PCR product were mixed and incubated for 10 min at room temperature. Samples were centrifuged for 15 min at 12,000×g, the supernatant was discarded, and the pellet was washed two times with 250 µL ethanol solution (c (v/v) = 70 %). After drying the pellet for 10 min at 65 °C, it was resolved in 20 µL water and transferred into 0.5-µL sample tubes (Applied Biosystems, Darmstadt, Germany). The analysis was carried out on an automated DNA sequencer (ABI Prism 310 Genetic Analyser, Applied Biosystems, Darmstadt, Germany). Resulting nucleic acid sequences were aligned with the help of Sequence Navigator software version 1.0.1 (Applied Biosystems, Darmstadt, Germany). The sequences were compared with sequences in GenBank® using the computer algorithm BLAST 2 [18].

ddPCR

The exact DNA copy number per microlitre in the DNA extract of sample 260601 (*Abramis brama*), used for the determination of the limit of detection, was determined by droplet digital PCR (ddPCR). A dilution series of three steps was analysed in four replicates. PCR products were produced by using 5 µL DNA template in a total reaction volume of 20 µL. The reaction conditions were as follows: 10 µL ddPCR Supermix for Probes (BioRad, München, Germany), 0.5 µmol/L of each primer and 0.2 µmol/L of TaqMan® probe. Water was added up to a total amount of 20 µL. 9.5 µL of the mastermix and 5.64 µL template were transferred into a cavity of a 96-well reaction plate and were mixed by repeated pipetting. The samples were centrifuged at 560×*g* for 2 min, and the mix was aliquoted into two 20 µL amounts and transferred into cavities of DG8 Cartridges for QX100/QX200 Droplet Generator (BioRad, München, Germany). 70 µL Droplet Generation Oil for Probes (BioRad, München, Germany) was pipetted in the intended cartridges. Droplets were generated by a droplet generator (QX200 Droplet Generator, BioRad, München, Germany) and transferred into a 96-well reaction plate. The plate was sealed with foil (Pierceable Foil Heat Seal, BioRad, München, Germany) at 180 °C by a PX1 PCR Plate Sealer (BioRad, München, Germany). Reactions were carried out on GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Darmstadt, Germany) using an initial denaturation step of 10 min at 95 °C, followed by 45 cycles of 30 s at 94 °C, 30 s at 60 °C, and a final elongation step of 10 min at 98 °C. The analysis was carried out by QX200 Droplet Reader (BioRad, München, Germany).

Real-time PCR

Oligonucleotides

The *Hox*c13 gene was selected as target gene for real-time PCR. Sequence data were obtained from public database (GenBank® [16]). Sequences of the *Hox*c13 gene from seven different fish were checked theoretically for homologous sections using the computer program ClustalΩ [19]. The accession numbers of the selected sequences are given in Table 2. A region at the 5′ end was chosen as target region for primers and probes. Deviations of only single basepairs between the compared species were observed. The alignment is shown in Fig. 1. Based on sequence data, primer and probe sequences were generated for the real-time PCR with the help of the Primer Express® 2.0 software (Applied Biosystems, Darmstadt, Germany). The received amplicon size is 141 or 134 bp according to the different locations of the reverse primers. Based on five samples [030901 (*Salmo salar*), 080901 (*Clupea harengus*), 300901 (*Cyprinus caprio*), 161006 (*Oreochromis niloticus*) and 201006 (*Esox lucius*)], the best primers and the best probe were chosen. These samples were amplified with the real-time PCR conditions described below using all possible primer combinations and one probe per reaction. By comparing the *C*q values, the most sensitive primers and probe combinations were determined. All oligonucleotides shown in Table 3 were synthesised by TIB MOLBIOL (Berlin, Germany).

Table 2 *Hox*c13 genes with GenBank® accession numbers

Species	Scientific name	GenBank number
Japanese rice fish	*Oryzias latipes*	AB_208012.1
Herring	*Clupea harengus*	XM_012836065.1
Guppy	*Poecillia reticulata*	XM_008413716.1
Zebrafish	*Danio rerio*	NM_131543.1
Tilapia	*Oreochromis niloticus*	XM_003448180.3
Northern pike	*Esox lucius*	XM_010875284.1
Salmon	*Salmo salar*	NM_001139531.1
Cattle	*Bos taurus*	NM_001083490.1
American alligator	*Alligator mississippiensis*	XM_006278444.1
Clawed frog	*Xenopus tropicalis*	XM_002936645.3
Chicken	*Gallus gallus*	XM_001235165.3

PCR conditions

Real-time PCRs were run in an ABI PRISM® 7900HT Sequence Detecting System (Applied Biosystems, Darmstadt, Germany) using an initial denaturation step for 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The temperature, time conditions and ramp rate are optimised for this instrument in the ABI reaction cycle 9600 mode, which corresponds to a ramp rate of approximately 1 °C/1 s. All reactions were run in a reaction volume of 25 µL. The reaction conditions were: 12.5 µL TaqMan® universal real-time PCR Mastermix (Applied Biosystems, Darmstadt, Germany), 300 nmol/µL of each primer, 100 nmol/µL of the TaqMan® probe and 5 µL template (*c* dsDNA = 1.8 ng/µL). The samples were brought up with water to a total volume of 25 µL.

Validation

Determination of specificity and sensitivity

The inclusivity of the PCR system was checked using 1.8 ng/µL template DNA of the fish samples. For testing the exclusivity, 1:10 dilutions of the extracted DNA of other animals [fly (*Calliphora vicina*), lobster (*Homarus americanus*), crocodile (*Crocodylus niloticus*), domestic duck (*Anas sparsa*), chicken (*Gallus gallus*), pigeon (*Columba palumbu*), mouse (*Mus musculus*), cattle (*Bos*

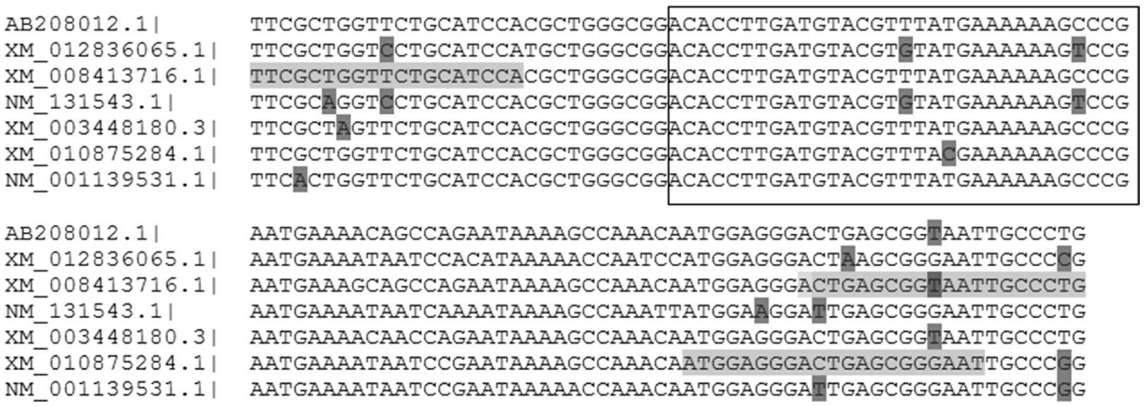

Fig. 1 Homologues sequences of the *Hoxc*13 gene. The homologues sequences of the target gene sequences from Japanese rice fish, Herring, Guppy, Zebrafish, Tilapia, Northern pike and Salmon are aligned. The bases which are not homologous to the others are *dark-shadowed*. The positions of the used primers are *light-shadowed*, and the probe sequence is framed

Table 3 Description of the used oligonucleotides

Oligonucleotide name	Oligonucleotide sequence (5′–3′)	Final concentration in PCR (nmol/µL)	References
L14735	5′-AAA AAC CAC CGT TGT TAT TCA ACT A-3′	500	[16]
H15149ad	5′-GCI CCT CAR AAT GAY ATT TGT CCT CA-3′	500	[16]
CTfish fwd1	5′-TTC GCT GGT TCT GCA TCC A-3′	300	This work
CTfish fwd7	5′-TTC GCT GGT CCT GCA TCC A-3′	300	This work
CTfish rev4	5′-CAG GGC AAT TCC CGC TCA AT-3′	300	This work
CTfish rev6	5′-ATT ACC GCT CAG TCC CTC CAT-3′	300	This work
CTfish rev7	5′-ATT CCC GCT TAG TCC CTC CAT-3′	300	This work
CTSeq fwd	5′-CGG CAR CCC STG ATA TG-3′	500	This work
CTSeq rev1	5′-GAG CAD GGC TTC TGC TGC ARG TT-3′	500	This work
CTSeq rev2	5′-GAA CAD GGT TTC TGC TGC ARG TT-3′	500	This work
CTfish1 TMP	5′-FAM- ACA CCT TGA TGT ACG TTT ATG AAA AAA GCC CGA–BHQ1-3′	100	This work

taurus), pig (*Sus scrofa*)] were amplified. The sensitivity was checked using 5 µL of each dilution of the spiked products. PCRs were performed as described above. The limit of detection was tested using a serial dilution of fish DNA (*Abramis brama*) in background DNA extracted from food to mimic real sample conditions (calves liver sausage; *c* dsDNA = 20 ng/µL) in 12 replicates. The PCR was performed using 2.5 µL 10× PCR buffer containing *c* ($MgCl_2$) = 15 mmol/L (Qiagen, Hilden, Germany), 0.5 µL dNTP-Mix [c (dATP, dCTP, dGTP, dTTP) = 200 µmol/L each] (Roche Diagnostics, Mannheim, Germany), 2.5 U/µL thermostable *Taq* DNA polymerase (Hot Star Taq Polymerase, Qiagen, Hilden, Germany), 300 nmol/µL of each primer, 100 nmol/µL of the TaqMan® probe and 5 µL template. The samples were brought up with water to a total volume of 25 µL. The reaction was run in LightCycler®480 (Roche, Mannheim, Germany) at the same temperature, time and ramp rate conditions as described above.

Robustness

The robustness of the method was checked by varying several parameters of the PCR reaction conditions like real-time instrument, PCR reagent kit, annealing temperature and primer and probe concentration. Table 4 lists the conditions used in the nine experiments. 5 µL DNA extract of the sample of the 10 mg/kg fish-containing proteinshake powder, which correspondents to 20 copies per reaction, was used as template.

Results and discussion

Specificity

The specificity of the method was assessed in silico and by practical tests. The exclusivity was checked in silico

Table 4 Conditions of the nine robustness experiments as orthogonal design

Factor	Combination								
	1	2	3	4	5	6	7	8	9
PCR equipment	x	A	A	x	x	x	x	x	A
PCR kit	B	x	B	x	x	x	x	B	B
Primer concentration (nmol/µL)	x	x	x	360	240	x	x	x	x
Probe concentration (nmol/µL)	x	x	x	x	x	120	80	x	x
Annealing temperature (°C)	x	x	x	x	x	x	x	58	62

x = standard conditions, A = LightCycler®480, Roche, Mannheim, Germany. B = HotStarTaq DNA Polymerase, Qiagen, Hilden, Germany

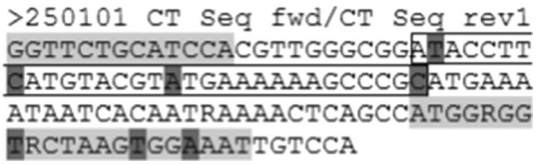

Fig. 2 Part of the *Hox*c13-gene sequence of Sturgeon (250101). The bases which are not homologous to the CTfish-system are *dark-shadowed*. The positions of the primers are *light-shadowed*, and the position of the probe is framed

by comparing *Hox*c13-gene sequences of animals belonging to other taxa than fish. The accession numbers of the selected sequences are given in Table 2. Practical evaluation was carried out by using DNA of different animals as template: fly (*Calliphora vicina*), lobster (*Homarus americanus*), crocodile (*Crocodylus niloticus*), duck (*Anas sparsa*), chicken (*Gallus gallus*), pigeon (*Columba palumbu*), mouse (*Mus musculus*), cattle (*Bos taurus*), pig (*Sus scrofa*). All results were negative, so false positive reactions by amplification of animals of other classes can be excluded. The primer–probe system consisting of CTfish1 TMP, CTfish fwd1, CTfish fwd7, CTfish rev4, CTfish rev6 and CTfish rev7 is specific for its target. The inclusivity was checked using 58 fish samples. The species was determined before by analysis of the cytochrome b gene. The results are shown in Table 1. Except for ray (030915), shark (201001) and sturgeon (250101) all tested fish species could be amplified with the primer probe system. To verify these results, the *Hox*c13 gene of ray, shark and sturgeon was sequenced using the sequence-primers created of the CTfish-system. The DNA sequences of the ray and the shark sample showed no sufficient homology with the CTfish-system. The sequence of the sturgeon differs in three to four positions in the probe and the reverse primers, as presented in Fig. 2. These results confirm that the developed primer probe system is not able to amplify ray, shark and sturgeon. Ray and shark are cartilaginous fish (Condrichthyes). All other tested fish are bony fish (Osteichthyes) belonging to the Teleostei. Sturgeons represent an own subclass (Chondrostei). So the CTfish-system can be used to detect fish of the subclass Teleostei, which compromises the most commonly consumed food fish [20]. Further developments are required to enable the detection of other fish species.

Sensitivity

The sensitivity of the CTfish-system was determined with dilution series in two types of food: food of animal origin and food of non-animal origin. A premanufactured proteinshake powder and a laboratory-made shrimp salad were used as matrix for spiking experiments. In shrimp salad sensitivity of 100 mg/kg and in proteinshake powder a sensitivity of 10 mg/kg relating to the fresh weight were reached. The results are shown in Table 5. Calculated from the weight per haploid genome of *Cyprinus caprio* of about 1.9 pg [21], the DNA content in white muscle meat of *Cyprinus caprio* of about 672 µg/g [22] and taking into consideration the extraction process, this approximately corresponds to 200 or 20 copies of the target gene.

The detection limit of allergens in food of 10–100 ppm as required by the German Federal Institute for Risk Assessment (BfR) was nearly reached. It has to be

Table 5 Sensitivity of the detection of fish in food matrices

Sample	Content of fish (mg/kg) (calculated copy number based on OD_{260} in brackets)						
	10,000 (20,000)	1000 (2000)	100 (200)	10 (20)	1 (2)	0.1 (0.2)	0.01 (0.02)
Proteinshake powder (*Cq* value)	28.1/28.3	31.3/31.6	35.7/36.0	39.6/40.0	n.a./n.a.	n.a./n.a.	n.a./n.a.
Shrimp salad (*Cq* value)	29.3/29.5	32.7/32.4	38.2/37.9	n.a./n.a.	n.a./n.a.	n.a./n.a.	n.a./n.a.

n.a. no amplification

Table 6 Assessment of the limit of detection by using a dilution series in background DNA from food of *Abramis brama*

Theoretical copy number	20	10	5	2	1	0:1
Number of PCR replicates	12	12	12	12	12	12
Number of positive test results	12	12	12	10	10	1

Table 7 Results of the robustness reactions using 20 copies per reaction in background DNA

Combination	1	2	3	4	5	6	7	8	9
*C*q value	36:7/36:7	35:6/34:6	33:7/33:8	35:8/35:7	37:1/35:7	35:8/36:2	37:2/36:6	33:6/33:7	35:7/35:4

considered that the proposed values are an average for all allergens. The EU-VITAL concept indicates individual detection limits for each allergen. A declaration of fish as ingredient is necessary at a content of 1.000 ppm and the labelling "may contain traces of fish" at 100 ppm [6]. The CTfish-system developed is capable of detecting these amounts of fish in food. A significant proportion of fish as allergen in food is co-processed with the original product. This could result in DNA degradation. Notwithstanding the PCR product, real-time PCR system is rather short and therefore the impact of processing should be limited; further experiments should be undertaken to verify the applicability to highly processed food. The limit of detection was determined using a dilution series of DNA copies in background DNA in twelve replicates. The results are shown in Table 6. The limit of detection ($LOD_{95\ \%}$) was calculated using the program Quodata [23]. $LOD_{95\ \%}$ is defined as the lowest concentration of fish DNA at which 95 % of positive samples are detected [24]. With the PCR method, a $LOD_{95\ \%}$ of 2.5 copies with a 95 % confidence interval of 1.5–4.0 can be reached. According to Poisson statistics, the theoretically lowest achievable $LOD_{95\ \%}$ is three copies, which was reached [25]. The difference showing a better $LOD_{95\ \%}$ than theoretically expected could be attributed to statistical bias. The requirement for a sensitive real-time PCR method is a $LOD_{95\ \%}$ of less than 20 copies [24].

Overall, the CTfish-system can be classified as a sensitive real-time PCR method which fulfils all requirements for the limit of detection.

The ligation-dependent probe amplification developed by Unterberger et al. [11] reached a sensitivity similar to the CTfish-system. The limit of detection described was 20 mg/kg fish in spiked sushi. Compared to other PCR systems published, the developed method is less sensitive. Benedetto et al. [9] described a real-time PCR method to detect fish DNA in feedstuff based on the mitochondrial 12S rRNA gene. With the primer–probe system generated, a limit of detection of 0.2 pg fish DNA diluted in plant DNA could be reached. The higher sensitivity of the method based on 12S rRNA can be attributed to the fact that each cell contains a lot of mitochondria and therefore several copies of target DNA. The number of mitochondria per cell and the DNA copies per mitochondria, however, is not constant but depends on the cell type and the individual. In the contrast to that, genomic genes are single copy gens, existing only one time per chromosome. Thus, the copy numbers detected correlate directly with the contained fish cells. These facts result in a subjective lower limit of detection compared to systems using mitochondrial genes. The advantage of this system, however, is the possibility to generate quantitative results. Another benefit of the developed real-time PCR system compared to the method of Benedetto et al. is that the CTfish-system can be used to detect fish in an animal DNA background.

Robustness

The robustness of a method is its capability to remain unaffected by small, randomly variations in the test conditions [24]. The altered variations comprising the type of instrument, the master mix, primer and probe concentration and annealing temperature are assessed by using an orthogonal design. The robustness test did not show any significant discrepancies (Table 7). The *C*q values of the single reactions only vary slightly. It can be concluded that the CTfish-system is robust.

Conclusion

The developed real-time PCR method is able to detect the presence of organisms belonging to the Teleostei in food. Teleostei represent the largest group within the taxonomy of fish, containing most of the edible fish, and cover the economically relevant species. Further analyses may be required to enable also the detection of fish spices of minor importance, like sturgeons, sharks and rays (cartilaginous fish). The method fulfils all requirements concerning the specificity, the sensitivity and the robustness defined by the "Guidelines for the single-laboratory validation of

qualitative real-time PCR methods" of the German Federal Office of Consumer Protection and Food Safety (BVL) [24].

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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