

Second intron in the protein-coding region of the fish parvalbumin gene – a promising platform for polymerase chain reaction-based discrimination of fish meat of various species

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Summary

An end-point polymerase chain reaction (PCR) for the discrimination of meat of various fish species is presented. The method is based on sequences of highly diverse intron regions within the parvalbumin gene. A procedure leading to such a sequence was developed through the use of a bioinformatic approach. A matrix describing the variability of DNA base pairs in each position of exons was constructed using available fish parvalbumin cDNA sequences, and this was used as an instrument for designing species-independent degenerate primers. Intron sequences suitable for designing species-specific primers were obtained from amplicons produced by PCR initiated by the degenerate primers. Pairs of primers designed to anneal within the second intron in the protein-coding region of the parvalbumin gene led to PCR stringent enough to distinguish black seabream (*Spondyliosoma cantharus*) from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).

Keywords

polymerase chain reaction; fish species identification; parvalbumin gene; sequence alignment; Black seabream; Atlantic salmon; Rainbow trout

Fish meat represents a considerable amount of the food consumed in coastal areas and countries. Even though the proportion is lower in landlocked states, it is increasing due to the known positive benefits of the consumption of fish on the health of the population. However, the protein parvalbumin, which is abundant in fish meat, can trigger an allergic reaction in sensitive individuals [1, 2]. In addition to other factors, the severity of the allergic reaction depends on the fish species [1, 3, 4]. Since a substantial amount of fish imported to landlocked countries comes in the form of frozen blocks of compressed meat or fillets, it is impossible to identify the particular fish species based on morphological traits [5]. Therefore, it is of great importance to have other tools for the identification of various fish species. Protein-based analytical methods for the determination of fish species are available, such as isoelectric focus-

ing [6], urea isoelectric focusing [7] or capillary electrophoresis [8]. Immunochemical methods are used as well [9]. Polymerase chain reaction (PCR)-based methods for food analysis have become widespread in recent years [10]. These include methods for identification of plant [11] or animal species in food [5, 12]. An important requirement for the development of PCR-based methods is knowledge of a characteristic DNA sequence unique to the particular taxon.

A scheme of the parvalbumin gene in fish can be drawn on the basis of that of mackerel (*Scomber japonicus*) parvalbumin gene sequence [12] (Fig. 1). The protein-coding part is composed of four exons, separated in a unique way by three introns. The length of the second intron seems to make it the most suitable for designing PCR for discrimination of fish species.

Only a few sequences of the parvalbumin gene

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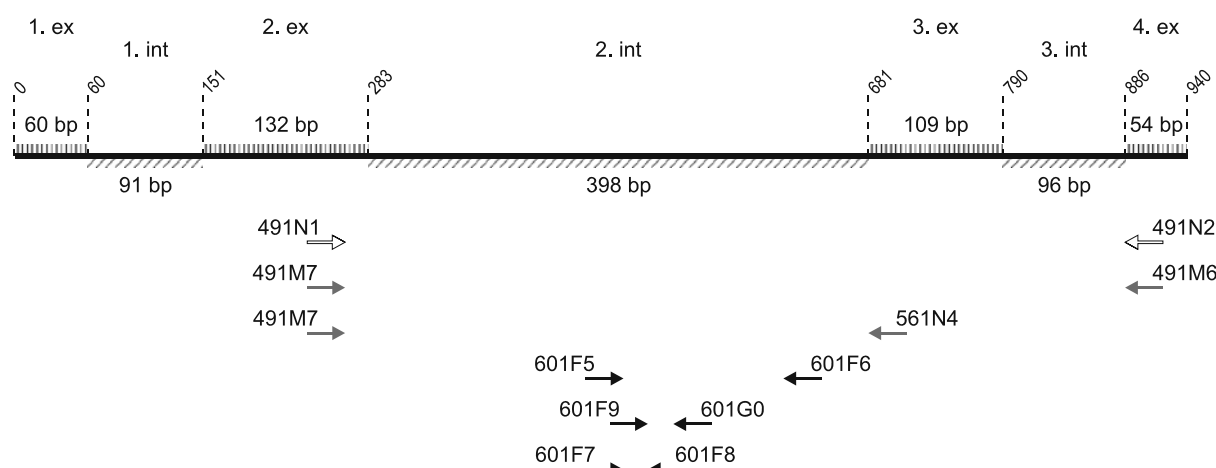


Fig. 1. Scheme of a protein-coding part of the fish parvalbumin gene based on mackerel (*Scomber japonicus*) parvalbumin gene sequence.

Second intron in this protein-coding part is most suitable for designing species-specific primer sets for discrimination of particular species. Black arrows symbolize these primers. Degenerate primers used to obtain amplicons usable for sequencing are symbolized by grey arrows. White arrows symbolize set of Atlantic salmon-specific primers used as a control of specificity of priming by one of the sets of degenerate primers. Exons are marked in the scheme as “ex”, introns as “int”.

or cDNA of commonly imported fish is available in the literature [13–15]. However, a homology search can provide a number of fish parvalbumin cDNA sequences. Based on *in silico* bioinformatic alignment of such sequences, degenerate primers spanning selected fragments of the gene can be designed. Such primers would initiate PCR amplification of templates derived from meat of various fish, leading to amplicons suitable for sequencing. This is facilitated by degenerate bases within the primer sequence on positions that are not conserved among various fish species. Degenerate base placed in such position pairs with all antiparallel bases present among various species-specific variants of the gene. Particular degenerate primer is a mixture of all respective combinatorial variants of oligonucleotide sequences. In such a way, degenerate primers are species-independent. Information on DNA sequences obtained from amplicons obtained by PCR with these primers contains highly homologous coding regions of the gene, but also quite diverse introns.

In this study, we designed species-specific primers within the region of the second intron in the protein-coding sequence of the parvalbumin gene. We demonstrated the capability of PCR initiated by the primers to distinguish black sea-bream (synonym, black bream) (*Spondyliosoma cantharus*) from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*).

MATERIALS AND METHODS

BLAST search in public DNA sequence databases

Fish parvalbumin cDNA sequences were obtained by a BLAST search in the databases of NCBI (National Center for Biotechnology Information, Bethesda, Maryland, USA) and the Wellcome Trust Sanger Institute (Hixton, United Kingdom). Salmon (accession numbers X97824 and X97825), mackerel (accession number FM994926) and carp (accession number AJ292212) parvalbumin sequences were used as a query [13–15]. A total of 20 fish parvalbumin cDNA or genomic sequences were obtained.

Bioinformatic processing

Alignment was done using the BioEdit Sequence Alignment Editor, version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, California, USA) with the ClustalW Multiple alignment function. The aligned cDNA sequences were fit to the pattern of the parvalbumin gene using the genomic sequence from mackerel (*Scomber japonicus*) (accession number AB091470) as a template [12].

Primer design

The designed primers were composed of two parts – the first corresponding to an antiparallel sequence of the template, the second serving as a template for sequencing and/or second-step

PCR. Second-step PCR was employed for further amplification of the product prior to sequencing. Degeneracies were incorporated in species-independent primers at positions where variability of bases was present in the twenty aligned sequences (Tab. 1). Inosine was used in degenerate primers in each case when the corresponding position in the alignment was represented by all four bases (A, T,

G, C). In this way, the number of species of oligomers included in the primer was kept to a minimum. Pairs of Atlantic salmon-specific primers were used with such a template as a control to the degenerate ones. Sequences of degenerate primers and the equivalent Atlantic salmon-specific ones are presented in Tab. 2. Tab. 3 overviews primers used for species discrimination. The length of the

Tab. 1. Alignment of the sequences of the parvalbumin gene region.

| Species | Accession number | Sequence |
|--------------------------------|------------------|--|
| <i>Gadus morhua</i> clone 1 | AY035584 | GACAAGAGTGGATTTCATTGAGG |
| <i>Gadus morhua</i> clone 2 | AY035585 | GACAAGAGTGACTTTGTTGAGG |
| <i>Theragra chalcogramma</i> | AY035586 | GACCAGAGCGGCTTCATTGAGG |
| <i>Lates calcarifer</i> | AY688372 | GACAAGAGTGGCTTCATTGAGG |
| <i>Rivulus marmoratus</i> | AY682949 | GACAAGAGTGGCTTCATTGAGG |
| <i>Sardinops melanostictus</i> | AB375262 | GACAAGAGTGGCTTCATTGAGG |
| <i>Sardinops sagax</i> | FM177701 | GACAAGAGTGGCTTCATTGAGG |
| <i>Ictalurus punctatus</i> | AF227795 | GACAAGAGTGGCTTCATTGAGG |
| <i>Oncorhynchus kisutch</i> | FN555150 | GATGCAAGTGGCTTTATTGAGG |
| <i>Trachurus japonicus</i> | AB211364 | GACAAGAGCGGCTTCATTGAGG |
| <i>Gasterosteus aculeatus</i> | BT028550 | GACAAGAGTGGCTTCATTGAGG |
| <i>Cyprinus carpio</i> | AJ292212 | GACAAGAGTGGCTTCATTGAGG |
| <i>Danio rerio</i> | BC0648961 | GACAAGAGTGGCTTCATTGAGG |
| <i>Salvelinus alpinus</i> | AF538282 | GACAAGAGTGGCTTCATTGAGG |
| <i>Salmo salar</i> „14.1“ | FN544080 | GATGCAAGTGGCTTTATTGAGG |
| <i>Salmo salar</i> „24.1“ | X97825 | GACAAGAGTGCCTTCATTGAGG |
| <i>Scomber japonicus</i> | AB091470 | GACAAGAGCGGCTTCATTGAGG |
| <i>Rivulus marmoratus</i> | AY682950 | GACAAGAGCGGCTTCATTGAGG |
| <i>Fundulus heteroclitus</i> | FJ696958 | GACAAGAGTGGCTTCATTGAGG |
| <i>Boreogadus saida</i> | FJ696956 | GACAAGAGCGGCTTCATTGAGG |
| Primer 491M7 | | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> GAYVMRAGYGRMTTYRTTGAGG |

The aligned sequences are reflected in the composition of the degenerate primer. The part of the primer used as a tag for sequencing or for second-step PCR is underlined.

Tab. 2. Overview of primers used in various fish species to amplify gene fragments.

| Primer | Species | Primer sequence | Primer length without tag [bp] | Primer length with tag [bp] |
|---------------|---------------------|--|--------------------------------|-----------------------------|
| 491M7 forward | species independent | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> GAYVMRAGYGRMTTYRTTGAGG | 22 | 52 |
| 561N4 reverse | species independent | <u>TTTACGACATAGGGAGCAGCTTACTATTCT</u> AAGDYCTGVAGGAASAG | 17 | 47 |
| 491M6 reverse | species independent | <u>TTTACGACATAGGGAGCAGCTTACTATTCT</u> YTADISYTTVAYMADDDBDIYVMA | 24 | 54 |
| 491N1 forward | Atlantic salmon | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> GACAAGAGTGCCTTCATTGAGG | 22 | 52 |
| 491N2 reverse | Atlantic salmon | <u>TTTACGACATAGGGAGCAGCTTACTATTCT</u> TTATCCCTTGATCATGGCAGCGAAC | 25 | 55 |

The part of the primer used as a tag for sequencing or for second-step PCR is underlined.

Tab. 3. Second intron species-specific PCR primers, lengths and expected amplicon lengths.

| Primer | Species | Primer sequence | Amplicon length without tag [bp] | Amplicon length with tags [bp] | Primer length without tag [bp] | Primer length with tag [bp] |
|---------------|-----------------|--|----------------------------------|--------------------------------|--------------------------------|-----------------------------|
| 601F5 forward | Atlantic salmon | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> GAATGTTCCCTTTATAGGGCT | 126 | 186 | 21 | 51 |
| 601F6 reverse | Atlantic salmon | TTTACGACATAGGGAGCAGCTTACTATTCT <u>GGAGGGGACTCTATTTTG</u> | | | 18 | 48 |
| 601F7 forward | Black seabream | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> CACTCAGGAAATGCACAC | 79 | 139 | 18 | 48 |
| 601F8 reverse | Black seabream | TTTACGACATAGGGAGCAGCTTACTATTCT <u>GTAAGTGTGTAACCAAGTTC</u> | | | 20 | 50 |
| 601F9 forward | Rainbow trout | <u>AGACAGAGACACAGGTTGGCTTACTATTCT</u> CCTACCCCTCTGTAGGGAAC | 75 | 135 | 20 | 50 |
| 601G0 reverse | Rainbow trout | TTTACGACATAGGGAGCAGCTTACTATTCT <u>CTACTCATATTTTGGGGGAG</u> | | | 20 | 50 |

The part of the primer used as a tag for sequencing or for second-step PCR is underlined.

amplicons, identified by the primers' positions on the template, was compared to the length based on the mobility of the PCR product in agarose gel electrophoresis. All primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic).

Samples of fish meat and taxonomical determination

Fish samples were purchased in food shops in Prague, Czech Republic. Collection of exemplars of each species was usually composed of two separate shoppings made at different time and in different supermarket chains. One of these shoppings represented a major part of the collection, the other one minor. Taxonomical identity of the meat was taken from the label. In parallel, taxonomical identification of each used exemplar was accomplished according following traits:

Oncorhynchus mykiss (Salmoniformes, Salmonidae)

Common name: Rainbow trout

Diagnosis: wide, pink to red stripe from head to caudal base (except in sea-run form), rays in D (dorsal fin) 10–12, A (anal fin) 8–12, V (ventral fin) 9–10, 115–130 total scales in midlateral row, 16–17 (16–22) gill rakers, breeding males without hump, juveniles with 5–10 parr marks, size up to 1000 mm SL (standard length) [16, 17].

Salmo salar (Salmoniformes, Salmonidae)

Common name: Atlantic salmon

Diagnosis: distinguished from other species of *Salmo* in Atlantic, North, White, Barents and Baltic Sea basins by: 10–13 scales between end of adipose base and lateral line, rays in D 10–12,

A 8–11, V 9–10, 109–121 total scales in midlateral row, lateral line decurved anteriorly, then straight, 17–24 (15–20) gill rakers. Additional characters distinguish salmon parrs from trouts: caudal deeply forked in individuals smaller than 200 mm SL, hyaline or adipose margin. Additionally, adult salmonids are distinguished from sea trout by: posterior part of vomer toothless, size up to 1000 mm [16, 17].

Spondyliosoma cantharus (Perciformes, Sparidae)

Common name: Black bream

Diagnosis: body deep, small head, maxilla reached anterior margin of eye, back grey, sides silver-grey usually with six or seven dark vertical stripes, fins grey with dark margin of caudal, rays in D XI, 11–13, A III, 8–10 (9–11), V I, 5, 66–75 lateral line scales, size up to 600 mm SL, in mean 20–30 mm SL [18–20].

When aquarium fish species were used as a control of specificity, fresh cadavers were collected from amateur breeders and frozen. DNA was isolated subsequently in the same way as from the food fish meat.

DNA extraction and quantification

The fish meat was homogenized in a mortar. DNA was isolated by non-chaotropic solid phase extraction (SPE) using Invisorb Spin Food Kit I (Invitek, Berlin, Germany). DNA was quantified spectrophotometrically at 260 nm using BioPhotometer 6131 (Eppendorf, Hamburg, Germany).

Polymerase chain reaction (PCR)

PCR was performed in a Touchgene Techne

gradient cycler (Techne, Cambridge, United Kingdom) in a total volume of 50 μl , containing 5 μl of template DNA, 500 $\text{nmol}\cdot\text{l}^{-1}$ of each species-specific primer or 4.2 $\mu\text{mol}\cdot\text{l}^{-1}$ of each degenerate primer, 200 $\mu\text{mol}\cdot\text{l}^{-1}$ dNTP mix (Eppendorf), 1.5 $\text{mmol}\cdot\text{l}^{-1}$ MgCl_2 (Invitrogen, Carlsbad, California, USA), 2.5 U Platinum Taq DNA polymerase (Invitrogen), 5 μl of 1x PCR buffer (Invitrogen).

The PCR amplifications were carried out under the following conditions: denaturation step at 94 °C for 5 min, followed by 45 and 28 cycles for degenerate primers and for species-specific primers, respectively. One cycle consisted of 1 min denaturation at 94 °C, 1 min annealing at 47 °C and 1 min extension at 72 °C. The final extension step was 10 min at 72 °C.

Electrophoresis, sequencing

Twenty or ten microliters of PCR product and 4 μl or 2 μl of loading buffer EliPhore (Elisabeth Pharmacon, Brno, Czech Republic) were mixed and loaded onto 2% Serva Premium agarose gel (Serva Electrophoresis, Heidelberg, Germany) containing 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ ethidium bromide, then the electrophoresis was run in TBE (Tris/borate/EDTA) buffer at 110 V for 100 min. The DNA bands were observed under ultraviolet light with a transilluminator (Herolab UVT-20, Wiesloch, Germany), compared with 50 bp molecular weight DNA ladder N3236 (New England BioLabs, Ipswich, Massachusetts, USA) with highlighted bands at 200 bp and 500 bp, and photodocumented with a Kodak digital camera and Kodak 1D software (Eastman Kodak, Rochester, New York, USA).

Selected DNA fragments of PCR products were extracted from the agarose gel using a DNA recovery chip TaKaRa RECOCHIP (Takara Bio, Otsu, Japan). The purified PCR products were sequenced on a commercial base by Geneti Biotech.

RESULTS AND DISCUSSION

By using the BLAST algorithm, twenty DNA sequences of the protein-coding region or cDNA of the parvalbumine gene from 18 different fish species were obtained by searching in DNA databases. The multiple alignment of these sequences enabled the design of degenerate, hence species-independent, primers (Tab. 1). Fragments of the gene defined by these primers were PCR-amplified in various fish species and subsequently sequenced. Information on the sequences of the region spanning second intron in parvalbumin gene of black seabream (*Spondyliosoma cantharus*,

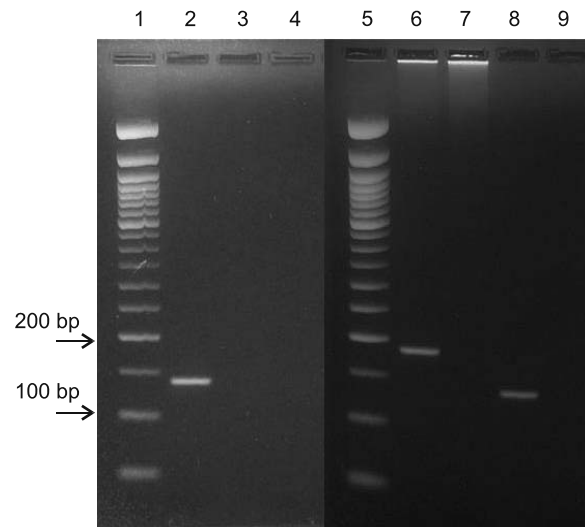


Fig. 2. Electrophoretic analysis of products of species-specific PCR.

Lanes: 1 – molecular weight standard, 2 – Black seabream, black seabream-specific primers (601F7 and 601F8), 3 – Black seabream, Atlantic salmon-specific primers (601F5 and 601F6), 4 – Black seabream, Rainbow trout-specific primers (601F9 and 601G0), 5 – molecular weight standard, 6 – Atlantic salmon, Atlantic salmon-specific primers (601F5 and 601F6), 7 – Atlantic salmon, Black seabream-specific primers (601F7 and 601F8), 8 – Rainbow trout, Rainbow trout-specific primers (601F9 and 601G0), 9 – Rainbow trout, black seabream-specific primers (601F7 and 601F8).

accession number JN671445), Atlantic salmon (*Salmo salar*, accession number JN671447) and rainbow trout (*Oncorhynchus mykiss*, accession number JN671446) was obtained. These sequences were composed of both exons and introns. Due to only minimal selection during the evolutionary process, intron sequences are highly diverse, and are therefore suitable for designing species-specific sets of primers. Using pairs of primers designed within the second intron in the protein-coding region of the parvalbumin gene of black seabream (*Spondyliosoma cantharus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), made it possible to distinguish black seabream from the two remaining fish species (Fig. 2).

A positive result was obtained for the sample of black seabream, a DNA fragment being amplified by primers specific for this species (601F7 – forward and 601F8 – reverse). The electrophoretic mobility of the product corresponded to a length of 139 bp, established according to the positions of both primers within the sequence of the second intron. Similar results were obtained when Atlantic salmon and rainbow trout genomic DNA were

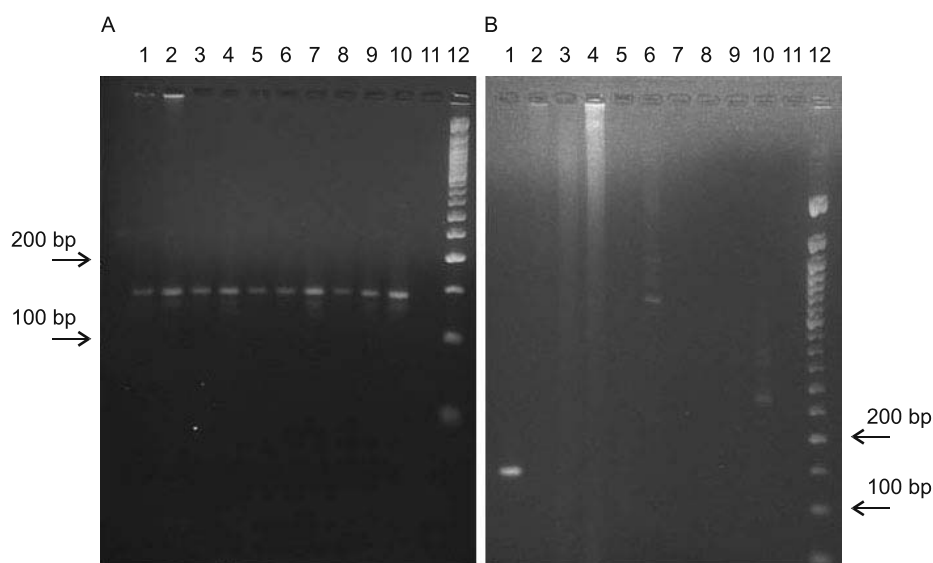


Fig. 3. PCR with black seabream species-specific primers (601F7 and 601F8).

A. Intra-species robustness of the method in the species of black seabream.

Lanes: 1–10 – different black seabream individuals, 11 – empty, 12 – molecular weight standard.

B. Specificity of the method tested on DNA from black seabream and from a number of other fish species.

Lanes: 1 – Black seabream (*Spondyliosoma cantharus*), 2 – Greenland halibut (*Reinhardtius hippoglossoides*), 3 – Guppy (*Poecilia reticulata*), 4 – Dwarf puffer (*Tetraodon travancoricus*), 5 – Common carp (*Cyprinus carpio*), 6 – Crucian carp (*Carassius carassius*), 7 – Zebrafish (*Brachidanio rerio*), 8 – Tench (*Tinca tinca*), 9 – Atlantic bluefin tuna (*Thunnus thynnus*), 10 – Silver carp (*Hypophthalmichthys molitrix*), 11 – Northern pike (*Esox lucius*), 12 – molecular weight standard.

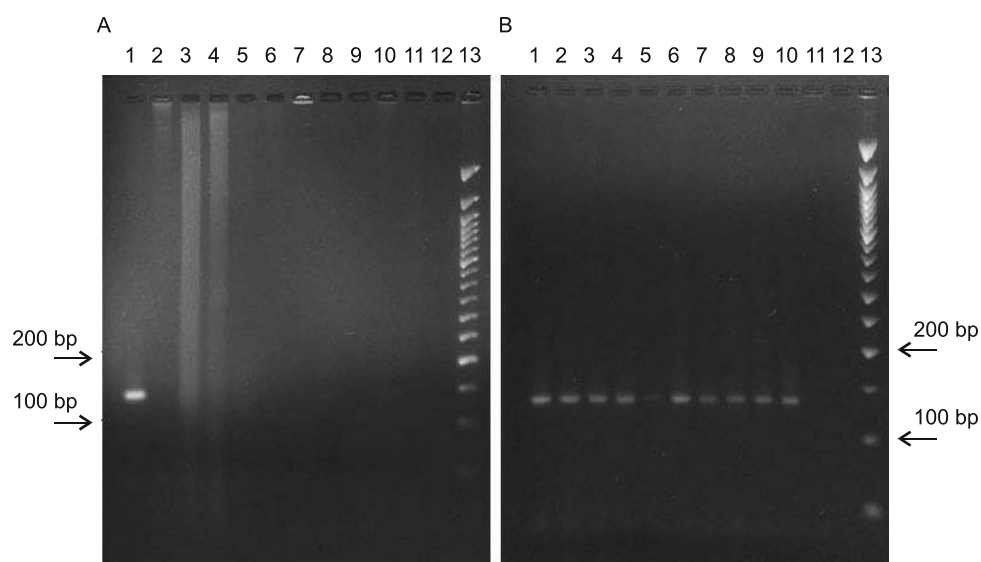


Fig. 4. PCR with rainbow trout species-specific primers (601F9 and 601G0).

A. Specificity of the method tested on DNA isolates from Rainbow trout and from a number of other fish species.

Lanes: 1 – Rainbow trout (*Oncorhynchus mykiss*), 2 – Greenland halibut (*Reinhardtius hippoglossoides*), 3 – Guppy (*Poecilia reticulata*), 4 – Dwarf puffer (*Tetraodon travancoricus*), 5 – Common carp (*Cyprinus carpio*), 6 – Crucian carp (*Carassius carassius*), 7 – Zebrafish (*Brachidanio rerio*), 8 – Tench (*Tinca tinca*), 9 – Atlantic bluefin tuna (*Thunnus thynnus*), 10 – Silver carp (*Hypophthalmichthys molitrix*), 11 – Northern pike (*Esox lucius*), 12 – empty, 13 – molecular weight standard.

B. Intra-species robustness of the method in the species of Rainbow trout.

Lanes: 1–10 – PCR with different rainbow trout individuals, 11–12 – empty, 13 – molecular weight standard.

amplified by their respective sets of primers. The electrophoretic mobilities in both cases confirmed the predicted length of the product – 186 bp for Atlantic salmon and 135 bp for rainbow trout. In accordance with the results of black seabream-specific PCR, assays specific for Atlantic salmon and for rainbow trout produced negative results when tested with black seabream-specific primers (Fig. 2).

Another set of experiments was accomplished to examine the robustness of the method in respect of the possible intra-species variability. DNA isolates from ten different individuals of black seabream were used as a template for PCR with species-specific primers of this fish (Fig. 3A).

Also, the specificity of black seabream-specific primers was tested in a broader setup when a panel of other fish was used (Fig. 3B). The results show that only the template from black seabream produced a positive signal (amplicon of 139 bp). No PCR product was detected all with other fish species. The specificity of primers specific for rainbow trout and Atlantic salmon was tested against the same panel of fish. Also in these experiments, species-specific primers were able to distinguish the species from all the other fish species from the panel (Fig. 4A, Fig. 5).

Also for rainbow trout, the intra species variability and robustness of the method were assessed. PCR amplicons of the corresponding length were obtained with 10 exemplars of rainbow trout, showing that also in this fish species the method was versatile in terms of intra-species variability (Fig. 4B).

Taken together, these results confirm the usefulness of the second intron from the protein-coding region of the parvalbumin gene as a platform for the development of species-specific PCR for fish species identification.

The bioinformatic approach that was chosen to bypass the initial lack of sequential information of particular fish species proved to be effective. The alignment of 20 fish sequences of the parvalbumin gene was representative enough to serve as a model for the variability of the sequence among different fish species. Thus, it could be used as an instrument for the design of degenerate primers. In addition, degenerate primers provide a versatile tool for the relatively rapid development of PCR-based methods for the detection of fish species being introduced as new commodities on the market. A decreased affinity of degenerate primers to the template is the main disadvantage compared to full-homology priming of PCR. As a consequence, increased level of background products is quite common in PCR with degenerate primers

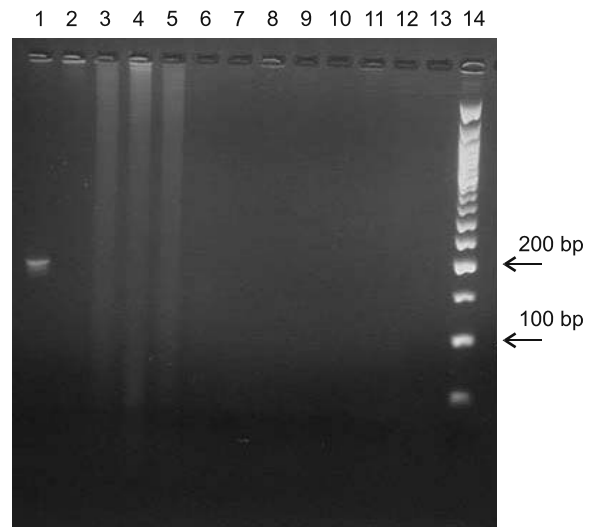


Fig. 5. PCR with Atlantic salmon-specific primers (601F5 and 601F6) on DNA from Atlantic salmon and from a number of other fish species, used to test specificity.

Lanes: 1 – Atlantic salmon (*Salmo salar*), 2 – Greenland halibut (*Reinhardtius hippoglossoides*), 3 – Guppy (*Poecilia reticulata*), 4 – Dwarf puffer (*Tetraodon travancoricus*), 5 – Common carp (*Cyprinus carpio*), 6 – Crucian carp (*Carassius carassius*), 7 – Zebrafish (*Brachidanio rerio*), 8 – Tench (*Tinca tinca*), 9 – Atlantic bluefin tuna (*Thunnus thynnus*), 10 – Silver carp (*Hypophthalmichthys molitrix*), 11 – Northern pike (*Esox lucius*), 12–13 – empty, 14 – molecular weight standard.

and thorough optimization of reaction conditions is required as a counter-measure. Also, touch-down setup of PCR is sometimes recommended to address this problem.

Further research is needed to discover if taxonomical distance or proximity plays a role in the sensitivity of the method. Only one pair of primers was needed to distinguish black seabream from two reference fish species and also to discriminate between black seabream and a panel of ten other fish species. The employment of sets of primers might provide an advantage in the discrimination of closely related species.

Pilot experiments with Atlantic salmon and rainbow trout confirmed the knowledge obtained with black seabream.

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