

Multiplexed identification of different fish species by detection of parvalbumin, a common fish allergen gene: a DNA application of multi-analyte profiling (xMAP™) technology

Sabine Hildebrandt

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Abstract Fish are a common cause of allergic reactions associated with food consumption, with parvalbumin being the major allergenic protein. Some fish-hypersensitive patients tolerate some fish species while being allergic to others. Reliable detection methods for allergenic fish species in foods are necessary to ensure compliance with food allergen labeling guidelines to protect fish-allergic consumers. The objective of this project was to develop a multi-analyte detection method for the presence of fish in food. Therefore, conserved parvalbumin exon sequences were utilized for the design of universal PCR primers amplifying intron DNA and small regions of exons flanking the enclosed intron from even very distantly related fish species. An assay for the identification of eight fish species was developed using xMAP™ technology with probes targeting species-specific parvalbumin intron regions. Additionally, a universal fish probe was designed targeting a highly conserved exon region located between the intron and the reverse primer region. The universal fish assay showed no cross-reactivity with other species, such as beef, pork, lamb, chicken, turkey, and shrimp. Importantly, with the exception of one notable case with fish in the same subfamily, species-specific detection showed no cross-

reactivity with other fish species. Limits of detection for these eight species were experimentally estimated to range from 0.01% to 0.04%, with potential to increase the detection sensitivity. This report introduces a newly developed method for the multiplex identification of at least eight allergenic fish species in food, which could conceivably be extended to detect up to 100 species simultaneously in one sample.

Keywords Fish allergy · Parvalbumin · PCR · Universal primer · xMAP

Introduction

Fish is one of the eight “major allergens” that are believed to account for more than 90% of all food allergies [1, 2]. The prevalence of fish allergies in the United States has been estimated to be 0.4% [3–5]. The clinical symptoms related to fish allergy are manifested in a variety of symptoms, ranging from mild (e.g., rash) to severe (anaphylaxis), which could culminate with death [6, 7]. Parvalbumin, a calcium-binding protein with low molecular weight (about 12 kDa), has been found to be the major allergen in fish [8, 9]. It is present in all vertebrates, including humans, but at higher quantities in the muscles of lower vertebrates, such as fish [8–10]. Parvalbumin is known as a cross-reactive allergen in different fish species, such as cod, salmon, carp, mackerel, and bigeye tuna [4, 6, 11–13]. Structural homology of parvalbumin in different fish species is believed to be responsible for the general cross-reactivity in fish-allergic individuals [14]. Nevertheless, some fish-hypersensitive patients can tolerate some fish species while being allergic to others [3, 5, 15, 16].

S. Hildebrandt
U.S. Food and Drug Administration, Center for Food Safety and
Applied Nutrition, Office of Regulatory Science,
Division of Bioanalytical Chemistry,
5100 Paint Branch Parkway,
College Park, MD 20740, USA

S. Hildebrandt (✉)
FDA, CFSAN,
HFS-716, 5100 Paint Branch Parkway,
College Park, MD 20740, USA
e-mail: s-hildebrandt@gmx.net

The Food Allergen Labeling and Consumer Protection Act of 2004 (P.L. 108-282, FALCPA) mandates that food products containing any type (tuna, salmon, etc.) or quantity of fish must be properly labeled, including the species name. Therefore, a sensitive method for detection of fish species in food products is necessary to ensure compliance with allergen food labeling regulations for consumer safety and protection. Almost all available fish parvalbumin DNA amplification methods use specific primers targeting only one fish species [17–19]. An assay developed by Sun et al. [20] detects parvalbumin DNA from several different fish using one set of primers, although it does not differentiate among the species. Presently, no method (commercial or non-commercial) is available that identifies several different allergenic fish species simultaneously in one assay.

Previous reports showed that the parvalbumin protein can be encoded by different gene isoforms that contain the same number of exons but are comprised of introns that differ in size and show nucleotide variability. Each analogous exon contains an identical number of nucleotides in all the paralogs [21–23]. The major objective of this project was to adopt conserved parvalbumin exon sequences to design universal PCR primers that amplify a species-specific intron, as well as regions of the exons flanking the intron, from even very distantly related fish species, so that the identification of the fish species could be achieved using probes targeting a species-specific intron region. Typically, probe-based PCR methods, such as TaqMan, are limited in their ability to detect more than five targets per sample in one well/tube, because of the limited number of filters in the instrument. To avoid this limitation, in this study, a fairly new and more multiplex-based technology was applied that uses the combination of the Luminex 100 flow analyzer and the MultiAnalyte Profiling (xMAP™) technology (Luminex, TX, USA, reviewed by Dunbar [24]). The technique takes advantage of 100 distinct sets of color-coded beads (microspheres) that can be coated with a specific capture probe, allowing the discrimination of up to 100 specific targets in one assay within a single reaction.

This report introduces a newly developed method for the identification of at least eight allergenic fish species in food using PCR for amplification of fish parvalbumin introns followed by xMAP™ for targeting species-specific sequences.

Experimental

Materials

Frozen samples of 49 fish species representing 40 genera, 27 families, and nine taxonomic orders were obtained from

and authenticated by the Smithsonian Institution (Washington, DC; see Table 1). Other samples, such as beef, pork, lamb, chicken, turkey, shrimp, and vegetable soup were obtained from a local supermarket and used for the analysis of assay specificity.

DNA extraction and quantitation

Test samples were prepared by homogenization in a laboratory blender (Waring, CT, USA). DNA was extracted from 30 mg of homogenized portions using the MasterPure™ DNA purification kit, as described for isolation of total nucleic acid from tissue samples (Epicentre Biotechnologies, WI, USA). The DNA pellets were resuspended in 20 µL ultrapure DNase/RNase-free distilled water and quantified spectrophotometrically with a Tecan infinite M200 Spectrophotometer and the i-control™ software (Tecan US Inc., NC, USA, Version 1.6).

Amplification of DNA sequences

Parvalbumin-encoding DNA was amplified with real-time PCR (Stratagene MX3005P, Agilent, USA) employing primers IFF 232 and IFF 233 described by Rehbein and Kress [17].

The real-time PCR assay contained 500 nM of each primer, 15 µL of Absolute™ Blue QPCR SYBR® Green Low ROX Mix (ABgene® Ltd, UK), and up to 1 µg of DNA template in a total volume of 30 µL. Amounts between 1 ng and 1 µg of total DNA were tested with the higher concentrations providing better results for complex food samples containing low amounts of fish. The PCR samples were heated at 95 °C for 15 min, followed by 40 cycles of 30 s/95 °C, 60 s/60 °C, and 45 s/72 °C. Amplification was monitored with a dissociation curve program of 1 min/95 °C, 30 s/55 °C with measurement performed while increasing to 95 °C. This helps to ensure that the correct product is being amplified. Amplicons were purified using the QIAquick PCR purification microcentrifuge protocol (Qiagen, CA, USA) according to the manufacturer's protocol and eluted with 15 µL of ultrapure water. One microliter of the purified amplicons were analyzed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent, Germany) with the DNA 7500 assay protocol.

DNA cloning and sequencing

Molecular cloning of parvalbumin DNA was accomplished by direct insertion of amplified PCR products into a plasmid vector using the TOPO TA Cloning® Kit for Sequencing (Version O, 10 April 2006, 25-0276, Invitrogen, CA, USA) following the manufacturer's protocol for

Table 1 Names and categories of fish samples studied

No.	Common name	Scientific Name		
		Genus / Species	Family	Order
1	Red snapper	<i>Lutjanus campechanus</i>	Lutjanidae	Perciformes
2	Gray snapper	<i>Lutjanus griseus</i>	Lutjanidae	Perciformes
3	Dog snapper	<i>Lutjanus jocu</i>	Lutjanidae	Perciformes
4	Yellow tail snapper	<i>Ocyurus chrysurus</i>	Lutjanidae	Perciformes
5 ^a	Vermillion snapper	<i>Rhomboplites aurorubens</i>	Lutjanidae	Perciformes
6	King mackerel	<i>Scomberomorus cavalla</i>	Scombridae	Perciformes
7	Spanish mackerel	<i>Scomberomorus maculatus</i>	Scombridae	Perciformes
8	Yellow fin tuna	<i>Thunnus albacares</i>	Scombridae	Perciformes
9	Black fin tuna	<i>Thunnus atlanticus</i>	Scombridae	Perciformes
10	Wahoo	<i>Acanthocybium solandri</i>	Scombridae	Perciformes
11	Sand sea trout	<i>Cynoscion arenarius</i>	Sciaenidae	Perciformes
12	Speckled trout	<i>Cynoscion nebulosus</i>	Sciaenidae	Perciformes
13 ^a	Spot	<i>Leiostomus xanthurus</i>	Sciaenidae	Perciformes
14 ^a	Atlantic croaker	<i>Micropogonias undulatus</i>	Sciaenidae	Perciformes
15	Blue runner	<i>Caranx crysos</i>	Carangidae	Perciformes
16	Almaco jack	<i>Seriola rivoliana</i>	Carangidae	Perciformes
17	Pompano	<i>Trachinotus carolinus</i>	Carangidae	Perciformes
18	White perch	<i>Morone americana</i>	Moronidae	Perciformes
19 ^a	Striped bass	<i>Morone saxatilis</i>	Moronidae	Perciformes
20	Hybrid striped bass	<i>M. saxatilis</i> x <i>M. chrysops</i>	Moronidae	Perciformes
21	White bone porgy	<i>Calamus leucosteus</i>	Sparidae	Perciformes
22 ^a	Spottail pinfish	<i>Diplodus holbrooki</i>	Sparidae	Perciformes
23	Red porgy	<i>Pagrus pagrus</i>	Sparidae	Perciformes
24	Longtail wrasse	<i>Hemanthisa leptus</i>	Serranidae	Perciformes
25	Gag grouper	<i>Mycteroperca microlepis</i>	Serranidae	Perciformes
26	Pomfret	<i>Brama dussumiere</i>	Bramidae	Perciformes
27	Barrelfish	<i>Hyperoglyphe bythites</i>	Centrolophidae	Perciformes
28 ^a	Nile tilapia	<i>Oreochromis nilotica</i>	Cichlidae	Perciformes
29	Spadefish	<i>Chaetodipterus faber</i>	Ephippidae	Perciformes
30	Bermuda chub	<i>Kyohosus sectatrix</i>	Kyphosidae	Perciformes
31	Triple tail	<i>Lobotes surinamensis</i>	Lobotidae	Perciformes
32	Goldenfau tilefish	<i>Caulolatilus chrysops</i>	Malacanthidae	Perciformes
33	Bluefish	<i>Pomatomus saltatrix</i>	Pomatomidae	Perciformes
34	Cobia	<i>Rachycentron canadum</i>	Rachycentridae	Perciformes
35	Gray triggerfish	<i>Balistes capricus</i>	Balistidae	Tetraodontiformes
36	Spotted triggerfish	<i>Canthidermis maculata</i>	Balistidae	Tetraodontiformes
37	Puffer	<i>Lagocephalus laevigatus</i>	Tetraodontidae	Tetraodontiformes
38	Ladyfish	<i>Elops saurus</i>	Elopidae	Elopiformes
39	Tarpon	<i>Megalops</i>	Megalopidae	Elopiformes
40	Gulf flounder	<i>Paralichthys albigutta</i>	Bothidae	Pleuronectiformes
41 ^a	Southern flounder	<i>Paralichthys lethostigma</i>	Paralichthyidae	Pleuronectiformes
42	Southern stingray	<i>Dasyatis americana</i>	Dasyatidae	Rajiformes
43	Atlantic stingray	<i>Dasyatis sabina</i>	Dasyatidae	Rajiformes
44	Sockeye salmon	<i>Oncorhynchus nerka</i>	Salmonidae	Salmoniformes
45 ^a	Atlantic salmon	<i>Salmo salar</i>	Salmonidae	Salmoniformes
46	Hardhead catfish	<i>Ariopsis felix</i>	Ariidae	Siluriformes
47	Gafftop Sail catfish	<i>Bagre marinus</i>	Ariidae	Siluriformes
48	Hammerhead shark	<i>Sphyrna tiburo</i>	Sphyrnidae	Carcharhiniformes
49	Ground mullet	<i>Menticirrhus americanus</i>	Mugilidae	Mugiliformes

All fish samples, except Yellowfin tuna, Blackfin tuna, Sockeye salmon, Wahoo, Speckled trout, and Tarpon were authenticated by the Smithsonian Institution (Washington, DC)

^a These species were used for the assay development. All other species were tested for cross-reactions

chemically competent TOP10 One Shot® cells. Positive clones were identified by PCR with T3 and T7 primers (300 nM each), 1× PCR buffer (containing 15 mM MgCl₂), 0.2 mM dNTPs (each), 2.5 units of HotStarTaq polymerase (all PCR components from Qiagen, Germany), and ~100 ng of template DNA in a total volume of 50 µL. The PCR reaction mixtures were incubated at 95 °C for 15 min, followed by 32 cycles of 30 s/95 °C, 45 s/53 °C, and 30 s/72 °C, followed by one cycle of 1 min/72 °C. Positive clone DNA was purified with the Wizard® Plus MidiPrep DNA Purification System (Promega, WI, USA) following the manufacturer's recommendations. Purified DNA was concentrated by ethanol precipitation and resuspended in 50 µl of pure water.

Plasmid constructs containing inserts were sequenced by Amplicon Express (WA, USA) using the T3 and T7 universal sequencing primers. Relevant nucleotide sequences were deposited into the GenBank database (www.ncbi.nlm.nih.gov) with Accession numbers: GU784816–GU784822 (see Table 2).

xMAP™ assay design

Sequence data were aligned using AlleleID® software (Premiere Biosoft, CA, USA) and used to design potential species-specific and universal fish capture probes that were synthesized with a 5'-AmMC12 amine group by Integrated DNA Technologies (IDT, USA). A schematic overview of the xMAP™ assay is shown in Fig. 1. Amine-modified capture oligonucleotides (see Table 3) were covalently coupled to MagPlex™-C magnetic carboxylated microspheres (Luminex Corporation, TX, USA) using a previously described carbodiimide coupling method [25]. Genomic DNA hybridization to the capture oligonucleotides was performed as described by Dunbar [24, 26]. Labeling reactions were incubated at 55 °C for 5 min.

Subsequently, 50 µL aliquots were analyzed on the BioPlex 200 analyzer (BioRad, CA, USA). A negative control was applied with every assay containing all components, except target sample, which was substituted for water. A cutoff value of greater than twice the background fluorescence of the negative control was used to indicate a positive reaction.

Results and discussion

Specificity of PCR primers

DNA from all 49 fish species could be amplified and detected with PCR (results not shown) and capillary electrophoresis (Fig. 2) using the primer set IFF 232/233 developed by Rehbein and Kress to detect parvalbumin DNA in *Gadoid* fish [17]. For most fish, more than one parvalbumin gene isoform was detected with this assay. Similar DNA band patterns were obtained for closely related species, including three species within the *Lutjanus* genera; red, gray, and dog snapper (see Table 1, Nos. 1 to 3) and two species within the *Thunnus* genera (see Table 1, Nos. 8 and 9). There have been conflicting reports regarding parvalbumin protein in some fish. Studies reported the absence of parvalbumin in tuna [20, 27, 28], whereas others have reported the presence of allergenic parvalbumin in tuna [29]. A study by Lim et al. [30] found the presence of parvalbumin in the white muscle but not in the red muscle of tuna. Parvalbumin DNA from both tuna species tested in this study (Nos. 8 and 9) could be amplified from and detected with the universal fish xMAP™ assay, confirming the presence of the parvalbumin gene in these tuna species. While the results do not necessarily prove the presence of the parvalbumin protein, the goal of this study was not to detect allergens, but

Table 2 Capture oligonucleotide design for parvalbumin gene sequences

Fish No.	Probe ^a 5'–3'	Bead No.	% of total bands ^b	Amplicon length (bps)	GenBank Accession No.
5	ATCTTGCTGCGTATCAGTGTTCTTT	52	47	259	GU784816
13	TCGGAATGATGTGCCTCAAAGA	38	80	451	GU784817
14	GACACTCGCTAATGATACTTCACAT	73	69	489	GU784815
19	CTTGAAATGAATGGAACTACACACA	36	72	497	GU784820
22	GTTCTGTGACACTGAGCCGAGG	56	72	379	GU784819
28	TGACTTTCTTATGGATCATTGTTG	45	22	397	GU784821
41	ACATTGACAGTCCAAAAGGAACATTAT	66	45	329	GU784818
45	TGTTTCTTAGAGTGAATGTTCCCTTTA	21	84	417	GU784822
All ^c	AGGCTGTTCTGCAGAACTTCTC	75	–	–	–

^a Capture oligonucleotides were amine-modified (5'-AmMC12 amine group) for covalent coupling to the MagPlex™-C magnetic carboxylated microspheres

^b The percentage of the sequence isoform used for the probe design was calculated with the Bioanalyzer method

^c This probe targets a sequence located on the exon of all tested fish parvalbumin sequences

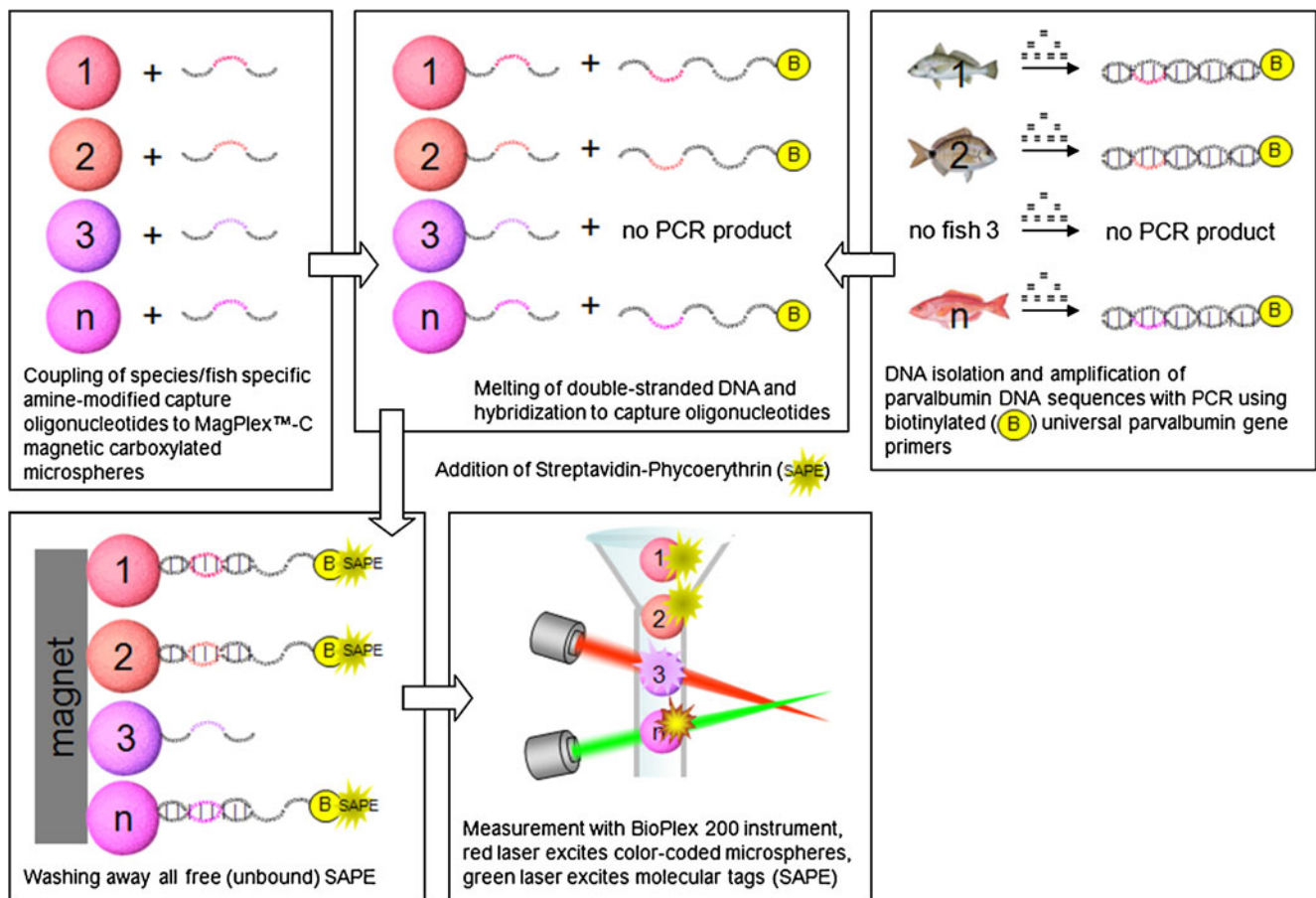


Fig. 1 Schematic overview of the xMAP™ assay. Amine-modified capture oligonucleotides were covalently coupled to color-coded MagPlex™-C magnetic carboxylated microspheres (Luminex Corporation, TX, USA) using a carbodiimide coupling method described by Fulton et al. [25]. Parvalbumin-encoding DNA was amplified with real-time PCR, whereas one of the primers was labeled with biotin. Biotin-labeled DNA strands were hybridized to their corresponding capture oligonucleotides. The fluorophore streptavidin–phycoerythrin (SAPE) was added and bound to the biotin molecules. Reaction tubes containing the magnetic microspheres were put on a magnet, unbound

oligonucleotides and SAPE was removed by washing. The reaction mixture was analyzed on the BioPlex 200 analyzer (BioRad, CA, USA). Microspheres were interrogated individually in a rapidly flowing fluid stream as they passed by two separate lasers. A red diode laser excited two fluorochromes contained within the color-coded microspheres and a green laser excited the reporter fluorochrome (SAPE) bound to the microsphere surface. High-speed digital signal processing classified the microsphere based on its spectral address and quantified the reaction on the surface

instead, the presence of organisms having an allergenic potential. Moreover, because every cell of an organism contains the same DNA, it is particularly advantageous to use DNA as a marker for potentially allergenic fish rather than the parvalbumin proteins which are differently distributed within different tissues of the organism.

xMAP™ assay

Isoforms of the parvalbumin sequences were isolated by molecular cloning and identified by sequencing for eight fish species in this study. Capture oligonucleotides were designed and coupled to the microspheres, as described previously for Vermillion snapper (No. 5), Spot (No. 13), Atlantic croaker (No. 14), Striped bass (No. 19), Spottail pinfish (No. 22), Nile tilapia (No. 28), Southern flounder

(No. 41), and Atlantic salmon (No. 45; Table 2) [25]. All species-specific probes were designed to target a unique intron region on the gene, whereas the universal probe for the detection of all tested fish samples targeted a highly conserved exon region within all of the different species. Figure 3 shows median fluorescence intensities (MFI) of the eight fish species using their specific capture probes with the xMAP™ assay. Experimentation was carried out in triplicate on three different days to establish reproducibility. The eight fish samples showed different response factors using their specific capture probes with 885 ± 10 MFI for species No. 5, 474 ± 77 MFI for species No. 13, 711 ± 22 MFI for species No. 14, $1,049 \pm 77$ MFI for species No. 19, 510 ± 52 MFI for species No. 22, 299 ± 59 MFI for species No. 28, 622 ± 43 MFI for species No. 41, and 894 ± 115 MFI for species No. 45. A background fluorescence of 106 ± 10

Table 3 Multiplex cross-reactivity tests

target	Capture probe no.								
	5	13	14	19	22	28	41	45	F ^a
1	–	–	–	–	–	–	–	–	+
2	–	–	–	–	–	–	–	–	+
3	–	–	–	–	–	–	–	–	+
4	–	–	–	–	–	–	–	–	+
5	+	–	–	–	–	–	–	–	+
6	–	–	–	–	–	–	–	–	+
7	–	–	–	–	–	–	–	–	+
8	–	–	–	–	–	–	–	–	+
9	–	–	–	–	–	–	–	–	+
10	–	–	–	–	–	–	–	–	+
11	–	–	–	–	–	–	–	–	+
12	–	–	–	–	–	–	–	–	+
13	–	+	–	–	–	–	–	–	+
14	–	–	+	–	–	–	–	–	+
15	–	–	–	–	–	–	–	–	+
16	–	–	–	–	–	–	–	–	+
17	–	–	–	–	–	–	–	–	+
18	–	–	–	–	–	–	–	–	+
19	–	–	–	+	–	–	–	–	+
20	–	–	–	+	–	–	–	–	+
21	–	–	–	–	–	–	–	–	+
22	–	–	–	–	+	–	–	–	+
23	–	–	–	–	–	–	–	–	+
24	–	–	–	–	–	–	–	–	+
25	–	–	–	–	–	–	–	–	+
26	–	–	–	–	–	–	–	–	+
27	–	–	–	–	–	–	–	–	+
28	–	–	–	–	–	+	–	–	+
29	–	–	–	–	–	–	–	–	+
30	–	–	–	–	–	–	–	–	+
31	–	–	–	–	–	–	–	–	+
32	–	–	–	–	–	–	–	–	+
33	–	–	–	–	–	–	–	–	+
34	–	–	–	–	–	–	–	–	+
35	–	–	–	–	–	–	–	–	+
36	–	–	–	–	–	–	–	–	+
37	–	–	–	–	–	–	–	–	+
38	–	–	–	–	–	–	–	–	+
39	–	–	–	–	–	–	–	–	+
40	–	–	–	–	–	–	–	–	+
41	–	–	–	–	–	–	+	–	+
42	–	–	–	–	–	–	–	–	+
43	–	–	–	–	–	–	–	–	+
44	–	–	–	–	–	–	–	+	+
45	–	–	–	–	–	–	–	+	+
46	–	–	–	–	–	–	–	–	+
47	–	–	–	–	–	–	–	–	+

Table 3 (continued)

target	Capture probe no.								
	5	13	14	19	22	28	41	45	F ^a
48	–	–	–	–	–	–	–	–	+
49	–	–	–	–	–	–	–	–	+
beef	–	–	–	–	–	–	–	–	–
Chicken	–	–	–	–	–	–	–	–	–
Lamb	–	–	–	–	–	–	–	–	–
Pork	–	–	–	–	–	–	–	–	–
Turkey	–	–	–	–	–	–	–	–	–
Shrimp	–	–	–	–	–	–	–	–	–
Veg. Soup ^c	–	–	–	–	–	–	–	–	–

Capture probes 5, 13, 14, 19, 22, 28, 41, and 45 were designed to specifically detect Vermillion snapper, Spot, Atlantic croaker, Striped bass, Spottail pinfish, Nile tilapia, Southern flounder and Atlantic salmon. Its nucleotide sequences target intron regions of the parvalbumin gene

^a Capture probe F was designed to detect all fish species. Its nucleotide sequence targets an exon region of the parvalbumin gene

^b Fluorescent values > than 2× the fluorescence value of the negative control (= cutoff, 212 MFI) were used to indicate a positive (+) reaction. Values in italics are expected positives

^c According to the label, the soup contained the following vegetables: peas, potatoes, celery, tomato, and corn

MFI was measured for the negative control, necessitating a cutoff value greater than 212 MFI to confirm a positive reaction (see “[Experimental](#)” section). The isoforms of the parvalbumin gene within one fish were detected in different concentrations of the total bands (see Fig. 2). Therefore, it was important to design the capture oligonucleotides to target the isoform with the highest content within one fish to get the strongest detection signal possible. For that reason, all capture probes were designed to target an isoform that represents a minimum of 20% of the total isoforms within one fish (compare Table 2). How critical this can be is shown by considering the results for fish No. 28 (Nile tilapia), which showed the lowest response, only slightly above the threshold cutoff value for a positive reaction (212 MFI), with 299±59 MFI detected with its specific capture probe. This could be explained by the low content (22%) of the target gene isoform (see Table 2). Interestingly, correlation of isoform content and detection signal did not remain constant for all eight species. For example the assay for fish No. 13 (Spot) targeted a gene isoform with 80% of the total detected bands but that resulted in only a medium signal of 474 MFI. The fish-specific capture probe designed to detect all eight fish also resulted in different responses for different fish. These findings suggest that in addition to the content of the target isoform in a fish species that other factors,

Fig. 2 Analysis of parvalbumin gene isoforms in fish species. Parvalbumin isoforms were amplified with PCR and analyzed with capillary electrophoresis using the Agilent 2100 Bioanalyzer. Sample numbers match the numbers described in Table 1. For each fish species, patterns of at least one parvalbumin gene isoform were amplified using the universal IFF 232/233 primers, with different ratios of DNA bands. Similar DNA band patterns were obtained for closely related species. Most isoforms show lengths ranging from 150 to 800 bp

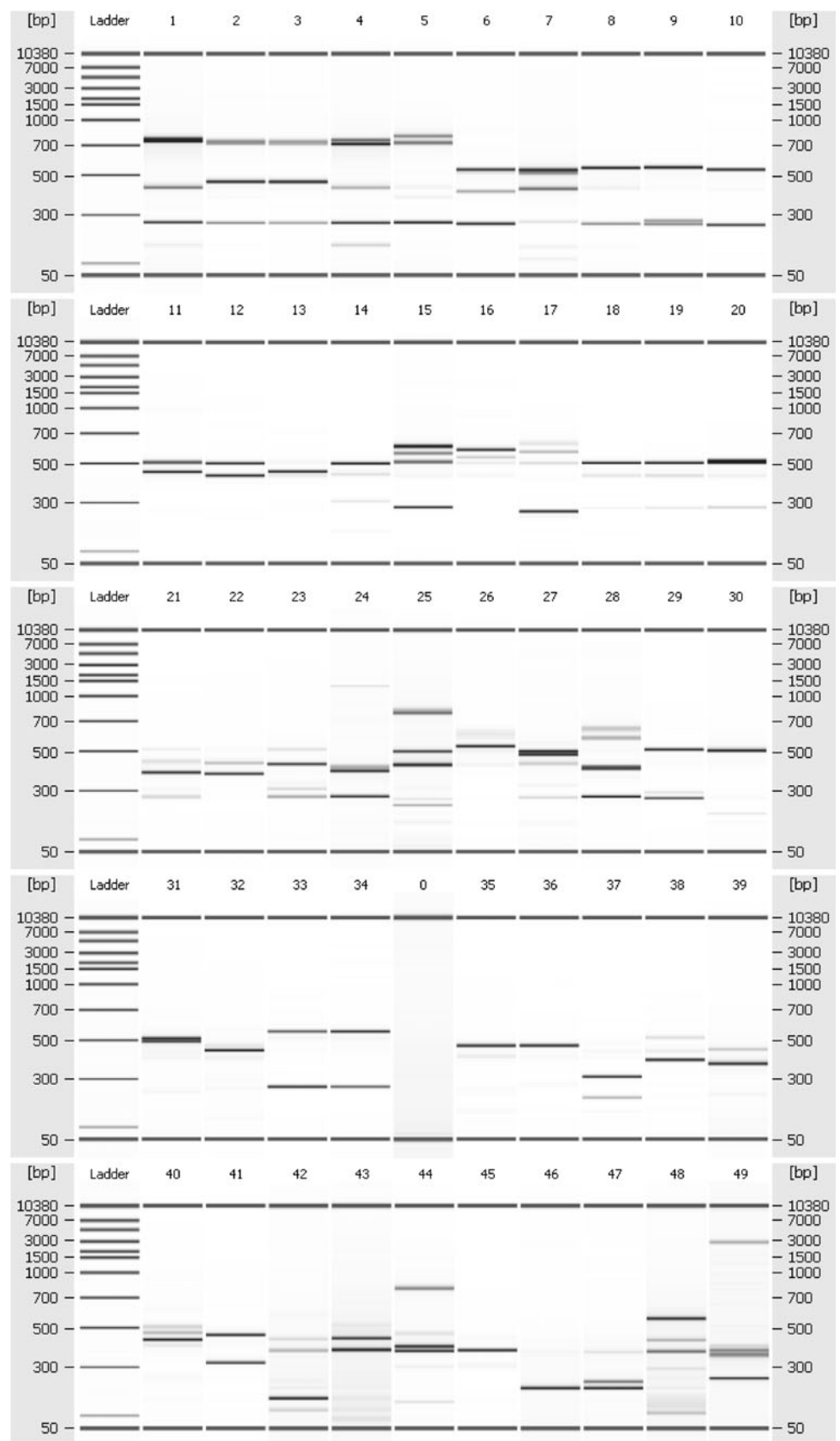
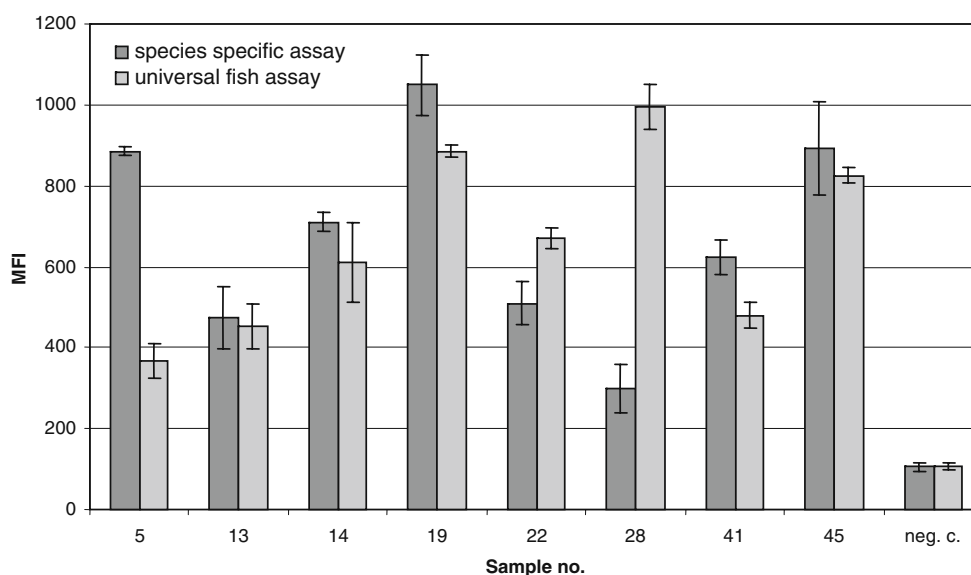


Fig. 3 Determination of capture probe-target specificity. Each target was determined in triplicate on three different days. Sample numbers match the numbers described in Table 1. Species-specific assays were designed to target a unique intron region on the parvalbumin gene (results shown in dark gray). A universal probe for the detection of all fish samples targeted a highly conserved exon region within different species (results shown in light gray). A cutoff value of 212 MFI was determined, indicating positive reactions for all tested samples



such as length, GC/AT-content or conformational factors of the target sequence, might affect the total signal response of the assay. A way to improve low signals could be to choose other parvalbumin target sequences, increase the capture probe density on the microspheres, or add a second capture probe to the same microsphere that targets another region of the gene or another isoform.

Cross-reactivity test results confirm a high specificity for the species detection assays and the universal fish assay. Corresponding data, presented in Table 3, showed that all 49 tested fish samples could be detected by the xMAP™ assay using the universal fish capture oligonucleotide. Other food samples, such as beef, lamb, chicken, pork, turkey, shrimp, and vegetable soup, did not cross-react with any of the fish-specific capture probes. The assays with species-specific capture probes 5, 13, 14, 22, 28, and 41 only detected the fish species they were designed for and none of the other tested fish. Using capture probe 19 for Striped bass, a hybrid of Striped bass and White bass (*Morone saxatilis* × *Morone chrysops*, No. 20) could also be detected confirming a close taxonomic relation of the hybrid to the target species. Using capture probe 45, which was designed to detect Atlantic salmon, Sockeye (Pacific) salmon (No. 44) was also detected. This was unanticipated because they are classified in different taxonomic genera [31], within the same subfamily. To establish the similarity of the parvalbumin DNA isoform used to design the Atlantic salmon capture probe to those of the Pacific salmon, the parvalbumin isoforms of Pacific salmon will need to be sequenced for direct comparison with the Atlantic salmon isoforms. To differentiate Pacific from Atlantic salmon, Pacific salmon-specific probes would be designed targeting regions of the Pacific salmon

isoforms that do not exist in Atlantic salmon, such as the 982 or 460 bp bands in Fig. 2. Presently, no information about the difference in allergic potential of Pacific and Atlantic salmon has been published. However, if it is shown that there is a different allergenic potential within a family, genera, or species, the expandability of this assay becomes more important and significantly enhances the practicality of this method.

Detection of Atlantic salmon in food

Spiking with different concentrations of Atlantic salmon meat in uncooked vegetable soup to simulate fish in a complex food matrix was performed in triplicate on three different days. The Atlantic salmon-specific assay was used to test the spiked soup samples. The amount of Atlantic salmon in the soup was plotted against the MFI (Fig. 4). The resulting sigmoid shaped curve shows a dynamic range between about 200 and 20,000 mg Atlantic salmon/kg food. The system becomes saturated at values greater than 20,000 mg of Atlantic salmon/kg food. The average coefficient of variation within one experiment (1 day) was 9.2% and ranged from 0.92% to 27.3% (data not shown), clearly falling within the range of acceptable limits for this type of analysis. The results for soup samples without fish were comparable to the results for the negative control (Fig. 3) and, therefore, fall into the range below the cutoff value ($2 \times$ background MFI). Using the cutoff value of 212 MFI, a detection limit of about 200 mg/kg was calculated for Atlantic salmon in vegetable soup (Fig. 4). This is equivalent to 0.02%. A threshold level for labeling of food containing fish has yet to be defined. Although a lowest observed adverse effect level

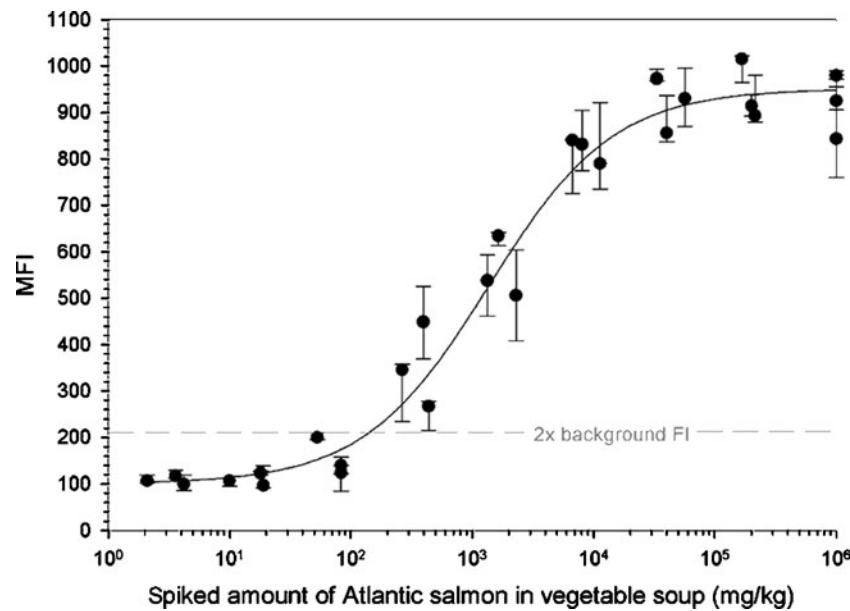


Fig. 4 Analysis of vegetable soup containing known quantities of Atlantic salmon. The experiment was performed on three different days. Each sample extract was measured in triplicate for each day. Results are shown as median values, including *error bars*. A background fluorescence of 106 ± 10 FI was measured using a

negative control containing all components except target sample. A cutoff value of greater than twice the background fluorescence of the negative control was used to indicate a positive reaction (=detection limit)

(LOAEL) for individuals with fish allergies has not been established for Atlantic salmon, values ranging from 5 mg to 6,000 mg have been published for other fish [15, 32]. Thus, the detection of 0.02% salmon in a complex food mixture, an amount equivalent to 20 mg in a 100 g serving, is within the lower range reported for the LOAEL. Based on the fluorescent values achieved for the other seven fish species, data suggest that similar detection limits between 10 and 40 mg in a 100 g serving could be expected for each of them. Increasing of the fluorescence signal is important to achieve detection limits equivalent to or lower than the lowest reported LOAEL. Presently, there are means by which the sensitivity of this method could be increased without loss of multiplexing. Conceivable approaches to improve sensitivity include the signal amplification strategies reviewed by Park et al. [33], or a means of increasing the efficiency of target capture [34].

Other complicated and diverse food matrices, including processed, cooked, canned, and pickled foods, still need to be evaluated and validated with this newly described multiplex detection assay. Furthermore, while this study presents data demonstrating the success of the method, the assay should be expanded to include additional species and tested to fully determine the versatility and practicality of the technology in detecting fish allergens in food samples.

Conclusion

This study presents a new xMAP™ technology-based method that can be successfully used for specific identification of eight allergenic fish species as well as the universal detection of fish simultaneously. This is the first study that takes advantage of the parvalbumin gene to amplify intron regions to detect fish in a species-specific manner. The salmon-specific assay was able to reliably detect approximately 20 mg of Atlantic salmon filet in 100 g of vegetable soup (0.02%), demonstrating that this assay is sufficiently sensitive to detect this fish in a complex food matrix at levels where only mild or no allergic reactions are to be expected. Detection limits of 0.01–0.04% are expected for the other seven tested species, based on their respective experimentally determined fluorescence values. The xMAP™ technology, which consists of 100 different microsphere sets that can be individually distinguished, makes this assay easily expandable to detect other fish species, providing an invaluable tool suitable for the detection of numerous fish species should the need be required.

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