

A method to detect allergenic fish, specifically cod and pollock, using quantitative real-time PCR and COI DNA barcoding sequences

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Abstract

BACKGROUND: Fish are one of eight major allergens defined in the US Food Allergen Labeling and Consumer Protection Act, and cod and pollock are two of the major fish allergens. This paper describes development and validation of a method to detect cod and pollock in complex food matrices using real-time polymerase chain reaction (PCR). Mitochondrial cytochrome oxidase I (COI) sequences obtained through DNA barcoding were used to design a single set of primers and probe which detected three species in the genus *Gadus*: Atlantic cod, Pacific cod, and walleye pollock.

RESULTS: Cod spiked into three different food matrices (cooking oil, clam chowder, and hushpuppy mix) yielded high linearity, dynamic range spanning six orders of magnitude, and lower limits of detection at 1–10 ppm (ppm; mg kg⁻¹). Frying had an adverse effect on the lower limit of detection, but not on linearity.

CONCLUSIONS: This work shows that COI DNA barcoding sequences can be used to effectively design real-time PCR assays for detection of food allergens in complex matrices. While full-length DNA barcodes distinguish individual species, the PCR assay designed here detected three different species. This is likely because real-time PCR assays are tolerant to basepair mismatches and do not utilize the full length of the DNA barcode.

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Keywords: real-time PCR; fish; allergen; COI; DNA barcoding

INTRODUCTION

The US Food Allergen Labeling and Consumer Protection Act mandates labeling for food products containing any of eight major allergenic foods or food groups, including fish. Fish allergy likely affects approximately 1.5 million Americans.^{1,2} However, individual fish species vary considerably in their levels of allergenicity. This has been attributed to differences in levels of the primary allergenic protein, parvalbumin, rather than to differences in the severity of patient reactions to it.³ Real-time polymerase chain reaction (PCR) data suggest variation in parvalbumin protein levels may be the result of variation in copy number of the parvalbumin gene between fish species.⁴ Compared to other fish species, cod and pollock contain higher levels of parvalbumin and are therefore more allergenic.^{5,6} Both consistently rank among the top ten seafood species consumed in the USA.⁷ Cod is also one of the most common fish species to cause reactions reported in clinical settings and is one of the most-studied allergenic fish.^{8,9}

Allergenic foods are often present in trace amounts resulting from cross-contamination which occurs, for example, in food processing plants through the use of shared equipment. In the case of seafood such as fish, cross-contamination through the use of shared frying oil appears to have caused allergic reactions in consumers.^{10–12} Protection of public health therefore requires highly sensitive and specific methods for accurate detection of seafood allergens in food products, including oils and foods subjected to high-temperature frying.

In recent years, PCR has been used to detect food allergens such as fish via detection of their DNA.⁸ While it does not detect allergenic proteins directly, PCR has proven to be more robust than protein-based detection methods in cases of food matrix interference and food processing.¹³ Detection of various fish species has been carried out using PCR-based assays for several targets, including the nuclear genes for parvalbumin, rhodopsin, Hoxc13, and 18S rRNA^{4,14–18} as well as the mitochondrial genes for 12S rRNA and 16S rRNA.^{19–21} Design of PCR-based detection assays depends on the availability of relevant DNA sequence data from the species of interest to ensure accuracy and, ideally, incorporates sequences from related nontarget species to help ensure that cross-reactivity does not occur. This requires inclusion of high-quality sequence data from the same genomic region of all species in assay design. In general, PCR-based detection assays which utilize high copy number targets, such as mitochondrial genes, are more sensitive than those utilizing lower copy number targets, such as nuclear genes.

Researchers in this field have cited the need for more work on DNA-based fish allergen detection, especially for gadiform species such as cod and pollock, as well as the limitations imposed on

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Table 1. Primer, probe, and IAC template sequences

Description	Sequence
Cod forward primer	CCGATATAGCTTCCCTCGAATAA
Cod reverse primer	CTAAAGGTGGGTAGACAGTTCAG
Cod probe	FAM-TCTCCCTCC/HEX/ATCTTCTGCTCCT-lowaBlack
IAC forward primer	CTAACCTTCGTGATGAGCAATCG
IAC reverse primer	GATCAGCTACGTGAGGTCCTAC
IAC probe	HEX-AGCTAGTCG ATG CACTCC AGT CCT CCT-lowaBlack
IAC template	TACAGCACCTAGCTTGGTAGAATCGATCAGC TACGTGAGGTCCTACGACGATGCCAAGCATG CCCTAGCTAAGATGCATCGATTGCTCATCAGT ACGTTAGGTCGACTAGGAGGCTGGAGTGTCAT CGACTAGCTAAGATGGTTCGATTGCTCATCAC GAAGGTTAGGTCGACTACGAACGAGTCGTAC GTTAGGTT

development of PCR assays by the lack of availability of sequence data.^{8,13,19,20} DNA barcoding generates DNA sequence data for an approximately 650 bp segment at the 5' end of the mitochondrial cytochrome oxidase I (COI) gene, and it has been used extensively for identification of animal species, including fish.^{22,23} A large number of sequences from this COI gene segment in fish have been deposited in public databases, including one generated and maintained by the US FDA.^{24,25} This paper describes development of a method for fish allergen detection with a real-time PCR assay developed using publicly available mitochondrial COI sequences from DNA barcoding, and validation of that method in food products.

MATERIALS AND METHODS

Sample preparation and DNA extraction

For optimization and cross-reactivity testing, DNA was extracted directly from fish tissue using the DNeasy Blood and Tissue Kit as per the manufacturer's instructions (Qiagen, Valencia, CA) and quantified using a Nanodrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, MA). PCR optimization was carried out using 10-fold dilutions of cod DNA in buffer ranging from 50 000 to 0.05 pg per reaction. Cross-reactivity testing was carried out using 2500 pg of DNA per reaction for all samples. For testing in food matrices, commercial products were spiked with Pacific cod at levels of 0.1–10⁵ mg of fish meat per kg of food matrix (ppm), and 5 g of spiked food matrix was homogenized with 20 mL of 1%

SDS (Fisher Scientific, Waltham, MA, catalog no. BP1311) using an IKA Tube Mill (IKA, Wilmington, NC). For frying experiments, samples were fried at 195 °C (385 °F) for 10 min in a Presto FryDaddy® filled with one quart of soybean oil. Temperature was monitored throughout the experiments. Aliquots of oil were taken between samples and assayed for fish content using the real-time PCR method. Fried samples were homogenized as described. DNA was extracted from 250 µL of homogenate using the DNeasy Blood and Tissue Kit as per the manufacturer's instructions with minor modifications: twice the recommended volumes of Buffer AL and ethanol were added to improve recovery of degraded DNA. Extracted DNA was quantified using a Nanodrop Lite (ThermoFisher Scientific, Waltham, MA) and normalized for concentration prior to PCR; a maximum of 50 ng of DNA was used per reaction.

Assay design and real-time PCR

Primers and probes for this assay were designed using PrimerQuest²⁶ and the mitochondrial COI sequence from Pacific cod, *Gadus macrocephalus*.²⁷ Several primer and probe sets were screened in laboratory tests of cross-reactivity, PCR efficiency, limits of detection, and linear range, resulting in selection of a single primer and probe set (Table 1) which amplified Pacific cod (*G. macrocephalus*), Atlantic cod (*G. morhua*), and walleye pollock (*G. chalcogrammus*). Concentrations of cod primers and probes were optimized prior to assay validation (Table 2). All reactions contained an internal amplification control (IAC) as previously described²⁸ (Table 1). Total reaction volume was 12.5 µL and each reaction contained 0.625 units of Platinum Taq Polymerase (Invitrogen, 10966-018; supplied with PCR buffer and MgCl₂), 1× PCR buffer, 1.2 mmol L⁻¹ dNTPs, 5 mmol L⁻¹ MgCl₂, 5 × 10⁵ copies of IAC template, 200 nmol L⁻¹ each IAC primer, 200 nmol L⁻¹ IAC probe, 2000 nmol L⁻¹ each cod primer, and 500 nmol L⁻¹ cod probe (Table 1). Each sample was run in triplicate on a 96-well PCR plate. Primers, probes, and IAC template DNA were obtained from Integrated DNA Technologies (Coralville, IA). PCR was carried out using a CFX96 (BioRad, Hercules, CA) with the following thermal profile: initial denaturation at 95 °C for 3 min, and 45 cycles of denaturation for 15 s at 95 °C, annealing at 65 °C for 30 s followed by reading of fluorescent signals, and extension at 65 °C for 2 min. PCR data were analyzed using CFX Manager software (BioRad, Hercules, CA). For the cod COI target, data were analyzed as standard curves and used to determine reaction efficiency, statistical R², and linear range.^{29–31} For the IAC, data were analyzed using the average and standard deviation of the C_T value for all samples regardless of fish spiking level: the IAC was added in equal amounts to all reactions and therefore expected to show equal levels of amplification.

Table 2. Assay optimization data

Primer concentration (nmol L ⁻¹)	Probe concentration (nmol L ⁻¹)	Linear range (pg per reaction)	Efficiency (%)	R ²	Max. RFU
200	200	5–50 000	95	0.98	1200
500	200	5–50 000	93	0.99	2200
1000	200	5–50 000	92	0.98	2200
2000	200	5–50 000 ^a	104	0.99	2000
2000	200	5–50 000 ^a	80	0.99	2800
2000	500	5–50 000 ^a	84	0.98	5600
2000	1000	5–50 000	78	0.99	7000

^a One well was positive at 0.5 pg per reaction.

RESULTS AND DISCUSSION

Optimization and cross-reactivity testing

PCR conditions, including cod primer and probe concentrations, were optimized using DNA diluted in TE buffer; data were evaluated based on linear range, reaction efficiency, statistical R^2 , and maximum fluorescent signal (Max. RFU).²⁹ It is optimal for real-time PCR methods to have a linear range spanning 6–8 orders of magnitude, reaction efficiencies of 90–110%, and R^2 values of 0.98–1.^{30,31} Run-to-run variability resulted in some reaction efficiencies outside the ideal range, but in general optimization data showed that the assay is robust across a wide range of primer and probe concentrations (Table 2). Relatively high primer and probe concentrations were chosen for the final assay because they yielded stronger amplification signals (higher max. RFU; Table 2), and to ensure robust amplification in complex food matrices.

Similar results were obtained when this assay was tested on Pacific cod, Atlantic cod, and walleye pollock. In cross-reactivity testing, the assay detected Pacific cod, Atlantic cod, and walleye pollock – all from the genus *Gadus* – equally well; no significant cross-reactivity was found with other fish species from other genera tested (Table 3). Average C_T values were similar across the three target species for equivalent amounts of DNA extracted from different individuals: 27.7 ± 2.7 cycles for walleye pollock, 28.7 ± 2.0 cycles for Pacific cod, and 28.2 ± 2.0 cycles for Atlantic cod. As previously stated, the assay was designed using a COI sequence from Pacific cod; sequence analysis (Fig. 1) shows that (i) the forward primer has a single basepair mismatch with Atlantic cod and walleye pollock, (ii) the reverse primer has a single basepair mismatch with Atlantic cod and walleye pollock, and (iii) the probe has a single basepair mismatch between individuals of Pacific cod, a single basepair mismatch with Atlantic cod, and a three basepair mismatch with walleye pollock. While similar C_T values and efficiencies were obtained for these three species, the walleye pollock tended to have lower fluorescence levels (data not shown) which may be a result of its greater level of mismatch with the probe. The single base mismatches in primers were likely well tolerated because they were in the middle and the 5' end; PCR amplification is most significantly affected by primer mismatches in the six bases at the 3' end, because that is where the polymerase binds to begin amplification. This analysis demonstrates that while full-length COI DNA barcodes can be effectively used to discriminate individual species, PCR assays developed from them are not necessarily species-specific because primer and probe sequence mismatches are tolerated in PCR. In addition, the full-length DNA barcodes are generally over 600 bp in length, which enables greater specificity than the shorter segments of approximately 100 bp which were used in this real-time PCR assay.

Assay validation: performance in complex food matrices and limits of detection

Validation in food matrices was conducted using three different types of food and two independent tests of each type according to the FDA's validation criteria³² with additional frying tests (Table 4). For the assay described here, linearity and R^2 values for the cod COI target were well within acceptable ranges, but as with optimization experiments in buffer some reaction efficiencies in food matrices were outside the ideal range (Table 4). Frying increased the lower limit of detection compared to uncooked foods, but some PCR wells remained positive at the lowest ppm levels tested (Table 4). Further limit of detection tests were carried out using Manhattan clam chowder as a model food matrix:

Table 3. Cross-reactivity data

No.	Common name	Scientific name ^a	C_T value
12	Walleye pollock	<i>Gadus chalcogrammus</i>	25.93
13	Walleye pollock	<i>Gadus chalcogrammus</i>	25.94
14	Walleye pollock	<i>Gadus chalcogrammus</i>	26.29
15	Walleye pollock	<i>Gadus chalcogrammus</i>	26.57
15	Walleye pollock	<i>Gadus chalcogrammus</i>	26.57
29	Walleye pollock	<i>Gadus chalcogrammus</i>	26.48
30	Walleye pollock	<i>Gadus chalcogrammus</i>	26.76
31a	Walleye pollock	<i>Gadus chalcogrammus</i>	34.5
32	Walleye pollock	<i>Gadus chalcogrammus</i>	29.98
3	Pacific cod	<i>Gadus macrocephalus</i>	25.99
27	Pacific cod	<i>Gadus macrocephalus</i>	27.65
28	Pacific cod	<i>Gadus macrocephalus</i>	30.24
34	Pacific cod	<i>Gadus macrocephalus</i>	30.95
5	Atlantic cod	<i>Gadus morhua</i>	28.99
6	Atlantic cod	<i>Gadus morhua</i>	29.09
7	Atlantic cod	<i>Gadus morhua</i>	28.25
9	Atlantic cod	<i>Gadus morhua</i>	25.19
10	Atlantic cod	<i>Gadus morhua</i>	24.39
26	Cod	<i>Gadus</i> sp.	27.44
21	Channel catfish	<i>Ictalurus punctatus</i>	No C_T
22	Channel catfish	<i>Ictalurus punctatus</i>	No C_T
35	Channel catfish	<i>Ictalurus punctatus</i>	No C_T
36	Channel catfish	<i>Ictalurus punctatus</i>	No C_T
4	Dover sole	<i>Microstomus pacificus</i>	No C_T
2	Flounder	No barcode ID	No C_T
1	Grouper or gag	<i>Mycteroperca microlepis</i>	No C_T
23	Sockeye salmon	<i>Oncorhynchus nerka</i>	No C_T
24	Sockeye salmon	<i>Oncorhynchus nerka</i>	No C_T
18	Blue tilapia	<i>Oreochromis aureus</i>	No C_T
17	Mozambique tilapia	<i>Oreochromis mossambicus</i>	No C_T
16	Nile tilapia	<i>Oreochromis niloticus</i>	41.52
19	Swai, Sutchi catfish	<i>Pangasianodon hypophthalmus</i>	No C_T
20	Swai, Sutchi catfish	<i>Pangasianodon hypophthalmus</i>	No C_T
11	Atlantic salmon	<i>Salmo salar</i>	No C_T
25	Atlantic salmon	<i>Salmo salar</i>	No C_T
33	Atlantic salmon	<i>Salmo salar</i>	No C_T
37	Sardine or pilchard	<i>Sardina pilchardus</i>	No C_T
38	Sardine or pilchard	<i>Sardina pilchardus</i>	No C_T
8	Argus grouper	<i>Cephalopholis argus</i>	No C_T

^a Scientific names were determined using DNA barcoding (<https://www.fda.gov/Food/FoodScienceResearch/DNASeafoodIdentification/default.htm>).

12 of 12 samples spiked at 10 ppm produced positive signals with an average C_T value of 37.4 ± 1.02 . Limit of detection tests were not carried out below 10 ppm, though several experiments with validation in food showed amplification at 1 ppm within the linear range (Table 4). Amplification of the internal control was highly consistent both within and between experiments, with nearly all samples having C_T values of 21–22 (Table 4).

CONCLUSIONS

This paper describes the design of a real-time PCR assay for fish allergen detection using COI DNA barcoding sequences. A single primer and probe set detected three different species within the genus *Gadus*. While full-length DNA barcodes are able to

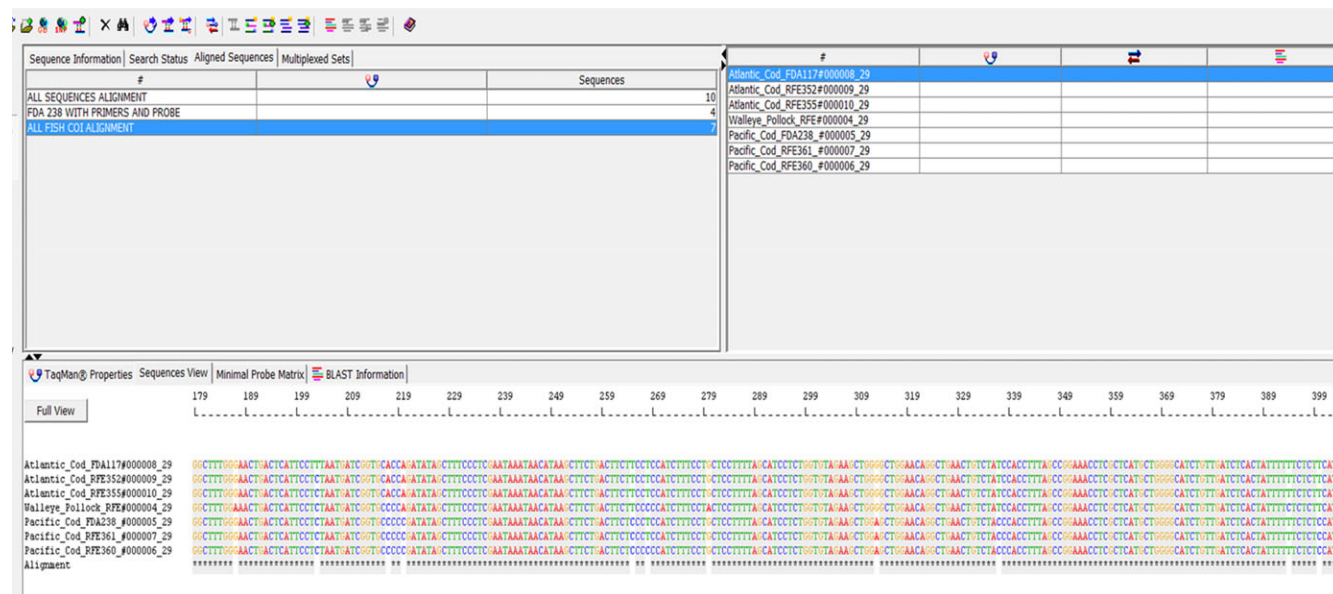


Figure 1. COI sequence alignments for Atlantic cod, Pacific cod, and walleye pollock. Forward primer (bp 219–242): CCGATATAGCTTCCCTCGAATAA; reverse primer: CTAAAGGTGGGTAGACAGTTTCAG; reverse complement of reverse primer (bp 325–347): CTGAAGTGTCTACCCACCTTTAG; probe (bp 262–285): TCTCCCTCCATCTTCTGCTCCT.

Table 4. Validation data from food matrices

Experiment/food matrix	Linear range (ppm)	Efficiency (%)	R ²	IAC (avg C _T ± SD)
Cooking oil, expt 1	1–10 ⁶	117	0.99	21.90 ± 0.120
Cooking oil, expt 2	1–10 ⁵	123	0.97	22.21 ± 0.044
Clam chowder, expt 1	1–10 ⁶	114	0.99	21.94 ± 0.067
Clam chowder, expt 2	10–10 ⁶	125	0.97	22.22 ± 0.055
Hushpuppy mix, expt 1	10–10 ⁵	117	0.99	21.83 ± 0.082
Hushpuppy mix, expt 2	1–10 ⁶	114	0.97	21.66 ± 0.080
Fried hushpuppies, expt 1	100–10 ^{5a}	98	0.99	22.24 ± 0.046
Fried hushpuppies, expt 2	100–10 ^{5b}	122	0.98	22.21 ± 0.056

^a One well positive at 1 ppm.

^b One well positive at 1 ppm and one well positive at 10 ppm.

discriminate individual species, the PCR assay designed from them was genus- but not species-specific. This has been attributed to the shorter target length and sequence mismatch tolerance of real-time PCR assays.

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