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Validation of FASTFISH-ID: A new commercial platform for rapid fish species authentication via universal closed-tube barcoding

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ABSTRACT

Seafood represents up to 20% of animal protein consumption in global food consumption and is a critical dietary and income resource for the world's population. Currently, over 30% of marine fish stocks are harvested at unsustainable levels, and the industry faces challenges related to Illegal, Unregulated and Unreported (IUU) fishing. Accurate species identification is one critical component of successful stock management and helps combat fraud. Existing DNA-based technologies permit identification of seafood even when morphological features are removed, but are either too time-consuming, too expensive, or too specific for widespread use throughout the seafood supply chain. FASTFISH-ID is an innovative commercial platform for fish species authentication, employing closed-tube barcoding in a portable device. This method begins with asymmetric PCR amplification of the full length DNA barcode sequence and subsequently interrogates the resulting single-stranded DNA with a universal set of Positive/Negative probes labeled in two fluorescent colors. Each closed-tube reaction generates two species-specific fluorescent signatures that are then compared to a cloud-based library of previously validated fluorescent signatures. This novel approach results in rapid, automated species authentication without the need for complex, time consuming, identification by DNA sequencing, or repeated analysis with a panel of species-specific tests. Performance of the FASTFISH-ID platform was assessed in a blinded study carried out in three laboratories located in the UK and North America. The method exhibited a 98% success rate among the participating laboratories when compared to species identification via conventional DNA barcoding by sequencing. Thus, FASTFISH-ID is a promising new platform for combating seafood fraud across the global seafood supply chain.

1. Introduction

Fish provides approximately 3.2 billion people with almost 20 percent of their average per capita intake of animal protein (FAO, 2018). In 2018 alone, over 151 million tons of seafood were consumed worldwide. The high demand has led to harvesting of over 30% of fish stocks at biologically unsustainable levels (FAO, 2018), as well as the exploitation of increasing numbers of fish species. In the United States alone, almost 2000 commercial species are currently listed for consumption (FDA, 2020) and 7 new species were added to the list in 2019 (FDA, 2019)

The high global demand for seafood has also increased the complexity of the seafood supply chains and has contributed to an increase in Illegal, Unreported, and Unregulated (IUU) fishing, along with

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fraudulent species mislabeling and substitution for economic gains (Cawthorn et al., 2018; Donlan & Luque, 2019; Watson et al., 2015). The most complete estimates suggest that up to 25% of global fish catches fall within IUU practices, identifying it as the single largest threat to achieving fisheries sustainability (Agnew et al., 2009; Helyar et al., 2014) and species mislabelling allows this practice to persits. To exacerbate the problem, over half of the world's catch is processed at sea or soon after landing, which renders species identification of the resulting products nearly impossible without forensic laboratory analysis. As a result, both distributors and consumers are becoming less and less familiar with the appearance of different fish species imported from different geographical regions.

Considering these facts, there is a pressing global need for increased science-based monitoring of traded fish species to ensure that this vital resource remains legal, available, and sustainable for generations to come. Both the Food and Agriculture Organization (2001) and the European Union (European Union Regulation (EC) No 1005/2008) have placed growing emphasis on the use of trade measures to prevent IUU-sourced fish and fish products from entering international trade. Species identification is a critical component required for establishment of quotas and labelling based on specific species scientific names (NRC, 1999). However, efficient, accurate, and affordable species identification remains a major technical challenge, particularly in remote regions where the incidences of IUU fishing are most likely (World Ocean Review, 2013).

Several nucleic acid-based tools for identification of commercial fish species have been developed. The most widely used method, DNA barcoding, involves sequencing of a standardized region of the mitochondrial COI gene shown to be species-specific for virtually all animal species on Earth, including most fish species (Hebert et al., 2003). The database of these DNA barcodes is an ever-expanding, internationally recognized resource for cataloguing and characterizing global biodiversity (Ratnasingham & Hebert, 2007). DNA barcoding has been adopted as a regulatory standard in some countries and has been used to expose and focus attention on seafood mislabeling and deliberate food fraud (e.g. Wong & Hanner, 2008; Miller & Mariani, 2010; Cawthorn et al., 2012; Khaskar et al. 2015; Vandamme et al., 2016; Di Muri et al., 2018). Although the reported levels of mislabeling are sometimes inflated (Luque & Donlan, 2019), it is widely accepted that this illegal activity is an ongoing concern for the seafood industry because of its negative economic and health consequences to consumers, as well as its impact on brand recognition and science-based stock management (Fox et al., 2018; Naaum & Hanner, 2016).

While DNA barcoding has helped expose and mitigate seafood fraud, this method is too slow and elaborate for routine widespread use in the seafood industry. Samples to be identified must be shipped to testing laboratories where the steps of DNA extraction, PCR amplification, DNA sequencing, and analysis take several days and involve the use of specialized equipment by trained scientists. These processes are ill-suited for the rapid identification needed for perishable, highly traded commodities such as fish. As a result, there is still a pressing need for new technologies that can generate fast, actionable answers in a convenient format and that can be used by anyone along the supply chain, regardless of their technical expertise.

One method for fast and portable species identification is real-time PCR analysis. This method uses species-specific tests to confirm the species identity of tested specimens on-site and in less than two hours (e.g. Rasmussen-Hellberg et al., 2011; Cardeñosa et al., 2019). Because this targeted analysis approach requires the development and use of a different test for each species, real-time PCR is best suited to screen large numbers of specimens from a specific species, rather than analysis of a wide range of species. This same limitation is true for other targeted approaches like lateral flow, HRM, and NanoTracer tests which only allow authentication of specific fish species (e.g. Baerwald et al., 2020; Fitzcharles, 2012; Fernandes et al., 2017; Valentini et al., 2017). Genome skimming, another emerging tool for species authentication, remains impractical for field applications due to its lengthy analysis times (up to 36 h) and technical expertise requirements (Johri et al., 2019). Given all of these limitations, there is still a need for a a rapid, convenient technology to identify the ever-increasing number of fish species in the seafood marketplace.

Closed-tube DNA barcoding was first developed for identification of nematodes and protozoan species (Rice et al., 2016; Siriani et al., 2016; Schiller et al., 2017) and, as described here, has now been adapted for fish species authentication. The method combines rapid asymmetric DNA amplification of the COI barcoding gene sequence found in the mitochondrial genomes of all animals, with highly informative closed-tube melt curve analysis of the resulting single-stranded DNA (Sanchez et al., 2004; Pierce et al., 2005). Positive-probes present in the amplification reaction increase their fluorescence when they hybridize to their target sequences following amplification, while Negative-probes decrease fluorescence when they hybridize adjacent to their paired Positive-probes (Rice et al., 2014). In the case of FASTFISH-ID, two separate sequences within the same COI gene product are distinguished by using Positive-probes of different colors, along with Negative-probes of different position. Thus, the method converts each species-specific DNA sequence into two unique fluorescent signatures. The identity of each specimen is determined in seconds by comparison of its fluorescent signature to all of the fluorescent signatures stored in a cloud-based library of signatures from vouchered specimens. Up to 48 samples can be analyzed in about two hours in a single run of a portable PCR device. The cost per specimen is $5.00 and each FASTFISH-ID kit comes with sufficient reagents for analysis of 260 specimens. These features of FASTFISH-ID clearly suggest that this platform, if shown to be reliable, will be faster, less expensive, and more convenient than analysis of the COI barcoding target by amplification followed by sequencing. Moreover, if proven reliable, FASTFISH-ID is likely to be more convenient and cost effective than species identification using sets of species-specific reactions, whether they are run in parallel or sequentially. This is because FASTFISH-ID uses the same set of versatile reagents for identification of all species, as well as many subspecies.

This report describes the results of a comparative blinded study using a common set of voucher specimens that was carried out in three independent, academic laboratories, one in Canada and two in the UK, to evaluate the performance of the FASTFISH-ID platform. The results provide evidence for the high efficacy and accuracy of this novel platform.

2. Materials and methods

2.1. FASTFISH-ID closed-tube barcoding protocol

2.1.1. Preparation of fish DNA

DNA extractions for FASTFISH-ID were carried out according to the method described by Tagliania et al. (2016). Briefly, a 1–2 mm^3 fish sample was collected with a sterile biopsy tool (EMS-Core Sampling Tool, Electron Microscopy Sciences, Hatfield, PA) or another suitable instrument, and then added to 100 μL 200 mM KOH, 2 mM EDTA pH 8.0, 0.2% Triton X-100 in 0.2 ml PCR tubes (Genesee Scientific, San Diego, CA). The sample was then incubated at 85 °C for 15 min in a PCR thermocycler (iCycler, Biorad, Hercules, CA) and then added to 300 μL 100 mM Tris-Cl pH 8.0 in a 1.5 ml low-adhesion Eppendorf tube (USA Scientific, Ocala, FL). Two microliters (2 μL) of the resulting lysate were used for PCR amplification as described below. Care was taken to clean the area where the fish was placed, to collect a sample from the inside of fish fillet rather than from the outside surface, and to clean the biopsy tool well in between collections to prevent cross-contamination between different fish species processed on the same day.

2.1.2. PCR reaction and amplification conditions

FASTFISH-ID uses asymmetric PCR to generate excess single stranded amplicons corresponding to the full-length internationally

recognized barcoding portion of the COI gene (Ratnasingham & Hebert, 2007). For maximal compatibility, FASTFISH-ID employs the same degenerate primers as those listed in the FDA-approved method for DNA barcoding of commercial fish species (Handy et al., 2011). Amplification reaction consisted of 1X PCR buffer (Thermo Fisher-Invitrogen, Carlsbad, CA), 2.5 mM MgCl2 (Thermo Fisher-Invitrogen, Carlsbad, CA) , 200 μM dNTPs (Bioline, Tauton, MA), 1 μM FDA degenerate primer FISH COI HBC_ts, 100 nM FDA primer FISH COI LBC_ts (both primers from Biosearch Technologies, Novato, CA), 1X FASTFISH-ID probe mix (ThermaGenix, Natick, MA; see Section 2.1.3 below), 1.25 units ThermaStop (ThermaGenix, Natick, MA), 25 nM Cal-Red ThermaMark (ThermaGenix, Inc, Natick, MA), 1.25 units MyTaq DNA polymerase (Bioline, Tauton, MA), and KOH-prepared fish DNA (2 μL) in a final volume of 12.5 μL. ThermaStop (ThermaGenix, Natick, MA) is a novel hot-start reagent that prevents non-specific amplification prior to the start of the reaction. ThermaMark (hereafter referred as TM) is a temperature-dependent marker for correction of melt-curve analysis (ThermaGenix, Natick, MA). PCR master mixes were prepared in low-adhesion Eppendorf tubes (USA Scientific, Ocala, FL). As in the FDA method, the PCR primer set included universal M13 tails for sequencing of the amplification products (Handy et al., 2011).

PCR amplification was carried out in a MIC real-time PCR thermocycler from Bio Molecular Systems (Upper Coomera, Queensland, Australia). Thermocycling conditions were 94 °C for 2 min, 5 cycles of 94 °C for 5 sec, 55 °C for 20 sec, 72 °C for 45 sec, then 65 cycles of 94 °C for 5 sec, 70 °C for 45 sec. After 70 total cycles of amplification, the reaction products are comprised of a low level of double-stranded DNA and a 10 to 20-fold excess of single stranded DNA that is available for probe/target hybridization in the same closed tube (Pierce et al., 2005; Sanchez et al., 2004). At the end of PCR, the temperature was lowered to 40 °C for 10 min to allow the fluorescent hybridization probes in the FASTFISH-ID probe mix to bind to the excess single stranded DNA products. This step was followed by melting curve analysis from 40 °C to 87 °C at 0.1 °C/sec with sequential fluorescent acquisition first in the MIC PCR Cycler's Orange Channel (suitable for detection of CalRed 610-labeled probes; max excitation: 590 nm; max emission 610 nm) and then detection in the Red Channel (suitable for detection of Quasar 670-labeled probes; max excitation: 647 nm; max emission 670 nm). Fluorescent signatures described in the text corresponded to the first derivative of the resulting melting curve fluorescent data. Each fish species or species variety is characterized by a unique combination of Cal-Red 610 and Quasar 670 fluorescent signatures. In this way, FASTFISH-ID can use the same reagents to authenticate a large number of species.

2.1.3. FASTFISH-D fluorescent hybridization probes

The FASTFISH-ID probe mix consisted of two sets of Positive/Negative probe pairs labeled in two different colors that hybridize along the length of two mini-barcode regions within the amplified COI target sequence, hereafter referred to as Barcoding Segment 1 (BS1) and Barcoding Segment 2 (BS2). Both BS1 and BS2 regions were selected for their high inter-species variation within the DNA barcode region, after extensive analysis of several thousand fish COI sequences in the iBOL database.

Positive/Negative probe pairs exhibit a higher degree of allele-discrimination than previously described Lights-On/Lights-Off probes (Siriani et al., 2016; Rice et al., 2014; Sanchez, unpublished). Each Positive-probes is comprised of a target binding sequence that is 20–35 nucleotides long and exhibits increased signal when bound to its target sequence, but low background when not bound. Negative-probes are quencher only probes that lower the signal when bound adjacent to their paired Positive-probe. Positive/Negative probe pairs are mismatch tolerant, meaning that they can anneal to both perfectly complementary strands and to target sequence variants having one or more nucleotide polymorphisms. Positive/Negative probe pairs are designed to hybridize to their single-stranded DNA targets after amplification. As a result, sets of Positive/Negative probe pairs can bind to a multitude of DNA variants over a temperature range of about 40 °C to 70 °C. Fluorescent signatures for similar-but-different target sequences that differ by even single nucleotide changes almost always exhibit different signatures. Thus Positive/Negative probe sets can distinguish many thousands of fish species and their variants.

In the case of FASTFISH-ID, sets of Positive/Negative probe pairs were designed as consensus probes generated via in silico sequence testing and statistical analysis of BS1 and BS2 regions in large numbers of COI target sequences selected from 200 commercial fish species listed by Shokralla et al. (2015). Probe sequences were carefully chosen so that at least one Positive-probe would bind to at least one Barcoding Segment for the most mismatched targets. Although none of the probes in a set of consensus Positive/Negative probe pairs is likely to be perfectly matched to any particular target, sequence variations among different targets nevertheless generate distinct patterns of probe/target hybridization. Positive-probes for BS1 are labeled with the Cal Red 610 fluorophore and Positives-probes for BS2 are labeled with Quasar 670. The Negative-probes are quencher-only probes labeled with Black-Hole Quencher 2 (Biosearch Technologies, Novato CA).

Although just one Barcoding Segment is often sufficient to uniquely identify species, FASTFISH-ID uses two Barcoding Segments and two differently colored probe sets for generation of two fluorescent signatures for each specimen. Hence, unambiguous species identification can be achieved even if two species have one identical Barcoding Segment sequence. In addition, sequence differences with BS1 and BS2 are sometimes due to variations within a species. In rare cases, the underlying sequence of one of the Barcoding Segments can differ from its complementary probe sequences to such an extent that the probes fail to bind to that segment. In these cases, the absence of a signal is regarded as a characteristic of that species and the signal from the other Barcode Segment is sufficient for species authentication.

Each composite melt curve of Positive/Negative probes pairs is called a fluorescent contour and is mathematically converted into a fluorescent signature by calculating its first derivative (Siriani et al., 2016; Rice et al., 2014, see Fig. 2). Each fluorescent signature is highly reproducible and is characteristic of the interrogated target sequences. Unknown sequences can be identified upon comparison of previously verified fluorescent signatures stored in a cloud-based reference library (Siriani et al., 2016).

2.1.4. DNA barcoding

Because FASTFISH-ID amplifies the entire COI DNA barcode sequence, the same single strand DNA products used to generate a fluorescent signature can also be sequenced by DNA barcoding for characterization at the nucleotide level, should this be required for regulatory purposes or for assigning species to unknown fluorescent signatures. The sequencing protocol uses the M13 tail sequence in the FASTFISH-ID FISH COI HBCts excess primer (5′ CACGACGTTGTAAAACGAC 3′, a modified version of the M13F primer) as a sequencing primer to generate the sequence of the excess primer strand. By design, the excess primer-strand sequence can be queried directly in the Barcode of Life Database for species identification (Ratnasingham & Hebert, 2007). For confirmatory purposes, the limiting primer strand of the same amplicon can also be sequenced using the M13R tail sequence in the FASTFISH-ID FISH COI LBCts as the sequencing primer. Sequencing was outsourced to MacrogenUSA (now Psomagen, Boston, MA). Samples were prepared according to the service provider protocols (https://www.macrogenusa.com/support/seq/sample_pre.jsp).

2.1.5. Data analysis

Species identification using FASTFISH-ID involves comparison of the bi-colored fluorescent signature to a cloud-based reference library of species-specific reference fluorescent signatures generated from taxonomically-verified or DNA-barcode-verified specimens. Construction of the reference library involved the use of fluorescent signatures from vouchered reference species. Specimens from these species

included the same reference samples used for construction of FDA Reference Standard Sequence Library for Seafood Identification (RSSL; https://www.accessdata.fda.gov/scripts/fdcc/?set=seafood_barcode_data) as well as samples procured from other sources (see Acknowledgements). In each instance, the species identity of the reference specimens was independently confirmed by DNA barcode sequencing.

Species-specific reference fluorescent signatures were generated in sets of 6–10 technical replicates and, whenever possible, at least three biological replicates. The BS1 and BS2 fluorescent signature data generated by the MIC PCR cycler were then exported as an Excel file via the MIC PCR Cycler software. The resulting fluorescent signature curves were first evaluated for signal intensity above a defined signal threshold to identify amplification failures/poorly amplified samples. Samples with fluorescent signatures below the signal threshold criteria were labeled as "Low Amplification". Strongly amplified fluorescent signatures were then mathematically smoothed by applying a moving average of 100 data points every 0.01 °C, corrected for potential temperature shifts caused by carry-over salt contamination from the fish DNA samples by first aligning the valley of the TM reference signal in the Cal Red 610 channel to 78 °C, then shifting the signals in the Quasar channel by a corresponding amount, and finally normalized against either the largest peak or the deepest valley in the fluorescent signature, whichever was the largest (TM is a temperature-dependent marker for correction of melt-curve analysis included in the FASTFISH-ID PCR master mix, see Section 2.1.2). Smoothed, corrected, and normalized replicate signatures for individual species were then averaged to generate species-specific reference signatures.

For species identification, the BS1 and BS2 fluorescent signature generated in the MIC PCR cycler for a tested specimen were exported to a proprietary cloud-based scoring algorithm at https://thermagenix-speciesid.shinyapps.io/Species-ID_FASTFISH-ID_Answers_1/. The R-based algorithm at that site analyzes the two fluorescence signatures from each specimen, as described above, and compares them to all reference signatures in the library by means of a numerical correlation function across the entire temperature range of the fluorescent signature. The algorithm assigns a specimen to a given species when both its BS1 and BS2 fluorescent signatures match those of a known species with a correlation factor greater than 90%. For cases in which two species share a fluorescent signature in one Barcoding Segment, the algorithm assigns species based on the combination of the common signature and the unique one of the second segment that has a correlation greater than 90%. As examples, Tilapia (*Oreochromis mossambicus*) and Channel Catfish (*Ictalurus punctatus*) do not generate a BS1 fluorescent signature, while Monkfish (*L. americanus*) does not have a BS2 signature because the probes fail to bind at 40 °C. Authentication of these species takes the absence for a signal in one segment into account.

2.2. Multi-center FASTFISH-ID validation

2.2.1. Sample collection

Seventy-five fish fillets from 18 different commercially important species were either purchased from stores in the Boston area or were procured from specimens available at ThermaGenix other than those used to build the reference library. These species were selected based on those tested by the FDA for evaluation of new DNA methods for identification of commercial fish species (Handy et al., 2011), or based on suggestions by the study participants as commonly substituted species in their regions. The specimen collection for Multi-Center FASTFISH-ID Validation Study comprised Red Snapper (*Lutjanus campechanus*; n = 7), Atlantic Salmon (*Salmo salar*; n = 6), Pandora (*Pagellus erythrinus*; n = 2), Pacific Ocean Perch (*Sebastes alutus*; n = 6), Tilapia (*Oreochromis mossambicus*; n = 5), Pacific Red Snapper (*Lutjanus peru*; n = 7), Monkfish (*Lophius americanus*; n = 1), Pacific Cod (*Gadus macrocephalus*; n = 3), Channel Catfish (*Ictalurus punctatus*; n = 2), Atlantic Cod (*Gadus morhua*; n = 8), Swordfish (*Xiphias gladius*; n = 5), Coho Salmon (*Oncorhynchus kisutch*; n = 5), Haddock (*Melanogrammus aeglefinus*; n = 4), Sockeye Salmon (*Oncorhynchus nerka*; n = 4), Atlantic Halibut (*Hippoglossus hippoglossus*; n = 2), Gilt-head Seabream (*Sparus aurata*; n = 1), Yellowtail Snapper (*Ocyurus chrysurus*; n = 5), and King Salmon (*Oncorhynchus tshawystscha*; n = 2).

2.2.2. Participants and roles

ThermaGenix performed the DNA extractions and preliminary evaluation of all the samples by FASTFISH-ID and conventional DNA barcoding sequencing approach as detailed above. DNA sequences from specimens were uploaded to BOLD "FFID FASTFISH-ID Multi-Center Validation Study" project (Code: FFID). Three additional laboratories participated in the blinded sample validation using FASTFISH-ID: Queen's University Belfast (UK1) and the University of Salford (UK2), both in the United Kingdom, and the University of Guelph in Canada (CA).

2.2.3. Shipment of DNA samples

DNA samples for the validation study extracted and tested as above were shipped blinded to the participating laboratories (UK1, UK2, CA) dried on punches made from Whatman N^{o} 1 filter paper (VWR, Radnor, PA). Briefly, 40 μL KOH-prepared fish DNA were dried overnight on 4 mm circles made with a conventional office holepunch. Filters were shipped in 0.2 ml PCR tubes marked only with sample numbers.

To elute DNA from the filter punches, each punch was completely submerged in 40 μL of 10 mM Tris-Cl, pH 8.0–8.3. Tubes were vortexed for 5 s to resuspend the DNA, centrifuged briefly to pool liquid, and heated for 15 min at 85 °C. DNA was then used immediately for PCR amplification using FASTFISH-ID protocols, or stored at 4 °C for later use.

2.2.4. Identification of samples with FASTFISH-ID

Each participating laboratory ran the blinded DNA samples in triplicate using the FASTFISH-ID protocol described. After PCR amplification and melt-curve analysis, fluorescent signature data was exported as an Excel file via the MIC PCR Cycler software, and entered into the FASTFISH-ID algorithm at https://thermagenix-speciesid.shinyapps.io/Species-ID_FASTFISH-ID_Answers_1/ to determine the identity of each sample. At the end of the study, FASTFISH-ID species identifications were un-blinded and the samples correctly identified were scored. The UK1 and UK2 laboratories each tested all 75 specimen DNA samples. CA tested a subset of 54 specimen DNA samples. For any DNA samples where any of the technical replicate tests returned a "Low Amplification" ID by the algorithm, the sample was evaluated a second time (UK1 = 0, UK2 = 4, CA = 13, Table 1) These higher number of amplification failures in the CA data were determined to be due to the initial use of non-low adhesive tubes at this site. Specimen DNA samples where none of the technical replicates amplified even after retesting were deemed degraded during shipping and removed from the study (CA = 2, UK2 = 2, Table 1). Reported test failures in Table 1 represent technical replicates from scorable samples where low amplification was observed even after this second analysis, or where an "unknown" ID was assigned by the algorithm.

3. Results

3.1. FASTFISH-ID description

Fig. 1 depicts the overall strategy for FASTFISH-ID asymmetric amplification of the 650 nucleotide COI DNA Barcode segment, followed by closed-tube analysis of two specific internal regions (BS1 and BS2) using differently colored pairs of Positive/Negative probes.

Data sent to the cloud-based FASTFISH-ID scoring algorithm is analyzed in seconds via comparison to a reference database of species-specific both BS1 and BS2 fluorescent signatures. Answers are reported as scientific names. Fluorescent signatures that are not in the reference database are reported as "Unknown" and the user can choose

Table 1
Combined Results of FASTFISH-ID Multi-Laboratory Evaluation. Results from all the participating laboratories are listed, with matches to DNA barcoding results reported per number of technical replicates. The scientific names were obtained from the DNA barcoding results for each sample. The common names correspond to the FDA Fish List for the listed scientific name. * indicates the samples that were omitted from the total counts due to DNA degradation during shipment.

Sample #	Market Name	Species Identity from iBOL	Match to DNA Barcoding Results			
			Reference Results USA	Blind UK1	Blind UK2	Blind Canada
1	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	3 of 3
2	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	Not tested
3	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	Not tested
4	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	3 of 3
5	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	Not tested
6	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	Not tested
7	Red Snapper	*Lutjanus campechanus*	3 of 3	3 of 3	3 of 3	Not tested
8	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	3 of 3	3 of 3
9	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	3 of 3	3 of 3
10	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	3 of 3	3 of 3
11	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	3 of 3	3 of 3
12	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	3 of 3	3 of 3
13	Atlantic Salmon	*Salmo salar*	3 of 3	3 of 3	0 of 3 *	Not tested
14	Pandora	*Pagellus erythrinus*	3 of 3	3 of 3	3 of 3	Not tested
15	Pandora	*Pagellus erythrinus*	3 of 3	3 of 3	0 of 3 *	Not tested
16	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
17	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
18	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
19	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
20	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
21	Pacific Ocean Perch	*Sebastes alutus*	3 of 3	3 of 3	3 of 3	3 of 3
22	Tilapia	*Oreochromis mossambicus*	3 of 3	3 of 3	3 of 3	3 of 3
23	Tilapia	*Oreochromis mossambicus*	3 of 3	3 of 3	3 of 3	3 of 3
24	Tilapia	*Oreochromis mossambicus*	3 of 3	3 of 3	3 of 3	3 of 3
25	Tilapia	*Oreochromis mossambicus*	3 of 3	3 of 3	3 of 3	3 of 3
26	Tilapia	*Oreochromis mossambicus*	3 of 3	3 of 3	3 of 3	3 of 3
27	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	Not tested
28	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	3 of 3
29	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	Not tested
30	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	3 of 3
31	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	Not tested
32	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	Not tested
33	Pacific Red Snapper	*Lutjanus peru*	3 of 3	3 of 3	3 of 3	3 of 3
34	Monkfish	*Lophius americanus*	3 of 3	3 of 3	3 of 3	3 of 3
35	Pacific Cod	*Gadus macrocephalus*	3 of 3	3 of 3	3 of 3	Not tested
36	Pacific Cod	*Gadus macrocephalus*	3 of 3	3 of 3	3 of 3	3 of 3
37	Pacific Cod	*Gadus macrocephalus*	3 of 3	3 of 3	3 of 3	Not tested
38	Catfish	*Ictalurus punctatus*	3 of 3	3 of 3	3 of 3	3 of 3
39	Catfish	*Ictalurus punctatus*	3 of 3	3 of 3	3 of 3	3 of 3
40	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
41	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
42	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
43	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
44	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
45	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	Not tested
46	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
47	Atlantic Cod	*Gadus morhua*	3 of 3	3 of 3	3 of 3	3 of 3
48	Swordfish	*Xiphias gladius*	3 of 3	3 of 3	3 of 3	3 of 3
49	Swordfish	*Xiphias gladius*	3 of 3	3 of 3	3 of 3	Not tested
50	Swordfish	*Xiphias gladius*	3 of 3	3 of 3	3 of 3	3 of 3
51	Swordfish	*Xiphias gladius*	3 of 3	3 of 3	3 of 3	3 of 3
52	Swordfish	*Xiphias gladius*	3 of 3	3 of 3	3 of 3	Not tested
53	Coho Salmon	*Oncorhynchus kisutch*	3 of 3	3 of 3	3 of 3	3 of 3
54	Coho Salmon	*Oncorhynchus kisutch*	3 of 3	3 of 3	3 of 3	1 of 3
55	Coho Salmon	*Oncorhynchus kisutch*	3 of 3	3 of 3	3 of 3	0 of 3*
56	Coho Salmon	*Oncorhynchus kisutch*	3 of 3	3 of 3	3 of 3	Not tested
57	Coho Salmon	*Oncorhynchus kisutch*	3 of 3	3 of 3	3 of 3	1 of 3
58	Haddock	*Melanogrammus aeglefinus*	3 of 3	3 of 3	3 of 3	Not tested
59	Haddock	*Melanogrammus aeglefinus*	3 of 3	3 of 3	1 of 3	2 of 3
60	Haddock	*Melanogrammus aeglefinus*	3 of 3	3 of 3	3 of 3	3 of 3
61	Haddock	*Melanogrammus aeglefinus*	3 of 3	3 of 3	3 of 3	3 of 3
62	Sockeye Salmon	*Oncorhynchus nerka*	3 of 3	3 of 3	3 of 3	Not tested
63	Sockeye Salmon	*Oncorhynchus nerka*	3 of 3	3 of 3	3 of 3	3 of 3
64	Sockeye Salmon	*Oncorhynchus nerka*	3 of 3	3 of 3	2 of 3	3 of 3
65	Sockeye Salmon	*Oncorhynchus nerka*	3 of 3	3 of 3	3 of 3	0 of 3*
66	Atlantic Halibut	*Hippoglossus hippoglossus*	3 of 3	3 of 3	3 of 3	3 of 3
67	Atlantic Halibut	*Hippoglossus hippoglossus*	3 of 3	3 of 3	3 of 3	2 of 3
68	Gilt-head Seabream	*Sparus aurata*	3 of 3	3 of 3	3 of 3	1 of 3
69	Yellow Tail Snapper	*Ocyurus chrysurus*	3 of 3	3 of 3	3 of 3	3 of 3
70	Yellow Tail Snapper	*Ocyurus chrysurus*	3 of 3	3 of 3	3 of 3	3 of 3
71	Yellow Tail Snapper	*Ocyurus chrysurus*	3 of 3	3 of 3	2 of 3	3 of 3

(*continued on next page*)

Table 1 (*continued*)

Sample #	Market Name	Species Identity from iBOL	Match to DNA Barcoding Results			
			Reference Results USA	Blind UK1	Blind UK2	Blind Canada
72	Yellow Tail Snapper	*Ocyurus chrysurus*	3 of 3	3 of 3	3 of 3	3 of 3
73	Yellow Tail Snapper	*Ocyurus chrysurus*	3 of 3	3 of 3	3 of 3	3 of 3
74	King Salmon	*Oncorhynchus tshawytscha*	3 of 3	3 of 3	3 of 3	3 of 3
75	King Salmon	*Oncorhynchus tshawytscha*	3 of 3	3 of 3	3 of 3	Not tested
		TOTAL	**225/225 (100%)**	**225/225 (100%)**	**215/219 (98.2%)**	**148/156 (94.9%)**
			COMBINED TOTAL	**588/600 (98.0%)**		

* Sample degraded during shipment.

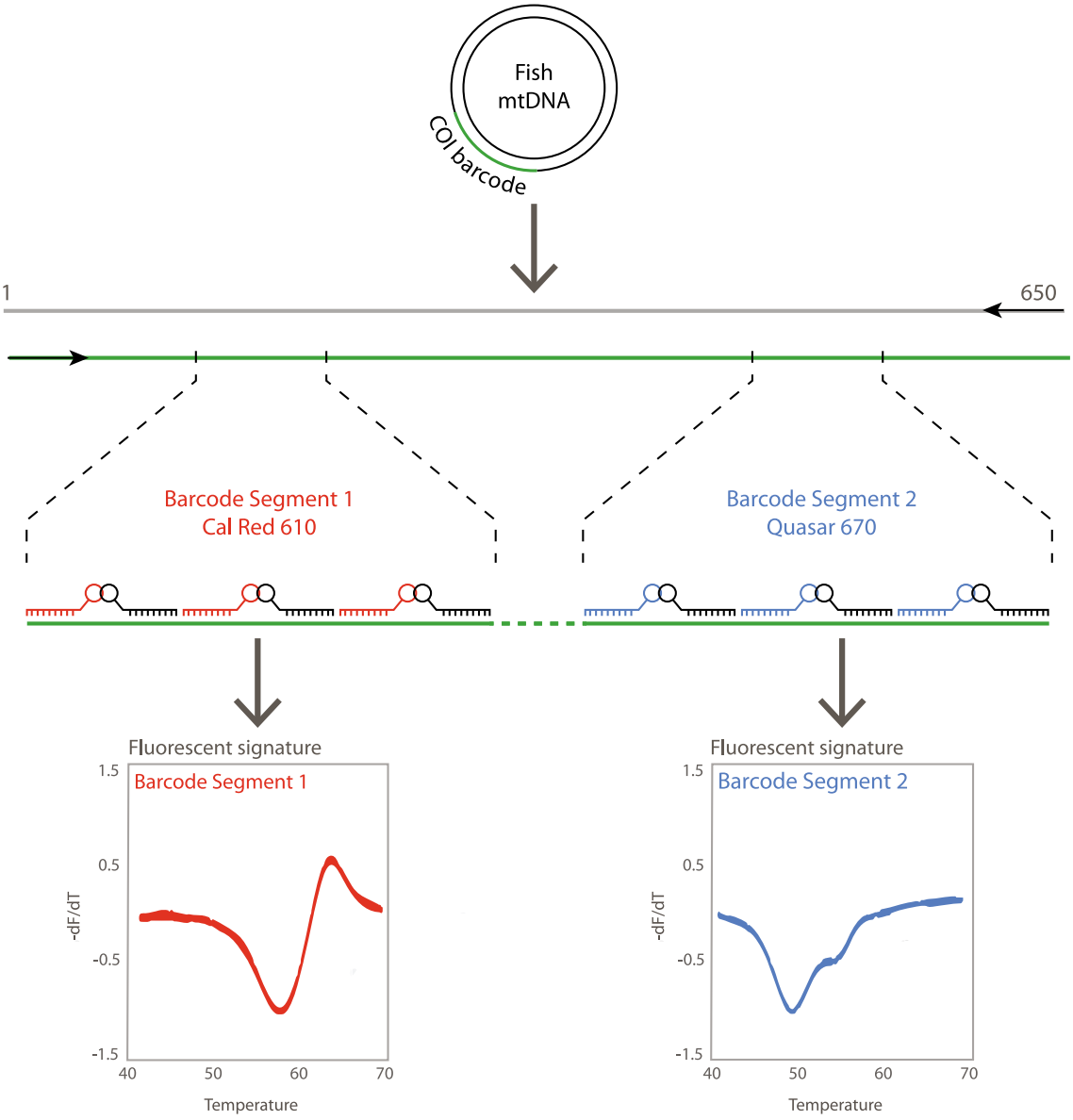

Fig. 1. Generation of Fluorescent Signatures for Fish Identification using FASTFISH-ID. The entire COI barcoding segment is amplified by asymmetric real-time PCR. Probes are designed to interrogate two different segments of the DNA barcode, chosen for their high inter-species variation within the DNA barcode region. A fluorescence signature is generated for each segment based on how the probes bind to the sample sequence. Due to the species-specific nature of the DNA barcode sequence, these fluorescent signatures can be used to differentiate species. The contents of the tube can also be used directly for subsequent DNA barcoding to confirm the species ID of the sample.

to send the corresponding tube to ThermaGenix, Inc. for DNA barcode sequencing and species identification. ThermaGenix then adds the fluorescent signature, and its identifying species information to the reference database for future species identification without sequencing. Thus, FASTFISH-ID eliminates the need for separate tests for each species, as well as the need to send samples to an analytical laboratory for time-consuming or expensive species identification via conventional DNA barcode sequencing (Fig. 2).

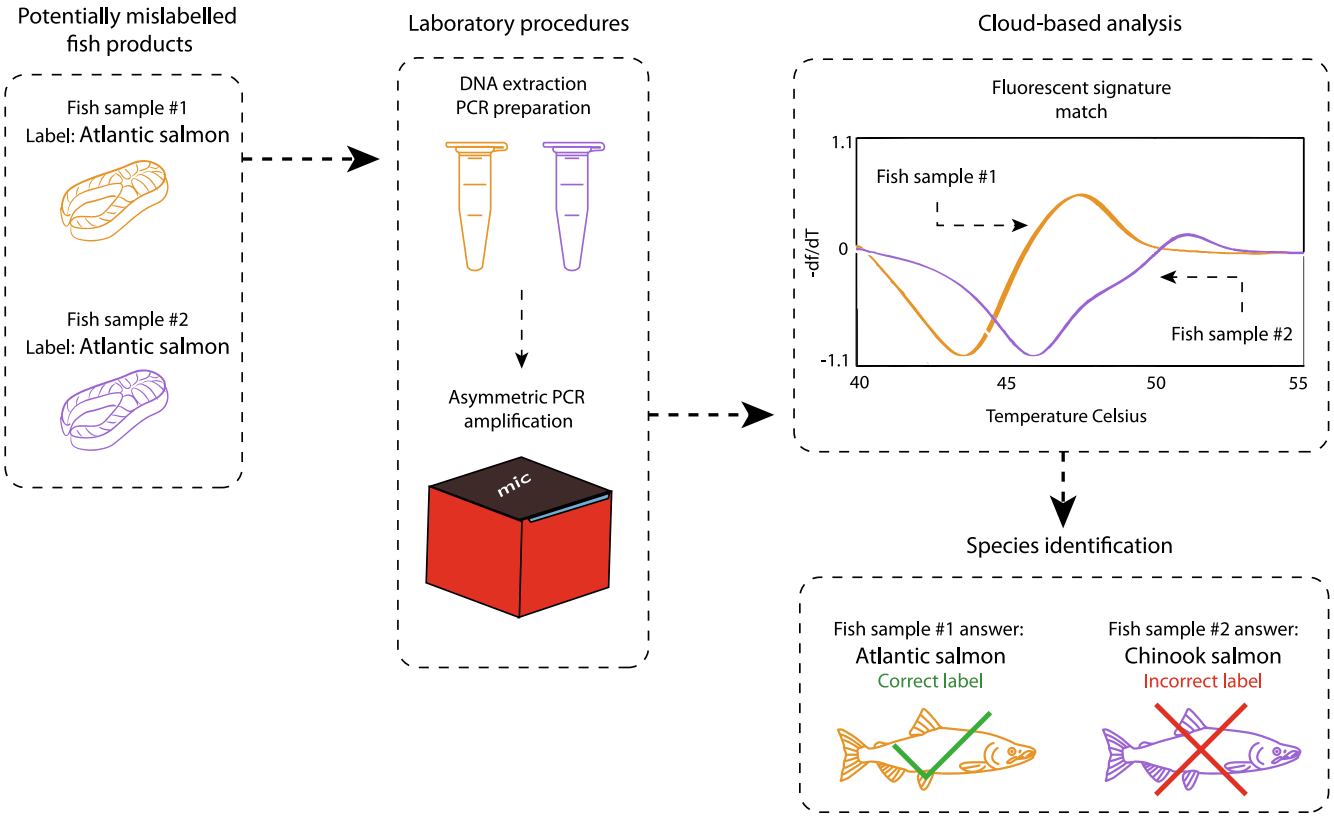

Fig. 2. Species Identification using FASTFISH-ID. DNA is extracted from fish samples and processed using standard laboratory procedures for FASTFISH-ID. Pre-programmed real-time PCR run templates are used on the MIC thermocycler to generate fluorescent signatures for each sample. Fig. 2 depicts just one of the two signatures used for FASTFISH-ID. Fluorescent signatures are then exported as an Excel document from the MIC PCR Cycler software and entered into the FASTFISH-ID algorithm online for automated signature comparison to the reference database. Species identification is made based on a match to two reference signatures. The scientific name of the identified species is then compared to the scientific name associated with the market label to determine the authenticity of the product. If a sample ID is returned as unknown, DNA barcoding can be conducted from the same sample tube post FAST-FISH-ID analysis to identify the sample.

3.2. Multi-center validation study

For independent validation of the commercial FASTFISH-ID method, 75 specimens encompassing 18 different commercial fish species were first authenticated by DNA Barcode sequencing (see Supplementary Materials, Table S1) and tested using FASTFISH-ID at Thermagenix in the US (Fig. 3). DNA samples from these specimens were then blinded and distributed to two separate laboratories in the United Kingdom (UK1, UK2) and one laboratory in Canada (CA) for rigorous independent validation of the FASTFISH-ID method. Due to sample DNA availability, the Canadian laboratory tested a subset of 54 of the original 75 samples. Upon completion of the analyses, the FASTFISH-ID test scores generated by the online algorithm were compared to the DNA barcode sequence identifications for accuracy.

The results established that FASTFISH-ID was accurate across technical and biological replicates for the samples tested in the study. The total success rate, measured as agreement between the FASTFISH-ID results with the DNA barcode identity for all the replicate tests of all the samples evaluated at the UK1, UK2, and CA sites, was 518/600 (98.0%, Table 1). Every FASTFISH-ID test performed at the US and the UK1 laboratories generated the correct result (100% accuracy) while 98.2% and 94.9% of the individual FASTFISH-ID test scores performed at the UK2 and the CA laboratories generated the correct species identifications, respectively. Replicate tests for any given DNA sample that were not scored as the correct species were scored as "unknown" instead. There were no instances of failures due to incorrect species identification by the FASTFISH-ID online algorithm.

4. Discussion

The results reported in this study established that FASTFISH-ID is a promising platform for authentication of fish species. The data generated by different investigators, working in different laboratories, demonstrate that the method performed with an overall success rate of 98.0% when data from all replicates of the same test samples were amassed (Table 1). There were not any false positives in among the participating testing laboratories that would have resulted in inaccurate authentication of species. This is important because the intended use for FASTFISH-ID is to detect cases of deliberate or inadvertent mislabeling of fish products where a failure to identify a sample would trigger a retest, but where an incorrect identification can have a far greater impact to brand image and economic loss due to erroneously rejected shipments.

The technology used for FASTFISH-ID is simple to use and requires minimal training. All it takes to conduct this test is to place a sample of fish is simply placed into a tube with lysis reagents to release DNA, transfer this material to another tube to prepare the DNA for analysis, add the resulting sample into a tube with DNA amplification reagents, and insert the tube into a portable PCR cycler. After a two-hour amplification, the resulting data-set is uploaded to the cloud-based library that archives all previously validated specimens (https://thermagenix.shinyapps.io/FastFishID-Answers1/) for automatic species scoring (see Materials and Methods). The use of a single device significantly reduces capital costs, as well as the risks of laboratory contamination and human errors that tend to occur in analytical process that employ multiple pieces of equipment. Importantly, the MIC PCR Cycler device on which FASTFISH-ID has been validated is a compact portable instrument. Its

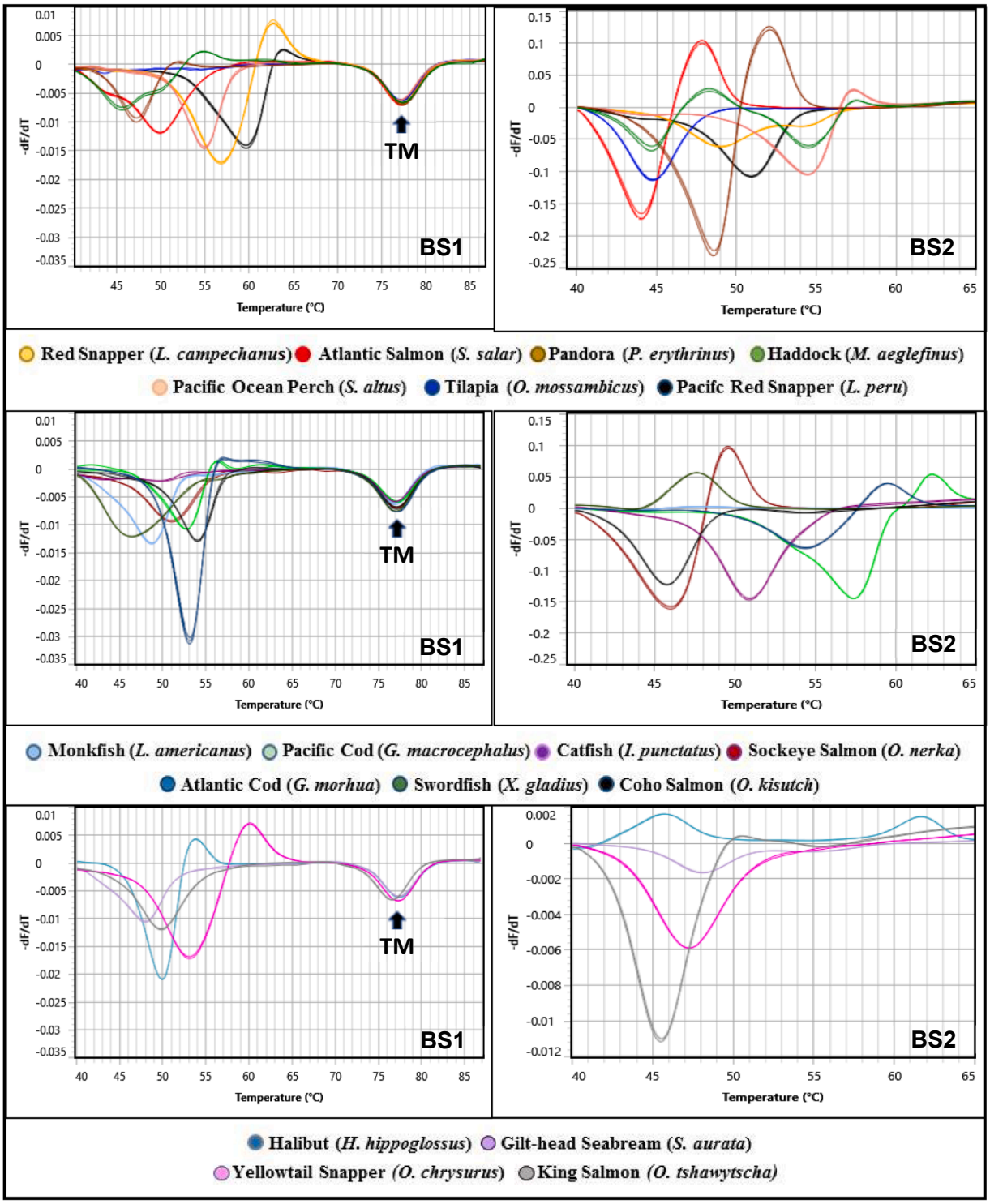

Fig. 3. Fluorescent Signatures Generated by FASTFISH-ID. This figure shows the raw BS1 and BS2 fluorescent signatures for the 18 fish species used in this study. Each species exhibits a unique combination of BS1 and BS2 signatures that identifies it. Although the signatures are shown in the figure in different color for display purposes, all the BS1 signatures were collected in the MIC PCR Cycler Orange Channel (fluorophore: Cal Red 510) while all the BS2 signatures were collected in the Red Fluorescent channel (fluorophore: QS670). The right-most valley in the BS1 fluorescent signature labelled "TM" corresponds to ThermaMark, an internal marker for correction of artifactual temperature variation. Signatures were obtained from 2 to 3 technical replicates. Note, Catfish (*I. punctatus*) and Tilapia (*O. mossambicus*) do not have a BS1 signature, while monkfish (*L. americanus*) does not have a BS2 signature (see Materials and Methods). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

relatively high throughput for portable instrumentation, 48 samples per run, allows for addition of positive and negative controls without drastically impacting analysis pipelines. FASTFISH-ID positive controls consist of synthetic oligonucleotides containing BS1 and BS2 sequences from specific fish species that generate known fluorescent signatures. As with many molecular assays, nuclease-free water serves as an effective negative control. Due to its portability and simplicity, FASTFISH-ID can be deployed at multiple points within the supply chain, from harvest to

consumer sale. The test is based on a suite of molecular chemistries that are very robust and require just one universal set of primers and probes for amplification and analysis of the full-length mitochondrial gene COI sequence that is used by the U.S. FDA for prosecution of cases of seafood fraud. At this time the test can only be run on the MIC, but potentially could be validated for use on other instruments with similar capabilities.

Because FASTFISH-ID is based on DNA barcoding, there could be limitations where species cannot be differentiated in cases where this region does not contain enough inter-species variation, or in cases where species hybridize. However, as with DNA barcoding, FASTFISH-ID would be suitable for the vast majority of commercial fish species (Shehata et al., 2018). In fact, in silico DNA sequence modeling showed that FASTFISH-ID identifies at least 200 commercial fish species (see Materials and Methods). Currently, 62 fish species have been authenticated with this method and 29 of them are presently in the FASTFISH-ID online reference database. Since FASTFISH-ID exhibits single nucleotide resolution (Rice et al., 2014) there is little risk that fluorescent signatures become overly similar as more species are added to the FASTFISH-ID reference library. When a specimen is encountered whose fluorescent signature is not yet in the FASTFISH-ID reference library, the species corresponding to those specimens must be identified by conventional DNA barcoding just once to assign the fluorescent signature to that species. Once the species assignment is made, future identification of such species can be based solely on their fluorescent signatures. This approach also ensures new haplotypes can be added as they are identified. Most specimens of the same species exhibit the same signature because their haplotype differences reside outside BS1 and BS2. However, specimens with haplotypes within either BS1 or BS2 would be expected to exhibit its own unique fluorescent signatures since each of these targets is decoded as a totality of all amplicons in the reaction. Species are scored based on the set of fluorescent signatures associated with their BS1 and BS2 haplotypes, so previously unknown haplotypes would be identified as "Unknown" in the same way species not yet in the database would be. Sequence analysis shows that, on the average, species are defined by 1–3 prevalent fluorescent signatures which together account for >90% of the known variants for that species.

The single-molecule detection sensitivity of FASTFISH-ID also makes possible species identification from DNA samples in extensively processed dried and smoked fish products (Wangh, unpublished). However, species identification with FASTFISH-ID is currently limited to testing of homogeneous samples from one source. Complex matrices such as fish cakes and other mixtures would generate composite species-specific fluorescent signature that would be difficult to resolve with the current methodology. This is being addressed with a current investigation into alternative approaches where DNA samples from these matrices would be diluted to the single molecule level, where mixtures no longer exist, and tested with FASTFISH-ID. Until these methods are established and automated, however, the only methods suitable for species identification of mixed fish products remain real-time PCR for testing for specific targets within a mixture (Naaum et al., 2019) and metabarcode sequencing (Haynes et al., 2019).

The BOLD database at http://www.boldsystems.org/ contains the COI DNA Barcode sequences more than 23,000 thousand species of fish that have been collected and analyzed by ichthyologists, taxonomists, and bioinformatic experts around the world. Most entries in this database have been isolated and sequenced multiple times, further validating their identities. Indeed, these repeated entries in the iBOL database can be used for rapid direct expansion of the FASTFISH-ID library, a process that is currently underway. Synthetic oligonucleotides for each sequence can be purchased and rapidly processed using FASTFAST-ID reagents to generate their corresponding fluorescent signatures. This can be done using the same reagents and protocols as from DNA barcoding, using synthetic genomic material as the template. The combined data can then be uploaded directly to the library without the need to catch a new specimen.

Non-targeted methods, such as FASTFISH-ID are increasingly sought after for food fraud detection. Seafood is consistently ranked as one of the most commonly mislabeled commodities (European Commission, 2020), and represents a particular challenge due to its complex supply chains (Martinsohn, 2011). For industry, impact from fraud events and reduced public trust can translate to significant financial loss (Jacquet & Pauly, 2008). From a regulatory perspective, estimates are that as little as 1% of imported seafood in the US is inspected (Lou, 2015) affecting the accurate monitoring and control of this commodity. Novel methods that increase accessibility of testing, by simplifying testing, making it more rapid, and lowering costs, can help to address these challenges. Better testing, geared towards the real needs of the industry, plays an important role in deterring food fraud and managing risks (Spink & Moyer, 2011). FASTFISH-ID offers a portable, turnkey solution for rapid on-site authentication of the thousands of species of fish that are legally and illegally caught and sold in the seafood markets of the world. The resulting data will help global efforts to manage and sustain fish populations that are important to human nutrition and ecological stability (Hilborn et al., 2020).

Use of the versatile FASTFISH-ID technology platform described here is not limited to measurement of edible teleosts. Proof-of-principle experiments have already established that the primers and probes contained in FASTFISH-ID kits can be used to recognize species of tropical reef fish, species of elasmobranch, and even species of mammals (Sanchez and LJ. Wangh, unpublished). While much bioinformatics work remains to be done, it is also already clear that modest changes in the design and locations of the primers and probes used in such kits will make it possible to construct closed-tube DNA barcoding kits for the analysis of large groups of animals in virtually any phylum on Earth (Siriani et al., 2016) making this a potential tool for all types of species-related food fraud.

5. Conclusion

FASTFISH-ID is a novel platform for rapid, portable and accurate identification of fish species using a single set of reagents and analysis tools. This multi-laboratory evaluation has shown FASTFISH-ID to be a robust approach, with 98% accuracy in identification across all participating labs. Based on asymmetric-PCR and closed-tube barcoding of a portion of the COI gene, this turnkey solution provides a new tool for combating seafood fraud across the global seafood supply chain by increasing the accessibility of non-targeted testing. The protocols are simple to follow, and the interpretation of results is automated through an online platform. The ability to easily sequence the resulting amplicon feeds into existing regulatory approaches. This allows independent confirmation of results when needed to either support a claim of potential fraud, or in cases where an identification is inconclusive. Since the portable MIC real-time PCR instrument is employed, the entire process can be completed almost anywhere, providing a testing option for any point in the supply chain. The commercially available FASTFISH-ID approach represents a means for non-targeted testing of seafood. This tool can be used to identify food fraud by assessing product labelling, and to combat IUU fishing by helping to monitor species landed.

CRediT authorship contribution statement

Amanda M. Naaum: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition. **Marine Cusa:** Methodology, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Maleeka Singh:** Validation, Investigation, Writing - review & editing. **Zoe Bleicher:** Software, Validation, Investigation, Writing - review & editing. **Christopher Elliott:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition. **Ian B. Goodhead:** Validation, Investigation, Writing - review & editing. **Robert H. Hanner:** Conceptualization, Methodology, Writing - review & editing,

Supervision, Funding acquisition. **Sarah J. Helyar:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Stefano Mariani:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **John E. Rice:** Software, Validation, Investigation, Writing - review & editing. **Lawrence J. Wangh:** Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition. **J. Aquiles Sanchez:** Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [AMN, MC, MS, ZB, CE, IBG, RHH, SJH, and SM declare no competing interests. The work described in this paper was initiated in the laboratory of LJW at Brandeis University and was completed at ThermaGenix, Inc. a biotech company which LJW founded. LJW, JER and JAS hold shares in this company.].

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J. Aquiles Sanchez, John Rice, and LJ Wangh wish to dedicate this paper in memory of Bill Hone, a wonderful friend and great supporter of closed-tube DNA barcoding.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.110035.

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