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COMPREHENSIVE REVIEW

Twenty-three years of PCR-based seafood authentication assay development: What have we learned?

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Abstract

Seafood is a prime target for fraudulent activities due to the complexity of its supply chain, high demand, and difficult discrimination among species once morphological characteristics are removed. Instances of seafood fraud are expected to increase due to growing demand. This manuscript reviews the application of DNA-based methods for commercial fish authentication and identification from 2000 to 2023. It explores (1) the most common types of commercial fish used in assay development, (2) the type of method used, (3) the gene region most often targeted, (4) provides a case study of currently published assays or primer-probe pairs used for DNA amplification, for specificity, and (5) makes recommendations for ensuring standardized assay-based reporting for future studies. A total of 313 original assays for the detection and authentication of commercial fish species from 191 primary articles published over the last 23 years were examined. The most explored DNA-based method was real-time polymerase chain reaction (qPCR), followed by DNA sequencing. The most targeted gene regions were *cytb* (cytochrome *b*) and COI (cytochrome *c* oxidase 1). Tuna was the most targeted commercial fish species. A case study of published tuna assays ($n = 19$) targeting the *cytb* region found that most assays were not species-specific through in silico testing. This was conducted by examining the primer mismatch for each assay using multiple sequence alignment. Therefore, there is need for more standardized DNA-based assay reporting in the literature to ensure specificity, reproducibility, and reliability of results. Factors, such as cost, sensitivity, quality of the DNA, and species, should be considered when designing assays.

KEYWORDS
cytb, DNA sequencing, food authentication, PCR, seafood fraud

1 | INTRODUCTION

The global demand for fish and fish products has increased from 141.5 million metric tons in 2005 to 184.1 million metric tons in 2022 (Food and Agriculture Organization [FAO], 2020, 2022; Rasmussen & Morrissey, 2009). The inability to meet this demand and fluctuations in supply and trade have led to fraud and adulterations (Silva et al., 2021). Oftentimes, fish and fish products lack external morphological features like fin position, body shape, scale size, or color pattern, which makes visually identifying one species from the other a difficult task (Teletchea, 2009). In addition, phenotypic similarities among some fish types/species further complicate the determination of origin and species identification (Kim et al., 2015). Therefore, (1) increased demand, (2) complexity of the supply chain, and (3) inability to determine species once morphological characteristics are removed make fish a prime target for fraudulent activities.

As a result, authentication methods have been developed to validate fish species. DNA-based methods used in food or fish authentication (Rasmussen & Morrissey, 2009) are considered more advantageous than protein-based methods, as DNA is less susceptible to degradation (Ward et al., 2009). DNA-based authentication methods use various gene target regions to "fingerprint" or uniquely identify fish species. The selected genetic markers tend to have high interspecies and low intraspecies polymorphism (Rasmussen & Morrissey, 2009). However, a variety of target genes are used to identify fish species more accurately.

This manuscript reviews the development and application of DNA-based methods, particularly PCR (polymerase chain reaction) for fish detection and authentication. In particular, it covers (1) the most common types of commercial fishes used in assay development, (2) the type of methods used (e.g., PCR), (3) the gene regions most often applied as targets, and (4) a case study of currently published assays for specificity.

2 | METHODS AND INCLUSION CRITERIA

Electronic databases, for example, Google Scholar, Scholar's Portal, and Primo, were searched for peer-reviewed articles published between January 2000 and mid-June 2023. The articles were retrieved from searches relevant to "fish primers," common names of commercial species of fish and "primers," "fish authentication," "fish assay," "fish primer for authentication," and "molecular fish identification methods." The criteria to include the articles in this manuscript were: (1) original articles with new PCR primers designed, (2) only targeted bony fish where DNA extraction occurred on muscle tissue (no eggs, shellfish, mollusks, etc.), (3) publication date between 2000 and mid-June 2023, (4) relevant to commercial species of fish, and (5) focus on fish authentication, forensics, and/or identification. Articles examining population genetics, environmental DNA (eDNA), fish species not commonly eaten (e.g., exotic species) or published prior to 2000 were excluded. In the end, 313 assays from 191 publications (Supporting Information Reference List) were found to be relevant for this review.

2.1 | Data recorded

Data captured from the articles included identified fish (species and common name), sample type (fresh, frozen, canned, etc.), target gene, reaction type (singleplex/multiplex), primer pairs and probe (if applicable), size of amplified DNA fragment, and detection method.

2.2 | Fish families/order groupings

Fish families and grouping into various orders were modified following Rasmussen and Morrissey (2009) in conjunction with the FishBase database retrieved from fishbase.org. Fish order and groupings are outlined in Table 1.

2.3 | Case study

2.3.1 | Inclusion/exclusion criteria

Commercial tuna assays ($n = 27$) were reviewed as a case study of primer/probe specificity. The assays included ($n = 19$) met the following criteria: (1) were from primary articles, with new PCR-specific primers, relevant to commercial species of tuna, over the last 23 years, (2) illustrated the most common tuna species explored in the literature, (3) represented the most common gene target used in primer design for tuna, *cytb* (cytochrome *b*), (4) targeted ≤3 species (for multiplex assays), (5) contained sufficient data to align primers and probes to both target and non-target taxa. Primer annealing temperatures and primer concentrations were not considered part of the in silico analysis. Table 2 summarizes the assays covered in the case study.

TABLE 1 Common species covered in the literature and their respective order.

Order	Taxonomic families	Common species (common name)
Acipenseriformes	Acipenseridae	Sturgeon
Beloniformes	Exocoetidae	Flying fish
Clupeiformes	Engraulidae and Clupeidae	Anchovies, herrings, shads, sardines, menhadens, and sprats
Cypriniformes	Cyprinidae	Common carp, Colden carp
Gadiformes	Gadidae, Phycidae, Lotidae, and Marluccidae	Hakes, pollock, codfish, and haddock
Gobiiformes	Gobiidae	Transparent goby
Lophiiformes	Lophiidae	Monkfish and anglerfish
Osmeriformes	Salangidae	Icefish
Perciformes	Apogonidae, Carangidae, Centropomidae, Lutjanidae, Moronidae, Percidae, Polyprionidae, Sciaenidae, Serranidae, and Sparidae	Perches, sunfish, groupers, snappers, jacks, porgies (seabream), bass, drums, wreckfish, dolphinfish, and billfish
Pleuronectiformes	Pleuronectidae and Soleidae	Flounder, halibut, plaices, soles, and turbots
Salmoniformes	Salmonidae	Salmon and rainbow trout
Scombroidei	Scombridae, Trichiuridae, Istiophoridae, and Xiphiidae	Mackerel, tuna, bonito, cutlassfish, hairtails, ribbonfish, billfish, and swordfish
Siluriformes	Clariidae, Ictaluridae, and Pangasiidae	Catfish
Tetraodontiformes	Tetraodontidae	Pufferfish

Note: Orders and families were retrieved from fishbase.ca for common species included in the literature between 2000 and 2023.

2.3.2 | Case study DNA reference database and alignment library for in silico specificity testing

DNA sequences were obtained for selected *taxa* from NCBI GenBank and the Barcode of Life Datasystems with the R (R Core Team, 2020) package MACER (V 2.1) (Young et al., 2021) using the auto_seq_download() function with default settings.

A total of 19 species of tuna and common substitutes were included in this study as outlined in Table 3. Species from the genus *Thunnus* included are outlined in Table 3. Bonito, a tuna-like fish species, is often substituted for or mixed with tuna products from the genus *Thunnus* (Gordoa et al., 2017; Lockley & Bardsley, 2000). Thus, the bonito were also included. Lastly, as escolar is commonly substituted for tuna species and can pose a risk to consumers' health (Warner et al., 2013), *Lepidocybium flavobrunneum* (escolar) was included.

Downloaded sequences were aligned in MAFFT using the automatic alignment strategy with all other default settings (Katoh & Standley, 2013). The multiple sequence alignment (MSA) was manually edited, and records not aligning and/or causing gaps or a stop codon after translation were removed. The final MSA contained 945 records. Primers and probes were aligned to the MSA, and nucleotide mismatches between primers/probes and each record in the MSA, when complete overlapping nucleotide sequence data was available, were individually assessed using a custom R script. Results, analysis, alignment, and script are outlined in the Supporting Information Results and Alignment files attached.

2.3.3 | Primer mismatch scoring

Various studies have explored the factors affecting primer binding and DNA extension. According to Kumar and Chordia (2015), primer efficiency depends on (1) annealing and extension temperature, primer kinetics, and dissociation, (2) location of primer mismatch and primer stability, and (3) polymerase recognition efficiency. However, a larger number of primer mismatches may significantly affect primer-annealing and, subsequently, efficiency, specificity, and reproducibility. Lefever et al. (2013) found that primers with ≥4 individual mismatches successfully inhibited amplification, whereas 3 mismatches in a primer and 2 in its primer-pair were also inhibitory regardless of location. As such, it is recommended that when primers are designed in silico, ≥4 nucleotide mismatches in a single primer or 3 mismatches in a primer and ≥2 in its primer-pair should be implemented to prevent nonspecific amplification (Lefever et al., 2013).

Therefore, this study scored the average number of nucleotide mismatches between primers and probes against the expected binding region for all unique hap-

TABLE 2 List of included/excluded assays for commercial tuna species over 23 years, using the *cytb* region.

Targeted species category	Identified species	Scientific name	Reaction type	Primer/Probe (5′–3′)	Product size (bp)	Detection group	Reference	Inclusion/ Exclusion and justification
Tuna	Yellowfin tuna	*Thunnus albacares*	Multiplex	Tuna F3: ATACGCAATTCTTCGGTCC Tuna B3: TTGTTCTCAGCTCAGCCT Tuna LF: GAAGTGTGCAGGAAGGGAA Tuna LB: GCGGAACAGCCCTTCATTA Tuna FIP: TGGTCGGAATGTTAGAGTT-CGCAGCCTCCATCCTTGTACTT Tuna BIP: TGCAGACGTAGCCATTCTT-ACCAGGCTACTTGGCCGATAA	–	LAMP	Ali et al. (2022)	Excluded as these are LAMP primers
Tuna	Albacore, yellowfin tuna, bigeye tuna, bluefin tuna, and skipjack	*Thunnus alalunga*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus thynnus*, and *Katsuwonus pelamis*	Multiplex	PCR Cytbs: AACGGGGCCTCTTTCTTCTT Cytbas: GAAGGCGGTCATCATAACTAG PER SP1: CCTCYTTCTTCTTYATCTG SP2: 7(T)CTTCTTTATCTGYATYTACT SP3: 16(T)CGGCTCYTACCTVTACAA SP4: 22(T)GAAACATCGGWGTAGTMCT	132	DNA sequence analysis, multiplex PCR	Bottero et al. (2007)	Excluded as species target >3 in a multiplex system
Tuna	Bigeye tuna	*Thunnus obesus*	Singleplex	BET588(F): AGGCTTTTCAGTAGACAATGC BET588(R): TGGATTATTTGAACCTGTTTCG BET588(P): HEX-CAATTCTTCATCTTCTTTTCCTT	127	qPCR	Chuang et al. (2012)	Included
Tuna	Southern bluefin tuna	*Thunnus maccoyii*	Singleplex	SBT875(F): AAACATGAAACATCGGAGTAGTACTC SBT875(R): CATATGGGACTGCGGATA SBT875(P): HEX-AGTTATGATAACCGCCTTC	135	qPCR	Chuang et al. (2012)	Included
Tuna	Yellowfin tuna	*Thunnus albacares*	Singleplex	YFT330(F): CGAGGACTTTACTACGGCTCTT YFT330(R): CGGTCATCATAACTAGGAGTAGGAGTAC YFT330(P): FAM-CCTATACAAGGAAACATGAAA	82	qPCR	Chuang et al. (2012)	Included
Tuna	Skipjack tuna	*Katsuwonus pelamis*	Singleplex	Kat-F: GGTCCTAGCTCTTCTTGCA Kat-R: TGCAAGTGGGAAGAAGATG	238	Conventional PCR	Lee et al. (2022)	Included

(Continues)

TABLE 2 (Continued)

Targeted species category	Identified species	Scientific name	Reaction type	Primer/Probe (5′–3′)	Product size (bp)	Detection group	Reference	Inclusion/ Exclusion and justification
Tuna	Albacore	*Thunnus alalunga*	Singleplex	Ala-F: GTTTCGTGATCCTGCTAGTG Ala-R: CCTCCTAGTTTGTTG GAATAGAT	178	Conventional PCR	Lee et al. (2022)	Included
Tuna/Bonito	Bullet tuna	*Auxis rochei*	Singleplex	AR333-L: GCTTGATGTGGGGTGGTGTAAC AR457-H: TCTCCTTGGCTTTGCAATC	121	Conventional PCR	Lin and Hwang (2008b)	Included
Tuna/Bonito	Frigate mackerel	*Auxis thazard*	Singleplex	AT363-L: TGGCGGGTGTAAAATTATCTGG AT541-H: ACACGAGACCGGGTCTAATAAC	156	Conventional PCR	Lin and Hwang (2008b)	Included
Tuna/Bonito	Eastern little tuna	*Euthynnus affinis*	Singleplex	EA642-L: CCCCTCAAATTCATTCAACAAG EA777-H: CTAGTGATGATAACTGCCTTCG	113	Conventional PCR	Lin and Hwang (2008b)	Included
Tuna/Bonito	Skipjack tuna	*Euthynnus pelamis*	Singleplex	EP844-L: GCCAATATGGGAGTAAATGCAG EP976-H: TACCCCTGACGTAGAATCAGCC	112	Conventional PCR	Lin and Hwang (2008b)	Included
Tuna/Bonito	Oriental bonito	*Sarda orientalis*	Singleplex	SO862-L: GCAAATGAAGAAAAAGGAGGCG SO994-H: ATTTCTAGCAATGCACTACACC	110	Conventional PCR	Lin and Hwang (2008b)	Included
Tuna/Bonito	Bluefin tuna, albacore, yellowfin tuna, bigeye tuna, skipjack, eastern little tuna, frigate mackerel, and oriental bonito	*Thunnus thynnus*, *Thunnus alalunga*, *Thunnus albacares* and *Thunnus obesus*, *Katsuwonus pelamis*, *E. affinis*, *Auxis thazard*, and *Sarda orientalis*	Multiplex	CbP2L: AAGGACGTAGCCAACGAAG CbP2H: TCAGTAGCCCACATTTGCC Cb126L: GCYTYTACTACGGYTCYTAC Cb126H: CCCCTCAGAATGATATTTGTCC Cb146L: CCTCGCAATACACTATACCCC Cb146H: CGATGTGGAAGTAGATGCAG	207, 126, 146	PCR-RFLP	Lin and Hwang (2007)	Excluded as species target >3 in a multiplex system

(Continues)

TABLE 2 (Continued)

Targeted species category	Identified species	Scientific name	Reaction type	Primer/Probe (5′–3′)	Product size (bp)	Detection group	Reference	Inclusion/ Exclusion and justification
Bonito	Skipjack, eastern little tuna, frigate mackerel, frigate tuna, and oriental bonito	*Euthynnus pelamis*, *Euthynnus affinis*, *Auxis rochei*, *Auxis thazard*, *Sarda orientalis*	Multiplex	AR333-L: GCTTGATGTGGGGTGGTGTAAC-(22-mer) AR457-H: TCTCCTTGGCTTTGCAATC-(19-mer) AT410-L: TAATGCTAGGGAAGCTAGTGCC-(22-mer) AT707-H: CATTACCAACCTCCTATCTGC-(21-mer) EA401-L: GGGGGAGAAAAGTGCTAAGG (20-mer) EA777-H: CTAGTGATGATAACTGCCTTCG-(22-mer) EP42-L: GGGAAGAAGATGAGGAATAACG-(22-mer) EP259-H: CTTGCATCCATTCTCGTCC-(19-mer) SO290-L: AATTGAGCGGAGAATGGCG-(19-mer) SO774-H: TAATAATAACTGCCTTCGTCGG-(22-mer)	143, 318, 398, 236, 506	DNA sequence analysis, multiplex PCR	Lin and Hwang (2008a)	Excluded as species target >3 in a multiplex system
Tuna/Bonito	Bluefin tuna, atlantic bonito	*Thunnus thynnus*, *Sarda sarda*	Multiplex	Bluefin: GCCGAGATGTCAACTTCGGTTGACTT Bonito: CCTCAGTCGCCCACATTTCACGA-GACGTTAATTTCGGCTGAATA Non-specific: ACATCTGTCCTCAGGGAAGAACGTA	207, 225	Conventional PCR	Lockley and Bardsley (2000)	Included
Tuna	Yellowfin, bigeye tuna, skipjack	*Thunnus albacares*, *Thunnus obesus*, *Katsuwonus pelamis*	Singleplex	TunaFor: CAGGACTATTCCTCGCAATACA TunaRev: CGAAACCAAGGAGGTCTTTGTA	577	DNA sequence analysis	Michelini et al. (2007)	Included
Tuna	Yellowfin, bigeye tuna, skipjack	*Thunnus albacares*, *Thunnus obesus*, *Katsuwonus pelamis*	Triplex	PrimerYF: CCGCAGTCCCATATGTTGGAACTACT PrimerBE: ATTACTAACCTTCTATCCGCAGTCC-CATACGTCGGAACTACC PrimerSK: CCACCTCCTATTCCTTCACGAAACCGGA TunaRev: CGAAACCAAGGAGGTCTTTGTA TunaRev2: CGAA**G**CCAAGGAGGTCTTTGTA	246, 262, 113	DNA sequence analysis	Michelini et al. (2007)	Excluded forward primer will not align

(Continues)

TABLE 2 (Continued)

Targeted species category	Identified species	Scientific name	Reaction type	Primer/Probe (5′–3′)	Product size (bp)	Detection group	Reference	Inclusion/ Exclusion and justification
Tuna	Albacore, Yellowfin tuna, bigeye tuna, bluefin tuna, and skipjack tuna	*Thunnus alalunga*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus thynnus*, *Katsuwonus pelamis*	Singleplex	H276: ACTAGGAGTAGGAGTACTACTC L276: ACTTTGGCTCACTACTTGGCC	276	PCR-RFLP	Pardo and Pérez-Villareal (2004)	Included
Tuna	Yellowfin tuna, skipkack tuna	*Thunnus albacares*, *Katsuwonus pelamis*	Duplex	ThunSpp F: CACCGTTGTTATTCAACTA ThunSpp R: ATCATGCAGARATATTAGAGGG ThunSpp Probe: [Biotin]—AACTCACCCGCTACTAAA Thun2 F: GCCGAGGACTTTACTACG Thun2 R: GTAAGGACGGTAGCTCCT ThunAlb probe[Biotin]—TCTTACCTATACAAGGAAACA ThunPel probe: [Biotin]—TGTAGTCCTACTTCTCCTAGTA	121, 145	Other	Santaclara et al. (2015)	Included
Tuna	Atlantic bluefin tuna, albacore	*Thunnus thynnus*, *Thunnus alalunga*	Singleplex	Thun3 F: GGCATCAGTTGACCTAAC Thun3 R: TAATTAGTACAGCTCATACAAACA ThunAla probe: [Biotin]—GTGTTTGATATTGAGAGATAGC Thun4 F: ACTTTGGCTCACTACTTGGC Thun4 R: CAGGGGTATAGTGTATTGCGAG ThunThy probe [Biotin]—CTTATTTCTCAGATCCTTACAG	152, 78	Other	Santaclara et al. (2015)	Thun3 F & R and Thun Ala probe excluded. Primers not able to align
Tuna	Skipjack tuna	*Katsuwonus pelamis*	Singleplex	Forward 5′-TAGACAACGCCACCCTTACC-3′ Reverse 5′-CGGTTTCGTGAAGGAATAGG-3′ Probe HEX-BHQ1 5′-TCCCCTTCGTCATCGCAGCC-3′	101	qPCR	Servusova and Piskata (2021)	Included
Tuna	Yellowfin tuna, bluefin tuna, and albacore	*Thunnus albacares*, *Thunnus thynnus*, *Thunnus alalunga*	Singleplex/ duplex	Ray-For: GCCCACATTTGCCGAGATG Ray-Rev: GAAAGAGGCCCCGTTTGC A/Y-Pb: FAM-CGGATGAGTCAACC-MGB R-Pb: VIC-TCCGGATAAGTCAACC-MGB	69	qPCR, PCR-RFLP, and DNA sequence analysis	Terio et al. (2010)	Included

(Continues)

TABLE 2 (Continued)

Targeted species category	Identified species	Scientific name	Reaction type	Primer/Probe (5′–3′)	Product size (bp)	Detection group	Reference	Inclusion/ Exclusion and justification
Tuna	Yellowfin tuna, bluefin tuna, and albacore	*Thunnus albacares*, *Thunnus thynnus*, and *Thunnus alalunga*	Singleplex/ duplex	Ayr-For: CCACTTCCTATTCCCATTCGTCAT Ayr-Rev: CCTGTTTCGTGAAGGAAAAGAAGGT A-Pb: VIC-AAGAATTGTCATAGCTGCG-MGB Y/R-Pb: FAM-AATTGTCATGGCTGCG-MGB	69	qPCR, PCR-RFLP, and DNA sequence analysis	Terio et al. (2010)	Included
Tuna	Yellowfin tuna, bluefin tuna, albacore	*Thunnus albacares*, *Thunnus thynnus*, *Thunnus alalunga*	Singleplex/ duplex	Yar-For: TTCTTCACCTTCTTTTCCTTCACGAA Yar-Rev: GTAAGAGAAGTATGGGTGGAATGAGATTT Y-Pb: VIC-TCCGATTGGATTATTTGA-MGB R/A-Pb: FAM-CCGATTGGATTGTTTGA-MGB	98	qPCR, PCR-RFLP, and DNA sequence analysis	Terio et al. (2010)	Included
Tuna	Yellowfin tuna, bigeye tuna, skipjack	*Thunnus albacares*, *Thunnus obesus*, *Katsuwonus pelamis*	Multiplex	AD: CCTTACAGGACTATTCCTCGC AR: CCGATGTTTCATGTTTCTTTG BD: GGCCGAGGCCTTTACTACGGC GA: GAAGAATCGGGTGAGGGTGGC CD: GTTGAATGAATCTGAGGAGGC CR: GATCACGAAACCAAGGAGG	528, 171	DNA sequence analysis	Terol et al. (2002)	Excluded as primer pairs were not intended to be specific
Tuna	Albacore, bigeye tuna	*Thunnus albacares*, *Thunnus obesus*	Singleplex	Tunal-F: CTYCTATCCGCAGTCCCATATGTYGG Tunal-R: GGAATAGGGAGAAGTAGAGGACG	500–750	DNA barcoding	Wulansari et al. (2015)	Included
Tuna	Skipjack tuna	*Katsuwonus pelamis*	Singleplex	KP-FIP: CCGATTCAGGTAAGGATTGC-CTTTTCACTTACATTCCGACCAGTC KP-BIP: CCTTCATTATCATCGGC-CAAGTTTTTCTGCAAGTGGGAAGAAGATG	242, 327, 570	Conventional PCR, DNA sequencing	Xiong et al. (2021)	Excluded as these are LAMP primers

TABLE 3 Species included in the reference library for the case study.

Species scientific name	Common name	Inclusion/Exclusion
Auxis rochei	Bullet tuna	Included
Auxis thazard	Frigate tuna	Included
Euthynnus affinis	Kawakawa	Included
Euthynnus alletteratus	Spotted tunny	Included
Katsuwonus pelamis	Skipjack tuna	Included
Lepidocybium flavobrunneum	Escolar	Included
Sarda chiliensis	Pacific bonito	Included
Sarda orientalis	Striped bonito	Included
Sarda sarda	Atlantic bonito	Included
Thunnus alalunga	Albacore	Included
Thunnus albacares	Yellowfin tuna	Included
Thunnus atlanticus	Blackfin tuna	Included
Thunnus maccoyii	Southern bluefin tuna	Included
Thunnus obesus	Bigeye tuna	Included
Thunnus thynnus	Bluefin tuna	Included
Thunnus orientalis	Pacific bluefin tuna	Included
Thunnus tonggol	Longtail tuna	Included

Note: Common names are based on the FDA Seafood List (https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set = SeafoodList).

lotypes for both target and nontarget taxa in the MSA. Only the number of mismatches was recorded, not the mismatch location or type. Further information on the scoring criteria can be found in Section 3.4.

2.3.4 | Considerations

Although the *cytb* locus of interest is approximately 1141 bp, some downloaded sequences were shorter (e.g., ~200 bp) or of various lengths (not the entire 1141 bp region); therefore, only records with full data for the area where the primers or probes align were studied for mismatch. For example, if a species had 10 unique haplotypes and the sequence associated with the primer-binding region was only available for 3 of the haplotypes, only those 3 haplotypes were examined in the sequence alignment.

3 | FISH ORDERS AND DETECTION METHODS

3.1 | Orders of fish in the literature

Table 4 summarizes the major fish orders for which DNA identification assays have been developed for fish authentication and their corresponding references. Sections 3.1.1–3.1.7 discuss each fish species' order and their susceptibility to fraud.

3.1.1 | Perciformes

Perciformes, comprising over 40% of bony fishes, is the largest and most diverse order, making it difficult to define due to its broad range of morphological characteristics (Bray & Gomon, 2020). *Percoidei*, a suborder of *Perciformes*, also known as percoids, include perches, snappers, groupers, sunfish, jacks, porgies (seabream), bass, drums, wreckfish, and dolphinfish (Rasmussen & Morrissey, 2009). Due to the large number of species included in this order, Section 3.1.1.1 will cover fish from the Scombridae family to adequately discuss assays developed for them.

This manuscript covers 42 assays (not including those for Scombridae) relevant to percoid authentication and detection (Table 4). The most discussed species in the literature are snappers, perches, and groupers. Although it is important to recognize that fish fraud affects multiple species within the order *Perciformes*, this article will only focus on snappers, Nile perch (*Lates niloticus*), wreckfish (*Polyprion americanus*), and groupers (*Epinephelus marginatus*) for the sake of conciseness and relevance.

Snappers are one of the most expensive and highly sought-after species of fish (Sivaraman et al., 2019). Red snapper (*Lutjanus campechanus*), a fish with a high price and value, is often substituted with cheaper, lower-value species (Rasmussen & Morrissey, 2009). Previous studies have reported mislabeling rates of 73%–100% for red snapper, which is often substituted with lower-valued snappers,

TABLE 4 Number of assays developed in the last 23 years, classified by fish order.

Order	Number of assays[a]	References[a]
Perciformes	42	[3, 4, 6, 10, 11, 12, 13, 26, 29, 33, 34, 37, 44, 50, 61, 62, 63, 80, 92, 94, 100, 101, 120, 136, 137, 147, 148, 149, 161, 162, 164, 186, 189, 191]
Perciformes (Scombroids)	97	[5, 6, 7, 20, 21, 25, 38, 39, 40, 45, 54, 60, 65, 74, 77, 84, 85, 95, 96, 99, 100, 103, 106, 107, 108, 109, 110, 111, 113, 114, 115, 125, 127, 129, 132, 141, 143, 150, 155, 157, 158, 164, 171, 175, 179, 181, 182]
Gadiformes	54	[2, 4, 8, 17, 28, 35, 42, 57, 58, 71, 75, 76, 77, 83, 90, 94, 102, 112, 120, 121, 123, 126, 130, 142, 145, 153, 154, 156, 160, 166, 176]
Salmoniformes	50	[43, 48, 51, 52, 55, 56, 66, 67, 69, 72, 94, 104, 105, 116, 119, 128, 135, 138, 150, 173, 174, 180, 183, 185, 188, 190]
Clupeiformes	14	[9, 18, 47, 64, 88, 89, 132, 134, 140, 178]
Tetraodontiformes	10	[1, 30, 81, 82, 122, 139, 184]
Pleuronectiformes	8	[27, 47, 49, 70, 76, 94, 124]
Others	49	[4, 9, 14, 15, 16, 19, 22, 24, 31, 32, 36, 46, 50, 53, 59, 61, 65, 66, 78, 79, 86, 93, 97, 98, 117, 118, 120, 128, 131, 133, 144, 146, 151, 152, 159, 163, 167, 168, 170, 187]

[a]Number of assays and number of references may differ as some articles have developed more than one assay reported in the same publication. List of references listed in the supplementary reference list.

seabream, rockfish, or mahi-mahi (Isaacs & Hellberg, 2020).

Grouper, an expensive, highly appreciated, and highly demanded meat, is often substituted with lower-value fish species like Nile perch or wreck fish (Asensio et al., 2008). Asensio et al. (2008) designed and tested an assay on 70 samples of commercial fish fillets and found that 58 of them were mislabeled. In their study, 34/70 samples of Nile perch and 13/70 wreck fish samples were mislabeled (Asensio et al., 2008). Ali, Di Taranto et al. (2022) and Chen et al. (2018) also did small-scale market validations of their assays on commercial samples labeled "grouper" and found that 4/4 and 3/18 samples, respectively, were mislabeled. Grouper is often substituted with Nile perch, which has been signaled by the Food and Agriculture Organization (FAO) as one of the leading causes of the increased demand, high prices, and shortage in the supply of Nile perch in European countries (FAO, 2012).

Scombroids

As mentioned earlier, scombroids belong to the order *Perciformes* and the suborder *Scombroidei*. Families within scombroids are Scombridae, which include tunas, mackerels, and bonito; Trichiuridae, which include hairtails, cutlassfish, and ribbonfish; Istiophoridae, which include billfish; and Xiphiidae, which include swordfish (Rasmussen & Morrissey, 2009). As outlined in this review, the Scombridae family has been the most researched group of scombroids for genetic identification, with 97 assays developed (Table 4).

One of the most commercially relevant fish in the Scombridae family is tuna. Tuna consumption and production are primarily driven by the canned tuna (>75% of all tuna catch) and sashimi/sushi industry (FAO, 2017). Skipjack (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) are the species most commonly used in canned tuna, whereas yellowfin tuna dominates the fresh/frozen market (FAO, 2017). The highly-priced yellowfin tuna is often substituted with the lower-value skipjack (FAO, 2017). Bluefin tuna (*Thunnus thynnus*) and bigeye tuna (*Thunnus obesus*) are usually preferred for sushi/sashimi (FAO, 2017).

A study of sushi mislabeling in Los Angeles, CA (USA), showed a high rate of mislabeling in tuna samples. Yellowfin tuna was most commonly substituted with bigeye tuna (58.3% substitution) (Willette et al., 2017). In addition, yellowfin tuna was found in products under the generic label "tuna" (Willette et al., 2017). This is concerning as yellowfin and bigeye tuna were classified as threatened and vulnerable species, respectively, at the time of the study (Willette et al., 2017). Similarly, Roungchun et al. (2022) reported that 20% of yellowfin tuna products were partially or completely substituted with bigeye tuna. Among the product categories tested, the highest mislabeling rate (42%) was found in dried (jerky and flakes) yellowfin tuna products. Servusova and Piskata (2021) found that 24% of products labeled as yellowfin tuna were mislabeled. Most products (84% of samples tested) were labeled "white tuna" when they were, in fact, escolar, which contains a toxin known to cause gastrointestinal illness (Warner et al., 2013).

Several assays reviewed in this article were applied to small-scale market surveys for tuna mislabeling. Liu et al. (2016) found that the mislabeled samples tested often contained a mixture of species or non-tuna species. Similarly, Klapper et al. (2023) observed that commercial samples

labeled "light tuna" were, in fact, mixed species. Mislabeling rate of tuna ranged from 5% to 24% depending on the assay and study (Mitchell & Hellberg, 2016; Servusova & Piskata, 2021).

It is important to note that mishandling Scombroids can cause scombroid poisoning, also known as scombrotoxin poisoning (Food and Drug Administration [FDA], 2017). It is among the top four reported seafood-related illnesses, causing symptoms like nausea, diarrhea, hives, and respiratory distress (FDA, 2017). Therefore, mislabeling of scombroids, like tuna, can severely impact public health. Furthermore, food safety issues due to mercury content in tuna should be considered. Some species of tuna, like skipjack, are considered "Best Choices" for at-risk consumers, such as pregnant women, whereas yellowfin and albacore are in the "Good Choices" category (FDA, 2017). Bigeye and bluefin tuna should be avoided by at-risk consumers due to their high mercury (FDA, 2017). As such, substitutions with these high-mercury fish can pose food safety risks.

3.1.2 | Gadiformes

Codfish, hakes, and pollock species belonging to the order *Gadiformes* are often referred to as Gadoids. *Gadiformes* include the families Gadidae, Merlucciidae, Phycidae, and Lotidae (Rasmussen & Morrissey, 2009). The order *Gadiformes* comprises over 18% of the total global fish catch (Fernandes et al., 2017). The Gadidae family represents some of the most commercially relevant species (Fernandes et al., 2017). Of this family, cod is one of the most consumed fish in the European Union, ranking only second to tuna (Helgoe et al., 2020). Of the Merlucciidae family, hake, with a reported mislabeling rate of 30%, is one of the most consumed and commercially relevant fish species in Spain (Garcia-Vazquez et al., 2011; Helgoe et al., 2020). Some commercially relevant *Gadiformes* species, like Atlantic cod, have been listed in the Red List of Threatened Species due to high consumption, exploitation, and shrinking global stock levels (Herrero et al., 2010).

This review covers 54 assays for the detection and authentication of *Gadiformes* (Table 4). Herrero et al. (2010) designed an assay able to detect mislabeling of 40 Gadoid species processed by different methods (e.g., fresh, frozen, and canned) and found that 20% of fish products sampled were mislabeled. In addition, 10 of the samples did not include the scientific name on the label and appeared under the ambiguous name "cod" (Herrero et al., 2010). Taboada, Sánchez, Sotelo et al. (2017) tested their assay on 31 commercially relevant ling (*Molva molva*) samples, of which 19.4% were mislabeled. In fact, three of the samples were substituted with other species, with two identified as lower-value species (*Brosme brosme* and *Molva dypterigia*) and one identified as a higher-value species (*Gadus morhua*) (Tabaoda, Sánchez, Sotelo et al., 2017). Substituting higher-value species may be a result of trying to meet fishing quotas. However, this can undermine the sustainability and management of cod stocks. Substituting with a lower-value species is economic fraud. Another study by Taboada, Sánchez, Perez-Martín et al. (2017) found that 22.6% of Pacific cod, Alaska pollock, and ling tested were mislabeled.

3.1.3 | Salmoniformes

Salmon, trout, and char species belonging to the family Salmonidae are often referred to as salmonids, under the order *Salmoniformes* (Rasmussen & Morrissey, 2009). The most common species are Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and coho salmon (*Oncorhynchus kisutch*), with estimated 2.7, 0.9, and 0.2 million tons sold globally in 2020, respectively (Espiñeira, Vieites et al., 2009; FAO, 2023). The commercial value of salmonids is driven by the species. For example, Atlantic salmon, sockeye salmon (*Oncorhynchus nerka*), and Chinook salmon (*Oncorhynchus tshawytscha*) are more expensive than other salmonid species (Espiñeira, Vieites et al., 2009), which drives fraudulent activities.

This review covers 50 assays designed for the detection of *Salmoniformes* (Table 4). In a small-scale application to commercial products, Herrero et al. (2011) tested their assay in 20 processed fish samples. No species' scientific name was present on any of the product labels, and 5% of the samples tested showed mislabeling (Herrero et al., 2011). Feng et al. (2017) also tested their designed assay on 11 samples of salmon and found that one of the samples could not be detected, indicating that it was not part of the Salmonidae family. Similar to the findings of Herrero et al. (2011), Feng et al. (2017) observed that the term salmon was used on labels ambiguously. Muñoz-Colmenero et al. (2019) indicated that in three different studies, the mislabeling and substitution rate were 4.7%, 8.3%, and 11.1%, respectively. The products were found to be substituted with another *Oncorhynchus* species associated with strong allergic reactions due to differences in amino acid composition (Muñoz-Colmenero et al., 2019). Hence, these substitutions can also pose health risks to consumers.

3.1.4 | Clupeiformes

Clupeiformes are small pelagic fishes, including anchovies, herring, sardines, and shads, which belong to the families Engraulidae and Clupeidae (Rasmussen & Morrissey,

2009). This review covers 14 assays developed for the detection of *Clupeiformes* (Table 4). Some of the species most frequently targeted are the European anchovy (*Engraulidae encrasicolus*), European pilchard (*Sardina pilchardus*), and round sardinella (*Sardinella aurita*). Although small pelagic fishes are usually used for bait in North America, they are often utilized in specialty products in European markets and make up a significant portion of the global seafood supply (Rasmussen & Morrissey, 2009).

In an investigation of small pelagic fishes, Giusti et al. (2019) reported a mislabeling rate of <1%. However, the type of processing affected the sample integrity and the ability for downstream authentication methods to validate species. Giusti et al. (2019) found that it was difficult to identify species in samples containing oil. As most small pelagic fishes are consumed as highly processed products, such as smoked, canned, salted, or cured, detection methods may be hindered.

3.1.5 | Tetraodontiformes

Tetraodontiformes accounted for 10 species-specific assays developed to authenticate the family Tetraodontidae (pufferfish), which is known for its toxicity. Tetrodotoxin poisoning has been primarily linked to species within the genus *Lagocephalus* and can cause vomiting, diarrhea, tachycardia, muscle weakness, and many other symptoms (Acar et al., 2017; Sangthong et al., 2014). Numerous cases of illness have been caused by the ingestion of pufferfish illegally mixed with other fish meat (Acar et al., 2017).

3.1.6 | Pleuronectiformes

Pleuronectiformes, commonly called flatfishes, include halibuts, soles, plaices, flounders, and turbots (Rasmussen & Morrissey, 2009). This review includes eight assays designed for the authentication and detection of *Pleuronectiformes* species (Table 4). Halibut (*Hippoglossus hippoglossus*) has been ranked as one of the most valued fish in this group, with high consumer demand (Rasmussen & Morrissey, 2009). Prior studies have found that Greenland halibut (*Reinhardtius hippoglossoides*) and common sole (*Solea solea*) are often substituted for halibut (Céspedes et al., 2000; Rasmussen & Morrissey, 2009).

Sole has a high market value and is in high demand, which can subsequently increase exploitation levels and cause stock levels to decrease (Herrero et al., 2012). In fact, when Herrero et al. (2012) tested their assay on 40 commercial samples of common sole, 30% were mislabeled. The study found that other sole species were used as substitutes (Herrero et al., 2012). Other mislabeling rates reported for this order were 24%, 15%, and 8% for common sole, yellowfin sole (*Limanda aspera*), and halibut, respectively (Paracchini et al., 2017). Halibut has also been reported to be substituted at a rate of 33% with olive flounder (*Panaeolus olivaceus*), which can cause gastroenteritis due to the presence of a species-specific parasite (Willette et al., 2017). Wong and Hanner (2008) also reported a high substitution rate of halibut with flounder, whereas Liou et al. (2020) reported mislabeling of 40% of halibut samples, which were substituted with California flounder.

3.1.7 | Others

Other fish types reviewed were *Cypriniformes* (common carp and golden carp), *Siluriformes* (catfish), *Acipenseriformes*, which include fish like sturgeon, *Beloniformes* (flying fish), *Beryciforms* (red sea marine fish), *Gobiiformes* (transparent goby), *Lophiiformes* (monkfish and anglerfish), *Moroniformes* (seabass), and *Osmeriformes* (icefish). Table 4 summarizes the assays for these categories.

3.2 | Assay type

Various DNA authentication technologies have been developed for species identification. While not encompassing every method or technique available on the market, Sections 3.2.1–3.2.9 include the most common detection methods used in the literature over the last 23 years for commercial fish species. These genetic identification methods are helpful in preventing illegal fishing, economic fraud, and maintaining fish stocks (Rasmussen & Morrissey, 2009). Table 5 summarizes the number of assays for each method discussed. Tables S1–S9 provide a detailed overview of all assays reviewed.

3.2.1 | Real-time polymerase chain reaction (real-time PCR or qPCR)

Of the 313 assays for fish identification reviewed in this study, 102 used real-time PCR. Real-time PCR, also known as quantitative PCR (qPCR) or real-time qPCR, is often used because it combines amplification and detection into a single step (Wong & Medrano, 2005), thereby eliminating the need for post-PCR handling and minimizing the risk of cross-contamination. Real-time PCR amplification is based on the cycle threshold, or the C_t value, which indicates the cycle number at which the fluorescence intensity exceeds the background fluorescence (Wong & Medrano, 2005). Real-time PCR is considered highly reliable and sensitive, as it can detect a single copy of a target gene.

TABLE 5 Assays developed using the most prominent DNA-based methods for fish authentication published from 2000 to 2023.

Technique (DNA-based method)	Sub type (detection method)	Number of assays[a]	References[a]
Real-time PCR (qPCR)		102	[3, 9, 17, 20, 22, 23, 24, 26, 29, 30, 33, 34, 35, 40, 41, 56, 57, 58, 63, 67, 69, 70, 71, 72, 73, 75, 76, 92, 99, 104, 109, 111, 118, 122, 129, 138, 143, 145, 156, 157, 160, 161, 163, 164, 173, 176, 178, 180, 186]
	TaqMan	58	[3, 20, 22, 33, 34, 40, 41, 56, 57, 67, 69, 70, 71, 72, 73, 75, 76, 99, 109, 111, 129, 154, 156, 161, 163, 164, 186]
	SYBR Green	16	[9, 17, 24, 26, 40, 92, 118, 145, 173, 176, 178, 180]
DNA sequencing		61	[4, 16, 21, 25, 35, 36, 39, 42, 43, 44, 45, 47, 54, 55, 64, 78, 82, 87, 89, 90, 91, 92, 96, 100, 107, 112, 119, 123, 126, 133, 134, 135, 137, 140, 148, 157, 158, 159, 172, 175, 191]
	Mini-sequencing	1	[42]
	Pyrosequencing	2	[47]
	FINS	13	[25, 54, 55, 78, 82, 92, 100, 140, 148]
	SNP/RAD sequencing	3	[90, 112]
PCR-RFLP		36	[2, 7, 8, 27, 31, 32, 51, 55, 80, 81, 88, 106, 113, 120, 125, 126, 130, 132, 140, 147, 149, 157, 170, 179, 189]
Conventional PCR		54	[1, 13, 14, 24, 29, 35, 38, 46, 59, 69, 83, 92, 93, 101, 102, 103, 108, 110, 114, 121, 169, 173, 174, 175, 176, 178]
DNA barcoding		38	[14, 19, 29, 30, 50, 52, 53, 57, 58, 60, 61, 65, 68, 79, 86, 93, 98, 115, 123, 124, 128, 144, 146, 152, 167, 171, 178, 184, 185, 187]
	Mini-barcoding	10	[19, 50, 60, 79, 115, 146, 152]
	High resolution melt (HRM)	7	[29, 30, 57, 58, 185]
Multiplex PCR		25	[6, 9, 10, 15, 18, 21, 25, 44, 62, 63, 66, 74, 84, 85, 107, 117, 127, 136, 139, 164]
PCR-SSCP		5	[11, 28, 148, 151, 168]
PCR-RAPD		3	[12, 37, 131]
Others		36	[5, 23, 37, 48, 49, 51, 77, 94, 95, 97, 105, 116, 141, 142, 150, 153, 155, 162, 166, 173, 181, 182, 183, 188, 190]
	LAMP	11	[5, 49, 105, 142, 166, 173, 181, 182]
	Lateral flow dipstick (LFD) PCR	5	[153]
	PCR-AFLP	2	[188, 190]
	Microarray assay	2	[94, 97]

Abbreviations: AFLP, amplified fragment length polymorphism; FINS, forensically informative nucleotide sequencing; LAMP, loop-mediated isothermal amplification; PCR (polymerase chain reaction); RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; qPCR, quantitative or real-time PCR; SSCP, single-strand conformation polymorphism.

[a]Number of assays and number of references may differ as some articles have developed more than one assay reported in the same publication. List of references listed in the Supporting Information Reference List.

However, it can be costly as expensive equipment and reagents are often required. Table S1 presents all the real-time PCR assays (using various master mixes such as FastStart TaqMan or SYBR Green) for commercial species of fish compiled in this study. Real-time PCR is often selected to authenticate species due to its high efficiency. Several studies have validated the efficiency of real-time PCR, with efficiencies ranging from 82.2% to 103.0% for tuna (Liu et al., 2016), 84.0%–100.0% for mackerel (Prado et al., 2013), 101% for cuttlefish (Velasco et al., 2020), and 100% for other species (Herrero et al., 2012; Liu et al., 2016). These studies, and others,c demonstrate that real-time PCR is an efficient and effective technique.

The ability of real-time PCR to amplify small fragment sizes and low levels of starting DNA material is advantageous for testing processed commercial fish products in

which DNA integrity may be affected. Some processing methods known to have an impact on DNA integrity are exposure to high pressure, high temperature, and acidic solutions (Velasco et al., 2013). For example, several articles reviewed (Table S1) amplified small fragments of the DNA target region. The smallest fragment size amplified was a 60-bp gene region in Atlantic mackerel by Velasco et al. (2013). Other studies, for example, Hellberg et al. (2010), Liu et al. (2016), Prado et al. (2013), Velasco et al. (2013), Kang (2019), and Servusova & Piskata (2021), developed assays to amplify fragment sizes <100 bp. Furthermore, Cao et al. (2013) showed that real-time PCR effectively detected sample mixtures of monkfish, with DNA as low as 0.01% in the final mixture. Prado et al. (2013) and Kang (2019) found that their real-time PCR assays showed high sensitivity, detecting as low as 5×10^{-3} ng of DNA for a mackerel assay and 1×10^{-3} ng/μL of DNA for a tilefish assay, respectively.

Another key advantage of using real-time PCR is the ability to multiplex. This allows identification and quantification for multiple species in the same tube, thereby saving time and money (due to fewer reagents and supplies required). Section 3.2.6 discusses multiplex PCR in further detail.

3.2.2 | DNA sequencing/analysis

DNA sequencing, DNA sequence analysis, or FINS (forensically informative nucleotide sequencing) allows for the visualization and determination of a precise sequence of nucleotides in an organic sample. DNA sequencing has enhanced the throughput, speed, and sensitivity of species authentication and genetic profiling. Although sequencing is time-consuming, requires high data handling capacity, and is technically demanding compared to other DNA-based methods, it provides the most information (Teletchea, 2009). Sanger sequencing uses reverse strand synthesis using deoxy-nucleotides and dideoxy-nucleotides, creating extension products with different lengths (Kircher & Kelso, 2010). As the chain-terminating nucleotide is labeled with a unique fluorescent dye, extension products can be read using DNA sequencing software after separation through capillary electrophoresis (Schoales, 2015). FINS uses DNA sequencing and phylogenetic analysis to identify a sample of interest based on informative nucleotide sequences (Li et al., 2011). This allows for the discrimination of closely from distantly related species by comparison against a sequence database (Li et al., 2011).

DNA sequencing has been effective in identifying species of fish for authentication purposes. Of the 313 assays reviewed, 61, as outlined in Table 5, used sequencing as either the sole method for fish genetic profiling/authentication or as a supplementary method to test the validity of other methods (see Table S2 for full list of assays). However, generating a complete genetic profile or sequence may be hindered in samples with highly degraded DNA (like canned fish). As such, assays that target a shorter region of nucleotides are important for detecting species in highly processed products.

Dalmasso et al. (2013) used mini-sequencing, a method based on traditional sequencing that targets a shorter oligonucleotide fragment, to authenticate 20 samples of highly processed cod/*Gadiformes* species. The study found that mini-sequencing was as accurate, precise, and reliable as traditional sequencing. Next-generation sequencing (NGS) or high-throughput sequencing (HTS) are terms used to describe modern sequencing technologies. HTS aims to reduce the cost of traditional DNA sequencing while producing thousands of sequences simultaneously. For example, Paracchini et al. (2019) used HTS to verify and authenticate 30 Gadoid species in lightly processed foods. This study found that HTS could also be applied to authenticate and identify mixtures in products.

3.2.3 | PCR-restriction fragment length polymorphism (PCR-RFLP)

PCR-restriction fragment length polymorphism (RFLP) amplifies a gene target or fragment by traditional PCR and then uses restriction enzymes to cut the amplicon into smaller fragments (Teletchea, 2009). As these fragments are of various sizes, they can be differentiated and visualized on an agarose gel through electrophoresis (Rasmussen, 2012). PCR-RFLP can identify numerous species with a single pair of primers depending on the type of restriction enzymes chosen (Teletchea, 2009). Compared to DNA sequencing, PCR-RFLP is cheaper and does not require advanced instrumentation. In addition, analysis can often be performed on publicly available software, which is relatively easy to navigate (Rasmussen, 2012). However, PCR-RFLP has several disadvantages; (1) incomplete digestion with restriction enzymes could lead to false results, (2) intraspecific variation may cause the addition or deletion of a single nucleotide polymorphism, necessary as a restriction enzyme binding/recognition site, (3) it is more labor intensive, and (4) it is not suitable for high-throughput (Rasmussen, 2012; Teletchea, 2009).

Of the 313 assays reviewed in this study, 36 were developed to use PCR-RFLP to authenticate or detect fish species (Table 5). Aranishi et al. (2005) designed an assay targeting the 558-bp fragment of a gene region (*cytb*) to successfully detect 4 unmarked cod samples using 4 different restriction enzymes. Espiñeira et al. (2009) used PCR-RFLP to amplify a 142-bp region of the *cytb* target

in salmon species. This method showed 100% specificity for the species tested and was used to authenticate 25 commercial products. This assay successfully amplified a fragment size <200 bp and could, therefore, be used for highly processed and canned samples (Espiñeira et al., 2009). Quinteiro et al. (2001) developed a PCR-RFLP assay for detecting hake species using the mtCR (mitochondrial control region), amplifying a fragment of approximately 197 bp using 4 restriction enzymes. The hake control region showed less variability than other teleosts, which may be a reason for its success (Quinteiro et al., 2001). This assay was also successful in identifying mixed species compared to DNA sequencing (Quinteiro et al., 2001). Lastly, Mata et al. (2020) designed a nested PCR-RFLP assay to detect four species of tuna, that is, skipjack, albacore, yellowfin, and bigeye tuna, using the COI (cytochrome *c* oxidase I) gene. This method could be used to detect as low as 10% skipjack in the sample (Mata et al., 2020), suggesting that this technique may be helpful in detecting sample mixes and canned fish. A full list of assays is provided in Table S3.

However, as mentioned earlier, there are numerous limitations to this method. A study on snappers found that blacktail snapper (*L. fulvus*) and blackspot snapper (*L. fulviflamma*) could not be distinguished using RFLP as they are closely related species (Sivaraman et al., 2018).

3.2.4 | Conventional PCR

Conventional PCR, or endpoint PCR, comprises (1) DNA amplification, (2) separation of DNA segments, and (3) detection. When applied to species identification, conventional PCR uses species-specific primers that amplify a genetic marker only in the target species. Unlike real-time PCR, where visualization and quantification occur in real-time, conventional PCR requires post-PCR handling, where products are separated and detected on an agarose gel by electrophoresis. As such, conventional PCR is more labor intensive and has an increased risk of cross-contamination than real-time PCR; however, it provides information faster than sequencing or methods requiring downstream testing. Therefore, conventional PCR is a cheap, rapid, sensitive, and efficient method to detect the presence of the target gene.

Of the 313 assays reviewed in this study, 54 used conventional PCR for fish authentication (Table 5). Table S4 summarizes all conventional PCR fish assays collected in this study. As mentioned previously, highly processed samples (thermal or high pressure treated) may exhibit low DNA integrity; as such, it is important that DNA-based assays can detect small fragment sizes (<200 bp). Many of the conventional PCR assays reviewed amplified DNA target regions <200 bp (Acar et al., 2017; Hellberg et al., 2010; Kang, 2019; Laknerová et al., 2014; Lin & Hwang, 2008a; Xiong et al., 2020). Xiong et al. (2020) assessed the sensitivity of conventional PCR. In this study, the species-specific band of cod samples was reported to be more sensitive than SYBR Green real-time PCR but less sensitive than TaqMan probe real-time PCR. Kang (2019) also found that tilefish samples could be visualized from serial dilutions of 1×10^{-2} ng/μL for some species of tilefish. As such, conventional PCR is sensitive to amplify small fragment sizes and low DNA quantities.

Primer dimers may also be detected in conventional PCR, which may obscure the 5′ end of the sequence and/or lead to false positives (Ivanova et al., 2007). Even though conventional PCR has fewer downstream steps than other DNA-based authenticating methods, it requires post-PCR handling, which can introduce cross-contamination. In addition, this detection technique is not automated like real-time PCR. Instead, detection requires gel electrophoresis and visualization of the gel under UV light. As such, nonspecific amplification, including cross-reactivity and faint bands, may produce false positive results, as reported by Hellberg et al. (2010). Furthermore, ethidium bromide, often used to visualize gels, requires special handling due to its toxic and mutagenic effects. Therefore, even though detection is rapid, conventional PCR has an increased risk of false positives and contamination, often requiring special handling.

3.2.5 | DNA barcoding and mini-barcoding

DNA barcoding is an authentication method where a short, standardized sequence is used to identify a particular species. This standardized target sequence acts as a "barcode," similar to a UPC (Universal Product Code) (Hellberg et al., 2016). The DNA barcode most often used for animals is a ~650-bp region of the COI gene, which usually shows sufficient divergence between species but remains relatively conserved within species (Hellberg et al., 2016; Rasmussen Hellberg & Morrissey, 2011). DNA barcoding uses DNA sequencing methodology as outlined in Section 3.2.2 to authenticate products. The resulting DNA sequence is then searched against a sequence database to identify the species. As it relates to fish, DNA barcoding provides an effective and reliable way of authenticating products. Its use of universal primers that amplify the COI gene in most fish species allows for broad application of the method across a wide range of fish categories. However, there have been some criticisms of using this technique with certain fish species groups, as

the COI or *cytb* regions may not exhibit adequate variation to differentiate between closely related species (Hellberg et al., 2016).

Another criticism stems from traditional DNA barcoding primary focus on mitochondrial DNA (mtDNA), which determines that it cannot be used to identify hybridized species (Rehbein, 2013). As such, nuclear targets such as ITS1 (internal transcribed spacer), rhodopsin, or 5S rRNA are preferable in cases of species hybridization (Hellberg et al., 2016). These gene targets are further discussed in Section 3.3.

Wulansari et al. (2015) found that barcoding with the *cytb* gene, in the range of 500–750 bp, for processed tuna was inadequate for authenticity testing due to damaged DNA. As such, shorter regions of the gene targets, or "mini-barcodes," are required for highly processed fish samples. As outlined in Table 5 and Table S5, this article reviewed 38 DNA barcoding (including mini-barcoding assays). Mitchell and Hellberg (2016) used both the mtCR and ITS1 to authenticate samples of tuna in a mini-barcoding assay. The study found that amplifying shorter regions of DNA was adequate for the authentication of tuna in 43% of canned products tested. The remaining samples likely could not be identified due to factors such as the presence of PCR inhibitors and/or multiple species, DNA fragmentation, or lack of primer binding.

3.2.6 | Multiplex PCR

Multiplex PCR is a method in which more than one locus is amplified simultaneously in the same reaction (Henegariu et al., 1997). Hence, multiplex PCR can detect multiple species at once. It is rapid, efficient, and versatile (Settanni & Corsetti, 2007). Multiplex PCR amplifies more than one target by mixing primer pairs at various specificities/concentrations and separating the species-specific amplicons on an agarose gel by electrophoresis (Settanni & Corsetti, 2007). However, there are some disadvantages to multiplex PCR as (1) it requires post-PCR handling, which can cause cross-contamination and exposure to toxic chemicals (see Section 3.2.4), (2) the likelihood of primer dimers is increased due to the use of multiple primers in one reaction, and (3) it is very sensitive to the magnesium ($MgCl_2$) concentration, as it affects specificity and efficiency of the reaction (Settanni & Corsetti, 2007).

Multiplex PCR is an effective approach to investigate species-specific amplification in complex mixtures (Settanni & Corsetti, 2007). Of the 313 assays reviewed in this study, 25 were unique multiplex assays for fish authentication (Table 5 and Table S6). Lin and Hwang (2008a) designed a multiplex PCR assay to authenticate five species of raw and cooked bonito (*Euthynnus pelamis*, *Euthynnus affinis*, *Auxis rochei*, *Auxis thazard*, and *Sarda orientalis*). These five bonito species could be accurately and successfully identified in a one-step multiplex PCR approach by examining the mitochondrial *cytb* (Lin & Hwang, 2008a). However, only five out of the eight cooked samples were identified in this study due to degraded DNA; therefore, this assay was only effective for mildly processed/heated fish (Lin & Hwang, 2008a). Damasceno et al. (2016) designed an assay for the detection of nine species of grouper using the COI gene. The assay was specific and effective for differentiating species (Damasceno et al., 2016). The technique is low-cost, rapid, and practical (as it does not require complicated laboratory equipment) (Damasceno et al., 2016). Georgiadis et al. (2014) and Rocha-Olivares and Chávez-González (2008) designed assays using a multiplex-haplotype-specific-PCR method that were specific enough to identify diagnostic point mutations.

3.2.7 | PCR-single strand conformation polymorphism (PCR-SSCP)

PCR-strand conformation polymorphism (SSCP) is a method based on the electrophoretic mobility of single stranded DNA, which corresponds to its nucleotide sequence (Teletchea, 2009). The amplified ssDNA strand is run on a non-denaturing polyacrylamide gel electrophoresis (PAGE), and the band pattern is visualized. PCR-SSCP is easy to perform and provides quick results. Furthermore, it can detect point mutations or changes to a single base in a sequence (Hayashi & Yandall, 1993). This allows close species to be accurately and efficiently discriminated as single base changes can lead to different folded conformations (Hayashi & Yandall, 1993; Teletchea, 2009). Sivaraman et al. (2019) used PCR-SSCP to differentiate between the closely related snapper species blacktail snapper (*L. fulvus*) and blackspot snapper (*L. fulviflamma*), which was not possible using PCR-RFLP.

Of the 313 assays reviewed in this study, only 5 were developed for fish authentication using PCR-SSCP (Table 5 and Table S7). Although this method can differentiate between species, intraspecies variation can lead to false identification (Teletchea, 2009). In addition, references/controls are required as detection depends on the native PAGE and multiple/false bands being differentially visualized based on the conformation of the ssDNA (Hayashi & Yandall, 1993; Teletchea, 2009). Chapela et al. (2007) developed a PCR-SSCP assay using a 588-bp *cytb* fragment to differentiate and authenticate various hake species. Species-specific band patterns were observed for 6 out of 11 species belonging to the genus *Merluccius*. When the Euro-African group of species was examined,

European hake (*M. merluccius*), Senegalese hake (*M. senegalensis*), and South-African hake (*M. capensis*) showed the same band patterns and were unable to be differentiated (Chapela et al., 2007). Argentine hake (*M. hubbsi*) and Benguela hake (*M. polli*) showed intraspecies variability (Chapela et al., 2007) that helped differentiation. Detection accuracy may be affected by fragment size, as 100–300 bp fragments exhibited 99% accuracy, whereas >400 bp fragments exhibited >80% accuracy (Girman, 1996).

3.2.8 | PCR-random amplified polymorphic DNA (PCR-RAPD)

PCR-random amplified polymorphic DNA (RAPD) uses a short, arbitrary primer sequence to amplify a short segment of the DNA (Teletchea, 2009). This method is easy to set up, as prior genetic knowledge of the species under study is not required (Asensio et al., 2002). In addition, it allows for the simultaneous amplification of multiple loci as it targets numerous sequences in a species' DNA (Asensio et al., 2002). Chiu et al. (2012) designed a PCR-RAPD assay to detect the presence of giant grouper (*Epinephelus lanceolatus*) in 14 fresh tissue samples. Among the 95 primers designed, 21 showed 13.3%–66.7% polymorphism, generating DNA fragments from 100 to 3000 bp (Chiu et al., 2012). One primer (RAPD115) showed high polymorphism, whereas another primer (RAPD73) was able to discriminate between wild and farmed giant grouper (Chiu et al., 2012). Asensio et al. (2002) designed a RAPD assay to differentiate grouper (*Epinephelus guaza*), wreck fish (*P. americanus*), and Nile perch (*L. niloticus*), which was tested against 15 fillets of each species. The study found that two primers (S1 and L1) could discriminate among the 3 species and successfully generate a unique fingerprint (Asensio et al., 2002). PCR-RAPD is useful for samples with limited quantities of DNA, where the DNA sequence is unknown, and/or to understand species-specific differences (Asensio et al., 2002).

However, PCR-RAPD may not be able to identify species in products containing multiple species or in severely degraded samples (Asensio et al., 2002). This limits the application of the technique because processed food products (e.g., canned) often contain a mixture of fish species. Furthermore, thermal treatments, for example, cooking or canning, can degrade DNA, which may negatively impact RAPD detection and analysis. Of the 3 assays reviewed (Table S8), DNA band fragments ranged from 100 to 3000 bp (Asensio et al., 2002; Chiu et al., 2012), which may indicate the necessity for larger fragment sizes non-retrievable from highly processed foods.

3.2.9 | Others

Other studies, as outlined in Table 5 and Table S9, used a variety of less common fish authentication and identification methods, such as loop-mediated isothermal amplification, lateral flow dipstick, and amplified fragment length polymorphism (PCR-AFLP).

3.3 | DNA target/assay region

A revision of DNA markers or gene regions targeted for fish identification, outlined in Table 6, revealed that most studies focused on mitochondrial DNA (mtDNA). There are several advantages to using mtDNA for fish species identification in commercial products. First, there are more copies of mtDNA than nuclear DNA (nDNA) inside a cell (Murugaiah et al., 2015). Therefore, it is more likely that a fragment within this genome will be amplified, especially in highly degraded DNA, as seen in processed products (Teletchea, 2009). This may be why most studies reviewed used mtDNA to identify and detect fish species in cooked, canned, and smoked samples. Second, mtDNA has a higher mutation rate than nDNA, which allows for closely related species to be identified and differentiated (Murugaiah et al., 2015). Lastly, as mtDNA is generally conserved (i.e., does not undergo recombination) as it is maternally inherited, the study and exploration of this target are more straightforward and more manageable than nDNA (Lin & Hwang, 2008b; Murugaiah et al., 2015). However, due to the maternal inheritance pattern of mtDNA, it cannot be used to differentiate hybridized species.

Intraspecific variability should be considered when studying species based on single-base polymorphisms (Pardo & Pérez-Villareal, 2004; Terol et al., 2002). Ideally, the most suitable DNA markers for species identification should be variable between species and display low to no-intraspecific variations (Teletchea, 2009). Some mtDNA markers, *cytb*, COI, and 16S, have been extensively researched for fish authentication, as described in the sections below and outlined in Table 6.

3.3.1 | Cytochrome *b* (*cytb*)

Cytb satisfies most of the criteria for a good DNA marker. Hence, it is widely studied for a large number of species and is the most used gene for species phylogeny (Teletchea, 2009). This gene is the most studied DNA region, with 94 assays designed for fish authentication. Lin and Hwang (2008b) found that the diversity between the 10 tested scombroid species was 9.4%–17.9%, which indicates that

TABLE 6 Common gene targets used in fish authentication assays from 2000 to 2023.

Target gene	Subgroup	Number of assays[a]	References[a]
COI		85	[1, 4, 14, 15, 16, 19, 22, 30, 33, 34, 35, 41, 43, 44, 50, 52, 53, 57, 58, 59, 61, 63, 65, 68, 69, 71, 72, 73, 74, 78, 82, 86, 89, 92, 93, 104, 113, 116, 122, 127, 128, 141, 143, 146, 152, 160, 163, 164, 167, 172, 173, 178, 179, 180, 186, 187]
	COII	1	[20]
cytb		94	[2, 5, 6, 8, 9, 18, 21, 24, 28, 31, 32, 35, 40, 42, 47, 49, 51, 54, 55, 57, 63, 64, 80, 81, 82, 88, 89, 94, 98, 100, 102, 103, 106, 107, 108, 110, 114, 116, 121, 125, 132, 135, 136, 137, 139, 140, 141, 142, 143, 144, 150, 153, 154, 157, 158, 164, 166, 168, 169, 170, 171, 172, 173, 174, 175, 180, 184, 185, 187, 190]
16S		36	[6, 17, 40, 47, 56, 62, 74, 79, 87, 89, 94, 97, 109, 111, 112, 120, 153, 161, 162, 164, 179, 186, 187]
	16S rRNA	31	[6, 17, 47, 56, 62, 74, 79, 87, 89, 94, 97, 109, 111, 112, 120, 161, 161, 164, 179, 186]
	16S rDNA	2	[153, 179]
12S		22	[11, 20, 22, 27, 85, 91, 99, 138, 139, 143, 145, 149, 166, 176, 179, 189, 191]
	12S rRNA	15	[11, 20, 22, 27, 85, 91, 99, 138, 139, 143, 145, 149, 176, 179, 189]
	12S rDNA	6	[166, 179, 191]
Mitochondrial control region (CR)		16	[25, 56, 59, 65, 96, 104, 115, 130, 179]
ATPase		10	[17, 40, 45, 103, 121, 143, 156, 179]
ND		11	[1, 20, 35, 26, 35, 74, 85, 99, 103]
Others		47	[3, 12, 26, 36, 37, 38, 39, 40, 46, 60, 66, 67, 70, 74, 75, 76, 83, 84, 90, 95, 101, 103, 112, 115, 117, 118, 119, 123, 124, 129, 133, 143, 144, 147, 148, 155, 188]
	Gene targets (growth hormone, transferrin gene, parvalbumin gene, pantophysin gene)	13	[3, 38, 67, 75, 76, 83, 101, 118, 119, 129, 144]
	5S	8	[7, 10, 60, 84, 126, 159]
	D-loop	5	[40, 117, 143, 147, 148]
	18S rRNA	4	[13, 77, 138, 187]
	ITS1	3	[39, 70, 115]
	Nuclear target	3	[123, 124, 133]
	SCAR marker	2	[37, 188]

Abbreviations: COI, cytochrome *c* oxidase 1; *cytb*, cytochrome *b*; ITS1, internal transcribed spacer; ND, NADH dehydrogenase; SCAR, sequence-characterized amplified region.

[a]Number of assays and number of references may differ as some articles have developed more than one assay reported in the same publication. List of references listed in the Supporting Information Reference List.

the distinguishable sequences of *cytb* were adequate for phylogeny and for the development of species identification methods (Lin & Hwang, 2008b).

Michelini et al. (2007) found that the genetic distances between the species tested (*T. albacares*, *T. obesus*, and *K. pelamis*) were 1 order of magnitude higher than the intraspecific distances, whereas the coefficient of differentiation was 0.9 ± 0.2. When a phylogenetic tree was created, the bootstrap values ranged from 90% to 100%, suggesting that *cytb* could be used to differentiate these three species (Michelini et al., 2007). Pardo and Pérez-Villareal (2004) found a high degree of conservation for the *cytb* target. *T. albacares*, *Thunnus alalunga*, and *T. thynnus* did not show any intraspecific variability, whereas the intraspecific variability was low for *T. obesus* and *K. pelamis* (Pardo & Pérez-Villareal, 2004). Similar results were reported by Terol et al. (2002), who observed low intraspecific variability among the three tuna species analyzed. Lastly, mini-barcoding with *cytb* offered improved results and performance than the COI gene for distinguish-

ing four fish species in the Gadidae family (Fernandes et al., 2017).

3.3.2 | Cytochrome *c* oxidase I (COI)

The COI region typically shows significant divergence between species but slight variation within species, making it ideal for species identification (Rasmussen & Morrissey, 2009). COI fragments have been able to distinguish and discriminate most animal and fish species effectively and reliably (Ward et al., 2005). Furthermore, DNA barcoding with COI has been adopted by the U.S. FDA for regulatory testing of fish species (Handy et al., 2011). Among the studies reviewed, 85 assays were designed utilizing the COI gene. An investigation into the COI barcoding sequences ($n = 874$) for commercially important trout and salmon species revealed that each species possesses a unique cluster of haplotypes, which are related but do not overlap (Rasmussen & Morrissey, 2009). Damasceno et al. (2016) found high bootstrap values for Epinephelidae species tested using the COI marker, indicating that the COI gene is reliable for identifying these species. High bootstrap values were also reported by Xu et al. (2016), where several tuna species were examined.

As the 650-bp region for the COI barcode may be degraded in highly processed samples, methods such as multiplex PCR and mini-barcoding, which target a shorter fragment of this gene region, are effective in species authentication. In particular, Fernandes et al. (2018) found that targeting a 102-bp region within the COI sequence by mini-barcoding could detect *Merluccius* spp. with confidence levels >99%. In fact, several studies targeted regions <200 bp within the COI gene (Acar et al., 2017; Cao et al., 2013; Chen et al., 2018; Hellberg et al., 2010; Kang, 2019; Servusova & Piskata, 2021).

3.3.3 | 16S rRNA

The 16S rRNA gene can be considered for fish authentication due to its inter-specific species polymorphism (Georgiadis et al., 2014). However, previous studies have found that the inter- and intra-specific divergence is lower for 16S rRNA than the *cytb* or COI gene targets (Nicolas et al., 2012). According to Nicolas et al. (2012), the 16S gene was 2.5 times less variable than both COI and *cytb*. Bootstrap values have also been reported to be lower for 16S rRNA than *cytb* and COI. Although Nicolas et al. (2012) identified difficulties in alignment due to insertions and deletions in the 16S gene region, of the 313 assays reviewed, 31 were based on the 16S rRNA region to authenticate fish.

Liu et al. (2016) found that 16S rRNA showed the most conservation, compared to COI and *cytb*, when tuna species (*Thunnus*) were tested. Feng et al. (2017) also reported that the 16S region was highly conserved among species of Salmonidae. Due to its high conservation, Lopez and Pardo (2005) used 16S rRNA for amplification of a conserved region in *Scombroidei* species as a positive control.

3.3.4 | 12S rRNA

The 12S rRNA mitochondrial gene has been reported as an effective target for fish detection and authentication due to its size (length), availability of sequence information in accessible databases, and mutation rate (Granata et al., 2012). Although 12S rRNA shows less degeneracy than other protein-coding genes belonging to the mitochondrial gene pool, its variation is sufficient for interspecies differentiation (Céspedes et al., 2000; Granata et al., 2012). As per Table 6, 15 assays were reviewed in this study. Céspedes et al. (2000) found that the 12S rRNA gene, when used with two restriction enzymes, *Aci*I and *Mwo*I, showed no intraspecific polymorphism for sole (*S. solea*) and halibut (*R. hippoglossoides*). In addition, high bootstrap values (98%) and high intraspecific homogeneity were reported for 1000 replicates of red snappers (*Lutjanus* spp.) (Zhang et al., 2007). When combined with molecular detection methods, this gene was effective in detecting and authenticating species.

3.3.5 | Mitochondrial control region (mtCR)

The mtCR is a noncoding region in DNA that exhibits high species variation and can be used to analyze variability within closely related species (Mitchell & Hellberg, 2016; Quinteiro et al., 2001). This review encompasses 16 assays using the mtCR region for fish authentication. Feng et al. (2017) found that the control region had the highest level of divergence than other regions like 16S rDNA, at 5.3%–14.3% for salmon species. The study also found that this region was less conserved than 16S rDNA (Feng et al., 2017). Liu et al. (2016) also reported that CR fragments showed more diversity and less conservation when species of tuna were examined. Similarly, Xu et al. (2016) found that mtCR allowed for the discrimination of tuna species and had a high bootstrap value. However, the phylogenetic tree created did not support the differentiation among *T. albacares*, *Thunnus Atlanticus*, or *T. tongol* but endorsed the monophyletic origin of other tuna species (Xu et al., 2016). Catanese et al. (2010) found that the mtCR was suit-

able and successful in detecting fraud within the *Scomber* genus.

3.3.6 | ATPase

ATPase (Adenosine triphosphatase) is another mtDNA target employed in identifying fish and fish products. As outlined in Table 6, 10 assays this gene target for species detection and authentication. This gene region is often targeted at the ATPase 6 and ATPase 8 loci. Prior studies have found high divergence among haplotypes when the ATPase genes were targeted. For example, Dammannagoda et al. (2011) reported a 1.5% mean divergence among clades when samples of skipjack tuna (*K. pelamis*) were examined. Xu et al. (2016) also identified higher divergence when using the ATPase gene rather than *cytb*, COI, 12S rDNA, and 16S rDNA in samples of tuna. This study found that after digestion with 2 restriction enzymes, the ATPase region was suitable for unambiguous and simultaneous identification of tuna or tuna-related species. Namikoshi et al. (2011) used the ATPase six region, in combination with PCR-RFLP, to effectively detect mixtures of cod species (*Gadus spp*. and *Micromesistius spp*.) as low as 0.5%.

3.3.7 | NADH dehydrogenase subunit

The NADH dehydrogenase subunits (ND) are mitochondrial genes often used for food authentication. In this review, 11 assays were identified for detecting and differentiating fish species, such as tuna or mackerel, using multiple ND subunits, primarily ND1, ND2, and ND5 as outlined in Table 6.

3.3.8 | Other

Other gene targets employed in fish assay development and authentication include the 5S, D-loop, 18S rRNA, ITS1, growth hormone, transferrin, parvalbumin, and pantophysin genes as outlined in Table 6.

3.4 | Case study

In silico results for mismatch and primer specificity of singleplex and multiplex assays are provided in Table 7, respectively. For ease of reporting, each assay was assigned a number from 1 to 19.

Primer and probe mismatches can be disruptive and cause variations in elongation efficiencies (Wright et al., 2014). For this review, assays were considered specific if the primer and probes: (1) were able to align with the target sequence(s) in the reference library, (2) showed <2 and <3 mismatches to the target sequence for a primer/probe pair or <4 mismatches to the target sequence for a single primer/probe, (3) did not exhibit a full match to nontarget record sequences, and (4) showed ≥3 and ≥2 mismatches to the primer/probe pair or ≥4 nucleotide mismatches to a single primer for nontarget references (through in silico analysis). Of the 19 assays reviewed in the study, 3 assays designed for the detection of *A. rochei, A. thazard*, and *S. orientalis* (Lin & Hwang, 2008b) showed in silico specificity based on the criteria set out herein, that is, by examining the match/mismatch count (Table 7). All other assays reviewed did not comply with one or more of the criteria. This indicates that there is a likelihood that these primers and/or probes may amplify nontarget species or may not amplify target species at all, generating false-positives or negatives. This is of particular concern in food products containing a mixture of species. There is also a risk that these nonspecific assays may amplify escolar (*L. flavobrunneum*), generating a false-positive for the target species and introducing a food safety risk to consumers. In fact, some primers and probes had <4 mismatches to escolar records and further investigation is required to understand whether they may be able to amplify in silico. Additional information such as the MSA and code can be found in the Supporting Information Files.

3.4.1 | Assay scoring

Due to the large number and variation in the validation techniques used in published assays, it can be challenging to test the reliability of an assay (Bruce et al., 2020). In silico analysis should be the first step in determining the efficiency of an assay. An assay scoring framework by Bruce et al. (2020) was modified to present the level of validation achieved for published assays. This framework was published for detecting eDNA, but it may be applied to numerous biological and forensic fields, including food authentication. As such, this modified framework was adapted for assays specific to food, as outlined in Table 8.

Interestingly, although all assays conducted in vitro testing against some off-target species, consistent with the potential for Level 2 validation, the in silico analysis conducted in the tuna case study showed the potential lack of assay specificity. Three assays for the detection of *A. rochei, A. thazard*, and *S. orientalis* from the Lin and Hwang (2008b) study showed no cross-reactivity with nontarget species (primers and/or probes were not able to align (or exhibit MSA) with any of the tested nontarget sequences

TABLE 7 Mismatch and primer specificity of the tuna assays reviewed.

Assay number	Assay type	Target	Reference*	Primer/Probe	Number of target records (full match)[a]	Average target nucleotide mismatch for records with <4 mismatches to primers/probes	Number of target records with ≥4 mismatches	Number of nontarget records with a full match[a] to primers/probes	Number of non- target records[a] with ≤4 mismatches to primers/probes[b]	Average nontarget nucleotide mismatch (≤4 mismatches to primers/probes)[b]	Potential in silico specificity
1	Singleplex	*Thunnus albacares*	Chuang et al. (2012)	YFT330(F): CGAGGACTTTAC-TACGGCTCTT YFT330(R): CGGTCATCATAAC-TAGGAGTAGGAGTAC YFT330(P): FAM-CCTATACAAGGAAACATGAAA	F: 131/150 R: 0/149 P: 147/150	F: 0.13 R: N/A P: 0.02	F: 0/150 R: 149/149 P: 0/150	F: 112/343 R: 0/700 P: 5/697	F: 297/343 R: 0/700 P: 651/697	F: 0.66 R: N/A P: 1.86	Not specific[c,d,e]
2	Singleplex	*Thunnus maccoyii*	Chuang et al. (2012)	SBT875(F): AAACATGAAA-CATCGGAGTAGTACTC SBT875(R): CATATGGGACTGCGGATA SBT875(P): HEX-AGTTATGATAACCGCCTTC	F: 8/33 R: 0/31 P: 5/34	F: 0.11 R: N/A P: 0.2	F: 24/33 R: 31/31 P: 24/34	F: 301/812 R: 0/734 P: 1/816	F: 789/812 R: 0/734 P: 766/816	F: 1.52 R: N/A P: 2.24	Not specific[c,d, e]
3	Singleplex	*Thunnus obesus*	Chuang et al. (2012)	BET588(F): AGGCTTTTCAGTAGACAATGC BET588(R): TGGAT-TATTTGAACCTGTTTCG BET588(P): HEX-CAATTCTTCATCTTCTTTTCCTT	F: 70/91 R: 84/91 P: 69/91	F: 0.24 R: 0.08 P: 0.25	F: 0/91 R: 0/91 P: 0/91	F: 98/680 R: 188/679 P: 4/641	F: 633/680 R: 537/679 P: 486/641	F: 1.28 R: 2.47 P: 1.88	Not specific[d,e]
4	Singleplex	*Katsuwonus pelamis*	Lee et al. (2022)	Kat-F: GGTCCTAGCTCTTCTTGCA Kat-R: TGCAAGTGGGAAGAAGATG	F: 0/1 R: 0/1	F: N/A R: N/A	F: 1/1 R: 1/1	F: 94/357 R: 0/352	F: 349/357 R: 0/352	F: 1.18 R: N/A	Not specific[c,d,e]
5	Singleplex	*Thunnus alalunga*	Lee et al. (2022)	Ala-F: GTTTCGTGATCCTGCTAGTG Ala-R: CCTCCTAGTTTGTTG GAATAGAT	F: 0/73 R: 0/71	F: N/A R: N/A	F: 73/73 R: 0/71	F: 1/280 R: 2/227	F: 7/280 R: 180/227	F: 2.71 R: 2.93	Not specific[c,d]

(Continues)

TABLE 7 (Continued)

Assay number	Assay type	Target	Reference*	Primer/Probe	Number of target records (full match)[a]	Average target nucleotide mismatch for records with <4 mismatches to primers/probes	Number of target records with ≥4 mismatches	Number of nontarget records with a full match[a] to primers/probes	Number of non- target records[a] with ≤4 mismatches to primers/probes[b]	Average nontarget nucleotide mismatch (≤4 mismatches to primers/probes)[b]	Potential in silico specificity
6	Singleplex	*Auxis rochei*	Lin & Hwang (2008b)	AR333-L: GCTTGATGTGGGGTGGTGTAAC AR457-H: TCTCCTTGGCTTTGCAATC	F: 1/1 R: 1/1	F. 0 R: 0	F: 0/1 R: 0/1	F: 0/357 R: 0/352	F: 4/357 R: 161/352	F: 3.00 R: 3.96	Specific
7	Singleplex	*Auxis thazard*	Lin & Hwang (2008b)	AT363-L: TGGCGGGTGTAAAATTATCTGG AT541-H: ACACGAGACCGGGTCTAATAAC	F: 1/17 R: 0/1	F. 1 R: 0	F: 0/17 R: 0/1	F: 0/753 R: 0/352	F: 124/753 R: 23/352	F. 3.65 R: 3.21	Specific
8	Singleplex	*Euthynnus affinis*	Lin & Hwang (2008b)	EA642-L: CCCCTCAAATTCATTCAACAAG EA777-H: CTAGTGATGATAACTGCCTTCG	F: 0/1 R: 0/1	F: N/A R: N/A	F: 1/1 R: 1/1	F: 0/348 R: 0/348	F: 0/348 R: 0/348	F: N/A R: N/A	Not specific[c]
9	Singleplex	*Euthynnus pelamis*	Lin & Hwang (2008b)	EP844-L: GCCAATATGGGAGTAAATGCAG EP976-H: TACCCCTGACGTAGAATCAGCC	F: 1/1 R: 0/1	F: 0 R: N/A	F: 0/1 R: 1/1	F: 1/494 R: 0/493	F: 447/494 R: 0/493	F: 2.64 R: N/A	Not specific[c,d,e]
10	Singleplex	*Sarda orientalis*	Lin & Hwang (2008b)	SO862-L: GCAAATGAAGAAAAAGGAGGCG SO994-H: ATTTCTAGCAATGCACTACACC	F: 1/1 R: 1/1	F. 0 R: 0	F: 0/1 R: 0/1	F: 0/473 R: 0/493	F: 425/473 R: 9/493	F. 3.35 R: 3.44	Specific
11	Singleplex	*Thunnus alalunga*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus thynnus*, and *Katsuwonus pelamis*	Pardo & Pérez-Villareal (2004)	H276: ACTAGGAGTAGGAGTACTACTC L276: ACTTTGGCTCACTACTTGGCC	F: 292/296 R: 169/576	F: 0.01 R: 0.55	F: 0/296 R: 2/576	F: 92/149 R: 13/272	F: 8/149 R: 18/272	F: 0.25 R: 2.01	Not specific[d,e]

(Continues)

TABLE 7 (Continued)

Assay number	Assay type	Target	Reference*	Primer/Probe	Number of target records (full match)[a]	Average target nucleotide mismatch for records with <4 mismatches to primers/probes	Number of target records with ≥4 mismatches	Number of nontarget records with a full match[a] to primers/probes	Number of non- target records[a] with ≤4 mismatches to primers/probes[b]	Average nontarget nucleotide mismatch (≤4 mismatches to primers/probes)[b]	Potential in silico specificity
12	Singleplex	*Thunnus Thynnus*	Santaclara et al. (2015)	Thun4 F: ACTTTGGCTCACTACTTGGC Thun4 R: CAGGGGTATAGTG-TATTGCGAG ThunThy probe [Biotin]—CTTATTTCTCAGATCCTTACAG	F: 96/97 R: 0/110 P: 16/99	F: 0.01 R: N/A P: 0.87	F:0/97 R: 110/110 P: 0/99	F: 288/348 R: 0/368 P: 1/356	F: 301/348 R: 0/368 P: 308/356	F: 0.9 R: N/A P: 1.08	Not specific[c,d,e]
13	Singleplex	*Katsuwonus pelamis*	Servusova & Piskata (2021)	Forward 5′-TAGACAACGCCACCCTTACC-3′ Reverse 5′-CGGTTTCGTGAAGGAATAGG-3′ Probe HEX-BHQ1 5′-TCCCCTTCGTCATCGCAGCC-3′	F: 228/241 R: 0/241 P: 233/241	F: 0.06 R: N/A P: 0.04	F:0/241 R: 241/241 P: 0/241	F: 27/492 R: 0/529 P: 0/491	F: 1/492 R: 0/529 P: 0/491	F: 1.98 R: N/A P: 1.77	Not specific[c,d,e]
14	Singleplex	*Thunnus albacares*, *Thunnus obesus*	Wulansari et al. (2015)	Tunal-F: CTYCTATCCGCAGTC-CCATATGTYGG Tunal-R: GGAATAGGGAGAAG-TAGAGGACG	F: 0/163 R: 0/67	F: 2.07 R: N/A	F: 0/163 R: 67/67	F: 0/602 R: 0/237	F: 429/602 R: 0/237	F: 3.09 R: N/A	Not specific[c,d]
15	Multiplex	*Thunnus thynnus*, *Sarda sarda*	Lockley & Bardsley (2000)	Bluefin: GCCGAGATGT-CAACTTCGGTTGACTT Bonito: CCTCAGTCGCCCA-CATTTCACGAGACGT-TAATTTCGGCTGAATA Nonspecific: ACATCTGTC-CTCAGGGAAGAACGTA	F1: 16/22 F2: 0/22 R: 17/42	F1: 0.32 F2: N/A R: 1.24	F1: 0/22 F2: 22/22 R: 0/42	F1: 8/473 F2: 0/473 R: 1/733	F1: 420/473 F2: 0/473 R: 681/733	F1: 1.19 F2: N/A R: 2.42	Not specific[c,d,e]

(Continues)

TABLE 7 (Continued)

Assay number	Assay type	Target	Reference*	Primer/Probe	Number of target records (full match)[a]	Average target nucleotide mismatch for records with <4 mismatches to primers/probes	Number of target records with ≥4 mismatches	Number of nontarget records with a full match[a] to primers/probes	Number of non- target records[a] with ≤4 mismatches to primers/probes[b]	Average nontarget nucleotide mismatch (≤4 mismatches to primers/probes)[b]	Potential in silico specificity
16	Multiplex	*Thunnus albacares*, *Katsuwonus pelamis*	Santaclara et al. (2015)	ThunSpp F: CACCGTTGTTATTCAACTA ThunSpp R: ATCATGCAGARATATTAGAGGG ThunSpp Probe: [Biotin]—AACTCACCCGCTACTAAA Thun2 F: GCCGAGGACTTTACTACG Thun2 R: GTAAGGACGGTAGCTCCT ThunAlb probe[Biotin]—TCTTACCTATACAAGGAAACA ThunPel probe: [Biotin]—TGTAGTCCTACTTCTCCTAGTA	F1: No data available R1: 0/139 P1: 70/71 F2: 147/151 R2: 0/313 P2: 132/391 P3: 227/390	F1: N/A R1: N/A P1: 0.03 F2: 1.88 R2: 0.07 P1: 1.88 P2: 0.06	F1: No data available R1: 139/139 P1: 0/71 F2: 0/151 R2: 313/313 P2: 0/391 P3: 150/390	F1: N/A R1: 0/303 P1: 231/290 F2:1/342 R2: 0/352 P1: 4/456 P2: 0/458	F1: 0/2 R1: 0/303 P1: 290/290 F2:296/342 R2: 0/352 P1: 410/456 P2: 119/458	F1: N/A R1: N/A P1: 0.07 F2: 0.67 R2: N/A P1: 1.88 P2: 3.92	Not specific[c,d,e,f]
17	Multiplex	*Thunnus albacares*, *Thunnus thynnus*, and *Thunnus alalunga*	Terio et al. (2010)	Ray-For: GCCCACATTTGCCGAGATG Ray-Rev: GAAAGAGGCCCCGTTTGC A/Y-Pb: FAM-CGGATGAGTCAACC-MGB R-Pb: VIC-TCCGGATAAGTCAACC-MGB	F: 256/262 R: 257/261 P1: 244/262 P2: 0/261	F: 0.03 R: 0.01 P1: 0.07 P2: N/A	F: 0/262 R: 0/261 P1: 0/262 P2: 261/261	F: 168/233 R: 166/233 P: 163/233 P2: 0/233	F: 233/233 R: 187/233 P: 232/233 P2: 0/233	F: 0.76 R: 0.24 P1: 0.91 P2: N/A	Not specific[c,d,e]

(Continues)

TABLE 7 (Continued)

Assay number	Assay type	Target	Reference*	Primer/Probe	Number of target records (full match)[a]	Average target nucleotide mismatch for records with <4 mismatches to primers/probes	Number of target records with ≥4 mismatches	Number of nontarget records with a full match[a] to primers/probes	Number of non- target records[a] with ≤4 mismatches to primers/probes[b]	Average nontarget nucleotide mismatch (≤4 mismatches to primers/probes)[b]	Potential in silico specificity
18	Multiplex	*Thunnus albacares*, *Thunnus thynnus*, and *Thunnus alalunga*	Terio et al. (2010)	Ayr-For: CCACTTCCTATTCC-CATTCGTCAT Ayr-Rev: CCTGTTTCGTGAAG-GAAAAGAAGGT A-Pb: VIC-AAGAATTGTCATAGCTGCG-MGB Y/R-Pb: FAM-AATTGTCATGGCTGCG-MGB	F: 136/140 R: 173/177 P1: 96/139 P2: 0/139	F: 0.03 R: 0.02 P1: 0.3 P2: N/A	F: 0/140 R: 0/177 P1: 0/139 P2: 139/139	F: 185/593 R: 113/593 P1: 109/593 P2: 0/593	F: 546/593 R: 516/593 P1: 533/593 P2: 0/593	F: 0.78 R: 2.14 P1: 1.74 P2: N/A	Not specific[c,d,e]
19	Multiplex	*Thunnus albacares*, *Thunnus thynnus*, and *Thunnus alalunga*	Terio et al. (2010)	Yar-For: TTCTTCAC-CTTCTTTTCCTTCACGAA Yar-Rev: GTAAGAGAAG-TATGGGTGGAATGAGATTT Y-Pb: VIC-TCCGATTGGATTATTTGA-MGB R/A-Pb: FAM-CCGATTGGATTGTTTGA-MGB	F: 173/177 R: 161/164 P1: 100/175 P2: 0/175	F: 0.02 R: 0.02 P1: 0.43 P2: N/A	F: 0/177 R: 0/164 P1: 0/175 P2: 175/175	F: 109/593 R: 176/587 P1: 168/589 P2: 0/589	F: 457/593 R: 540/587 P1: 539/589 P2: 0/589	F: 1.90 R: 1.54 P1: 1.82 P2: N/A	Not specific[c,d,e]

Abbreviations: F, forward primer; P, probe; R, reverse primer.

[a]Number of target records = number of records examined/total number of records. Records were only examined if the primers and probes were able to align in the region.

[b]Dataset also includes the number of nontarget records with ≤3 and ≤2 mismatches to the primer/probe pair.

[c]A single primer or probe not aligned to any target sequence(s) in the reference library or a single primer or probe contains ≥4 mismatches to the target or the primer/probe pair contains ≥3 and ≥2 mismatches to the target.

[d]Not specific due to full match for nontarget records.

[e]Primer/probe combination does not contain ≥4 nucleotide mismatches in a single primer or ≥3 and ≥2 mismatches in its primer/probe pair with nontarget species.

[f]No data available for target haplotypes.

*List of references listed in the Supporting Information Reference List.

TABLE 8 Assay validation scale for commercial fish authentication.

Level 1	In silico analysis	PCR protocol applied but not optimized				
Level 2	In silico analysis	PCR protocol applied and optimized	Some in vitro testing of species (target and off-target) for assay specificity			
Level 3	In silico analysis	PCR protocol applied and optimized	Testing in vitro species (target and off-target) for some closely related species to test for assay specificity	Some commercial/ market validation testing of samples and comparison with traditional DNA sequencing		
Level 4	In silico analysis	PCR protocol applied and optimized	Testing in vitro species (target and off-target) for all closely related species to test for assay specificity	Commercial/ market validation testing of samples and comparison with traditional DNA sequencing	Limits of detection and limits of quantification established	
Level 5	In silico analysis	PCR protocol applied and optimized	Testing in vitro species (target and off-target) for all closely related species to test for assay specificity	Commercial/ market validation testing of samples and comparison with traditional DNA sequencing	Limits of detection and limits of quantification established	Statistical modelling and understanding factors affecting detectability, for example various processing methods.

Abbreviation: PCR, polymerase chain reaction.
Source: Adapted from Bruce et al. (2020).

in silico, that is, mismatch averages ≥3 and ≥2 in each primer pair) and showed specificity for the target reference sequence(s) (with mismatch averages <3). These 3 assays achieved a score of Level 3 as they were tested against commercial samples, and samples were sequenced and compared to the sequence database. However, the LODs and quantification, which are needed to achieve Level 4 validation, were not established for these assays. Overall, this indicates a lack of standardization of validation techniques for fish species identification wherein the published assays do not perform as expected, leading to the potential for false negatives and false positives, which may also impact specificity and reproducibility.

3.4.2 | Limitations of the case study

The average primer mismatches presented in this case study may not account for all unique haplotypes as (1) not all sequences included in the DNA reference library spanned the entire *cytb* region and (2) there may be a lack of sampling/data available for a wide range of haplotypes. Furthermore, misidentified sequences in GenBank may have led to the inclusion of erroneous sequence data in the case study. Therefore, standardization of testing parameters, as well as collecting more sequence reference data, will be important for future studies.

Finally, this study treated all assays the same, regardless of whether they were intended to be species-specific at the initial PCR step or whether there were downstream differentiation methods. In vitro factors that may affect primer annealing and extension, such as primer concentration, location and type of primer mismatches, melting temperatures, and type of master mix (Wu et al., 2009), were not examined. This may make these results flawed, as a one-step approach was applied to all published tuna assays. However, this case study enforces the argument that (1) a standardized testing method for food authentication should be developed and (2) regardless of downstream methods, a lack of specificity in the primer/probe binding could have inhibitory effects, delay the elongation period, and/or amplify off-target species.

4 | CHALLENGES AND OPPORTUNITIES

Several assays have been designed to target specific, at-risk commercial fishes in recent years. Each DNA detection method and target loci present pros and cons that must be weighed prior to designing an assay. Cost is a primary factor in developing and using food authentication tools. However, as research progresses, the industry can improve protocols and techniques to allow for rapid, efficient, and cost-effective methods. Table 9 presents some characteristics of the most popular detection methods used in fish authentication and outlines the pros and cons (limitations) of each method. From the 313 original assays developed for commercial species of fish over the last 23 years, the most explored DNA-based method is real-time PCR, followed by DNA sequencing. The high efficiency, sensitivity, reproducibility, low intraspecies variation errors, ability to multiplex, high throughput, and ability to quantify short DNA fragments make real-time PCR an essential tool in

TABLE 9 Main characteristics of DNA-based methods, most commonly used in fish authentication, are organized from most to least recommended for commercial samples.

DNA-based method	Efficiency	Sensitivity	Reproducibility	Robustness to DNA degradation	Intraspecies variation errors	Ability to identify species mixtures	Ability to multiplex	Experimental steps/design	Cost
Real-time PCR (qPCR)	Highly efficient	Highly sensitive	Highly reproducible	High	Low	Yes	Able to multiplex and view fluorescence in real-time	Post-PCR handling not required	Can be expensive for reagents and equipment
DNA sequencing/analysis	Highly efficient	Highly sensitive	Highly reproducible	Medium-high	Low	Yes	Yes	Post-PCR handling required	Very expensive
PCR-RFLP	Highly efficient	Sensitive	Highly reproducible	Medium-high	Medium	Yes	Yes: Not able to handle a large number of samples/ high throughput	Requires post-PCR handling. Extensive knowledge of sample	Relatively cheap. Restriction enzymes can be costly
DNA barcoding, mini-barcoding	Efficient for longer fragment size.	Sensitive	Highly reproducible	Traditional barcoding: Low mini-barcoding: medium	Low-medium	Yes	Yes	Some handling required	Expensive
Multiplex PCR	Efficient	Sensitive	Reproducible	Medium-high	Medium	Yes	Yes	Requires post-PCR handling. EtBr may require special handling	Relatively cheap. May require more time to optimize assay
Conventional PCR	Efficient	Sensitive	Reproducible	Low	High as quantification is visualized on a gel	No	N/A	Requires post-PCR handling. EtBr may require special handling	Cheap
PCR-SSCP	Efficient	Sensitive	Reproducible	Medium-high	Low-medium	No	No	Post-PCR handling required	Intermediate expense
PCR-RAPD	Low efficiency	Low sensitivity	Some reproducibility	Low	Low-medium	No	Yes	Post-PCR handling required	Expensive

Note: This table was adapted and modified from Rasmussen and Morrissey (2008), Da-cheng et al. (2010), and Levin et al. (2018) as well as information reviewed in this article. Full references are in Supporting Information Reference List.

Abbreviations: EtBr, ethidium bromide; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

food/seafood authentication. For these reasons, it is listed as the most recommended DNA-based detection method in this review. However, as mentioned prior, designing multiplex assays can pose challenges.

The second most used detection method has been DNA sequencing; however, having the whole DNA sequence for the gene target may be difficult due to the quality and quantity of DNA present in food. As such, methods that amplify smaller fragment sizes, such as mini-barcoding, mini-sequencing, and NGS, may be preferable for testing highly processed products. Additionally, DNA sequencing is costly and requires more labor and time compared to real-time PCR. DNA detection methods should be chosen depending on the food product being tested and the capabilities of the lab (equipment, cost, etc.). For example, highly processed fish, including smoked, canned, or dried, products, are common in the supply chain. Therefore, detection methods should consider the type of sample and the amount of DNA present.

mtDNA has been the most common region targeted for identification, as it exists in circular form and is less susceptible to DNA degradation than nDNA. In addition, there are a lot more copies of mtDNA than nDNA inside a cell. However, intraspecific variability of the target fish species, as well as interspecific variation, should be considered when choosing the gene target.

Given the advantages and limitations of various detection methods described in this review, it may be beneficial to target multiple gene loci as well as to use multiple DNA analytical technologies to identify species. This will ensure that results are reported with more accuracy and confidence. There have also been criticisms in regards to using a single genetic marker for differentiating species (Xu et al., 2016). However, this can be a costly and time-consuming process. Researchers should thoroughly understand the limitations of their methods as they relate to technology and the fish species of interest. This is because the degree of species intra and inter-specificity will differ on various factors. Furthermore, it is important to note that in some cases a negative test result for a particular species may be due to a limitation of the assay (e.g., inability to amplify degraded DNA) rather than the absence of the target species.

In addition, the most studied gene regions were *cytb*, followed by COI; however, species may differ in their inter-species variability at various gene regions. The advantages and disadvantages of each DNA detection method and gene region, as they relate to efficiency, cost, sensitivity, reproducibility, inter-species variability, and intra-species variability, should be explored before designing and developing assays. Furthermore, primer and probe specificity have a significant impact on the sensitivity and quality of an assay. A case study of 19 commercial tuna assays targeting the *cytb* region showed a lack of standardization for specificity and assay validation (Table 8). For example, many of the assays did not show in silico specificity or did not receive higher than Level 3 on the assay validation scale. This ultimately affects the reliability, efficiency, and reproducibility of some of the published assays reviewed in this study. These results indicate the need to improve the standardization of food assay design and validation techniques, as the goal is to protect consumers' health. It is recommended that in silico tools be used to test primers for specificity prior to conducting laboratory tests. This will help save time, reduce the need for troubleshooting, decrease overall costs, and ensure assay specificity.

One of the limitations of this review is that assays surveyed herein tested anywhere between 1 and 20+ individual species using multiple targets and methods. Furthermore, many articles were missing specificity data, were unable to align with the reference library, and/or the types of specificity testing and reporting varied depending on the detection method used or researcher preference. Therefore, a valid statistical comparison among species, gene targets, and detection methods was difficult. This emphasizes the need for more focused and streamlined research to explore the reproducibility and reliability of DNA-based identification methods (Rasmussen & Morrissey, 2009). It is also important to note that this review does not cover the actual quality of an assay using either DNA technology or gene target loci. Although there are benefits and disadvantages of using various methods, the quality of an assay primarily depends on its primers' specificity.

5 | CONCLUSION

As the supply chain grows, there is a need for authentication of commercial species of fish due to environmental, economic, and food safety concerns. Fish fraud causes a breakdown in product traceability, which makes it a risk to consumers should there be a food safety event. Over the last 23 years, 313 original assays were reviewed for the authentication of commercial species of fish. The most studied gene region was *cytb*, followed by COI; however, species may differ on their inter-species variability, which may make other gene regions more suitable. The most explored DNA-based method was real-time PCR, followed by DNA sequencing. However, various factors, such as efficiency, sensitivity, reproducibility, sample type, and cost, must be considered when developing assays for fish authenticity.

Primer and probe specificity may impact the sensitivity and quality of an assay. A case study of 19 commercial tuna assays targeting the *cytb* region showed a lack of standardization for specificity and assay validation, as per the parameters outlined in this article. This questions the reli-

ability, efficiency, and reproducibility of published assays for fish authentication. Therefore, food assay design and validation techniques need to be standardized, and in silico tools should be used to test primers for specificity prior to laboratory implementation.

AUTHOR CONTRIBUTIONS

Maleeka Singh: Conceptualization; investigation; writing—original draft; methodology; validation; visualization; writing—review and editing; formal analysis; data curation. **Robert G. Young**: Investigation; methodology; validation; visualization; writing—review and editing; software; formal analysis; data curation. **Rosalee S. Hellberg**: Writing—original draft; methodology; validation; visualization; writing—review and editing; supervision. **Robert H. Hanner**: Conceptualization; funding acquisition; writing—original draft; writing—review and editing; visualization; methodology; project administration; supervision; resources. **Maria G. Corradini**: Funding acquisition; validation; visualization; writing—review and editing; project administration; resources; supervision. **Jeffrey M. Farber**: Conceptualization; funding acquisition; writing—original draft; writing—review and editing; visualization; methodology; project administration; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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