DNA barcoding of commercial fish products using dual mitochondrial markers exposes evidence for mislabelling and trade of endangered species

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Abstract. Fish fraud has been extensively reported in world fish trade. The fraud includes IUCN Red List and CITES-listed species. Hence, there is a growing need to identify the trade of endangered and threatened species that has been misused to satisfy consumer needs. Here, we apply DNA barcoding by using dual mitochondrial marker; cytochrome b (*Cytb*) polymorphic fragment and cytochrome c oxidase subunit I (*COI*) to authenticate 50 commercial fish products collected from the Malaysian market. The dual marker system improves species detection in tested fish products even in highly processed food and exposes the trade of one critically endangered (also CITES-listed) and three endangered or near threatened species under the IUCN red-list status. Our result also indicates that 36% of fish products in the Malaysian market is mislabelled and might cause concern for food safety. The newly developed *Cytb* primer pair also shows a higher success rate by identifying 92% of the tested samples compared to 40% for *COI* primer. This work suggests the dual-marker DNA barcoding approach is more effective in detecting food mislabelling and is indeed a promising tool to help regulatory bodies obtain a clearer standpoint for monitoring endangered fish trade to prevent further biodiversity loss.

1. Introduction

Fish is the main source of animal protein for 3.2 billion people worldwide with an annual consumption of over 151 million tonnes [1]. The increasing demand has expanded the manufacturing of diversified fish products. As one of the highest traded food commodities, food security in fish products is often challenging as it is prone to be substituted and mislabelled [2,3]. These deliberate practices offer numerous opportunities to gain profits by exchanging the valuable species with less valuable ingredients, promote illegal, unreported and unregulated (IUU) fishing, and also overexploitation [4,5]. Consequently, these illegal practices could inflate the species validity in the catch, misinterpret the stock numbers subsequently, causing a major decline in some fish populations [6]. The overexploitation also jeopardizes the species long-term sustainability, particularly for the International Union for the Conservation of Nature (IUCN) Red List of Threatened species such as sharks, rays, tuna and eels [7–11]. Along with IUCN, the international agreement of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has been implemented to combat overexploitation and to monitor international trade in the fisheries sector including species used in the fish products.

The fact that the flesh of many fish species are similar in appearance, taste and texture [1], means that the fraudulent practices could easily go unnoticed especially in processed fish products which are indistinguishable after processing and freezing [12]. The hindrance in species verification from canned

products, fillets, deep fried, and heavily processed fish products such as fish balls and crab sticks have hampered the conservation efforts targeted at IUCN Red List of Threatened Species and CITES. Considering the urgency to address fish fraud and the frequent collapse of fish populations [13,14], DNA barcoding has stayed ahead of the curve to be one of the most promising tools to assist species identification, improve food authentication methods and eventually reveal trade of threatened and endangered species [7,10]. This method relies on comparisons of DNA barcode sequences generated against the reference sequences deposited in the reference library, GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and another worldwide gaining popularity database, Barcode of Life Database (BOLD) (http://www.boldsystems.org/).

In Malaysia, all food products sold must comply with the Malaysian Food Act 1983 and Food Regulations 1985. The administration and regulation of food safety are under the authority of the Food Safety and Quality Division (FSQD) at the Ministry of Health (MOH) [15]. In 2012, the Malaysian fisheries sector produced 1.7 million tons of fish valued at RM10.8 billion and generated trade worth RM6 billion [16]. Meanwhile, the National Agro-Food Policy (NAFP) 2011-2020 estimated that the annual demand for fish will increase to 1.7 million tons in 2011 and further to 1.93 million tons by 2020 (http://www.kada.gov.my). Despite being one of the highest fish consumers in the world [16], there is a gap in understanding the extent in which the fish products in Malaysia markets have been adulterated [17–19] thus compromising its safety and how it affects fish conservation efforts in Malaysia. Therefore, in this work, a dual mitochondrial DNA (mtDNA) marker system, *COI* and newly developed *Cytb*, was used to authenticate fish products in the Malaysian market. Our work exposes the level of both mislabelling and substitution of fish products with species listed as threatened, endangered as well as critically endangered species which are surprisingly widely available and consumed.

2. Materials and methods

2.1 Sample collection

A total of 50 commercial fish products representing a variety of species and product types (sliced filleted, canned fish, salted and dried fish, smoked, marinated, pre-cooked, sushi products and frozen fish products) were collected. Products were purchased from several supermarkets, fresh marts and sushi restaurants in Johor and Penang, Malaysia. Samples purchased from restaurants were ordered for take-away and information on the main ingredient used was based on menus or from the details orally reported by the restaurant staff. Samples were transported under ice-chilled to the lab immediately and were stored according to the manufacturer's instruction at 4°C, -20°C or room temperature until further analysis.

2.2 *DNA extraction*

DNA extractions were performed using DNeasy® Blood & Tissue Kit (#Cat. No. 69506, QIAGEN GmbH, Hilden, Germany) as per standard protocols following the manufacturer's instruction. A negative extraction control with no added tissue was included to verify the purity of the extraction reagents. The DNA concentration and purity of extracted DNA samples were evaluated using NanoDrop ™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The DNA quality was further assessed by means of 1% (w/v) agarose gel electrophoresis (Vivantis Inc., USA) in 1X TAE buffer, stained with Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Germany), and visualised via AlphaImager gel documentation systems (ProteinSimple, California, USA). The degree of DNA fragmentation was estimated by comparing to the standard marker 1Kb Plus DNA Ladder (TransGen). The extracted DNA was stored at -20°C until further analysis.

2.3 Primer design

To amplify the *Cytb* barcode, a universal primer pair was designed. The sequences of the mitochondrial *Cytb* region for 40 species of fish from various families and genera were aligned using Clustal Omega (EMBL-EBI) to determine the conserved regions applicable for primer design. The alignment

parameters were kept as default. The conserved region obtained from the multiple sequence alignment (MSA) was used as an input for designing primers using PrimerQuest Tool (Integrated DNA Technologies). A primer pair (Forward: 5' CGGCGCATCATTCTTCTTYATC 3' and Reverse: 5' AGGCRAAGAATCGGGTTARGG 3') amplifying a 287 bp of *Cytb* mini barcode region was constructed according to the parameters proposed by [20]. For amplifying *COI* barcode region, a set of previously reported universal fish primers (Forward: ATCACAAAGACATTGGCACCCT and Reverse: AATGAAGGGGGGAGGAGTCAGAA) targeting a fragment of 295 bp was used [19]. Both the primers were synthesised by IDT (Integrated DNA Technologies, Singapore) and were supplied by Apical Scientific (Selangor, Malaysia).

2.4 PCR amplification and sequencing

The optimal thermal cycling of *Cytb* design primer pair were evaluated using a gradient PCR approach, resulting in the selected condition: an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 52°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 5 min and hold at 4°C. The PCR cycling for *COI* was identical as *Cytb* except for the annealing temperature at 59°C. All the PCR reactions were performed in a final volume of 25 µl containing 5 µl of 5X Green GoTaq Flexi Buffer (Promega, Madison, USA), 1 µl of each forward and reverse primers (10 mM), 2 µl of 25 mM MgCl2 (Promega, Madison, USA), 0.5 µl of 10 mM dNTPs mix (Promega, Madison, USA), 0.625 U of GoTaq Flexi DNA Polymerase (Promega, Madison, USA), 10-50 ng DNA template and sterilised ultrapure water to final volume. PCR amplifications were carried out using Mastercycle nexus Gradient Thermal Cycler (Eppendorf, Germany). A negative control (without DNA template) was included in all PCR runs to validate the reliability of PCR results. PCR success was verified on a 2% (w/v) agarose gel electrophoresis and the size of PCR amplicons were accessed by comparison with the standard marker 100bp Plus II DNA Ladder (TransGen). Successful PCR products were purified and sequenced by Apical Scientific Sdn Bhd (Selangor, Malaysia) on an ABI 3730xl Genetic Analyser (Applied Biosystems, Foster City, USA).

2.5 Sequence data analysis

The obtained sequences were analysed and edited using Sequence Scanner v2.0 software (Applied Biosystems). Fine adjustments were manually made after visual inspection against their chromatograms and trimmed the sequence ends. All the *COI* and *Cytb* sequences were compared to reference sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using the basic local alignment search tool (BLAST) for species identification. Identification results for the *COI* sequences were cross-referenced within the Barcode of Life Database (BOLD) (http://www.boldsystems.org/) using Identification System (IDs) against species level barcode records only. The evolutionary analysis of the samples was inferred using the Neighbour-Joining method [21]. A phylogenetic tree was constructed in MEGA 7 (Molecular Evolutionary Genetics Analysis) with distances computed using the Kimura 2-parameter model [22]. The robustness of the inferred tree was evaluated by 1000 bootstrap re-samplings to obtain confidence node support. The conservation status of each identified species was further compared against the IUCN Red List of Threatened Species (http://www.iucnredlist.org) and CITES (https://www.cites.org/).

3. Results

3.1 Species identification via DNA barcoding

The use of dual mtDNA markers (*Cytb* and *COI*) had successfully identified 25 fish products (50%) up to species level. This consists of 18 species which include one nearly threatened species (S31: Narrow-barred Spanish mackerel, *Scomberomorus commerson*), two endangered species (S19: Japanese eel, *Anguilla japonica* and S14: Atlantic bluefin tuna, *Thunnus thynnus*) and one critically endangered species (S17 & S43: European eel, *Anguilla anguilla*). The identified critically endangered European eel is also categorized as CITES-listed under Appendix II. The complete IUCN red list status, CITES-

listed species and population trend for all 18 identified species are described in Table 1. Out of the 25 authenticated products (total of 33 barcodes), eight products were identified with dual markers, while 15 and two products were identified solely by the *Cytb* barcode and the *COI* barcode, respectively. A more detail analysis of the dual barcode revealed that *Cytb* resulted as a better barcode with 92% (23/25 samples) success amplification rate, compared to only 40% (10/25 samples) for *COI*. Further sequence comparison with GenBank and BOLD reference database confirms nine mislabeling cases which resort to 36% mislabeling rate in the fish products analyzed in this work.

Table 1. The IUCN red list status and CITES-listed species identified in this work

Species	Common name	Number of sample (Sample ID)	IUCN Red list status	Population trend	CITES-listed
Priacanthus macracanthus	Red bigeye	1 (S1)	LC	Unknown	No
Lutjanus griseus	Grey snapper	1 (S2)	LC	Unknown	No
Thunnus thynnus	Atlantic bluefin tuna	1 (S14)	EN	Decreasing	No
Seriola quinqueradiata	Japanese amberjack	1 (S15)	LC	Unknown	No
Salmo salar	Atlantic salmon	3 (S16, S18, S36)	LC	Unspecified	No
Anguilla anguilla	European eel	2 (S17, S43)	CR	Decreasing	Yes
Anguilla japonica	Japanese eel	1 (S19)	EN	Decreasing	No
Lepidocybium flavobrunneum	Escolar	2 (S20, S38)	LC	Unknown	No
Oreochromis niloticus	Nile tilapia	2 (S21, S32)	LC	Stable	No
Gadus chalcogrammus	Alaska pollock	2 (S22, S23)	NE	Unknown	No
Decapterus maruadsi	Japanese scad	2 (S27, S44)	LC	Unknown	No
Abudefduf lorenzi	Black-tail sergeant	1 (S28)	LC	Stable	No
Trichiurus lepturus	Largehead hairtail	1 (S29)	LC	Stable	No
Scomberomorus commerson	Narrow-barred Spanish mackerel	1 (S31)	NT	Decreasing	No
Nemipterus hexodon	Ornate threadfin bream	1 (S40)	LC	Unknown	No
Ruvettus pretiosus	Oilfish	1 (S41)	LC	Stable	No
Oncorhynchus mykiss	Rainbow trout	1 (S42)	LC	Unknown	No
Siniperca knerii	Chinese perch	1 (S45)	NE	Unknown	No

Note: CR=critically endangered, EN=endangered, NT=near threatened, LC=least concern, NE=not evaluated; The common names were based on Fishbase (www.fishbase.org/)

Table 2 summarised the comprehensive species identification result for 25 fish products based on GenBank and BOLD databases. The remaining 50% which failed to amplify are excluded from the table. For the *Cytb* barcodes (n=23), GenBank database revealed definitive identity scores of more than 97% (range 97%-100%) for consensus sequences for most of the species except S2, S28, S40, S44 and S45 with identity scores of less than 97% (range 75.71%-89.47%) where relatively low sequence quality was observed (data not shown). A maximum identity in the range of 97.25-100% was obtained for *COI* barcodes (n=10) through the BLAST search in GenBank. Of the 10 *COI* barcodes, all of them returned a close match up to species level with exception of three barcodes identified only to genus level; S21 (*Oreochromis* sp. with 98.05% maximum identity), S29 (*Trichiurus* sp. with 99.21% maximum identity)

and S32 (*Oreochromis* sp. with 97.25% maximum identity). Overall, GenBank results and BOLD ID's results are consistent for all *COI* barcodes analyzed in this work except for one sample. One specific discrepancy between GenBank and BOLD is illustrated in sample S29 where GenBank BLAST result indicated this sample as *Trichiurus* sp. (99.21%) but was identified as *Trichiurus lepturus* with 98.82% in BOLD suggesting BOLD yielded greater species resolution compared to GenBank. Species identifications were further verified via phylogenetic analysis using distance NJ tree approaches with validated reference sequences from GenBank. Phylogenetic analysis of the full dataset for both *Cytb* (Figure 1) and *COI* barcodes (Figure 2) showed clear and well-defined subclusters separation at both genus and species level, which is parallel with GenBank and BOLD analysis (Table 2).

Table 2. GenBank and BOLD results from query barcodes retrieved from the 25 success amplified fish products

Sample ID	Product label	Declared ingredient	Gene target	Genbank (BLAST)				BOLD	
				Species identification	Ident.	Query cover	Accession number	Species identification	Ident.
S1	Mini fish cake	Threadfin Bream	*Cytb*	*Priacanthus macracanthus* (Red bigeye)	97.18%	97%	KT897925.1	N/A	
			COI	Failed to amplify					
S2	White fish ball	Surimi	*Cytb*	*Lutjanus griseus* (Grey snapper)	75.71%	93%	HQ162426.1	N/A	
			COI	Failed to amplify					
S14	Maguro sushi	Bluefin tuna	*Cytb*	*Thunnus thynnus* (Atlantic bluefin tuna)	98.35%	98%	MG017705.1	N/A	
			COI	*Thunnus thynnus* (Atlantic bluefin tuna)	99.60%	98%	KU168655.1	*Thunnus thynnus* (Atlantic bluefin tuna)	99.20%
S15	Hamachi sushi	Japanese amberjack	*Cytb*	Failed to amplify					
			COI	*Seriola quinqueradiata* (Japanese amberjack)	98.85%	98%	KU168712.1	*Seriola quinqueradiata* (Japanese amberjack)	99.22%
S16	Fresh salmon nigiri	Salmon	*Cytb*	*Salmo salar* (Atlantic salmon)	99.18%	98%	KY122206.1	N/A	
			COI	Failed to amplify					
S17	Roasted eel sushi	Eel	*Cytb*	*Anguilla anguilla* (European eel)	98.31%	98%	HG794918.1	N/A	
			COI	*Anguilla anguilla* (European eel)	98.45%	98%	KU168676.1	*Anguilla anguilla* (European eel)	98.37%
S18	Norwegian salmon sushi	Salmon	*Cytb*	*Salmo salar* (Atlantic salmon)	99.18%	97%	KY122206.1	N/A	
			COI	Failed to amplify					
S19	Unagi slice sushi	Eel	*Cytb*	*Anguilla japonica* (Japanese eel)	99.17%	98%	MH050933.1	N/A	
			COI	*Anguilla japonica* (Japanese eel)	99.58%	92%	KU168677.1	*Anguilla japonica* (Japanese eel)	98%
S20	White tuna sushi	White tuna	*Cytb*	*Lepidocybium flavobrunneum*	99.19%	98%	AP012519.1	N/A	

				(Escolar)					
			COI		Failed to amplify				
S21	Chunky fish fillets	Tilapia	*Cytb*	*Oreochromis niloticus* (Nile tilapia)	98.39%	99%	MH041454.1	N/A	
			COI	*Oreochromis* sp.	98.05%	99%	MH515294.1	*Oreochromis* sp.	98.81%
S22	Tempura fish fillets	Alaska Pollock	*Cytb*	*Gadus chalcogrammus* (Alaska pollock)	99.61%	100%	KP644331.1	N/A	
			COI		Failed to amplify				
S23	Fish & chips	Pollock	*Cytb*	*Gadus chalcogrammus* (Alaska pollock)	99.61%	100%	KP644331.1	N/A	
			COI		Failed to amplify				
S27	Breaded fish nugget	Surimi	*Cytb*	*Decapterus maruadsi* (Japanese scad)	97.00%	96%	KX212078.1	N/A	
			COI		Failed to amplify				
S28	Salmon fish ball	Fish meat	*Cytb*	*Abudefduf lorenzi* (Black-tail sergeant)	86.17%	99%	KU553498.1	N/A	
			COI		Failed to amplify				
S29	Yellow tail fish ball	Surimi	*Cytb*		Failed to amplify				
			COI	*Trichiurus* sp.	99.21%	97%	LC269236.1	*Trichiurus* lepturus (Largehead hairtail)	98.82%
S31	Otak-otak spicy fish paste	Fish	*Cytb*	*Scomberomorus commerson* (Narrow-barred Spanish mackerel)	98.34%	97%	EF141176.1	N/A	
			COI		Failed to amplify				
S32	Tilapia kabayaki	Taiwanese Tilapia	*Cytb*	*Oreochromis niloticus* (Nile tilapia)	98.39%	99%	MH041458.1	N/A	
			COI	*Oreochromis* sp.	97.25%	98%	MH515294.1	*Oreochromis* sp.	98.76%
S36	Frozen salmon fillet	Salmon	*Cytb*	*Salmo salar* (Atlantic salmon)	99.18%	99%	KY122206.1	N/A	
			COI		Failed to amplify				
S38	Butterfish portion cut	Butterfish	*Cytb*	*Lepidocybium flavobrunneum* (Escolar)	99.20%	98%	AP012519.1	N/A	
			COI		Failed to amplify				
S40	Fish snack	Fish meat	*Cytb*	*Nemipterus hexodon* (Ornate threadfin bream)	81.45%	93%	EU672446.1	N/A	
			COI		Failed to amplify				
S41	Shiro maguro zuke sushi	White tuna	*Cytb*	*Ruvettus pretiosus* (Oilfish)	99.20%	98%	AP012506.1	N/A	
			COI	*Ruvettus pretiosus* (Oilfish)	98.82%	98%	HQ945992.1	*Ruvettus pretiosus* (Oilfish)	98.43%

S42	Sake belly sushi	Salmon	*Cytb*	*Oncorhynchus mykiss* (Rainbow trout)	98.77%	99%	MG434732.1	N/A	
			COI	*Oncorhynchus mykiss* (Rainbow trout)	100.00%	95%	FJ999050.1	*Oncorhynchus mykiss* (Rainbow trout)	98.78%
S43	Unagi sushi	Eel	*Cytb*	*Anguilla anguilla* (European eel)	98.76%	97%	HG794917.1	N/A	
			COI	*Anguilla anguilla* (European eel)	98.37%	98%	KU168680.1	*Anguilla anguilla* (European eel)	98.35%
S44	Fish ball	Threadfin Bream (wild)	*Cytb*	*Decapterus maruadsi* (Japanese scad)	89.47%	95%	MG457153.1	N/A	
			COI	Failed to amplify					
S45	Otak-otak	Mackerel fish	*Cytb*	*Siniperca knerii* (Chinese perch)	84.75%	93%	KU884502.1	N/A	
			COI	Failed to amplify					

Note: Shaded sample indicates mislabelled. N/A= not available

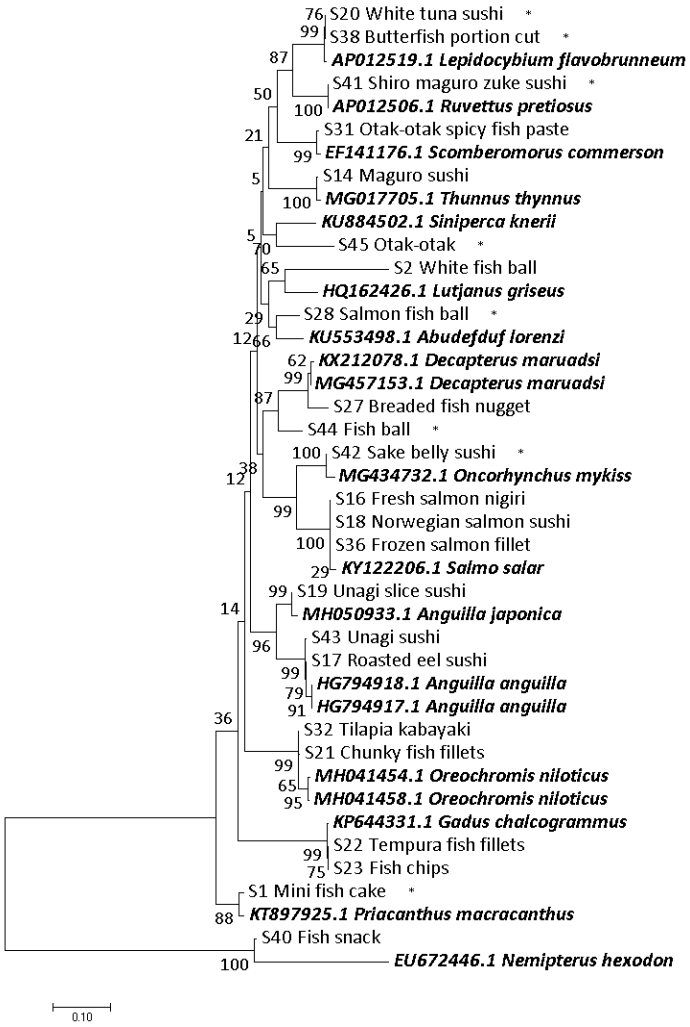

Figure 1. NJ tree of 23 *Cytb* barcode sequences generated from fish products with validated reference available in GenBank. Samples with * indicates mislabelled products

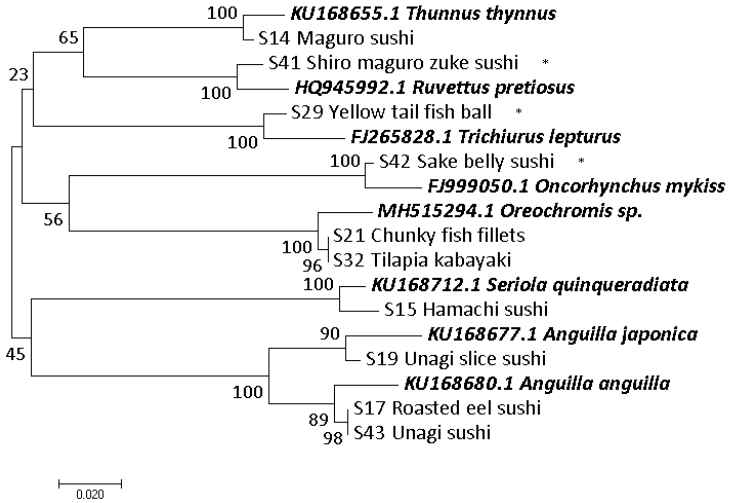

Figure 2. NJ tree of 10 *COI* barcode sequences generated from fish products with validated reference available in GenBank. Samples with * indicates mislabelled products

4. Discussion

4.1 DNA barcoding

An effective DNA barcoding approach for food product authentication highly relies on the quality of DNA extracted. However, DNA extraction from highly processed samples such as fish products used in this work is often challenging as they are typically associated with high DNA degradation [23]. In addition, the presence of multiple additives, preservatives and flavours might affect the DNA quality and quantity [24]. The application of DNA barcoding targeting amplification of full-length barcode with ~650 bp is therefore highly restricted. Instead, the mini barcoding approach which focuses the analysis on relatively short DNA fragments ranging from 100 to 300 bp as genetic marker, could help to increase the efficiency in successful PCR amplification from degraded DNA samples [19,24–26].

Generally, the mitochondrial *COI* gene is the marker of choice for developing mini barcoding due to its higher interspecies than intraspecies variability, which enables accurate identification of a wide range of fish species [24,27]. Nonetheless, the ability of the *Cytb* gene to discriminate differences in sequences between closely related species has also been proven as a suitable marker for fish species identification [28]. In addition, there is a quite comprehensive collection of *Cytb* reference genes with more than 60,000 sequences available in GenBank solely for fishes which will further magnify species detection (including IUCN-listed species) and increase the reliability of this marker for fish product authentication [28]. Here, both of the mtDNA markers (*Cytb* and *COI*) were employed for the identification of various types of fish products in the Malaysian market. Higher amplification rate was shown in *Cytb* barcode (92%) as compared to *COI* barcode (40%). The use of *Cytb* gene had successfully discriminated 23 samples up to species level where the *COI* region failed to amplify in 15 samples. Furthermore, the amplification of *Cytb* barcodes were necessary for the analysis of samples identified as *Oreochromis niloticus* (S21 and S32), of which *COI* barcode failed to provide sufficient resolution to species level (identified only as *Oreochromis* sp. in both cases). Despite the low amplification success rate of the *COI* barcode primer set, it was the only method that enabled the identification of two samples (S15 and S29). Such findings demonstrate the necessity of using more than one marker to allow identification of a wider range of species and the advantages of using shorter barcodes on highly processed samples containing degraded DNA.

Nevertheless, no amplification was detected from the other 50% samples despite repeated attempts even though mini barcode barcoding was applied. Surprisingly, the low PCR amplification success is mainly observed in less processed frozen products (portion cut and battered fillet). Although these products exhibit lower DNA denaturation compared to other highly processed products such as fish

balls, fish cake or cooked products, their PCR amplification still failed. Similarly, all the samples under categories of canned, salted and smoked products also failed to be amplified either by *Cytb* or *COI* barcode. This could be ascribed to the significant degradation of DNA leaving an insufficient amount of DNA template due to high thermal and pressure treatment during preservation or presence of inhibitors (e.g. lipids and salts) which might interfere with DNA amplification [29]. Therefore, for such samples, alternative approaches such as quantitative PCR using species-specific primers, shorter barcodes (< 200 bp) or metabarcoding techniques may represent efficient alternatives [23].

4.2 Mislabelling rates

The species identification results were compared to the expected scientific names based on the declared general descriptions on product label or information given onsite to detect mislabeling and substitution. Overall, of the 25 product samples identified up to species level, nine samples (36%) were found to be mislabeled (Table 2). The mislabeling rate is in accordance with previous similar studies conducted concerning mislabeling 16% in [17] and 55% in [19], suggesting considerable enhancement in the current functional regulation and monitoring of fisheries products is still needed in Malaysia.

In particular, species substitution were highlighted for S20, S38 and S41, involving the use of species that may lead to significant food safety risks. Two samples labelled as "white tuna sushi" (S20 White tuna sushi and S41 Shiro maguro zuke sushi), presumably a more valuable sushi made from "*Thunnus alalunga*" (albacore tuna) were instead detected to be substituted by a much less valuable fish, *Lepidocybium flavobrunneum* (escolar) as in S20 and *Ruvettus pretiosus* (oilfish) as in S41. Similarly, the cases of escolar and oilfish being sold under the name "white tuna" had been documented in the works of [30], [31] and [5], indicating the fraudulent white tuna marketing practices for economic gain were prevalent. On the other hand, one sample (S38) sold as "butterfish" was identified as *Ruvettus pretiosus* (oilfish). Both aspects of mislabeling could be considered as serious intentional fraud under both economic and nutritional perspective. Escolar and oilfish belong to the Gempylidae (snake mackerel) family and contain high levels of indigestible wax esters, gempylotoxin that can cause significant gastrointestinal distress termed keriorrhea [4,32]. Due to their potential hazardous toxicity, Italy and Japan have banned their import and sale [33]. Though the sale of both escolar and oilfish is not prohibited in Malaysia and no regulations have drawn up for the marketing of these two species, the accidentally consumption of these fishes could potentially leads to episodes of unpleasant keriorrhea especially for those with higher susceptibility such as pregnant women, the elderly, children and individuals with bowel sensitivity [34]. This calls for a more detailed and accurate labeling of escolar and oilfish to alert consumers for health hazards prevention.

Another substitution incident was characterized by the swapping of *Salmo salar* (Atlantic salmon) with *Oncorhynchus mykiss* (rainbow trout) in S42. Their closely identical morphological characteristics often make them vulnerable to accidental mislabeling or intentionally substitution. *Salmo salar* has a higher commercial interest as compared to *Oncorhynchus mykiss* [35]. The differentials between these two species where the substitution of rainbow trout as salmon has a potential economic gain of up to $3.02 per kilogram has therefore encouraged such deliberate substitution for the operators' economic benefit [36].

Furthermore, the other five mislabeled products (S1, S28, S29, S44 and S45) are all surimi-based. Species substitution was detected in S1 where the sample was expected to be *Nemipterus hexodon* (Threadfin bream), but was instead identified as *Priacanthus macracanthus* (Red bigeye). Sample S28 labelled as salmon was found to contain *Abudefduf lorenzi* (Black-tail sergeant); whereas sample S29 sold as "yellowtail fish ball" with expected species of *Seriola quinqueradiata* was verified to originate from *Trichiurus lepturus* (Largehead hairtail). On the other hand, *Cytb* barcode of S44 revealed to contain *Decapterus maruadsi* (Japanese scad) instead of expected species of *Nemipterus hexodon* (Threadfin bream), confirming it as substituted. Lastly, the DNA barcode of S45 returned a close match to *Siniperca knerii* (Chinese perch) in GenBank though the sample was declared to contain mackerel (family Scombridae). Compared to other fish products, surimi-based products particularly have higher susceptibility toward substitution because of their highly processing nature, making them nearly

impossible to differentiate by morphological characters and without laboratory analyses [37]. This finding is consistent with the previous studies where high frequent incidences of intentional adulterations in surimi-based fish products were reported, i.e. 84.2% as in Pepe et al. (2007) and 40% as in Sultana et al. (2018).

4.3 Conservation issues

The drastically increasing population in the world has led to the fast-growing demand for fish or fish related products which yielded 171 million tons total fishery in global in 2016 [1]. According to the Food and Agriculture Organization of the United Nations, over 70% of fish populations are fully used, overused or depleted, causing significant effects on biodiversity and conservation of species and fragile populations. Aware of the growing demand for fish and fish related products in feeding the world, the present study highlights the critical importance of fish product authentication via DNA barcoding to aid in the sustainable management of aquatic resources. It is unfortunate to note that our study also detected the presence of several critically endangered, endangered and near threatened species in Malaysian fish products.

Amongst the 18 species that have been identified, 5.56% (*Anguilla anguilla*, European eel) (S17 and S43) is listed as critically endangered by IUCN Red List and also a CITES Appendix II-listed species [39]. The severe declining of *A. anguilla* population has been formally reported since 1998 due to increasing fishing activities along the coasts and the effect of increased abundance of predators such as ichthyophagous birds [8,40]. Meanwhile, the Japanese eel, *Anguilla japonica* (S19) is listed as endangered species. Apart from overfishing, the decreasing trends in eel fisheries are also caused by loss of habitat due to the land reclamation, dam construction and deterioration in water quality [41]. Moreover, this study also revealed another endangered species, *Thunnus thynnus* (Atlantic bluefin tuna) (S14) and nearly threatened species, *Scomberomorus commerson* (Narrow-barred Spanish mackerel) (S31) which belongs to the Scombridae family that is widely consumed in the Malaysian market. Scombridae (mackerels, tunas, and bonitos) are well known due to high commercial value. Along with mackerel, the endangered global Atlantic bluefin tuna populations are declining as a result of overexploitation and heavy fishing pressure [42,43]. However, several conservation efforts have been done to increase the species population of this family and starting to display promising outcomes where recovery of migrations and return of bluefin tuna have been spotted in the northern North Sea and Norwegian Sea [44,45].

In short, the limited understanding in the level of usage and substitution of Malaysian fish products with IUCN status and CITES-listed species is clearly affecting conservation efforts for monitoring the ever declining fish population. This work validates the effectiveness of DNA barcoding approach with dual mtDNA marker system as a reliable tool in species identification and further provides a standpoint of the current situation of the studied market concerning food safety and conservation. The developed *Cytb* improved species detection in tested fish products, as shown by its robust reference dataset (60,000 fish *Cytb* sequences) in GenBank and its higher amplification success even in highly processed. The result also suggests that despite having two reference databases (GenBank and BOLD), *COI* is not guaranteed to be a better marker due to its lower PCR amplification rate, thus hampering species detection. Apart from the detection of 36% mislabeled products and revealing that fish fraud remains a prevalence issue that require much effort to conquer, the discovery of near threatened, endangered and critically endangered species under the IUCN red-list status and CITES-listed within the studied samples make it a good time to revisit our current fish supply chain management concerning biodiversity loss. A better traceability system of fish products to facilitate a more effective national response is needed to safeguard our biodiversity and secure our food quality. In this regard, dual marker DNA barcoding could serve as a promising tool for such monitoring work. Together with the implementation of a more systematic and stringent regulation, this will lead towards more sustainable fishing to prevent further biodiversity loss of protected species as well as significantly reduce fish fraud in the food industry.

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