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Application of a DNA Mini-Barcoding System based on the Mitochondrial Control Region for the Identification of Raw and Processed Tuna Products

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ABSTRACT

Application of an Optimized DNA Mini-Barcoding System based on the Mitochondrial Control Region for the Identification of Raw and Processed Tuna Products

by Jiahleen Barazon Roungchun

Accurate species identification methods are needed to combat tuna fraud, improve tuna stock regulation, and mitigate health risks associated with mislabeled tuna products. The objective of this study was to conduct a market survey of raw and processed tuna products using a DNA mini-barcoding system based on the mitochondrial control region (CR). A total of 80 samples of raw, dried, and canned tuna products were collected at the retail level for CR mini-barcoding analysis. The samples underwent DNA extraction, polymerase chain reaction (PCR), and DNA sequencing of the 236-bp CR mini-barcode. The resulting sequences were searched against GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST) to determine the species. The study achieved species identification for 100% of the raw samples, 95% of the dried samples, and 50% of the canned samples for an overall success rate of 82% (n = 69 samples). Mislabeling occurred in 11 of the identified samples (16%), including 8 products (raw, dried, and canned) marketed as yellowfin tuna, 2 samples (dried and canned) labeled as skipjack tuna, and 1 raw fillet sold as bluefin tuna. PCR amplification was successful in all 80 samples, but sequencing was unsuccessful for half of the canned products. The reduced success in canned products may have been due to highly fragmented DNA caused by the canning process and/or the presence of multiple species in these products. Overall, the DNA mini-barcoding system proved to be a promising method in identifying tuna species in both raw and processed samples. Future research should explore optimization of this method for improved identification of canned tuna samples.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

BOLD: Barcode of Life Data Systems

BP: Base pairs

CFR: Code of Federal Regulations

COI: Cytochrome c oxidase subunit 1

CR: mitochondrial control region

CYTB: cytochrome b

FDA: U.S. Food and Drug Administration

FINS: forensically informative nucleotide sequencing

FISH-BOL: Fish Barcode of Life

IEF: isoelectric focusing

ITS1: first internal transcribed spacer region

IUU: illegal, unreported, and unregulated

NCBI: National Center for Biotechnology Information

PCR: polymerase chain reaction

RFLP: restriction fragment length polymorphism

1. Introduction

Seafood is a staple of the global food supply, with close to 180 million tonnes produced in 2018 (FAO, 2020). Global seafood production is dominated by finfish, with the most captured groups—small pelagics, gadiformes, tuna and tuna-like species—making up 85% of total production (FAO, 2020). Tuna captures have consistently increased each year, reaching approximately 8 million tonnes in 2019. Around 58% of these tuna captures consisted of skipjack (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) (FAO, 2020). In 2019, the total landings and imports of fresh and frozen tuna in the US was 500 thousand tonnes, 62% of which was used for canning. Canned tuna ranks as the third-most consumed seafood in the United States after shrimp and salmon, with per capita annual consumption at 1.0 kg in 2019 (NFI, 2021; NMFS, 2021).

With an increasing amount and variety of seafood in the global marketplace, seafood fraud has become a major concern throughout the seafood supply chain (FAO, 2018; Pardo et al., 2016). The intentional substitution of fish species for economic gain is a form of seafood fraud that has been challenging to combat due to the similar appearances of various species and fluctuations in quality, supply, and demand of specific seafood products (FAO, 2018). Tuna, in particular, is susceptible to fraudulent activity due to its high production, growing popularity, and disparate prices between species. For instance, in 2018, the average ex-vessel price for skipjack tuna was $1.23/kg, while the average ex-vessel price for bluefin tuna was $11.90/kg (NMFS, 2020). In addition to the economic deception associated with tuna fraud, there are health risks as well. For example, some types of tuna have elevated levels of mercury and therefore, at-risk individuals are advised to eat only one serving per week of yellowfin or albacore (*Thunnus alalunga*), and to avoid bigeye tuna (*Thunnus obesus*) (FDA/EPA, 2019). Mislabeling of these

tuna species as a low-mercury fish, such as skipjack tuna, could lead to potentially unsafe levels of mercury exposure in these at-risk consumers. Furthermore, "white tuna" sold at sushi restaurants in the U.S. has frequently been identified as escolar (*Lepidocybium flavobrunneum*), an oily fish that contains high levels of gempylotoxins and can cause gastrointestinal distress in sensitive individuals (FDA, 2020; Lowenstein et al., 2009; Warner et al., 2013). Substitution of closely related tuna species also hinders the effective recording and conservation of certain species, such as the endangered Atlantic bluefin tuna (*Thunnus thynnus*) and the critically endangered Southern bluefin tuna (*Thunnus maccoyii*) (FAO, 2018; Liou et al., 2020; Vinas & Tudela, 2009).

Morphological characteristics, such as color, fin shape, and head structure, are often used to distinguish species of whole fish from one another. However, morphological features are removed during processing, making it difficult to visually identify fish species that are similar in appearance (Mitchell & Hellberg, 2016; Pollack et al., 2018). In these instances, analytical methods, such as those based on protein or DNA analysis, are required for the accurate identification of fish species (Silva & Hellberg, 2021). In the United States, the Food and Drug Administration (FDA) has implemented a method based on DNA barcoding for the regulatory identification of fish species (Handy et al., 2011). DNA barcoding differentiates species by analyzing the genetic diversity in standardized sequences of DNA, referred to as DNA barcodes (Handy et al., 2011). The standard DNA barcoding procedure for fish species identification targets a ~650 base pair (bp) region within the cytochrome *c* oxidase subunit 1 (CO1) mitochondrial gene (Ward et al., 2005). While standard (full-length) DNA barcoding has been effective for identification of raw or minimally processed fish, it is challenging to obtain a full-length sequence in products that have been canned because the DNA is highly fragmented. For

example, Ward et al. (2005) discriminated between all 207 raw fish species they analyzed with DNA barcoding, compared to a later study that successfully barcoded only 21% of the heavily processed fish products tested with full barcoding (Shokralla et al., 2015). One way to overcome the limitations of full DNA barcoding in processed seafood has been the implementation of DNA mini-barcoding systems that use shorter genetic regions (~150-300 bp) to identify fish species (Mitchell & Hellberg, 2016; Pollack et al., 2018; Shokralla et al., 2015).

While the combined use of full and mini-barcoding based on COI has been highly successful in identifying fish species, these methods are often inadequate in discriminating closely related tuna species due to low genetic divergences (Lowenstein et al., 2009; Mitchell & Hellberg, 2016; Pollack et al., 2018; Shokralla et al., 2015). The inability to consistently identify the species of these samples combined with ambiguous market labeling prevents a comprehensive assessment of tuna substitution on the commercial market (Hanner et al., 2011). To overcome these challenges, researchers have explored the use of the mitochondrial control region (CR) and the nuclear first internal transcribed spacer region (ITS1) (Mitchell & Hellberg, 2016; Vinas & Tudela, 2009). Vinas & Tudela (2009) targeted a 450-bp region of CR supplemented with ITS1, a genetic marker able to differentiate introgressed tuna species, to successfully sequence and identify the species of all 26 tuna samples they analyzed. In contrast, the COI region was found to be less robust and was unable to distinguish between Pacific bluefin tuna (*Thunnus orientalis*) and Atlantic bluefin tuna (Vinas & Tudela, 2009). While their study demonstrated the viability of using the CR and ITS1 to differentiate between tuna species, the genetic regions they targeted were too long for reliable use with canned tuna.

To address this issue, Mitchell & Hellberg (2016) developed a mini-barcoding system for the differentiation of canned tuna species targeting a shorter 236-bp fragment within the CR and

a 179-bp fragment of ITS1. The primers target a short (<250 bp) fragment of the CR in 15 tuna species: Atlantic bluefin tuna, Southern bluefin tuna, albacore tuna, bigeye tuna, yellowfin tuna, skipjack tuna, Pacific bluefin tuna, blackfin tuna (*Thunnus atlanticus*), longtail tuna (*Thunnus tonggol*), spotted tunny (*Euthynnus alletteratus*), black skipjack tuna (*Euthynnus lineatus*), Kawakawa (*Euthynnus affinis*), slender tuna (*Allothunnus fallai*), bullet tuna (*Auxis rochei*), and frigate tuna (*Auxis thazard*). The method was able to identify species in 23 of the 53 canned products tested, including one instance of mislabeling. Although the species identification rate was only 43%, it was a significant improvement compared to previous studies that were either unable to amplify DNA extracted from tuna samples or only identified tuna samples to the genus level using COI mini-barcoding (Armani et al., 2017; Chin et al., 2016; Pollack et al., 2018; Shokralla et al., 2015). In addition to its applications in canned products, this mini-barcoding system has shown high potential for use in raw and lightly processed products. For example, Frigerio et al. (2021) used these CR primers to successfully identify tuna in all 20 raw, frozen, and dried samples tested of *bottarga,* a product made of salted and dried tuna roe. In another study, Liou et al. (2020) used CR mini-barcoding to identify 9 out of 10 raw tuna samples to the species level (yellowfin tuna, albacore tuna, Pacific bluefin tuna, and Southern bluefin tuna), with the remaining sample identified to the genus level (*Thunnus* spp.).

Despite the potential applications of CR mini-barcoding, it has yet to be widely applied to investigating tuna species identification in the marketplace. Therefore, the objective of the current study was to apply CR mini-barcoding to the identification of species in raw, dried, and canned tuna products sold on the commercial market.

2. Review of Literature

2.1. Seafood fraud

2.1.1. Seafood consumption and production rates

Global consumption of seafood has steadily increased at an average rate of 3.1% every year since 1961, with a record 21 kg per capita in 2018 (FAO, 2020). The United States is the second-largest global consumer of seafood and consumed 8.7 kg per capita in 2019, the highest consumption level seen in the country since 2007 (NMFS, 2021). Global seafood production reached 180 million tonnes in 2018 and was valued at $400 billion (FAO, 2020). This continued growth has led to a decline in marine fishery resources and increased concerns over sustainability, primarily due to overfishing. Overfishing occurred in a record 34% of fish stocks in 2017 and, in particular, 33% of the major tuna species stocks—albacore, bigeye, bluefin, skipjack, and yellowfin—experienced a biologically unsustainable level of fishing (FAO, 2020). Unsustainable fishing occurs when fish stocks are depleted beyond the level needed to achieve the maximum sustainable yield each year. In 2019, the most captured fish species groups in the world were cyprinids (31 million tonnes), followed by anchovies (20 million tonnes), haddocks (9.3 million tonnes), and billfishes (8.0 million tonnes), which include tunas (NMFS, 2021). The total supply of fresh and frozen tuna in the US was over 500 thousand tonnes, 62% of which was used for canning in 2019. Manufacturers produced 170 thousand tonnes of canned tuna worth $800 million in 2019 alone (NMFS, 2021). Americans consumed more canned tuna than any other canned seafood product, at a rate of 1.0 kg per capita in the same year (NMFS, 2021). The growing popularity and production of seafood renders it susceptible to seafood fraud, an ongoing issue in the seafood industry.

2.1.2. Seafood substitution

Seafood substitution is the intentional act of substituting a more economically valuable species of fish with a cheaper fish species (Pardo et al., 2016). Seafood substitution is a form of food fraud, which refers to the intentional misrepresentation of a food or food ingredient for economic gain. The growing demand for specific types of fish and the existence of complicated international supply chains provide the incentives and opportunities for species substitution to occur, along with other deceitful practices, such as short-weighting, trans-shipping, and glazing (Handy et al., 2011). Fish and fish products are considered by INTERPOL/EUROPOL as the third-highest food group at risk for food fraud after condiments, such as oils and spices, and fruits and vegetables (Europol, 2016). The prevalence of seafood fraud throughout the seafood supply chain has impacted overall consumer acceptability and can reduce the demand for seafood products (Khaksar et al., 2015). There is a general loss of trust in the seafood supply system if consumers receive a less valuable product than they paid for or if there are ingredients in the product not listed on the label.

2.1.3. Health and marine conservation concerns over seafood fraud

In addition to the economic impact of seafood fraud, substituted or mislabeled seafood products may also negatively affect consumer health and marine conservation efforts. Certain fish species, such as pufferfish, tuna, and escolar, contain toxins or dangerous levels of mercury and other contaminants that can cause adverse health effects, especially in at-risk individuals (Armani et al., 2015; Civera, 2003). Pufferfish contains tetrodotoxin, a paralytic and deadly substance to humans (Mosher & Fuhrman, 1984). Consumers have died or become ill after being exposed to tetrodotoxin in pufferfish that was mislabeled as other fish, such as monkfish or squid (FAO, 2018). Fish of the Scombridae family, including tuna species, naturally produce high

levels of histidine that can be converted by bacteria to histamine. High levels of histamine consumption can lead to histamine toxicity, a severe allergic-type reaction (Civera, 2003). Escolar is known to contain gempylotoxins, or wax esters, that produce negative gastrointestinal reactions when eaten. Another serious health concern is the risk of veterinary drug residues being present in farmed fish sold as wild species (FAO, 2018). Several tuna species contain elevated amounts of mercury, including albacore, yellowfin, and bigeye tuna, that may cause adverse health effects in at-risk consumers (Civera, 2003). Furthermore, seafood conservation programs rely on the proper reporting of fish captures in order to effectively regulate fishery production and population recovery (Kyle & Wilson, 2007).

2.1.4. *Investigation of seafood fraud*

Seafood fraud has been detected worldwide, with many studies investigating its prevalence in Europe. Among the studies conducted in Europe, wide ranges of seafood mislabeling (3.4-68%) have been identified (**Table 1;** Di Pinto et al., 2015; Guardone et al., 2017; Nedunoori et al., 2017; Pardo et al., 2018). For example, in Italy, Armani et al. (2015) assessed 68 products from various seafood markets and identified 49% of samples tested to be mislabeled or lacking a country of origin. A separate Italian study discovered that 68% of fish fillet products lacked complete labelling with proper scientific names, geographical origins, production methods, and commercial designations (Di Pinto et al., 2015).

In Russia, fish product labels were found to use vague language to declare the fish in the products, often using the more generic names of fish such as “flounder” and “halibut” rather than stating the species used and its origin (Nedunoori et al., 2017). “Salmon” may refer to either Atlantic or Pacific salmon, with the latter garnering a higher sale price. Khaksar et al. (2015) discovered that of the 25 samples generically labelled as “salmon” from American restaurants,

24 were Atlantic salmon, which could still be sold at a higher price point due to the perceived value of "salmon" as an entire species. This type of unspecified product labeling has the potential of misleading consumers, as these generic names can represent multiple species from different origins.

Table 1. Seafood mislabeling rate ranges reported globally from selected studies conducted between 2011-2020.

Quantity and type of products tested	Mislabeling rate range (%)	Region	Source
1,443 raw and processed fish, species variety	16.3 – 41.0	North America	(Hanner et al., 2011; Hu et al., 2018; Khaksar et al., 2015; Liou et al., 2020; Warner et al., 2013)
813 raw and processed seafood, species variety	3.4 – 68.0	Europe	(Armani et al., 2015, 2017; Di Pinto et al., 2015; Guardone et al., 2017; Pardo et al., 2018)
235 raw and processed seafood, species variety	16.0 – 58.0	Asia	(Chin et al., 2016; Sultana et al., 2018; Xiong et al., 2018)
22 raw and processed fish, species variety	23.0	Russia	(Nedunoori et al., 2017)

Mislabeling or species substitution could occur in seafood products that are filleted or otherwise heavily processed, such as canned fish and fish balls (Nedunoori et al., 2017; Sultana et al., 2018). Processed fish have their defining morphological traits removed, making visual species identification difficult, especially for fish with flesh that is similar in taste, look, and texture (FAO, 2018). For instance, Xiong et al. (2018) discovered that *xue yu*, a type of heavily processed, roasted cod fillet product sold in China, had a high mislabeling rate of 58%. Among the multiple different species found in these products was *Lagocephalus* spp., or pufferfish,

which contains tetrodotoxin and may pose a health threat (Xiong et al., 2018). Malaysian fish balls and fish nuggets were mislabeled 75-80% of the time and 100% of surimi-based fish products contained cheaper fish species than what was listed on the label (Sultana et al., 2018). In Ireland, smoked fish had a higher mislabeling rate (73%) compared to non-smoked fish (13%) (FSAI, 2011). These studies indicate that a higher degree of processing allows for increased levels of mislabeling to occur due to the loss of morphological features.

Species substitution occurs often with higher valued fish species where there is a greater economic incentive for substitution. Snapper, an expensive fish, was found to be substituted by 33 different fish species in a single study conducted in the USA, clearly demonstrating the extent to which a single fish species may be substituted by multiple others without consumers discerning the difference (Warner et al., 2013). Similarly, bluefin tuna is the most valuable tuna species worldwide due to its scarcity and perceived superior flavor and is frequently substituted by two cheaper tuna species, yellowfin and bigeye tuna (Pardo et al., 2018).

2.2. Tuna species substitution

2.2.1. Prevalence of tuna species substitution

There have been several studies that have investigated tuna species substitution with varying degrees of success (**Table 2**). A number of studies have used COI barcoding to confirm the presence of tuna in products labeled as "tuna"; however, many of the samples were not identified to the species level. For example, Armani et al. (2017) identified 41 out of 47 fresh tuna sushi samples as *Thunnus* sp. with COI DNA barcoding, while the remaining 6 samples could only be identified to the family level (Scombridae). Genus-level identification was sufficient to confirm the 41 samples were tuna, but family-level identification was inadequate for the remaining 6 samples and thus were excluded. Similarly, Carvalho et al. (2015) identified 2

tuna samples (cooked and raw) only as *Thunnus* sp. with COI barcoding. None of the 36 tuna samples in a study by Khaksar et al. (2015) were found to be mislabeled, but 34 samples were sold as "tuna" and were identified only as *Thunnus* spp. with COI barcoding. In another COI barcoding study, out of 17 tuna samples, all 5 canned products failed full DNA barcode amplification and 11 fresh samples were identified only as *Thunnus* sp. (Cawthorn et al., 2012). The remaining sample was identified as skipjack tuna, but it was labeled as yellowfin tuna, a more valuable species. Likewise, Gunther et al. (2017) identified the species of 4 samples (2 fillets, 2 canned) out of 11 samples using COI DNA barcoding (fillets) and mini-barcoding (canned). They discovered that one of the yellowfin tuna fillet samples was in fact bigeye tuna based on full barcoding. The remaining 7 samples failed both full and mini-barcoding, most likely attributed to the mixture of ingredients present in the product and high levels of processing (pizza, pickled, canned). Chin et al. (2016) was also unable to sequence the 2 canned tuna samples in their study with COI mini-barcoding but identified a fresh sample with full COI barcoding as Southern bluefin tuna. While this is not fraudulent because it was sold as "tuna sushi," Southern bluefin tuna is an endangered species and its stock status must be closely evaluated.

In a study by Hanner et al. (2011), the researchers were able to identify 34 raw and smoked tuna samples to at least the genus level with COI DNA barcoding, 8 of which were to the species level. All 6 "red tuna" samples were identified as bigeye tuna and the remaining 2 samples were identified as a species other than tuna. The sample labeled as "white tuna" was in fact Mozambique tilapia and a "yellowfin" sample was actually Japanese amberjack. Notably, the 26 samples identified as simply *Thunnus* sp. were considered as unresolved mislabeling cases because 12 samples were sold under the inclusive term of "tuna" while 14 samples were sold as

albacore, yellowfin, tuna ahi, or white tuna. The inability to identify the species of these samples combined with ambiguous market labeling prevents the exhaustive review of tuna substitution (Hanner et al., 2011). Wong & Hanner (2008) used COI barcoding to identify 3 out of 4 tuna samples to the genus level and, similar to the previous study, the last sample was found to be Mozambique tilapia instead of "white tuna." Additionally, Hu et al. (2018), with the use of COI barcoding, confirmed 3 cases of substitution out of 45 tuna samples. Escolar was found to be sold as "white tuna," Atlantic or Pacific bluefin tuna as bigeye tuna, and blackfin or Southern bluefin tuna as "ahi tuna." The two instances of expensive bluefin tuna marketed as less valuable tuna species may be attributed to by-catch or illegal, unreported, and unregulated (IUU) fishing, while the substitution of white tuna with escolar is likely an intentional fraudulent act (Hu et al., 2018). In yet another study, Lowenstein et al. (2009) identified the species of all 68 of their tuna samples with COI barcoding. The authors discovered that 5 "white tuna" samples were actually escolar and 48 samples were sold under ambiguous terms such as "tuna" or "*toro* (the fatty part of the tuna)"; 13% of these samples were Northern or Southern bluefin tuna despite their labels failing to indicate these very important species. Similarly, Warner et al. (2013) used COI barcoding to identify 114 tuna samples to either the genus or species label and revealed 59% of the samples were mislabeled, including 52 "white tuna" samples that were escolar.

A few studies have supplemented the use of the COI with other barcoding regions. For example, Pardo et al. (2018) used COI and CYTB (cytochrome b) sequencing to identify the species of 26 samples. They detected substitution in 54% of samples labeled as bluefin or yellowfin tuna: yellowfin and bigeye tuna were sold as bluefin tuna and bigeye tuna was sold as yellowfin tuna. Mitchell & Hellberg (2016) used CR mini-barcoding to identify 23 out of 53 canned tuna products and discovered that a product labeled as canned tongol tuna was actually

striped bonito. Likewise, Liou et al. (2020) identified the genus of 9 out of 10 tuna samples with COI barcoding and used CR mini-barcoding to further identify the species of 8 of those samples. One sample labeled as "yellowfin ahi tuna" was found to be Southern bluefin tuna, which may have been an accidental substitution due to by-catch or an instance of IUU. Finally, Vinas and Tudela (2009) confirmed the species of all 26 tuna samples they analyzed with the CR and ITS1 regions. They found that 2 of the samples were Atlantic bluefin tuna, an endangered species, even though they were labeled as Pacific bluefin tuna (Vinas & Tudela, 2009).

Table 2. Summary of studies that have investigated substitution of tuna products on the market. *n* is the number of samples.

Number of identified samples/total samples	Sample type (*n)*	Substitution (*n)*	Level of identification (*n*)	Source
4/11	Fresh (1), frozen (5), canned (4), pickled (1)	Bigeye tuna labeled as yellowfin tuna (1)	Species (4)	(Gunther et al., 2017)
41/47	Fresh (44), canned (3)	No substitution detected; identified samples were *Thunnus* sp.	Genus (41), Family (6)	(Armani et al., 2017)
2/2	Fresh (1), frozen (1)	No substitution detected; all identified samples were *Thunnus* sp.	Genus (2)	(Carvalho et al., 2015)
12/17	Fresh (12), canned (5)	Skipjack tuna labeled as yellowfin tuna (1)	Genus (11), Species (1)	(Cawthorn et al., 2012)
1/3	Fresh (1), canned (2)	No substitution detected; identified sample was Southern bluefin tuna	Species (1)	(Chin et al., 2016)

34/34	Fresh (13), frozen (7), unknown (14)	Mozambique tilapia labeled as “white tuna” (1); Japanese amberjack labeled as yellowfin tuna (1)	Genus (26), Species (8)	(Hanner et al., 2011)
45/45	Fresh, cooked (unspecified)	Escolar labeled as “white tuna” (1); Northern or Atlantic bluefin tuna labeled as bigeye tuna (1); Blackfin or Southern bluefin tuna labeled as “ahi tuna” (1)	Unspecified	(Hu et al., 2018)
36/36	Fresh, frozen (unspecified)	No substitution detected; all identified samples were either yellowfin tuna or *Thunnus* sp.	Genus (31), Species (5)	(Khaksar et al., 2015)
23/53	Canned (53)	Striped bonito labeled as tongol tuna (1)	Species (23)	(Mitchell & Hellberg, 2016)
10/10	Fresh, frozen (unspecified)	Southern bluefin tuna labeled as yellowfin tuna (1)	Genus (1), Species (9)	(Liou et al., 2020)
68/68	Fresh (68)	Escolar labeled as “white tuna” (5)	Species (68)	(Lowenstein et al., 2009)
26/unspecified	Unspecified	Yellowfin labeled as bluefin tuna (6); bigeye tuna labeled as bluefin tuna (5); bigeye tuna labeled as yellowfin tuna (3)	Species (unspecified)	(Pardo et al., 2018)

114/114	Unspecified	Escolar labeled as "white tuna" (52); Albacore or other tuna labeled as "white tuna" (10); Pacific bluefin tuna labeled as yellowfin tuna (1); unspecified tuna substitution for another species (4)	Genus, Species (unspecified)	(Warner et al., 2013)
4/4	Unspecified	Mozambique tilapia labeled as "white tuna" (1)	Genus (3), Species (1)	(Wong & Hanner, 2008)
26/26	Raw (26)	Atlantic bluefin tuna labeled as Pacific bluefin tuna (2)	Species (26)	(Vinas & Tudela, 2009)

2.2.2. *Consequences of tuna species substitution*

There is a need for tools to combat tuna species substitution because the consequences of fraudulence negatively affect the health and trust of consumers and marine conservation efforts. Mislabeling of tuna species known to have elevated mercury levels such as albacore, yellowfin, and bigeye tuna, poses a risk for individuals who should limit their mercury intake (FDA/EPA, 2019). Elevated mercury consumption may lead to mercury poisoning, which can result in long-term neurological damage. At-risk consumers, such as pregnant women and young children, are recommended to limit consumption of albacore tuna and yellowfin tuna to 1 serving per week and to avoid bigeye tuna altogether (FDA/EPA, 2019). Among commercial tuna products, fresh/frozen bigeye tuna and fresh bluefin tuna have the highest mercury content, followed by fresh/frozen albacore, fresh/frozen yellowfin, and canned albacore, with the lowest levels in fresh/frozen skipjack and canned light tuna (FDA, 2014; Lowenstein et al., 2010). In addition, escolar is a species of fish in the family Gempylidae that is often mislabeled as "white tuna"

(Warner et al., 2013). This is a health concern since escolar contains high levels of indigestible wax esters that can cause diarrhea, abdominal cramps, headache, and vomiting in sensitive individuals (FDA, 2020). Scombrotoxin, which causes histamine poisoning, has also been found in improperly handled escolar (FDA, 2020).

The ability to differentiate between closely related tuna species is crucial for the conservation of certain species, such as the endangered Atlantic bluefin tuna and the critically endangered Southern bluefin tuna (Collette, Amorim, et al., 2011; Collette, Chang, et al., 2011). A third species of bluefin tuna, Pacific bluefin, is not endangered but is considered a threatened species. All 3 bluefin species are highly prized fish that hold far more economic value when sold minimally processed to restaurants. They are rarely found in canned form and their presence in a canned product is ascribed to accidental inclusion, rather than intentional seafood substitution (Pollack et al., 2018). However, efficient detection methods of tuna species are valuable to identify when these instances do occur. Proper identification of tuna served in restaurants is also important for consumers who are concerned with the conservation of endangered species. Warner et al. (2013) discovered Atlantic and Southern bluefin tuna sold merely as "bluefin tuna," a broad market name that does not differentiate between the endangered and less threatened bluefin species. In addition, Pacific bluefin tuna was found to be sold as yellowfin tuna, a significantly cheaper and abundant species (Warner et al., 2013). Tuna substitution prevents consumers from making informed decisions and hinders effective fishery management since each tuna species varies widely in market pricing, nutritional and metal contents, stock status, and vulnerability to overfishing.

2.2.3. Canned tuna specifications

According to the Code of Federal Regulations (CFR), there are 14 tuna species allowed for use in canned tuna products: *Thunnus thynnus* (Northern, or Atlantic, bluefin tuna), *Thunnus maccoyii* (Southern bluefin tuna), *Thunnus alalunga* (albacore), *Thunnus atlanticus* (blackfin tuna), *Thunnus obesus* (bigeye tuna), *Thunnus albacares* (yellowfin tuna), *Thunnus tonggol* (longtail tuna), *Katsuwonus pelamis* (skipjack tuna), *Euthynnus alletteratus* (spotted tunny), *Euthynnus lineatus* (black skipjack tuna), *Euthynnus affinis* (Kawakawa), *Allothunnus fallai* (slender tuna), *Auxis rochei* (Bullet tuna), and *Auxis thazard* (Frigate tuna) (21 CFR 161.190). *Thunnus orientalis* (Pacific bluefin tuna) is not listed in the CFR, which is likely an issue of nomenclature, as *T. thynnus* and *T. orientalis* were not considered to be separate species until 1999 (Collette, 1999).

"White" canned tuna only consists of albacore with a Munsell value higher than 6.3. Munsell values indicate the lightness of colors, with 0 as pure black and 10 as pure white (*Munsell Value*, 2020). "Light" canned tuna signifies any tuna with meat with a Munsell value higher than 5.3, typically skipjack, yellowfin, and bigeye, though bluefin is also permitted; and "dark" canned tuna designates all tuna with Munsell values lower than 5.3 (21 CFR 161.190; NMFS, 2020). Producers may pack canned tuna in a variety of liquids and seasonings, such as vegetable and olive oils, water, salt, monosodium glutamate, spices, lemon flavoring, and vegetable broth (21 CFR 161.190). Canned tuna is especially vulnerable to seafood fraud due to its popularity, the extensive range of tuna species used, and the high levels of processing (Chapela et al., 2007).

2.3. Protein versus DNA-based methods for species identification

Morphological characteristics such as fins, heads, and color are often used to identify the species of whole, unprocessed fish. These physical traits are lost once fish are processed into fillets and other products, and analytical approaches are often required to differentiate fish species (Handy et al., 2011). Methods conventionally used in species identification are based on analyzing specific proteins with high-performance chromatography or immunoassay-based applications (Rasmussen & Morrissey, 2008). However, these traditional methods are not applicable to all sample types. Protein-based methods can fail to differentiate between closely related species, thermal processing or drying can denature the protein structures or biochemical attributes needed for analysis, and proteins can be highly variable depending on age, tissue type, and other traits (Rasmussen & Morrissey, 2008). More advanced and versatile DNA-based methods have become widely used to authenticate seafood specimens accurately and prevent mislabeling (Handy et al., 2011; Kyle & Wilson, 2007; Pardo et al., 2018; Silva & Hellberg, 2021). DNA-based methods take advantage of species-specific genetic polymorphisms and the diversity between DNA sequences for the differentiation of fish species (Griffiths et al., 2014). One of the reasons that DNA analysis is so versatile is due to the fact that DNA exists in practically all cells, regardless of age, tissue type, or processing and can provide greater amounts of information as compared to protein analysis (Civera, 2003; Hebert et al., 2003). Although processing degrades DNA into smaller fragments, DNA fragments up to 300 bp can still be recovered with appropriate extraction techniques and PCR amplification to identify species (Mackie et al., 1999; Pollack et al., 2018).

Some of the most widely used methods using DNA sequencing, such as forensically informative nucleotide sequencing (FINS) or DNA barcoding, are considered highly informative

and reliable for species identification (Civera, 2003; Hebert et al., 2003; Mackie et al., 1999; Rasmussen & Morrissey, 2008). While both nuclear and mitochondrial DNA targets are used, mitochondrial genes pose several advantages over nuclear genes for species identification: a faster mutation rate, shorter regions, multiple units available in every cell, a robust library of fully sequenced mitochondrial genomes for numerous fish species, a simpler, smaller structure due to the lack of large stretches of non-coding DNA (introns), and maternal lineage (Civera, 2003; Mackie et al., 1999). Most significantly, the faster mutation rate helps elucidate when speciation occurs, while the matrilineal nature of mitochondrial DNA provides a much more straightforward lineage without conflation from heterozygous genotypes (Civera, 2003). However, introgression can occur within mitochondrial DNA due to this matrilineal heritage, which means that some hybridized or introgressed species cannot be differentiated by mitochondrial genes alone. In these cases, a nuclear target such as the ITS1 must be used to resolve mitochondrial genome introgression (Vinas & Tudela, 2009).

Commonly targeted mitochondrial DNA regions include the genes coding for CYTB, COI, and 16s ribosomal DNA (Civera, 2003; Rasmussen & Morrissey, 2008; Vinas & Tudela, 2009). While these markers are highly successful for the identification of most fish species, it has been challenging to differentiate some groups of closely related fish species, such as tuna. The mitochondrial control region (CR) holds greater genetic diversity than the COI, is a shorter region of ~400 bp, and has been able to discriminate between closely related tuna species (Mitchell & Hellberg, 2016; Vinas & Tudela, 2009).

2.4. Identification of fish species with DNA barcoding

2.4.1. DNA barcoding

DNA barcoding is a standardized, molecular-based approach to identify fish species (**Figure 1**). Specifically, it is used to identify fish species from all forms of tissue, from larvae or eggs to whole fish and fins (Ward et al., 2009). Full DNA barcoding utilizes universal primers to amplify DNA fragments that are approximately 500-650 base pairs (bp) long. The resulting amplicons from the PCR amplification are Sanger sequenced and compared to reference sequences in a database to identify species (Casiraghi et al., 2010). Examples of databases that are used include the Barcode of Life Data Systems (BOLD; http://www.barcodinglife.org) and GenBank by National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genbank/). BOLD is primarily used for DNA barcodes retrieved from the COI region, while GenBank is referenced for all genetic markers (Ratnasingham & Hebert, 2007).

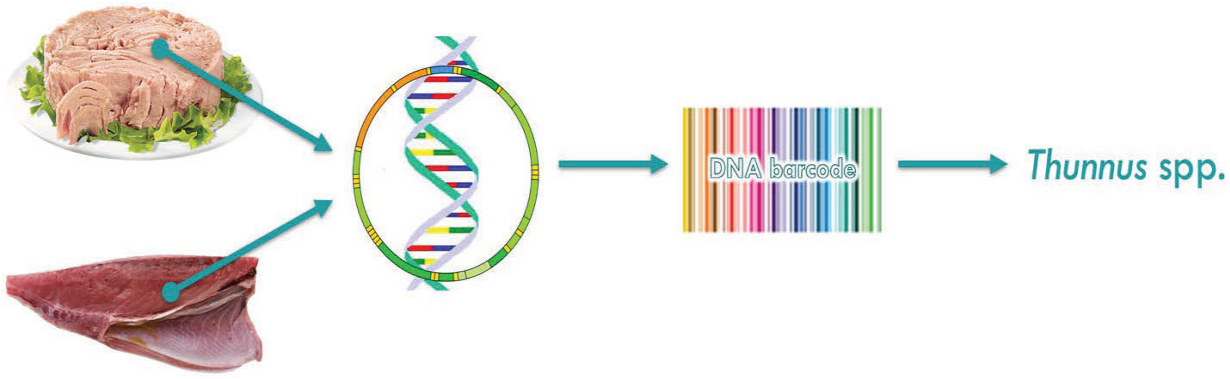

Figure 1. Schematic representation of DNA barcoding based on the mitochondrial gene. Steps include the extraction of DNA from processed or raw samples; PCR amplification of DNA to obtain its unique DNA barcode; and a comparison of barcode to databased sequences to identify species.

DNA barcoding relies on what is called the 'barcoding gap,' or the genetic threshold value of intra- and inter-species differences (Casiraghi et al., 2010). Specifically, the divergence value of inter-specific differences should be greater than that of intra-specific variations in order to discriminate between taxon (Ratnasingham & Hebert, 2007). The divergence value indicates the degree to which genetic codes differ from one another; higher values signify a separation of species, while lower values may point to the same species or a closely related species that originated recently (Hebert et al., 2003). The success of DNA barcoding and BOLD has led to the Fish Barcode of Life campaign (FISH-BOL), an international scientific effort to barcode all of the 30,000 fish species in the world (Ward et al., 2009). As part of this effort, over 19,000 fish species have been successfully barcoded (Panprommin et al., 2019).

2.4.2. *Full DNA barcoding of fish species*

Full DNA barcoding using the mitochondrial COI region is the US Food and Drug Administration's (FDA) standard identification procedure to authenticate fish samples (Handy et al., 2011). Numerous researchers have successfully sequenced fish barcodes using full DNA barcoding (**Table 3**). Ward et al. (2005) tested 207 mostly Australian marine fish species using full DNA barcoding of the COI gene. They found a 100% success rate in species identification, illustrating the diversity of the COI region for species discrimination. However, the researchers discovered the genetic differences within tuna species were extremely small (0.11%), as well as the interspecies differences (1.04%), revealing the difficulty in delineating between tuna species due to their very similar genetic codes as there is typically at least a 2% genetic divergence between species (Handy et al., 2011; Ward et al., 2005).

Table 3. Summary of studies that have used full DNA barcoding of various DNA regions for the identification of seafood species.

Samples and (quantity) tested	Target region	Barcoding success rate (%)	Source
Fish variety (207)	COI (655 bp)	100	(Ward et al., 2005)
Tuna (8)	COI (655 bp)	95	(Lowenstein et al., 2009)
Fish variety (55)	COI (652 bp)	93	(Hanner et al., 2011)
Fish variety (1,247)	COI (655 bp)	97	(Warner et al., 2013)
Seafood variety (23)	COI (650 bp)	94.7	(Khaksar et al., 2015)
Fish variety (5)	COI (234-645 bp)	100	(Nedunoori et al., 2017)
Seafood variety (39)	COI (655), 16S rRNA (588), PEPCK* (598)	84.5	(Guardone et al., 2017)
Fish variety (80)	COI (655) CYT-B (528)	97	(Pardo et al., 2018)

* PEPCK = nuclear gene encoding the enzyme phosphoenolpyruvate carboxykinase

In particular, tuna species identification using the COI region has been tested in various seafood marketplaces around the world (Hanner et al., 2011; Khaksar et al., 2015; Lowenstein et al., 2009; Nedunoori et al., 2017; Pardo et al., 2018; Warner et al., 2013). Lowenstein et al. (2009) sequenced COI barcodes for 68 tuna sushi samples obtained in New York and Colorado, USA. Using the BOLD Identification Engine, they identified a complete match for four species from a single sequence obtained from a yellowfin tuna sample: bigeye tuna, blackfin tuna, yellowfin tuna, and dogtooth tuna. The inability to identify the sequence to the species level may have been due to the species being closely related and/or previous incorrect reference sequences uploaded by other researchers (Lowenstein et al., 2009). Guardone et al. (2017) used primers for the COI, 16S rRNA, and nuclear gene encoding the enzyme phosphoenolpyruvate carboxykinase

(PEPCK) regions and properly identified 84.5% of the 277 seafood products they tested in Italy. These products included frozen, canned, salted or smoked fish, cephalopods, crustaceans, and bivalves. However, in regards to the frozen fish samples, the researchers were able to identify the species of only 49.3% of the fish products they analyzed with primers targeting the COI (Guardone et al., 2017).

2.4.3. *Limitations of full DNA barcoding for canned tuna identification*

The canning process of tuna, which includes high heat, pressure, and may incorporate acidic ingredients and/or nucleases, results in enzymatic degradation and hydrolysis of DNA (Chapela et al., 2007). During the canning process, DNA can degrade into fragments of <350 bp and may be further damaged by the brine in which it is packed, making it difficult to identify species with full barcoding (Chapela et al., 2007; Mackie et al., 1999; Pollack et al., 2018; Ram et al., 1996). In addition, the COI fragment alone is often unable to differentiate between tuna species that share similar genetic codes and exhibit low genetic divergences, such as albacore and Pacific bluefin tuna (Cawthorn et al., 2011; Hellberg & Morrissey, 2011; Ward et al., 2009). Tuna species introgression also creates challenges, as these resulting hybrid species would only have maternal mitochondrial DNA, and thus limits the genetic targets with which they could be discriminated (Ward et al., 2005). Introgression has been reported to occur between Atlantic bluefin tuna and albacore tuna, as well as Atlantic bluefin tuna and Pacific bluefin tuna (Lowenstein et al., 2009; Mitchell & Hellberg, 2016).

2.4.4. *DNA mini-barcoding*

DNA mini-barcoding targeting short (<300 bp) regions of the COI gene has been successfully used for the identification of fish species in processed products (Armani et al., 2017; Pollack et al., 2018; Shokralla et al., 2015). Mini-barcoding has been especially employed to

overcome the challenges of tuna species identification in processed products (**Table 4**). For example, Shokralla et al. (2015) tested a variety of 44 processed fish products with multiple mini-barcoding primers based on the COI region, achieving species or genus level identification for 41 (93.2%) of their samples. However, sequencing was unsuccessful in 3 of the 13 of the tuna products they analyzed and the remaining 10 tuna samples were only identified to the genus level. In comparison, species identification of all other samples was accomplished with at least one set of primers. The relatively low sequencing and species identification success rate of these tuna products was most likely due to primer binding issues, interference from packing ingredients, multiple species within the product, DNA degradation, and the minimal genetic variation between tuna species (Shokralla et al., 2015).

Table 4. Summary of studies that have used DNA mini-barcoding for the identification of species in canned tuna.

Number of canned tuna products	Target region	Sequencing success rate (%)	Level of identification (success rate %)	Source
13	COI (127-314 bp)	77	Genus (77)	(Shokralla et al., 2015)
2	COI (~150 bp)	0	Unsuccessful	(Chin et al., 2016)
53	CR (236 bp), ITS1 nuclear region (179 bp)	45	Species (43) Genus (2)	(Mitchell & Hellberg, 2016)
3	COI (139 bp)	100	Unsuccessful (top matches for *Thunnus* sp. and *K. pelamis*)	(Armani et al., 2017)
1	COI (226 bp)	100	Genus (100)	(Pollack et al., 2018)

Other researchers have also obtained varying sequencing success from canned fish samples with the use of mini-barcoding (Armani et al., 2017; Pollack et al., 2018). Mini-barcode sequences were acquired for most canned tuna samples in these studies, but complete species identification was not achieved. There were multiple top matches for *Thunnus spp.* and

Katsuwonus pelamis (Armani et al., 2017) and for several species within the *Thunnus* genus (Pollack et al., 2018) due to the lack of discriminatory genetic information from the targeted COI fragments.

Because mini-barcoding of the COI region alone is insufficient for tuna species identification, use of other regions from the mitochondrial or nuclear genome is required for testing of processed tuna species (Armani et al., 2017; Mitchell & Hellberg, 2016; Pollack et al., 2018; Vinas & Tudela, 2009). The CR (~450 bp) and ITS1 (~600 bp) target regions were successfully used to identify 8 *Thunnus* species in 26 raw tuna samples. These regions demonstrated greater genetic diversity than the COI region, as they were able to differentiate between each introgressed tuna species (Atlantic bluefin, Pacific bluefin, and albacore tuna) (Vinas & Tudela, 2009). The success of this study led to Mitchell and Hellberg (2016) targeting the CR (236 bp) and ITS1 (179 bp) regions for the species identification of 53 canned tuna samples using a mini-barcoding system. However, they reported a relatively low (43%) sequencing success rate. For the 23 successfully sequenced tuna samples, 21 samples were identified at the species level with BLAST, including albacore tuna, yellowfin tuna, and skipjack tuna, while 2 samples required further analysis with ITS1 primers or phylogenetic results to determine their species (albacore and yellowfin). Sequencing failure was attributed to DNA fragmentation and/or inhibition of PCR by additives in the products, the possibility of multiple species being present, and low primer-binding success (Mitchell & Hellberg, 2016). Identification of sequenced samples to the species level was more successful with the mini-barcoding of the CR and ITS1 (43%) compared to other studies that have utilized mini-barcoding of the COI region with only genus level identification (Armani et al., 2017; Pollack et al., 2018; Shokralla et al., 2015).

A consecutive study by Frigerio et al. (2021) utilized the CR primers developed by Mitchell & Hellberg (2016) for the species identification of *bottarga*, a product made of salted and dried tuna roe. Complete species identification was attained for all 14 fresh and frozen reference samples and 6 dried market samples, indicating the efficacy of these primers for identifying tuna samples of varying life phases and product types (Frigerio et al., 2021). Optimizing the methodology for amplification of the CR mini-barcode region may improve sequencing and identification rates for the authentication of highly processed tuna specimens.

2.5. Rationale and significance

The prevalence of tuna fraud continues to grow as the international market for seafood flourishes. *There is a need* for effective DNA-based methods to identify fish species in order to combat species substitution in seafood products, protect consumer health, and improve the regulation of fish stocks. Processed tuna species are especially difficult to differentiate with current DNA barcoding methods. *This study aims* to conduct a market survey of raw, dried, and canned tuna products on the market using mini-barcoding primers based on short (<250 bp) fragments of the CR. *It is hypothesized* that the CR will provide sufficient genetic information to identify all tuna species present in raw and processed products. *Results from this study will help determine* the possibility of using DNA mini-barcoding to detect and distinguish between tuna species in processed samples despite drying or high heat processing, which both degrade the fish DNA present in the finished product. *A DNA mini-barcoding system to identify tuna species in processed products would contribute to more accurate detection methods of fraudulent species substitution and mislabeling in processed tuna products. Improved detection of seafood fraud will provide* necessary information to control fraud more effectively, restoring consumer

confidence in producers, guarding consumer health, improving management of marine resources, and furthering seafood product quality. *This DNA mini-barcoding system will also contribute to* the understanding of the slight genetic differences between tuna species and the prospect of using the CR, in addition to the COI gene, to barcode other fish and animal species.

3. Materials and Methods

3.1. Sample collection

A total of 80 raw and processed tuna products were collected for testing, including 20 raw tuna fillets, 20 raw sushi samples, 20 canned tuna products, and 20 dried tuna products (**Figure 2**). Products were collected from 12 grocery stores and 10 sushi restaurants in Orange County, CA, as well as 3 online retailers. Fillets and sushi samples purchased at retail outlets were transported in coolers with ice packs and stored at -80 °C upon arrival at Chapman University. Fish tissue samples (~25 mg each) were collected from the centers of each sample after defrosting in a refrigerator (Nor-lake Scientific, WI) at 4 °C for 24 h. Tissue samples were obtained with sterile forceps and stored in sterile 1.5 ml microcentrifuge tubes (Eppendorf, Germany) for DNA extraction. The remaining fillet portions were stored in plastic freezer bags (Ziploc, USA) at -80°C.

A variety of canned tuna samples was obtained, including tuna processed in cans and retort pouches with an array of ingredients (water, oil, salt, seasonings, and marinades). Dried tuna samples consisted of plain dried flakes and dried jerky produced with a variety of seasonings. Canned and dried fish tissue samples (~25 mg each) were obtained with sterile forceps and retained in sterile 1.5 ml microcentrifuge tubes (Eppendorf, Germany) for DNA extraction. The remaining canned portions were stored in plastic freezer bags (Ziploc) at -80°C,

while the remaining dried samples were stored in plastic freezer bags (Ziploc) at ambient room temperature (~23°C).

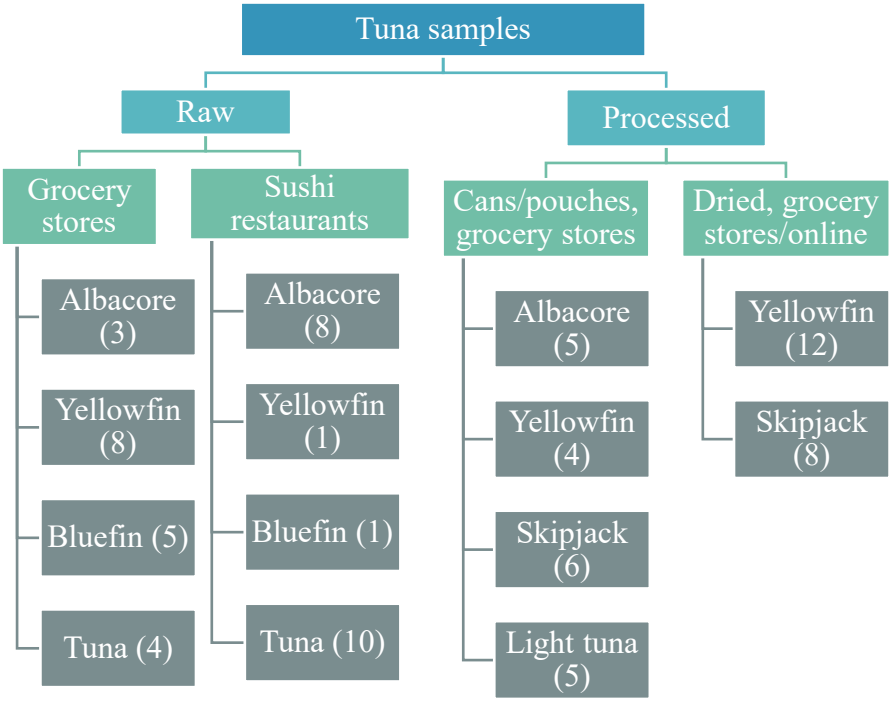

Figure 2. Sampling scheme of 80 tuna products for DNA analysis.

3.2. DNA extraction

The DNeasy Blood and Tissue Kit, Spin-Column protocol (Qiagen, Valencia, CA, USA) was used to extract DNA from all fish samples, according to the manufacturer's instructions. A negative extraction control was included with each batch of samples. Buffer ATL (180 µl) and Proteinase K (20 µl) were added to each microcentrifuge tube containing 25 mg of fish tissue. Each tube was then incubated at 56 °C in a ThermoMixer C (Eppendorf, Hamburg, Germany) set at 300 rpm for 4 h. Next, Buffer AL (200 µl) and ethanol (200 µl) were added to the samples followed immediately with vortexing. The tube contents were pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min at 8000 rpm. The flow-through

and collection tube was discarded and the DNeasy Mini spin column was transferred to a new 2 ml collection tube, followed by addition of Buffer AW1 (500 µl). This was centrifuged for 1 min at 8000 rpm again and the resulting flow-through and collection tube was discarded. The DNeasy Mini spin column was transferred to a new 2 ml collection tube followed by addition of Buffer AW2 (500 µl) and centrifuged for 3 min at 14000 rpm to completely dry the membrane. The resulting flow-through and collection tube was discarded and the DNeasy Mini spin column was transferred to a new 2 ml collection tube with Buffer AE (50 µl) and centrifuged at 8000 rpm. The resulting eluted liquid containing the DNA was stored at -20 °C until PCR amplification.

Table 5. Primers used in this study.

Primer set	Primer name	Primer direction	Primer sequence (5'-3')	Described	Target fragment length
CR	Tuna CR_F	Forward	CAC GAC GTT GTA AAA CGA CGC AYG TAC ATA TAT GTA AYT ACA CC	Mitchell & Hellberg (2016)	236 bp
	Tuna CR_R1	Reverse	GGA TAA CAA TTT CAC ACA GGC TGG TTG GTR GKC TCT TAC TRC A		
	Tuna CR_R2	Reverse	GGA TAA CAA TTT CAC ACA GGC TGG ATG GTA GGY TCT TAC TGC G		

3.3. PCR and sequencing

The protocol for PCR and DNA sequencing of the extracted tuna DNA samples was as described by Mitchell & Hellberg (2016). The reaction mixture included 0.5 OmniMix HS PCR bead (Cepheid, Sunnyvale, CA, USA), 0.5 µl of each 10-µM primer or primer cocktail (**Table 5**), 3 µl DNA template or negative control, and 21.5 µl molecular grade water for a total volume of 25 µl. Cycling conditions were as follows: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 49 °C for 40 s, and 72 °C for 1 min; and a final extension of 72 °C for 10 min. A Mastercycler nexus

gradient thermal cycler (Eppendorf) was used for thermocycling and a non-template PCR control was included alongside each batch of samples. Precast 2.0% E-Gel agarose gels with ethidium bromide (Invitrogen, Carlsbad, CA, USA) were used to confirm DNA amplification. The gels were loaded with 10 µl of PCR product from raw samples or 20 µl of PCR product from dried and canned products and run for 30 min with an E-Gel Powerbase (Life Technologies). A ChemiDoc Imager (Bio-Rad, Hercules, CA, USA) was used to visualize and photograph the agarose gel results. Any samples that failed the initial round of PCR amplification underwent a repeat DNA extraction with an extended 12 h lysing period and were re-amplified with PCR. Cleanup of PCR products was carried out with ExoSAP-IT (Applied Biosystems, Santa Clara, CA, USA) following the manufacturer's instructions. Bi-directional sequencing of all positive samples was carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730xl DNA Analyzer (Applied Biosystems) by Eurofins Genomics (Louisville, KY, USA).

3.4. Species identification

Raw sequence data was assembled and edited using Geneious R7 (Biomatters, Ltd., Auckland, NZ; http://www.geneious.com; Kearse et al., 2012). QC parameters were determined as explained by Pollack et al. (2018). Samples were considered successfully sequenced if they generated bidirectional sequences that were ≥76% of the target length (236 bp) and had <2% ambiguities or single reads that were ≥76% of the target length and had ≥98% HQ. The resulting consensus sequences were searched against GenBank using the nucleotide Basic Local Alignment Search Tool (BLAST), megablast algorithm. Sequences were identified based on the top species matches in GenBank. In instances where a sequence showed less than 90% identity to the top species match in GenBank, that sample underwent repeat DNA extraction with a 12 h

lysing period, PCR amplification, and DNA sequencing. Samples that had <90% identity to any other sequences in GenBank or that showed multiple equivalent top matches underwent phylogenetic analysis using Geneious R7. The sequences were aligned using ClustalW with a subset of sequences representing each target species (Mitchell & Hellberg, 2016). Genetic divergence was calculated using the Jukes Cantor distance method and a neighbor-joining (NJ) tree was compiled (Saitou & Nei, 1987). The robustness of the tree was evaluated using bootstrap analysis with 1000 iterations and a consensus tree was created using a 70% threshold. In the case of any potential mislabeling, samples in question were subjected to another round of DNA extraction, PCR, and DNA sequencing.

4. Results and Discussion

4.1. PCR amplification and sequencing results

Initially, 59 samples exhibited PCR amplification success based on gel electrophoresis results. The samples that failed amplification consisted of 17 canned products and 4 dried products. However, following re-extraction with an extended 12 h lysing period and PCR amplification, all 21 re-extracted products showed bands in gel electrophoresis. Based on these results, an extended lysing period is recommended for improved PCR amplification of processed (dried and canned) tuna products. Of the 80 samples successfully amplified, 69 samples generated sequences that passed the QC parameters established by Pollack et al. (2018). The successful sequences had an average sequence length of 235 ± 3 bp, average HQ% of 92.68 ± 17.66%, and 0.30 ± 0.00% ambiguities. According to the QC parameters, raw products yielded the highest quality of sequences with an average HQ% of 98.22 ± 2.28%, followed by dried (95.52 ± 6.42%) and canned (65.14 ± 35.44%) products, respectively.

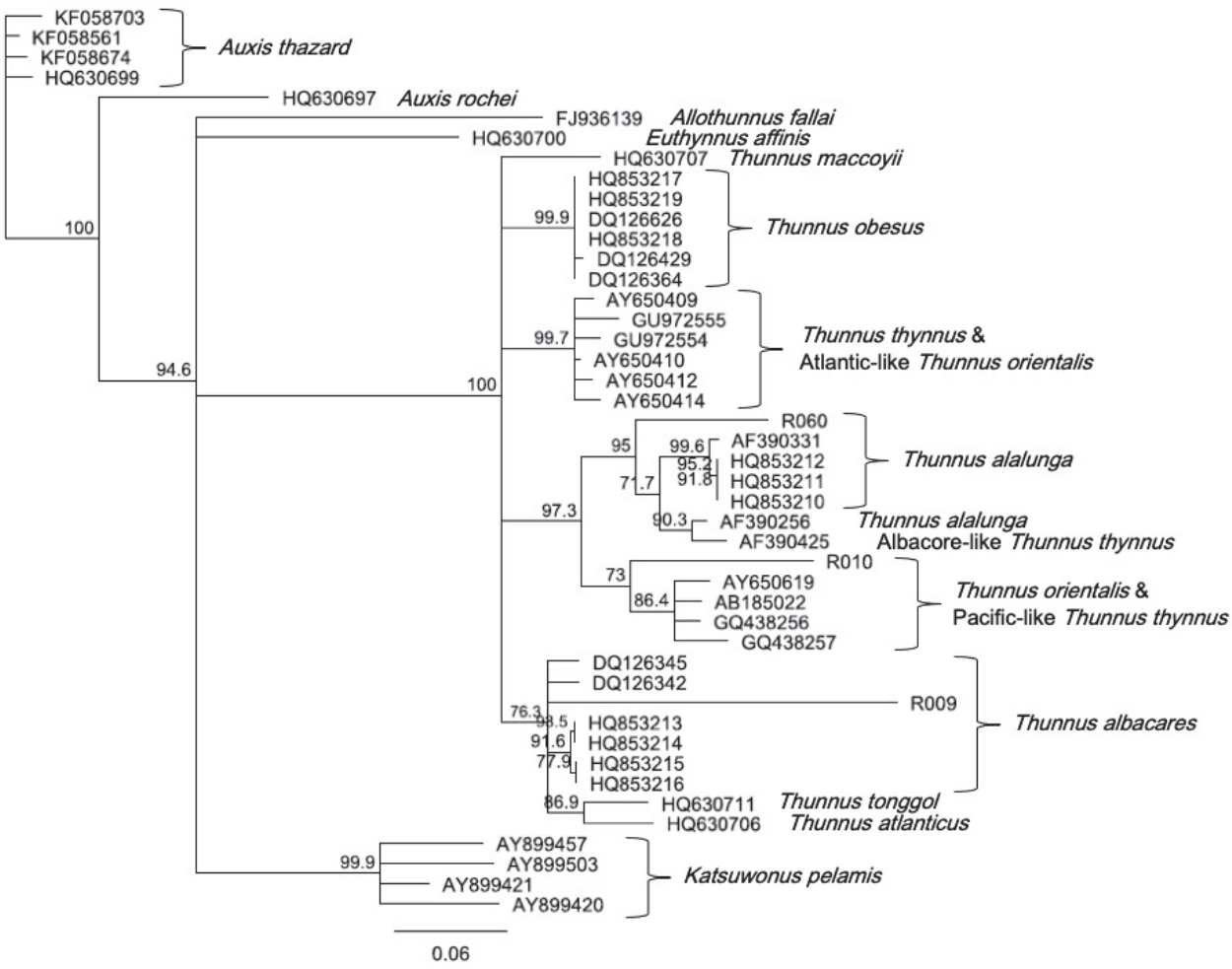

Figure 3. Neighbor-joining tree showing samples R009, R010, and R060 and reference sequences for each tuna species targeted by the CR mini-barcode. GenBank accession numbers are shown for all reference sequences. The Jukes Cantor method was used to calculate genetic distances, bootstrap analysis was conducted with 1000 replicates, and a consensus tree with a 70% threshold was created.

All 69 sequences obtained were identified to the species level using BLAST, with the top sequence matches for 67 of these sequences showing >94% identity and >98% query coverage. There were 2 successfully sequenced canned samples with 84% and 88% identity to yellowfin tuna (R009) and Pacific bluefin tuna (R010), respectively. Due to the relatively low percent identity (<90%) of these samples, they underwent phylogenetic analysis (**Figure 3**) and were subjected to repeat DNA extraction, amplification, and sequencing. As shown in **Figure 3**, the sample sequences grouped with the same species identified through GenBank, with R009

grouping with the yellowfin tuna sequences and R010 grouping with the Pacific bluefin tuna sequences. The repeat analysis for these two samples resulted in sequencing failure for sample R010 and a positive identification for R009 to bigeye tuna, with 100% identity and 100% query coverage. The identification of both yellowfin and bigeye tuna in R009 indicates the possibility of a species mixture in this product.

Within the product categories, all 40 raw samples from grocery stores and restaurants were identified to the species level, 19 out of the 20 dried samples were identified to the species level, and 10 out of 20 canned samples were identified to the species level (**Table 6**). Species identification for all raw samples was similar to the results of Liou et al. (2020), who identified 9 out of 10 raw tuna samples to the species level with this CR mini-barcoding system. Likewise, species identification for 95% of the dried samples was comparable to the 100% identification success of dried tuna roe products achieved by Frigerio et al. (2021). The 100% PCR amplification success rate and 50% sequencing rate for the canned samples in this study were improvements to the 49% PCR amplification success and 45% sequencing rate obtained by Mitchell & Hellberg (2016) using the same CR primers. The improved PCR amplification success in the current study was attributed to the increased (12 h) lysing period used for DNA extraction for repeat samples, as compared to a 1-3 h lysing period in Mitchell & Hellberg (2016). The extended lysing period may have improved the ability of Proteinase K to fully digest the denatured proteins of the processed samples and release the DNA in the cells (Ebeling et al., 1974). The difference between the amplification success (100%) and sequencing success (50%) for canned samples in this study may have been due to the presence of multiple species, which can co-amplify and generate a mixed chromatogram (Shokralla et al., 2015). Indeed, many of the

samples that failed the QC sequencing parameters showed multiple, overlapping peaks in the sequencing chromatogram, which may indicate the presence of multiple species.

Table 6. Summary of the species identification results for the 80 tuna products analyzed in this study with the CR mini-barcode.

Category	Labeled species	Sample size	Identified to species level	Mislabeled samples	% of samples identified in each category
Raw, grocery store	Albacore	3	3		100
	Yellowfin	8	8		
	Bluefin	5	5	1	
	Tuna	4	4		
Raw, sushi restaurant	Albacore	8	8		100
	Yellowfin	1	1	1	
	Bluefin	1	1		
	Tuna	10	10		
Dried	Yellowfin	12	12	5	95
	Skipjack	7	6	1	
	Tuna	1	1		
Canned	Albacore	5	3		50
	Yellowfin	4	3	2	
	Skipjack	6	1	1	
	Light tuna	5	3		

All samples showed a single top species match in GenBank with the exception of R060, which was a raw sample labeled as albacore tuna. This sample yielded a high-quality sequence with 100% HQ that matched 2 albacore tuna sequences in BLAST with the same % identity (97.03%) and query coverage (100%) as a singular bigeye tuna sequence (Accession ID KM055385.1). All other 97 matches were to albacore tuna entries. While introgression between these two species is possible, it has not been reported in previous research. The bigeye sequence entry was from unpublished research and it is possible that it was mistakenly identified and

uploaded to GenBank. Previous studies have also noted the possibility of researchers uploading inaccurate reference sequences onto genetic databases (Hanner et al., 2011; Lowenstein et al., 2009; Mitchell & Hellberg, 2016). Moreover, R060 clearly grouped with the albacore tuna clade when its CR mini-barcode was further analyzed against reference tuna sequences (**Figure 3**). Therefore, considering the BLAST results and phylogenetic analysis, R060 was determined to be albacore tuna.

4.2. Mislabeled samples

Out of the 69 samples that were identified, 11 were determined to be mislabeled (15.9%) (**Table 7**). All potentially mislabeled samples underwent DNA extraction, PCR, and sequencing a second time for confirmation. Upon repeat testing, mislabeling was confirmed for 8 of the samples, while sample R015 failed the second round of PCR amplification and thus remained an unconfirmed case of mislabeling. In addition, samples R018 and R044 yielded different species matches on the second run of testing than the initial sequencing, leaving these as unconfirmed mislabeling cases of species mixtures. The majority (73%) of mislabeled products were advertised as "yellowfin tuna," followed by products advertised as skipjack tuna (18%) and bluefin tuna (9%). Of the identified samples across product categories, 5% of the raw samples, 26% of the dried samples, and 20% of the canned samples were found to be mislabeled. Multiple samples labeled as yellowfin tuna (R009, R015, R067, R074-R076, R078, R080) were found to be substituted with bigeye or Pacific bluefin tuna. In the case of R009 (canned), it was labeled as only containing yellowfin tuna but was found to include both yellowfin and bigeye tuna after

Table 7. Summary of the 11 mislabeled tuna products found in this study. Top species matches were determined using BLAST.

Mislabeled sample	Category, product type	Expected species based on product label	Species determination	Price paid (US $/kg)
R009	Canned, chunk	Yellowfin tuna (*T. albacares*)	Yellowfin tuna (*T. albacares*) and bigeye tuna (*T. obesus*)	23.27
R015	Canned, chunk	Yellowfin tuna (*T. albacares*)	Bigeye tuna[b] (*T. obesus*)	40.41
R018	Canned, chunk	Skipjack tuna (*K. pelamis*)	Bigeye tuna (*T. obesus*) and skipjack tuna[b] (*K. pelamis*)	14.01
R035	Raw (grocery store), fillet	Bluefin tuna[a] (*T. maccoyii/ orientalis/thynnus*)	Yellowfin tuna (*T. albacares*)	55.10
R067	Raw (restaurant), sushi	Yellowfin tuna (*T. albacares*)	Bigeye tuna (*T. obesus*)	423.29
R044	Dried, flakes	Skipjack tuna (*K. pelamis*)	Yellowfin tuna[b] (*T. albacares*) and skipjack tuna (*K. pelamis*)	87.57
R074	Dried, jerky strip	Yellowfin tuna (*T. albacares*)	Pacific bluefin tuna (*T. orientalis*)	117.65
R075	Dried, jerky strip	Yellowfin tuna (*T. albacares*)	Pacific bluefin tuna (*T. orientalis*)	117.65
R076	Dried, jerky strip	Yellowfin tuna (*T. albacares*)	Pacific bluefin tuna (*T. orientalis*)	117.65
R078	Dried, jerky	Yellowfin tuna (*T. albacares*)	Bigeye tuna (*T. obesus*)	164.67
R080	Dried, jerky	Yellowfin tuna (*T. albacares*)	Bigeye tuna (*T. obesus*)	164.67

[a] Labeled as "B/F Tuna" (considered to be an abbreviated description for bluefin tuna)
[b] Unconfirmed; repeat testing failed

repeat sequencing. Yellowfin tuna is considered to be lower in mercury and safe to eat once a week for at-risk consumers, but bigeye and Pacific bluefin tuna both have elevated mercury levels and can pose a health risk to vulnerable individuals (FDA/EPA, 2019; Lowenstein et al., 2010). Bigeye and yellowfin tuna inhabit the same tropical waters, look similar to one another, and are difficult to accurately distinguish morphologically without trained personnel (NOAA, 2020). However, the ex-vessel price of bigeye tuna was $6.12/kg in 2019, making it more economically valuable than yellowfin tuna ($1.76/kg) (NMFS, 2021). Therefore, it is possible that bigeye tuna catches were misidentified as yellowfin tuna and were mistakenly processed into canned and dried products alongside yellowfin tuna. Similar to the results of the current study, substitution of yellowfin tuna with bigeye or bluefin tuna has been reported in numerous studies (Gunther et al., 2017; Hu et al., 2018; Liou et al., 2020; Pardo et al., 2018; Warner et al., 2013).

Samples R074-R076 were packaged as single strips of dried “yellowfin” tuna and were all identified as Pacific bluefin tuna. These three samples were from the same company but contained different flavors, indicating that this mislabeling issue impacted several product lines. The ex-vessel price for Pacific bluefin tuna ($8.36/kg) is significantly higher than that of yellowfin tuna so there does not appear to be an economic incentive for the company to intentionally mislabel their product (NMFS, 2021). However, mislabeling may be a method to conceal instances of IUU and to provide a legal market for these catches (Hu et al., 2018; Warner et al., 2013). Pacific bluefin tuna continue to be an overfished, threatened species and their inclusion in mislabeled products deters conservation efforts to preserve the species.

Two samples labeled as skipjack tuna (R018 and R044) were found to contain other tuna species. Specifically, R018 (canned) was identified as bigeye tuna during its first sequencing run and then skipjack tuna after repeat sequencing. Similarly, R044 (dried) yielded both yellowfin

and skipjack tuna identifications after multiple testing rounds. These results suggest that both products contained a mixture of species but remain unconfirmed cases of mislabeling. Although unconfirmed, these mislabeling events introduce a health concern because skipjack tuna is part of the "Best Choices" category for consumers at risk of mercury exposure, meaning that it can be consumed multiple times a week. On the other hand, yellowfin tuna is in the "Good Choices" category for seafood that may be eaten once a week, while consumption of bigeye tuna should be completely avoided by at-risk consumers (FDA/EPA, 2019). With an ex-vessel price of $1.21/kg in 2019, skipjack tuna is a cheaper species compared to yellowfin ($1.76/kg) and bigeye ($6.12/kg) tuna (NMFS, 2021), suggesting that this mislabeling event was not associated with an economic incentive. Skipjack tuna is morphologically distinct from yellowfin and bigeye tuna, but all three species inhabit the same waters and bycatch may occur (NOAA, 2020). Therefore, it is possible that the undeclared species were present due to accidental inclusion.

One sample (R035) was identified to be yellowfin tuna even though it was a raw fillet labeled as "B/F Tuna," which was considered to be an abbreviated description for "bluefin tuna". It was priced at $55.10/kg, a similar price level as raw sample R034 ($61.72/kg) that was explicitly labeled "bluefin tuna chutoro" and identified as Southern bluefin tuna from the same grocery store (**Figure 4**). "Chutoro" refers to a valuable cut of tuna consisting of the upper belly area of the fish and often garners a higher price (Shimose et al., 2018). Furthermore, sample R037 was also from the same grocery store and was labeled as "Tuna Steak" with a price tag of $28.64/kg. R037 was identified as yellowfin tuna and was almost half the price of the other samples labeled as bluefin tuna, which demonstrates the economic incentive associated with these kinds of mislabeling cases (Hu et al., 2018; Pardo et al., 2018). These factors suggest that

the mislabeled sample R035 was indeed intended to be sold as bluefin tuna at a premium price instead of the lower price yielded for yellowfin tuna.

Figure 4. Product labels for raw samples (a) R034, (b) R035, and (c) R037 which were all purchased from the same grocery store.

5. Conclusion

Accurate methods for the reliable detection of tuna species in food products facilitate the long-term goals of detecting seafood fraud, advancing tuna stock regulation, and protecting consumer health. This study investigated the efficacy of a CR mini-barcoding method to identify a variety of raw and processed tuna products. PCR amplification was successful for all samples and species identification was achieved for the majority of tested products. However, there was

limited success in identifying canned tuna samples, which is likely due to fragmented DNA and/or the presence of multiple species within the product. Overall, 15.9% of identified samples were determined to be mislabeled, with mislabeling occurring most frequently in dried products, followed by canned and raw products. Most of the mislabeled products were marketed as yellowfin tuna, followed by skipjack and bluefin tuna. These products were likely mislabeled for a variety of reasons, including accidental inclusion due to by-catch, provision of a legal market for IUU catches, and substitution of more expensive species with a cheaper species for economic gain. Overall, this research shows the utility of the CR mini-barcoding method for the detection of species in raw and processed tuna products. Future research should examine optimization of the method to further improve identification of species in canned tuna samples.

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