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Identification of Animal Species by Utilization of Processed Remains

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IDENTIFICATION OF ANIMAL SPECIES BY UTILIZATION OF PROCESSED
REMAINS

A Thesis Presented in Partial Fulfillment of the Requirements for the Master of Science
in Forensic Science

John Jay College of Criminal Justice

City University of New York

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May 2018

IDENTIFICATION OF ANIMAL SPECIES BY UTILIZATION OF PROCESSED
REMAINS

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This thesis has been presented to and accepted by the office of Graduate Studies, John Jay College of Criminal Justice in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science.

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PREFACE

This thesis is submitted for the degree of Master of Science in Forensic Science at John Jay College of New York. The research described herein was conducted under the supervision of Professor Richard Li in the Molecular Biology Department, John Jay College, between September 2014 and June 2017.

Part of this work has been presented in the following publications:

M. Gaud, *An Enzymatic Method to Process Decomposed Non-Human Bone for Forensic DNA Analysis.*, Forensic Research Volume 5 Issue 2.

I would like to thank Dr. Richard Li for his assistance in the laboratory facilities in the Department of Molecular Biology at John Jay College, I am extremely grateful and indebted to Dr. Li for his constant guidance, support, knowledge, and contributions to the formulation of this thesis. I would also like to thank Smriti Nair, Benjamin Bernstein, Wendy Chen, Larry Chan, and Roger Arteaga for their technical assistance. Special thanks to Artem Domashevskiy and Andrew Schweighardt for serving on the thesis committee. Thank you to all my colleagues and friends at John Jay College. In conclusion, I would like to thank my family for their support as I worked towards my master's Degree.

**CHAPTER1: AN ENZYMATIC METHOD TO PROCESS
DECOMPOSED NON-HUMAN BONE FOR FORENSIC DNA
ANALYSIS**

ABSTRACT

Forensic analysis of DNA from non-human bones can be important in investigating a variety of forensic cases. However, decomposed bone is difficult to process for isolating DNA. In this study, a previously established enzymatic method was utilized to process bone samples that simulate decomposed specimens. Our results demonstrated that this enzymatic processing method is effective for removing decomposed soft tissues and outer surface materials such as mineralized bone connective tissue of bone fragment samples. Our data suggested that this method can be used in the initial sample preparation for cleaning the outer surface of decomposed non-human skeletal fragments. This study introduced an alternative method for processing decomposed non-human bone evidence prior to DNA isolation. Such a method can potentially be used to process various samples of different sizes and conditions for the investigation of a wide variety of criminal cases involving animals.

CHAPTER 2: IDENTIFICATION OF RED SNAPPER SPECIES BY SEQUENCING OF THE COI GENE

ABSTRACT

Red snapper is one of the most common fish substituted by other fish in instances of illegal seafood mislabeling. The only species legally considered Red Snapper is *Lutjanus campechanus*, but in filet form it is virtually impossible to distinguish *L. campechanus* from other snappers. *L. campechanus* is often substituted by the less expensive fish *Lutjanus peru* and *Lutjanus synagris*. The objective of the research was to find a way to distinguish other *Lutjanus* species from *L. campechanus* by identifying acute differences in their genetic codes. DNA was extracted from eighteen collected samples. The samples were sequenced and analyzed using barcoding technology. After analyzing these sequences, it was found that there is one single nucleotide polymorphism (SNP) that can differentiate the species; the SNP analyzed is at position 359. The study elucidated that a reliable testing method of Red Snappers is possible with barcoding technology paving the way for possible rapid testing. If successful rapid testing is created, the procedure could allow for prompt investigations of a wide variety of cases involving illegal seafood mislabeling.

CHAPTER 1: AN ENZYMATIC METHOD TO PROCESS DECOMPOSED NON-
HUMAN BONE FOR FORENSIC DNA ANALYSIS

INTRODUCTION

The forensic analysis of non-human bone DNA is a useful tool in investigating a variety of cases. Animal evidence associated to human victims or suspects and the killing, trade, and possession of an animal or animal products derived from a species that is protected from illegal hunting are two common applications of forensic investigations. The evidence is often examined using forensic DNA analysis to determine the species of the animal evidence. However, the success of DNA analysis of animal remains depends on the quality of extracted DNA. An animal killed illegally is often found partially consumed or decomposed in the field. Remains with postmortem decomposition pose a great challenge to forensic DNA analysis. The DNA extracted from the decomposed soft tissues is often degraded, rendering it unsuitable for species identification. Hard tissues such as bones are the preferred source for forensic DNA identification because the DNA of hard tissues can be protected from degradation. Thus, the forensic analysis of DNA from bone is important in species identification of non-human bone evidence. It is required that the processing of non-human bone evidence follow the same standards as any other forensic investigation (Linacre et al., 2011). One of the major problems affecting the quality of forensic analysis is comingled remains, contamination by animal scavenging, environment borne inhibitors, and bacterial contamination. As a result, the outer surface of the bone fragment must be removed (Ogden, Dawnay, & McEwing, 2009). Currently, limited methodologies are available for processing decomposed samples used in the forensic DNA analysis of non-human bone evidence. Most skeletal preparation techniques may cause DNA degradation, which is not ideal for processing evidence intended for DNA analysis (Rennick, Fenton, & Foran, 2005). The processing

of bones may be carried out using a mechanical method (Davoren et al., 2007.). However, to avoid cross-contamination between samples, the bone dust generated by the mechanical method (with single-use sanding discs attached to a rotary sanding tool during bone sanding) must be cleaned and removed. Thus, processing bone evidence obtained from a severely decomposed animal is sometimes a laborious and a time-consuming task (Linacre, 2009). Developing a simple and reliable processing method for processing decomposed evidence is highly desired. An enzymatic method, using a proteolytic trypsin enzyme to degrade various types of proteins (Buck, Vithayathil, Bier, & Nord, 1962; Walsh, 1970) has been utilized in the maceration of bone samples in skeletal preparation (Hangay & Dingley, 1985; Hendry, 1999). In our previous study, the trypsin maceration technique was adapted to prepare samples prior to DNA isolation from human fresh bone samples (Li, Chapman, Thompson, & Schwartz, 2009; Li & Liriano, 2011) and human burial bone samples (Li & Klempner, 2013). Additionally, the effects of this technique on the yield of DNA isolated were compared to that of a mechanical method (Li & Klempner, 2013). Comparable values of DNA yields between the two methods were observed (Li & Klempner, 2013). This study adapted the enzymatic trypsin method to process decomposed nonhuman bone prior to DNA isolation. Swine (*Sus scrofa domesticus*) bone was used in this study as they are a useful model system for simulating various animal bones. Additionally, the bone sample studied was prepared to reflect more typically encountered samples in forensic cases. In this study, the effects of trypsin treatment on the yield of DNA isolated and on the quality of DNA analysis were examined.

MATERIALS AND METHODS

Sample Preparation and Processing

The fragments of swine femur and scapula (approximately 250 g) were dissected. Experiments were prepared by placing a piece of bone fragment with soft tissue, protected by a metal cage, outdoors for seven days (average daily high temperature, 32°C; humidity, 49%).

The surface cleaning of bone samples was processed using the trypsin method as previously described (Li & Liriano, 2011). Trypsin (laboratory grade powder) was obtained from Fisher Scientific. The trypsin treatment was carried out by placing a piece of bone fragment in 500 ml of trypsin solution (30 μ g/ μ l, 10 mM Tris, pH7.5) and then was incubated with gentle agitation at 55 °C overnight. After incubation, the liquid was removed. The cleaned bone fragments were further processed by inversion for 30 seconds in distilled water, 0.5% sodium hypochloride, and 96% ethanol as described in Davoren et al. (2007). The bone fragments were then air dried.

For scanning electron microscopy (SEM) observation, samples were cut, dehydrated and coated with gold under a vacuum according to the standard procedures. The samples were observed and photographed using a variable pressure scanning electron microscope (Vega 5136 mm) to confirm the cleaning effects.

DNA Extraction and Quantitation

Bone powder was prepared by drilling, as described in Courts and Madea (2011) using a rotary tool (Dremel, Racine, WI). Demineralization of bone powder was carried out as described in Loreille, Diegoli, Irwin, Coble, and Parsons (2007). For each sample, 0.2 g of pulverized bone powder was decalcified by incubating in 3.2 ml of extraction buffer (0.5 M EDTA, 1% lauryl-sarcosinate) and 200 μ l of 20 mg/ml proteinase K overnight at 56 °C with gentle agitation.

The DNA from each sample was extracted using the method previously described. The volume of the demineralized sample was reduced to approximately 400 μ L using an Amicon Ultra-4 (30 kD) column (Millipore, Billerica, MA). DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The final volume of eluted DNA was 60 μ l. Extraction negative controls were employed to monitor the potential contaminations. DNA quantitation was performed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) according to the manufacturer's protocols (Li, Gaud, & Nair, 2014). The final DNA yield was the mean of six determinations.

Species Identification by Sequencing Swine Mitochondrial Cytochrome b Locus

The amplification of specific fragments of the swine mitochondrial cytochrome b (*Cytb*) gene was carried out. A 0.5 ng of DNA template was used. PCR reactions were performed in reaction volumes of 25 μ L containing GeneAmp PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5 mmol/l $MgCl_2$, 200 M each dNTP, 1 mM bovine serum albumin (Sigma-Aldrich) and 2 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems). Additionally, 0.4 M each forward (5'-TCA CAC GAT TCT TCG CCT TCC ACT-3') and reverse primer (5'-TGA TGA ACG GGT GTT CTA CGG GTT-3') was used (Steadman, DiAntonio, Wilson, Sheridan, & Tammariello, 2006). The expected size of the amplicon was a 521bp fragment of the swine mitochondrial *Cytb* gene (at nucleotide position 524 – 1022; GenBank Accession Number: AY237533). The reactions were initiated with an 11-minute activation step at 95°C. For each cycle, the parameters included a 30 seconds denaturation step at 94°C, a 30 seconds primer annealing step at 50 °C, and a 30-s extension step at 72°C. The PCR was performed for a total of 34 cycles. As a positive control, amplification with 0.5 ng of genomic DNA of known mitochondrial DNA sequence was carried out. To monitor contamination, PCR negative controls were included with each amplification experiment.

To identify and to quantify the PCR products, DNA separations were performed using the DNA 1000 Lab-on-Chips Assay kit with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. The Agilent DNA 1000 ladder (Agilent Technologies) was used as a sizing standard. The data was analyzed to determine DNA fragment size based on the sizing ladder and internal standards. The quantitation of each PCR product was performed using the manufacturer's software

provided with the Agilent Bioanalyzer 2100 system.

The 521bp amplicon fragment of the swine mitochondrial *Cytb* gene was sequenced. The ExoSap-IT reagent (Affymetrix, Santa Clara, CA) was used to remove unincorporated primers and nucleotides. The cycle sequencing reaction was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The total reaction volume was of 20 μ L including 5 ng template. The reactions were initiated with a 60 seconds soak at 96°C. For each cycle, the cycling parameters included a 15 seconds denaturation step at 96°C, a 15 seconds primer annealing step at 50°C, and a 60 seconds extension step at 60°C. The cycle sequencing was performed for a total of 25 cycles. Post-amplification sample clean-up was carried out using the DyeEx spin columns (Qiagen, Valencia, CA). The cycle sequencing products were separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and were analyzed with the Sequencher software (Gene Codes, Ann Arbor, MI). The DNA sequence obtained was compared with the *BLAST* database. (Li, Gaud, & Nair, 2014)

Results and Discussions

The trypsin-treated bone fragments were examined after incubation. The removal of the decomposed soft tissue of the bone sample was observed after incubation (Figure 3). The surface cleaning effect of the trypsin treatment was further examined using SEM observation. Figure 3 shows the intact outer surface of untreated bone surface. The removal of the outer surface layer of the bone sample was observed after the trypsin treatment.

DNA was isolated from trypsin treated samples according to the procedure as described in the Materials and Methods. DNA quantitation was performed, and the DNA yield of trypsin-treated bone samples was 1.68 mg DNA/g bone (the values were the mean of six determinations), which was sufficient for subsequent DNA analysis. No DNA contamination was detected in negative controls. To evaluate the quality of DNA isolated from the trypsin-processed bone samples, species identification using mitochondrial DNA analysis was performed. In species identification, the most commonly used are the mitochondrial Cytb, cytochrome c oxidase I (COI), and D-loop loci (Linacre et al., 2011). In this study, a segment (521 bp) of Cytb gene was analyzed because it was applied to the identification of various vertebrates (Parson, Pegoraro, Niederstatter, Foger, & Steinlechner, 2000; Hsieh et al., 2001). The Cytb fragment was amplified and quantified using a microfluid electrophoresis device: Agilent Bioanalyzer 2100. Successful amplification (average yield was 780 ng) was detected in all DNA samples tested. No adverse effects of trypsin treatment on PCR were observed compared to control samples (Figure 4). A cycle sequencing reaction usually requires

approximately 5 ng of amplified product (Schwark, Heinrich, Preusse-Prange, & von Wurmb-Schwark 2011). Thus, all amplified samples yielded sufficient quantities of PCR products for subsequent sequencing analysis. The amplified fragment at the *Cytb* locus was successfully sequenced (Figure 5). No adverse effect of trypsin treatment on sequencing was observed compared to control samples (Figure 5). Results from the sequence analysis confirmed that the origin of the samples was *Sus scrofa domestica* (465 bp, E-value = 0.0).

Currently, the cleaning of the outer surface of bone fragments (removing approximately 1–2 mm of surface bone materials) for forensic DNA analysis is usually carried out using mechanical methods, such as sanding, which uses sanding discs attached to a rotary tool (Davoren et al., 2007; Courts & Madea, 2011; Edson, Ross, Coble, Parsons, & Barritt, 2004) or sandpaper (Anslinger, Weichhold, Keil, Bayer, & Eisenmenger, 2001; Miazato Iwamura, Oliveira, Soares-Vieira, Nascimento, & Muñoz, 2005). However, mechanical methods cannot be used to process multiple samples simultaneously. Additionally, mechanical methods cannot be used to process bone samples that are porous or fragile (Schwark et al., 2011).

In a previous study, an enzymatic method using trypsin solution was adapted to clean bone samples prior to DNA isolation from fresh swine and human bone samples (Li, Chapman, Thompson, & Schwartz, 2009). It was demonstrated that this trypsin method can remove outer surface materials such as the mineralized bone connective tissue of fresh bone samples (Li, Chapman, Thompson, & Schwartz, 2009). A separate study revealed that the yield of DNA isolated from trypsin treated fresh human bone

samples was sufficient for forensic short tandem repeat (STR) analysis (Li & Liriano, 2011). In a subsequent study, the trypsin method was evaluated in samples that are more typically encountered in forensic cases such as buried human bones (over 50 years postmortem). Comparable values of DNA yields and Internal Positive Controls (monitoring the presence of PCR inhibitors) between the mechanical sanding and enzymatic trypsin method were observed. Additionally, the effects of the trypsin method on the quality of STR profiling were also studied. The percentage of the allele calls of STR profiles and the signal intensities of STR alleles were comparable between the two methods (Li & Klempner 2013).

In this study, the feasibility of using the enzymatic trypsin method for cleaning decomposed bones prior to DNA isolation was examined. Our results demonstrated that this method was effective for removing decomposed soft tissues attached to bone samples and the outer surface materials such as the mineralized bone connective tissue of bone fragment samples. Our data suggested that this method can be used in the initial sample preparation for cleaning the outer surface of decomposed non-human skeletal fragments. This study introduced a new method for processing decomposed non-human bone evidence prior to DNA isolation. Our method can be advantageous over conventional methods first because it is not labor-intensive for processing bone samples. Second, this potentially automatable method can be used to process multiple samples simultaneously to improve the throughput. Additionally, such a method may be used to process various samples of different sizes and conditions (i.e. porous surface or fragile) for the investigation of a wide variety of criminal cases involving animals.

FIGURES

SAMPLE PREPARATION OF SWINE BONES



Figure 1: Sample preparation of swine bones investigated in this study. Experiments were prepared by placing a piece of bone fragment (a fragment of swine scapula is shown), protected by a metal cage, outdoors for seven days.

ENZYMATIC TREATMENT OF BONE FRAGMENTS

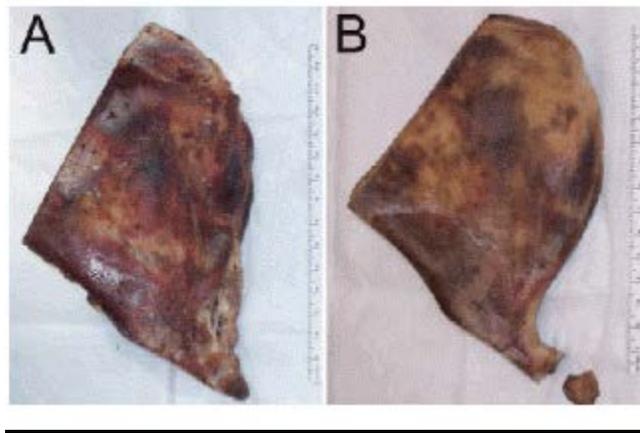


Figure 2: Enzymatic treatment of bone fragments. The enzymatic treatment was carried out by placing a piece of decomposed bone fragment in 500 ml of trypsin solution ($30 \mu\text{g}/\mu\text{L}$). The sample was then incubated overnight at 55°C . The trypsin-treated bone fragment was examined and photographed: A) Before, and B) after the trypsin treatment.

SCANNING ELECTRON MICROGRAPHS OF CONTROL AND TRYPSIN-TREATED SAMPLES.

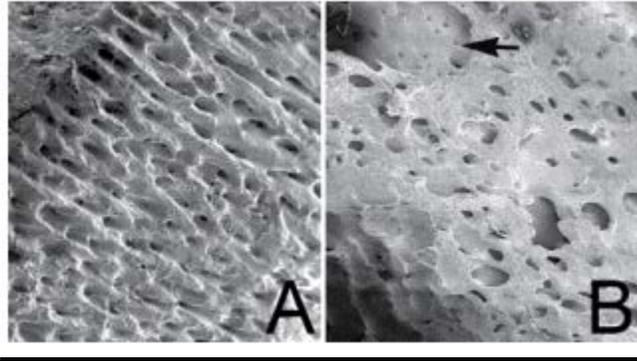


Figure 3: Scanning electron micrographs of control and trypsin-treated samples. Swine bone chips (Outer surface of cortical bones; 0.2g) were collected and examined using SEM: A) Untreated control sample. The control sample showed the outer surface of intact plexiform bone tissue, and B) the trypsin ($30\mu\text{g}/\mu\text{L}$) treated sample showed that the exposure of the vascular spaces of plexus (arrow), due to the removal of the surface layer of the bone sample, was observed. Field width: 18mm.

**RESULTS FROM THE AGILENT BIOANALYZER 2100 SHOWING
ELECTROPHEROGRAMS WITH THE MTDNA CYTB AMPLICONS**

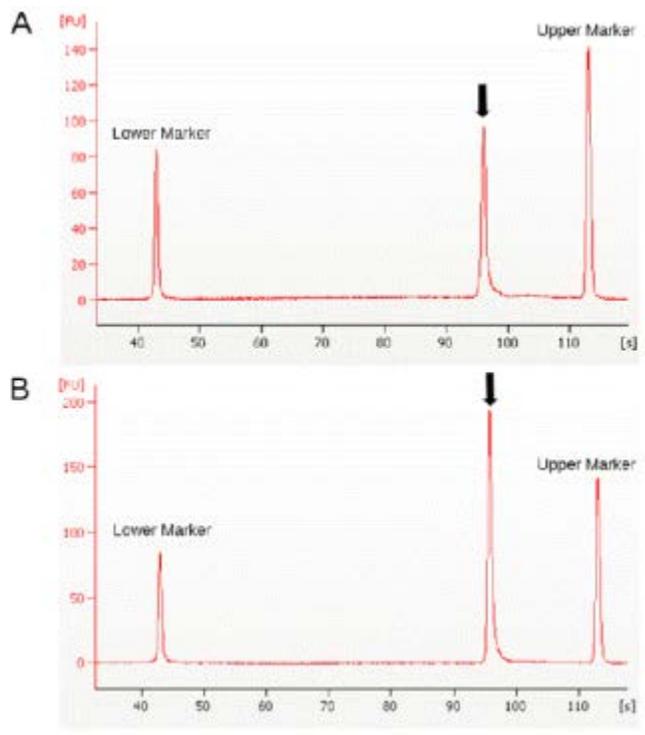


Figure 4: Results from the Agilent Bioanalyzer 2100 showing electropherograms with the mtDNA Cytb amplicons (arrows). The x-axis on the electropherogram represents the migration time of the amplicon and the y-axis represents the fluorescence intensity of the amplicon. RU: relative fluorescence unit. S: second. Lower marker (15bp) and upper marker (1500 bp) are the internal size standards. A) Untreated control sample and B) the trypsin (30 μ g/ μ l) treated sample.

**CONFIRMATORY DNA ANALYSIS USING DIRECT SEQUENCING OF
AMPLIFIED FRAGMENT AT CYTB LOCUS.**

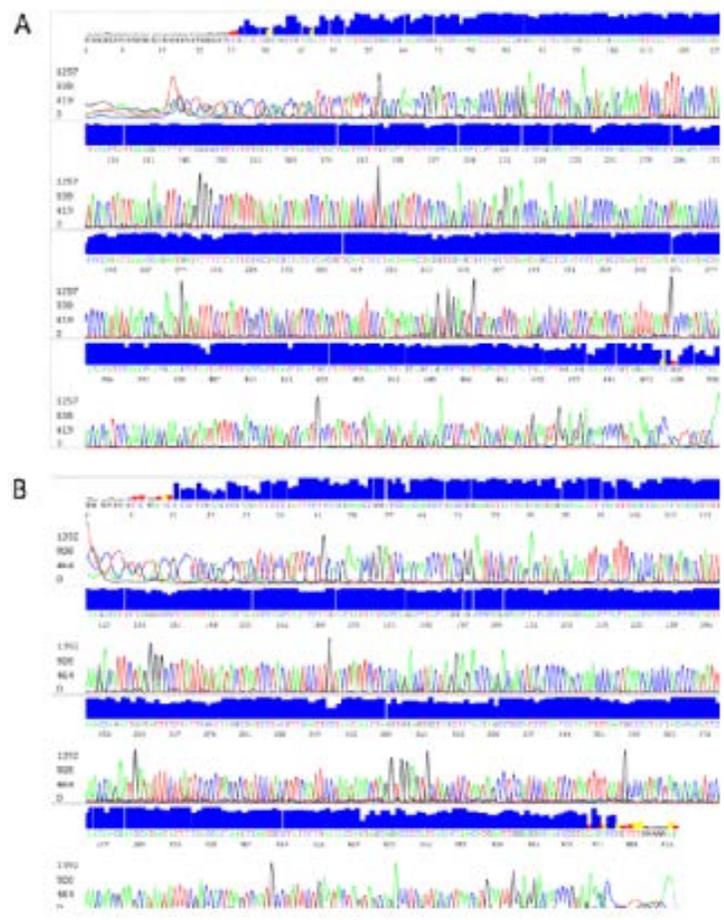


Figure 5: Confirmatory DNA analysis using direct sequencing of amplified fragment at Cytb locus. The electropherograms of A) the untreated control sample and B) the trypsin (30 μg/μl) treated sample.

CHAPTER 2: IDENTIFICATION OF RED SNAPPER SPECIES
BY SEQUENCING OF THE COI GENE

INTRODUCTION

The counterfeiting of food products

The practice of counterfeiting food products affects consumer trust and involves making a profit by consumer deception. Consumers typically take labeling of food at face value. Unfortunately, what is on the label is not always completely accurate. Mislabeling can occur out of ignorance or malicious intent. When fisheries identify fish they commonly use color, fins, size, and other meristic characteristics to identify the fish, but one cannot employ this method of identification if the organism is already in filet form due to the process removing distinguishing marks. The filet form is usually what the consumer observes. Therefore, a consumer is by far less likely to be able to distinguish what is written on the label versus what the item is. The fisheries which purposely mislabel an item tend to do so because they want the industry to believe that a stock is not depleted, or they wish to make a profit by selling a cheaper product as a more desirable or expensive one.

Under the Food, Drug, and Cosmetic act of 1938, the Food and Drug Administration (FDA) has attempted to address mislabeling in varying markets. In 2010, a report was written to congress to combat fraud and deception in the seafood market. The report identified incidents of fraud and examined policy issues within the seafood market and how the market itself was being monitored (Congressional Research Service, 2010). The report identified that misidentification, whether on purpose or otherwise, can occur anywhere in the consumer chain. The fishermen may associate a certain region where they made the catch with a specific fish and mistakenly identify the wrong species. The manufacturer can actively mislabel a fish to receive higher payments. The fishermen

often feel compelled to meet the demand, thus selling cheaper fish that are labeled as more expensive; the everlasting bait and switch tactic. A fish market may mislabel a fish to meet the demand of its consumers, or perhaps a restaurant may knowingly misrepresent items to their patrons by substituting cheaper fish for higher priced ones (Congressional Research Service, 2010). This behavior causes honest food service entities to be financially impacted as competitors will profit by the bait and switch tactic. Thus, the food service entity loses business to the deceitful competitor (Congressional Research Service, 2010).

Instances of counterfeiting

The FDA once intercepted 550 kilograms of fish labeled as Red Snapper from Canada, which proved to be rockfish. In Wong and Hanner (2008) two samples labeled as “Red Snapper” were in fact “Acadian Redfish” where according to US fisheries Red Snapper in 2006 was valued at \$2.93/lb. versus \$0.72/lb. for Redfish (National Marine Fisheries Service, 2011). Jacquet and Pauly (2007) postulated that up to 80% of Red Snapper sold is mislabeled. On the East coast alone, it was found that approximately 77% of fish products labeled as Red Snapper were actually different species. In Wong and Hanner (2008) seven of nine samples from varying New York City markets were identified as not being *Lutjanus campechanus*, despite being labeled as “Red Snapper”. Worst still, the same species was not consistently used as a substitution for the Red Snapper; of the seven mislabeled specimens each belonged to five different species, each from a different genus. The findings confirm inferences made by Marko et al. (2004) that approximately three fourths of all “Red Snappers” sold in the US are in fact other species.

The indication is that widespread overfishing has fully exploited, over-exploited or depleted up to 75% of global fish stocks (Food and Agriculture Organization of the United Nations, 2008) and has deleterious effects on aquatic ecosystems (Pauly, Watson, & Alder, 2005; Worm, Barbier, & Beaumont, 2006). Fisheries could potentially lose profits if consumers gained information that those fisheries admittedly tap into fish stocks which are depleting. Red Snapper has been in a steady decline, but it is still widely sold in markets and restaurants. Unsurprisingly, this fish is commonly substituted because the supply simply cannot reach the demand. Also, fisheries may be afraid that conscientious consumers will begin to take notice that the stock is depleting and purchase less Red Snapper in a personal attempt to conserve the population (“The label”, 1992). Another implication, which comes from mislabeling, is that substitutions made after landing data is collected cause an overestimation of the most desirable fish and an underestimate of the less desirable fish (Jacquet & Pauly, 2007). Mislabeling directly undermines import/export seafood regulations and its documentation.

The Snapper fish species

Fish have scientific names, as well as, colloquial names that are used in the fishing community. Many fish species are commonly referred to as “Red Snapper” however the only species which is legally referred to as the “Red Snapper” is *Lutjanus campechanus*. *L.campechanus* commonly found in the Southern Atlantic and Gulf of Mexico. *L.campechanus* is also known by the following English language common names: northern Red Snapper, Sow Snapper, Rat Snapper, Mule Snapper, Chicken Snapper, Gulf Red Snapper, American Red Snapper, Caribbean Red Snapper, Pensacola

Red Snapper, Mexican Red Snapper, Red Snapper, Mutton Snapper, and bream (Bester, 2015). “This snapper has long pectoral fins and a truncate caudal fin. The first and second dorsal fins are continuous with a slight notch in between the two and the anal fin tapers to a point posteriorly. The pectoral fins are long and reach the anus when pressed against the body. They have a large head with small red eyes and a somewhat pointed snout” (Bester, 2015). Their schools are commonly found close to the ocean floor on rocky outcrops, ledges, artificial reefs and oil drilling platforms, usually at depths between 30-200 feet.

The Red Snapper is a highly desirable, expensive fish found commonly on restaurant menus. Due to the economic importance of the *L. campechanus* fishery in the Gulf of Mexico, in 1996 the Gulf of Mexico Fishery Management Council and the United States Department of Commerce declared that *L. campechanus* was being obscenely overfished. Intense overfishing in the 1980s and 1990s led to a deeply depleted stock and led to older fish being observed less and less (Saari, Cowan, & Boswell, 2014). Strict management measures, to restore stocks to sustainable levels, were implemented. The restrictions that resulted appeared to conclude in an economic incentive for seafood substitution. Thus, the more expensive *L. campechanus* is substituted by the less valuable ones, which are typically closely related fish making them more difficult to identify after the filleting process; common substitutes for *L. campechanus* are *Lutjanus purpureus*, *Lutjanus peru*, and *Lutjanus synagris*.

L. purpureus, a deep red fish with a rosy underside, silvery sheen, and red fins, commonly known as the Southern American Red Snapper, is found throughout most of the Caribbean Sea from Cuba southward to northeastern Brazil. *L. purpureus* is not as highly valued as the *L. campechanus* but has been interchanged frequently in fish

markets, especially in the New York City area. There is an ongoing controversy regarding the two fish species, some scientists believe that the two species are the same species that were separated by varying geographies. The fish are often morphologically indistinguishable; they share a red color pattern and some meristic characteristics. The primary article questioning whether the species are truly different “Can *Lutjanus purpureus* (South red snapper) be “legally” considered a red snapper (*Lutjanus campechanus*)?” (Gomes et al., 2008) utilized DNA sequences of the mitochondrial control region of *L. purpureus* and compared it with the same sequences submitted into Genbank for *L. campechanus*. Employing only the mitochondrial DNA and composing a phylogenetic tree with the data, the study was unable to differentiate the two Atlantic red Snapper species.

A current hypothesis suggests that the varying morphologies, mainly size, are due to the food indigenous to the native areas of *L. campechanus* and *L. purpureus*. Such instances of large morphological differences seen in the same species due to geographic variances have been observed in *L. campechanus*. In South Texas smaller, fast-growing individuals dominated and in Alabama and Louisiana slower-growing larger catches dominated (Saari, Cowan, & Boswell, 2014). The article suggests that the most plausible hypothesis is that the two snappers simply represent varying populations of a single species with a large geographical distribution.

L. peru, or the Pacific Red Snapper, is usually found from Southern California to the central Gulf of California to Peru, the Revillagigedos and Malpelo. *L. peru* is reddish-pink with a silvery sheen and an oval body, teeth which can be conical to caniniform, with the teeth in front of jaws appearing fang-like. *L. peru* possesses a spiny dorsal fin, a

truncated caudal fin, one anal fin, and spiny pectoral pelvic fins. ("Pacific Red Snapper - *Lutjanus peru* - Details - Encyclopedia of Life", 2018)

L. synagris, or the Lane Snapper, is found in the western Atlantic Ocean, from North Carolina to southern Brazil, with occasional sightings in Bermuda and the Gulf of Mexico. The Lane Snapper is almond shaped, pink-red on top, with yellow to red fins, and silver bodies with pink to yellow lines. It has a spiny double dorsal fin, with a rounded anal fin and short pectoral fins. The caudal fin is emarginate to slightly truncated ("Lutjanus synagris-Florida Museum of Natural History", 2018). The restrictions on the Gulf Red Snapper have increased, thus amplifying the likelihood that the Lane Snapper will be utilized to fraudulently deceive consumers (Karlsson, Saillant, & Gold, 2009).

As illustrated above it is plausible for an experienced fisherman to differentiate *L. synagris*, *L. peru*, and *L. campechanus* by utilizing morphological means while the fish are whole, allowing for observance of their determining structures. On the contrary, when the fish are processed and filleted it is nearly impossible to differentiate the Snapper species; even more so for *L. purpureus* and *L. campechanus* who are often indistinguishable in nature, making it imperative to distinguish the two utilizing DNA.

Authenticating fish products using DNA

The analysis of DNA has introduced the ability to use DNA-based methods of authentication on animal meat products (Wong & Hanner, 2008). DNA barcoding is based on the designation of a mitochondrial DNA fragment of cytochrome c oxidase I (COI) gene or cytochrome b (Cyt b) to act as a “barcode” to identify the organism. The DNA barcode is then compared to ever-growing libraries of known organisms for assistance in identification. In 2005, due to the socio-economic importance of fish and their proper identification, over 5000 species were barcoded to be utilized as reference materials for future experimentation (Wong & Hanner, 2008). In testing of reference materials, results indicated that the short fragment of COI used in barcoding contains enough variation to speciate a large variety of animals (Waugh, 2007). Other sources of DNA, such as 16S or 12S ribosomal DNA, were contemplated, but it was deduced that the aforementioned types of DNA are predominantly used to analyze the diversity and structure of bacterial communities and does not have a large database associated with organisms such as fish.

Barcoding has emerged as a source for food authentication and confirmation of food safety, as well as other aspects of fishery management that affects the public (Costa & Carvalho, 2007). In “Molecular barcoding reveals mislabeling of commercial fish products in Italy” a fragment of 300 bp of cytochrome b gene (Cyt b) and a COI gene fragment was amplified using PCR. The fragments were then separated and sequenced. Of the sixty-nine samples, twenty-two, or 32% did not match the declared species and of the twenty-two mislabeled specimens 26% were serious variances and accounted for a large economic fraud.

The efficacy of barcoding has been compared with the utilization of rDNA 5S banding patterns “where non-transcribed spacers (NTSs) with variations within them should lead to differences in the fragment sizes that are amplified, resulting in identification of species” (Veneza, et al., 2014). Distinct banding patterns are seen in organisms such as cephalopods (Bráullio de Luna Sales, Fernando da Silva Rodrigues-Filho, Haimovici, Sampaio & Schneider, 2011) , salmonids (Pendas, Moran, Martinez, & Garcia-Vazquez, 1995), and sharks (Pinhal, Araki, Gadig, & Martins, 2009).

Continuing with the success of barcoding on other species of fish, it is anticipated that barcoding could differentiate other species of the genus *Lutjanus* from the Red Snapper, particularly by utilizing the COI gene. The objective is to find a single nucleotide polymorphism (SNP) of the COI sequence that will differentiate *L. campechanus* from *L. peru* and *L. synagris*, allowing for refined investigations into illegal seafood mislabeling.

MATERIALS AND METHODS

Processing of fish found in filet form from local retailers

Samples of fish filet labeled as Red Snapper were collected from multiple fish markets in New York and New Jersey. The fish were then cut into small thinly sliced increments. The extraction of DNA was completed utilizing eighteen tissue samples.

DNA Extraction and Quantitation

DNA from tissue was extracted using the QIAmp DNA Micro Kit as per manufacturer's protocol.

DNA quantitation was performed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE) according to the manufacturer's protocols (Li, Gaud, & Nair, 2014)

Species Identification by Sequencing *Lutjanus* COI gene

The amplification of specific fragments of the mitochondrial COI gene was carried out. A 2 μ L aliquot of DNA was used with 23 μ L of primers (05 μ M) in NEB Taq Master Mix. The forward primer was vF2_t1 (5'-CAA CCA ACC ACA AAG ACA TTG GCA C-3') and reverse primer FishR2_t1 (5'-ACT TCA GGG TGA CCG AAG AAT CAG AA-3') was used. The initial step for the PCR was at 94°C for one minute. For each cycle, the cycling parameters included a 15 seconds denaturation step at 94°C, a 15 seconds primer annealing step at 54 °C, and a 30 seconds extension step at 72°C. The PCR was performed for a total of 35 cycles. As a positive control, amplification with 0.5 ng of genomic DNA of known *Lutjanus* COI gene DNA sequence was carried out. To

monitor contamination, PCR negative controls were included with each amplification experiment.

The cycle sequencing products were separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and were analyzed with the Sequencher software (Gene Codes, Ann Arbor, MI). The DNA sequence obtained was compared with the *BLAST* database (Li, Gaud, & Nair, 2014).

RESULTS AND DISCUSSION

Upon analysis of variances in the COI gene amongst the three species in question of the *Lutjanus* genus, it was observed, that at position 359, *L. campechanus*, *L. peru*, and *L. synagris* each exhibited one single nucleotide polymorphism, that could be utilized in differentiating the three in future instances (Figures 2-1 and 2-2). At position 359, the nucleotides for each organism is as follows: adenine for *L. synagris*, thymine for *L. peru*, and cytosine for *L. campechanus*. The SNP could be a defining feature in other species of the *Lutjanus* genus, thus serving as a mode of identification, amongst the interrelated species.

The samples were accurately identified using the single nucleotide polymorphism and database searches; of the eighteen samples only two samples were genetically confirmed to be the species they were marketed as. The North American Red Snapper is commercially more expensive than the Pacific Red Snapper (*L. peru*) and Lane Snapper (*L. synagris*). Morphologically, the consumer would not be able to decipher the identity of the fish, especially if in filet form, but, genetically, the fish can be differentiated with a single nucleotide polymorphism at position 359. The distinguishing SNP could serve as a screening process to determine offenders with a pattern of substitution; a means of confirmation of identity would allow a sense of confidence in each supplier's stock.

Table 1 exhibits samples of fish which were marketed as "Red Snapper" across varying fish retailers. Of the eighteen samples, only two samples were *L. campechanus*. The species most often substituted for *L. campechanus* was *L. peru*, the Pacific Red Snapper, and *L. synagris*, the Lane Snapper. The alignment length served as the source of comparison between the samples and the database used to identify the fish species. The

BIT score is a normalized score that expresses the magnitude of the search space one would look through before it is expected to find a better match than the one found solely by chance. Therefore, the larger the BIT number the larger the area that needs to be searched to find a random match. The E-value serves to describe how likely a random “match” would be seen in a database of a specific size. The E-value decreases as the score of the match increases; therefore, the closer the E-value is to zero, the more notable the match is.

The phylogenetic tree, Figure 2-3, displays comparisons of *L. synagris*, *L. peru*, and *L. campechanus*. The tree exhibits the correlation and evolutionary trends hypothesized for the *Lutjanus* species compared in the study. The DNA Subway software was used to obtain the phylogenetic analysis, using PHYLIP NJ. A phylogenetic tree is a graph that represents an inference about the evolutionary history of different organisms. The tree branches out from oldest (on the left) to most recently seen species (on the right). Each length of the branch is proportional to the number of changes that have taken place since a divergence from a common ancestor. The nodes indicate the point of divergence for the individual species. Any sequence change beyond a node are specific for each branch, or species. The neighbors or branches next to each other are determined by the amount of changes in sequence, equating relative distances between the species; thus, the closer the sequence, the closer the species, the closer the neighboring branch (Orr, 2007).

The evolutionary tree comparing *L. campechanus*, *L. peru*, and *L. synagris* exhibits that *L. campechanus* and *L. peru* diverged from one common ancestor, who itself diverged from the same common ancestor as *L. synagris*. The three fish species exhibit

similar physical characteristics such as color, dorsal, and anal fins, lateral line scales, and gill rakers with some morphological variances as expected by the evolutionary divergence (Figure 2-4). *L. campechanus* and *L. peru* diverged from the same ancestor resulting in the morphological similarities seen between the two today. *L. synagris* varies morphologically to the other two species because it diverged from an ancestor further up the evolutionary time line. The barcoding of the COI region of each species proved to differentiate one from the other despite physical similarities.

On a worldwide spectrum, the barcoding of the COI region has been used to identify species of a fish, emphasize their similarities, identify common ancestors amongst fish, and ultimately elucidate misidentifications. Markets in Italy have identified substitutions by sequencing the mitochondrial genes COI and CYTB regions (Cutarelli, et al. 2014; Filonzi, Chiesa, Vaghi, & Nonnis Marzano, 2010). The Italian markets were experiencing instances of misidentification of species occurring with transformed foodstuff that were processed into filet, slice form, or other methods that may destroy or damage morphological characteristics. “COI standard barcode region (around 655bp) is indeed relatively conserved within species, but at the same time shows sufficient variation to allow differentiation between species” (Cutarelli, et al., 2014). In Cutarelli et al. the Italian researchers conducted testing on fifty-eight samples, all of which were identified utilizing the COI region and fifty-six out of fifty-eight samples identified using the CYTB region. The Filonzi et al. (2010) researchers were able to directly sequence and amplify sixty-nine out of seventy-two samples utilizing the COI and CYTB genes. Of the sixty-nine amplified samples twenty-two did not match the previously declared fish species.

The successes of the researchers alluded to above and our own research amplifies the possibility of DNA barcoding serving as a screening process to correctly identify morphologically ambiguous species or samples which have been modified or destroyed in any manner. Our research specifically exhibited its capability in differentiating specific species within the *Lutjanus* genus. The research could be utilized to fine tune a reliable method of screening applied to more general fish species specimens that leave the warehouses and other suppliers, thus identifying those who are unknowingly or maliciously deceiving their consumers. In addition, further research into other genes and methodologies should be conducted to establish a method of differentiation between the still indistinguishable *L. campechanus* and *L. purpureus*, to determine if the two species deviate from each other at all. Other methods that are used to authenticate meat products that should be explored to speciate the two as identified by Lockley and Bardsley (2000) and Kumar, Singh, Karabasanavar, Singh and Umapathi (2012) are DNA hybridization, species- specific polymerase chain reaction (PCR) primers, restriction fragment length polymorphism (RFLP) analysis, single-strand conformational polymorphism (SSCP) analysis, random amplified polymorphic DNA (RAPD) analysis, PCR product sequencing, and other methods that may exploit nuclear DNA versus mitochondrial DNA. If a differentiation between the two Snapper species is established, the method could be applied to other Snapper species. Nevertheless, the research should be expanded to include development of a rapid screening process for real time use on the fish market.

FIGURES

Sequence Analysis of the species closely related to *Lutjanus campechanus*.

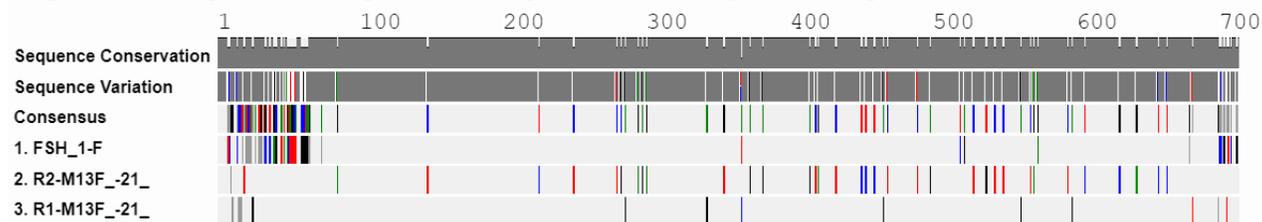


Figure 2-1 The DNA Subway software was used to obtain the analysis.

Sequence analysis of the COI region of *Lutjanus* species *Lutjanus peru*, *Lutjanus synagris*, and *Lutjanus campechanus* respectively.

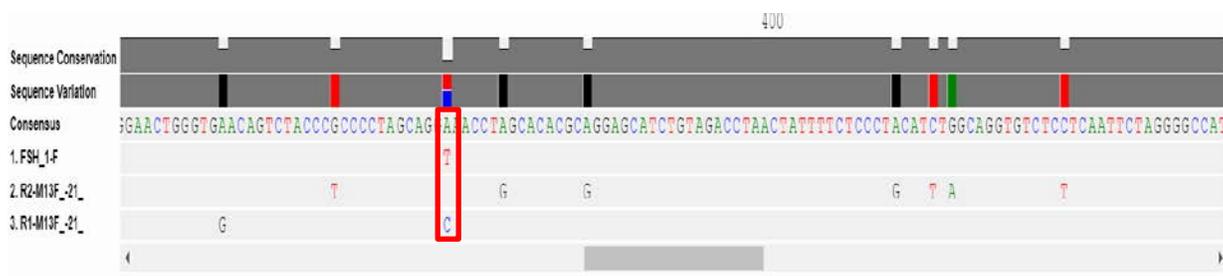


Figure 2-2 exhibits that at position 359 there is one single nucleotide polymorphism (SNP) that differs amongst all three species. At position 359, the nucleotides for the samples are adenine for *Lutjanus synagris* (R2-M13F_-21_), thymine for *Lutjanus peru* (FSH_1-F), and cytosine for *Lutjanus campechanus* (R1-M13F-21). The samples R2-M13F_-21_, FSH_1-F, and R1-M13F-21 were chosen to directly analyze arbitrarily, they exemplified the most common sequences of the *Lutjanus synagris*, *Lutjanus peru*, and *Lutjanus campechanus*.

TABLE 1: IDENTIFICATION OF SAMPLES COLLECTED

GenBank® Identification	Sample Analyzed	Alignment Length (bp)	Bit Score	E-value
<i>L. campechanus</i> (Red Snapper)	2	696-712	1157-1201	0.0
<i>L. peru</i> (Pacific Red Snapper)	12	695-742	1125-1234	0.0
<i>Lutjanus synagris</i> (Lane Snapper)	4	688-695	610-1206	2e-1.70-0.0

Figure 2-3: Neighbor Joining Phylogenetic tree of *Lutjanus* species

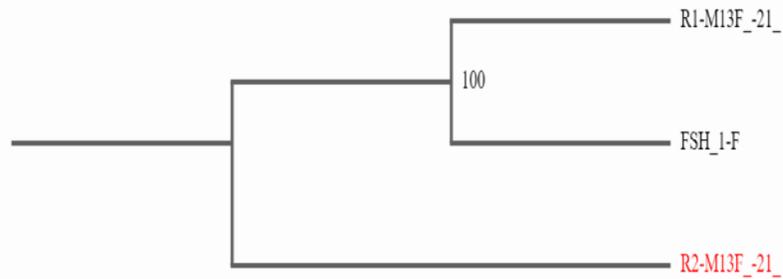


Figure 2-3: Neighbor Joining Phylogenetic tree of *Lutjanus* species. An evolutionary tree comparing *Lutjanus campechanus* (R1-M13F_-21_), *Lutjanus peru* (FSH_1-F), and *Lutjanus synagris* (R2-M13F_-21_). *Lutjanus campechanus* and *Lutjanus peru* diverged from one common ancestor, who itself diverged from the same common ancestor as *Lutjanus synagris*. All three species arose at approximately the same time and still inhabit the Earth.

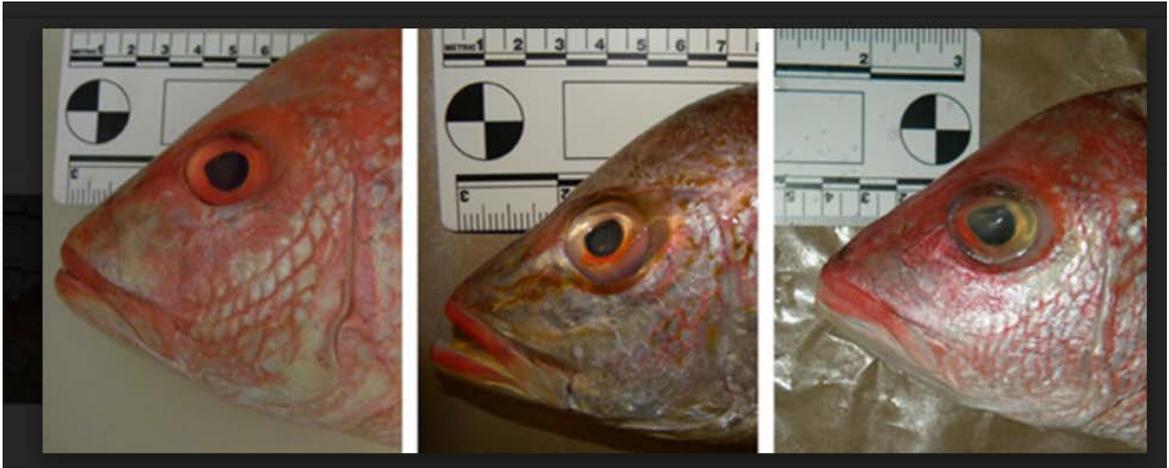
FIGURE 2-4: PHOTOGRAPHS OF FISH SAMPLES

FIGURE 2-4: Photographs of fish samples from left to right: *L. campechanus*, *L. synagris*, and *L. peru*. The figure displays the facial morphology of the three species, each exhibit angular faces with red tinted scales and a slight silver sheen.

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