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Detection of Mislabeled Commercial Fishery By-Products in the Philippines using DNA Barcodes and its Implications to Food Traceability and Safety

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ABSTRACT

Global trade negotiations require a stringent line of certifications on accurate labeling and species traceability. National trade policies should therefore, comply with these requirements, not only to increase international competitiveness, but also to ensure food security, sustainability and safety. However, this is difficult to achieve without a strong basis for monitoring strategies and enforcement. In this study, issues on the identities of several species of sardines, pangasius, fish sold as fillets and choice cuts and shrimps were shown using DNA barcodes. Indications of mislabeling were found in frozen “tawilis” samples and “bluefin” tuna fillets. Some products have been identified at the species level. Finally, fish labeled as gindara steaks have been found to be a different fish which can cause human health problems. These results highlight the importance of increasing national concern and government effort in food traceability and that DNA barcoding provides a robust method of assessment for species identification and authenticity testing of commercial fishery products.

Keywords: DNA Barcoding; fish products; Philippines; fillet; market survey; food traceability

1. Introduction

Considering the importance of fish trade in the globalization era, technological developments in food production, handling, processing and distribution by a global network of operators make it necessary to ensure the authenticity and the origin of fish and seafood products (Filonzi et al., 2010; Marko et al., 2004). Because species substitution of fish occurs frequently, particularly for imported products which are not recognizable visually and are indistinguishable on the morphological basis after processing and freezing (Filonzi et al., 2010), precautionary measures are therefore, necessary. Certain issues that may arise from this are health problems that occur primarily through consumption of cryptic species from contaminated areas (van Leeuwen et al., 2009). Because of this, Global Trade Operations require a stringent line of certifications with regards to fish labels and other related aspects. For instance, the European Union law EC No. 2065/2001 requests appropriate species traceability and accurate labeling. In the Philippines, RA no. 7394, known as the Consumer Act of the Philippines, mandates that all products be properly labeled as to its accurate nature, quality and quantity. However, it is often difficult to comply and because of this, many monitoring agencies are looking for innovatives and safe technologies to assess species identification and authenticity testing (Dawnay et al., 2007; Maldini et al., 2006).

DNA barcoding is a rapidly emerging global initiative which involves characterizing species using a short arbitrary DNA sequence. This is based on the premise that species are generally well delineated by a particular sequence or by a tight cluster of very similar sequences that allow unambiguous identifications (Hebert et al., 2003). The primary goals of DNA barcoding focus on the assembly of reference libraries of barcode sequences for known species in order to develop reliable, molecular tools for species identification in nature

(Hubert et al., 2008). The cytochrome c oxidase subunit 1 mitochondrial region (COI) is the most popular barcode for animals and a lot of studies have established the usefulness of barcoding in several large groups of animals, such as birds (Hebert et al., 2004a), fish (Collins et al., 2012; Ward et al., 2005; Hubert et. al., 2008), cowries (Meyer and Paulay, 2005), spiders (Barrett and Hebert, 2005), and Lepidoptera (Hajibabaei et al., 2006).

Numerous straightforward benefits from the use of a standardized molecular approach for identification have been shown (Hebert et al., 2004a; Ward et al., 2005; Hubert et al., 2008; Meyer and Paulay, 2005; Barrett and Hebert, 2005; Hajibabaei, et. al., 2006). In recent years, molecular barcoding has been the favorite methodology in forensic taxonomy (Dawnay et al., 2007) because DNA barcodes are almost always effective whatever the condition of the samples under scrutiny is. Barcoding is particularly useful in taxonomic studies where intra-specific phenotypic variation often overlaps that of sister taxa which can lead to incorrect identifications if based on phenotype only (Pfenninger et al., 2006). Likewise, cryptic variation and often high levels of undetected taxonomic diversity have been frequently reported (Hebert et al., 2004b).

In this study, we highlight the importance of awareness for monitoring programs and strategies on a local and national scale for proper food labeling, and for adopting molecular techniques as tools to do this. Thus, the study aims to use DNA barcoding in identifying the source of labeled fishery products in the Philippines like fillets and choice cuts. It is also the goal of the study to assess label accuracy using a direct sequencing method of the mitochondrial gene cytochrome oxidase subunit I (CO1). Specifically, the study aims to determine if frozen "*tawilis*" products are actually *Sardinella tawilis*, assess label accuracy of Blue fin tuna fillets, and identify fish and shrimp in several fish fillets/choice cuts and whole specimens up to the species level.

2. Methodology

2.1 Sample Collection

Samples of fresh and frozen fish fillets / choice cuts were obtained from several supermarkets in Quezon City and Manila or wet markets from Cebu City and General Santos City, Philippines as indicated in Table 1. The name of the store cannot be disclosed in this study. As a reference for phylogenetic analyses, shrimp samples from a wet market in Manila, a shrimp farm in Batangas and a lake in Leyte were collected, morphologically identified and authenticated (Location details were used and indicated in Figure 5). Specimens obtained from the field were chilled on ice until reaching the laboratory for tissue sampling. Samples bought from stores, whether frozen or fresh, were also chilled on ice until reaching the laboratory for tissue sampling. A small amount of muscle tissue (about 150 mg) was kept in absolute ethanol and stored at -20 °C until DNA extraction. A detailed description of analyzed specimens is presented in Table 1.

2.2 DNA Extraction

Ethanol preserved tissues were rinsed with de-ionized H_2O. The tissues were then minced and placed in a properly marked 1.5 mL Eppendorf tubes containing Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction buffer (1.9% CTAB pH 8.5, 0.4% Proteinase K) . DNA extraction was conducted using modified CTAB method (Santos et al., 2010).

2.3 PCR Amplification

A 25 uL reaction mixture was prepared containing water, 1x PCR Buffer, 0.2 mM dNTP’s, 0.8 uM each of Forward primer LCO1490 and Reverse primer HCO2198 for CO1 amplification (Folmer et al., 1994): 2 mM $MgCl_2$, 1 unit Taq polymerase (Kapa Biosystems, USA) and approximately 0.5 to 1 µg of DNA template. They were run on a thermal cycler with the following PCR cycling parameters: 94 °C initial denaturation for 1 min followed by 5 cycles of 94 °C for 1 minute, 45 °C for 1 minute and 30 seconds, 72 °C for 1 minute and 30 seconds; another 35 cycles of 94 °C 1 minute, 50 °C for 1 minute and 30 seconds, 72 °C for 1 minute; and a final extension of 72 °C for 5 minutes. After the reaction, amplicons were run in 1 % agarose gel stained with Ethidium Bromide and submerged in TAE buffer. No cloning was done before sequencing. Amplicons were sent to Macrogen, Inc., Korea (www.macrogen.com) for purification and bi-directional sequencing using Big Dye Terminator method. PCR amplification forward and reverse primers were used as sequencing primers.

2.4 Genetic Analysis

Representative CO1 sequences were obtained from Genbank for comparison except for CO1 sequences of *Sardinella fimbriata*, which were obtained and sequenced by this study. If the sequences were obtained from an existing database, a label, either GENBANK (http://www.ncbi.nlm.nih.gov/genbank/) or BOLD (www.barcodinglife.com), was indicated before each sequence name and accession number in the phylogenetic trees. In the case of sequences determined by this study, a consensus was generated by aligning the obtained sequences using forward and reverse sequencing primers. If the sequences are in disagreement at a site, the signal from the chromatogram with a higher quality was used in the consensus. All DNA sequences were edited and aligned using alignment explorer packaged in MEGA version 5.0 (Tamura et al. 2011) using ClustalW default parameters. Species classification was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions considered were frames 1, 2, and 3 including the non-coding region. All positions containing gaps and missing data were eliminated. Analyses were conducted in MEGA5 (Tamura et al., 2011).

3. Results and Discussion

Table 1 lists all the samples under consideration for barcoding and monitoring. Genetic Analysis was done separately for 6 groups namely 1) sardines, 2) blue fin tuna, 3) sashimi tuna, 4) cream dory, 5) shrimp and 6) gindara steak. It is important to note that the Neighbor Joining method used in this study requires strict monophyly of each species, which may result in a situation where the inclusion of a single misidentified specimen renders all queries in that species as misidentifications (Collins et al, 2012). The separation in the analysis of these specific groups was necessary in order strengthen the results and avoid taxonomic complexities caused by intra-specific variations (especially for the tuna and sardine analyses) that lie far beyond the bounds of this study.

Figure 1 shows the Neighbor Joining Tree of CO1 sequences from frozen t*awilis* samples using Kimura 2-parameter model. Here, the GenBank sequence of *Escualosa thorocata* was used as outgroup. The analysis involved 32 nucleotide sequences. There were a total of 545 positions in the final dataset where 172 are parsimony informative. The mean genetic distance (Kimura 2-parameter model) within the group is 0.143. All frozen “tawilis” samples grouped into one clade with morphologically identified and authenticated *Sardinella fimbriata* representative specimen (this study). Together, they form a much larger OTU at

100% bootstrap with *Sardinella melanura* GenBank sequence. They did not group with the known *S. tawilis* GenBank sequences. These clearly indicate that the "tawilis" samples are not *S. tawilis* but are actually *S. fimbriata*. To confirm, we did additional analysis using BOLD (Barcode of Life Database) Animal Identification and identified that the frozen "*tawilis*" had a 100% maximum identity with *S. fimbriata* samples. These data strongly suggest that the "*tawilis*" samples being sold in major supermarkets in Quezon City, Philippines are not *S. tawilis*. This is a clear case of mislabeling of the fishery product. It is possible that this is being done since *S. tawilis* commands a high price in the market being the only freshwater sardine in the world. A number of implications can be deduced from such practice including consumer fraud and wrong information on the real stock status of *S. tawilis*.

Figure 2 is the Neighbor Joining Tree of CO1 sequences from *Bluefin Tuna Fillet* samples using Kimura 2-parameter model. This time, *Euthynnus affinis* GenBank sequence was used as outgroup. The analysis involved 27 nucleotide sequences with 638 positions each in the final dataset, which contained 20 parsimony informative characters. The mean K2 genetic distance within the group is 0.015. It has been shown that all *bluefin tuna fillet* samples are grouped in one clade with *Thunnus tonggol* (longtail tuna) at 82% bootstrap support. Animal identification using CO1 sequence through BOLD systems also revealed *T. tonggol* as a nearest match for the *BF CO1* sequences. Based on the results from BLAST, BOLD identification and Neighbor Joining, this clearly indicate mislabeling of the *BF* samples because they are labeled as blue fin tuna fillet instead of longtail tuna fillet. Similar to *S. tawilis,* this is another possible case of mislabeling *T. tonggol* with the bluefin tuna since the latter commands a very high price in the market. This again has important implications to consumer welfare and conservation of the bluefin tuna species, which now considered to be highly threatened and has been already proposed to be included in the CITES Appendix.

Figure 3 is the Neighbor Joining Tree of CO1 sequences from sashimi tuna fillet samples where *Euthynnus affinis* was assigned as outgroup. The analysis involved 16 nucleotide sequences with 639 positions each in the final dataset, which contained 4 parsimony informative characters. The mean K2 genetic distance within the group is 0.019. Results showed that the sashimi tuna sample belongs to yellowfin tuna, *Thunnus albacares* species. The sashimi tuna (*C0012*) sample was clearly identified as *T. albacares* because it forms one operational taxonomic unit (OTU), separate from other *Thunnus* spp. Moreover, based on BLAST and BOLD identification, the sashimi tuna sample, C0012 is 94% homologous with *T. albacares* sequences in GenBank. In this context, no issue of mislabeling is present as all *Thunnus* species are being used sashimi fillets. Furthermore, this sashimi sample was clearly identified as coming from yellowfin tuna, *T. albacares*.

Analysis of cream dory in Figure 4 used 6 nucleotide sequences with 607 positions in the final dataset. This set, where 74 positions were parsimony informative characters, has a mean K2 genetic distance of 0.102. C0008 Cream dory was found to group with *Pangasionodon hypophthalmus* with 100% bootstrap support suggesting that it is *P. hypophthalamus*, a species of iridescent shark catfish originating from the Mekong River in Vietnam. Thus, cream dory sample (*C008)* has been correctly labeled based on Neighbor joining in accordance with BLAST and BOLD matches.

Figure 5 shows the genetic analysis of frozen shrimp samples with the Green porcelain crab (*Petrolisthes armatus)* as outgroup. In the analysis, a total of 9 nucleotide sequences were used and a total of 557 positions where included in the final dataset with 16 parsimony informative characters. Overall genetic distance is 0.185. All GenBank sequences formed highly supported clades with their corresponding morphologically identified counterparts. The shrimp samples S7 and S10; and SSL13 were correctly labeled as tiger prawn *Penaeus monodon*, and Pacific white shrimp *P. vannamei*, respectively. Interestingly, however, the 2 unknown samples U1 and U3 grouped with the *Metapenaeus* sp. at 89% bootstrap values. The frozen shrimp samples formed a highly supported clade by morphological and genetic analysis. The U1 and U3 samples are likely to be *Metapenaeus* sp. but species identification warrants further studies. A more detailed phylogenetic analysis may be needed. However, in the context of this study, this again indicates an issue in correct labeling this time with shrimp products.

C0002 Gindara Steak sample formed a single clade with 100% bootstrap support to *Lepidocybium flavobrunneum* (Escolar) as seen in Figure 6 suggesting that this sample is *L. flavobrunneum* or oilfish. In this set, other species in related families were used as outgroups. The analysis involved 7 nucleotide sequences with 597 positions each in the final dataset, where 132 are parsimony informative. The sequences have an overall K2 distance of 0.200. According to this result including BLAST search and BOLD matching, *C0002* sample labeled as Gindara Steak comes from a fish, *Lepidocybium flavobrunneum*. In the market, gindara fish commonly refers to sablefish or *Anoplopoma fimbria*. However, aside from this fish species, other alternatives such as *Lepidocybium flavobrunneum* (escolar) and *Ruvettus pretiosus* (oilfish) are being sold as gindara steak / fillet, either as a misidentification or a form of adulteration, because they have the same characteristic white meat. The main concern is that the latter two species can cause mild keriorrhea, a condition characterized by excretion of an orange to brown oil without causing loss of body fluid as in ordinary diarrhea (Berman et al., 1981), after consumption. This calls for a more detailed and accurate labeling of gindara steaks, whether they are from sablefish, escolar or oilfish.

Generally from an economical point of view, most cases of described mislabeling in this study were examples of species with a scarce or lower market value but are sold as other species more expensive and valuable (Filonzi et al., 2010). These cases may or may not describe serious commercial frauds because they may be a result of misguided identifications.

However, it is important that this issue be taken seriously by the government for consumer welfare. As in the case of gindara steaks, substituted or mislabeled fishes offered in markets, fisheries and restaurants may be potentially dangerous, due to the presence of unknown toxic or allergenic substances that are hurtful to consumers (Collins et al., 2012). We have seen the potential of DNA barcoding in the cases presented and would like to note that no matter how morphologically unidentifiable our fish product samples or food in general were, as seen especially in the shrimp samples, the species where they came from can easily be traced genetically as long as the DNA is preserved in the sample.

4. Conclusion

The results of the study reveal a high probability of incorrect species declaration in the "*tawilis" and "Bluefin Tuna fillet* products and insufficient labeling information for gindara steaks / fillets. Meanwhile, sashimi tuna fillets and cream dory products are correctly labeled based on CO1 barcoding and identification. The cases presented add more evidence urging for increased traceability of food products and the national assessment for authenticity of raw materials for commercial packaging and selling in the country as regulated by RA no. 7394, or the Consumer Act of the Philippines. The study further proves molecular investigations based on DNA barcoding to be one of the most powerful tools for the assessment of species identity, food traceability, safety and fraud. A valuable effort should then be placed to create a strong and standardized monitoring program or strategy, and finally, to evoke consumer awareness on several aspects of accurate labeling information.

5. Acknowledgement

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Tables and Figures

Table 1. Sample Distribution and Collection Sites

Sample Code	Product Label	Sampling Date / Location
TW01	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW02	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW03	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW04	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW05	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW06	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
TW07	Frozen Tawilis	7-11-2011 / Quezon City, Philippines
BF01	Blue Fin Fish Fillet	7-11-2011 / Quezon City, Philippines
BF02	Blue Fin Fish Fillet	7-11-2011 / Quezon City, Philippines
BF03	Blue Fin Fish Fillet	7-11-2011 / Quezon City, Philippines
BF04	Blue Fin Fish Fillet	7-11-2011 / Quezon City, Philippines
C0012	Sashimi Tuna Fillet	7-11-2011 / Quezon City, Philippines
C0008	Cream Dory Choice Cuts	7-11-2011 / Quezon City, Philippines
S7	Tiger Prawn	07-22-2010 / Quezon City, Philippines
S10	Tiger Prawn	07-23-2010 / Quezon City, Philippines
SSL13	Pacific White Shrimp	11-02-2010 / Cebu City, Philippines
U1	Frozen Headless Shrimp	01-05-2011 / Manila, Philippines
U3	Frozen Headless Shrimp	01-14-2011 / Manila, Philippines
C0002	Gindara Steak / Fish Fillet	11-03-2009 / General Santos City, Philippines

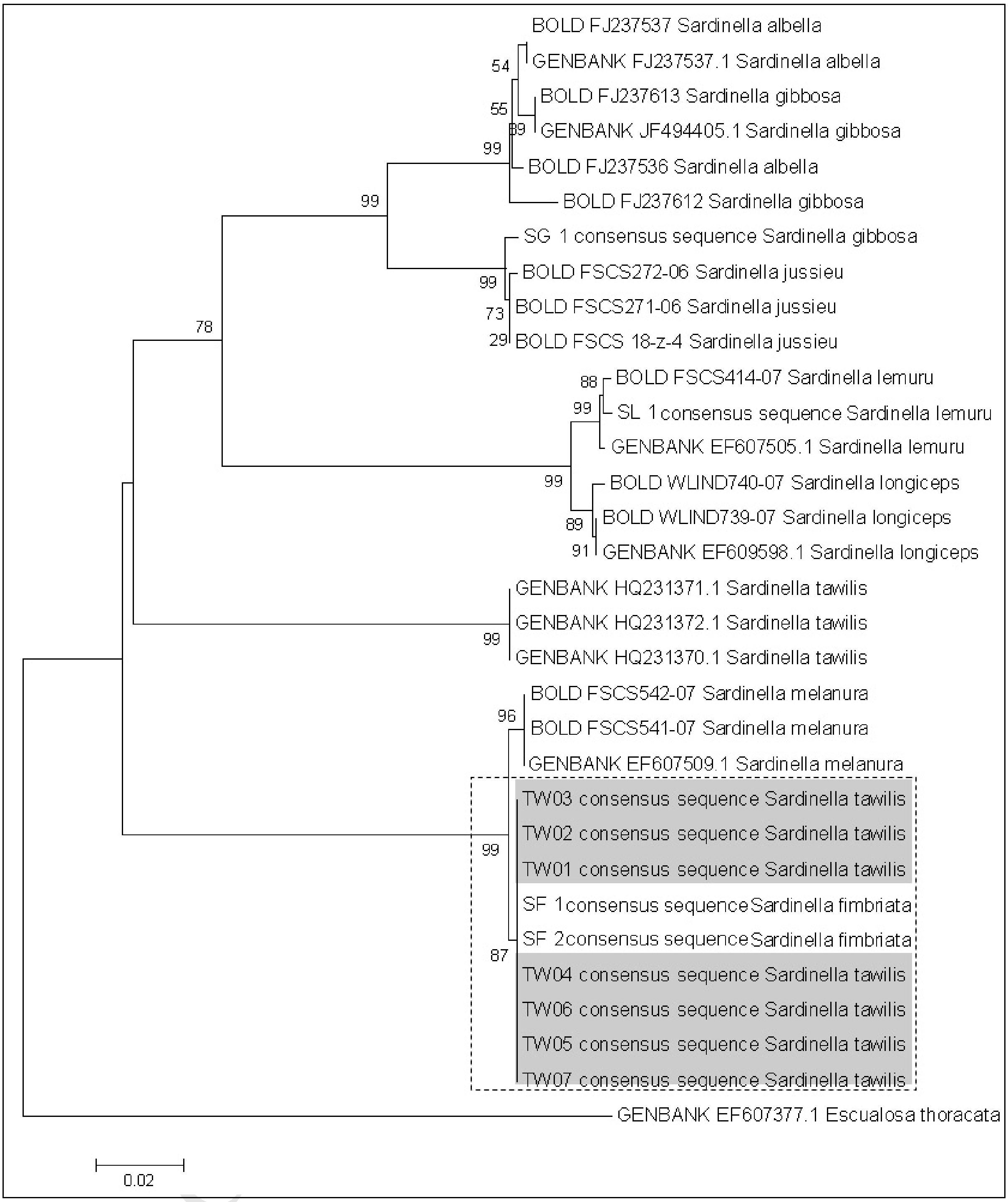

Figure 1. Neighbor Joining Tree of CO1 sequences from *Tawilis* samples and other sardines using Kimura 2-parameter model. *TW* samples were obtained from whole fish products labeled as "*tawilis*." GENBANK or BOLD labels indicate the database from which sequences were obtained followed by database accession number.

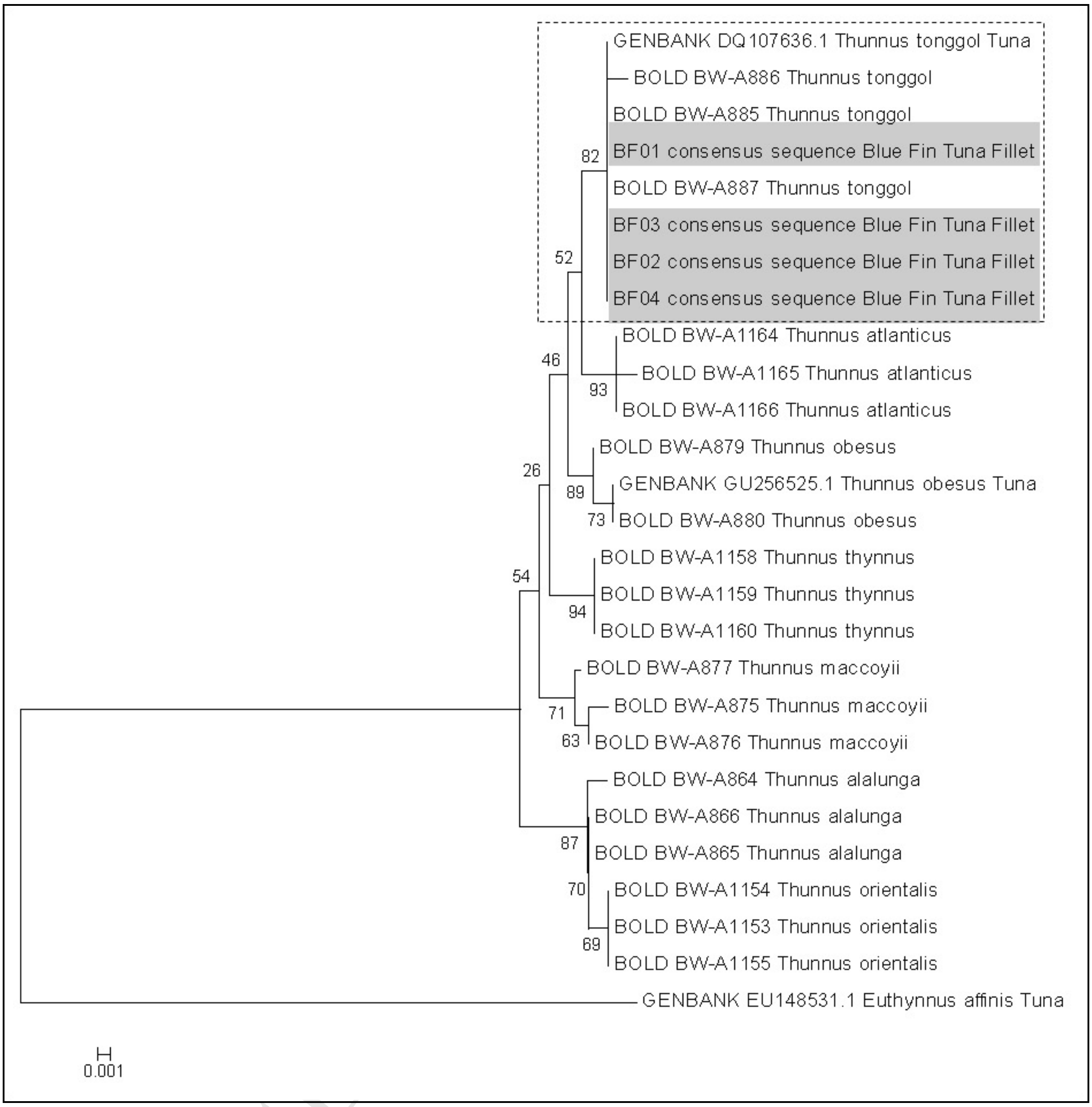

Figure 2. Neighbor Joining Tree of CO1 sequences from Blue fin Tuna Fillet samples using Kimura 2-parameter model. GENBANK or BOLD labels indicate the database from which sequences were obtained followed by database accession number.

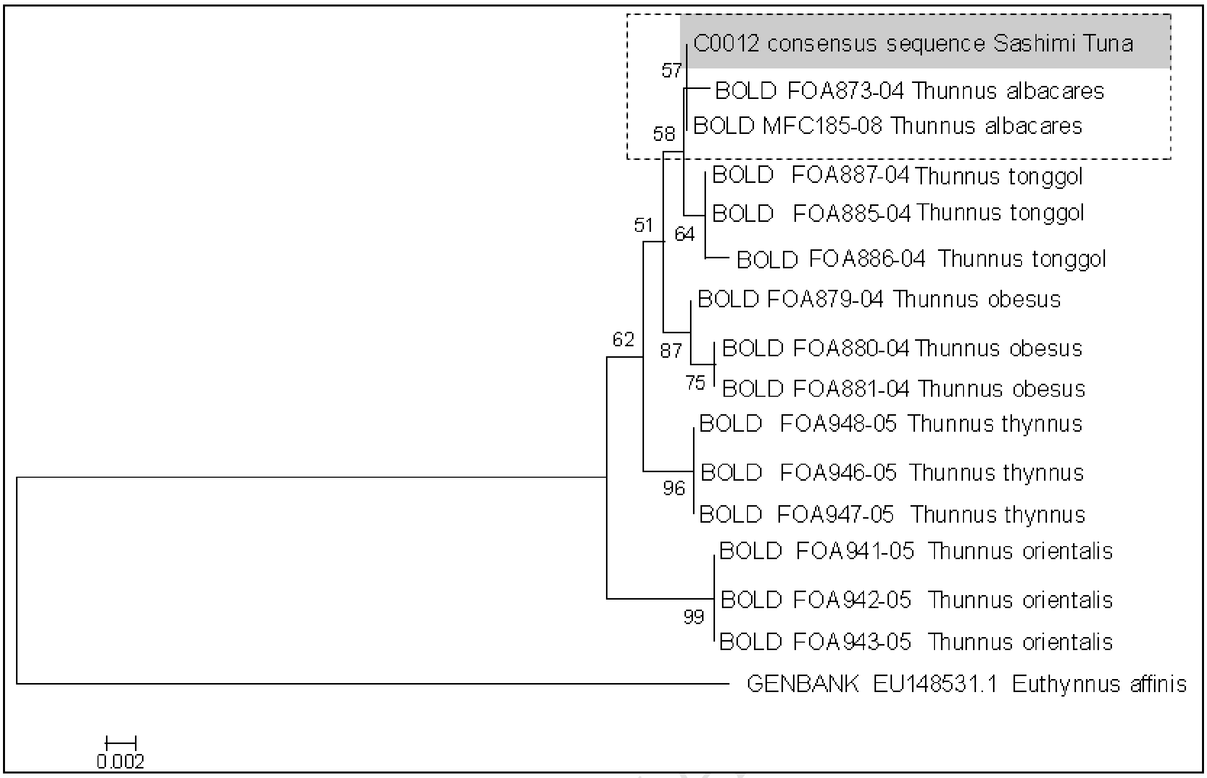

Figure 3. Neighbor Joining Tree of CO1 sequences from sashimi tuna Fillet samples using Kimura 2-parameter model. GENBANK or BOLD labels indicate the database from which sequences were obtained followed by database accession number.

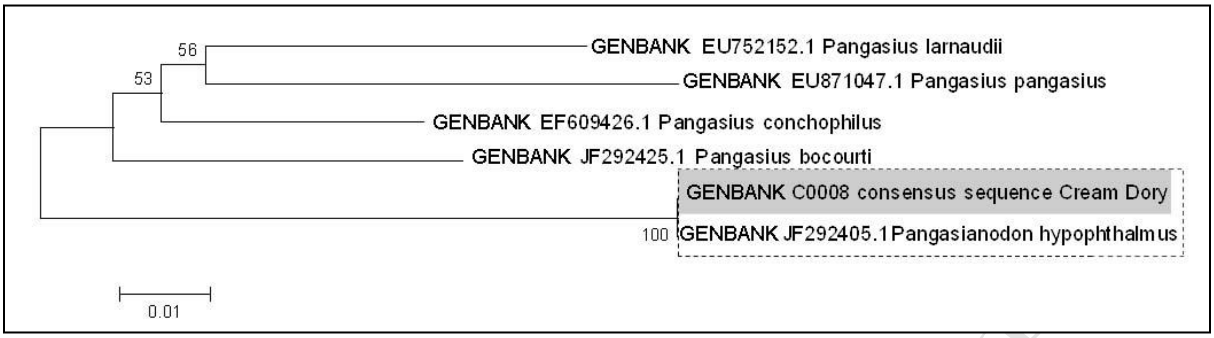

Figure 4. Neighbor Joining Tree of CO1 sequences from (Cream Dory) Pangasius Fillet samples usıng Kimura 2-parameter model. GENBANK label indicate the database from which sequences were obtained followed by database accession number.

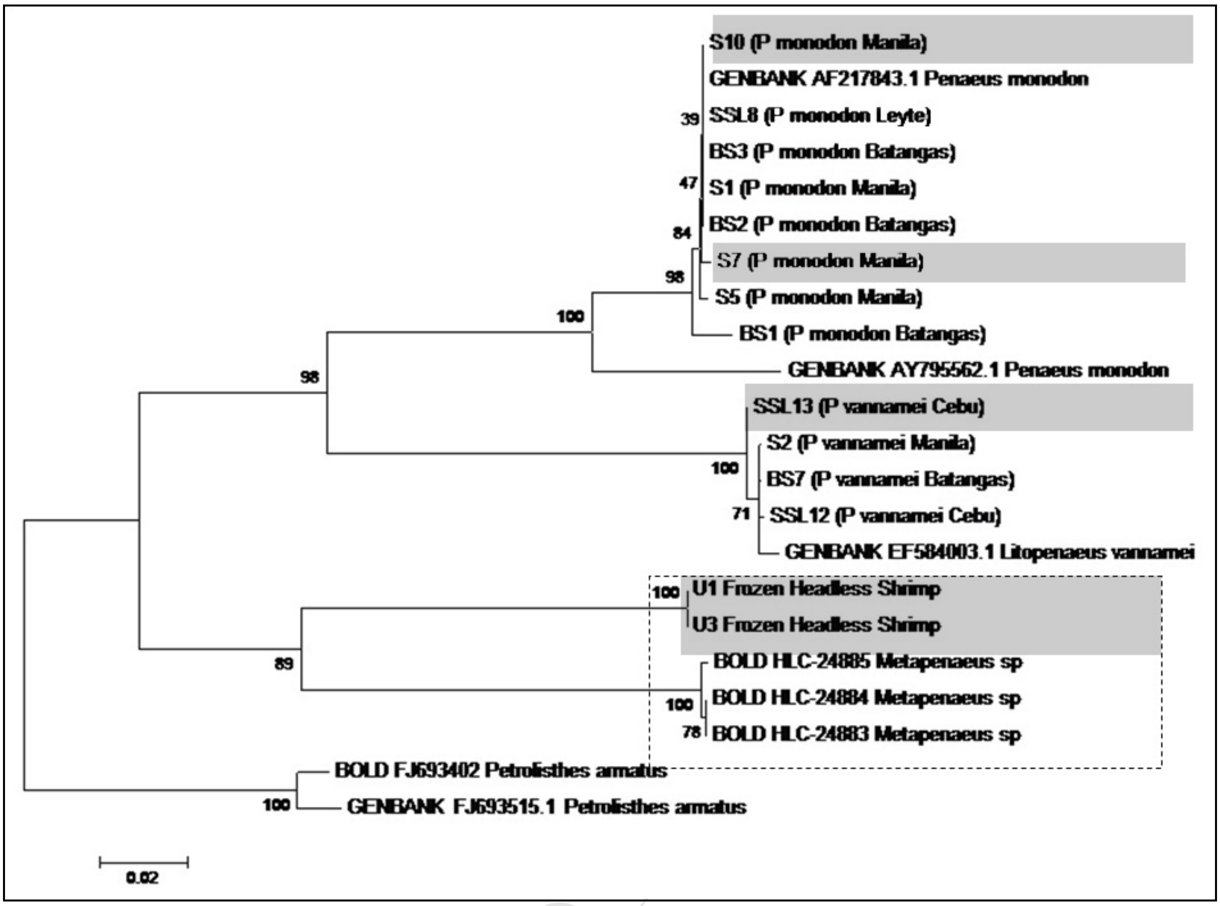

Figure 5. Neighbor Joining Tree of CO1 sequences from Frozen Shrimp samples using Kimura 2-parameter model. GENBANK or BOLD labels indicate the database from which sequences were obtained followed by database accession number.

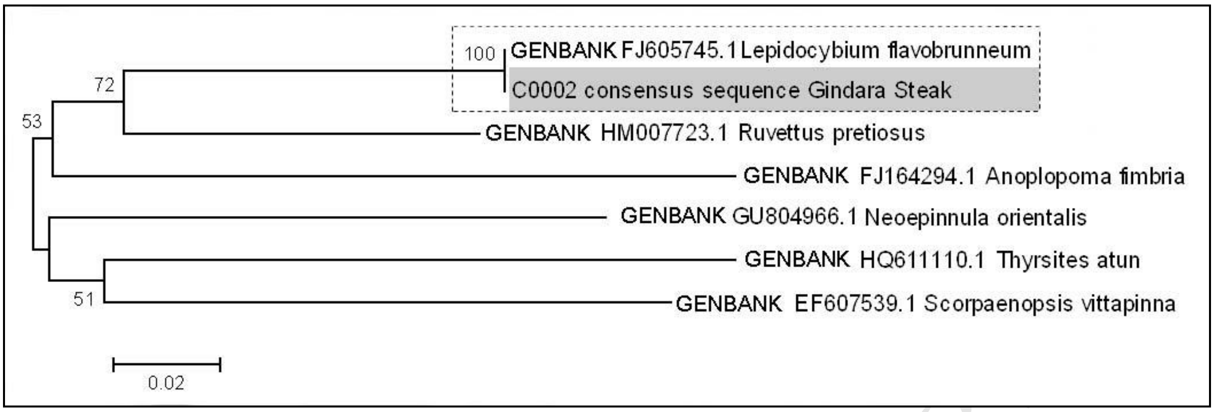

Figure 6. Neighbor Joining Tree of CO1 sequences from Gindara Steak / Fillet samples using Kimura 2-parameter model. GENBANK label indicate the database from which sequences were obtained followed by database accession number.

Research Highlights

- We highlight DNA barcoding as one of the most powerful tools for the assessment of species identity, food safety and fraud.
- There is a high probability of incorrect species declaration in the "*tawilis"* and *"Bluefin Tuna fillet* products in the Philippines
- Sashimi tuna fillets and cream dory products sold in the Philippines are correctly labelled.
- Labelling information for gindara steaks / fillets sold in Philippine markets is insufficient.