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RESEARCH ARTICLE

# Identification of Mislabelling in Frozen Fish Fillets Based on DNA Barcoding Analysis

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**ABSTRACT**

A number of studies have shown that mislabelling and species substitutions in fish products are very common worldwide. This fraud has two major aspects: economics and health. Moreover, poor trading, and neglecting the species conservation status are growing threats for fish stocks. First the type and extend of this fraud in fish must be detected in order to take proper actions. As some markers (e.g. protein analysis and morphological features) can fail, DNA markers, especially sequencing of cytochrome oxidase I gene (or DNA barcoding), is becoming a more widely preferred methodology for species identification. In this study, DNA barcoding technique was employed to confirm the species names written on the product packages of fish fillets purchased from the market. The fillets were labeled as Nile tilapia (*Oreochromis niloticus*). Among the 15 fillet samples analyzed, only 4 of them were labeled correctly. Seven (47%) of them were found to originate from pangasius (*Pangasianodon hypophthalmus*) and three of them were found to originate from a different tilapia species (*Oreochromis mossambicus*). This paper revealed a significant mislabelling of frozen fish fillets in Turkey. Customers are making informed decisions based on many reasons (like health issues or palate) and they have the right to eat what they think they are paying for. The results indicate the necessity for taking immediate actions and regulations against fraud in food items to sustain food quality and safety.

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## Introduction

There is an increasing awareness in the world against fraud, mislabelling and species substitutions in food items due to health and safety problems as well as economic issues. These issues interfere with the traceability in the production chain as well as with the applications of national and international regulations. Fish are an important component of the present biodiversity with more than 30000 species existing worldwide. They are an important protein source in the human diet and they possess direct economic value. Like other food items, mislabelling and species substitutions are also major concerns in the fishery products market (FAO, 2018a). In general, substitution and mislabelling rates vary with the species and whether the product is processed or not. Moreover, the rates can be dependent on the countries, like France having one of the lowest mislabelling/substitution rates reported (Bénard-Capelle et al., 2015).

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Today, the food industry and consumers are largely focused on food safety, quality and sustainability (Di Pinto et al., 2015). Accurate labeling of domestic and imported seafood; reporting the true origin, content, and the species contained within the product are very important both for the consumer and the seller companies in order to prevent possible economic losses as well as health problems, having allergic reactions being the most important as it can be life-threatening.

Misidentifications are often made in differentiating between the species with similar morphological characteristics and in the nomenclature of species with different names for the same local name or for the same species (Cawthorn et al., 2012). Since fish meat is a perishable food, it is a common strategy filleting its meat for fish trade (Staffen et al., 2017). However, most of the morphological features used in the identification of fish species during filleting may be lost during this process. Di Pinto et al. (2013) stated that in trading fish products, aquaculture products with low economic value can be sold, in whole or in part, substituting higher quality products. For example, it has been reported in Iran that, catfish fillets can be sold instead of sturgeon fillets (Changizi et al., 2013). This is an important issue from the economic, health and food safety aspects.

There are different markers and methods used in species identification and authentication based on morphology, proteins, and DNA. Although morphological characters can be used for species identification (Strauss and Bond, 1990), they can be misleading/uninformative in discriminating especially between closely related species. Furthermore, they become useless when it comes to processed food as the characters are lost. Protein markers used in identification can be of considerable value in certain instances (Rehbein, 1990; Hubalkova et al., 2007; Asensio et al., 2008). However, like morphological characters, protein analyses can fail species identification, too. Because, proteins start losing their biological activity once the animal is dead, and they denature once they are subjected to heat. As accurate identification of fish fillets based on morphologic or allozyme markers is neither easy nor feasible, employing DNA based molecular markers for accurate identification and regular inspection of fillet products is inevitable (Smith et al., 2008).

DNA sequencing analysis is one of the most widely used molecular marker in species identification for the last two decades. But; different laboratories may prefer different DNA regions for the same taxonomic groups, or different markers are used for different taxonomic groups. In this case, conducting comparative analyses of DNA sequences within and across species cannot be carried out. In order to overcome these problems, Hebert et al. (2003) indicated that a single gene sequence would be sufficient to differentiate all or at least a large majority of animal species. For this purpose, they suggested employing the mitochondrial DNA (mtDNA) cytochrome oxidase gene subunit I (COI) as a universal biological identification system for animals. In this methodology, based on the partial sequence of the mtDNA COI region, each species is represented by a specific sequence and the sequences of individuals of the same species form a cluster. These DNA sequences are compared to databases to assign the individual sample of interest to corresponding species. This resembles the barcoding system used in stores for tracking the trade items. That is why this system is called "DNA Barcoding". Since it has been first proposed by Hebert et al. (2003), the mtDNA COI gene has been used extensively in the determination of the species belonging to a sample of unknown origin, the determination of new sequences of species and the determination of whether or not a new species of unknown origin. It has been reported that this partial nucleotide sequence analysis of COI region in species identification has a discrimination power of 98% in marine fish and 93% in freshwater fish (Ward et al., 2009).

Currently, about 40% of the total production of aquatic products is met from aquaculture, worldwide. It is reported that aquaculture production (fish, crustaceans and mollusks) in the world was 170.35 million tons in 2015 and 76.4 million tons of this production was obtained through aquaculture (FAO, 2018b), but in 1980 aquaculture was reported to be only 4.7 million tons (FAO, 2010). The estimated amount of products obtained from aquaculture by 2030 is expected to reach or exceed the production from capture fisheries (World Bank, 2013). According to TUIK data (TurkStat, 2018), Turkey has imported 82074 tons of aquatic products in 2016, 100444 tons in 2017 and 98314 tons in 2018. Among these products, imported frozen fish fillets in 2018, which is approximately 5836 tons, comprises about 6% of the total imported products (TurkStat, 2018). Nile tilapia (*Oreochromis niloticus*) is one of the imported freshwater fish species in Turkey, as fish fillets. TUIK have reported that 400 tons (~7%) of fish fillets imported in 2018 were Nile tilapia. This fish is also known as aquatic chicken (Maclean, 1984) and its production is increasing sharply in each year throughout the world (Özcan-Gökçek et al., 2012).

In the present study, we aimed at testing the accuracy of the species declarations on the package of tilapia (*O. niloticus*) products sold as fillets in the markets by using DNA barcoding method, which is accepted as a universal method in species identification.

## Material and Methods

Fifteen commercially packed fish fillets were purchased from three different supermarkets, which were labeled as *O. niloticus* as the species name (Figure 1). The fillets were taken to Ege University Molecular Biology Laboratory. Approximately 100 mg of muscle tissue samples were taken from each fillet and transferred into 2 ml eppendorf tubes. Both the fillets and the tissue samples were stored at -20°C.

**Figure 1.** Fillet samples purchased from the supermarkets.

For the laboratory analysis, first DNAs were isolated from each tissue sample using a column-based DNA purification kit (EURX,

Molecular Biology Products). This kit was preferred because it was suitable to isolate DNA from fish muscle tissue providing high-quality DNA with repeatable results. Then, the quality and quantity of DNA samples were measured by MAESTROGEN™ spectrophotometer. In addition, the agarose gel (0.5% TBE) electrophoresis method was used to check whether the quality and quantity of DNA samples were suitable for the DNA analysis. Since all the extracted DNA samples were found to be of good quality, they were all used in the following PCR amplification reactions. For PCR amplification of the mtDNA COI gene region, primers listed in Ward et al. (2005) were used (FishF2: 5'-TCGACTAATCATAAAGATATCGGCAC-3'; FishR1:5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'). The PCR reactions were performed on Applied Biosystems™ brand SimpliAmp™ thermal cycling device. The total volume of the reaction mixture was 25 µl; which contained 50 ng genomic DNA, 1X *Taq* buffer, 5 pmol from each primer, 2 mM $MgCl_2$, 0,2 mM dNTPs, 0.8 unit *Taq* DNA polymerase and ultra pure water. The cycling protocol was as follows: 1 cycle of initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for 45 s, 54°C for 45 s, and 72°C for 60s, which then followed by the final extension at 72°C for 5 min. When performing PCR amplification, negative control without template DNA was used to check for possible contamination in every PCR reaction.

PCR amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose (Sigma A5093) gel in 0.5x TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) and stained with SafeView DNA Stain (5 uL/100 mL) (GeneMark, Taiwan). A GeneRuler™ 100 bp DNA Ladder Plus (MBI Fermentas,Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using Vilber Lourmat transilluminator. All the amplicons were about 700 bp in length as expected (Figure 2).

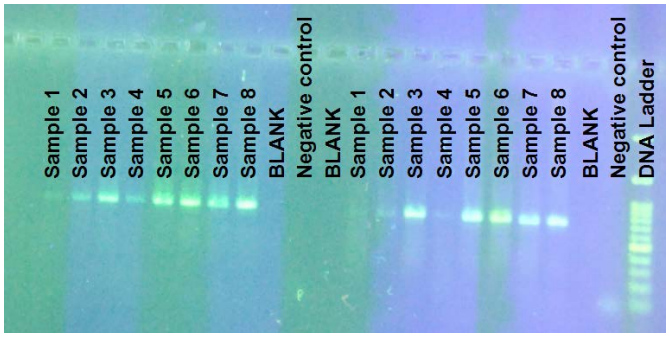


**Figure 2.** Imaging of the result of PCR amplification of mtDNA COI gene region of eight fish fillet samples by agarose gel electrophoresis method

Having checked the PCR products by agarose gel electrophoresis, the amplicons were transferred to LetgenBio Ltd. for bidirectional sequencing using the PCR primers given above (Ward et al., 2005). The company performs Sanger sequencing reactions using BigDye™ (Applied Biosytems™) and uses capillary-based automatic DNA Analyzers (ABI DNA Analyzer) for collecting the chromatograms.

After receiving the chromatogram results from the LetgenBio Ltd., first, they were all checked by using ChromasPro software (ChromasPro Version 2.1.8, Technelysium Pty. Ltd., Australia) for their quality. Then the chromatograms of forward and reverse sequences for each sample were aligned forming contigs. After analysis of the contigs, consensus sequences were exported in Fasta format for each sample for data analysis. The generated sequences were all subjected to BLASTn analysis at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the identity of generated DNA barcodes to previously deposited sequences in order to assign the DNA samples of the study to appropriate species.

Taking the BLAST results into consideration, reference sequences were selected from the NCBI database; which are: KU565839 *Oreochromis mossambicus*, DQ426667 *Oreochromis niloticus*, LC052672 *Oreochromis niloticus*, and KR080263 *Pangasianodon hypophthalmus*. Afterward, project samples and reference samples were analyzed to find the best nucleotide substitution model using the module implemented in the MEGA 7 version (Kumar et al., 2016). The best model turned out to be HKY (Hasegawa et al., 1985) with zero gamma distribution. Finally, MEGA software was used to construct a Neighbor-joining (NJ) tree with 1000 bootstrapping based on the best model to assess the phylogenetic relationships within and among the study samples and reference samples.

## Results

The mtDNA COI gene barcode sequences of 15 samples obtained during the analyses were compared with the database using the BLAST program and the species to which each of the samples belongs to were detected. As a result of the BLAST comparison, a total of 8 (eight) samples were revealed as Tilapia (*O. niloticus* and *O. mossambicus*) and 7 (seven) samples were revealed as Panga (*P. hypophthalmus*). The findings for each fillet are given in Table 1 below.

**Table 1.** Species detected by DNA barcode analysis of 15 fish fillet samples analyzed

| Sample No | Species name on the package | Species name based on DNA barcoding analysis |
|---|---|---|
| 1 | *O. niloticus* | *O. niloticus* |
| 2 | *O. niloticus* | *O. niloticus* |
| 3 | *O. niloticus* | *O. mossambicus* |
| 4 | *O. niloticus* | *O. niloticus* |
| 5 | *O. niloticus* | *O. niloticus* |
| 6 | *O. niloticus* | *O. mossambicus* |
| 7 | *O. niloticus* | *O. niloticus* |
| 8 | *O. niloticus* | *O. mossambicus* |
| 9 | *O. niloticus* | *P. hypophthalmus* |
| 10 | *O. niloticus* | *P. hypophthalmus* |
| 11 | *O. niloticus* | *P. hypophthalmus* |
| 12 | *O. niloticus* | *P. hypophthalmus* |
| 13 | *O. niloticus* | *P. hypophthalmus* |
| 14 | *O. niloticus* | *P. hypophthalmus* |
| 15 | *O. niloticus* | *P. hypophthalmus* |

The actual species names of the fish fillets revealed by the BLAST software were also prominent in the evolutionary relationship tree reconstructed by using MEGA 7 software (Figure 3).

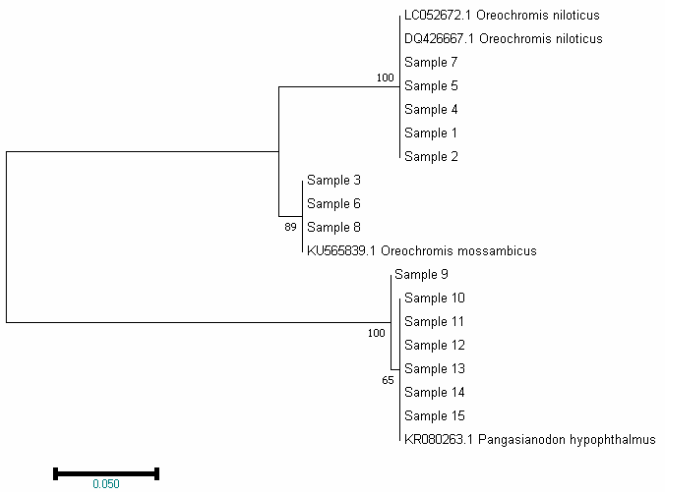


**Figure 3.** Phylogenetic relationship tree of the barcoding sequences of the 15 samples and the reference sequences, reconstructed by Neighbor-Joining method with 1000 bootstrap, based on HKY substitution model.

There were three clades revealed by phylogenetic tree. *P. hypophthalmus* reference sample and seven of the analyzed samples formed a distinct clade separating from Tilapia species (*O. niloticus* (Sample 1, 2, 4, 5, 7) and *O. mossambicus* (Sample 3, 6, 8)) with a 100% branch support. Moreover, reference samples from the two Tilapia species are divided into two different clades with high branch supports. The reference sample for *O. mossambicus* and three of the analyzed samples formed one clade with an 89% branch support and the reference samples for *O. niloticus* and five of the analyzed samples formed the other clade with a 100% branch support. These results were in consisted with the BLAST analysis.

## Discussion

The studies around the world (e.g. the USA, Brazil, Italy, Iran, etc.) have reported to have a high rate of substitution in fish products (Barbuto et al., 2010; Filonzi et al., 2010; Changizi et al., 2013; Di Pinto et al., 2013, 2015; Staffen et al., 2017; Willette et al., 2017). For example, Neto (2013) has reported that in the labeling of seafood, tilapia is substituted by pangasius.

Understanding the incentive behind the mislabeling in fish products can be difficult to quantify because mislabelling may happen at any stage of the process. It may result from the fraud of the manufacturer, vendor, restaurateur or shop owner. However, it might also be resulted from the confusion of labeling laws, from misidentification based on morphological characters, or from using common vernacular. Being independent of the reason, what is often detected is that products of less value are substituted for more valuable fish suggesting an economic incentive for illegally substituting fish (e.g. Barbuto et al., 2010; Filonzi et al., 2010; Changizi et al., 2013; Di Pinto et al., 2013, 2015; Staffen et al., 2017; Willette et al., 2017).

In the present study, it was aimed at detecting whether the name of the species indicated on the frozen fish fillet packages was correct using DNA Barcoding technique. The BLAST analyses of the 15 DNA barcoding sequences obtained from the samples purchased from the shops have revealed that seven of them (47%) were indeed pangasius despite their label as being *O. niloticus*. Moreover, three of the eight tilapia fillets were of *O. mossambicus* origin; again, despite their labels as being *O. niloticus*. When a phylogenetic relationship tree was reconstructed including reference DNA barcoding sequences taken from NCBI (KU565839, DQ426667, LC052672, KR080263), the samples of the present study have grouped in three clades, as expected based on the BLAST search (Figure 3). In this phylogenetic tree, each clade included reference sequences of one of the three species: *O. niloticus*, *O. mossambicus* or *P. hypophthalmus*. These results provided evidence that commercial fraud and mislabelling can be observed in fillet fish products; one species substituting for another one as observed commonly across the world (Barbuto et al., 2010; Filonzi et al., 2010; Changizi et al., 2013; Di Pinto et al., 2013, 2015; Staffen et al., 2017). In this study, it was pangasius substituting for tilapia. Neto (2013) have proposed that substituting tilapia by pangasius is a marketing strategy to promote the consumption of these products. Furthermore, for the three of the samples, the genus name on the label was correct (*Oreochromis*), but not the species name (true species name is *O. mossambicus*).

There are few studies in Turkey reporting similar commercial fraud and mislabelling. In one of the studies, DNA barcoding analysis was carried out on surimi products sold in the markets, all of which were labeled as Alaskan Pollock (*Theragra chalcogramma*) on their packages (Keskin and Atar, 2012). Among these 50 surimi-based products, only 8 of them (%16) were found to be of Alaskan Pollock origin as declared on the product. Two of the samples were found to originate from different species and the rest of the samples were found to originate from different families like Sciaenidae, Synodontidae, Merlucciidae, Nemipteridae, etc. (Keskin and Atar, 2012). Another species identification study based on DNA barcoding analysis has been carried out on the 10 processed squid products purchased from the markets (Keskin and Atar, 2011). Among these, labels on the 6 of the squid products were reported as having lacked the information about the species origin of the products. The sequencing results revealed that the products were originated from 7 different species confined in two families: one is commercially high-valued Loliginidae family and the other one is commercially lower-valued Ommastrephidae family. Among the 10 samples, 4 of them were composed of the species belonging to the high-valued Loliginidae family and 6 of them were composed of the species belonging to the lower-valued Ommastrephidae family. Furthermore, they revealed that one of the four products informing the genus name on the label was mislabelled.

There are non-governmental organizations like Oceana (https://oceana.org) reporting fraud in seafood worldwide. One of the major concerns in such reports is that higher mercury levels detected in fraud fish products. Unfortunately, not all the countries, especially Asian countries, have the same chemical and handling regulations in aquaculturing fish. One might purchase a fish product thinking that he is buying a local high value fish, but instead it might turn out to be a substitute fish imported from Asia. Being uninformed/misinformed about what fish is on your plate may present a high health risk for you and your family in terms of heavy metal exposure or allergic reactions.

## Conclusion

The increasing number of studies worldwide has proven that commercial fraud and mislabelling on aquatic food products and species substitutions are major problems in terms of food quality and food safety. Despite the conflicts caused by morphological identifications (especially for processed food), DNA barcoding analysis is a highly successful and applicable technique in seafood safety. The number of existing aquatic species is quite high such that the discrimination power of DNA barcoding was proven to be high, even for the identification of the local varieties (Galimberti et al., 2013). Strict monitoring based on DNA barcoding for end-to-end tracking supply chains and publicizing the results may help increased awareness in customers. Moreover, stricker regulations / laws on and increased consumer awareness may exert pressure on vendors to avoid fraud, which eventually would help decrease the rate of the fraud observed worldwide.

## Conflict of Interest

The author declares that there is no conflict of interest.

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