Species Identification of Some Fish Processing Products in Iran by DNA Barcoding

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ABSTRACT

This study pursued the molecular identification of fish species from processed products for human consumption which, a priori, belonged to nine species. DNA barcoding using direct sequencing of about 650 bp of the mitochondrial gene cytochrome oxidase subunit I (COI) revealed incorrect labeling in the three Alaska Pollack samples (11\% of all samples). Substitution of fish species constitutes serious economic fraud, and our results increase concern regarding the trading of processed fish products in Iran from both health and conservation points of view.

Keyword: Biotechnology, Fish product, Food traceability, Forensic genetics.

INTRODUCTION

Trade in aquaculture and fisheries products, especially in particular species of fishes, has increased dramatically in recent decades. According to FAO statistics, in 2009, this trade totaled 144 million tons. This value is equal to 599 thousand tons in Iran, of which about 30 thousand tons are related to non-canned imported fish. With the development of processing industries and food processing, especially in marine products, which have high diversity, there is always the possibility that manufacturers of such products use low-value species rather than more expensive consumer-friendly species and use fake labels on their products to receive more profit. One of the problems to identify the species used in processed marine products is the absence of morphological features such as skin pattern, body shape and size, shape and number of the fins, etc. Therefore, developing some techniques to determine the species in such products is absolutely essential (Teletchea, 2009). Thus, consumers with no awareness pay more than the actual value of the products. This happens especially for marine products which are in frozen or fillet forms. Because some fishes in such fraudulent products could be captured from polluted marine areas and are not marketable, they may cause health problems for consumers (Cespedes et al., 1998). Therefore, precise quality control and identifying the species used in the products is absolutely essential. Among the different methods of fish identification, using molecular genetics techniques are widely used and considered (Teletchea, 2009).

In the past, most applied molecular methods were PCR–RFLP of Cytochrome b gene (Cespedes et al., 1998; Hold et al., 2001; Sanjuan and Comesana, 2002), random amplified polymorphic DNA

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(RAPD) fingerprinting (Asensio et al., 2002), single strand conformation polymorphism (SSCP) (Cespedes et al., 1998). Recently, Amplified Fragment Length Polymorphisms (AFLPs) have been used to investigate genomes of different complexities (Gonzales Fortes et al., 2008, Papa et al., 2005, Watanabe et al., 2004).

In recent years, molecular barcoding has been recognized as the favorite and the best methodology in forensic science for species identification (Dawnay et al., 2007). DNA barcoding is based on the sequencing of a mtDNA fragment of the cytochrome oxidase I (COI) gene to act as a “barcode” to identify and delineate all animal life (Roe and Sperling, 2007; Ward et al., 2005). Nowadays, by choosing a standard DNA fragment shared among multiple research groups, efforts have been coordinated, and a more comprehensive library of DNA sequences of thousands of species is available. DNA barcoding has been used to identify specific groups of fish species, such as tuna (Terol et al., 2002), flatfish (Espiñeira et al., 2008), anchovy (Jérôme et al., 2008) and sharks (Barbuto et al., 2010a).

In this study, for the first time in Iran, the identification of nine species of imported fish including Alaska Pollack (Theragra chalcogramma), Red Cod (Pseudophycis bachus), Warehou (Seriolella brama), Hoki (Macruronus novaezelandiae), Atlantic salmon (Salmo salar), Southern blue whiting (Micromesistius australis), White fish (Coregonus clupeaformis), Nile Perch (Lates niloticus), and Tilapia (Oreochromis mossambicus) was conducted. Such efforts should be made for other food products, especially other processed fish species; and regulatory agencies should design and perform DNA barcoding in a systematic and comprehensive manner to prevent fraud in the food products.

**MATERIALS AND METHODS**

**Collecting Samples**

Samples of processed aquatic products were collected in 2010 from the Shahrvand, Refah, and Hyperstar department stores in Tehran. To determine the number of samples, a Lot Tolerance Percent Defective (LTPD) protocol was used (Montgomery, 2008).

This sampling design is used to assess compliance to product specifications. It is useful in cases where the total number of products is too large for every individual product to be inspected manually. Small samples of a particular size are taken and, if a defective unit is observed, the entire “lot” is rejected. The sampling design is based on a known relationship between the total number of products and the number that will be accepted despite being defective (the acceptance number), say 10%. In other words, for a certain LTPD lot number, the probability of acceptance is 0.01. The size of the sampling lot is determined based on a geometric distribution. The steps are outlined below:

1- The total product number $N$ is determined: In our case, this was equal to 3,000 kg (3 stores considered in this study, with a total of approximately 1,000 kg of fish each).

2- level or PL (Performance Level) is determined. This is the level of quality that we're going to be confident of achieving through this design.

3 - The Defective ($D$) = the total product number ($NPL$) value is calculated as $D = 3000 \times 0.05 = 150$.

4- The closest value to the calculated $D$ is found in the LTPD table (Table1). The value

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Table 1. Lot tolerance percent defective (LTPD) table.

<table>
<thead>
<tr>
<th>f</th>
<th>0.00</th>
<th>0.01</th>
<th>0.02</th>
<th>0.03</th>
<th>0.04</th>
<th>0.05</th>
<th>0.06</th>
<th>0.07</th>
<th>0.08</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>1.0000</td>
<td>0.9562</td>
<td>0.9117</td>
<td>0.8659</td>
<td>0.8184</td>
<td>0.7686</td>
<td>0.7153</td>
<td>0.6567</td>
<td>0.5886</td>
</tr>
<tr>
<td>0.8</td>
<td>1.437</td>
<td>1.3865</td>
<td>1.3428</td>
<td>1.2995</td>
<td>1.2565</td>
<td>1.2137</td>
<td>1.1711</td>
<td>1.1286</td>
<td>1.0860</td>
</tr>
<tr>
<td>0.7</td>
<td>1.9125</td>
<td>1.8601</td>
<td>1.8088</td>
<td>1.7586</td>
<td>1.7093</td>
<td>1.6610</td>
<td>1.6135</td>
<td>1.5667</td>
<td>1.5207</td>
</tr>
<tr>
<td>0.6</td>
<td>2.5129</td>
<td>2.4454</td>
<td>2.3797</td>
<td>2.3159</td>
<td>2.2538</td>
<td>2.1933</td>
<td>2.1344</td>
<td>2.0769</td>
<td>2.0208</td>
</tr>
<tr>
<td>0.5</td>
<td>3.3219</td>
<td>3.2278</td>
<td>3.1372</td>
<td>3.0497</td>
<td>2.9652</td>
<td>2.8836</td>
<td>2.8047</td>
<td>2.7283</td>
<td>2.6543</td>
</tr>
<tr>
<td>0.4</td>
<td>4.5076</td>
<td>4.364.</td>
<td>4.2270</td>
<td>4.0963</td>
<td>3.9712</td>
<td>3.8515</td>
<td>3.7368</td>
<td>3.6268</td>
<td>3.5212</td>
</tr>
<tr>
<td>0.3</td>
<td>6.4557</td>
<td>6.2054</td>
<td>5.9705</td>
<td>5.7496</td>
<td>5.5415</td>
<td>5.3451</td>
<td>5.1594</td>
<td>4.9836</td>
<td>4.8168</td>
</tr>
<tr>
<td>0.2</td>
<td>10.3189</td>
<td>9.7682</td>
<td>9.2674</td>
<td>8.8099</td>
<td>8.3902</td>
<td>8.0039</td>
<td>7.6471</td>
<td>7.3165</td>
<td>7.0093</td>
</tr>
<tr>
<td>0.0</td>
<td>*229.1053</td>
<td>213.9741</td>
<td>175.5957</td>
<td>156.4055</td>
<td>13.4488</td>
<td>11.3494</td>
<td>9.9432</td>
<td>8.6068</td>
<td>7.3493</td>
</tr>
</tbody>
</table>

*a For value of f<0.01, Use f= 2.303/D.

The number 200 has a value of f<0.01. Based on the parameters in the table, the formula f= 2.303/D should be used:

f= 2.303/D = 2.303×150 = 0.0153
n= f×N = 0.0153×3000 = 46 kg

According to the calculations showing a 3,000 kg total product number for three stores, a 46 kg sampling lot would be required. The number of samples required from each store would equal 13.5 kg for whole fish (that is, one of each of the nine samples is equal to 1.5 kg. All muscle tissue samples were fixed in 100% ethanol alcohol after collection, and were sent to the Biotechnology Laboratory in Science and Research Campus, Azad University.

**DNA Extraction**

27 muscle samples of 20 mg from 27 different individuals were used to extract DNA. Twenty mg starting material was transferred to a 1.5 ml centrifuge tube containing digestion buffer according to the classical SDS-proteinase K and phenol–chloroform technique described by Infante (2006). DNA quality and extraction yield were assessed by means of 1.2% agarose gel electrophoresis in TE buffer.

**PCR Amplification**

Approximately 650 bp were amplified from the Cytochrome oxidase I in mitochondrial DNA using different combinations of two newly designed primers (Ward et al., 2005):

- FishF1- 5’TCAACCAACCACAAAGACATTGGC
- FishR1- 5’TAGACTTCTGGGTGGCCAAAGAAT

The 25 µL PCR reaction mixes included 18.75 µL of ultrapure water, 2.25 µl of 10X PCR buffer, 1.25 µL of MgCl₂ (50 mM), 0.25 µL of each primer (0.01 mM), 0.125 µL of each dNTP (0.05mM), 0.625 U of Taq polymerase and 0.5–2.0 µL of DNA template. Amplifications were performed using a Mastercycler Eppendorf gradient thermal cycler (Brinkmann Instruments,Inc.). The thermal regime consisted of an initial step of 5 minutes at 94°C followed by 30 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, followed in turn by 10 minutes at 72°C and then held at 4°C. PCR products were visualized on 1.2% agarose gels and the most intense products were selected for sequencing.

**Sequencing and Species Identification**
Products were labelled using a BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.), and were sequenced bidirectionally using an ABI 3730 capillary sequencer according to the manufacturer’s instructions.

Sequences were aligned using CLUSTAL X ver.2.0 software (Applied Biosystems, Inc.). The Barcode of Life Database (www.boldsystems.org) is designed to Sequence divergences.

**RESULTS**

The mitochondrial Cytochrome oxidase I region of all samples was successfully amplified using PCR. Twenty seven market samples were subsequently sequenced bi-directionally to assemble a 650bp length Cytochrome oxidase I barcode. When the BOLD identification engine was employed, 24 of the 27 sequences, representing an estimated 8 species, had 99 to 100 percent maximum identity with the species as labeled. The only mismatched samples were the three labeled as Alaska Pollock, which matched to a very different species via BOLD, at 99.20-100% similarity (Table 2, Figure 1).

**DISCUSSION**

Today, especially in developed countries, the use of molecular techniques is highly recommended. Therefore, standard methods such as molecular techniques for DNA Barcoding, which have higher resolution compared to other methods, are recommended for species detection in such processed products (Wong and Hanner, 2008). In the present study, for the first time

Table 2. List of all samples analyzed in this study.

<table>
<thead>
<tr>
<th>Sampling store</th>
<th>Sold as</th>
<th>BOLD reference subset</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shahrvand Refah</td>
<td>Alaska Pollock fillet</td>
<td>Theragra chalcogramma Micromesistius australis (99.2%)</td>
<td>Mislabeled</td>
</tr>
<tr>
<td>Hyper Star</td>
<td>Red Cod fillet</td>
<td>Pseudophycis bachus Pseudophycis bachus (99.7%)</td>
<td>OK</td>
</tr>
<tr>
<td>Shahrvand Refah</td>
<td>Warehou fillet</td>
<td>Seriolella brama Seriolella brama (100%)</td>
<td>OK</td>
</tr>
<tr>
<td>Hyper Star</td>
<td>Hokifillet</td>
<td>Macruronus novaezelandiae Macruronus novaezelandiae (100%)</td>
<td>OK</td>
</tr>
<tr>
<td>Shahrvand Refah</td>
<td>Atlantic Salmon fillet</td>
<td>Salmo salar Salmo salar (99.8%)</td>
<td>OK</td>
</tr>
<tr>
<td>Hyper Star</td>
<td>Southern Blue Whiting fillet</td>
<td>Micromesistius australis Micromesistius australis (100%)</td>
<td>OK</td>
</tr>
<tr>
<td>Shahrvand Refah</td>
<td>White fish fillet</td>
<td>Coregonus clupeaformis Coregonus clupeaformis (99.8%)</td>
<td>OK</td>
</tr>
<tr>
<td>Hyper Star</td>
<td>Nile Perch fillet</td>
<td>Lates niloticus Lates niloticus (98.19%)</td>
<td>OK</td>
</tr>
<tr>
<td>Shahrvand Refah</td>
<td>Tilapia fillet</td>
<td>Oreochromis mossambicus Oreochromis mossambicus (100%)</td>
<td>OK</td>
</tr>
</tbody>
</table>

*Barcode of Life Data Systems*
in Iran, some techniques with higher resolution than other methods were used in non-canned fishes.

A considerable portion (11%) of the analyzed samples revealed an incorrect species labeling, demonstrating insufficient control and security of fish products derived from local and foreign fisheries. Major frauds concerned the Alaska Pollock (no labeled scientific name) substituted by the Southern Blue Whiting.

From an economic point of view, this major fraud concerned a low market value product being sold as the expensive and valuable ones. This should be considered as serious commercial frauds.

In addition, the species identified through our molecular investigations have different nutritional properties compared to those
declared. Another example is the short fin mako, a cartilaginous fish similar to the smooth-hound, sold as swordfish, which is considered particularly suitable for low fat diets (Filonzi et al., 2010). There is always the possibility that manufacturers of such products replace expensive consumer-friendly species by low value species and use fake labels on their products to receive more profit; so consumers with no awareness pay more than the actual value of the products. For example, Atlantic mackerel (*Scomber scombrus*) is a very market-friendly and expensive species in Spain and is used in canned tuna production industry, but, due to high price of this species, some of the factories use less valuable species of tuna fish instead and sell their fraud products with Atlantic mackerel label at the market (Infante et al., 2006). There is always concern that labels of some marine fish products such as Alaska Pollock, which is a valuable fish, do not comply with contents and supply of food products. In addition to economic issues, fraud in the production and supply of food products should be considered because of social and religious aspects of view (Rastogi et al., 2007). Recently, the presence of pork sausages imported into Malaysia has been reported, using DNA-based molecular methods (Aida et al., 2004). This may also occur in aquatic products. For example, it is possible that some profit-seekers sell Catfish filet instead of valuable species filets such as sturgeons, and in this way Moslem consumers use religiously forbidden meat without any awareness. This case is especially more important in species imported from Western countries. It is noteworthy that, in most cases, fish products come from extra-European areas provenance, from polluted, without the same standards of sanitary controls of farming sites, pathogens and bioaccumulation of heavy metals. For example, the Nile perch is one of the most diffused species in fish frauds, and in recent years was subjected to repeated commercial prohibitions, because of its provenience in polluted African waters. In particular, the poisoning by Methylmercury, a neurotoxin, occurs primarily through consumption of fish, the bioaccumulation of this metal could increase the risk of myocardial infarction (Gualar et al., 2002) and neurological damages. The identification of fish species is also important for conservation of biodiversity: the substitutions of commercial species with endangered or vulnerable species could be considered a wildlife crime. These kinds of substitutions are frequent in some country markets (Barbuto et al., 2010b). In conclusion, DNA barcoding is emerging as an invaluable tool to regulatory agencies and fisheries managers for species authentication, food safety, conservation management, as well as consumer health and support (Costa and Carvalho, 2007). Here, we have used DNA barcoding techniques and consensus sequences for the identification of important species of fish in Iranian market. Our results indicate that DNA barcoding is a powerful technique, accurately identifying samples regardless of sample source. The developed barcodes will aid in upcoming efforts to heighten Iran fish products inspection and regulation requirements by ensuring accurate labeling of frozen and processed fish products. Our results add up to other evidence urging for increased traceability of food products and the authenticity of raw material to be assessed in Standard Organization of Iran. Molecular investigations based on DNA barcoding are one of the most powerful tools to assess species identity, food safety, protection of wildlife fauna and sustainable fishery and should be urgently applied to Iranian market.

REFERENCES


DNA

نخستین گونه‌ای برخی از محصولات عمل آوری شده آریزونا در ایران بوسیله

پارکدینگ

ر. چنگیزی ع. فرهنم، م. سلطانی ف. درویش ع. علم دوست

چکیده

این مطالعه در راستای شناسایی 9 گونه فیله ماهی عمل آوری شده مورد مصرف در بازار ایران می‌باشد. این تحقیق با کارگیری تکنیک بارکدینگ و استفاده از زن سیتوکروم اکسیداز (خطوط شماره‌شده) به جهت راستی آزمایش گونه‌ها صورت یافته‌است. بر اساس نتایج بدست آمده نام درج شده بر روی فیله آلاساپولاک اشتباه بوده و با توجه به تعداد نمونه‌ها می‌توان 11 درصد

محصولات را نقلی دانست.