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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 whithing (*Micromesistius australis*), White fish (*Coregonus clupeaformi*), Nile Perch (*Lates niloticus*), and Tilapia (*Oreochromis mossambicus*) was conducted. Verification for the label of the packaged product was conducted, using mitochondrial Cytochrome Oxisidase sequencing method. Species used for DNA barcoding are mainly caught from Atlantic Ocean and the east coast of Pacific Ocean and are provided as the bestselling frozen or fillet products in the stores. The objective of the present study is a starting point of this method and the use of DNA barcoding to identify some of the imported fish in Iran. 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 $D= 3000\times0.05= 150$.

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.

f^a	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08
0.9	1.0000	0.9562	0.9117	0.8659	0.8184	0.7686	0.7153	0.6567	0.5886
0.8	1.43.7	1.3865	1.3428	1.2995	1.2565	1.2137	1.1711	1.1286	1.0860
0.7	1.9125	1.8601	1.8088	1.7586	1.7093	1.6610	1.6135	1.5667	1.5207
0.6	2.5129	2.4454	2.3797	2.3159	2.2538	2.1933	2.1344	2.0769	2.0208
0.5	3.3219	3.2278	3.1372	3.0497	2.9652	2.8836	2.8047	2.7283	2.6543
0.4	4.5076	4.364.	4.2270	4.0963	3.9712	3.8515	3.7368	3.6268	3.5212
0.3	6.4557	6.2054	5.9705	5.7496	5.5415	5.3451	5.1594	4.9836	4.8168
0.2	10.3189	9.7682	9.2674	8.8099	8.3902	8.0039	7.6471	7.3165	7.0093
0.1	21.8543	19.7589	18.0124	16.5342	15.2668	14.1681	13.2064	12.3576	11.6028
0.0	*	229.1053	113.9741	75.5957	56.4055	44.8906	37.2133	31.7289	27.6150

[a] For value of $f < 0.01$, Use $f = 2.303/D$.

of f is determined from the corresponding row and column of the table.

5- The lot number is determined by $n = Sample\ size = f \times N$

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:

$f = 2.303/D = 2.303 \div 150 = 0.0153$

$n = f \times N = 0.0153 \times 3000 = 46$ 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 Cytochromec oxidase I in mitochondrial DNA using different combinations of two newly designed primers (Ward *et al*., 2005):

FishF1-
5“TCAACCAACCACAAAGACATTGGC AC3”,

FishR1-
5“TAGACTTCTGGGTGGCCAAAGAAT CA3”,

The 25 µL PCR reaction mixes included 18.75 µL of ultrapure water, 2.25 µl of 10X PCR buffer, 1.25 µL of $MgCl_2$ (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

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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.

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

[a] Barcode of Life Data Systems

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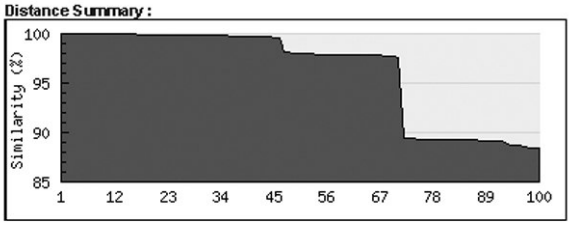
Search Result:

Identification Summary :

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Actinopterygii	100
order	Gadiformes	100
family	Gadidae	100
genus	Micromesistius	100
species	Micromesistius australis	100

A species level match has been made. This identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed. Such cases are rare.

Distance Summary :



Similarity scores of the top 100 matches

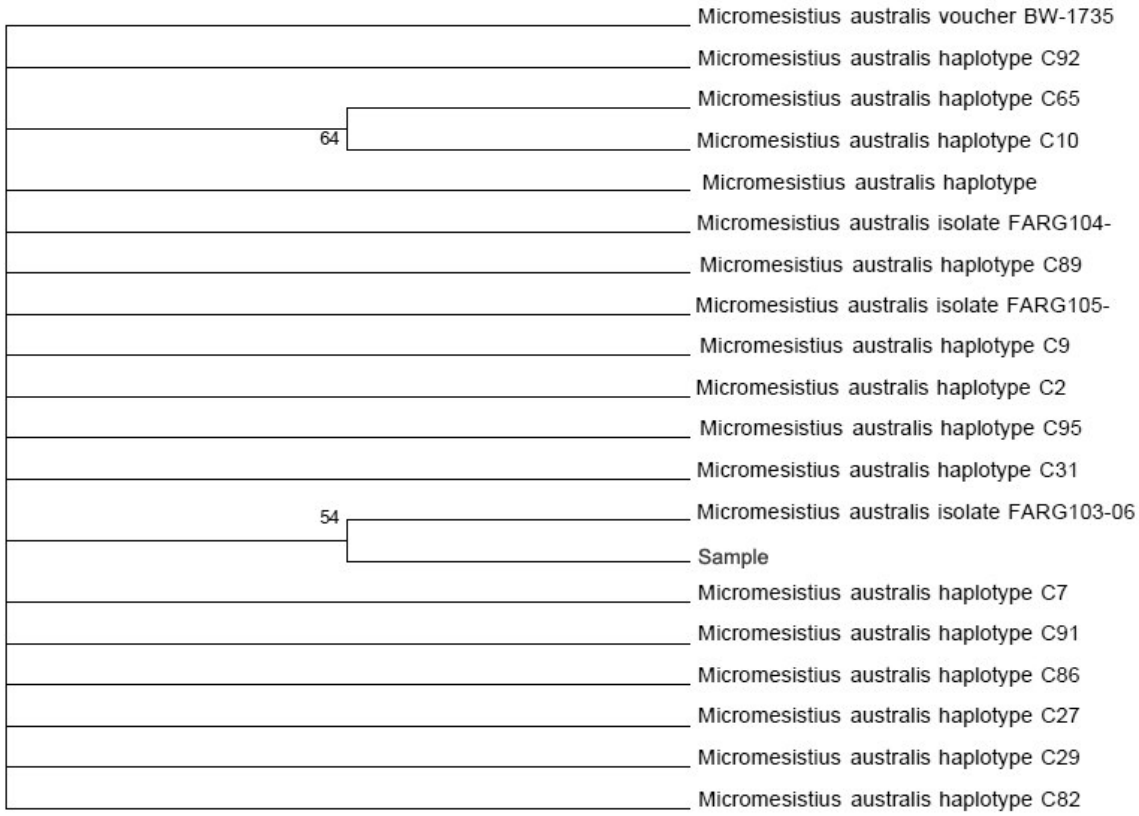


Figure 1. Phylogeny tree for mislabel sample.

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

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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 (*Scombers 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 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 (Guallar *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.

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تشخیص گونه‌ای برخی از محصولات عمل آوری شده آبزیان در ایران بوسیله DNA بارکدینگ

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چکیده

این مطالعه در راستای شناسایی ۹ گونه فیله ماهی عمل آوری شده مورد مصرف در بازار ایران می‌باشد. این تحقیق با بکارگیری تکنیک DNA بارکدینگ و استفاده از ژن سیتوکروم اکسیداز (زیرواحد شماره۱) به جهت راستی آزمایی گونه‌ها صورت پذیرفت. بر اساس نتایج بدست آمده نام درج شده بر روی فیله آلاسکاپولاک اشتباه بوده و با توجه به تعداد نمونه‌ها می‌توان ۱۱ درصد محصولات را تقلبی دانست.

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