Species identification reveals mislabeling of important fish products in Iran by DNA barcoding

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Abstract

This study reports on the molecular identification of fish species from processed products which had a priori been classified as belonging to 5 important species in Iran for human consumption. DNA barcoding using direct sequencing of an approximately 650bp of mitochondrial Cytochrome oxidase subunit I (COI) gene revealed incorrect labeling of Narrow-barred Spanish mackerel samples. High occurrence of fraudulent fishery products, if left unchecked, can pose a negative impact on the economy. This investigation adds further concern on the trading of processed fish products in Iran from both health and conservation points of view.

Keywords: Food traceability, Forensic genetics, DNA barcoding, Fish product

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Introduction

Trade in aquaculture products especially in particular species of fishes has increased dramatically in recent decades. According to FAO statistics, in 2009 totaled 144 million tons(Barbuto et al., 2010a). The development of food processing industries, especially in diverse types of marine provide possibilities of unscrupulous manufacturers of such products to substitute more expensive consumer-friendly species with low-value species. Products are given fake labels and sold to consumers who are unaware that they were not getting value for their money. This is especially the situation for marine products which are in frozen or fillet forms. In some instances the fishes processed into such fraudulent products are captured from polluted marine areas and are not marketable fresh, they may also cause health problems to the consumers. The need for precise quality control and identifying the species used in the products is absolutely essential. Among the different methods of fish identification, molecular genetics techniques are now widely used.

In the past, some of the most applied molecular markers were PCR–RFLP of Cytochrome b gene of Cytochrome b gene, random amplified polymorphic DNA (RAPD) fingerprinting (Asensio et al., 2002), single strand conformation polymorphism (SSCP) (Cespedes et al., 1998; Hold et al., 2001; Sanjuan and Comesana, 2002). More recently Amplified Fragment Length Polymorphisms (AFLPs) have been used to investigate genomes of different complexities (Watanabe et al., 2004; Papa et al., 2005; Gonzales Fortes et al., 2008).

In recent years molecular barcoding has been universally regarded as the best methodology in forensic taxonomy (Dawnay et al., 2007). The DNA barcoding is based on the designation of a 650 bp mtDNA fragment of Cytochrome oxidase I (COI) gene to act as a‘‘barcode” to identify and delineate all animal life (Ward et al., 2005; Roe and Sperling, 2007) Nowadays, by choosing this standard DNA fragment shared among multiple research groups, the efforts have been coordinated, and a more comprehensive library of DNA sequences of thousands of species is available. The DNA barcoding has successfully 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, which is the first of its kind to be performed in Iran, five species of fish, including the Narrow-barred Spanish mackerel, Gold-spotted Sweetlips, Southern meagre, Black pomfret and Japanese threadfin bream were selected.

Verification for the identity of the packaged product was conducted, using mitochondrial Cytochrome oxidase sequencing method. The products analyzed in this DNA barcoding study were mainly caught from the Persian Gulf and Oman Sea and are the best selling frozen or fillet products in the stores. The present study

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act as a starting point of to test the utility of this method in identifying fraudulent imported fish products into Iran. Such efforts should be done for other food products; especially other processed fish species and regulatory agencies should implement and enforce DNA barcoding in a systematic and comprehensive manner to prevent fraud in food products.

Materials and methods

The 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 such as 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 3000 kg (3 stores were considered in this study, with a total of approximately 1000 kg of fish each).

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

3 – The D = NPL value is calculated as D = 3000 × 0.05 = 150.

4- The closest value to the calculated D is found in the LTPD table. The value of f is determined from the corresponding row and column of the table.

5- The Lot number is determined by n = sample size = f × N

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

f = 2.303/D = 2.303 ÷ 150 = 0.0153

n = f × N = 0.0153 × 3000 = 46kg

According to the calculations showing a 3000 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 9 samples is equal to 2.7 kg. All muscle tissue samples were fixed in 100% ethanol alcohol after collection, and were sent to the laboratory.

Muscle tissues were dissected then treated with proteinase K. DNA extraction was carried out using phenol-chloroform then the DNA was precipitated with absolute ethanol(Infante et al., 2006).

DNA quality and extraction yield were assessed by means of 1.2% agarose gel electrophoresis in TE buffer.

An approximately 650 bp of the mitochondrial Cytochromec oxidase I were amplified using different combinations of the primers (Ward et al., 2005):

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FishF1-
50TCAACCAACCACAAAGACATTGGCAC
30,
FishR1-
50TAGACTTCTGGGTGGCCAAAGAATCA
30,

The 25µl PCR reaction final volume included 18.75µl of ultrapure water, 2.25µl of 10X PCR buffer, 1.25µl of $MgCl_2$ (50mM), 0.25µl of each primer (0.01mM), 0.125 µl of each dNTP (0.05mM), 0.625 U of *Taq* polymerase and 0.5–2.0µl 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 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed in turn by 10 min 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 ABI sequencing.

Products were labelled using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems Inc.) and sequenced bidirectionally using an ABI 3730 capillary sequencer following the manufacturer's instructions. Sequences were aligned using CLUSTAL X v.2.0 software (Applied Biosystems Inc.). Sequence divergences were calculated using the Barcode of Life Data Systems (BOLD) identification engine.

Results

The mitochondrial Cytochrome oxidase I region of all samples were successfully PCR-amplified. 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, 15 sequences, representing nine presumed species, had a range of 99 to 100 % maximum identity with the database with a single exception. These were three samples labeled as Narrow-barred Spanish mackerel sample which was matched to a different species namely the Japanese Spanish mackerel, *Scomberomorus niphonius* via BOLD at 99.6% similarity (Table 1, Fig. 1).

Table1: List of all samples in this case

Sample No.	Sold as		BOLD reference subset	Note
A1	Narrow-barred	*Scomberomorus*	*Scomberomorus*	**Mislabeled**
A2	Spanish mackerel	*commerson*	*niphonius*	
A3	fillet		(99.6%)	
B1	Gold-spotted	*Pomadasys hasta*	*Pomadasys hasta*	**OK**
B2	Sweetlips fillet		(99.7%)	
B3				
C1	Southern meagre	*Argyrosomus*	*Argyrosomus hololepidotus*	**OK**
C2	fillet	*hololepidotus*	(100%)	
C3				
D1	Black pomfret fillet	*Parastromateus*	*Parastromateus niger* (100%)	**OK**
D2		*niger*		
D3				
E1	Japanese threadfin	*Nemipterus*	*Nemipterus japonicus* (99.8%)	**OK**
E2	bream fillet	*japonicus*		

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E3

Search Request:

Type : COI SPECIES DATABASE

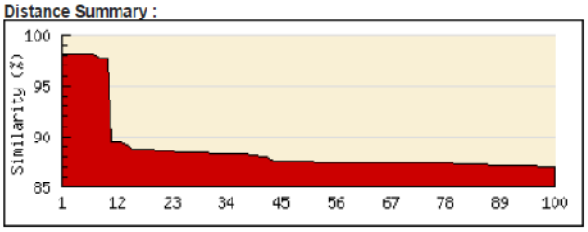
Search Result:

Identification Summary :

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
phylum	Chordata	100
class	Actinopterygii	100
order	Perciformes	100
family	Scombridae	100
genus	Scomberomorus	98.1

A species level match could not be made. The nearest neighbor is Scomberomorus niphonius.

Distance Summary :



Similarity scores of the top 100 matches

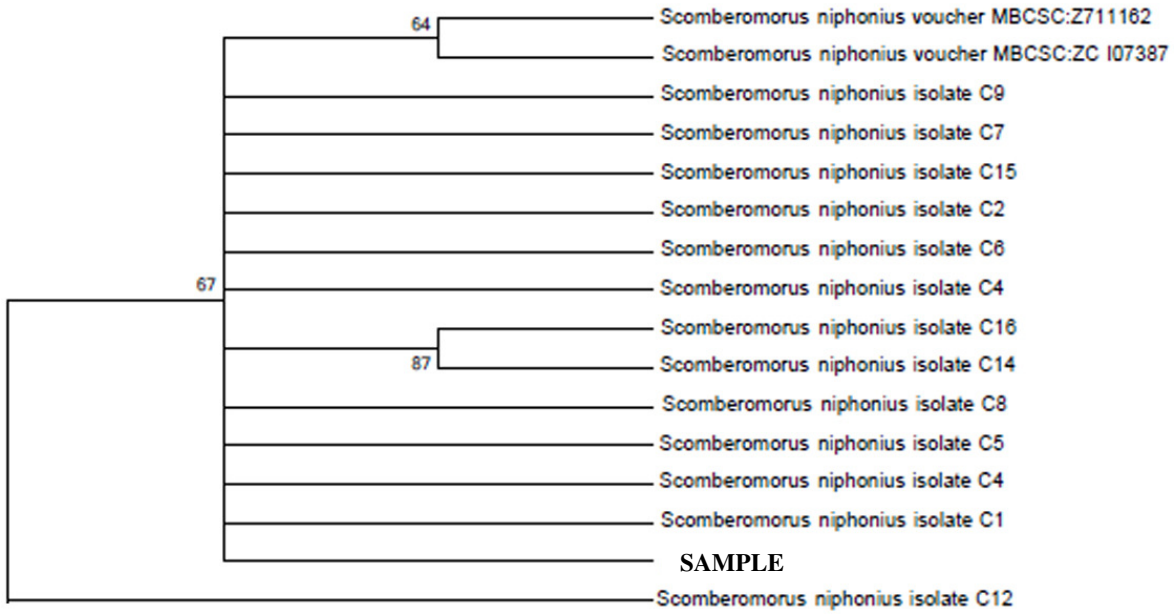


Fig.1: Phylogeny tree for mislabeled sample

Discussion

One of the problems in identifying processed marine products is the absence of diagnostic morphological features such as skin pattern, and shape body skin pattern, shape and body size, shape and number of the fins etc. Thus, developing techniques which enable accurate species identification in such products is absolutely essential. Today, especially in developed countries the use of molecular techniques such as DNA Barcoding which have higher resolution compared to other methods are recommended for species detection in such processed products (Wong and Hanner, 2008). This method was used in this study for the first time on imported fish fillet.

Our study revealed that all products investigated were correctly labeled except for Narrow-barred Spanish mackerel fillet (label

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did not include scientific name). This had been substituted by the Japanese Spanish mackerel (*Scomberomorus niphonius*), demonstrating insufficient control and security of fish products derived from local and foreign fisheries. From an economical point of view, most cases of described mislabeling involved species with a low market value, which were sold as expensive and valuable ones? Such cases should be considered as serious commercial frauds.

In addition, the species identified through our molecular investigations have different nutritional properties compared to those declared. As for short fin mako, a cartilagineous fish similar to the smooth-hound, sold as swordfish which is considered particularly suitable for low fat diets (Wong and Hanner, 2008).

There is always the possibility that manufacturers of such products replace low-value species rather than more expensive consumer-friendly species and use fake labels on their products to receive more profit, so unsuspecting consumers pay more than the actual value of the products. For example, Atlantic mackerel (*Scomber combrus*) is a common market friendly and expensive species in Spain, and it is used in the canned tuna industry. However, due to the high price of this species, some factories use less valuable species of tuna fish instead, and sell their fraudulent products labeled as Atlantic mackerel (Infante et al., 2006).There is always concern that labels of some marine fish products such as, Alaska Pollock which is an imported and valuable fish does not comply with its contents. 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, presences of pork sausages imported into Malaysia have 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 fillet instead of valuable species fillets 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 to be mentioned that in most cases fish products came from outside the European region, without stringent 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 have been subjected to repeated commercial prohibitions, because of its provenience from polluted African waters. In particular, the poisoning by Methyl mercury, 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

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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 important imported 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 مهمترین محصولات عمل آوری شده آبزیان در ایران بوسیله DNA بارکدینگ

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چکیده

این مطالعه به شناسایی ۵ گونه فیله ماهی داخلی عمل آوری شده مورد مصرف در بازار ایران پرداخت. 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 ماهی شیر اشتباه بوده و از فیله ماهی قباد در آن استفاده شده که با توجه به تعداد نمونه‌ها می‌توان ۱۱ درصد محصولات را تقلبی دانست.

کلمات کلیدی: ردیابی غذایی ، DNA Barcoding ، فیله آبزیان

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