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RESEARCH ARTICLE
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Investigating the Diversity of Fish used in Canned Tuna in Iran using Cytochrome Oxidase 1 Marker

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ABSTRACT

Canned fish production industry is one of the big industries of Iran, especially in the southern coastal provinces of Iran. A wide variety of types of fish are caught in the Oman Sea and the Persian Gulf. In the meantime, various types of fish, especially tuna, are used to produce canned food. Some fish can be canned in spite of processing by improving their rheological characteristics in the factory, which unfortunately are sometimes offered instead of tuna in canned food. In this study, the aim is to accurately identify the fish species used in canned tuna, which may not match the information on the product. For this purpose, identification of fish meat was done by DNA barcoding method using cytochrome oxidase 1 marker. Samples were collected from different Iranian cities from different canned tuna brands. DNA extraction from the studied tissue was done from each studied canned sample. Then, using the primers designed for the cytochrome oxidase 1 gene, the PCR reaction was performed to amplify the desired gene. Thus, after DNA extraction, its quality and quantity were checked by agarose gel electrophoresis. The extracted DNA was amplified using appropriate primers. The PCR product was analyzed and sequenced using agarose gel electrophoresis. The fishes identified in the cans include *Thonnus tonggol, Thonus albacares, Thonus abesus, Sarda orientalis, Sillago sihima, Sillago indica, Sillago robusta, Sillago arabica, Sillago attenuata*. These species differed from the species listed on the can, which mainly included *Gaider, hoover*. According to these results, it is suggested that the fish species observed by the DNA barcoding method in canned tuna should be included on the cans with their own title due to their sensory acceptability by consumers and in the list of fish that can be caught and canned be offered to fishermen.

Keywords: DNA barcoding, Canned fish, Species diversity, Cytochrome oxidase 1.

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INTRODUCTION

Tuna is one of the types of saltwater fish, which is one of the most consumed fish in the world and has a very high economic value. Tuna is sold as frozen, canned and fresh fish in more than 70 countries, which is mostly consumed in canned form in Iran (Aberoumand, 2024). Like other countries, in Iran, the consumption of canned fish, especially canned tuna fish is preferred to the consumption of fish due to the easy and convenient use of tuna fish instead of fresh fish (Ganjavi et al., 2010, Aberoumand & Baesi, 2023).

The canned fish industry in Iran has a production capacity of 134 units, with almost 717 million cans potentially produced annually. The industry's operational capacity is 564 million cans per year. The industry's development has been such that out of 33 fishery processed foods, as many as 12 products are canned ones (Iranian Fisheries Statistical Yearbook, 2018; Adeli et al., 2024).

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The fish species processed to canned tuna products in Iran mainly include yellowfin tuna (*Thunnus albacares), long-tailed tuna (Thunnus tonggol), Katsuwonus pelamis* and *Euthynnus affinis (*Kawakawa*)*. Different species of canned tuna have completely different costs. In addition economic value, there are also various contents of toxic substances in different species of canned tuna, such as mercury and histamine.

According to food and drugs administration (FDA), producers must mention the species of fish used in producing canned foods. Reliable methods to identify fish species for preventing from wrong labelling in fish market is an issue that seems vital (Cermakova et al., 2023; Pecoraro et al., 2020). Recently, a food fraud regarding tuna samples has been detected in Europe (Hernández et al., 2023). Seafood fraud involves mislabeling and false claims. To avoid this deceptive practice, authentication techniques, including DNA-based methods, have been developed (Lorusso et al., 2024).

DNA barcoding is a method that implements genetically dynamic sequences of DNA with low intra-species but high inter-species diversity in order to differentiate between the species. It is used as a practical method for tracking food-related materials. That is why different biological indicators are used for detecting the fish (Dawnay et al., 2007; Razavai, 2018; Jin and Gil, 2023; Gostel and Kress, 2022). Using cytochrome genes is a wise choice and decision for various species of fish (Cortes-Miranda et al., 2024; Galimberti et al., 2013). In comparison to core genes, mitochondrial DNA (mtDNA) is more suitable for DNA barcoding due to massive reproduction and multiplication, lack of intron, low recombination and maternal heredity (Figura et al., 2024; Fernandes et al., 2017).

Technology of stuffing canned tuna is done in brine and in oil. In Canned prepared in brine, 4% brine used instead of oil. They are autoclaved at the end of processing phase. This does not exist in canned tuna that is produced in oil. Similarly, in the production of this type of canned tuna, two types of meat are used. Those canned foods are like a piece of meat. This is how the fish meat is molded and placed in metal cans after cooking. Regarding the canned tuna with chopped meat, the extra pieces of meat are put and piled in a big tray after being molded for one piece of cans and then are placed in metal cans either automatically or manually by special workforce (Nicolè et al., 2013).

In this study we developed PCR method to identify the species of fish for two types of canned tuna in oil and brine, and then used this method to investigate species of several commercial canned tuna products in Iran.

MATERIALS & METHODS

Collecting Samples

For this purpose, common brands existing in canned tuna fish were collected. After investigating the features on the cans, information such as type of the species as well as method of processing the canned tuna such as the conserved tuna in brine or in the oil along with type of the meat (one piece or chopped meat) were recorded. Out of any brands, at least three samples were obtained. All samples were stored in - 20°C until use (Eszterbauer et al., 2025; Maleki et al., 2017).

DNA Extraction

100 canned tuna were used to extract DNA. 25 milligrams of canned meat were crashed by liquid nitrogen. Afterwards, it was extracted by xPLUS produced by Cynoclone Company and was extracted based on the instruction of the manufacturing company (Tumerkan, 2022; Ward et al., 2005).

Constructing the PCR Primers

For this purpose, cytochrome oxidase 1 gene was used, which is a key region in detecting individuals and is mainly used in barcoding. Primers were synthesized based on the information available in different sources. Sequencing of this gene is almost universal in all the aquatic organisms. These primers included FishF2_t1, FishR2_t1, FR1d_t1, L5956, H6558, VF2_t1 and Mini Barcode, which were obtained from previous studies (Inoue et al., 2001; Parkhemi-nejad et al., 2013). The sequences of the primers are given in Table 1.

Evaluation of the Quantity of DNA

For this purpose, spectrophotometric method was used. The extent of light absorbance of the samples in the wavelength of 260-280 nanometers and in relation to A280/260 was measured by the machine and recorded. If the amount of dilation A1/A2 is1.8, the extracted DNA has a good quality. If this is more than 1.8, DNA has RNA impurity. However, if this is less than 1.8, it shows it is a sign of phenol and protein impurity. Protein has high absorption in 280 nanometers and polysaccharides have high absorption in 230 nanometers. Therefore, the degree of contamination of the product to protein and carbon hydrates was detected through the average of these absorption (Reinmuth-Selzle et al., 2022; Aryainejad et al., 2017).

Evaluation of the Quality of DNA

For this purpose, horizontal electrophoresis was used. For qualitative evaluation of the extracted DNA, 1% of agarose gel was used. 0.4 grams of agarose were mixed with 40 milliliters of TAE (Tris-Acetate-EDTA) buffer and the gel was prepared by means of microwave. Afterwards, it was poured into the tray after its temperature was reduced. The sample of DNA was transferred to small holes located on the gel. Based on DNA electrical load, its movement from negative to positive was examined. To observe DNA on the gel, DNA Safe Stain was used (Miandare et al., 2013; Kitpit et al., 2014).

PCR Amlification

For PCR reaction, Master Mix Kit for PCR was used. Preparation of the samples for PCR was made based on the instruction of the manufacturing company and The PCR reaction mixes included 15µl of PCR Master Mix, 1µl of template DNA, 1µl of each primer and 7µl of the sterilized

Table 1: Primers used in investigation of DNA barcoding for detecting any frauds in canned tuna

Code	Primer	Sequence
VT	VF2_t1	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC
FFT	FishF2_t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC
FRT	FishR2_t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA
FT	FR1d_t1	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA
LC	L5956-COI	ACAAAGACATTGGCACCCT
HC	H6558-COI	CCTCCTGCAGGGTCAAAGAA
MB	MiniBarcode	ATCACAAAGACATTGGCACCCT

deionized water. The mixture was mixed for 10 seconds, and then, the micro tube consisting of the materials were placed into PCR machine. Polymerase chain reaction was implemented as follows:

Five minutes of primary Denaturation phase and then 35cycles consisting of 30 seconds in 94°C, 45 seconds in 50°C , 1 minute in 72°C. Eventually, we had 10 minute of final Extension step in 72°C. Finally, PCR product was loaded on Agarose gel containing DNA Safe Stain. After that, electrophoresis was run in TBE buffer for 40 minute and DNA bands photographed by GEL DOC (Biord .XR model). Finally Purified PCR products sent for sequencing (Kolengi et al., 2012; Parkhemi-nejad et al., 2013).

Sequencing and Species Identification

After sequencing the samples, the sequences were evaluated by Bioedit and MEGA. Likewise, in order to measure the authentication of the sequences, the sequences were first evaluated in the gen bank by means of BLAST. Finally, the data was compared by means of BOLD, and the species of the fish used in the canned tuna were determined (Miandare et al., 2013).

Analytical Methods

This research is a qualitative research in terms of data analysis in order to express or explain the degree of mislabeling. Also, because this research describes the characteristics and specifications of canned tuna and sought the possibility or impossibility of detecting meat in each product by DNA barcoding method, and also the presence or absence of expected fish in each canned food, this is a descriptive research. Excel software was used to draw graphs and calculate percentages (Miandare et al., 2013).

RESULTS

The primers used for PCR in this research were examined in previous studies. It was revealed that among the primer used, the Mini barcoding primer was the specific primer and all the reproductions were based on this primer. The results showed that when the sizes of pieces (particles) were 700bp, they were not reproduced appropriately. Therefore, the DNA was cut into smaller fragments of 260-280bp in length.

The electrophoresis of the PCR product using the MB primer worked specifically for most of the samples and this issue was observed in the gel electrophoresis images (Fig. 1). Fig. 1 showed that the pieces of reproduced DNA were appropriate pieces with no fracture or interruption and the pieces obtained from this reproduction had a good quality. So the samples were sequenced. The results of the sequencing showed that out of 100 canned samples examined, 20 mislabeling were reported. In 85% of the cases, this discrepancy was seen in the canned stuffed with minced meat. And 15% were observed in canned tuna stuffed with one piece meat. According to the results out of 100 cans examined, 80 cases had a 97% of genetic similarity to tuna fish. However, in 18 cases, 90% of similarity with *Sillago sihama* was observed. This type of fish lives in low-depth areas of bays, gulfs and seas.

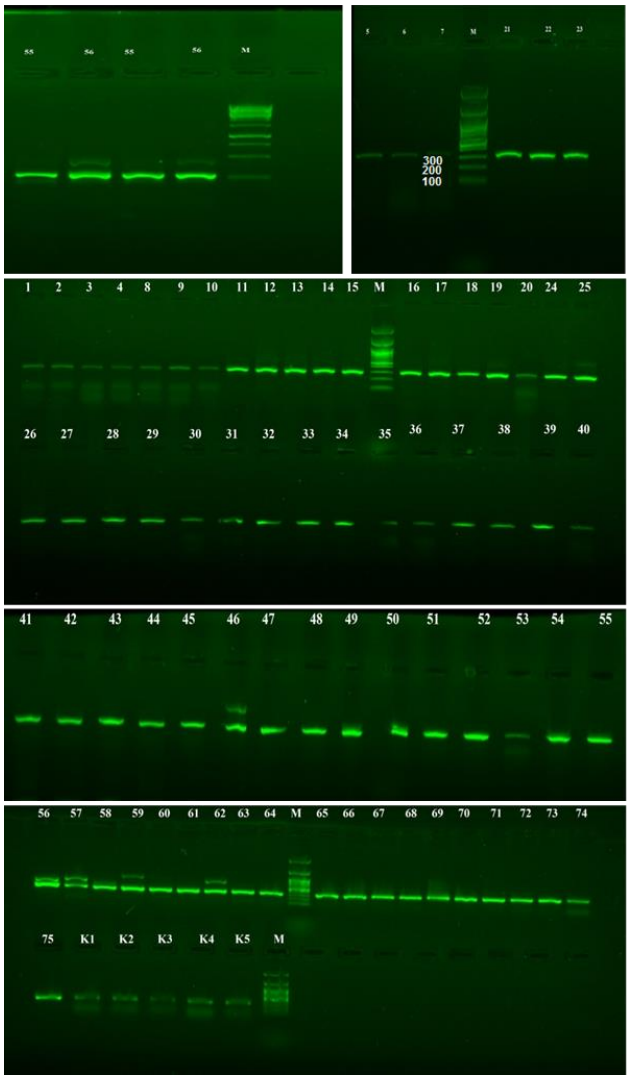

Fig. 1: The results obtained from gel electrophorese of the samples.

One sample had 83% of similarity to *Pristolepis rubripinni*. This type of fish was detected by Britz in 2012 in the waters of southern Indian Ocean. This type of fish is closest to Gourami fish. Likewise, 1 sample out of 20 cases had 75% of similarity to *Halichoeres hortulanus* belongs to waters of Indian Ocean that sometimes randomly enter

into Persian Gulf and Sea of Oman waters. Generally, in this authentication, the labels on the canned tuna were 80% true, and 20% mislabeling had been observed. It was observed that the most fraudulent cases belong to *Sillago sihama*. Since these types of fish live in low-depth areas, it is natural to get fished with tuna fish (Fig. 1).

Also, the results of investigation of non-compliance of canned fish, based on the type of meat and the type of processing are given in Table 2 and 3. It was observed out of 11 fraudulent cases in the canned tuna fish with crashed meat and processed in brine, 9 cases belonged to *Sillago sihama*, 1 case belonged to *Halichoeres hortulanu* and 1 case belonged to *Pristolepis rubripinni*. These cases show that the fraud was unintentional, because *Sillago sihama* in tuna fishing areas is more than other species and the low number of other species is negligible (Table 4).

Table 2: The results of the investigation of the non-compliance of canned tuna with the label of different processing

Rejected samples (%)	Non matched samples	Total number	Product
24	12	50	Tuna fish in brin
16	8	50	Tuna fish in oil

Table 3: The results of the investigation of the non-compliance of canned tuna with the label of different kind of meat

Rejected samples (%)	Non matched samples	Total number	Product
32	16	50	Chopped meat
8	4	50	One piece meat

Out of 6 cases of fraud in the canned tuna stuffed with chopped meat in oil, all the cases belong to *Sillago sihama* (Table 5) and out of 2 cases of fraud in canned tuna fish with one piece meat in brin, both cases belong to *Sillago sihama* (Table 6). Only one case of fraud in the canned tuna fish with one piece meat in oil belongs to *Sillago sihama* (Table 7). It was revealed that the most common fraud was in the canned tuna with crashed meat in brine (Fig. 2).

DISCUSSION

Authenticity and traceability of food products in all the levels of production process, from raw materials to final products are substantially important. But the problem is that some manufacturers replace expensive products by low value spices to get more profit (Hoffman et al., 2024; Jil and Gil, 2023). In recent years, DNA barcoding has been used to investigate unrelated labels of marine fish products (Senathipathi et al., 2024; Barcaccia et al., 2015; Fiorino et al., 2018; Fernandes et al., 2021). In addition, DNA barcoding has promising and useable potential for detecting species in processed products like canned tuna (Emmi et al., 2023; Mirkhani et al., 2012).

The advantage of this technique over protein analysis is that although DNA can be changed with different processing (such as conserved and heat treatment), it has a higher resistance against heat in comparison with proteins. Furthermore, DNA can potentially be revived from any sample, because it is present in almost all living cells (Kitpit et al., 2014, Wang et al., 2024). So far, the detection of existing species in foods via DNA barcoding has been successfully done in many different studies regarding sea products (Filonzi et al., 2023; Barcaccia et al., 2015; Fernandes et al., 2021).

Out of 9 main DNA indicators, apparently the majority of studies have focused on mitochondrial genes, specifically cytochrome oxidase 1. Since there are various

Table 4: The fraudulent detected species in samples of canned tuna fish with chopped meat of fish prepared in brine

tuna fish	Type of the meat	Features of the sample	Length of the piece	The degree of similarity (%)	The detected species	Access code of gene bank
89-141686-1-R	chopped	tuna fish	266	75	*Halichoeres hortulanus*	JF434990.1
90-141687-2-R	chopped	tuna fish	269	94/78	*Sillago sihama*	JF494530.1
91-141688-3-R	chopped	tuna fish	266	90/32	*Sillago sihama*	MN512097.1
92-141689-4-R	chopped	tuna fish	268	96/94	*Sillago sihama*	92-141689-4-R
42-144989-5-R	chopped	tuna fish	266	97/90	*Sillago sihama*	JF494530.1
29-143150-16-R	chopped	tuna fish	263	99/18	*Sillago sihama*	JF494530.1
30-143151-17-R	chopped	tuna fish	262	98/35	*Sillago sihama*	JF494530.1
20-143155-18-R	chopped	tuna fish	263	99/18	*Sillago sihama*	JF494530.1
21-1413156-19-R	chopped	tuna fish	261	83/13	*Pristolepis rubripinnis*	MG923398.1
22-143157-20-R	chopped	tuna fish	267	97	*Sillago sihama*	JF494530.1
24-143159-22-R	chopped	tuna fish	263	99/57	*Sillago sihama*	JF494530.1

Table 5: The detected fraudulent species in the samples of canned foods with chopped meat of tuna fish prepared in oil

tuna fish	Type of the meat	Features of the sample	Length of the piece	The degree of similarity (%)	The detected species	Access code of gene bank
93-141690-5-R	chopped	tuna fish	267	99/57	*Sillago sihama*	JF494530.1
32-143066-6-R	chopped	tuna fish	267	98/72	*Sillago sihama*	EF609617.1
31-143070-8-R	chopped	tuna fish	266	96/17	*Sillago sihama*	EF609617.1
26-143147-13-R	chopped	tuna fish	266	96/17	*Sillago sihama*	EF609617.1
27-143148-14-R	chopped	tuna fish	265	96	*Sillago sihama*	EF609617.1
28-143149-15-R	chopped	tuna fish	265	95	*Sillago sihama*	EF609617.1

Table 6: The detected fraudulent cases in the samples of canned fish with one piece meat of tuna fish prepared in brine

tuna fish	Type of the meat	Features of the sample	Length of the piece	The degree of similarity (%)	The detected species	Access code of gene bank
43-144990-20-R	One piece	tuna fish	271	99/95	*Sillago sihama*	JF494530.1
23-143158-21-R	One piece	tuna fish	263	99/57	*Sillago sihama*	JF494530.1

Table 7: The detected fraudulent cases in the samples of canned foods with one piece meat of tuna fish prepared in oil

tuna fish	Type of the meat	Features of the sample	Length of the piece	The degree of similarity	The detected species	Access code of gene bank
25-143160-23-R	One piece	tuna fish	263	99/57%	*Sillago sihama*	JF494530.1

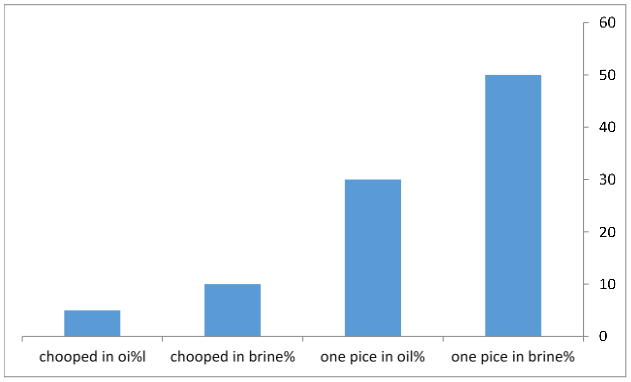

Fig. 2: The amount of incompatibility in Can contents with label information in the studied canned tuna in different treatment.

versions of mitochondrial DNA inside a cell, it most probably reinforces the piece inside this genome more than core genome. Generally, mitochondrial DNA evolves very much faster than core DNA. Therefore, it provides segregation and even detection of very close species to each other. For the above reasons, the majority of studies focus on mitochondrial DNA genome (mtDNA) rather than core DNA (Dowling and Wolff, 2023; Pazhenkova & Lukhtanov 2019; Riley et al., 2020).

In this authentication, the labels on the canned tuna were 80% true and 20% were observed as incompatible. In compliance with the results of this research, Mirkhani et al (2012) investigated the existence of ordinary long-tailed fish in 48 samples of canned tuna in Tehran fish market by means of DNA barcoding. In this method, DNAs of the samples were extracted and the quantity and quality of the extracted DNA were determined by spectrophotometry and agarose gel methods. Polymerase chain reaction was done by fish general primers and, then, PCR products were sequenced. The results suggest the existence of *Platypterus istiphorus*, *Coryphaena hipprus* and *Auxis thazard* species. The main sample that should exist in canned foods was 23% (Mirkhani et al., 2012). Of course, in the current research, the most common type of fraud was observed in the canned tuna with chopped meat

The main reason for this result is that in this method, the leftover pieces of meat from other species left during processing have a good potential to be preserved and are combined with the pieces of tuna meat without being detected.Since the detection of meat after being cooked is difficult due to its appearance changed (Razavai 2018). This fraud might have been unintentional. For this problem, it is recommended that a section be devised in canned tuna manufacturing companies so that the conserved (canned) foods are randomly used for investigating the unintentional fraud and the tests are implemented with different molecular methods.

In the present research, although DNA pieces might have been broken due to high temperature caused by the autoclaves of the canned and osmotic pressure due to brine, mitochondrial DNA (Cytochrome Oxidase 1) is much more durable and resistant than cellular DNA. In the present study and by means of cytochrome oxidase1 gene, which was a resistant mitochondrial DNA piece, we were able to find out about the frauds in different canned tuna fish in many different brands existing in the Iranian market. Although these frauds, are 20% and are probably unintentional, since it was incompatible with what had been written on the cans, serious pursuit is required to be made.

Also, in research conducted in Indonesia in 2016 for four tuna products (canned sushi, fish meat ball, fish floss), the cytochrome oxidase gene was used to identify the type of fish used in the product. And miss lable was reported only for canned tuna.

Among the examined primers, one primer could work specifically for the examined samples.

In compliance with the findings of the present research, Kelengi et al. (2012) investigated the detection of fraud among all types of caviar fish. For this purpose, the DNA extracted from the samples by means of three primers, including R1, F1a and F2a, that were designed based on Cytochrome b gene, were reproduced through PCR (Nurilmala et al., 2016).

Conclusion

In conclusion the osmotic pressure and heat during the processing of canned tunna in brine reduces the quality and quantity of extracted DNA, and this reduces the accuracy of the PCR method in detecting the type of species. But the observation of this research confirmed that the materials and conditions used in the canning technology did not have a negative effect on the polymerase chain reaction and the identification of the fish species, which is due to the thermal stability of DNA (mitochondrial gene). Finally, the findings of the research showed that there is fraud in canned tuna products in the country and DNA barcoding is a powerful and valuable method to identify the species of canned tuna and should be applied in Iranian market.

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Approval of the Submitted Manuscript: Mona Aivaz, Mehdi Zolfaghari, Mojtaba Nasr Esfahani, Hamed Paknejad

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