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Identification of Gadoid Species in Fish Meat by Polymerase Chain Reaction (PCR) on Genomic DNA

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Identification of fish species is significant due to the increasing interest of consumers in the meat of sea fish. Methods focusing on fish species identification help to reveal fraudulent substitution among economically important gadoid species in commercial seafood products. The objective of this work was to develop a conventional PCR method for the differentiation of the following gadoid fish species in fish products: Alaska pollack (*Theragra chalcogramma*), blue whiting (*Micromesistius poutassou*), hake spp. (*Merluccius* spp.), Atlantic cod (*Gadus morhua*), saithe (*Pollachius virens*), and whiting (*Merlangius merlangus*). The species-specific primer pairs for gadoid species determination were based on the partial *pantophysin I* (*PanI*) genomic sequence. Sequence identification was confirmed by cloning and sequencing of the PCR products obtained from the species considered. For the simultaneous detection of Alaska pollack, blue whiting, and hake spp., a quadruplex PCR system was constructed. Other gadoid species were detected in separate PCR reactions. After optimization of the reactions, the developed PCR systems were used for the analysis of codfish samples obtained from the Czech market and the customs' laboratories. This method represents an alternative approach in the use of genomic DNA for the identification of fish species. This method is rapid, simple, and reliable without the need for further confirmative methods. Furthermore, the identification of a mixture of more than one species is possible. The PCR system has been optimized for routine diagnostic purposes.

KEYWORDS: Codfish; fish species identification; pantophysin I gene; conventional PCR; food adulteration

INTRODUCTION

Seafood and their products present a commodity in which all of the characteristics are defined by law and where occurrence of adulteration is controlled. Particularly, it is concerned with the distribution of lower quality fish species labeled as high-quality ones. Because most of the fish products are imported to the Czech Republic as frozen blocks of the compressed meat or fillets, morphological identification is impossible. Council Regulation 104/2000 of the European Parliament (*1*) with effect from January 2002 in the European Union directs obligatory data in labeling fishery products, mainly the name of the species of fish, the geographical location where they were captured, and the production method (wild or cultivated).

Inspection authorities need fast and reliable methods that allow the processing of a large number of samples for speciation analysis to avoid this type of economic fraud and protect consumer rights. The prevention and control of substitution and adulteration is complicated by the growth of international trade and the increasing use of fillets or minced fish as raw material for the manufacture of seafood products by the processing industry (*2*).

With regard to the amount of gadoid fish imported to the Czech Republic and problems with taxonomy and different values of respective fish species, substitution of species of high commercial value (Alaska pollack) for species of less commercial value (hake species) is possible. Hake sp., Alaska pollack, and blue whiting are most frequently imported. The importance of the species varies with the type of product and country.

Gadoid fish species identification methods based on morphological characteristics are suitable for whole or lightly processed fish; however, the identification of fish species becomes more difficult after their processing and related removal of morphological features (*3–5*). Therefore, analytical methods have been developed for fish species identification. Gadoid fish species identification based on protein analysis, such as isoelectric focusing (*6–8*), SDS-PAGE, or urea IEF (*9, 10*), capillary electrophoresis (*11*), or application of immunological methods

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Table 1. Authentic Species Used in the Study

English name	scientific name	abbreviation	source
Alaska pollack	*Theragra chalcogramma*	Tc	Czech market
blue whiting	*Micromesistius poutassou*	Mp	
whiting	*Merlangius merlangus*	Mm	
hake sp.	*Merluccius* sp.	Msp	
saithe	*Pollachius virens*	Pv	Swedish market
Atlantic cod	*Gadus morhua*	Gm	

(*12*) is still considered to be effective for fish species determination. However, these techniques become less reliable with heat-treated products as with canned or smoked fish (*13*).

DNA based techniques for species identification have recently started to be applied toward a wide variety of fish, including closely related species belonging to the same family or genus. Methods based on DNA analysis, however, present advantages over protein analysis because they are largely independent of tissue source, age, or sample damage (*13, 14*). Additionally, the DNA molecule is more stable to food-processing technologies including heat treatment than proteins (*15*).

DNA analysis, especially Polymerase Chain Reaction (PCR) and its modifications [restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), random amplification of polymorphic DNA (RAPD)], based on the amplification of specific DNA segments, represents a significant improvement in gadoid fish species identification. Mitochondrial DNA is most often used as a template for the PCR analysis. Identification of gadoid fish species using RFLP analysis with various combinations of restriction enzymes has already been reported (*16–20*). In recent years the results of gadoid fish species identification using RAPD (*21*) or SSCP (*22*) were published. A real-time method as a quantitative analysis was developed for the detection and quantification of *Melanogrammus aeglefinus* (*23*). Sequencing of the PCR product allows the most complete information, including fish species identification (*24*), to be obtained. The other purpose of the PCR product sequencing is to confirm the results obtained by other PCR methods (*24–26*).

The main objective of this study was to develop an alternative conventional PCR approach based on the different DNA template rather than the mitochondrial DNA for the identification of six important gadoid species. After searching sequence databases, the partial DNA sequence encoding the integral membrane protein of cytoplasmatic microvesicules *pantophysin I* was chosen (*27–30*). Specific primers were designed and optimized for use in mono- and multiplex PCR reactions. Developed PCR systems were then used for the identification of gadoid fish products that were obtained from the Czech market and from customs' laboratories.

MATERIALS AND METHODS

Gadoid Samples. Reference samples of six gadoid species (one individual per species) were chilled or frozen fillets obtained in the Czech or Swedish market (**Table 1**). Due to the scarce occurrence or even absence of a broader range of seafish species (*Gadiformes*) in the Czech market, the spectrum of fish species analyzed was limited. Due to the absence of morphological features, three different independent methods for the authentication of the reference species were chosen: sequencing, RFLP typing (*17*), and isoelectric focusing. All methods provided us identical results, and we consider them as strong as the morphological identification. Eighty-one commercial gadoid fish products were investigated, of which 64 samples were purchased in the local markets and 17 samples were obtained from the customs' laboratories. All samples were stored at –20 °C until DNA extraction.

Sampling. Commercially available gadoid fish products (fillets or compressed blocks of meat) in original packages were purchased in several supermarkets in the area of Brno during July 2005. Each package was opened, and two randomly selected fillets or meat blocks served as the source of the samples. The customs' samples were taken from three deliveries of fish meat to the Czech Republic, and the samples were taken randomly from different containers containing frozen meat. Each piece of frozen meat was packed separately and was considered to be an independent sample. For the DNA isolation, samples were taken from the middle part of meat after the removal of the surface, resulting in the elimination of contamination caused by handling.

DNA Extraction. The isolation of total genomic DNA from fish muscle was carried out using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The protocol for the purification of total DNA from animal tissues was used with the following modifications: the amount of starting material was increased to 100 mg, the volume of the tissue lysis buffer (ATL), lysis buffer (AL), ethanol, and proteinase K was doubled, RNase treatment of the samples was made by adding a doubled volume of RNase A (100 mg/mL). Washing steps included in the original protocol were performed twice. For the DNA elution 2 × 100 μL of elution buffer (AE) preheated to 56 °C was used.

Estimation of the DNA Quality. The DNA concentration in the extracts was measured by the absorbance at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The quality and purity of isolated DNA were determined by the ratio A_{260}/A_{280}. Only samples with ratios between 1.8 and 2.0 were used for the PCR.

Monoplex PCR Systems. Six sets of primers for the determination of six different gadoid species determination based on the partial *pantophysin I* (*PanI*) genomic sequence were designed (**Table 2**). Thus, each sample was analyzed by six independent monoplex PCR reactions. As a positive control the DNA from the reference species was used. The PCR reaction was performed in a final volume of 20 μL using the ThermoCycler Machine DYAD (Bio-Rad, Munich, Germany). The reaction mix contained 10 μL of PPP Master Mix (Top Bio, Prague, Czech Republic), 10 pmol of each primer set, and 4 μL of the DNA template. The amplification was carried out with an initial denaturing step at 95 °C for 3 min, followed by 35 cycles and each cycle with denaturation at 95 °C for 10 s, annealing at 65 °C for 20 s, extension at 72 °C for 2 min, and then a final extension at 72 °C for 5 min (Czech patent application PV 2007-794).

Quadruplex PCR System. Following the results obtained from the monoplex PCRs, a quadruplex PCR reaction for the identification of Alaska pollack, blue whiting, and hake sp. was constructed. These three species were the only ones detected. Additionally, an internal amplification control (plasmid *F57* BEREN) was used to avoid false-negative results. The plasmid was derived from the *F57* fragment of *Mycobacterium avium* subsp. *paratuberculosis* (Czech patent application PV 2007-295). The optimized reaction was performed in a total volume of 40 μL, including 20 μL of PPP Master Mix (Top Bio, Prague, Czech Republic), 10 pmol of primers for Alaska pollack, 10 pmol of primers for blue whiting, 5 pmol of primers for hake sp., and 10 pmol of primers for plasmid *F57* BEREN, 1000 copies of plasmid *F57* BEREN, and 5 μL of extracted DNA. The reaction mix was enriched with 1.5 mM $MgCl_2$ and 5 mM $(NH_4)_2SO_4$. Ten microliters of each defined DNA of Alaska pollack, blue whiting, hake sp. (10 ng/μL), and plasmid *F57* BEREN (10000 copies) was mixed in 60 μL of AE buffer and served as the PCR positive control. PCR amplification (without initial denaturation) was performed in 45 cycles consisting of denaturing (95 °C for 10 s), annealing (65 °C for 20 s), extension (72 °C for 1 min), and final extension at 72 °C for 10 min (Czech patent application PV 2007-794).

Sensitivity of the Quadruplex PCR System. To determine the LOD of the quadruplex PCR system, DNA from each sample was prepared to a concentration of 1 ng of DNA per microliter. From this solution the decimal dilution up to 1 fg/μL was prepared. Samples of fish fillets from two gadoid species (Alaska pollack/blue whiting, Alaska pollack/hake sp., and hake sp./blue whiting) were mixed together in the total amount of 20 g of muscle. Only binary mixtures were prepared due to the low probability of the occurrence of a mixture of three species in one fish product. Thereby the samples contained 50, 25, 10, 5, and 1%

Table 2. Sequences of Primers Used[a]

organism	name	sequence 5′ → 3′	length of the PCR product	relative position of primers	reference sequence	ref
Theragra chalcogramma	TcPanF1	GCTGGTTGGTTGATATGTTTGG	492	659–680	AY292495	*29, 30*
	TcPanR1	GCAGTAGTTTTAAAATGGGGAACA		1127–1150		
Gadus morhua	GmPanF1	GGCAACTACACTTCGTCAGCAG	361	782–803	AF288943	*27, 28*
	GmPanR2	GCAGTAGTTTYAAAATGGGGAACT		1119–1142		
Micromesistius poutassou	MpPanF	TTCTCATTGTCTCTCCTAACTCTCCTG	350	640–666	AY292496	*29*
	MpPanR	TCGCCATTTAATGTTCCTGAAAGA		966–989		
Merlangius merlangus	MmPanF	GGCATATGTTCATGACTTGTTTCTCC	549	676–701	AY292488	*29*
	MmPanR	TGATGACACTTGGTAAGCAGTCG		1202–1224		
Pollachius virens	PvPanF	GGCAATGTTTCTCCTACTTTAGGTTG	737	699–724	AY292491	*29*
	PvPanR	TACATTAGACAGTGCAGACGTGCAG		1411–1435		
Merluccius sp.	TrPanF	CCCGTTTAGGTTTGTGGTTGC	201	1–21	EU301630	this paper
	TrPanR2	TGTGCAGACGAACTGTGGTTC		181–201		

[a] All primer pairs were designed according to the Primer3 (*32*) synthesized by Generi Biotech (Czech Republic) and are covered by the patent PV2007-794.

Table 3. Preparation of DNA and Tissue Admixtures

%	DNA concentration	percentage by weight of tissue
50:50	1 ng/1 ng	50 g/50 g
25:75	0.5 ng/1.5 ng	25 g/75 g
10:90	0.1 ng/1.9 ng	10 g/90 g
5:95	50 pg/1.95 ng	5 g/95 g
1:99	10 pg/1.99 ng	1 g/99 g

of one species in another. The mixing was performed by grinding with mortar and pestle. In addition, DNA extracts (1 ng/μL) from the same authentic samples were used to prepare DNA admixtures. The percentage rate was identical to that in the tissue admixtures (**Table 3**).

Separation and Visualization of the PCR Products. Separation and visualization of the PCR products were carried out using horizontal electrophoresis in 1.5% agarose gel (quadruplex PCR products in 2.5% gel) on a 0.5× TBE containing 1 μL/mL ethidium bromide. A 200 bp ladder (Top Bio) was used as a marker. The amplification fragments were analyzed under UV light using an Ultraviolet Transluminator (Spectroline, Westbury, NY).

Cloning and Sequencing of the PCR Products. The authenticity of six gadoid species was confirmed by sequencing. The species-specific *PanI* amplicons obtained from six gadoid species were cloned into TA vector pCR2.1 (Invitrogene, Groningen, The Netherlands) according to the manufacturer's instructions. Transformation was performed into *Escherichia coli* Top10F′ competent cells. A blue−white screening test was used for the differentiation of positive and negative colonies. Positive clones (randomly chosen white colonies) were checked by PCR for the presence of the insert using specific and M13 universal primers. Subsequently, positive colonies were put into liquid LB medium. After purification using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), PCR products were sent for sequencing to a commercial facility (MWG Biotech, Ebersberg, Germany).

RESULTS AND DISCUSSION

Species Specificity. Six gadoid species were selectively amplified using species-specific primers and could be differentiated according to the length of the PCR product. **Figure 1** shows the PCR amplification of *PanI* partial sequences from DNA of all six species mixed in one DNA sample (10 ng/μL for each species) using the appropriate set of specific primers. No cross-reaction was observed. The sequences of Alaska pollack, blue whiting, Atlantic cod, whiting, and saithe were identical with the sequences deposited in GenBank. De novo obtained sequences of three hake species were deposited in GenBank (accession no. EU301630, EU301631, and EU301632).

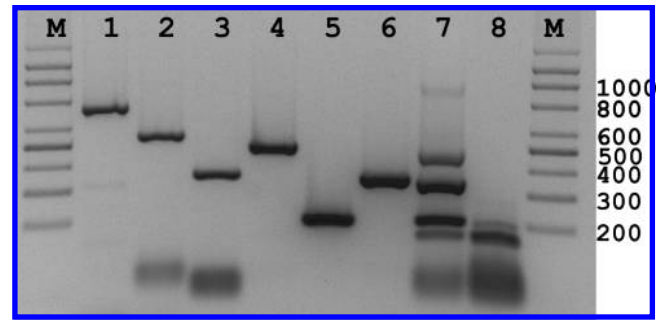

Figure 1. Primer specificity for gadoid species identification: amplification of *PanI* partial sequences from DNA of all six species mixed in one DNA sample (10 ng/μL for each species) using the appropriate set of specific primers. Lane M, molecular size marker (200 bp); lane 1, primers for saithe (737 bp); lane 2, primers for whiting (549 bp); lane 3, primers for Atlantic cod (361 bp); lane 4, primers for Alaska pollack (492 bp); lane 5, primers for hake sp. (201 bp); lane 6, primers for blue whiting (350 bp); lane 7, quadruplex system; lane 8, IAC only (147 bp).

Hake Species Identification. During the development of the method some negative results were observed. No primers for Alaska pollack, blue whiting, Atlantic cod, whiting, or saithe amplified these samples within the stringent reaction conditions. At less stringent conditions primers for Alaska pollack amplified a partial sequence of the *PanI* gene, which was not present in GenBank. This sequence was used for the construction of primers amplifying this unknown sequence. With RFLP analysis of the mitochondrial cytochrome *b* gene followed by restriction cleavage (*17*), three hake species were identified: North Pacific hake (*Merluccius productus*), South Pacific hake (*M. gayi*), and Argentine hake (*M. hubbsi*). These species were confirmed by sequencing of the PCR products obtained by cytochrome *b* amplification (*31*). The PCR system based on *PanI* gene amplification was not able to differentiate individual species of hake. The interspecific identification of hake species using this method could be included in further studies. From the commercial point of view (the substitution of Alaska pollack for hake) the identification of hake species is considered to be sufficient.

Analysis of 81 Gadoid Fish Products by Monoplex PCR Systems. The conventional PCR method was tested using 81 commercial gadoid fish products from different manufacturers and distributors. These samples were either fillets or compressed

blocks of meat. This fact was important because the fillets that originated from one fish showed typical morphological features, whereas the compact blocks of meat were compressed muscle of fish, so there was a possibility of using a mixture of species.

The species identification of all 81 samples is shown in **Table 5**. Fillets from two different species were detected in six packages. Probably, the fillets of more gadoid species were contained in the packing. The remaining 18 samples (from the total 24 samples in which mixed species were detected) were compressed blocks of meat. From the total of 81 samples, 46 were declared as Alaska pollack. Only 6 (13%) of total 46 samples declared as Alaska pollack were confirmed as Alaska pollack. A mixture of Alaska pollack and blue whiting or a mixture of Alaska pollack and hake sp. was investigated in 18 samples (39%). In 22 samples (48%) completely different gadoid species were detected (blue whiting or hake sp.), despite the declaration (Alaska pollack). Fifteen samples were labeled as blue whiting, but only 9 samples (60%) were confirmed as blue whiting. Four samples (27%) were identified as a mixture of blue whiting and hake sp., and one sample (6.5%) was identified as a hake species. Species identification in one gadoid fish product declared as blue whiting (6.5%) was not assigned. Three samples were labeled as hake species, which were confirmed in two cases; the other sample was identified as blue whiting. Only one sample was declared as Atlantic cod, but this declaration was not confirmed. In the remaining 16 samples labeled in common as gadoid fish, the species identification was confirmed except in 1 case. No whiting, saithe, or Atlantic cod was detected.

Analysis of 81 Gadoid Fish Products by Quadruplex PCR System. Only three gadoid fish species (Alaska pollack, blue whiting, and hake sp.) were identified in gadoid fish products using monoplex PCRs. Therefore, a quadruplex PCR system for their detection was constructed. The use of an internal amplification control prevents the detection of false-negative results, which can be caused by the presence of PCR inhibitors or deficiencies in pipetting. If amplification was performed correctly, the amplicon was visible in the gel. If the PCR reaction was inhibited, the amplicon did not appear, and the reaction was of no validity. The quadruplex PCR system was applied to the investigation of 81 commercial samples, previously analyzed by monoplex PCRs. The results of the monoplex PCRs were confirmed by the quadruplex PCR. The IAC was amplified in all reactions, so the negative results received by monoplex PCR were confirmed. This implies that another fish species could be contained in the product.

Sensitivity of the Quadruplex PCR System. A mixture of extracted DNA from Alaska pollack, blue whiting, and hake sp. in a final concentration 1 ng of DNA per microliter was prepared. From this solution the decimal dilution up to 1 fg/μL was provided. The experimental limit of detection of the quadruplex PCR system was determined to be 100 pg/μL of DNA extracted from these three species (**Figure 2**). The application of this method for the detection of gadoid fish species in samples containing more than one species was tested using DNA and tissue admixtures. DNA admixtures were prepared as model samples (**Tables 3** and **4**). For comparison and practical application of this approach tissue admixtures were also produced. In this case thorough homogenization of the mixtures was essential to avoid unequal distribution of the two mixing gadoid species. However, this was not always achieved. In the case of DNA admixtures the detection level of 1% of one species in another was demonstrated. Results of tissue admixture analysis were not always comparable to those

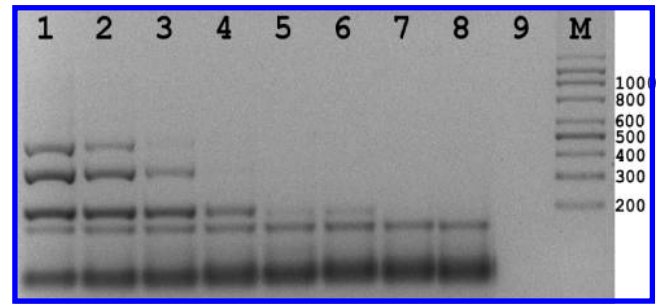

Figure 2. Sensitivity of quadruplex PCR method. Lanes 1–7 contain decreasing DNA concentration in 1 μL of DNA template: lane 1, 1 ng; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 100 fg; lane 6, 10 fg; line 7, 1 fg; lane 8, IAC only; lane 9, negative control; lane M, molecular size marker (200 bp).

Table 4. Results of the DNA Mixtures[a]

	Tc/Mp	Mp/Tc	Tc/Msp	Msp/Tc	Mp/Msp	Msp/Mp
50:50	+/+	+/+	+/+	+/+	+/+	+/+
25:75	+/+	+/+	+/+	+/+	+/+	+/+
10:90	+/+	+/+	+/+	+/+	+/+	+/+
5:95	+/+	−/+	+/+	+/+	−/+	+/+
1:99	+/+	−/+	−/+	+/+	−/+	+/+

[a] +, identifiable; −, not identifiable.

Table 5. Results of 81 Investigated Gadoid Samples

		results						
declared as	no. of samples	Tc	Mp	Msp	Tc + Mp	Tc + Msp	Mp + Msp	−
Alaska pollack	46	6	9	13	10	8		
blue whiting	15		9	1			4	1
hake sp.	3		1	2				
Atlantic cod	1							1
gadoid fish	16		9	4			2	1
total	81	6	28	20	10	8	6	3

received from the DNA admixture analysis. It was possible to detect as little as 1% hake sp. in Alaska pollack or hake sp. in blue whiting. In the cases of mixtures of blue whiting in Alaska pollack and Alaska pollack in blue whiting a limit of 5% was achieved. However, the mixtures of blue whiting in hake sp. and Alaska pollack in hake sp. were not consistent in all percentage rates, although the level of 1% was well detected. These results correspond with those obtained by another study (*4*). The authors prepared DNA admixtures and admixtures from freeze-dried samples of fish fillets. A detection limit of 5% (in some samples even 2%) was assessed. To detect fish species in samples containing more than one species substantial preparation is necessary.

In this study a conventional PCR method based on genomic DNA for the identification of six gadoid fish species has been developed. For this purpose species-specific primer pairs based on the amplification of the partial genomic sequence of the *pantophysin I* gene (*PanI*) were designed. Pantophysin is an integral membrane protein found in small (<100 nm) cytoplasmatic microvesicles that are thought to have a function in a variety of intracellular shuttling pathways (*27–30*). The system has been optimized for the routine diagnostic purpose of authenticating the presence of gadoid fish species in fish products. The method has been applied to imported gadoid fish products. The usefulness of the method for the identification of a broad range of gadoid fish species has been demonstrated.

The identification of multiple species within the products was also shown to be easily achievable.

Every sample is to be analyzed using all four PCR systems (the quadruplex PCR system for the simultaneous identification of Alaska pollack, blue whiting, and hake sp. and three monoplex PCR reactions for the detection of Atlantic cod, whiting, and saithe). The quadruplex PCR system represents the tool of first choice, because the three gadoid species (Alaska pollack, blue whiting, and hake sp.) are the most common in the Czech market. When a negative result is observed, the application of monoplex PCRs for the detection of whiting, saithe, and Atlantic cod is performed. These monoplex PCR systems do not require the use of an IAC. The PCR protocol for the monoplex PCR systems is identical, which is convenient in the analysis of large numbers of samples. In one experiment the simultaneous analysis of the samples by all monoplex PCR systems is feasible. This means a significant reduction of time and cost required for the examination. The main advantages of this method are its affordable applicability and the reduction of time and cost needed for the analysis in comparison with the PCR-RFLP technique. Another convenience of the method developed is the ability to detect more than one gadoid species contained in one fish product. On the other hand, the significance of RFLP analysis for intraspecies differentiation of hake species is indisputable. However, the presented PCR method is specific enough for hake differentiation among other gadoid species. Developed PCR methods, in addition, do not require the use of commercial reference standards, contrary to techniques based on protein analysis as another approach for gadoid species identification.

Because gadoid fish products can be prepared using fish species of different commercial value, it is important to have a method that can identify various gadoid species. With regard to the increasing consumer demand for sea fish and their products, it would be advisable to have available methods that would reveal fish species substitution and allow the right information about data declared on the product to be obtained.

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