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One-Step Triplex-Polymerase Chain Reaction Assay for the Authentication of Yellowfin (*Thunnus albacares*), Bigeye (*Thunnus obesus*), and Skipjack (*Katsuwonus pelamis*) Tuna DNA from Fresh, Frozen, and Canned Tuna Samples

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A one-step triplex-polymerase chain reaction (PCR)-based assay was developed to discriminate between three tuna species, *Thunnus albacares*, *Thunnus obesus*, and *Katsuwonus pelamis*, even in highly processed food samples such as canned or cooked tuna. Diagnostic nucleotides were identified by direct sequencing and alignment of part of the mitochondrial cytochrome *b* gene of 30 authenticated exemplars, which allowed us to evaluate intraspecific variation and the genetic distance between three tuna species. The assay relies on a one-step triplex-PCR reaction in which in a single tube species-specific amplification products are generated only in the presence of the correct template nucleic acid and the species of origin of the DNA is indicated by the distinctive size of the PCR product. The identification of tuna species can be performed with a good accuracy, low cost, and with potential automation for large-scale high-throughput screenings in small in-house laboratories.

KEYWORDS: Species identification; triplex-PCR; mitochondrial cytochrome *b* gene; *Thunnus albacares*; *Thunnus obesus*; *Katsuwonus pelamis*; misdescription of tuna products; food forensics

INTRODUCTION

The authenticity of food labeling is a serious issue that involves not only the consumer but also the manufacturers and everyone involved in the food chain (*1*). Fraudulent misdescription and other food frauds have always been present at every level of the food industry, and they represent a challenge for the nowadays available analytical techniques. The use of high accurate methods, for example, based on protein electrophoresis and mass spectrometry techniques, is not always appropriate, due to the requirement of expensive instrumentation, pretreatment of the sample, and skilled personnel. In addition, protein analysis is not suitable since proteins often do not endure the processes used in the food industry (thermal treatments, vacuum drying, etc).

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New straightforward, sensitive, and high-throughput strategies suitable for extralaboratory use by nonskilled personnel are thus urgently required in food inspection activity to guarantee the authenticity of food labeling (*2*). An honest and accurate description of a food product is of particular importance when processing, cooking, canning, and other treatments have hindered the ability to identify the precise composition of the finished product (*3*). This is of particular interest in the fish industry when species identification of raw or processed fish products is required to prevent commercial frauds and to identify the presence of hazardous species, protected species, or species other than those listed in the label.

The difficulties in fish species identification in processed food derive from many factors, such as the absence of morphological features (e.g., fins, head, and skin) in the processed product, the denaturation of proteins or other material important for the analysis during heat treatments, the presence of a large number of possible species present in the food sample, and finally the presence of contaminants (e.g., olive and other vegetal oils), which may interfere with the analysis. We focused our attention on the development of an easy and accurate method for the

rapid analysis of tuna even in highly processed products such as canned tuna.

Tunas are all large species widely found in oceanic habitats and are commercialized in different forms, either fresh or canned. According to the European Union labeling legislation, the collective name "tuna" refers to several closely related members of the genus *Thunnus*, including the yellowfin tuna (*Thunnus albacares*), albacore tuna (*Thunnus alalunga*), bigeye tuna (*Thunnus obesus*), bluefin (*Thunnus thynnus*), and other species. Also, skipjack tuna (*Katsuwonus pelamis*) is included in the commercial designation "tuna". Prices obviously vary depending on species, reflecting tuna catches, market preferences, and the final use. The market for tuna served raw (e.g., for sashimi) requires high-quality fish and is usually the most demanding; indeed, a 200 kg bluefin tuna may cost as much as $35000 at Tsukiji market, the world's largest fish market. On the contrary, markets for cooked, canned, frozen, and smoked tuna have lower quality requirements. In the European fish market, due to less demand, skipjack prices are much lower than bigeye and yellowfin.

Because of different prices and European Union import levies, recent attention has been devoted to methods able to identify commercial fish species according to labeling legislation (*4*). Dissimilarly to raw fish, which is easy to identify with isoelectric focusing (IEF) of water-soluble proteins, the identification of highly processed tuna requires alternative methods. The presence of thermal treatments such as smoking, canning, and cooking, in fact, causes an irreversible lost of water solubility of the proteins (*5, 6*). In such methods, water-soluble proteins of tissues are separated and the profile obtained is further compared with those of the authenticated species for the establishment of the identity. Antibody-based methods would be most appropriate, although only a limited number of immunoassays have been developed (*7*), and none are available for wide-scale commercial use.

Nevertheless, protein profiles depend on the cell type, as different organs or tissues express different proteins. All of these factors make it preferable to analyze DNA rather than proteins. Recently, several DNA-based methods have been applied for fish identification based on polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) (*8, 9*), sequencing of PCR-amplified mitochondrial DNA (mtDNA) fragments (*10*), PCR–single-strand conformation polymorphism (PCR-SSCP) (*11*), and real-time PCR (*12*).

In particular, the use of a mitochondrial gene may present advantages over nuclear DNA because of its relatively high abundance, allowing an easier detection, and because mitochondria remain intact after processing, loss of DNA is avoided. Most of the DNA-based assays analyze sequences derived from the gene encoding cytochrome *b*, which is located on the mitochondrial genome. Because mtDNA shows high intraspecific variability, a preliminary analysis is always required to evaluate the presence of intraspecific polymorphisms in individuals of the same species (*13*). Terol et al. used a short sequence of mtDNA cytochrome *b* gene to demonstrate, by sequencing, the importance of calculating bootstrap values as an indicator of the statistical validation of species assignation (*10*). Although sequencing methods provide a reliable assignment of the species to which samples belong, they require expensive instrumentation and sophisticated research facilities, which are not suitable for a rapid and cost-effective analysis to be performed as routine screening directly by the industrial unit personnel.

Another approach is based on multiplex PCR reaction, which allows the amplification of multiple DNA templates in a single reaction tube, and the specific products are distinguished by the band size on agarose gel electrophoresis. Previous multiplex PCR-based assay reactions were developed to discriminate *T. thynnus* from *Sarda sarda* (*14*) and for the authentication of *Scomber colias* (*15*), but no methods are available to discriminate three closely related tuna species in a single PCR reaction.

In this paper, we present a new method for the identification of three tuna species: *T. albacares*, *T. obesus*, and *K. pelamis* in fresh, frozen, cooked, and canned samples. These three species are the most commercialized in the European market, and undeclared substitutions of *T. albacares* with the cheaper species *K. pelamis* and *T. obesus* have been reported. A rapid and cost-saving analytical method to investigate the accuracy of labeling descriptions of tuna species would be therefore very useful to reveal fraudulent substitutions and to protect the consumer.

Considering the high rate of intraspecies polymorphisms previously reported in literature, we decided to perform a preliminary sequencing of an internal region of the mitochondrial cytochrome *b* gene of different exemplars of these three tuna species. A rapid triplex-PCR assay was developed with primers specific for the three tuna species to allow the amplification of a region of the cytochrome *b* mtDNA and to distinguish the three species on the basis of PCR product lengths.

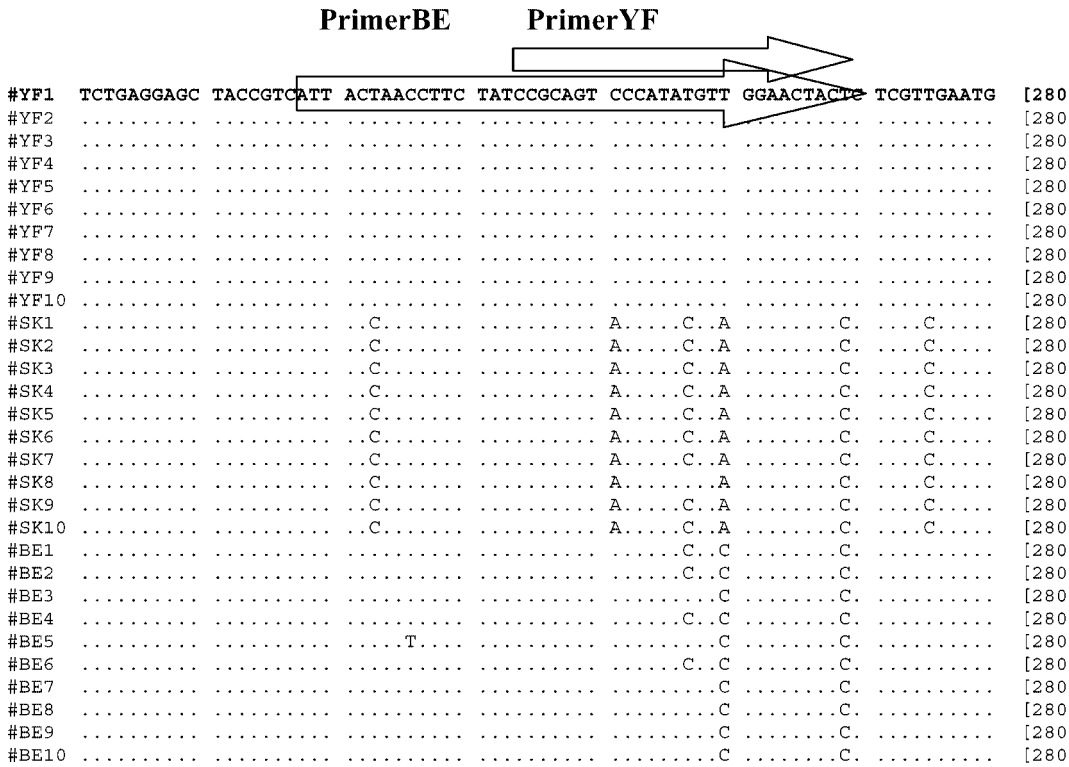
MATERIALS AND METHODS

Sample Collection. Thirty muscle samples were obtained from authenticated *T. albacares*, *T. obesus*, and *K. pelamis* specimens (10 exemplars for each species) supplied by Palmera S.p.A. (Olbia, Italy), one of the main Italian canned tuna producers. For validating the system, Palmera S.p.A. provided us with eight different preparations obtained from one exemplar for each of the three above-mentioned species: a portion of crude fish muscle, a cooked muscle portion, three tuna cans in olive oil, and three tuna cans in water. Eleven authenticated samples (five portions of frozen crude fish muscle and six canned samples, three in olive oil and three in water) of *Thunnus alalunga* were also provided by Palmera S.p.A. Seven canned products of different main commercial brands and presentations (prepared in olive oil, water, or sunflower oil), three labeled as tuna, two yellowfin tuna, one salmon, and one sardines, were also purchased at local supermarkets. Ten fresh fish samples, seven labeled as tuna, one as salmon, and two as sardines, were bought in local supermarkets and fish markets of Northern Italy.

DNA Extraction. To solubilize the tissues, 0.1 g was minced with a sterile surgical blade and transferred to a 1.5 mL Eppendorf tube. DNA was isolated from the supernatant using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of extracted DNA was confirmed by means of spectrophotometer measurements using a Cary 50 UV–vis spectrophotometer (Varian, Palo Alto, United States).

For the canned tuna samples, a small portion, approximately 0.2 g of sample, was placed on filter paper for oil and water removal and washed with bidistilled water. Oil and lipids were completely removed by hatching the muscle tissue overnight in chloroform/methanol/water (1:2:0.8) (*11*). The tissue was then recovered and employed for DNA extraction as described above. DNA suspensions were stored at −20 °C until use for PCR amplification.

Cytochrome *b* Gene Fragment Amplification and Sequence Analysis. The NCBI Entrez Nucleotide Database was searched for mitochondrial cytochrome *b* sequences for yellowfin tuna (*T.* albacares, L11557), bigeye tuna (*T. obesus*, L11559), and skipjack (*K. pelamis*, L11539). The three sequences were aligned with AlignX software (VectorNTI Suite 5.5, Informax Inc., North Bethesda, MD). Primers were designed to bind the three species cytochrome *b* genes and to ensure amplification of a 577 bp region in mtDNA: TunaFor, 5′-CAGGACTATTCCTCGCAATACA-3′, and TunaRev, 5′-CGAAAC-CAAGGAGGTCTTTGTA-3′. All of the bases are conserved in the three species except for the underlined base in TunaRev, which is G


PrimerBE
PrimerYF
#YF1 TCTGAGGAGC TACCGTCATT ACTAACCTTC TATCCGCAGT CCCATATGTT GGAACTACTC TCGTTGAATG [280]
#YF2 [280]
#YF3 [280]
#YF4 [280]
#YF5 [280]
#YF6 [280]
#YF7 [280]
#YF8 [280]
#YF9 [280]
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#SK7 [280]
#SK8 [280]
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#SK10 [280]
#BE1 [280]
#BE2 [280]
#BE3 [280]
#BE4 [280]
#BE5 [280]
#BE6 [280]
#BE7 [280]
#BE8 [280]
#BE9 [280]
#BE10 [280]
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#YF2 [350]
#YF3 [350]
#YF4 [350]
#YF5 [350]
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#BE6 [350]
#BE7 [350]
#BE8 [350]
#BE9 [350]
#BE10 [350]
PrimerSK
#YF1 TTCGTCATCG CAGCTATGAC AATTCTTCAC CTTCTTTTCC TTCACGAAAC AGGTTCAAAC AATCCAATCG [420]
#YF2 [420]
#YF3 [420]
#YF4 [420]
#YF5 [420]
#YF6 [420]
#YF7 [420]
#YF8 [420]
#YF9 [420]
#YF10 [420]
#SK1 [420]

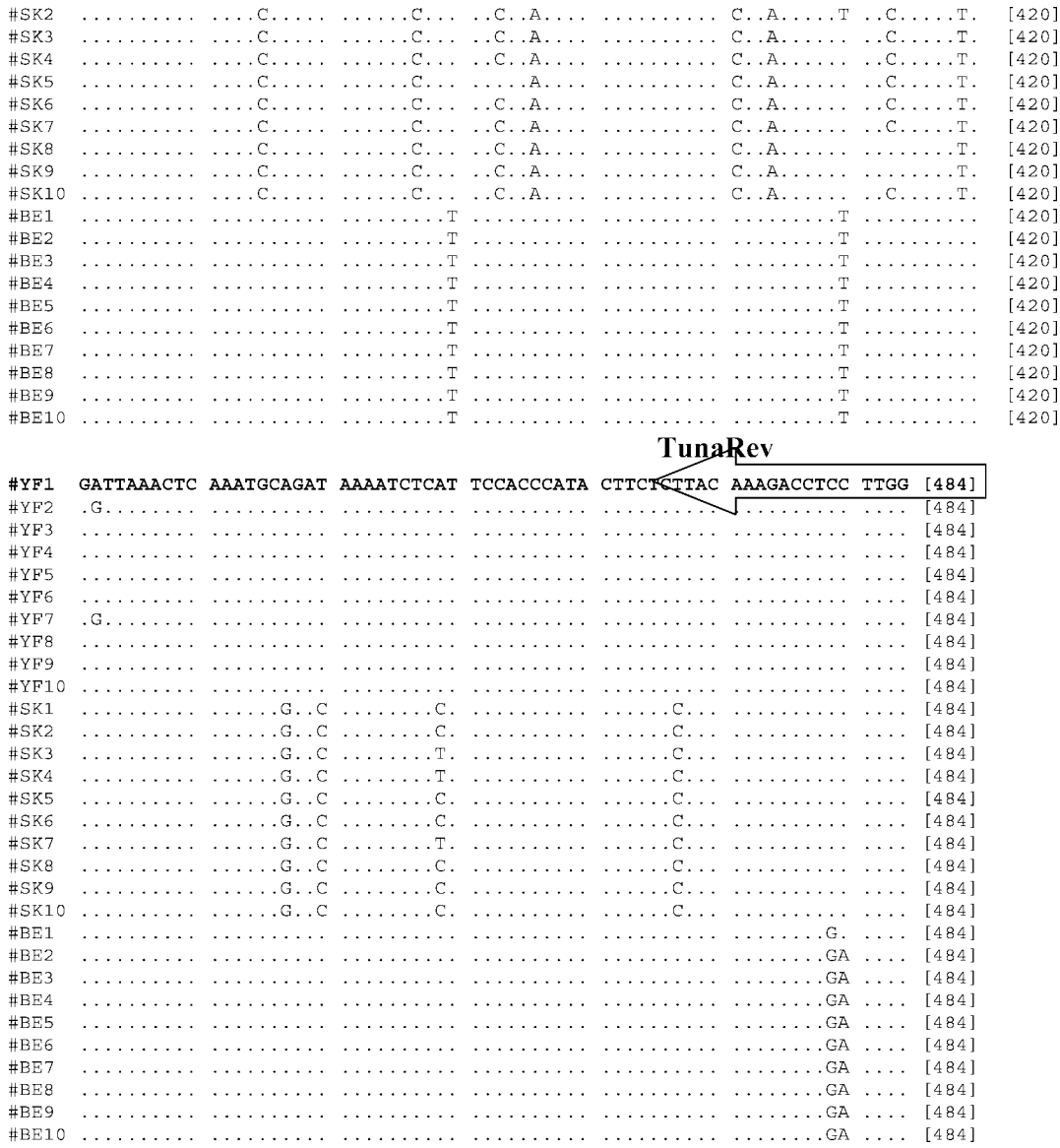

Figure 1. Multiple alignment of the cytochrome *b* gene fragment amplified from the 30 exemplars. The alignment was performed with MEGA version 3.1. Species-specific primers are shown with an arrow. Primer TunaRev was the common reverse primer used to amplify all of the species. The bigeye specific primer is in the same position as the yellowfin-specific primer, but it has 16 additional bases upstream.

in *K. pelamis*. PCR amplifications were carried out using a MJ Research PTC 100 thermal cycler (Perkin Elmer Inc., Wellesley, MA) with 50 μL as the final volume.

The PCR reaction was carried out with an initial denaturation at 95 °C for 1 min followed by 30 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 68 °C for 1 min, with a final extension at 68 °C for 5 min) using Platinum Pfx DNA polymerase (Gibco, Inc., Gaithersburg, MD). The PCR products were purified using Qiagen columns (Qiagen) according to the manufacturer's instructions.

Prior to sequencing, PCR products were purified with a Qiagen QIAquick kit according to the manufacturer's protocol. DNA sequencing of the PCR-amplified DNA was then performed with a capillary array sequencer CEQ2000XL System (Beckman Coulter Inc.).

Database Searches. The GenBank database was searched for mitochondrial cytochrome *b* sequences from related tuna species, corresponding to *Thunnus maccoyii* (no. EF141183), *Thunnus tonggol* (no. EF141181), *Thunnus alalunga* (no. L11556), and *Thunnus thynnus* (no. X81563). The alignment was performed with MEGA version 3.1.

Genetic Distances and Phylogenetic Analysis. Sequences were compared and aligned with those already present in the database using the program AlignX (Vector NTI suite 5.5 InforMax). Phylogenetic analysis was conducted using MEGA version 3.1 (*16*). A phylogenetic tree was built using the neighbor-joining method to infer an evolutionary relationship between the sequences in the alignment; the robustness of topology nodes was determined by the bootstrap method with 2000 iterations.

Triplex-PCR. After sequence alignment, intraspecies polymorphisms were evaluated and diagnostic sites were detected for the design of species-specific primers to ensure amplification from fresh, frozen, and canned samples, where the size of the fragment is critical. DNA from each tuna fresh sample was amplified in a single multiplex reaction with three species-specific forward primers and a third common reverse primer. The former primers are of different lengths, thus enabling PCR products of different lengths to be discriminated by a simple agarose gel electrophoresis.

We designed primers that terminate precisely at the point of the single base polymorphism so, in theory, if there is not perfect complementarity between the 3′-terminal base of the primer and the DNA template, no PCR product will be observed (*17*). The following primers were used to amplify the three species: TunaREV (nonselective primer common to all of the species) has been previously defined, PrimerYF (*T. albacares*-specific primer): 5′-CCGCAGTCCCATATGTTGGAACTACT-3′, PrimerBE (*T. obesus*-specific primer): 5′-ATTACTAACCTTCTATCCGCAGTCCCATACGTCGGAACTACC-3′, and PrimerSK

(*K. pelamis*-specific primer): 5′-CCACCTCCTATTCCTTCACGAAAC-CGGA-3′. Variable sites that differ among species are shown underlined in the primers. By using these primers, the predicted sizes of PCR products were 246 bp for *T. albacares*, 262 bp for *T. obesus*, and 113 bp for *K. pelamis*.

Normal separate PCR reactions were first performed using the same annealing temperature (57 °C) and yellowfin, bigeye, and skipjack DNA as templates. Then, duplex-PCR reactions were developed using two species-specific forward primers and TunaRev as the common reverse primer. Three different combinations of the primers were assayed as follows: PrimerBE, PrimerYF, and TunaRev; PrimerYF, PrimerSK, and TunaRev; Primer SK, PrimerBE, and TunaRev.

The triplex-PCR reaction was carried out using the four primers PrimerSK, PrimerYF, PrimerBE, and TunaREV in the same reaction mixture and same cycling regime. The optimized conditions for the triplex-PCR were as follows: 1× Pfx buffer, 2× Enhancer solution, 1.5 mM $MgSO_4$, 0.3 mM dNTPs, 0.15 μM TunaREV, 0.1 μM PrimerYF, 0.3 μM PrimerSK, 0.3 μM PrimerBE, between 0.5 and 1 μg of DNA template, and 2 units of Platinum Pfx DNA polymerase (Gibco, Inc.). The final volume of the PCR mixtures was 50 μL, and they were carried out with a MJ Research PTC 100 thermal cycler (Perkin Elmer). The PCR reactions were carried out with an initial denaturation at 95 °C for 1 min followed by 30 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, and extension at 68 °C for 45 s, with a final extension at 68 °C for 5 min). The triplex-PCR reaction was also carried out using Pfu DNA Polymerase (Promega, Madison, WI) to confirm the high reproducibility of the reaction. The reaction conditions used with Pfu DNA Polymerase were the following: 200 mM Tris-HCl, 100 mM KCl, 20 mM $MgSO_2$, 100 mM $(NH_4)_2SO_4$, 0.3 mM dNTP, 0.15 μM TunaREV, 0.1 μM PrimerYF, 0.3 μM PrimerSK, 0.3 μM PrimerBE, between 0.5 and 1 μg of DNA template, and 2 units of Pfu DNA polymerase. The reaction was carried out with an initial denaturation at 95 °C for 1 min followed by 30 cycles of amplification (denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min).

The PCR products were purified using Qiagen columns (Qiagen), and sequencing was performed to confirm the results as described above. PCR product sizes were determined against a O'GeneRuler 1 kb and 100 bp DNA ladders (Fermentas, Vilnius, Lituania) by electrophoresis of 5 μL of the product through 1.5% agarose gels using TAE buffer containing ethidium bromide for band characterization via ultraviolet transillumination. With few canned products, if a faint band was obtained as a PCR product at the expected size, it was used as a template for a new PCR round to enhance the yield of PCR product.

RESULTS AND DISCUSSION

The purpose of this work was first to investigate the genetic divergence between three tuna species and to successively develop an easy to perform and rapid triplex-PCR-based assay to authenticate three tuna species from commercial tuna products. Because of their great commercial value in the fish market, we focused our attention on three tuna species: *T. albacares* (yellowfin tuna), *T. obesus* (bigeye tuna), and *K. pelamis* (skipjack tuna), representing the main tuna species commercialized in the European market.

The three species were analyzed to determine the intraspecific variation and the positions with diagnostic value. Polymorphic sites between the species that did not present intraspecific variation were given a diagnostic value. According to these diagnostic nucleotides, a set of species-specific primers were designed to obtain a species-specific PCR amplified of 246 bp fragment for yellowfin, 262 for bigeye, and 113 for skipjack.

A simple agarose gel electrophoresis analysis of the amplicons proved to be capable of leading to unambiguous identification of the three species. All of the analyzed samples were correctly assigned to the corresponding species. The sequencing of PCR products was used as the confirming analysis.

Sequence Analysis. A preliminary sequencing was performed to individuate intraspecies polymorphisms not yet reported in the literature and to localize positions with diagnostic value. mtDNA was obtained from fresh samples of 30 exemplars, and a 577 bp fragment of the cytrocrome *b* gene was amplified as described in the Materials and Methods. We obtained a total of 30 sequences corresponding to 10 yellowfin, 10 bigeye, and 10 skipjack exemplars. Sequences were then aligned with AlignX and MEGA version 3.1 to the sequences obtained from the GenBank database, corresponding to *T. albacares* (L11557), *T. obesus* (L11559), and *K. pelamis* (L11539).

The sequencing of yellowfin, bigeye, and skipjack DNA extracted in our laboratory indicated that only some of the polymorphisms reported in the database also held true for these samples (*10*). The analysis of the alignment showed that 74 positions, of 484, were variable ones (**Figure 1**). Intraspecific variation was found in the different exemplars of the three species analyzed, with four polymorphic variations in the yellowfin tuna, eight in the bigeye ones, and 16 in the skipjacks. Once defined the intraspecific polymorphisms, we identified diagnostic positions able to differentiate the three species: 54 variable sites were found between skipjack and bigeye, 11 positions were found between yellowfin and bigeye, and 48 variable sites were found between skipjack and yellowfin. The diagnostic positions were chosen because they did not exhibit intraspecific variations in the analyzed sequences. To obtain an accurate species identification, we evaluated the genetic distances between the cytochrome *b* sequences with the Tamura and Nei method (*18*). This model allows the analysis of the differences in nucleotide sequence by considering the different weights of transitions and transversions, and also, each base frequency is taken into account to define the relevance of any base substitution. **Table 1** shows intraspecific distances estimated with this method: They ranged from 0 to 0.019 in *K. pelamis*, from 0 to 0.010 in *T. albacares*, and from 0 to 0.008 in *T. obesus*. These genetic distances, when compared to previously reported ones (*10*), confirmed the higher variability found in *K. pelamis*, but we reported a bigger variability in all of the species. The genetic distances between the three species were 1 order of magnitude bigger than the intraspecific ones; the mean distance between *K. pelamis* and *T. obesus* was 0.114 ± 0.015, 0.023 ± 0.007 between *T. albacares* and *T. obesus*, and 0.119 ± 0.016 between *K. pelamis* and *T. albacares*. The coefficient of differentiation was calculated to be 0.899 ± 0.019.

A phylogenetic tree was constructed using the Tamura–Nei distance matrix with the neighbor-joining method (*19*). The tree, shown in **Figure 2**, shows that all exemplars belonging to the same species cluster together with high bootstrap values, 90% for yellowfin, 96% for bigeye, and 100% for skipjack, confirming the results reported by Terol et al. (*10*). Therefore, the reported high bootstrap values statistically validate the cytochrome *b* fragment sequencing as an accurate tool for species identification. However, such a sequence-based assay is not suitable for a food company's in-house laboratory.

In fact, with the routine quality controls, such as chemical testing for allergens and for food health, required by new legislation and European regulation, many food companies who could not update their laboratory equipment and train staff have outsourced many services to external specialists. Laboratory outsourcing may be bypassed, with the obvious advantages of saving time and money, when dedicated in-house laboratories provide analytical services for performing essential routine

Table 1. Genetic Distances between the Sequences Amplified from 30 Exemplars (10 Yellowfin, 10 Bigeye, and 10 Skipjack Tuna) Estimated by the Tamura and Nei Method[a]

	SK1	SK2	SK3	SK4	SK5	SK6	SK7	SK8	SK9	SK0	YF1	YF2	YF3	YF4
[SK1]														
[SK2]	**0.006**													
[SK3]	**0.015**	**0.013**												
[SK4]	**0.015**	**0.013**	0.000											
[SK5]	**0.008**	**0.010**	**0.019**	**0.019**										
[SK6]	**0.006**	**0.008**	**0.013**	**0.013**	**0.010**									
[SK7]	**0.013**	**0.010**	**0.002**	**0.002**	**0.017**	**0.010**								
[SK8]	**0.013**	**0.015**	**0.015**	**0.015**	**0.017**	**0.010**	**0.017**							
[SK9]	**0.010**	**0.008**	**0.013**	**0.013**	**0.015**	**0.008**	**0.011**	**0.006**						
[SK0]	**0.004**	**0.006**	**0.015**	**0.015**	**0.008**	**0.006**	**0.013**	**0.013**	0.010					
[YF1]	0.121	0.121	0.113	0.113	0.123	0.121	0.111	0.118	0.116	0.124				
[YF2]	0.123	0.124	0.116	0.116	0.126	0.124	0.113	0.121	0.119	0.126	**0.002**			
[YF3]	0.121	0.121	0.113	0.113	0.123	0.121	0.111	0.118	0.116	0.124	**0.000**	**0.002**		
[YF4]	0.124	0.124	0.116	0.116	0.126	0.124	0.114	0.121	0.119	0.126	**0.002**	**0.004**	**0.002**	
[YF5]	0.124	0.124	0.116	0.116	0.126	0.124	0.114	0.121	0.119	0.126	**0.002**	**0.004**	**0.002**	**0.000**
[YF6]	0.118	0.119	0.111	0.111	0.121	0.119	0.108	0.116	0.114	0.121	**0.002**	**0.004**	**0.002**	**0.004**
[YF7]	0.123	0.124	0.116	0.116	0.126	0.124	0.113	0.121	0.119	0.126	**0.002**	**0.000**	**0.002**	**0.004**
[YF8]	0.131	0.131	0.123	0.123	0.133	0.131	0.121	0.128	0.126	0.134	**0.008**	**0.010**	**0.008**	**0.010**
[YF9]	0.121	0.121	0.113	0.113	0.123	0.121	0.111	0.118	0.116	0.124	**0.000**	**0.002**	**0.000**	**0.002**
[YF0]	0.124	0.124	0.116	0.116	0.126	0.124	0.114	0.121	0.119	0.126	**0.002**	**0.004**	**0.002**	**0.000**
[BE1]	0.121	0.116	0.114	0.114	0.124	0.122	0.112	0.124	0.117	0.124	0.023	0.025	0.023	0.026
[BE2]	0.124	0.119	0.116	0.116	0.126	0.124	0.114	0.127	0.119	0.127	0.025	0.028	0.025	0.028
[BE3]	0.129	0.124	0.121	0.121	0.131	0.129	0.119	0.126	0.124	0.132	0.025	0.027	0.025	0.028
[BE4]	0.124	0.119	0.116	0.116	0.126	0.124	0.114	0.127	0.119	0.127	0.025	0.028	0.025	0.028
[BE5]	0.127	0.122	0.119	0.119	0.129	0.127	0.117	0.124	0.122	0.129	0.028	0.030	0.028	0.030
[BE6]	0.124	0.119	0.116	0.116	0.126	0.124	0.114	0.127	0.119	0.127	0.025	0.028	0.025	0.028
[BE7]	0.127	0.122	0.119	0.119	0.129	0.127	0.117	0.124	0.122	0.129	0.023	0.025	0.023	0.025
[BE8]	0.127	0.122	0.119	0.119	0.129	0.127	0.117	0.124	0.122	0.129	0.027	0.030	0.027	0.030
[BE9]	0.129	0.124	0.121	0.121	0.131	0.129	0.119	0.126	0.124	0.132	0.025	0.027	0.025	0.028
[BE0]	0.129	0.124	0.122	0.122	0.132	0.130	0.119	0.127	0.125	0.132	0.025	0.028	0.025	0.028

	YF5	YF6	YF7	YF8	YF9	YF0	BE1	BE2	BE3	BE4	BE5	BE6	BE7	BE8	BE9	BE0
[YF5]																
[YF6]	0.004															
[YF7]	0.004	0.004														
[YF8]	0.010	0.010	0.010													
[YF9]	0.002	0.002	0.002	0.008												
[YF0]	0.000	0.004	0.004	0.010	0.002											
[BE1]	0.026	0.026	0.025	0.030	0.023	0.026										
[BE2]	0.028	0.028	0.028	0.032	0.025	0.028	**0.002**									
[BE3]	0.028	0.028	0.027	0.032	0.025	0.028	**0.006**	**0.004**								
[BE4]	0.028	0.028	0.028	0.032	0.025	0.028	**0.002**	**0.000**	**0.004**							
[BE5]	0.030	0.030	0.030	0.034	0.028	0.030	**0.008**	**0.006**	**0.006**	**0.006**						
[BE6]	0.028	0.028	0.028	0.032	0.025	0.028	**0.002**	**0.000**	**0.004**	**0.000**	**0.006**					
[BE7]	0.025	0.025	0.025	0.030	0.023	0.025	**0.004**	**0.002**	**0.002**	**0.002**	**0.004**	**0.002**				
[BE8]	0.030	0.030	0.030	0.034	0.027	0.030	**0.008**	**0.006**	**0.006**	**0.006**	**0.008**	**0.006**	**0.004**			
[BE9]	0.028	0.028	0.027	0.032	0.025	0.028	**0.006**	**0.004**	**0.004**	**0.004**	**0.006**	**0.004**	**0.002**	**0.002**		
[BE0]	0.028	0.028	0.028	0.032	0.025	0.028	**0.006**	**0.004**	**0.004**	**0.004**	**0.006**	**0.004**	**0.002**	**0.006**	**0.004**	

[a] Bold numbers show distances between exemplars belonging to the same species.

quality controls. For this purpose, a single reaction step based on a PCR assay would definitely be more suitable to identify tuna species in fresh, frozen, and processed tuna samples.

Triplex Species-Specific PCR. By aligning the 30 sequences alongside available tuna cytochrome *b* sequences, we were able to design primers to differentiate the three tuna species utilizing a common reverse primer to all three species and a specific forward primer for each species. A one-step triplex-PCR was developed employing different primer pairs in the same amplification reaction: one common reverse primer and three distinct forward primers specific for yellowfin, bigeye, and skipjack, shown in **Figure 1**. The bigeye specific primer is in the same position as the yellowfin-specific primer, but it has 16 additional bases upstream.

In addition, species-specific primers were also aligned with cytochrome *b* sequences of other related tuna species (*T. maccoyii*, *T. tonggol*, *T. alalunga*, and *T. thynnus*) to avoid the risk of aspecific amplification of other tuna sequences. As shown in **Figure 3**, the three species-specific forward primers bind only to the corresponding target sequence. Because the 3′-terminal positions in PCR primers are essential for priming polymerization of the target DNA, primer specificity was in fact increased by selecting a 3′-terminal sequence with high interspecies variability. In this way, the ability of the triplex-PCR to discriminate between species is favored.

The primers were then combined in a one-step triplex-PCR reaction, optimized stepwise, and validated with authenticated samples. Single base differences were found in all sequenced exemplars in contrast with the deposited sequences in Pubmed. For example, in position 296, there is a polymorphism, not previously reported, shown underlined; three yellowfin exemplars out of 10 showed A instead of T. The three primer pairs were then combined together in the same reaction mix, to get

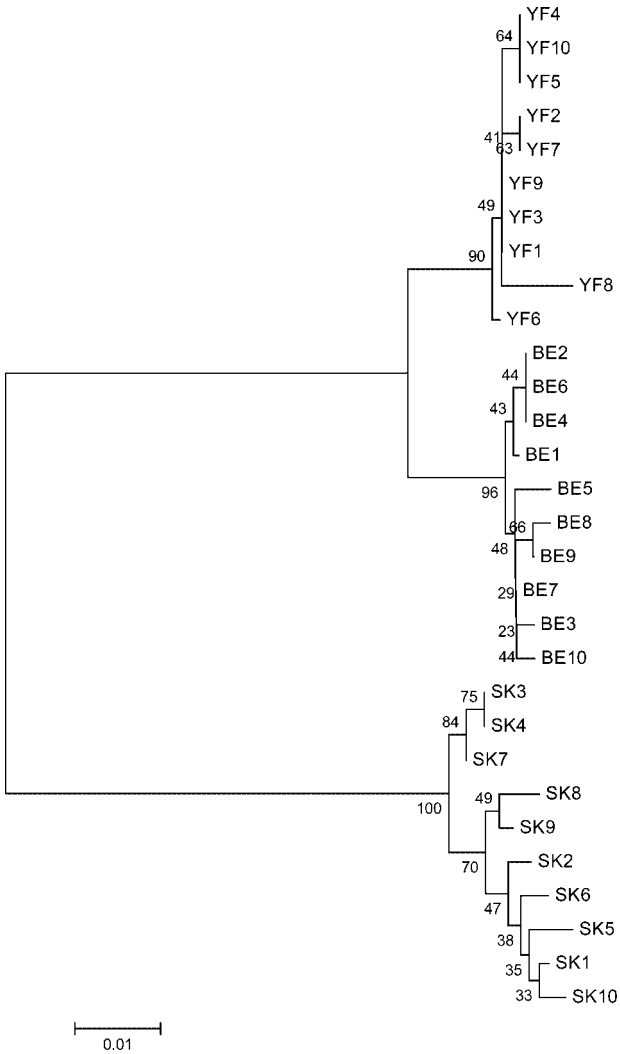

Figure 2. Phylogenetic tree for the sequences obtained from the 30 exemplars, constructed with the neighbor-joining method, on the basis of genetic distances determined with the Tamura and Nei method from the multiple alignment.

simultaneously a specific amplification and to distinguish the tuna species on the basis of band size on analytical agarose gel.

PCR conditions were optimized using fresh tuna DNA samples. The optimal annealing temperature was determined to be 60 °C using Pfx Platinum Polymerase and 57 °C using Pfu DNA polymerase; no aspecific amplification was detected.

This set of primers used in a multiplex PCR assay amplified three specific fragments of different sizes for each species. The quick identification of the three groups was therefore possible, given the different amplicon sizes detected through agarose gel electrophoresis: 246 bp for *T. albacares*, 262 bp for *T. obesus*, and 113 bp for *K. pelamis*. Before using the combination of primers described before, the three species-specific primers were first used with the common reverse primer in simple PCR reactions to confirm the specificity of the templates and to identify a common annealing temperature that allowed the amplification of all of the templates. **Figure 4** shows the three PCR products obtained from yellowfin, bigeye, and skipjack DNA subjected to amplification using the same annealing temperature (57 °C) in normal separate PCR reactions. Second, duplex-PCR reactions were developed using two species-specific forward primers and the common reverse primer. Three different combination of the primers were assayed as follows: PrimerBE, PrimerYF, and Tuna Rev; PrimerYF, PrimerSK, and TunaRev; and Primer SK, PrimerBE, and TunaRev. Results are shown in **Figure 5**. As expected, the combination of yellowfin/skipjack- and bigeye/skipjack-specific primers did not yield a specific amplification since a band was observed even in the lane corresponding to bigeye DNA and yellowfin DNA, respectively. Most likely, this is due to the low nucleotide variability between these two species; no cross-amplification was in fact observed using bigeye- or yellowfin-specific primers with skipjack DNA as the template.

Figure 6 shows the PCR products obtained from the triplex-PCR reaction. The correct amplification was obtained in all of the exemplars, allowing a reliable discrimination of the three species. Different amplicon sizes were in fact observed through agarose gel electrophoresis: 246 bp for *T. albacares*, 262 bp for *T. obesus*, and 113 bp for *K. pelamis*. The competition between different templates allowed a clean amplification of the specific product. Sequencing of the obtained bands confirmed primer specificity. Primer binding sites were selected to obtain specific amplimers of less than 300 bp in length. The analysis of short fragments, usually between 100 and 300 base pairs, is preferable when assaying degraded substrates such as canned or highly processed food. Thermal treatments and food production processing involving high pressure, drying, irradiation, and pH variations cause DNA degradation and, therefore, may affect the suitability of PCR.

Method Validation. To assess reproducibility and reliability of the developed method, several samples, including fresh and highly processed samples (frozen, canned, and smoked), were analyzed and sequencing was performed to confirm the results of the triplex-PCR. Authenticated tuna samples were analyzed to validate the triplex-PCR based assay; in particular, eight different preparations of one exemplar for each of the three tuna species were analyzed as follows: a muscle portion of frozen crude meat, a cooked frozen muscle portion, three canned tuna in olive oil, and three canned tuna in water. Both of the DNA samples extracted from cooked and uncooked fish gave the expected bands; all canned tunas, except two samples, gave the correct amplicon after the preliminary treatment to remove PCR inhibitors as described in the Materials and Methods. For these two samples, a nested PCR with the same primers was sufficient to allow a species-specific PCR and an unambiguous identification.

In addition, authenticated *T. alalunga* samples were analyzed to better evaluate the specificity of the triplex-PCR assay and to exclude the risk of aspecific amplification of other tuna species. Five frozen crude samples of different *T. alalunga* exemplars were analyzed with the triplex-PCR assay, and no amplification at all was reported. Authenticated canned *T. alalunga* samples, three in olive oil and three in water, were also assayed with the method, and again, no PCR product was observed even with a nested PCR (data not shown).

Method Application. Besides the authenticated samples, we analyzed real samples: 10 samples of fresh fish bought in local supermarkets and a total of seven canned products of different commercial brands (prepared in olive oil, water, or sunflower oil), labeled as tuna, yellowfin tuna, salmon, and sardines, to validate the method (**Table 2**). The 10 fresh fish samples, seven labeled as tuna, one as salmon, and two as sardines, were assayed with the triplex-PCR reaction. Concerning the seven fresh tuna samples, five tested positive for skipjack and two for yellowfin, whereas no PCR product was obtained with the

```
                                    PrimerBE                PrimerYF
#T.albacares TCTGAGGAGC TACCGTCATT ACTAACCTTC TATCCGCAGT CCCATATGTT GGAACTACTC TCGTTGAATG  [280]
#K.pelamis   .......... .......... ..C....... .......... A....C..A ........C. ....C.....  [280]
#T.obesus    .......... .......... .......... .......... ......C..C ........C. ..........  [280]
#T.maccoyii  .......... .......... ........C. .......... .........C ........C. ..........  [280]
#T.tonggol   .......... .......... .......... .......... .........C .......... ..........  [280]
#T.alalunga  .......... .......... ........C. .......... .......... ........C. ..........  [280]
#T.thynnus   .......... .......... ........C. .......... .........C .......... ..........  [280]

#T.albacares AATCTGAGGA GGCTTTTCAG TAGACAATGC CACCCTCACC CGATTCTTCG CATTCCACTT CCTATTCCCA  [350]
#K.pelamis   .......... .......... .......C.. ......T... .......... .G........ .........C  [350]
#T.obesus    .......... .......... .......... .......... .......... .......... ..........  [350]
#T.maccoyii  .......... .......... .......... .......... .......... .......... ..........  [350]
#T.tonggol   .......... .......... .......... .......... .......... .......... ..........  [350]
#T.alalunga  .........G .......... .......... T.....T... .......... .......... ..........  [350]
#T.thynnus   .......... .......... .......... .......... ..T....... .......... ..........  [350]

                                    PrimerSK
#T.albacares TTCGTCATCG CAGCTATGAC AATTCTTCAC CTTCTTTTCC TTCACGAAAC AGGTTCAAAC AATCCAATCG  [420]
#K.pelamis   .......... ....C..... ......C... ..C..A.... .......... C..A...... ..C.....T.  [420]
#T.obesus    .......... .......... .........T .......... .......... .........T ..........  [420]
#T.maccoyii  .......... .......... .......... .......... .......... .........T ..........  [420]
#T.tonggol   .......... .......... .......... .......... .......... .........T ..........  [420]
#T.alalunga  .......... ....C..... .......... .......... .......... .........T ..........  [420]
#T.thynnus   .......... .......... .......... .......... .......... .........T ..........  [420]
```

Figure 3. Multiple alignment of the cytochrome *b* gene fragment obtained from analyzed samples (*T. albacares*, *T. obesus*, and *K. pelamis*) with the sequences obtained from the GenBank database, corresponding to *T. maccoyii* (no. EF141183), *T. tonggol* (no. EF141181), *T. alalunga* (no. L11556), and *T. thynnus* (no. X81563). The alignment was performed with MEGA version 3.1. Only variable sites are shown. Forward species-specific primers are shown in a rectangle.

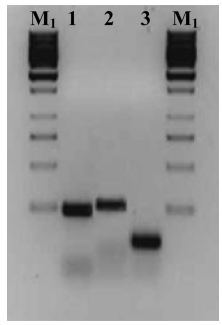

Figure 4. PCR products from yellowfin (lane 1), bigeye (lane 2), and skipjack (lane 3) DNA subjected to species-specific amplification using TunaRev as a common reverse primer and PrimerYF, PrimerBE, or PrimerSK as specific primers. Lane M_1 contains molecular weight marker (O'GeneRuler 1 kb DNA ladder). Lane 1 shows a 246 bp fragment resulting from amplification of yellowfin DNA. Lane 2 shows a 262 bp fragment resulting from amplification of bigeye DNA, and lane 3 shows a 113 bp fragment generated by amplification of skipjack DNA. A triplex-PCR reaction was therefore developed using all the three specific primers and the common reverse primer.

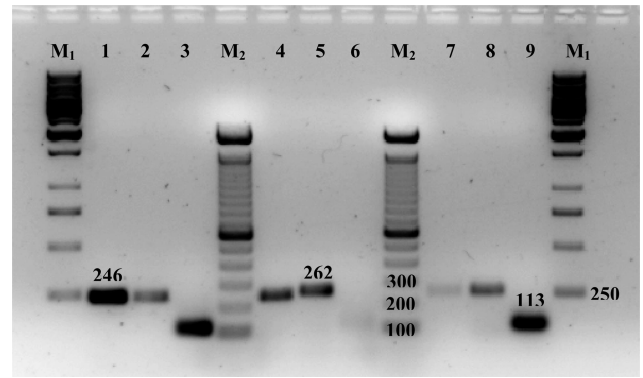

Figure 5. Duplex-PCR products obtained using two species-specific forward primers and a nonselective reverse primer using as templates yellowfin (lanes 1, 4, and 7), bigeye (lanes 2, 5, and 8), and skipjack DNA (lanes 3, 6, and 9). Lanes 1, 2, and 3 show duplex-PCR products obtained with PrimerYF, PrimerSK, and TunaRev. Lanes 4, 5, and 6 show duplex-PCR products obtained with PrimerYF, PrimerBE, and TunaRev. Lanes 7, 8, and 9 show duplex-PCR products obtained with PrimerBE, PrimerSK, and TunaRev. Lane M_1 contains molecular weight marker (O'GeneRuler 1 kb DNA ladder); lane M_2 contains molecular weight marker (100 bp DNA ladder).

salmon and the two sardines sample, demonstrating the absence of aspecific amplification.

Results obtained with the seven analyzed canned samples, three labeled as tuna, two as yellowfin tuna, one as salmon, and one as sardines are shown in **Table 2**. Both of the samples labeled as yellowfin tuna produced the PCR product corresponding to yellowfin, whereas the two products generally labeled as "tuna" contained skipjack and one sample resulted positive for skipjack and bigeye; probably a mixture of the two species was present in the canned sample. Other canned items did not generate any PCR product, even with a nested PCR. In the majority of tested samples, the triplex-PCR assay was able to positively identify the species present in the product, and these species were as declared on the product label.

We analyzed a total of 52 samples (35 authenticated tuna samples and 17 commercial samples), and only in a few instances (three canned samples, 5.8% of the total), it was not possible to fully identify the species present in the product with the one-step triplex-PCR. For these samples, inadequate PCR product was obtained resulting in patterns that were not clear enough to be able to positively identify the individual species present. In all three cases, a nested PCR with the same primers was sufficient to give PCR products with expected bands. In practice, a positive result is always indicative of the presence of a particular species, whereas the occurrence of occasional false negative may be overcome by simply performing a nested PCR. As previous works dealing with PCR-based assays on canned tuna (*20*), we did not face a PCR inhibition in the analyzed canned samples. Nevertheless, thermal treatments may reduce the maximum size of the obtained DNA fragments, thus the possibility of having degraded DNA or the presence of additives that may inhibit the PCR reaction cannot be ruled out

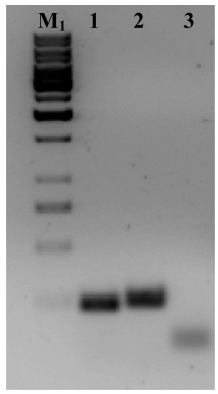

Figure 6. Triplex-PCR products from yellowfin (lane 1), bigeye (lane 2), and skipjack (lane 3) DNA subjected to species-specific amplification using TunaRev as a common reverse primer and PrimerYF, PrimerSK, and PrimerBE as specific primers in the same reaction tube. Lane M_1 contains molecular weight marker (O'GeneRuler 1kb DNA ladder).

Table 2. Results of Triplex-PCR Performed on Commercial Products[a]

commercial product	label	triplex-PCR result
fresh tuna	tuna	SK
fresh tuna	tuna	SK
fresh tuna	tuna	SK
smoked tuna	tuna	SK
tuna fillets	tuna	SK
tuna salad	tuna	YF
tuna fillets	tuna	YF
fresh salmon	salmon	no PCR product
fresh sardines	sardines	no PCR product
sardine fillets	sardines	no PCR product
canned tuna in olive oil	tuna	SK + BE
canned tuna in olive oil	yellowfin tuna	YF*
canned tuna in water	yellowfin tuna	YF
canned tuna in water	tuna	SK
canned tuna in water	tuna	SK
canned salmon	salmon	no PCR product
canned sardines	sardines	no PCR product

[a] An asterisk indicates products that needed two consecutive PCR reactions with the same primers; SK indicates skipjack tuna, YF indicates yellowfin tuna, and BE indicates bigeye tuna.

(*11*). For this reason, some authors recommend two independent DNA-based analytical methods to obtain a reliable species identification. The presence of intraspecific variation, due to its high degree in mtDNA, may in fact account for false negative/positives.

In conclusion, the proposed triplex-PCR assay represents a reliable, low cost, and simple method for the routine identification of three tuna fish species. Genetic intraspecies variability was first evaluated, to minimize the risk of false positives/negatives due to minute differences in conserved sequences.

Because the discrimination is obtained simply during the course of a PCR reaction, without the need of secondary steps, such as sequencing or RFLP analysis, the method is very rapid and inexpensive, more so than any other method previously developed to discriminate between tuna species. The method can be in fact performed in a 96 well microtiter format for high-throughput applications, requiring for the whole analysis hundreds of samples, from DNA extraction to the gel electrophoresis, only a working day (6–8 h). The method was applied to diverse real samples, and it was able to positively identify the species present in the commercial product; these species were as declared on the product label. Analogous approaches were previously reported to identify two fish species with a duplex-PCR (*14, 20, 21*) for feedstuff analysis (*22*) and for the identification of potato virus (*23*), but to the best of our knowledge, this is the first triplex-PCR-based assay developed to distinguish three fish species and, most interestingly, three closely related tuna species.

The applicability of the method for real samples analysis suggests its possible use in panels for the authentication of commercial canned products and for the identification of animals species in feedstuffs. The method thus represents a valuable tool to determine and confirm tuna identity with potential applications for routine works such as surveys of quality and labeling of commercial tuna preparations even in small in-house laboratories.

ABBREVIATIONS USED

PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; RFLP, restriction fragment length polymorphism.

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