JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 7435−7443 **7435**

Novel Method for the Authentication of Frigate Tunas (*Auxis thazard* and *Auxis rochei*) in Commercial Canned Products

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A novel procedure for the authentication of frigate tunas (*Auxis thazard* and *Auxis rochei*) in commercially canned products has been developed. Three mitochondrial regions were simultaneously amplified by multiplex-Polymerase Chain Reaction, one corresponding to the small rRNA 12S subunit as a positive amplification control and two species-specific fragments corresponding to cytochrome *b* for *A. rochei* and ATPase 6 for *A. thazard,* respectively. Testing of two different detection systems revealed the fluorescence-based approach as the most sensitive. The results demonstrate that this rapid, low-cost methodology is a reliable molecular tool for direct application in the authentication of canned products.

KEYWORDS: ***Auxis thazard*; *Auxis rochei*; cytochrome *b*; ATPase 6; multiplex-PCR; canned products**

INTRODUCTION

The past few years have been witness to a tremendous growth in quality food consumption and a resultant change in attitudes, especially in the area of raw and processed fish products. Nowadays, consumers demand quality products that are correctly labeled. However, fraudulent and unintentional mislabeling exists and may go undetected, resulting in a misrepresentation of the actual quality of that product. Consumers can seldom identify the species of a fish when the morphological characteristics such as shape, size, or appearance are removed during processing. The increasing availability of cuts, either fresh or frozen, processed or unprocessed, often makes the species unrecognizable and opens the possibility of fraudulent adulteration and substitution of high-priced species with others of lesser value. In this context, falsification is commonplace due to the enormous profits that can be realized (*1*).

Frigate tunas of the genus *Auxis* are cosmopolitan species and the smallest members of the tribe Thunnini, the true tunas. Currently most workers have recognized the existence of two distinct species, the narrow-corseleted *Auxis thazard* (Lacepède, 1800) and the wide-corseleted *Auxis rochei* (Risso, 1810) (*2*, *3*). Morphologically, they are differentiated primarily by the width of the corselet under the origin of the second dorsal fin and by the anterior extent of the dorsal scaleless area above the pectoral fin. In Spain, and particularly in Andalucia (southern Spain), frigate tunas have been largely exploited due to the excellent properties of the meat, with its mild taste and low cholesterol content. It is significant to note that from 1997 to 2000 nearly 3000 tons were off-loaded at Andalusian fishing ports. The high commercial interest of these species has been recognized by the recent passage of regional legislation to protect the denomination of "Melva de Andalucía" as specific when referring to *A. rochei* and *A. thazard* canned products. This denomination protects a traditional way of fish processing, including steps such as manual skinning of samples and the fillet presentation of the final product. Nevertheless, due to their similarity of flesh in both appearance and texture, lower market price species such as the skipjack (*Katsuwonus pelamis*), little tuny (*Euthynnus alletteratus*), or yellowfin tuna (*Thunnus albacares*) are not easily distinguishable from one another in the absence of morphological markers. Hence, substitution for the more expensive *Auxis* species can occur, and consequently the development of a procedure for frigate tuna authentication should be of the highest priority.

Fish species identification has been achieved by several methods based on protein analysis, including electrophoretic and chromatographic techniques (*4*−*8*). In these methods, water-soluble proteins of tissues are separated and the profile obtained is further compared with those of the authentic species for the establishment of the identity. In other cases, antibodies raised against particular proteins have been successfully used to identify the species in standard immunoassay procedures (*9*−*11*). Nevertheless, the major constraint of these protein-based methods is that when the flesh is cooked, the proteins become irreversibly denatured, hindering comparisons of cooked and raw products. Moreover, these techniques are not convenient for routine analyses because of the relatively high cost, the time required to obtain the results, and the proper technical complexity. In addition, 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

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10.1021/jf0492868 CCC: $27.50 © 2004 American Chemical Society
Published on Web 11/10/2004

Table 1. Primers Used in This Study[a]

name	sequence	amplicon (bp)
RNA12S•1	5′-GACAGCTACGACACAAACTGCGATTAGATACC-3′	534
RNA12S•2	5′-TGCACCTTCCAGTACACTTACCATGTTACGAC-3′	
ATP•1	5′-GCTAAACCTGAACCCTGAAACTGACCATGA-3′	492
ATP•2	5′-CGAGCGGACGGATAAAAAGGCTAATTG-3′	
cytb•1	5′-CCACATCGCCCGAGGTCTTTACTACG-3′	423
cytb•4	5′-ACTAGCAGGATCACGAAACCAAGGAGGTCT-3′	
RNA12spp•1	5′-HEX-CGCATGAGAGGGGAAGAAATGGGCTA-3′	146
RNA12spp•2	5′-CGGTGTGTACGCACTTCAGAGCCGATT-3′	
ATPath•1	5′-GTCTCCTTCCCTACACTTTCACGCCTACAACC-3′	128
ATPath•2	5′-FAM-AAGGTGGCCTAAAGCTTCTGTTGGCTGA-3′	
cytbAro•1	5′-TGAAACATCGGTGTAGTCCTCCTTCTACTGGTC-3′	95
cytbAro•2	5′-FAM-GTGGCACCTCAGAATGACATTTGTCCTCAT-3′	

[a] Upper: primer sequences for PCR amplification of partial cyt *b* (cytb•1 and cytb•4), ATPase 6 (ATP•1 and ATP•2), and rRNA 12S (RNA12S•1 and RNA12S•2) mitochondrial genes. Lower: primer sequences for multiplex-PCR amplification of species-specific products for *A. thazard* (ATPath•1 and ATPath•2) and *A. rochei* (cytbAro•1 and cytbAro•2) and for the universal rRNA 12S fragment (RNA12spp•1 and RNA12spp•2). For GeneScan analysis, primers modified with HEX or FAM (underlined) were used. Amplicon size is indicated in each case.

when identifying the species origin of an unknown sample: genetic information is the same in all cell types of an animal; DNA is a remarkably stable molecule, even at high temperatures; and the information content of DNA is greater than that of proteins because of the degeneracy of the genetic code. As a result, several DNA-based methods have recently been applied for fish identification, including sequencing of Polymerase Chain Reaction (PCR)-amplified mitochondrial DNA fragments (*12*, *13*), PCR-restriction fragment length polymorphism (PCR-RFLP) (*14*−*19*), PCR-single-strand conformation polymorphism (PCR-SSCP) (*20*), random amplified polymorphic DNA (RAPD) (*21*, *22*), fragment size analysis of nuclear 5S rDNA gene (*23*−*26*), and microsatellite loci (*27*).

It is well-known that processes such as canning involve a thermal treatment for sterilization, in which the DNA is severely degraded. In fact, only residues of an average size ranging from 100 to 200 bp are found (*28*−*30*). To authenticate commercially canned products, this restriction has been overcome with the amplification and further analysis of short DNA fragments using the same techniques cited above. Consequently, the identification of closely related tuna species or canned sardine and sardine-type products can be determined by direct sequencing of PCR amplified products (*29*, *31*, *32*), PCR-RFLP (*28*, *30*, *33*, *34*), or PCR-SSCP (*35*, *36*).

To date, most of the surveys aimed at identifying the species origin of canned fish samples have employed the mitochondrial genome as their target. Mitochondrial DNA has several advantages over nuclear DNA, including its higher abundance, mutation rate, and number of copies inside the cell (*1*). Particularly, the cytochrome *b* (cyt *b*) gene has been the most widely used mitochondrial gene in the identification of canned tuna. The cyt *b* gene shows enough interspecific variation in nucleotide sequence to allow differentiation even between the closely related tuna and bonito species (*1*, *12*, *13*). Moreover, the cyt *b*, as well as the ATPase, gene has been used to resolve phylogenetic relationships among species of the *Thunnus* genus (*29*, *37*), establishing them as suitable genes for frigate tuna species differentiation.

In this study, a multiplex-PCR-based methodology for the authentication of frigate tuna canned products is presented. It relies on *A. thazard* and *A. rochei* specific amplification products of ATPase 6 and cyt *b* genes, respectively, being generated only in the presence of the correct template nucleic acid. Besides this, a primer pair capable of amplifying a fragment corresponding to the small rRNA 12S subunit in tuna species has been included to serve as positive amplification control. The protocol, involving a one-step procedure, is reliable, sensitive, and quick to perform, enabling it to be used to identify any commercially canned frigate tuna samples in an accurate, expeditious manner.

MATERIALS AND METHODS

Sample Collection. Frigate tuna specimens included in the present study were supplied by Conservas Ubago, S.L. (Spain): 3 examples of *A. thazard* (Ath 1, Ath 2, and Ath 3) and 11 of *A. rochei*, 3 of which were from the Pacific (Aro 5, Aro 6, and Aro 7) and 8 from the Atlantic−Mediterranean Sea (Aro 1−Aro 4 and Aro 8−Aro 11). The example of the species *Thunnus alalunga* (Tal) was also supplied by Conservas Ubago, S.L. The Atlantic Bluefin tuna (*Thunnus thynnus*), little tunny (*Euthynnus alletteratus*), and skipjack tuna (*Katsuwonus pelamis*) specimens (Tth 1 and Tth 2; Eal 1 and Eal 2; Kpe 1, Kpe 2, and Kpe 3, respectively) were captured in the Atlantic waters of the Gulf of Cádiz (Spain). A muscular portion of each was excised and kept at −80 °C.

Canned products, all labeled as frigate tuna fillets (“filetes de melva”), were purchased at local supermarkets. A total of 20 canned products of different commercial brands and presentations (prepared in sunflower oil, olive oil, or extra virgin olive oil), were analyzed.

DNA Isolation, Amplification, and Sequencing. Total genomic DNA was isolated from 150 mg of raw or processed muscle sections using a FastDNA kit for 40 s at speed setting 5 in the Fastprep FG120 instrument (Bio101, Inc., Vista, CA). All DNA isolation procedures were performed in accordance with the manufacturer’s protocol.

A fragment of the rRNA 12S (534 bp), cyt *b* (423 bp), and ATPase subunit 6 (492 bp) mitochondrial genes was amplified in all of the examples of tuna cited above using the primers shown in **Table 1**. Primer pairs were designed by employing the software Oligo v 6.82 (Medprobe, Oslo, Norway) from known teleost sequences for each of these genes. Reactions were carried out in 25 μL of reaction volume: 1 μL of DNA template (∼30−50 ng) was added to 24 μL of PCR mix consisting of 17.25 μL of sterile distilled water, 2.5 μL of 10 mM dNTP mix, 2.5 μL of 10× buffer, 1 μL of 50 mM $MgCl_2$, and 0.25 μL (1.25 units) of BioTaq DNA polymerase (Bioline, London, U.K.) plus 0.5 μL of each primer (10 μM). The thermal cycle profile was identical for all of the amplified fragments. An initial denaturation step of 96 °C for 2 min was followed by 35 cycles of 96 °C for 30 s, 60 °C (rRNA 12S) or 55 °C (ATPase and cyt *b*) for 30 s, and 72 °C for 1 min. A final extension for 10 min at 72 °C was also included. PCR products were electrophoresed on a 1.5% agarose gel and visualized via ultraviolet transillumination before sequencing.

Double-stranded DNA products were purified using a PCR product purification kit (Marlingen Bioscience, Ijamsville, MD) and subsequently used for direct cycle sequencing with a BigDye Terminator v

Table 2. GenBank/EMBL/DDBJ Accession Numbers of the Partial Cyt *b*, ATPase 6, and rRNA 12S Mitochondrial Sequences Obtained in this Work

species	individuals	cyt *b*	ATPase 6	rRNA 12S
T. alalunga	Tal	AB176812	AB176809	AB176804
T. thynnus	Tth 1	AB176813	AB176820	AB176805
	Tth 2	AB176814	AB176820	AB176805
E. alletteratus	Eal 1	AB176815	AB176821	AB176806
	Eal 2	AB176815	AB176822	AB176807
K. pelamis	Kpe 1	AB176816	AB176823	AB176808
	Kpe 2	AB176817	AB176824	AB176808
	Kpe 3	AB176818	AB176823	AB176808
A. thazard	Ath 1	AB106835	AB176825	AB176809
	Ath 2	AB106834	AB176826	AB176809
	Ath 3	AB106833	AB176827	AB176809
A. rochei	Aro 1	AB106327	AB106990	AB176810
	Aro 2	AB106328	AB176828	AB176811
	Aro 3	AB106329	AB176829	AB176811
	Aro 4	AB106330	AB176830	AB176811
	Aro 5	AB106843	AB106995	AB176811
	Aro 6	AB106844	AB106996	AB176811
	Aro 7	AB106845	AB106991	AB176811
	Aro 8	AB106841	AB106991	AB176811
	Aro 9	AB106840	AB106992	AB176811
	Aro 10	AB106837	AB106991	AB176811
	Aro 11	AB106838	AB106993	AB176811

3.1 kit (Applied Biosystems, Foster City, CA). All sequencing reactions were performed in accordance with the manufacturer's instructions on a 377 DNA sequencer (Applied Biosystems). Primers used were the same as those used for PCR.

Nucleotide sequences were analyzed using the computer programs Sequencing Analysis v 3.4.1 (Applied Biosystems) and Seqman v 5.51 (DNASTAR, Madison, WI), respectively, and further aligned with the Megalign 5.51 package (DNASTAR). The sequences obtained have been deposited in the GenBank/EMBL/DDBJ with the accession numbers indicated in **Table 2**.

Multiplex-PCR and Electrophoresis. Reactions were carried out in a 25 μL reaction volume containing 1 μL of DNA template, 17.63 μL of sterilized distilled water, 2.5 μL of 10 mM dNTPs, 2.5 μL of 10× buffer, 0.2 μL of each of the tuna species primers RNA12spp•1 and RNA12spp•2 (10 μM), 0.18 μL of each of the *A. rochei* specific primers cytbAro•1 and cytbAro•2 (10 μM), 0.18 μL of each of the *A. thazard* specific primers ATPath•1 and ATPath•2 (10 μM), and 0.25 μL (1.25 units) of HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany). These sets of primers (**Table 1**) were also designed using the software Oligo v 6.82 (Medprobe, Oslo, Norway). Following an initial step of 2 min at 96 °C to activate the HotMaster Taq DNA polymerase, the thermal cycle profile was 40 cycles of denaturation at 96 °C for 30 s, annealing at 66 °C for 30 s, and extension at 72 °C for 30 s.

For analysis, PCR products (10 μL each) were electrophoresed on a 2.5% Ultrapure Agarose 1000 (Invitrogen, Carlsbad, CA) gel using 1× TBE buffer, and visualized via ultraviolet transillumination after staining of the gel with ethidium bromide. The 25 bp DNA Ladder (Invitrogen) was used as the molecular weight standard. Alternatively, PCR products (0.07 μL of each reaction) were loaded for fluorescence-based electrophoresis analysis on a 4.25% AutoPAGE high-throughput acrylamide gel (Sigma, St. Louis, MO). The size of the different fragments was determined with the software GeneScan v 3.1.2 (Applied Biosystems) using GeneScan-350 TAMRA (Applied Biosystems) as an internal lane size standard.

Phylogenetic Analyses. Neighbor-joining phylogenetic analyses were performed using MEGA (*38*). The neighbor-joining trees were built from a distance matrix construction using the Tamura–Nei model (*39*), which takes into account both transition/transversion and compositional content biases. The degree of confidence assigned to nodes in trees was achieved by bootstrapping (*40*) with 2000 replicates (*41*). The species *Pagrus auriga* (Perciformes, Sparidae) was used as an outgroup (GenBank/EMBL/DDBJ accession number of the complete mitochondrial genome: AB124801) to root trees.

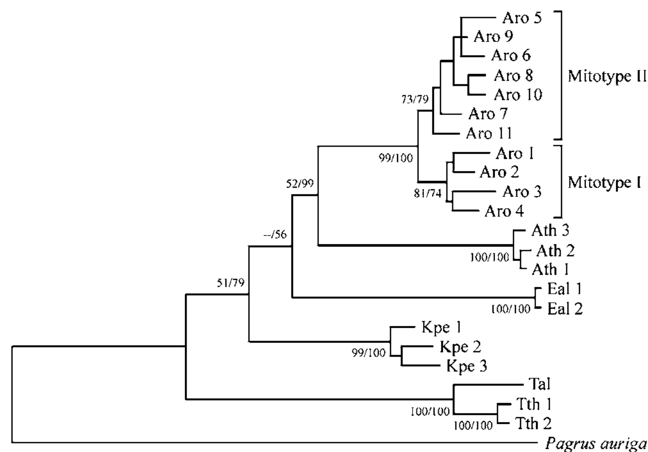

Figure 1. Neighbor-joining phylogenetic tree inferred from Tamura-Nei genetic distances among partial sequences of cyt *b* and ATPase 6 mitochondrial genes. Bootstrap values >50% are indicated on the tree for cyt *b*/ATPase 6 sequences. The species *Pagrus auriga* (Perciformes, Sparidae) was used at outgroup.

RESULTS AND DISCUSSION

This study, involving a multiplex-PCR-based method, shows that amplification of species-specific products corresponding to *A. thazard* and *A. rochei* can be used to authenticate processed commercial products of frigate tunas. The procedure is reliable and less time-consuming than any of the molecular techniques developed to date, making it of practical value to all laboratories concerned with the authentication of these kinds of products.

Sequence Data, Phylogenetic Analysis, and Primer Design. For the development of the multiplex-PCR-based method of authentication presented in this study, three pairs of primers (**Table 1**), corresponding to highly conserved regions of each of the cyt *b*, ATPase 6, and rRNA 12S mitochondrial genes, were designed. PCR fragments of each of the genes were further sequenced in all samples included in this work (**Table 2**) and aligned for pairwise comparisons.

A neighbor-joining tree was inferred using Tamura–Nei genetic distances from the cyt *b* and ATPase 6 sequences (**Figure 1**). In each case, bootstrap analysis was used to obtain support for the different nodes. All examples from *A. rochei* species were grouped in the same cluster, with bootstrap values of 99 and 100% for cyt b and ATPase 6, respectively. Similar results were obtained with respect to examples of *A. thazard*, *E. alletteratus*, *K. pelamis*, and *T. alalunga*/*T. thynnus*. Surprisingly, inside the *A. rochei* clade, a well-supported subdivision into two separate branches was observed. The first one, named Mitotype II, included examples from Atlantic–Mediterranean (Aro 8–Aro 11) and Pacific (Aro 5–Aro 7) origins, whereas the second one, Mitotype I, included examples from only the Atlantic–Mediterranean (Aro 1–Aro 4). This repetitive phylogenetic pattern corresponded to conserved nucleotide differences along the partial sequences obtained from each of the genes (see **Figures 2** and **3**). The complete mitogenome sequence of these two mitotypes has been obtained in our laboratory and deposited in the GenBank/EMBL/DDBJ under accession numbers AB103467 (mitotype I) and AB103468 (mitotype II).

In the case of cyt *b*, specific primers for *A. rochei* were designed (**Figure 2**). Due to the restrictions in band size imposed by the canning process (*28*–*30*), only fragments between 80 and 150 bp were considered. Positions 56 and 89 were chosen as optimal for two reasons. First, they allowed for observation

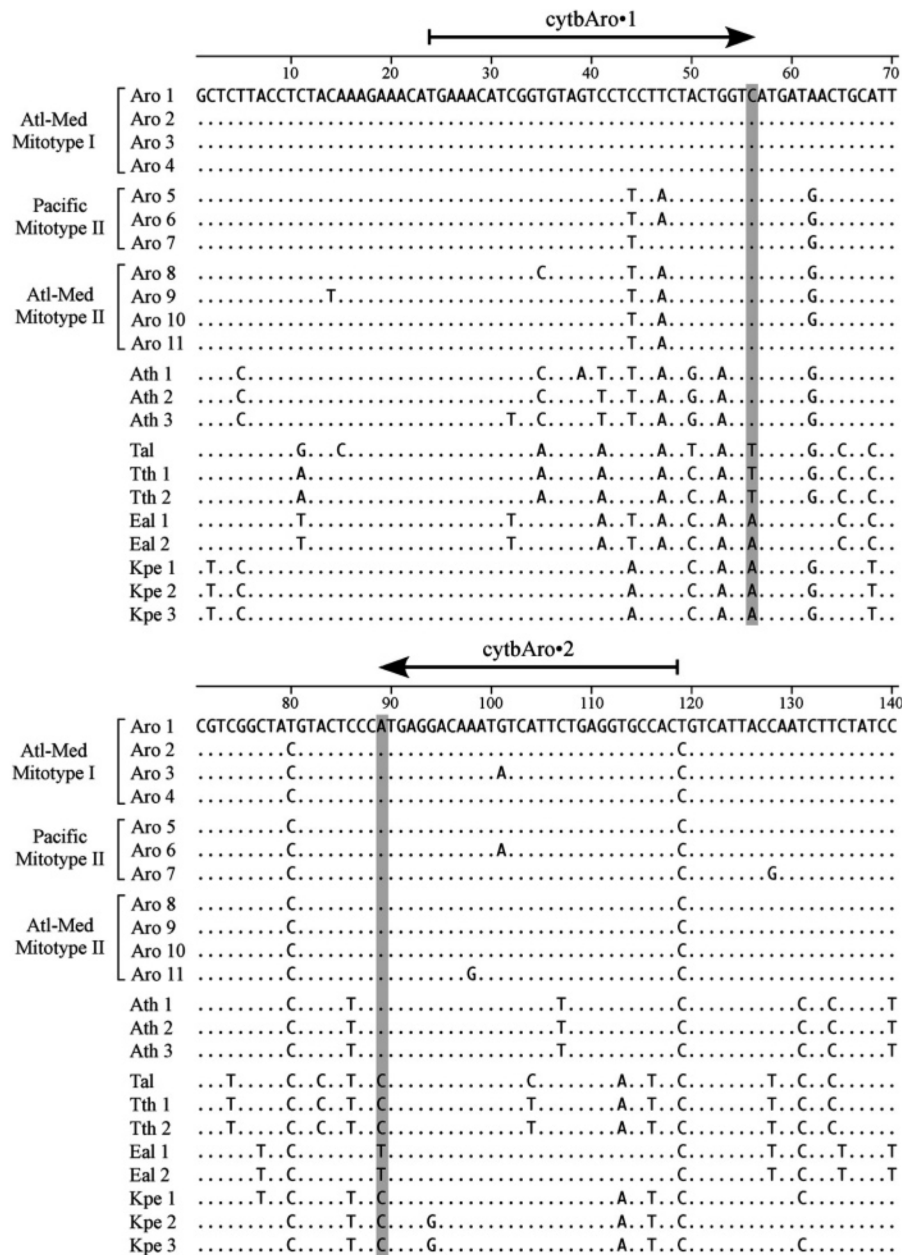

Figure 2. Location of the forward (cytbAro•1) and reverse (cytbAro•2) primers for the amplification of the *A. rochei* specific product. Positions 56 and 89, corresponding to the 3′-ends of the primers, are shaded in gray in each of the sequences obtained.

of the *Auxis*-specific nucleotide differences at the critical 3′-end in both primers cytbAro•1 (C in *Auxis* species, T or A in tuna species) and cytbAro•2 (A in *Auxis* species, C or T in tuna species), respectively. Second, these primers showed the best thermodynamic characteristics as selected by the program Oligo v 6.82 (Medprobe). The amplicon expected was 95 bp in length (**Table 1**).

A similar strategy was used to design specific primers for *A. thazard* but with ATPase 6 partial sequences (**Figure 3**). In this case, positions 287 and 356 corresponding to the 3′-ends of the primers ATPath•1 (C in *Auxis* species, A or G in tuna species) and ATPath•2 (T in *Auxis* species, C in tuna species) were selected, respectively. The size of the expected amplified fragment was 128 bp in length in all samples (**Table 1**).

In some cases, the existence of PCR inhibition has been reported for canning products (*33*). Consequently, for the multiplex-PCR reaction, a third primer pair was included to amplify a fragment of the highly conserved mitochondrial rRNA 12S gene to provide positive control of DNA amplification (**Figure 4**). There were no nucleotide differences among sequences with regard to these primers to ensure efficient amplification in all tuna species. The amplicon expected was 146 bp (145 bp for *T. alalunga* and *T. thynnus*) in length (**Table 1**).

Test Development. Once the three primer pairs were designed, the PCR conditions were first optimized using fresh fish DNA samples. An optimal annealing temperature of 66 °C was determined, as there was no amplification observed in the specific *Auxis* fragments in tuna species (**Figure 5**). In the same way, no cross-amplification between *A. thazard* and *A. rochei* was detected, indicating that the existing nucleotide differences were sufficient to provide specificity with the PCR conditions used, because the positions corresponding to the 3′-ends of the primers were the same in the two species. It is worth noting that, even with the observed nucleotide differences between the *A. rochei* mitotype sequences in relation to the specific cyt *b*

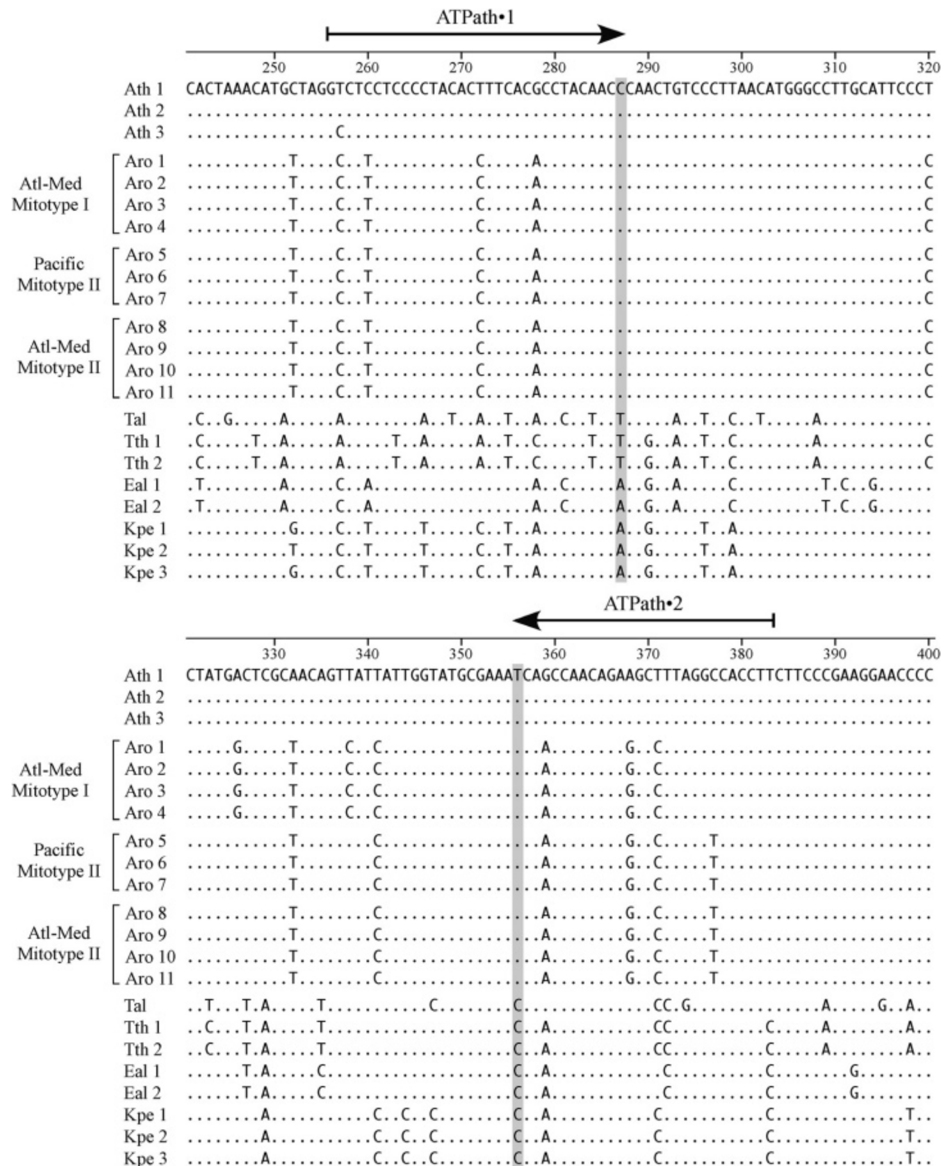

Figure 3. Location of the forward (ATPath•1) and reverse (ATPath•2) primers for the amplification of the *A. thazard* specific product. Positions 287 and 356, corresponding to the 3′-ends of the primers, are shaded in gray in each of the sequences obtained.

primers, the optimized annealing temperature of the PCR reaction allowed successful amplification in all *A. rochei* samples.

The same PCR conditions were assayed in 20 commercially canned frigate tuna products. As shown in **Figure 6**, 5 samples tested positive for *A. thazard* and 13 tested positive for *A. rochei*. However, negative results were obtained in two samples (6 and 16). Further sequencing of the rRNA 12S product allowed identifying these samples as *K. pelamis* (not shown). This is an important advantage of this system, as there is no need of amplifying and analyzing a new fragment to detect commercial fraud. Consequently, because the discrimination is achieved during the course of the PCR reaction, for the purpose of obtaining positive amplification, no more analytical methods such as sequencing, RFLP, or SSCP are required.

One of the major problems with techniques such as PCR-RFLP and PCR-SSCP is the existence of intraspecific variability, giving rise to possible mutations and genetic variability within a species and, consequently, to the loss or gain of restriction recognition sequences (*18*, *28*) and changes in the representative pattern of single-stranded DNA (*36*), respectively. Yet, it should be highlighted that our analytical method, analogous to a mutagenically separated PCR, would be affected only if the specific base at the 3′-end of the primers was altered in relation to the template DNA of *A. thazard* or *A. rochei*. To date, intraspecific variation has not been found to present a problem in relation to the cyt *b* and ATPase 6 genes, although the 3′-ends of the primers are located in the third position of a codon, where the majority of the polymorphism in protein-coding genes is detected. In fact, nearly 200 commercial frigate tuna samples have already been tested in our laboratory, and no false negatives have been obtained as confirmed by sequencing. Thus, our protocol constitutes a rapid screening method for detecting positive signals. Samples giving a negative result require identification by a different DNA analysis technique (sequencing, PCR-RFLP, or PCR-SSCP). Furthermore, the possibility of successful amplification of the specific *Auxis* DNA fragments in tuna species (false positives) must be rejected, even if identity of bases at the 3′-end of any of the primer pairs is found, owing to the high stringent PCR conditions. In this sense, the simplicity, sensitivity, and confidence in the results of species-specific PCR amplification of DNA products for authentication of commercial products contribute to the development of an increasing number of similar systems (*42*−*44*).

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                                   ARN12spp•1
                              250       260       270       280       290       300       310       320       330
Ath 1                         CGTCAGGTCGAGGTGTAGCGCATGAGAGGGGAAGAAATGGGCTACATTCGCTACTACTAGCGAATACGAACGATGCACTGAAAACGTTCA
Ath 2                         ..........................................................................................
Ath 3                         ..........................................................................................
Atl-Med Mitotype I     Aro 1  ..........................................................................................
                       Aro 2  ..........................................................................................
                       Aro 3  ..........................................................................................
                       Aro 4  ..........................................................................................
Pacific Mitotype II    Aro 5  ..........................................................................................
                       Aro 6  ..........................................................................................
                       Aro 7  ..........................................................................................
Atl-Med Mitotype II    Aro 8  ..........................................................................................
                       Aro 9  ..........................................................................................
                       Aro 10 ..........................................................................................
                       Aro 11 ..........................................................................................
Tal                           .....................................................A..-..............................C..
Tth 1                         .....................................................AC.-.................................
Tth 2                         .....................................................AC.-.................................
Eal 1                         ........................................................T.................................
Eal 2                         ........................................................T.................................
Kpe 1                         ......................................................C.T.................................
Kpe 2                         ......................................................C.T.................................
Kpe 3                         ......................................................C.T.................................

                                                                                   ARN12spp•2
                              340       350       360       370       380       390       400       410       420
Ath 1                         TCTGAAGGAGGATTTAGCAGTAAGTGGAAAATAGAGTGTTCCGCTGAAATCGGCTCTGAAGTGCGTACACACCGCCCGTCACTCTCCCCA
Ath 2                         ..........................................................................................
Ath 3                         ..........................................................................................
Atl-Med Mitotype I     Aro 1  ..........................................................................................
                       Aro 2  ..........................................................................................
                       Aro 3  ..........................................................................................
                       Aro 4  ..........................................................................................
Pacific Mitotype II    Aro 5  ..........................................................................................
                       Aro 6  ..........................................................................................
                       Aro 7  ..........................................................................................
Atl-Med Mitotype II    Aro 8  ..........................................................................................
                       Aro 9  ..........................................................................................
                       Aro 10 ..........................................................................................
                       Aro 11 ..........................................................................................
Tal                           ..........................................A...............................................
Tth 1                         ..........................................A...............................................
Tth 2                         ..........................................A...............................................
Eal 1                         ..........................................................................................
Eal 2                         ..........................................................................................
Kpe 1                         ..........................................................................................
Kpe 2                         ..........................................................................................
Kpe 3                         ..........................................................................................
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Figure 4. Location of the forward (RNA12spp•1) and reverse (RNA12spp•2) primers for the amplification of the universal product. Shaded in gray is shown the total coincidence of the partial rRNA 12S nucleotide sequences between examples with those of the primers designed.

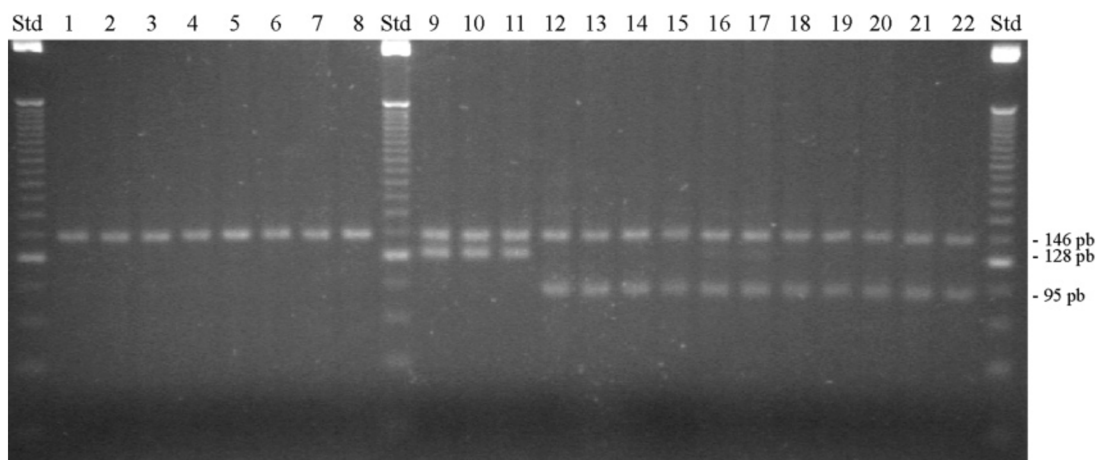

Figure 5. Multiplex-PCR products from fresh DNA samples subjected to species-specific amplification. Electrophoresis was performed on a 2.5% Ultrapure Agarose 1000 (Invitrogen) and stained with ethidium bromide. Std, lanes containing the 25 bp DNA Ladder (Invitrogen); lane 1, Tal; lane 2, Tth 1; lane 3, Tth 2; lane 4, Eal 1; lane 5, Eal 2; lanes 6–8, Kpe 1–Kpe 3; lanes 9–11, Ath 1–Ath 3; lanes 12–22, Aro 1–Aro 11. The size of the generated products is indicated on the right.

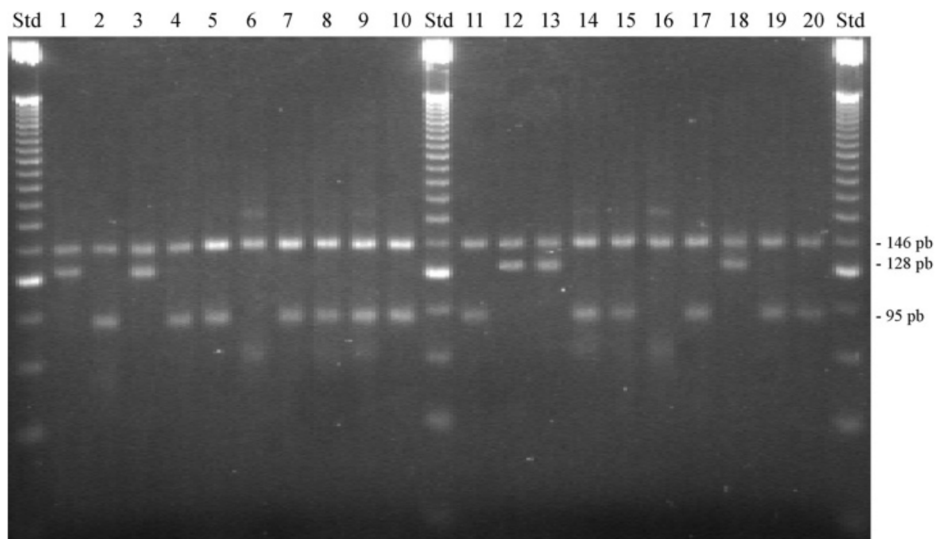

Figure 6. Multiplex-PCR products from 20 different commercial canned DNA samples subjected to species-specific amplification. Electrophoresis was performed on a 2.5% Ultrapure Agarose 1000 (Invitrogen) and stained with ethidium bromide. Std, lanes containing the 25 bp DNA Ladder (Invitrogen). The size of the generated products is indicated on the right.

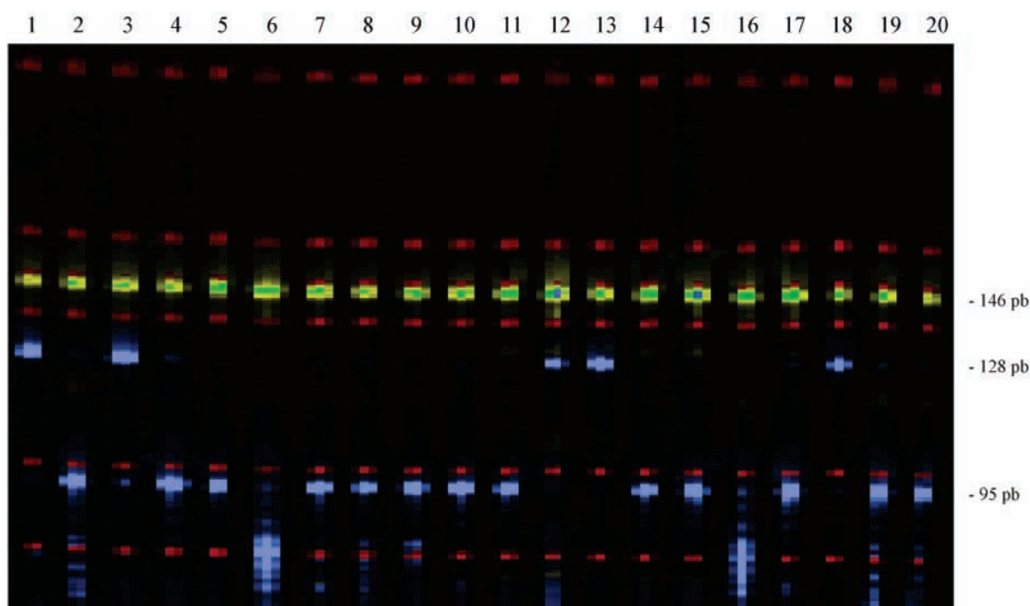

Figure 7. Multiplex-PCR products from 20 different commercial canned DNA samples subjected to species-specific amplification. Samples were electrophoresed on a 4.25% AutoPAGE high-throughput acrylamide gel (Sigma). The size of the different fragments, indicated on the right, was determined with the software GeneScan v 3.1.2 (Applied Biosystems) using GeneScan-350 TAMRA (Applied Biosystems) as internal lane size standard.

Figure 7 shows the same image as **Figure 6**, but, instead, using fluorescently labeled primers. One of the major advantages of employing fluorescent, versus UV, detection of PCR amplified bands is the higher sensibility of the system. In our case, the DNA quantity necessary to obtain an appropriately measurable signal is reduced by ~100-fold (0.07 μL instead of 10 μL of the PCR products). This is a very important issue to take in account because it has been determined that, in commercially canned tuna, the ability to acquire and subsequently analyze a PCR product may be affected by canning conditions (*33*). In some cases, the lack of UV−visible PCR products has been attributed to the high degree of DNA degradation produced, as result of the thermal treatment, or to the presence of PCR inhibitors, such as additives used in the food industry as well as those generated in the canning process itself (*33*). In fact, PCR inhibitors are common in degraded DNA (*45*, *46*). Increasing the amount of DNA template in the PCR reaction has not proven to be the best solution in resolving this problem, as it has been closely related to an excess of inhibitors (*30*, *33*). Nevertheless, in such situations, an amplicon of 276 bp corresponding to cyt *b* gene has been obtained in commercially canned tuna species using nested primers in two consecutive PCR reactions (*30*). The results of this survey demonstrate that, even in the presence of inhibitors, the first PCR product was in fact amplified, albeit in an extremely low amount to be detected in ethidium bromide gel. Therefore, the use of fluorescently labeled primers in authentication analyses of canned products in general, and frigate tunas in particular, could be a good alternative when identification of a particular species is confirmed if an appropriately sized amplicon is detected on the gel. Although there was not detected inhibition of the PCR reactions due to additives in the 20 canned samples analyzed in this study, this possibility cannot be ruled out in any case.

As previously stated, the analytical methods employed in

species identification should be rapid and easy to perform, without being cost prohibitive (*1*). The one-step protocol presented here fulfils all of these criteria. Moreover, if fluorescently labeled primers were used, inconveniences caused by PCR inhibition could be overcome, as a result of the ability of the system to detect very small amounts of DNA. Given the possibility of intraspecific variability in mitochondrial DNA sequences, it has also been recommended that the identification of species should be done by two independent DNA analysis techniques (*1*). The method described here is a powerful tool for the detection of mislabeling or fraudulent substitution of frigate tuna species in canned products and constitutes an undoubtedly valuable addition to the range of methodologies currently available for *A. thazard* and *A. rochei* identification.

ACKNOWLEDGMENT

We thank José María Naranjo Márquez his continuous support to the laboratory. C.I. is grateful to Marc Jérôme for kindly supplying papers.

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Received for review May 5, 2004. Revised manuscript received September 13, 2004. Accepted September 22, 2004. This work has been funded by IFOP.

JF0492868