Use of mtDNA Direct Polymerase Chain Reaction (PCR) Sequencing and PCR–Restriction Fragment Length Polymorphism Methodologies in Species Identification of Canned Tuna

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Identification of six canned tuna species using DNA-based methodology was studied. DNA was degraded during the canning process of fish muscle: DNA fragment sizes that ranged from <100 up to 200 bp were obtained from canned tuna muscle, whereas DNA sizes for frozen tuna muscle ranged from <100 up to 20 000 bp. Amplification of DNA from canned tuna muscle was carried out using primers flanking a region of cytochrome *b* gene of 126 bp. Sequences from PCR-amplified DNA of six tuna species were studied for polymorphic sites; seven diagnostic positions were identified in this fragment for the species studied. The suitability of a genetic distance measurement with phylogenetic tree construction method for the identification of canned tuna species using two cytochrome *b* sequences (299 and 126 bp) was studied. PCR-amplified DNA from canned tuna was also analyzed by using three restriction endonucleases, *Bsi*YI, *Mbo*I, and *Mnl*I. The restriction fragments allowed for the identification of the six tuna species studied.

Keywords: *Canned tuna; species identification; cytochrome b; genetic distance; RFLP–PCR*

INTRODUCTION

The identification of fish species of commercial importance becomes a problem when the usual external characteristics of the fish are removed by processing into fillets or portions of flesh devoid of any species-specific characteristics.

Raw fish is easily identified by the use of isoelectric focusing (IEF) of water-soluble proteins, with specific profiles being obtained by IEF. However, when elaboration processes involve a certain degree of thermal treatment, such as smoking, cooking, frying, or canning, the water solubility of these proteins is irreversibly lost, necessitating the use of alternative approaches to identify species in such products (Mackie, 1990; Rehbein, 1990; Sotelo et al., 1993).

In the case of canned fish, at least one sterilization step is involved in the thermal treatment applied, which results in complete denaturation of muscle proteins and hindrance of protein recovery for further analyses. One possible way of solving this problem is the use of specific enzymes or chemical cleavage of heat-denatured muscle proteins (Mackie et al., 1992). However, this methodology is not entirely satisfactory when one is trying to differentiate and identify species belonging to the same genus.

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Tuna species include fish belonging to the *Thunnus* genus as well as to the genera *Sarda*, *Katsuwonus*, and *Euthynnus*, species that can have different market prices, depending on the country. Likewise, there is a commercial labeling difference among *Thunnus* species. Usually, the “white tuna” label means that the can should contain *Thunnus alalunga*, “light tuna” refers to canned *Thunnus albacares*, and “tuna” can be any *Thunnus* or similar species. It is quite obvious that mislabeling of these species of different price and quality must be avoided and that a tool allowing for the enforcement of labeling regulations is a necessity for the canning industry as well as for government food analysis laboratories.

Since species identification of heat-sterilized tunas is extremely difficult through the use of protein analyses, the suitability of deoxyribonucleic acid (DNA) analysis techniques was considered to be worthy of study in trying to solve the problem of identifying fish species in food items. In fact, it had been shown that analysis of mitochondrial DNA was successful in differentiating species (Borgo et al., 1996; DeSalle and Birstein, 1996). Also, Bartlett and Davidson (1991) showed that by polymerase chain reaction (PCR) sequencing of a certain fragment of cytochrome *b* [350 base pairs (bp) long] it was possible to differentiate four raw *Thunnus* species.

The problem of identifying species in canned muscle is that the severe thermal treatment applied to the muscle leads to alteration of the DNA, with less DNA being extracted from the tissue and with this DNA being degraded. Unseld et al. (1995) first dealt with this problem by extracting DNA from canned muscle and

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amplifying a rather short mitochondrial cytochrome *b* sequence (59 bp). However, the sequences obtained from this short fragment did not allow for the differentiation of all tuna species: two species of great commercial importance, *T. albacares* and *T. thynnus*, were not distinguished.

The main objective of this study was to obtain DNA from canned tuna and to produce an amplified DNA fragment suitable for sequencing and for obtaining species-specific sequences of six tuna species. Another PCR-based technique was also tested in an attempt to provide a simpler method for the analysis of canned tuna species.

MATERIALS AND METHODS

Collection and Preparation of Samples. Raw albacore (*T. alalunga*), Atlantic bonito (*Sarda sarda*), yellowfin tuna (*T. albacares*), skipjack (*Katsuwonus pelamis*), bluefin tuna (*T. thynnus*), and bigeye tuna (*Thunnus obesus*) were obtained from the Vigo (Spain) local market. The fish were gutted immediately and then frozen in an air-blast freezer at −40 °C. After freezing, each specimen of each species was cut to provide a frozen sample, with the remaining part being employed for the preparation of two or three cans. The numbers of specimens used in this study were as follows: four albacores, one Atlantic bonito, two yellowfin tunas, four skipjacks, four bluefin tunas, and three bigeye tunas.

Canned samples were prepared by steam cooking and then filling circular cans with white muscle (60.5 diameter × 30.5 mm depth). Once the muscle was inside the can, salt and vegetable oil were added and lids placed on each can. The cans were sterilized at 115 °C for 55 min.

Extraction of DNA. Oil and lipids were removed from canned muscle by soaking in chloroform/methanol/water (1:2:0.8) overnight. The defatted muscle was recovered by filtration and employed for DNA extraction.

The method is a modification of that of Kocher et al. (1989). Two hundred milligrams of muscle was ground to fine powder with the aid of liquid nitrogen. This powder was incubated, at 37 °C overnight, with 1 mL of TENSD buffer (100 mM Tris-HCl, pH 8.0; 30 mM EDTA; 100 mM NaCl; 5% SDS; 50 mM dithiothreitol; 300 μg/mL proteinase K). DNA was isolated by three extractions with phenol/chloroform/isoamyl alcohol (24:24:1). The DNA present in the aqueous phase was ultrafiltrated by using a Microcon-100 (Amicon).

Mitochondrial Cytochrome *b* Gene Fragment Amplification. Primers, used for amplification of part of the mitochondrial cytochrome *b*, were those described by Bartlett and Davidson (1991). These primers were designated cytBH 5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′ and cytBL 5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′. Also, another set of primers was used in this study: H15573 5′-AAT AGG AAG TAT CAT TCG GGT TTG ATG-3′ and L15424 5′-ATC CCA TTC CAC CCA TAC TAC TC-3′; these primers correspond with those described by Meyer (1993), and the fragment amplified using these primers is designated here B126.

Reactions were prepared as follows: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each dNTP, 2.5 mM $MgCl_2$, 0.625 units/reaction AmpliTaq DNA polymerase (Perkin-Elmer), 0.06 μM each primer, and 0.2−1 μg of template DNA. Reaction volumes were of 50 μL, and no mineral oil was employed to overlay reactions. Amplification reactions were carried out in a Gene Amp 2400 PCR system from Perkin-Elmer. Amplification of a DNA fragment of ≈350 bp was intended with cytBH and cytBL primers, and 30 cycles were employed (92 °C for 20 s, 50 °C for 20 s, and 72 °C for 50 s); for H15573 and L15424 35 cycles were employed (94 °C for 20 s, 52 °C for 20 s, and 72 °C for 50 s).

Sequence Analyses. Direct sequencing of PCR products was carried out using Sequenase PCR product sequencing kit (USB, Cleveland, OH). Autoradiograms were read in a Bio-image image analyzer and sequences analyzed with Gene-Jockey software (Biosoft, Cambridge, MA).

PCR−Restriction Fragment Length Polymorphism (RFLP) Analysis of the B126 Fragment. Once the B126 fragment sequences for the different tuna species had been obtained, a search was undertaken for restriction endonuclease sites; eventually, a set of enzymes was chosen on the basis of the predictable specific pattern that they would produce.

B126 PCR products were concentrated to a volume of 6 μL by using Microcon-50 microconcentrators (Amicon, Beverly, MA) with a nucleotide cutoff of 100 bp of double-stranded DNA. Aliquots from concentrated PCR amplicons were digested separately with *Bsi*YI (Boehringer Mannheim) and *Mbo*I and *Mnl*I (Amersham) at 55, 37, and 37 °C, respectively. Digestions were carried out in 20 μL volumes, consisting of 11 μL of water, 2 μL of 10× specific enzyme buffer (supplied by the manufacturers with enzymes), and 1 μL of each enzyme solution, giving final concentrations of 1−2 units per reaction. The fragments generated from the digestion were separated by PAGE.

Electrophoresis of DNA Fragments. Agarose electrophoresis (LE analytical grade agarose from Promega Corp., Madison, WI) was used to check DNA extraction and PCR products, 2 and 4% agarose gels using TAE buffer (2 mM EDTA, 40 mM Tris acetate pH 8.5) with of ethidium bromide (0.5 μg/mL).

DNA restriction fragments were separated in rehydrated 36S Clean Gels from Pharmacia Biotech. The gels were rehydrated in 112 mM Tris acetate, pH 6.4, and as electrode buffer 0.2 M Tricine, 0.2 M Tris, and 0.55% SDS, pH 8.3, was used. Electrophoresis was carried out at 300 V, 15 mA, and 15 W for ∼3 h. DNA fragments were visualized by staining with the silver staining kit DNA from Pharmacia Biotech.

Measurement of Genetic Distance. Genetic distances and phylogenetic trees were calculated by employing the Tamura−Nei model included in the MEGA program (Kumar et al., 1993).

RESULTS AND DISCUSSION

Extraction of mtDNA from Frozen and Canned Tuna. One of the standard procedures for DNA extraction is the generally termed proteinase K−SDS digestion method (Kocher et al., 1989). Bartlett and Davidson (1991) reported some difficulties in obtaining undegraded high molecular weight DNA using this methodology. However, in our experience, the yield and quality of DNA recovery were good, allowing for the obtention of amplification product. This procedure was simplified and improved by the inclusion of an ultrafiltration step, thus eliminating the ethanol precipitation, saving time and increasing the yield of DNA recovery. Using this alternative, a concentration step is gained, and this could be very important in samples with low amounts of degraded DNA, such as canned tuna samples. An additional benefit is that DNA samples are purified from interfering material such as nucleic acids fragments shorter than 100 bp, which are washed out with this procedure. This proteinase K method has been used in this study as a standard method for DNA extraction from both frozen and canned tuna muscle.

One of the main features of DNA extracted from canned tuna muscle is the degradation, produced as a result of thermal treatments employed in the processing of canned tuna. In this case, the fragment size of the DNA which was detected with ethidium bromide is ∼100 bp; the smear of degraded DNA ranged from <100 up to 200 bp. Frozen samples, depending on the conditions of frozen storage, gave fragments of variable length (20 000−100 bp) (Figure 1).

Amplification of mtDNA from Frozen and Canned Tuna. The DNA extracted from both frozen

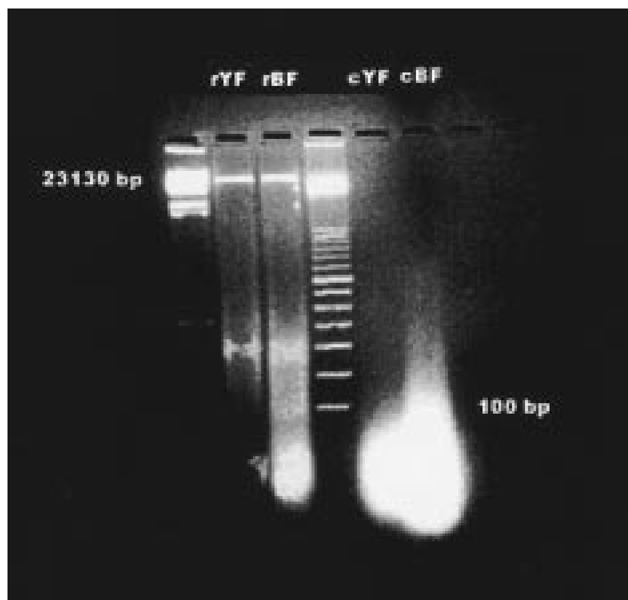

Figure 1. Electrophoretic analysis of DNA extracted from frozen and canned tuna on 2% agarose gel: (from left to right) λ DNA−*Hin*dIII digest MW marker; frozen *T. albacares* (rYF); frozen *T. thynnus* (rBF); 100 bp DNA ladder (MW); canned *T. albacares* (cYF); canned *T. thynnus* (cBF).

and canned tuna was tested for amplification using two sets of primers, the first being the CytbL/H primers, which should produce a 350 bp fragment, and the other being the H15573/L15424 primers, which should produce a 176 bp fragment (B126 fragment).

The DNA extracted from frozen samples resulted in a PCR product with both sets of primers (Figure 2A). In the case of canned tuna DNA, the amplification of the CytbH/L usually failed (Figure 2B).

The first requirement for authentication of canned tuna is to obtain DNA suitable for amplification and containing enough information to enable the differentiation of tuna species. The proteinase K method appeared to be appropriate for extracting both frozen and thermally processed tissues, due to the high lysis power of proteinase K and SDS and the concentration step introduced in place of the alcohol precipitation.

Another important aspect in the analysis of canned fish species is the DNA fragment size: to be amplified, it should be of the same length or shorter than the longest fragment present in the sample. This maximum size has to ensure that in every case the amplification will not be impeded by too low a number of DNA template molecules of adequate size. In this study this size was estimated to be 176 bp. Similar results were obtained by Unseld et al. (1995); the amplification of canned tuna DNA was successful only when the amplified fragment (123 bp) was of a length similar or inferior to the average size of extracted DNA.

Finally, the amplification should be carried out with conserved primers, which means that a PCR product has to be generated for every species within the study with similar yields. Amplification with conserved primers might even allow the detection of mixed species sequences present in an individual can of fish.

Sequencing of mtDNA from Canned Tuna. DNA sequences were obtained for the B126 fragment, which was amplifiable in every tuna species analyzed. This fragment had not been entirely sequenced before, and sequences for different individuals of six canned tuna species are given in Table 1.

The direct PCR sequencing method, using the Sequenase PCR product sequencing kit from USB, proved to be very useful with canned tuna mtDNA fragments, as it was accurate and few ambiguities were found. In all samples, sequences in both directions were obtained, with those from canned and frozen samples being identical in every case.

After comparing these DNA sequences in different individuals, it was observed that 94 of the 126 bases of the sequence were monomorphic bases, whereas 32 were polymorphic; 25 of these were polymorphic sites with only two variants (positions 4, 13, 22, 24, 25, 28, 31, 34, 40, 46, 55, 56, 64, 70, 73, 76, 82, 91, 97, 100, 103, 112, 115, 121, and 124), whereas the other 7 polymorphic nucleotides had three variants (positions 1, 19, 37, 43, 67, 109, and 118). Among these polymorphic sites, seven were selected as positions with diagnostic value (Table 2). These diagnostic positions were selected using two criteria: first, that they had to be useful for the differentiation of all the tuna species included in this study, and, second, that these positions did not show intraspecific variability. In addition to these diagnostic positions, position 64 can be useful for the differentiation of *T. obesus* and *T. albacares*. Using the B126 diagnostic positions and position 64, *T. obesus* and *T. albacares* were differentiated with two nucleotides. With these diagnostic positions *T. thynnus* and *T. albacares* were differentiated with two nucleotides, whereas with the cytochrome *b* fragment sequence used by Unseld et al. (1995) there were no differences.

One important step in designing a strategy for species identification is the selection of the sequence to be employed as the diagnostic sequence. One of the first requirements that the sequence data has to fulfill is that it must be of sufficient length to contain species information and also be present as intact DNA template in the canned sample. In the literature, we have found short sequences that were suitable for amplification in canned tuna, but they were devoid of sufficient species information to allow for the identification of all tuna species (Unseld et al., 1995). The use of sequences from mtDNA for identification has been recognized by several authors. However, among the cytochrome *b* gene fragments, differences regarding interspecific variability can be found (Whitmore et al., 1994). With the B126 fragment six tuna species have been differentiated using the diagnostic positions provided within this fragment.

Another requirement that the sequence data has to fulfill is a low level of intraspecific variability to avoid the misidentification of samples. Reduced intraspecific variation of sequences was found in the number of samples analyzed. Each tuna species analyzed presents an exclusive nucleotide sequence pattern at certain positions, which could be considered diagnostic positions. Furthermore, heteroplasmy was not observed (different mtDNA sequences in the same individual).

Measurement of Genetic Distances for Fish Species Identification. Genetic distance measurement is commonly used for estimating the level of similarity between two particular sequences. The application of genetic distance measurements to the identification of an unknown sample, by building a matrix of pairwise distances between all of the nucleotide sequences introduced in the analysis, permits a more accurate species identification. The explanation for this lies in the fact that, in a typical analysis, the sequence of a particular DNA fragment of an unknown

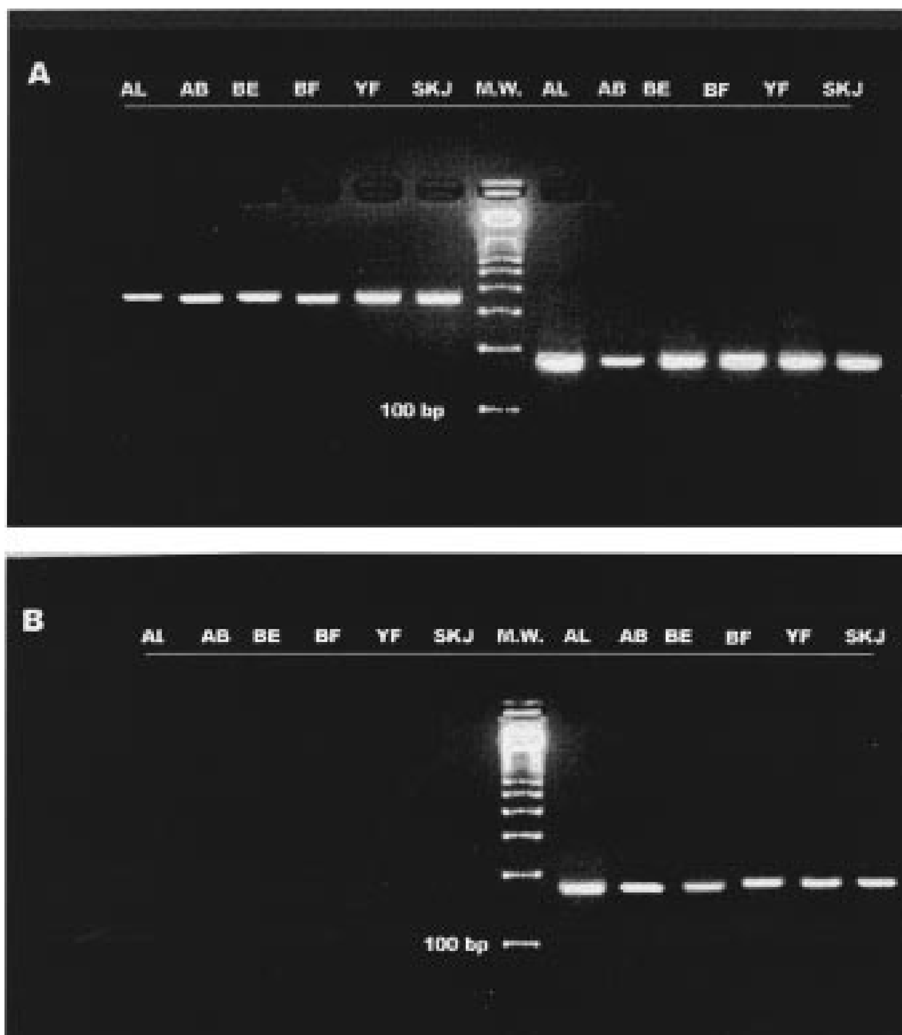

Figure 2. PCR products of six frozen and canned tuna species. Electrophoresis was performed on 4% agarose gels and stained with ethidium bromide. (A) PCR products obtained with frozen tuna muscle DNA: (from left to right) PCR products from six tuna species obtained with the CytBH/L primers; 100 bp ladder (MW); PCR products from six tuna species obtained with the B126 primers. (B) PCR products obtained with canned tuna muscle DNA: (from left to right) PCR products from six tuna species obtained with the CytBH/L primers; 100 bp ladder (MW); PCR products from six tuna species obtained with the B126 primers. Species abbreviations are the same as in Table 1.

sample is introduced in the analysis for comparison with the sequences of that particular fragment of different individuals of reference species. The unknown sequence will show low distance values with the phylogenetic group to which it belongs among the pool of reference sequences.

A similar technique was employed by Bartlett and Davidson (1992), employing phylogenetic tree-building methods using parsimony criteria. Other criteria based on genetic distances were used for meat species identification. In this case, a distance matrix was employed for evaluating the similarity of one unknown sequence with a pool of reference meat samples (Forrest and Carnegie, 1994). The use of distance-based methods seems more appropriate than parsimony methods for the evaluation of the identity of a particular unknown sample because less computing time is involved in the distance methods and phylogenetic information is not as relevant for identification purposes (Forrest and Carnegie, 1994).

The diagnostic character of a particular DNA fragment is greatly influenced by its intraspecific variability. If the number of diagnostic sites is low, the intraspecific variability could mask the ability of these sites to differentiate among species, leading to misidentification of a particular individual. This could be especially important to individuals of the same species belonging to different populations.

Table 3 gives the Tamura−Nei distance matrix (Tamura and Nei, 1993) between the canned tuna B126 sequences. This model permits the analysis of the differences in nucleotide sequence by evaluating the different weights of transitions and transversions and by taking into account each base frequency to determine the importance of any base substitution. Same-species individuals show a low score (below 0.016). When unknown samples are included in the analysis, they will have the lowest scores with the sequences of their own species. As can be seen in the table, scores for the *Thunnus* genus lie between 0.024 and 0.068. The lowest score between *Thunnus* species is presented by *T. albacares* and *T. obesus* (0.024). When different genera are compared, these distance scores are high, like those observed for *Sarda*, *Thunnus*, and *Katsuwonus* that range from 0.122 to 0.238.

Figure 3 shows a phylogenetic tree constructed using the Tamura−Nei distance matrix with a neighbor-joining (Saitou and Nei, 1987) method. In this tree it

Table 1. B126 Sequence Alignment of Six Canned Tuna Species[a]

				111	111	111	122	222	222	223	333	333	333	444	444	444	455	555	555	556	666
	123	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123
AL1	TTA	CAA	AGA	CCT	CCT	TGG	TTT	CGT	GAT	CCT	ACT	AGT	GGC	ACT	CGC	CTC	TCT	AGC	ACT	ATT	CTC
AL2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
AL4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
AL6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
SKJ1	C..	...	...	...	...	...	C..	..C	A..	T..	...	...	A..	T..	...	T..	...	...	...	...	...
SKJ2	C..	...	...	...	...	...	C..	..C	A..	T..	...	...	A..	T..	T..	T..	...	...	...	...	...
SKJ3	C..	...	...	...	...	...	C..	..C	A..	T..	...	...	A..	T..	...	T..	...	...	...	...	...
SKJ6	C..	...	...	...	...	...	C..	..C	A..	T..	...	...	A..	T..	...	T..	...	...	...	...	...
BE4	...	...	...	...	...	...	...	...	...	T..	G..	...	A..	...	...	...	...	...	...	...	...
BE8	...	...	...	...	...	...	...	...	...	T..	G..	...	A..	...	...	...	...	...	...	...	...
BE10	...	...	...	...	...	...	...	...	...	T..	G..	...	A..	...	...	...	...	...	...	...	...
YF1	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
YF12	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
BF2	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
BF7	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
BF9	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
BF13	...	...	...	...	...	...	...	...	...	...	G..	...	A..	...	...	...	...	...	...	...	...
AB2	A..	T..	...	...	T..	...	A..	T.C	A..	T..	G..	T..	T..	...	A..	...	...	...	GT.	...	...

													111	111	111	111	111	111	111	111	111
	666	666	777	777	777	788	888	888	889	999	999	999	000	000	000	011	111	111	112	222	222
	456	789	012	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456
AL1	CCC	TAA	CCT	CCT	GGG	AGA	CCC	AGA	CAA	CTT	CAC	CCC	TGC	CAA	CCC	AAT	GGT	CAC	CCC	ACC	TCA
AL2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
AL4	...	...	...	...	A..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
AL6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
SKJ1	...	...	...	...	A..	...	...	...	...	T..	...	G..	A..	...	...	C..	...	T..	G..	T..	C..
SKJ2	...	...	...	...	A..	...	...	...	...	T..	...	G..	A..	...	...	C..	...	T..	G..	T..	C..
SKJ3	...	C..	...	...	A..	...	...	...	...	T..	...	G..	A..	...	...	C..	...	T..	G..	T..	C..
SKJ6	...	...	...	...	A..	...	...	...	...	T..	...	G..	A..	...	...	C..	...	T..	G..	T..	C..
BE4	T..	C..	...	...	A..	...	...	...	...	...	...	...	...	...	...	C..	...	T..	...	...	...
BE8	T..	C..	...	...	A..	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	...	...
BE10	T..	C..	...	...	A..	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	...	...
YF1	...	C..	...	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	T..	...	...	...
YF12	...	C..	...	...	A..	...	...	...	...	...	...	...	...	...	...	T..	...	T..	...	...	...
BF2	...	...	...	...	A..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	T..	...	...
BF7	...	...	...	...	A..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	T..	...	...
BF9	...	...	...	...	A..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	T..	...	...
BF13	T..	...	...	...	A..	...	T..	...	...	...	...	...	...	...	...	...	...	T..	T..	...	...
AB2	...	A..	T..	T..	A..	...	...	...	...	T..	...	G..	...	T..	...	...	A..	T..	T..	T..	...

[a] AL, albacore; SKJ, skipjack; BE, bigeye tuna; YF, yellowfin tuna; BF, bluefin tuna; AB, Atlantic bonito. The number after the species label denotes different individuals. A dot (.) indicates identity with the sequence directly above.

Table 2. Polymorphic Sites in B126 Fragment with Diagnostic Value[a]

species	position (from 3′ end)						
	28	31	37	82	115	118	121
AL (*T. alalunga*)	C	A	G	C	C	C	A
SKJ (*K. pelamis*)	T	A	A	C	T	G	T
BE (*T. obesus*)	T	G	A	C	T	C	A
YF (*T. albacares*)	C	G	A	C	T	C	A
BF (*T. thynnus*)	C	G	A	T	T	T	A
AB (*S. sarda*)	T	G	T	C	T	T	T

[a] Species abbreviations are the same as in Table 1.

was observed that each tuna species analyzed was well separated in a different cluster. Closer species, such as the ones belonging to the same genus, were also grouped under the same branch. Bigeye and yellowfin appeared as the closest species within the *Thunnus* genus. As mentioned above, some intraspecific variability was observed, especially in the case of *T. alalunga* individuals. When a sample is to be identified, the determined sequence is introduced in the distance matrix analysis and a tree is constructed with the results. The unknown sample will be clustered with those sequences of the reference species to which the sample is most phylogenetically related.

In the analysis of the sequences described by Bartlett and Davidson (1991), corresponding to 299 bp of mitochondrial cytochrome *b* DNA, it was found that 21 positions were variable. This could be an indication of a lower mutation rate in this fragment compared with the B126 fragment (Whitmore et al., 1994). If the aim of the study is the identification of *Thunnus* genus species, it would be necessary to have a higher number of variable positions, which both allows the clear differentiation of closely related species and buffers the possible intraspecific variability.

When the same sort of distance measurement analysis is made with the sequences provided by the Bartlett

Table 3. Genetic Distances Matrix between the B126 Sequences of Six Canned Tuna Species, Using the Tamura−Nei Model[a]

	AL1	AL2	AL4	AL6	SKJ1	SKJ2	SKJ3	SKJ6	BE4	BE8	BE10	YF1	YF12	BF2	BF7	BF9	BF13	AB2
AL1																		
AL2	0.000																	
AL4	0.008	0.008																
AL6	0.000	0.000	0.008															
SKJ1	0.151	0.152	0.142	0.152														
SKJ2	0.162	0.162	0.153	0.162	0.008													
SKJ3	0.163	0.163	0.153	0.163	0.008	0.016												
SKJ6	0.152	0.152	0.142	0.152	0.000	0.008	0.008											
BE4	0.068	0.068	0.059	0.068	0.133	0.143	0.122	0.133										
BE8	0.060	0.059	0.050	0.059	0.142	0.153	0.132	0.142	0.008									
BE10	0.060	0.059	0.050	0.059	0.142	0.153	0.132	0.142	0.008	0.000								
YF1	0.041	0.041	0.050	0.041	0.153	0.164	0.142	0.153	0.033	0.033	0.033							
YF12	0.050	0.050	0.041	0.050	0.143	0.154	0.133	0.143	0.025	0.024	0.024	0.008						
BF2	0.050	0.050	0.042	0.050	0.142	0.153	0.153	0.142	0.051	0.043	0.043	0.041	0.033					
BF7	0.050	0.050	0.042	0.050	0.142	0.153	0.153	0.142	0.051	0.043	0.043	0.041	0.033	0.000				
BF9	0.050	0.050	0.042	0.050	0.142	0.153	0.153	0.142	0.051	0.043	0.043	0.041	0.033	0.000	0.000			
BF13	0.059	0.059	0.050	0.059	0.153	0.164	0.164	0.153	0.042	0.034	0.034	0.050	0.042	0.008	0.008	0.008		
AB2	0.238	0.238	0.227	0.238	0.202	0.202	0.202	0.202	0.214	0.204	0.204	0.225	0.214	0.204	0.204	0.204	0.216	

[a] Species abbreviations are the same as in Table 1. A box around a number indicates that the distance is mentioned in the text.

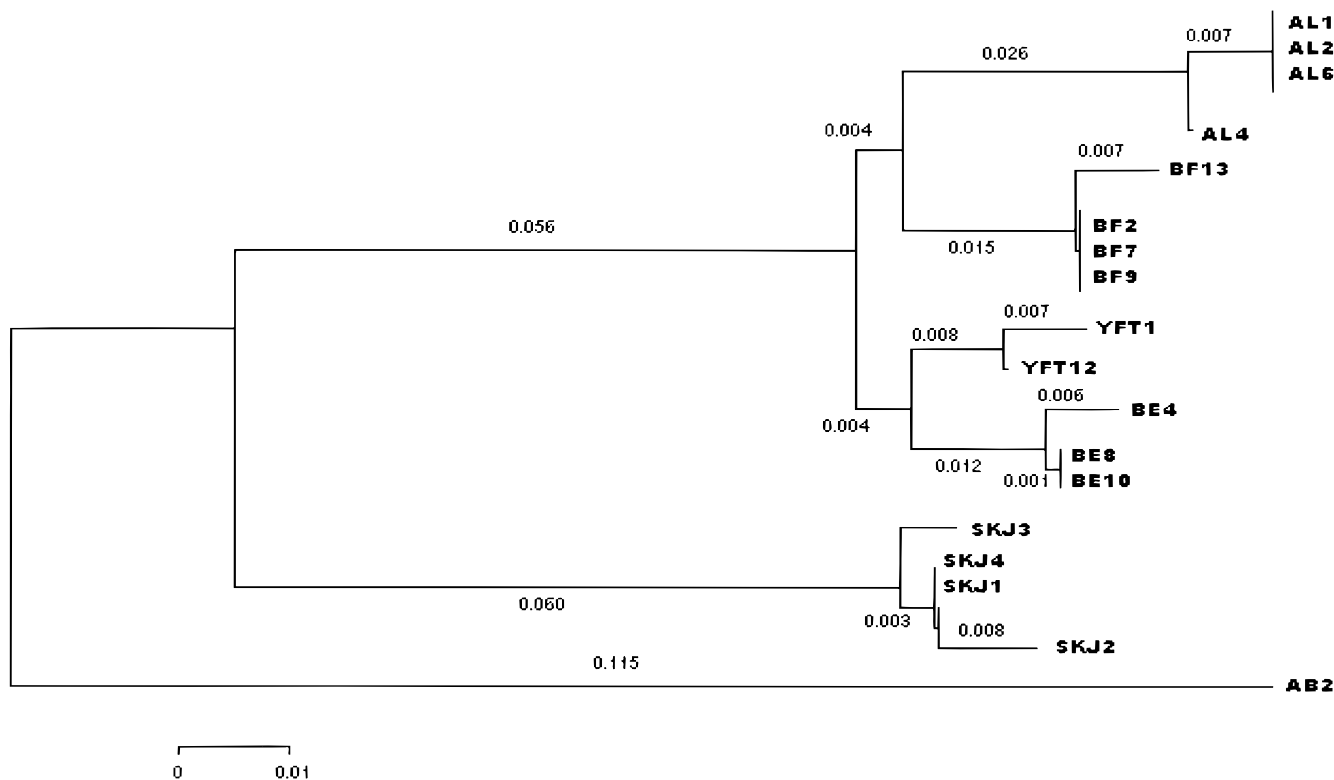

Figure 3. Phylogenetic tree constructed with the Tamura−Nei distance matrix. Species abbreviations are the same as in Table 1.

and Davidson DNA fragment (data not shown), the intraspecific variability ranges from 0.0034 to 0.0206, whereas interspecific values lie between 0.0068 and 0.038. This means that there is an overlap of distance values between and within species. In the case of an unknown sample with distance values ranging from 0.0068 and 0.0206, it would be impossible to classify this sample, using this genetic distance based methodology, in any of the groups because this distance could be due to either intra- or interspecific variability.

PCR−RFLP Analysis of B126 PCR Products. A search for restriction endonuclease cutting sites was undertaken in the sequences of the B126 fragment, obtained from the individuals analyzed in this study. Once a set of restriction enzymes was obtained, a selection of the minimum enzymes that would produce diagnostic restriction profiles was made. Another criterion employed was the lack of intraspecific variability in their recognition sequences. This minimum number was three for the six tuna species analyzed. Table 4 shows the predictable restriction fragments for the six tuna species with *Bsi*YI, *Mbo*I, and *Mnl*I.

PCR products were digested using these three restriction endonucleases, and the generated fragments were separated by PAGE. After silver nitrate staining, different restriction fragments were detected (Figure 4).

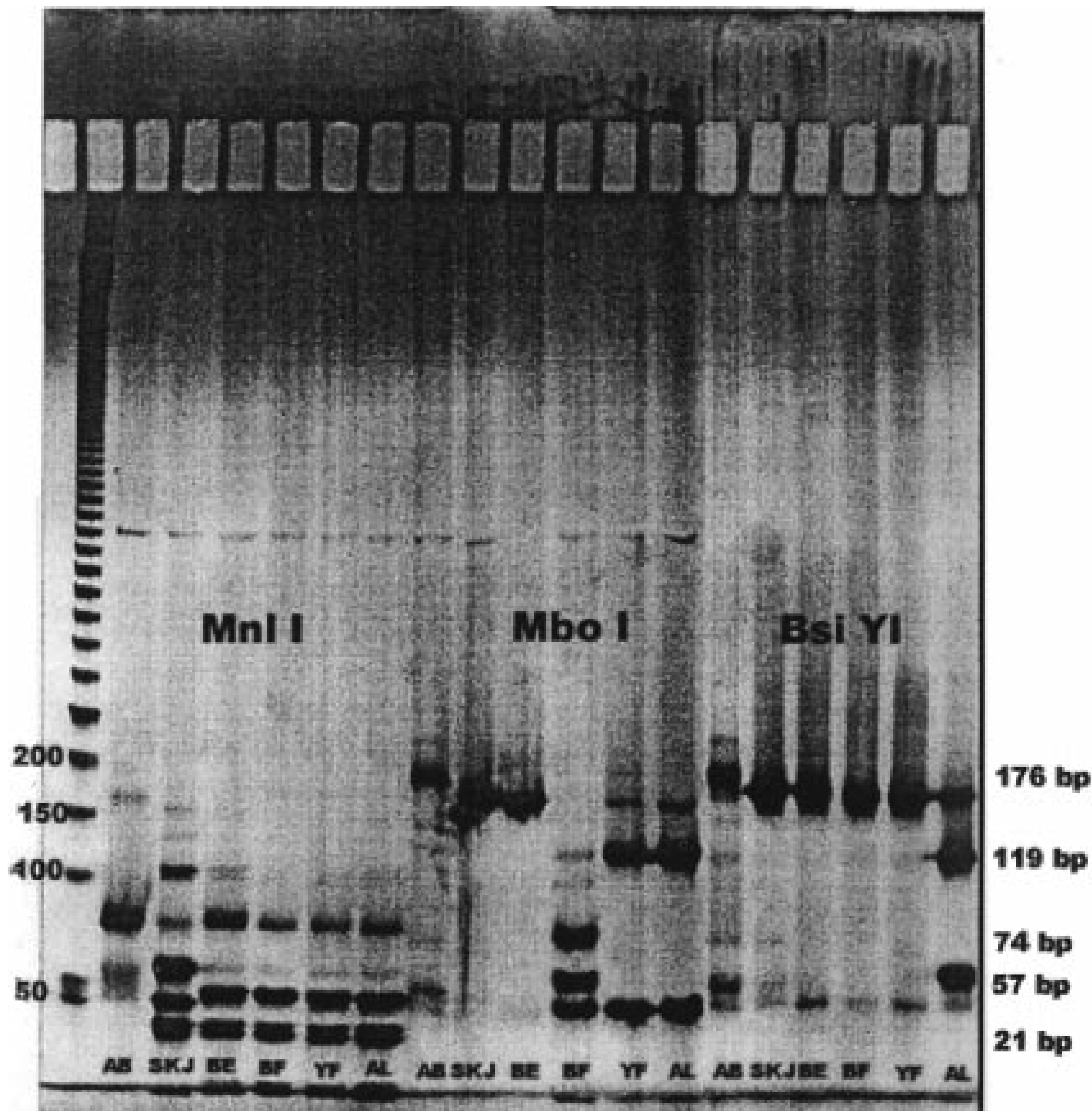

Figure 4. RFLP patterns of six tuna species: (from left to right) 50 bp ladder; *Mnl*I digestion of B126 from the six tuna species; *Mbo*I digestion of B126 from the six tuna species; *Bsi*YI digestion of B126 from the six tuna species. Species abbreviations are the same as in Table 1.

Table 4. Expected Size of Restriction Fragments of Six Tuna Species after Digestion of B126 with Three Enzymes

	*Bsi*YI	*Mbo*I	*Mnl*I
AL	57/119	47/129	21/25/35/43/52
YF	176	47/129	21/25/35/43/52
BF	176	47/55/74	21/25/35/43/52
BE	176	176	21/25/35/43/52
SKJ	176	176	24/43/49/60
AB	176	176	20/77/79

The restriction pattern generated by *Bsi*YI allowed the differentiation between albacore and the rest of the tunas; however, some undigested PCR product can be observed. Since the same result was obtained with several individuals, it seems most likely that the existence of secondary conformation, which could make enzyme access to target site difficult, can be the cause of the undigested product. By using *Mbo*I, bluefin was distinguished from the rest of the tunas. Yellowfin was distinguished by the existence of two fragments after digestion with *Mbo*I and remained undigested with *Bsi*YI. Bigeye, skipjack, and Atlantic bonito were not digested by *Mbo*I, and they had to be differentiated by the use of the third enzyme, *Mnl*I, which gave specific restriction patterns for Atlantic bonito and skipjack. The number of bands obtained did not match the predicted restriction fragments, probably because the sizes of some of the fragments are very similar and they are not discriminated in the gel. For instance, the pattern for Atlantic bonito was one band of 79 bp, skipjack gave a 60 bp fragment and a band composed by the two 43 and 49 bp fragments and the fragment of 24 bp. The four *Thunnus* species presented the same pattern: a band composed by the smallest fragments, 21 and 25 bp, and another band that was made up by three fragments of 35, 43, and 52 bp. An unexpected fragment of ~70 bp also appeared in these four species (albacore, bigeye, bluefin, yellowfin) and skipjack, which could be the result of intermediate digestion products.

The PCR–RFLP method has been used before for the authentication of canned tuna by Ram et al. (1996); these authors used two shorter cytochrome *b* sequences (59 and 60 bp long) and four restriction enzymes. This methodology works for the analysis of two *Thunnus*

species (*T. alalunga* and *T. albacares*), together with *Euthynnus affinis* and *Katsuwonus pelamis*. However, in the case of the 59 bp sequence (R211), which is the same 59-bp cytochrome *b* gene sequence described by Unseld et al. (1995), the sequences of *T. albacares* and *T. thynnus* are identical, which impedes the authentication of these two commercially important species using the PCR−RFLP method with this fragment.

The use of a PCR−RFLP method provides a simpler, quicker, and cheaper alternative to sequencing for tuna species identification. One of the drawbacks of this technique at the moment, also stated by Ram et al. (1996), is the existence of intraspecific variability. Although intraspecific variability was absent at the restriction targets of the enzymes in the B126 sequences used in this study, the existence of undigested PCR products or unexpected digestion products could be due to the existence of nonpreviously detected variability; more work is needed to determine nucleotide substitutions at these target sites to confirm the technique as a method of choice for canned tuna species identification.

ABBREVIATIONS USED

IEF, isoelectric focusing; bp, base pair; PCR, polymerase chain reaction; dNTP, deoxynucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; RFLP, restriction fragment length polymorphism.

LITERATURE CITED

Bartlett, S. E.; Davidson, W. S. Identification of *Thunnus* tuna species by the Polymerase Chain Reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. *Can. J. Fish. Aquat. Sci.* **1991**, *48*, 309−317.

Bartlett, S. E.; Davidson, W. S. FINS (Forensically Informative Nucleotide Sequencing): A procedure for identifying the animal origin of biological specimens. *BioTechniques* **1992**, *12*, 408−411.

Borgo, R.; Souty-Grosset, C.; Bouchon, D.; Gomot, L. PCR−RFLP analysis of mitochondrial DNA for identification of snail meat species. *J. Food Sci.* **1996**, *61* (1), 1−4.

DeSalle, R.; Birstein, V. J. PCR identification of black caviar. *Nature* **1996**, *361* (6579), 197−198.

Forrest, A. R. R.; Carnegie, P. R. Identification of gourmet meat using FINS (Foresincally Informative Nucleotide Sequencing). *BioTechniques* **1994**, *17* (1), 24−25.

Kocher, T. D.; Thomas, W. K.; Meyer, A.; Edwards, S. V.; Pääbo, S.; Villablanca, F. X.; Wilson, A. C. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6196−6200.

Kumar, S.; Tamura, K.; Nei, M. *MEGA: Molecular Evolutionary Genetics Analysis*, version 1.0.; The Pennsylvania State University: University Park, PA, 1993.

Mackie, I. M. Identifying species of fish. *Anal. Proc.* **1990**, *27*, 89−92.

Mackie, I. M.; Chalmers, M.; Reece, P.; Scobbie, A. E.; Ritchie, A. H. The application of electrophoretic techniques to the identification of species of canned tuna and bonito. In *Pelagic Fish: The Resource and Its Exploitation*; Burt, J. R., Hardy, R., Whittle, K. J., Eds.; Fishing News Books: Oxford, U.K., 1992.

Meyer, A. Evolution of mitochondrial DNA in fishes. In *Biochemistry and Molecular Biology of Fishes*; Hochachka, P. W., Mommsem, T. P., Eds.; Elsevier Science: Amsterdam, 1993; Vol. 2.

Ram, J. L.; Ram, M. L.; Baidoun, F. F. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *J. Agric. Food Chem.* **1996**, *44*, 2460−2467.

Rehbein, H. Electrophoretic techniques for species identification of fishery products. *Z. Lebensm. Unters. Forsch.* **1990**, *191*, 1−10.

Saitou, M.; Nei, M. The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406−425.

Sotelo, C.; Piñeiro, C.; Gallardo, J. M.; Pérez-Martín, R. I. Fish species identification in seafood products. *Trends Food Sci. Technol.* **1993**, *4* (12), 395−401.

Tamura, K.; Nei M. Estimation of the number of nucleotides substitutions in the control region of mitochondrial DNA in humans in chimpazees. *Mol. Biol. Evol.* **1993**, *10*, 512−526.

Unseld, M.; Beyermann, B.; Brandt, P.; Hiesel, R. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods Appl.* **1995**, *4*, 241−243.

Whitmore, D. H.; Thai, T. H.; Craft, C. M. The largemouth bass cytochrome *b* gene. *J. Fish Biol.* **1994**, *44*, 637−645.

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