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Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR

Miguel A. Pardo *, Begoña Pérez-Villareal

Department of Food Technology, AZTI-Fisheries and Food Technological Institute, Txatxarramendi ugartea z/g, E-48395 Sukarrieta (Bizkaia), Spain

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Abstract

In this work, the authentication of five different tuna species from commercial canned tuna by Nested Primer PCR–RFLP has been developed. Species identification of commercial canned tuna by techniques based on PCR is rather difficult due to the presence of additives as well as to the fact that the DNA is usually severely degraded. The utilization of Nested Primer PCR, a technique which increases considerably the specificity and sensitive of the reactions, has allowed us to obtain an amplicon of 276 bp (TUN276) from commercial canned tuna in spite of the presence of additives. Consequently, a very useful tool to authenticate canned tuna in brine, oil, pickled, sauced and spiced is presented here. To our knowledge, this 276 bp amplicon is the longest fragment obtained so far from canned tuna. In this study, five diagnosis sites are described to discriminate the most common tuna species processed in the canning industry.

Keywords: PCR; RFLP; Canned tuna; Mitochondrial DNA; Cytochrome *b*

1. Introduction

In order to avoid possible fraud in the labelling of seafood, the identification of fish species is becoming a topic of growing concern. Authentication of these food products becomes an unsolvable problem when the external morphological characteristics of the fish are removed during filleting or processing.

According to the EU Regulation 1536/92, the term *white tuna* includes exclusively *Thunnus alalunga*, the *light tuna* label refers to *Thunnus albacares*, and the label denominated as *tuna* includes any *Thunnus* or similar species (e.g., *Katsuwonus pelamis*). Therefore, it is necessary to have an easy and reliable assay to authenticate the raw material in the canning industry.

Fish identification is feasible using biochemical assays that determine the water-soluble protein composition, such as isoelectring focusing (IEF) which reveals protein polymorphism that, in turn, can be used for unequivocal identification of species (Sotelo, Pineiro, Gallardo, & Pérez-Martín, 1993). Nonetheless, the utilization of electrophoretic analyses of proteins obtained from canned tuna is unsuitable for species identification since the canning process involves a thermal treatment that changes irreversibly the proteins water solubility. Consequently, during the last few years, deoxyribonucleic acid (DNA) analysis techniques have been used in this respect. These techniques are based on the polymorphism analysis of different genetic markers amplified by the polymerase chain reaction technique (PCR). Despite of the DNA analysis being more complex than protein analyses, more information can be obtained with the former. Moreover, the DNA molecule appears to be much more stable to the thermal treatment than proteins themselves (Mackie et al., 1999). Analysis of mitochondrial genome (mainly of control regions, cytochrome *b* and ATPase genes) has been extensively used to resolve evolutionary relationships among closed species of the *Thunnus* genus

* Corresponding author. Tel.: +34-94-602-99-00; fax: +34-94-687-00-06.
E-mail address: mpardo@suk.azti.es (M.A. Pardo).

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(Alvarado-Bremer, Naseri, & Ely, 1997; Chow & Kishino, 1995; Terol, Mascarell, Fernández-Pedrosa, & Pérez-Alonso, 2002).

Pre-canning and canning conditions usually involve the utilization of frozen fish which, in many cases, is subsequently thawed. These conditions might affect the average size of extracted DNA because nucleases can survive freezing, as other enzymes do. The duration of the thermal treatment is also a crucial point to obtain DNA of an average size ranging from 100 to 200 bp (Quinteiro et al., 1998). To date, several works have reported the existence of genetic markers (amplified by PCR) not much longer than 200 bp, in order to discriminate among very closed tuna species by restriction site analysis of polymorphic fragments (RFLP) (Quinteiro et al., 1998) and other methods (Lockley & Bardsley, 2000; Rehbein, Kress, & Schmidt, 1997; Rehbein et al., 1999; Terol et al., 2002). These works authenticated canned tuna samples elaborated in their own laboratories. To our knowledge, up to now, only Ram, Ram, and Baidoun (1996) have described experiments using commercial canned tuna. However, these authors were not able to identify some of their samples due to the inhibitory effect of additives.

As a matter of fact, the use of additives, such as spices or sauces, in seafood inhibits the PCR reaction (Ram et al., 1996). Besides, nowadays, it is very common to consume smoked canned tuna. In any case, the number of additives susceptible of exerting an inhibitory effect appears to increase steadily.

In this study, an amplification of DNA fragments using nested primers in two consecutive PCRs (Nested Primer PCR) was carried out. An amplicon of 276 bp (TUN276) was obtained and, subsequently, analysed by RFLP to identify the tuna species. To date, this amplicon seems to be the longest fragment amplified from the mitochondrial cytocrome *b* gene of canned tuna. This analytical method could be used to identify any commercial canned tuna samples.

2. Materials and methods

2.1. Sample preparation

Specimens of five tuna species were obtained from a local market and then morphologically identified attending to external characters. The distinction between juveniles of bigeye tuna and albacore was carried out comparing their liver shape. In this way, three albacores (*T. alalunga*), three yellowfin tunas (*T. albacares*), three bigeye tunas (*T. obesus*), one bluefin tuna (*T. thynnus*), and three skipjacks (*K. pelamis*) were characterized. Aliquots of light muscle of each fish were stored at −20 °C in 96% (v/v) ethanol.

To prepare the canned samples, the fish were gutted, and the chunks (diameter: 60 cm, height: 30 cm) obtained were steam-cooked (102–103 °C) for 45 min in brine. Then, after placing the light muscle in the cans, vegetable oil was added to it. The cans were sterilized at 110 °C for 60 min.

To test Nested Primer PCR, 23 different commercial canned tunas were purchased at the local market. These tunas had been subjected to different treatments such as brine, vegetable oil, olive oil, pickled, cooked in sauce, spiced or smoked.

Before proceeding towards the DNA extraction, the oil and lipids were removed by blotting with filter paper and incubating the samples in a solution of chloroform/methanol/water (1:2:0.8; v:v:v) overnight at room temperature. The defatted muscle was stored at −20 °C in 90% (v/v) ethanol.

2.2. DNA extraction

The method used is a modification of that reported by Rehbein et al. (1997). The muscle tissue (0.1–0.3 g) was solubilized in 0.45 ml of an extraction buffer [1% (w/v) SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris–HCl, pH 8.0, supplemented with 50 μl of 5 M guanidinium thiocyanate and 40 μl of proteinase K solution (600 U ml^{-1}) (Bioline, London UK). The mixture was incubated at 56 °C for 1–2 h and then centrifuged at 8000*g* for 5 min, as many times as necessary until a clear supernatant was obtained. The supernatant was treated with the Wizard-DNA Clean-Up Extraction Kit (Promega, Madison USA). The purified DNA was resuspended in bidistilled sterile water and stored at −20 °C.

2.3. Mitochondrial cytochrome b gene fragment amplification

In order to amplify a cytochrome *b* gene fragment, two pairs of primers were used for the Nested Primer PCR. The primers H276 (5′-ACT AGG AGT AGG AGT ACT ACT C-3′) and L276 (5′-ACT TTG GCT CAC TAC TTG GCC-3′) were used to amplify fragment TUN276 (276 bp) from the frozen samples. However, when the template was obtained from canned tuna, TUN276 was amplified using two different sets of primers: (i) those described by Bartlett and Davidson (1991), i.e., cytBH (5′-CCC CTC AGA ATG ATA TTT GTC CTC A-3′) and L14735 (5′-AAA AAC CAC CGT TGT TAT TCA ACT A-3′); and (ii) H276 and L276 again.

Reactions were carried out as follows: 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.2 mM dNTPs, 3.5 mM $MgCl_2$, 1 μM of primer, and 0.1–1 μg of template DNA. The reaction was started by adding 1 U/reaction Taq DNA Polymerase (Amersham Biosciences AB, Buckinghamshire, UK) to the reaction mixture. Reaction

volumes of 50 μl were used. Amplification reactions were developed in a Mastercycler Personal from Eppendorf. In the case of canned samples, TUN276 was obtained by Nested Primer PCR after two sequential reactions. The first reaction was carried out in 40 cycles (92 °C for 60 s, 54 °C for 30 s and 72 °C for 30 s) with cytBH and L14735 primers, and the second reaction consisted of 25 cycles (92 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s) with H276 and L276 primers.

The amplicons were purified with the GFX–PCR–DNA and Gel Band Purification Kit (Amersham Biosciences AB, Buckinghamshire, UK).

2.4. DNA sequencing

The sequencing was carried out directly on the purified fragments with a 3700 DNA Analyzer ABI PRISM, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0 (Applied Biosystems, Foster City, USA).

2.5. Restriction fragment length polymorphism (RFLP) analysis of the TUN276 fragment

For this analysis, a set of restriction enzymes was chosen according to the different tuna species studied here: *Bsi*YI, *Nde*II (Roche Applied Science, Basel, Switzerland), *Bsa*I, *Stu*I and *Tsp*509I (New England Biolabs, Beverly, USA). The reactions were carried out in a volume of 10–15 μl, at each enzyme optimum temperature. The reactions were started by adding 1–5 U of enzymatic activity per reaction mixture for 1–2 h.

2.6. Electrophoresis of DNA fragments

The DNA fragments obtained were separated by electrophoresis in 1–3% (w/v) agarose using TBE buffer (45 mM Tris-Borato, 1 mM EDTA, pH 8.0) for 1–3 h at 3–5 V cm^{-1}. The gels were stained with ethidium bromide as described by Sambrook, Fristch, and Maniatis (1989). In order to estimate the size of the fragments, a 100 bp Molecular Ruler (BIO-RAD, Hercules, USA) and a λ DNA Hind III digest MW marker (Amersham Biosciences AB, Buckinghamshire, UK) were used as controls.

2.7. Sequence analysis

The multiple alignment was carried out using the Clustal X program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Genetic distances and phylogenetic trees were calculated by inferring the Neighbour Joining method (Saitou & Nei, 1987) based on the percentage of substitutions in the nucleotides with the Clustal X program.

The restriction sites analysis of the sequences was carried out using the Webcutter 2.0 program.

3. Results and discussion

3.1. DNA extraction from frozen and canned tuna

As already mentioned in Section 2, the DNA extraction was carried out as described by Rehbein et al. (1997). This method is based on the high lysis power of proteinase K and SDS, which are both adequate to extract DNA from muscles (Kocher et al., 1989). Although Rehbein et al. (1997) reported a much longer incubation time (i.e., overnight), in our study, the mixture was incubated for just 1–2 h without observing any loss of efficiency. Thus, the incubation time was shortened considerably. By contrast, Bartlett and Davidson (1991) indicated the extreme difficulty of obtaining intact high molecular weight DNA from tuna muscle using the standard protocol of proteinase K and SDS. However, and attending to the distribution of DNA fragments obtained from frozen and canned tuna (Fig. 1), this method appears suitable. As seen in Fig. 1, the DNA extracted from canned tuna samples was strongly degraded, most likely due to the thermal treatment applied. As previously reported, the size of most of the fragments obtained from canned tuna was smaller than 200 bp (Quinteiro et al., 1998; Terol et al., 2002). Although not distinguished in this figure, some of the fragments had sizes longer than 200 bp (see below).

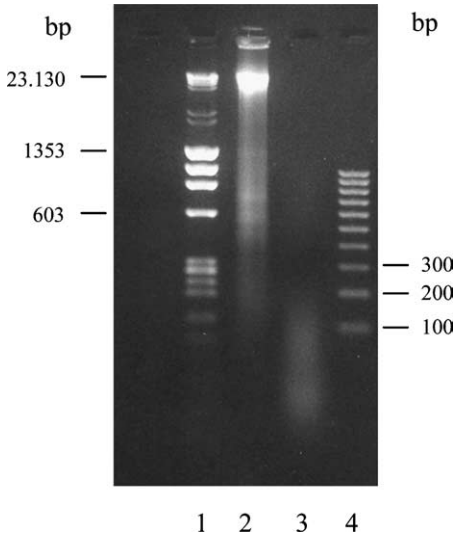

Fig. 1. Electrophoretic analysis of DNA purified from frozen and canned tuna on a 1% (w/v) agarose gel stained with ethidium bromide. Lane 1, λ DNA *Hin*dIII digest MW marker; lane 2, frozen tuna; lane 3, canned tuna; lane 4, 100 bp Molecular Ruler.

3.2. Amplification of TUN276 from commercial canned tuna

Initially, the amplification of TUN276 from frozen samples was carried out using only one PCR reaction step, so that reference sequences which could be used to find diagnosis sites were obtained (see below). When the DNA template was extracted from the tuna samples canned in our laboratory (Fig. 2, lane 2), the amplification of TUN276 in one PCR reaction step was possible. On the other hand, with commercially available canned tuna, the amplicon was not obtained in this way (with only one PCR reaction step) (Fig. 2, lanes 3–5). This problem might be due to the average DNA fragment size and/or the presence of additives that inhibit the PCR.

The average DNA fragment size delimits the size of the amplicon. For canned tuna, several studies have considered an average fragment size of 176 bp (Bartlett & Davidson, 1991; Quinteiro et al., 1998), although it is true that this size could be smaller depending on the canning process. The amplification of fragments shorter than 176 bp is usually the case since the extracted DNA is highly degraded. Quinteiro et al. (1998) tried unsuccessfully to amplify a 299 bp fragment from canned tuna.

To solve this problem, in our study, initially the DNA template concentration was increased so that the longest possible fragment could be obtained. Unfortunately, this increment did not lead to any observable improvement. A higher quantity of DNA in the PCR reaction has been described as being closely related to an excess of inhibitors (Ram et al., 1996). The presence of additives used in the food industry as well as in the canning process itself might exert an inhibitory effect on the PCR. Since they could not amplify a 123 bp fragment from every commercial canned fish samples tested, Ram et al. (1996) rejected the possibility of using PCR as a suitable technique for the identification of fish. In addition, Ram et al. (1996) as well as other authors (Quinteiro et al., 1998; Rehbein et al., 1997, 1999) worked only with commercial canned fish in oil or in vinegar. On the contrary, in this work, apart from those kind of samples, smoked tuna and even cans supplemented with spices and sauces were used. In consequence, the number of possible additives susceptible of exerting an inhibitory effect on the PCR has been increased considerably.

Nested Primer PCR provides a way to avoid these inhibitory effects, because it allows the amplification of fragments from low DNA concentrations with high sensitivity and efficiency. This technique was tested in those tuna samples canned in our own laboratory. After the first PCR reaction, the maximum size of fragments (Fig. 3, lanes 2, 3) was increased considerably. The utilization of a second PCR reaction led to the longest fragment obtained so far, to our knowledge, from canned tuna fish (TUN276). As shown in Fig. 3, despite the amplicon size, the efficiency obtained for frozen (lanes 7, 10) and canned (lanes 8, 9) tuna samples was very alike. Thus, this process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second either. In addition, this process increases the PCR sensitivity, since two pairs of primers are required to amplify the target sequence for a final product to be generated.

After carrying out the Nested Primer PCR with tuna canned in our laboratory, we proceeded to test it using commercial canned tuna in brine, vegetable oil, olive oil, pickled, cooked in spicy sauce and smoked. As described

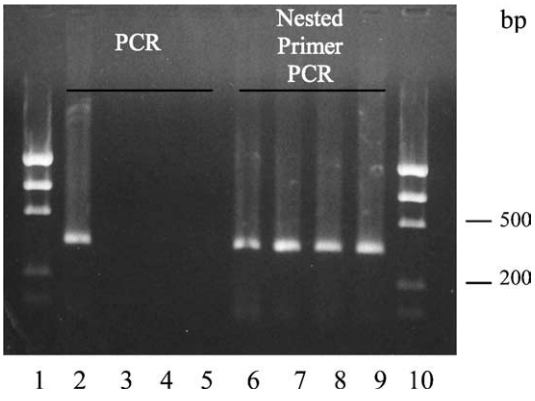

Fig. 2. PCR products for cytochrome *b* region electrophoresed on a 2% (w/v) agarose gel stained with ethidium bromide. Lanes 2 and 5, TUN276 after only one PCR reaction step; lanes 6–9, TUN276 after Nested Primer PCR; lanes 2 and 6 from tuna canned in our own laboratory; lanes 3–5 and 7–9, from commercial canned tuna; lanes 3 & 7, in oil olive; Lanes 4 and 8, supplemented with sauce; lanes 5, 9, smoked canned tuna. Lanes 1 and 10, Molecular Ruler.

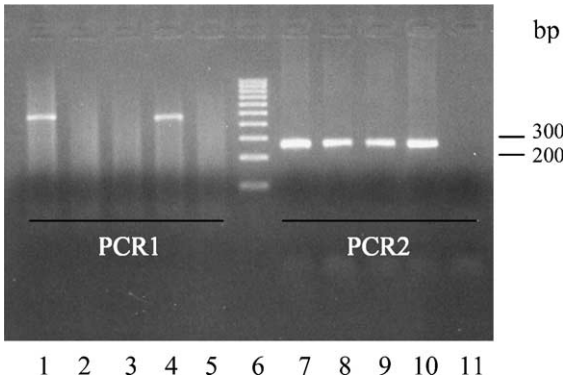

Fig. 3. PCR products from cytochrome *b* region by Nested Primer PCR electrophoresed on a 2% (w/v) agarose gel stained with ethidium bromide. Lanes 1–5, products after first PCR reaction; lanes 1 and 4, PCR positive controls; lanes 2 & 3, from canned tuna; lane 5, PCR negative control. lane 6, 100 bp Molecular Ruler; lanes 7–11, amplicons of 276 bp after second PCR reaction (TUN 276), using the products from the first PCR reaction (from 1 to 5) as templates (i.e., 1 template for 7; 2 templates for 8; 3 templates for 9; 4 templates for 10; 5 templates for 11).

above, when the DNA template was extracted from commercial canned tuna samples, the amplification of TUN276 in one PCR reaction step was not possible (Fig. 2). However, when Nested Primer PCR was applied to commercial samples, TUN276 was amplified with a good efficiency from each of the samples, in spite of the presence of inhibitors (Fig. 2, lanes 6–9).

The Nested Primer PCR was validated using 23 commercial samples with optimal results in all cases. Only four samples out of these 23 have been represented (Fig. 2) for clarity proposes.

3.3. Sequencing of partial mitochondrial cytochrome b gene from tuna

DNA sequencing is the most direct means of obtaining information from PCR products, and extensively to identify species. The use of sequences from mtDNA for tuna identification has been reviewed by several authors (Alvarado-Bremer et al., 1997; Bartlett & Davidson, 1991). Mitochondrial DNA has some advantages over nuclear DNA which has also been used to resolve evolutionary relationships among closely related species of the *Thunnus* genus (Alvarado-Bremer et al., 1997; Bartlett & Davidson, 1991; Chow & Kishino, 1995). In any case, as mtDNA exhibits a certain degree of intraspecific variability, one should always be careful when studying differences among organisms based on single base polymorphisms (Unseld, Beyermann, Brandt, & Hiesel, 1995). Terol et al. (2002) reported the analysis of partial sequence of the mitochondrial cytochrome *b* gene to identify three tuna species. Those polymorphic sites of these sequences that did not present intraspecific variation were given a diagnostic value (Terol et al., 2002).

Although DNA sequencing is time consuming, expensive and certainly technically demanding, it produces a large amount of information. However, there are other techniques to analyse DNA that are much more convenient for routine analysis. In these cases, the DNA sequences can be obtained from available GenBank databases. Unfortunately, some of the sequences described in the GenBank are ambiguous, most likely due to inaccuracies (Ram et al., 1996).

Sequencing of amplicon TUN276 showed that 48 polymorphic sites could be found when comparing the DNA sequences of five tuna species. Forty-three of them had only two variants, whereas the remaining five had three (4 sites) or four (1 site) variants (data not showed). As expected, 39 of these polymorphic sites corresponded to skipjack, which belongs to *Katsuwonus* (not *Thunnus*) genus. According to these data, a high degree of conservation was accounted for cytochrome *b* gene. Similar results had been described elsewhere (Terol et al., 2002).

Two significant points are always needed to develop a proper strategy for species identification: at least one diagnosis position for each species, and a low degree of intraspecific variability (Quinteiro et al., 1998).

Table 1
Diagnostic polymorphic sites in TUN276 fragment

Species	Position (from 3′ end)				
	38	128	209	212	236
T. alalunga (ALB)	A	C	C	T	A
T. albacares (YFT)	A	C	A	T	G
T. obesus (BET)	A	T	A	T	A
K. pelamis (SKJ)	A	C	T	C	A
T. thynnus (BFT)	G	C	A	T	A

In our study, three diagnosis sites were described for *T. thynnus*, two for *T. albacares*, two for *T. alalunga*, two for *T. obesus*, and 39 for *K. pelamis*. The diagnosis position for each tuna species showing the lowest intraspecific variability was chosen. Consequently, five diagnostic sites, one belonging to each species, were studied (i.e., 38, 128, 209, 212 and 236) (Table 1). Position 38 corresponds to *T. thynnus* (BFT), 128 to *T. obesus* (BET), 209 to *T. alalunga* (ALB), 212 to *K. pelamis* (SKJ) and 236 to *T. albacares* (YFT).

Table 2 shows multiple alignment (143 sequences analyzed) of these five positions carried out with the sequences here obtained as well as with those sequences compiled from GenBank and bibliography (Bartlett & Davidson, 1991; Block et al., 1993; Cantatore et al., 1994; Chow & Inoue, 1993; Terol et al., 2002). Regarding intraspecific variability, *T. albacares*, *T. alalunga* and *T. thynnus* did not seem to present any. By contrast, a very low intraspecific variability was found for *K. pelamis* and *T. obesus* (Table 2).

As already mentioned, so far, DNA sequencing is the most robust method for species identification. This method consists of building a matrix of genetic pairwise distances between all the nucleotide sequences introduced in the analysis. In this way, those sequences showing a low distance value among them might constitute a phylogenetic group. When a sequence obtained from an unknown sample is introduced into this analysis, a degree of similarity between the unknown sequence and those of the phylogenetic group will indicate to which group that sample belongs (Chow & Inoue, 1993; Quinteiro et al., 1998; Terol et al., 2002). This method was used in our study to validate the results obtained by RFLP analysis from canned tuna species.

3.4. RFLP analysis of TUN276 fragment from canned tuna species

After sequencing TUN276, the sequences were scrutinized so that sites for restriction enzymes with diagnosis value could be detected. Table 2 shows grey boxes around the restriction sites of *Nde*II (GATC), *Tsp*509I (AATT), *Stu*I (AGGCCT), *Bsa*I (GGTCTCN) and

Table 2
Multiple alignment of five diagnosis values of 143 sequences belonging to TUN276 fragment from frozen tuna samples[a]

Species	*N*	Position (from 3′ end)				
		38–41	126–129	206–211	207–212	227–237
ALB	1	AATC	AACT	AGGCCT	GGCCTT	CCTCTACAAAG
ALB	2	AATC	AACT	AGGCCT	GGCCTT	CCTGTACAAAG
ALB[d]	1	AATC	AACT	AGGCCT	GGCCTT	CCTGTACAAAG
ALB[c]	1		AACT	AGGCCT	GGCCTT	CCTATACAAAG
ALB[b]	12	AATC	AACT	AGGCCT	GGCCTT	CCTATACAAAG
YFT	3	AATC	AACT	AGGACT	GGACTT	CCTATACAAGG
YFT[c]	1	AATC	AACT	**AGGCCT**	GGCCTT	CCTATACAAGG
YFT[f]	7		AACT	AGGACT	GGACTT	CCTATACAAGG
YFT[b]	33	AATC	AACT	AGGACT	GGACTT	CCTATACAAGG
BKT[d]	2	AATC	AACT	AGGACT	GGACTT	CCTATACAAAG
SBT[d]	1	AATC	AACT	AGGACT	GGACTT	CCTATACAAAG
SBT[c]	1		AACT	AGGACT	GGACTT	CCTATACAAAG
SKJ	1	AATC	AACT	**AGGCCT**	GGCCTC	CCTCTACAAAG
SKJ	2	AATC	AACT	AGGTCT	GGTCTC	CCTCTACAAAG
SKJ[c]	1		AACT	AGGTCT	GGTCTC	CCTCTACAAAG
SKJ[d]	1	AATC	AACT	AGGTCT	GGTCTC	CCTCTACAAAG
SKJ[f]	8		AACT	AGGTCT	GGTCTC	CCTCTACAAAG
SKJ[f]	1		**AATT**	AGGTCT	GGTCTC	CCTCTACAAAG
SKJ[f]	1		AACT	AGGTCT	GGACTC	CCTCTACAAAG
BFT	1	GATC	AACT	AGGACT	GGACTT	CCTATACAAAG
BFT[e]	1	GATC	AACT	AGGACT	GGACTT	CCTATACAAAG
BFT[d]	1	GATC	**AATT**	**AGGCCT**	GGCCTT	CCTGTACAAAG
BFT[b]	33	GATC	AACT	AGGACT	GGCCTT	CCTGTACAAAG
BET	1	AATC	AACT	AGGACT	GGACTT	CCTATACAAAG
BET	2	AATC	AATT	AGGACT	GGACTT	CCTATACAAAG
BET[c]	1	AATC	AATT	AGGACT	GGACTT	CCTATACAAAG
BET[f]	13		AATT	AGGACT	GGACTT	CCTATACAAAG
BET[b]	10	**GATC**	AATT	AGGACT	GGACTT	CCTATACAAAG

[a] *N* number of individuals analyzed. Each diagnosis site is delimited by its position (bp) in the sequence (38–41, 126–129, 206–211, 207–212 and 227–237) which corresponds to the recognition site of *Nde*II, *Tsp*509I, *Stu*I, *Bsa*I, *Bsi*YI enzymes, respectively. Grey boxes around sequences show recognition sites. Bold sequences indicate the diagnosis sites coincidences among species. Abbreviations are the same as in Table 1, except for *T. atlanticus* (BKT) and *T. macoii* (SBT).
[b] Bartlett and Davidson (1991).
[c] Block, Finnerty, Stewart, and Kidd (1993).
[d] Chow and Inoue (1993).
[e] Cantatore et al. (1994).
[f] Terol et al. (2002).

*Bsi*YI (CCN_7GG). Although the interspecific variability detected was surely low (less than 3%), when the diagnosis values among different species were identical (see bold sequences in Table 2), the combination of different restriction enzymes was sufficient to resolve the coincidences.

Table 3 shows the predictable restriction fragments for the five tuna species obtained with the five endonucleases enzymes. In this work, the resulting fragments were separated on 3% (w/v) agarose gels and the bands were visualized staining with ethidium bromide. Other authors have reported the utilization of the more sen-

Table 3
Expected size of restriction fragments of five canned tuna species after digestion of TUN276 fragment with five restriction enzymes[a]

	*Bsa*I	*Nde*II	*Bsi*YI	*Tsp*509I	*Stu*I
ALB	276	276	276	276	210 + 70
YFT	276	276	236 + 40	276	276
BET	276	276	276	146 + 130	276
SKJ	210 + 70	276	276	276	276
BFT	276	236 + 40	276	276	276

[a] Abbreviations are the same as in Table 1.

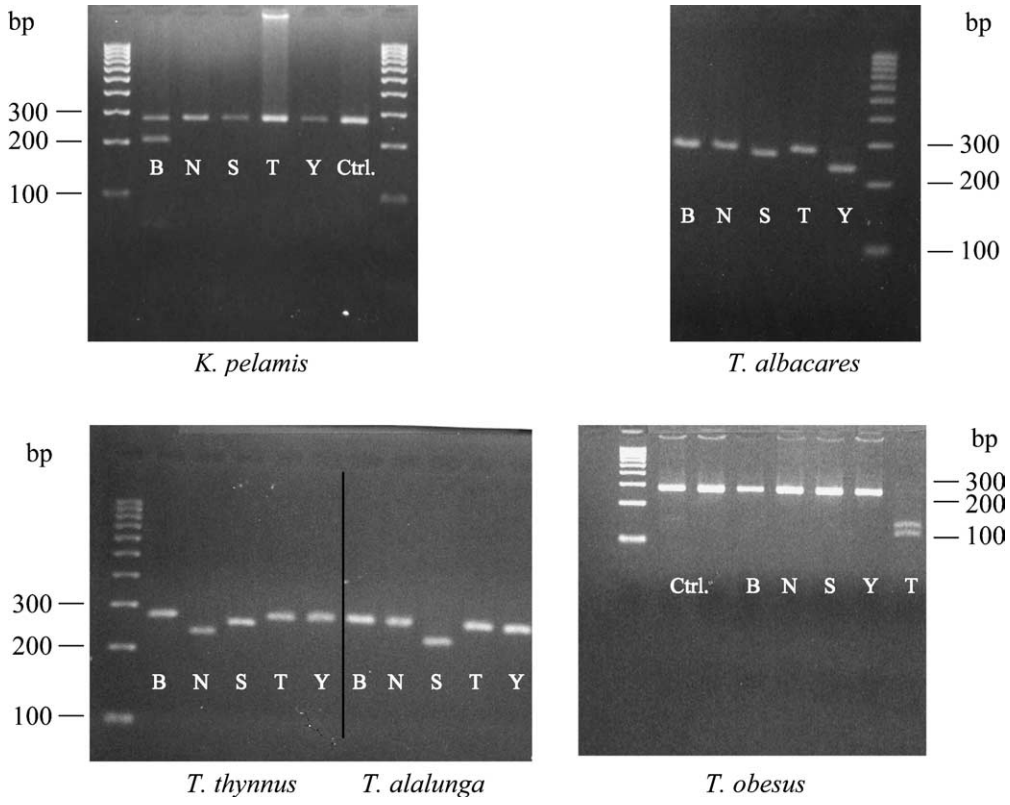

Fig. 4. RFLP patterns of five tuna species. The TUN276 obtained from canned tuna species were digested with *Bsa*I (B), *Nde*II (N), *Stu*I (S), *Tsp*509I (T) and *Bsi*YI (Y) as described in Section 2. Ctrl: non-digested fragment.

sitive silver nitrate staining method that allows the detection of shorter fragments (Quinteiro et al., 1998). Due to the large size of TUN276, this more sensitive silver nitrate staining technique was not needed in our case (Fig. 4), making it more adequate for routine analyses.

Fig. 4 shows the restriction patterns generated after digestion of amplified TUN276 from canned tuna, allowing us to differentiate among the five tuna species. This PCR–RFLP technique has already been used for the identification of *Thunnus* genus (Ram et al., 1996). Quinteiro et al. (1998) identified six tuna species from non-commercially available canned samples by restriction patterns of a 126 bp sequence.

4. Conclusion

The utilization of Nested Primer PCR–RFLP provides a very useful tool to authenticate commercial canned tuna in brine, oil, pickled, smoked or even supplemented with spice and sauce. This method discriminates the most common tuna species processed in the canning industry.

To our knowledge, fragment TUN276 appears to be the longest amplicon obtained so far from canned tuna. Although it is certainly true that the presence of polymorphism in the genetic marker is more important than the length of the fragment itself, a longer fragment (such as TUN276) increases the probability of finding diagnosis sites for other tuna species. Since, nowadays, most markets have started to import new less valuable tuna species from other countries, it is becoming more and more important to have an easy, cheap and reliable method, such as the one presented here, to be able to detect fraud.

The results presented here are of great benefit for both the canning industry and the governmental food control laboratories.

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