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Fish species identification in canned tuna by PCR-SSCP: validation by a collaborative study and investigation of intra-species variability of the DNA-patterns

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Abstract

Analysis of single strand conformation polymorphism (SSCP) of an amplicon (123 bp) obtained by polymerase chain reaction (PCR) of the mitochondrial cytochrome *b* gene was used to identify the fish species in canned tuna. Single-stranded DNA (ssDNA) was separated by polyacrylamide gel electrophoresis, and visualised by silver staining. The reliability of the method was tested by a collaborative study in which eight European laboratories participated. Seven unknown samples (five from individual species and two mixtures of two tuna species) of canned tuna had to be identified by comparison with reference material. From a total of 72 cases, 65 (90.3%) were assigned correctly. Intra-species variability of SSCIP patterns was found in the case of *Katsuwonus pelamis* and *Sarda sarda*. As specimens from various fishing grounds gave two or three different patterns of ssDNA, the possibility of some variability of the DNA patterns has to be considered in SSCP analysis of these species.

Keywords: Fish species identification; Canned tuna; PCR-SSCP

1. Introduction

The study of single strand conformation polymorphism (SSCP) of mitochondrial DNA is becoming more and more popular for differentiation of fish species or populations (Ostellari, Bargelloni, Penzo, Patarnello, & Patarnello, 1996; Oohara, 1997; Rehbein, Kress, & Schmidt, 1997). The technique is simple, fast and sensitive enough to detect one base exchange or a few differences in the sequence of short (100–400 bp) DNA fragments (Hayashi, 1996).

In the case of many fishery products. the DNA is severely degraded. In canned tuna only residues of the order of 100 base pairs are found (Mackie, 1997). Amplification of short DNA fragments by polymerase chain reaction (PCR) and analysis of the amplicons by SSCP using polyacrylamide gel electrophoresis (native PAGE) followed by silver staining of DNA bands was used to identify the fish species in canned tuna (Rehbein, Mackie, Pryde, Gonzales-Sotelo, Perez-Martin, Quinteiro, & Rey-Mendez, 1995).

The reliability of this method and its suitability for food control laboratories was tested by a validation exercise, the results of which are described here.

2. Materials and methods

The following laboratories participated in the collaborative study:

1. TÜV Südwest DLG, Freiburg im Breisgau, Germany.

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Abbreviations: ds DNA: double-stranded DNA; mt: mitochondrial; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; RAPD: random amplified polymorphic DNA; RFLP: restriction fragment length polymorphism; SSCP: single strand conformation polymorphism; ssDNA: single-stranded DNA.

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2. Rowett Research Institute, Aberdeen, Scotland, UK.
3. Laboratory of the Government Chemist, Teddington, UK.
4. Institute of Biochemistry and Technology, Federal Research Centre for Fisheries, Hamburg, Germany.
5. Consejo Superior de Investigaciones Scientificas, Institute de Investigacions Marinas, Vigo, Spain.
6. Universidad de Santiago de Compostela, Santiago de Compostela, Spain.
7. Central Science Laboratory, Food Science Laboratory, Norwich, UK.
8. Chemische Landesuntersuchungsanstalt Freiburg, Freiburg im Breisgau, Germany.

The order of succession of this list does not correspond to the numbers for the laboratories in Table 2 describing the results.

2.1. Preparation of samples

Specimens of eight tuna species (Table 1) were morphologically identified and canned in the Institute de Investigacions Marinas.

Gutted fish were steam-cooked (102–103°C) for 90 min, the temperature in the core of the fish being about 65°C. After being cooked and then allowed to cool, the fish were skinned and filleted. Light muscle from the fillets was taken for filling RO-100 type cans (diameter: 6.52 cm, height: 3 cm), and 2 g of sodium chloride were added. Oil was poured in to completely cover the flesh. The cans were hermetically closed and then sterilised at 115°C for 60 min.

Table 1
Samples of tuna

1. Reference samples	
A	Albacore (*Thunnus alalunga*)
Rb	Yellowfin tuna (*Thunnus albacares*)
L	Skipjack tuna (*Katsuwonus pelamis*)
Ro	Bluefin tuna (*Thunnus thynnus*)
M	Frigate mackerel (*Auxis thazard*)
B	Little tunny (*Euthynnus alleteratus*)
S	Atlantic bonito (*Sarda sarda*)
P	Bigeye tuna (*Thunnus obesus*)
2. Unknown samples from individual species	
1.	Yellowfin tuna
2.	Skipjack tuna
3.	Bluefin tuna
4.	Albacore
5.	Atlantic bonito
3. Unknown mixtures of two tuna species	
M1	Yellowfin tuna + skipjack tuna
M2	Albacore + Atlantic bonito

As it was found more practical to distribute the fish flesh preserved in ethanol, instead of in cans, samples of canned tuna muscle were processed as in the following paragraphs.

At first oil was removed by blotting the flesh with filter paper, then lipids were extracted by incubating the flesh for 24 h in a mixture of chloroform/methanol/water, 1/2/0.8 (v/v/v). To one part by weight of defatted tuna muscle, two parts by volume of absolute ethanol were added; after stirring for 2 h, the ethanol was decanted and fresh ethanol was added. The ethanol was then poured off and the muscle pieces were divided into 0.5 g portions and placed in cryogenic vials (2 ml). To these 0.5 g portions of muscle, 1 ml volumes of ethanol were applied. Samples were then sent to the participants by ordinary (air) mail. Mixtures of tuna flesh from two species were prepared by weighing equal amounts of canned, defatted light muscle of each species, adding ethanol and stirring the mixture for 30 min. Ethanol was poured off and the mixed muscle was allowed to dry before weighing it in vials. Fresh ethanol was then added.

For each tuna species, reference material and sample were from the same canned specimen.

2.2. Extraction of DNA

The extraction of DNA was performed according to the procedure described by Meyer, Candrian, & Lüthy, (1994) using the Wizard DNA Clean Up System (Promega No. A 7280).

Solubilisation of muscle tissue: 0.1 g (raw) or 0.3 g (canned) muscle was cut into small pieces and transferred into a 1.5 ml Eppendorf tube; 0.43 ml of extraction buffer (1%, w/v, SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris–HCl, pH 8.0) were added.

Fifty microlitres of 5 M guanidinium thiocyanate and 20 μl of a proteinase K solution (20 mg ml^{-1} distilled water, i.e. 600 units ml^{-1}), were added. The content of the tubes was mixed by inversion and incubated overnight at 56°C. After a period of 2–3 h, another 20 μl aliquot of proteinase K solution was added before overnight incubation.

Undissolved material was removed by centrifugation (e.g. in an Eppendorf Table Centrifuge 5412) for two 5 min periods. Isolation of DNA: the supernatant was treated as described in the Technical Bulletin no. 141 of Promega. One millilitre of Wizard DNA Clean Up Resin and the supernatant were added to an Eppendorf tube and mixed by gently inverting several times. The resin containing the bound DNA was pipetted into the barrel of a 2 ml disposable syringe. The syringe plunger was slowly inserted and the slurry was gently pushed into the Wizard minicolumn.

The column was washed by gently pushing 2 ml of 80% (v/v) isopropanol through the column. This

procedure was repeated once. Then the column was transferred to a 1.5 ml Eppendorf tube and centrifuged for 20 s to dry the resin. For evaporation of residual isopropanol the column was placed at room temperature for 10 min.

The column was transferred to a new Eppendorf tube; 0.1 ml of prewarmed (70°C) TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) was applied to the column, and after 1 minute the DNA was eluted by centrifugation for 20 s. The purified DNA was stored at 4°C or at −25°C.

2.3. PCR conditions

For PCR the PCR Master (Boehringer Mannheim) or equivalent kit was used. The PCR assay (PCR Master) contained 1.25 U Taq DNA polymerase in Brij 35 (0.005%, v/v), dATP, dCTP, dGTP, dTTP, each 0.2 mM, 10 mM Tris–HCl (final pH 8.3), 2 mM $MgCl_2$ per 0.05 ml of total volume. The concentration of primers was 1 μM, and that of DNA was 50–100 ng per assay.

Preheating step: 5 min at 94°C; cycling parameters: 40 s at 94°C, 80 s at 50°C, 80 s at 72°C, 35 cycles; final extinction step: 7 min at 72°C.

The primers were selected to amplify a 123 bp (length including primers) region of the mitochondrial cytochrome *b* gene (Unseld et al., 1995):

59 − 3 :5′ − AAA CTG CAG CCC CTC AGA
ATG ATA TTT GTC CTC A − 3′34mer

59 − 5 :5′ − GCT GGT ACC TCT ACA AAG
AAA CAT GAA ACA − 3′30mer.

2.4. Preparation of single stranded DNA

After completion of PCR, 5 μl of the assay were mixed with 15 μl of denaturing solution heated for 5 min at 95°C, and placed immediately in iced water. The samples were then loaded onto the polyacrylamide gel without delay.

2.5. Preparation of denaturing solution

Ninety-five millilitres of formamide (analytical grade) and 5 ml of 0.2 M NaOH were mixed; bromphenol blue and xylene cyanol were added to a concentration of each of 0.05% (w/v).

2.6. Gel electrophoresis

CleanGel 10% 48S (Pharmacia Biotech, Freiburg) was used for native PAGE, generally following the operating instructions given by Pharmacia. Rehydration buffer: 112 mM Tris acetate pH 6.4, electrode buffer 0.2 M Tris: 0.2 M Tricine, 0.55% (w/v) sodium dodecylsulfate, pH 8.3. The plate of the electrophoresis chamber was cooled with water of 10°C by means of a thermostatic circulator.

DNA bands were visualised by silver staining either according to the procedure outlined below or by using the Plus One DNA Silver Staining Kit (Pharmacia Biotech).

Silver staining procedure:

1. Fixation:	30 min	200 ml	of 10% (v/v) acetic acid
2. Washing:	3×2 min	200 ml	of distilled water
3. Silvering:	30 min	200 ml	of 0.1% (w/v) $AgNO_3$ + 0.2 ml formaldehyde (37%)
4. The gel, film backing and tray were thoroughly washed with distilled water using a squeeze bottle.			
5. Developing:	30 s	200 ml	2.5% (w/v) Na_2CO_3 + 0.1 ml formaldehyde + 0.2 ml sodium thiosulfate 2% (w/v);
	2–5 min	200 ml	2.5% Na_2CO_3 + 0.1 ml formaldehyde + 0.2 ml thiosulfate. The Na_2CO_3 solution should be precooled (10°C)
6. Stopping:	10 min	200 ml	10% (v/v) acetic acid
7. Impregnation:	10 min	200 ml	10% acetic acid/10% (v/v) glycerol
8. Drying:	overnight at ambient temperature.		

2.7. Quantification of DNA

The DNA content of solutions was measured using the fluorescence-enhancement assay with bisbenzimidazole (Hoechst 33258) (Downs & Wilfinger, 1983).

Major deviations from the procedures described above by participants of the study are detailed below.

3. Results and discussion

3.1. Collaborative study

The results of the collaborative study are compiled in Table 2. From a total of 72 cases, only seven (9.7%) were incorrectly assigned. More than half of the wrong identifications were made by one laboratory (no. 7), where the differentiation between skipjack and albacore had fai led.

The participants had closely followed the prescribed procedure. Nevertheless, some variation of patterns of

ssDNA became obvious when the results of the different laboratories were compared.

Representative patterns of single-stranded DNA (ssDNA), obtained by two different laboratories (no. 1 and no. 3), are shown in Figs. 1 and 2. Each species gave a characteristic pattern of ssDNA, with the exception of bluefin and yellowfin tuna, which gave the same pattern. The amplicon of the two latter species has the same DNA sequence (Unseld, Beyermann, Brandt, & Hiesel, 1995).

Table 2
Identification of unknown samples by SSCP

Lab. no.	Sample						
	1 Rb	2 L	3 Ro	4 A	5 S	M1 Rb+L	M2 A+S
1	+	+	+	+	+	+ +	+ +
2	+	+	+	+	+	− −	+ +
3	+	+	+	+	+	− +	+ +
4	+	+	+	+	+	+ +	+ +
5	+	+	+	+	+	+ +	+ +
6	+	+	+	+	+	+ +	+ +
7	+	−	+	+	−	+ −	− +
8	+	+	+	+	+	+ +	+ +

+: Correct identification; in case of sample 1 and 3, both of the statements 'yellowfin' or 'bluefin' were classified as being correct, because the amplicon of both species has the same sequence (for most specimens) (Unseld et al., 1995).
−: Incorrect identification.
Laboratory no. 5 had performed native PAGE by means of the BioRad Minigel electrophoresis unit without a cooling device, but using the same buffers as described in 'Materials and methods'.

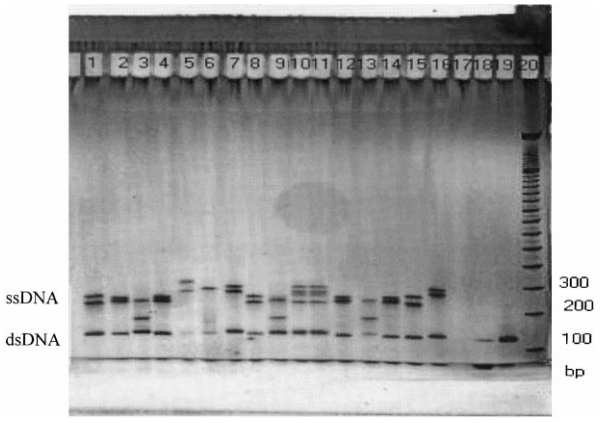

Fig. 1. SSCP patterns of samples and references of canned tuna obtained by laboratory 1. Preparation of single-stranded DNA, and rehydration, running and staining of CleanGel 10% 48S was exactly performed as described in 'Materials and methods'. Lanes 1–8: references, lanes 9–16: unknown samples, lane 17: no sample applied, lane 18: PCR control (without DNA), lane 19: PCR of sample M1 (i.e. non-denatured amplicon), lane 20: 100 bp ladder. Lane 1: *T. alalunga*, 2: *T. albacares*, 3: *K. pelamis*, 4: *T. thynnus*, 5: *A. thazard*, 6: *E. alletteratus*, 7: *S. sarda*, 8: *T. obesus*, 9: sample M1 (*T. albacares* + *K. pelamis*), 10 and 11: sample M2 (*T. alalunga* + *S. sarda*), 12: sample 1 (*T. albacares*), 13: sample 2 (*K. pelamis*), 14: sample 3 (*T. thynnus*), 15: sample 4 (*T. alalunga*), 16: sample 5 (*S. sarda*).

The patterns of Fig. 1 are characterized by one or two bands of ssDNA for each species. The patterns of Fig. 2 show several additional bands within the zone of ssDNA for nearly all species. The patterns reported by the other participants were between these extremes.

Laboratory no. 1 had used the silver staining procedure described under 'Materials and methods', whereas laboratory no. 3 had used the Plus One DNA Silver Staining Kit, which was found to be more sensitive. The other conditions of electrophoresis were the same in both laboratories.

Depending upon the conditions of PCR, denaturation and electrophoresis, more than one conformational state of ssDNA may be formed during the procedure (Hayashi, 1991). It has also been demonstrated that interaction between residual PCR primers and ssDNAs may occur, resulting in the formation of mixed bands (Cai & Touitou, 1993; Kasuga, Cheng, & Mitchelson, 1995). However, as long as samples and references are amplified and denatured under identical conditions, and run side-by-side on the same gel, inter-laboratory differences do not hamper the reliability of PCR–SSCP for species identification, as demonstrated by the results of this exercise.

On the other hand, the dependence of SSCP patterns on electrophoretic conditions can be utilised to optimise

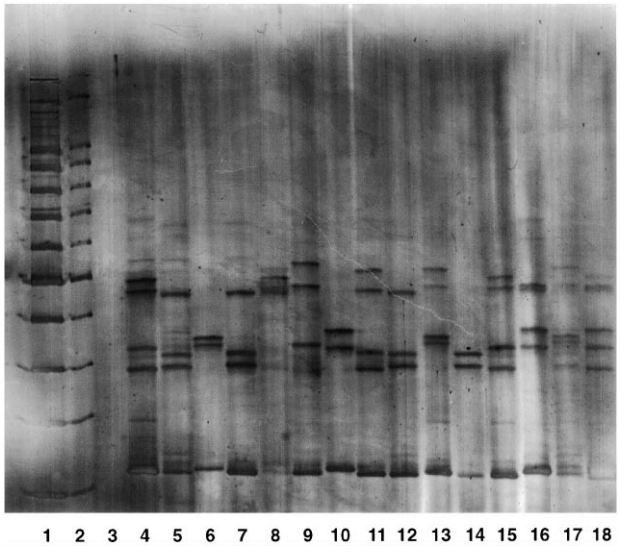

Fig. 2. SSCP patterns of samples and references of canned tuna obtained by laboratory 3. CleanGel 10% 48S was rehydrated and run as described in 'Materials and methods'. The Plus One DNA Silver Staining Kit was used for staining of DNA bands. The main bands of ssDNA are located in the same position as in Fig. 1. and additional bands, representing other conformations of ssDNA, are found in a zone above the main bands. Lanes 1 and 2: 100 bp ladder; 3: control (without DNA), 4: *T. alalunga* (reference, r). 5: *T. albacares* (r), 6: *K. pelamis* (r), 7. *T. thynnus* (r), 8: *A. thazard* (r), 9: *E. alletteratus* (r), 10: *S. sarda* (r), 11: *T. obesus* (r), 12: samp1e 1 (*T. albacares*), 13: sample 2 (*K. pelamis*), 14: sample 3 (*T. thynnus*), 15: sample 4 (*T. alalunga*), 16: sample 5 (*S. sarda*), 17: sample M1 (*T. albacares* + *K. pelamis*), 18: sample M2 (*T. alalunga* + *S. sarda*).

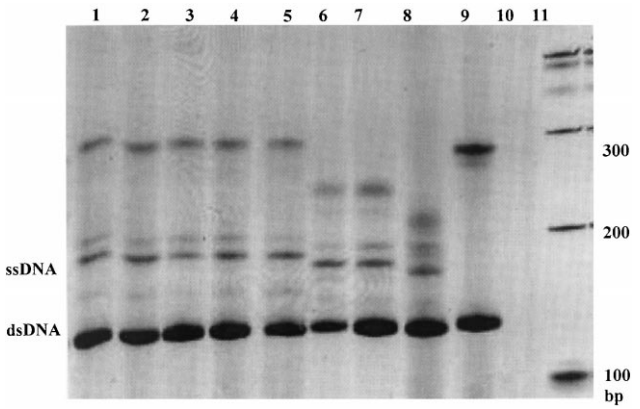

Fig. 3. Variation of the ssDNA patterns of *K. pelamis*. Samples of skipjack were obtained from institutes and traders without detailed knowledge of the geographical origin (fishing ground). Lane 1 : Skipjack in o. 1 (France, raw), lanes 2 and 3: skipjack no. 2 (Spain, lane 2: raw, lane 3: canned), lanes 4 and 5: skipjack no. 3 (Spain, lane 4: raw, lane 5: canned), lane 6: skipjack no. 4 (Spain, raw), lane 7: skipjack no. 5 (Thailand, raw), lane 8: skipjack no. 6 (Thailand, raw), lane 9: *T. albacares*, lane 10: control without DNA, lane 11: 100 bp ladder.

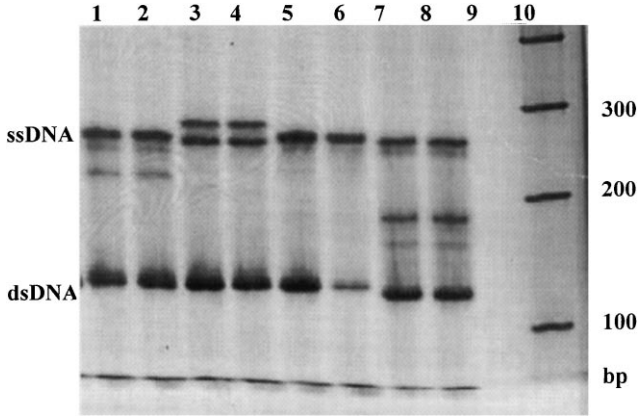

Fig. 4. Variation of ssDNA patterns of *S. sarda*. The patterns of two specimens of Atlantic bonito (*Sarda sarda*) (obtained from Spain) are compared with the patterns of Australian bonito (*S. australis*) (Australia) and Pacific bonito (*S. chiliensis*) (Thailand). Lanes 1 and 2: *S. sarda*, specimen no. 1, lanes 3 and 4: *S. sarda*, no. 2, lanes 5 and 6: *S. chiliensis*, lanes 7 and 8: *S. australis*, lane 9: no sample, lane 10: 100 bp ladder.

the procedure for differentiation of DNA fragments to be analysed (Teschauer, Mussack, Braun, Waldner, & Fink, 1996).

As shown by this study, SSCP can also be used to identify the fish species in mixed products. The patterns are very distinct compared to the fingerprints obtained by the random amplified polymorphic DNA (RAPD) technique, which has also been used for species identification of fishery products (Partis & Wells, 1996).

3.2. Intra-species variability of ssDNA patterns

During analysis of a large number of authenticated or commercial samples of raw or canned tuna by SSCP, we did observe some variability in the patterns, especially for two species, skipjack (*Katsuwonus pelamis*) and Atlantic bonito (*Sarda sarda*).

In the case of skipjack, three types of patterns were found (Fig. 3). whereas various samples of Atlantic bonito have expressed two patterns to date (Fig. 4).

From the results of sequencing the amplicons made by the 59primers (Unseld et al., 1995) and other regions of the cytochrome b gene of tunas (Bartlett & Davidson, 1991), it has to be expected that SSCP is influenced to some extent by the origin, i.e. the population, of the fish.

However, we have found that the ssDNA patterns of tunas, especially those of the genus *Thunnus*, are generally very constant when using the 59-primers.

In case of difficulties in identifying an unknown sample, different regions of mitochondrial DNA (mtDNA) can be utilised for SSCP (Rehbein et al., 1997). In addition to SSCP, more sophisticated and time-consuming techniques such as restriction fragment length polymorphism (RFLP) (Quinteiro, Sotelo, Rehbein, Pryde, Medina, Perez-Martin, Rey-Mendey, & Mackie, 1998) or sequencing of amplicons are available.

4. Conclusion

It was demonstrated by a collaborative study that PCR-SSCP using a short fragment of the cytochrome *b* gene is a reliable method of identifying the species in canned tuna. As the technique is fast, cheap and straightforward, it can be recommended for use in food control laboratories.

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