ELSEVIER
Trends in Food Science & Technology 10 (1999) 9–14
TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Challenges in the identification of species of canned fish

I.M. Mackie,[*†] S.E. Pryde,[*] C. Gonzales-Sotelo,[‡] I. Medina,[‡] R. Peréz-Martín,[‡] J. Quinteiro,[§] M. Rey-Mendez[§] and H. Rehbein[¶]

[*]Rowett Research Institute, Aberdeen, UK
[‡]Instituto de Investigacions Marinas, Vigo, Spain
[§]University of Santiago de Compostela, Santiago de Compostela, Spain
[¶]Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Hamburg, Germany

The identification of fish species becomes a problem when the usual identifying characteristics are removed on processing and only a portion of flesh is available. When the flesh is raw or cooked under normal conditions, the species is readily established by electrophoresis of the muscle proteins. The procedure cannot be used for heat-sterilised canned fish as the proteins are severely denatured. DNA is also degraded but techniques are now available for targeting and amplifying species-specific fragments. The amplified products can then be analysed by a range of techniques some of which are suitable for food control laboratories.
© 1999 Elsevier Science Ltd. All rights reserved.

Authenticity testing

Identifying the species of a plant or animal can be uncertain whenever the usual species characteristics such as shape, size, appearance, etc. (the morphological characters) are removed on processing and only a portion of flesh is available. It is then necessary to use methods of species identification which depend upon the analysis of species-specific components of the flesh such as proteins or DNA, itself. As the morphological characters are themselves a reflection of genetic differences and are the basis of systematics [1] it is evident that analytical techniques which can reveal those genetic differences at the molecular level will be suitable means of identifying species.

As far as proteins are concerned, physico-chemical differences in size or charge are revealed as differences in electrophoretic mobility, isoelectric points or chromatographic elution times. Thus when water-soluble proteins of tissue such as muscle are separated by electrophoresis, isoelectric focusing or by liquid chromatography, separation profiles unique to each species are obtained [2–7]. By comparing a profile with those of authentic species the identity of the species can then be established.

The specificity of interaction between antibodies and antigens, as utilised in the immunoassay analysis, offers an alternative means of differentiating proteins and thereby of identifying the species [3]. Antibodies raised against a particular protein or group of proteins can then be used to identify the species in a standard immunoassay procedure. The assay, however, gives no indication of the presence or absence of other species as it is specifically an analysis for a pre-selected species for which an antibody has been produced. As far as fish are concerned, it is largely impractical because of the potentially large number of species that might be involved.

When the flesh is cooked, the proteins become irreversibly denatured and can no longer be examined by techniques suitable for their native states. It is, however, possible to solubilise heat-denatured proteins in denaturing solvents such as 2% sodium dodecylsulphate or 8 M urea and to obtain species-specific profiles on electrophoresis [3]. Immunoassay systems are not generally of value for cooked flesh products as the antibodies are normally raised against undenatured proteins [3].

While electrophoresis in SDS or urea solvents is suitable for the identification of species which have been cooked under normal conditions, it is of no value for heat-sterilised products because of the severe denaturation suffered by the proteins. Protein zones on electrophorograms become indistinct and strong background

[†]Corresponding author.

0924-2244/99/$ - see front matter Copyright © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0924-2244(99)00013-8

staining develops depending upon the degree of denaturation, making reliable identification of species impossible. An alternative procedure is to digest the canned flesh with a protein-splitting agent cyanogen bromide which cleaves proteins at methionine residues [8]. The water-soluble protein residues released can then be analysed by isoelectric focusing and the protein profiles obtained used to identify the species. However, as protein profiles of closely related species cannot be differentiated, the method is of no value for identifying species within such families as those of sardine, salmon and tuna.

In principle, the most direct method of identifying species is by analysis of the DNA, which is more thermostable. Prior to method development, it is necessary to identify DNA fragments that show differences in sequence of the nucleotides among closely related species. Fragments can be selected for different levels of differentiation either of individuals or of populations, species or families [9, 10]. It is therefore important that the fragment shows the greatest difference between species and the least difference among individuals and populations of a species. Once the complete nucleotide sequence of the selected fragment is determined it can then be used to identify the species directly or to devise faster, simpler secondary methods of DNA analysis more suitable for use in food control laboratories.

DNA from canned fish

Although DNA is also degraded during the heat-sterilisation step of the canning process, it is still possible to obtain small fragments with sufficient differences in sequence to enable even closely related species to be differentiated and identified. Basic to the identification procedure is the polymerase chain reaction (PCR) in which selected primers with complimentary sequences to the opposing ends of the two single strands of a species-specific fragment of DNA, enable the fragment to be targeted and the double strand DNA to be enzymatically synthesised. The enzyme DNA polymerase is required for the initial synthesis and for the subsequent chain reaction by means of which millions of copies of the desired DNA fragment are synthesised to give sufficient product (amplicon) for sequencing or other DNA analysis.

In a cell, both nuclear and mitochondrial DNA are potentially available for species identification purposes but of the two forms, mitochondrial DNA is preferred for the following reasons:

(1) Mitochondrial DNA generally evolves much faster than nuclear DNA.
(2) Mitochondrial DNA is much smaller than nuclear DNA.
(3) There are several copies of mitochondrial DNA inside a cell.
(4) The complete sequence of mitochondrial DNA is known for several aquatic organisms.
(5) Large non-coding stretches of DNA (introns) are absent.

One of the most used fragments is the gene for cytochrome b which shows considerable inter- and intraspecies variation in nucleotide sequence. The amount of variation within a species, however, is less than between species which makes analysis of fragments of this gene suitable for the identification of species of different organisms [11, 12].

As far as the identification of canned fish is concerned, it is, first of all, necessary by means of PCR to produce amplicons of species-specific regions of the cytochrome b gene or other potential species-differentiating part of mitochondrial DNA. These fragments of DNA can then be analysed by sequencing and by secondary DNA analysis techniques such as single strand conformation polymorphism (SSCP) or restriction fragment length polymorphism (RFLP). The secondary DNA analysis technique or techniques selected for routine use will depend upon the specificity of the selected sequence for the species in question. In some cases more than one secondary technique will be required to establish the species unequivocally.

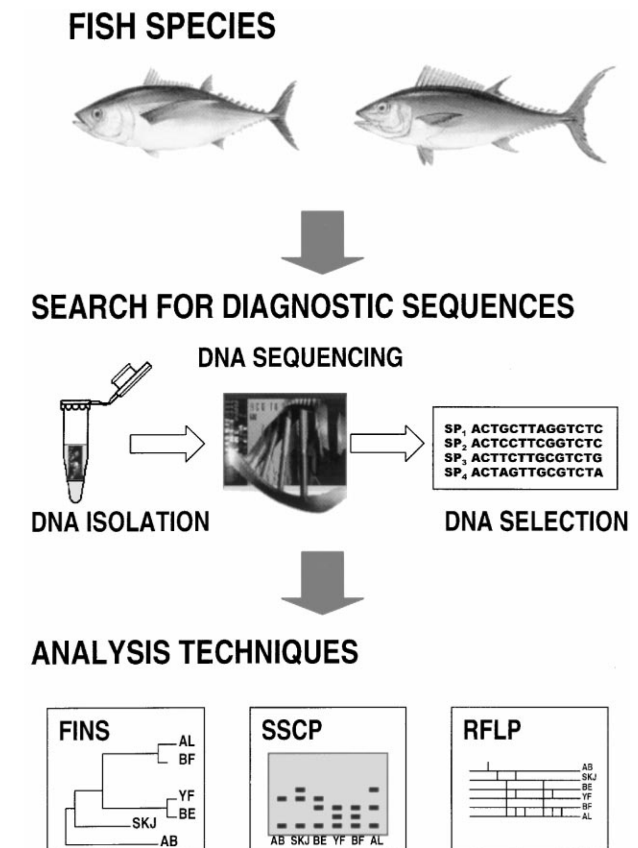

Fig. 1. Strategies for development of tuna species identification techniques. FINS: Forensically informative nucleotide sequencing, SSCP: Single strand conformation polymorphism, RFLP: Restriction fragment length polymorphism.

Table 1. Names of tuna and bonito species

Scientific name	English name	Spanish name
Auxis thazard	Frigate tuna	Melva
Euthynnus affinis	Kawakawa	Bonito del Pacifico
Euthynnus alletteratus	Little tunny	Bacoreta
Euthynnus lineatus	Black skipjack tuna	Bonito negra
Euthynnus (Katsuwonus) pelamis	Skipjack tuna	Listado
Sarda australis	Australian bonito	Bonito Austral
Sarda chiliensis	Pacific bonito	Bonito Chileno
Sarda orientalis	Oriental bonito	Bonito del Pacifico
Sarda sarda	Atlantic bonito	Sarda
Thunnus albacares	Yellowfin tuna	Rabil
Thunnus alalunga (Thunnus germo)	Albacore	Albacora
Thunnus atlanticus	Blackfin tuna	Atun de Aleta Negra
Thunnus maccoyii	Southern Bluefin tuna	Atun rojo del Sur
Thunnus obesus	Bigeye tuna	Patudo
Thunnus thynnus orientalis	Pacific Bluefin tuna	Atun (Rojo) del Pacifico
Thunnus thynnus thynnus	Atlantic Bluefin tuna	Atun (Rojo)
Thunnus tonggol	Long tail tuna	Atun tongol

A case study with tunas and bonitos

The tuna and bonito species are of great commercial importance worldwide accounting for more than 4.0% of the total world catch [13]. Their perceived eating qualities and hence commercial values vary considerably, ranging from Bluefin tuna (*T. thynnus*), the most expensive species which is much in demand for the sushi and sashimi outlets in Japan, to the cheaper species of Skipjack tuna, Frigate tuna and bonitos. In all, as many as 17 species (Table 1) are of commercial importance and as the flesh of some is similar in appearance and texture, particularly when canned, there is inevitably the possibility of cheaper species being mixed with or substituted for more expensive species. For example, Skipjack tuna or bonito species could be substituted for the true *Thunnus* species. Bonito, in particular, has a lower import tariff into the EU than tuna and not surprisingly it is believed that some of the canned species entering the EU are more likely to be bonito than tuna. For commercial reasons there is also a need to differentiate some of the true *Thunnus* species—Yellowfin tuna, and Albacore for example are sold by name as canned products in Spain, France, Germany, the UK, and Japan. Although regulations on labelling of canned tunas and bonitos exist in many countries [3, 14] there has been no reliable means of identifying the species and thus of enabling regulations to be enforced. Following recent work by ourselves [15–18] and others [19, 20] it is now possible to do so.

Previous relevant work

It had been shown by Bartlett and Davidson [12] for raw samples of four commercially important species—Bluefin tuna, Bigeye tuna, Yellowfin tuna and Albacore, that sequence analysis of a 358 base pairs (bp) amplicon of the PCR reaction could be used to differentiate them. When this procedure was applied by ourselves to canned tuna and bonito species, inconsistent results were obtained primarily because the bulk of the DNA had been reduced, on heating, to residues of less than 300 bp. Unseld *et al.* [19] dealt with the problem by amplifying a much shorter 59 bp fragment and were able to identify nine out of 11 species of tuna and bonito by sequence analysis. Two species of great commercial importance, Yellowfin tuna and Bluefin tuna could not be differentiated because of complete sequence identity within the respective fragments of DNA. To identify all tuna and bonito species longer segments of the cytochrome b gene or other species-variable sequence have to be amplified using alternative sets of primers.

Recent developments

The main objective of our recent studies was to develop DNA-based techniques for routine identification of all species of commercially canned tunas and bonitos. Our approach was to amplify DNA segments of different length from within the cytochrome b gene and to determine their sequences. The aim of this research was to find sequences which had sufficient species information to enable all tuna and bonito species to be clearly differentiated. It was also important that the fragments were of a size that could be amplified in good yield from the degraded DNA of a canned product.

From studies on DNA isolated from eight species of tuna (raw and canned), it was possible to identify four segments of cytochrome b which met the requirements.

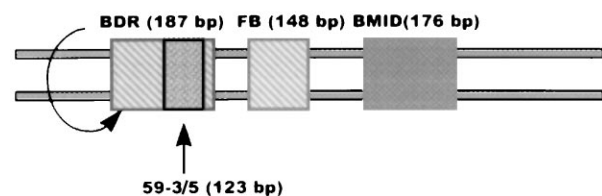

Fig. 2. Cytochrome b fragments selected for studying the identification of tuna and bonito species.

Depending upon the species they could be used either singly or in combination to identify all the species. Their fragment sizes ranged from 123 to 187 bp. A comparison of the sequences obtained for the eight species enabled genetic differences as well as the levels of intra-specific and inter-specific variability to be established, thereby confirming the diagnostic value of the selected fragments. The possible existence of polymorphisms due to geographic variation must be taken into consideration when selecting DNA fragments. Comparison of sequences of a limited number of tuna and bonito species from the Atlantic, Pacific and Indian oceans showed that there is little of any geographic variation. However there is some degree of introgression among Pacific Bluefin tuna and Albacore. This means that mitochondrial sequences from Albacore might be found in some specimens of the oriental sub-species of Bluefin tuna and that misidentification problems might arise when using cytochrome b-based, DNA techniques.

Once the intra-specific and inter-specific levels of variability were established for the selected DNA fragments the next step was to develop alternative secondary analysis techniques. These included SSCP, RFLP, random amplified DNA profiles (RAPDs) and microsatellites. Only SSCP and RFLP were found to be suitable for the identification of canned tunas and bonitos. These techniques are less time-consuming and cheaper than sequencing and are therefore more convenient for routine use in species identification.

PCR–SSCP

In this procedure, single strands (SSs) of DNA are formed from the double strand (DS) amplicon usually by heating in a denaturing solvent, such as formamide [18]. The SS DNA obtained moves in an electric field adopting a sequence-dependent secondary conformation which, in turn, affects its electrophoretic mobility: small differences in nucleotide sequence produce a different SS migration. SSs are then fixed in their positions and visualised by silver staining. Comparison of the profiles obtained with those of authentic species enables the identities to be established. Once denaturation and separation conditions are optimised this can be a very sensitive method of differentiating closely related species.

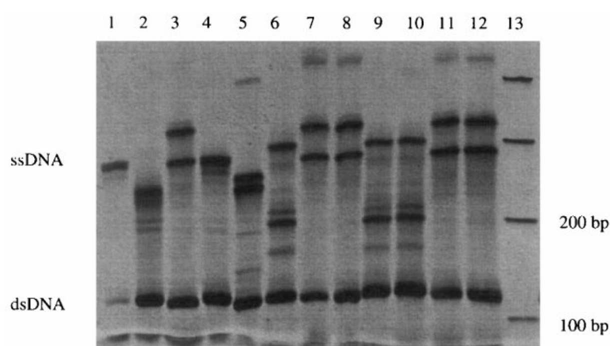

Fig. 3. SSCP of canned tuna, commercial cans from the German market and references. Lane 1: *Sarda sarda*; lane 2: *Euthynnus affinis*; lane 3: *Auxis thazard*; lane 4: *Sarda chiliensis*; lane 5: *Thunnus albacares*; lane 6: *Katsuwonus pelamis*; lane 7: Commercial can identified as *A. thazard Lane 8: Commercial can identified as* A. thazard*; lane 9: Commercial can, identified as* K. pelamis*; lane 10: Commercial can, identified as* K. pelamis*; lane 11: Commercial can, identified as* A. thazard*; lane 12: Commercial can, identified as* A. thazard*; lane 13: 100 bp ladder.*

Although SSCP analysis of the 59-3/59-5 amplicon could not differentiate Yellowfin tuna from Bluefin tuna, it was possible to differentiate them by analysis of the amplicons of the primer sets for BMID and BDR regions of cytochrome b. The validity of PCR–SSCP analysis using the 59-3/59-5 primers was tested in a collaborative trial among European laboratories [17]. It was confirmed that the method was a reliable and robust means of identifying tuna and bonito species and that it was particularly useful for detecting mixed species products. As some intra-specific variability had been detected in the selected sequences some small differences in the SSCP pattern was observed for some species. This problem should be resolved by studying the sequence of a significant number of authentic individuals to enable SSCP intra-specific variability to be established.

PCR–RFLP

PCR–RFLP is a more robust but also a more time-consuming procedure. Sets of enzymes (endo-nucleases) are chosen on the basis of their ability to recognise and cut stretches of nucleotide sequences within a particular DNA fragment. These stretches of DNA are then separated by size on gel electrophoresis rendering species-specific profiles. In our study once the sequences of the cytochrome b amplicons were obtained and analysed, fragments with a high number of inter-specific sites were selected. Using the sequences of those fragments (BDR or BMID) from the different tuna species, it was possible to generate theoretical restriction sites by computer software (DNASIS from Hitachi Software Engineering Co. Ltd.; Gene Jockey from Biosoft). In this way enzymes generating specific fragments were identified, the number of enzymes required to produce species-differentiating profiles, depending upon the intra-specific variability of the particular fragment. Three enzymes are required to differentiate eight tuna species using BMID [16] whereas five are needed using BDR [21]. With the latter, however, it was not possible to differentiate Bluefin and Bigeye tunas because of the high level of similarity in their sequences. Figure 4 shows a typical RFLP analysis of tuna samples using the BMID fragment in which the fragments of different size indicate the different cutting positions. As with PCR–SSCP an advantage of the technique is that it allows the detection of mixed species samples (Fig. 5).

Analysis of short amplicons such as the 59-3/59-5, is often useful for differentiating tuna from bonito species,

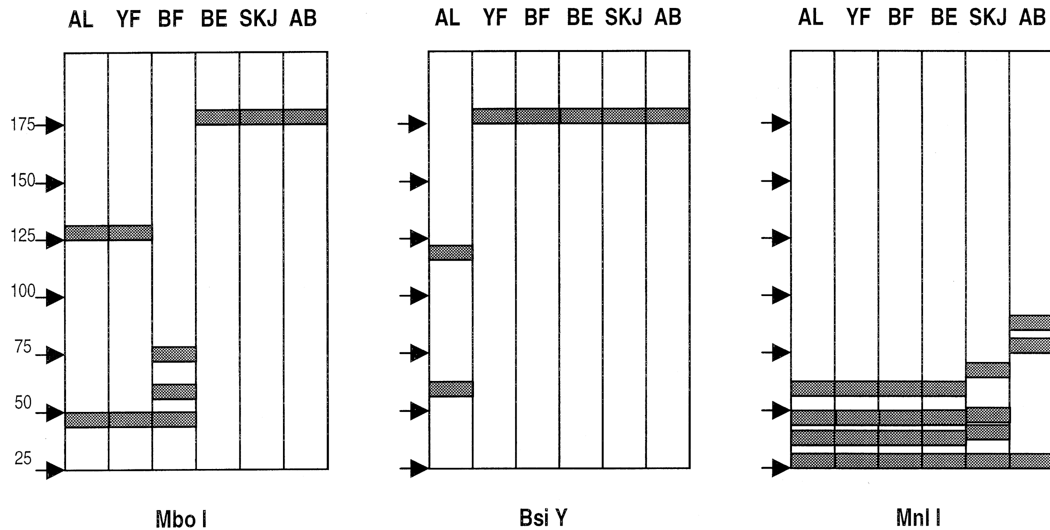

Fig. 4. Computer simulated RFLP analysis of tuna samples using the BMID amplicon.

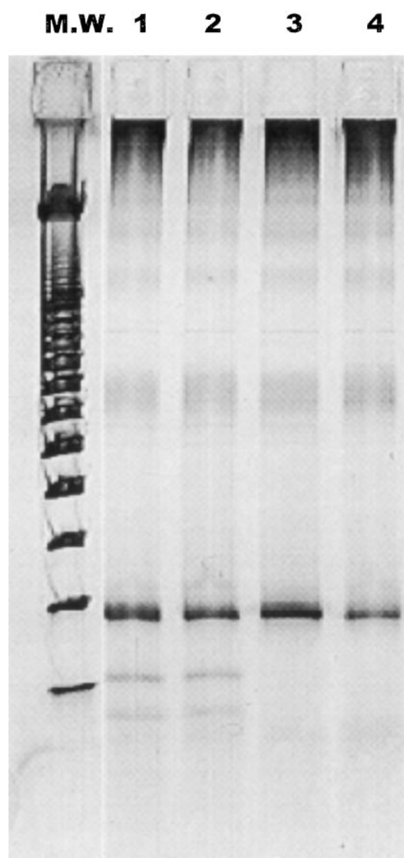

Fig. 5. RFLP gel showing restriction fragments of the BDR amplicon after digesting with Eco NI. Lanes 1 and 2 show a digestion pattern of a mixed species sample (*Thunnus alalunga* + *Thunnus albacares*); lanes 3 and 4 show the uncut amplicon for *Thunnus alalunga* which is not digested by Eco NI.

sometimes all that is required to satisfy some labelling requirements (e.g. import distinctions for tariff purposes).

One of the main drawbacks of this technique relates to the existence of intra-specific variability which means that the same species can present different nucleotide patterns at a particular restriction target site. This problem was resolved by studying a significant number of authentic species and selecting a target site with no polymorphism. Until more information is built up on the sequence variability of species, RFLP analysis should be used with caution.

It is therefore recommended that the identification of species be done by two independent DNA analysis techniques, e.g. RFLP of BDR and RFLP of BMID or RFLP and SSCP of either amplicon as it gives greater confidence in the results.

RFLP was shown to be a useful and rapid tool to identify accurately not only tuna species but also species which can present an appearance and texture similar to canned tuna muscle. Such species could be the phylogenetically related mackerels which present very different profiles from those of the tuna and bonito species.

In extreme situations, where it is not possible to identify a species using fragments of the cytochome b gene, recourse can be made to sequence analysis of part of the Control region (D1 fragment) [21]. As the number of variable positions is higher than in the cytochrome b gene, closely related species such as Long tail tuna, Blackfin tuna, Yellowfin tuna and Bluefin tuna can be differentiated unequivocally. However, because of high sequence variability, amplicons of this region are not suitable for SSCP or RFLP analysis.

Commercial samples

Analysis of a small number of commercial samples from the participants' countries and from other countries within and outside the EU showed that this was evidence of direct substitution of one species by another and of mixed species products. There is thus a strong need for reliable methods of identifying canned tuna and bonito species. The use of such techniques will help to protect consumers' rights by enabling the enforcement of labelling regulations in member countries and within the framework of the EU. It will also help to regulate imports from producer countries outside the EU and, not least, it will assist in the protection of endangered species such as the Bluefin tuna [22].

Future perspectives

For the first time, reliable methods based on the analysis of mitochondrial DNA are available for identifying the species of all commercially important tunas and bonitos, when canned.

There is a need to have a complete map of diagnostic sequences (BMID, BDR or D-loop) for a significant number of authentic specimens of different species. This will enable more accurate information to be obtained about intra-species variation. The influences of geographical location and of migrations on these sequences also need to be studied.

There is a need to make DNA analytical techniques available to potential users in forms that are rapid, easy to perform and not excessively expensive. The secondary DNA analytical techniques used in the study (PCR–SSCP and PCR–RFLP) meet these criteria and are likely to be used routinely in food control laboratories.

Further developments are likely in the search for specific DNA fragments for constructing species-specific probes. These would be used to hybridise with a target sequence and so identify the species. This technique is already being used for other diagnostic applications.

Acknowledgements

We thank the European Commission for the award of the project AIR2-CT94-11.26.

References

1. Ayala, F. (1983) Protein Polymorphism: Adaptive and Taxonomic Significance in *Enzymes as Taxonomic Characters. Systematics Association Special Volume*, No. 24 (Oxford, G.S. and Rollinson, D., eds.), Academic Press, London and New York
2. Sotelo, C., Pineiro, C., Gallardo, J.M. and Peréz-Martín, R.I. (1993) 'Fish Species Identification in Seafood Products' in *Trends Food Sci. Technol* 4, 395–401
3. Mackie, I.M. (1997) 'Identifying Fish' in *Food Authenticity* (Dennis, M.J. and Ashurst, P.R., eds.), pp. 140–170, Blackie Academic and Professional, London
4. Rehbein, J. (1990) 'Electrophoretic Techniques for Species Identification of Fishery Products' in *Z. Lebensm. Unters Forsch*. 191, 1–10
5. Osman, M.A., Ashoor, S.H. and Marsh, P.C. (1987) 'Liquid Chromatographic Identification of Common Fish Species' in *J. Assoc. Off. Anal. Chem*. 70, 618–625
6. Pineiro, C., Sotelo, C.G., Medina, I., Gallardo, J.M. and Peréz-Martín, R.I. (1997) 'Reversed-Phase HPLC as a Method of Identification of Gadoid Species' in *Z. Lebensm Unters Forsch* 204, 411–416
7. Gallardo, J.M., Sotelo, C.G., Pinciro, C. and Peréz-Martín, R.I. (1995) 'Use of Capillary Zone Electrophoresis for Fish Species Identification. Differentiation of Flat Fish Species' in *J. Agric. Food Chem* 43, 1238–1244
8. Gross, E. (1966) 'The Cyanogen Bromide Reaction' in *Meth. Enzymol* II, 238–255
9. Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X. and Wilson, A.C. (1989) 'Dynamics of Mitochondrial DNA Evolution in Animals: Amplification and Sequencing with Conserved Primers' in *Proc. Natl. Acad. Sci. USA* 86, 6196–6200
10. Meyer, A. (1993) 'Evolution of Mitochondrial DNA in Fishes' in *Biochemistry and Molecular Biology of Fishes* (Hochachka, P.W. and Mommsen, T.P., eds.), pp. 617–622, Elsevier
11. Bartlett, S.E. and Davidson, W.S. (1992) 'FINS (Forensically Informative Nucleotide Sequencing): A Procedure for Identifying the Animal Origin of Biological Specimens' in *Biotechniques* 12, 408–411
12. Bartlett, S.E. and Davidson, W.S. (1991) 'Identification of *Thunnus* Tuna Species by the Polymerase Chain Reaction and Direct Sequence Analysis of their Mitochondrial Cytochrome b Genes' in *Can. J. Fish Aquat. Sci*. 48, 309–317
13. F.A.O. (1997) '1995 Yearbook. FAO Fishery Statistics—Catches and Landings, Vol. 80, FAO, Rome
14. The Food Labelling Regulations (1966) No. 1499, HMSO, London
15. Rehbein, H., Mackie, I.M., Pryde, S., Gonzales-Sotelo, C., Peréz-Martín, R.I., Quinteiro, J. and Rey-Mendez, M. (1995) 'Fish Species Identification in Canned Tuna by DNA Analysis (PCR-RFLP)' in *Inf. Fischwirtsch*. 42, 209–212
16. Quinteiro, L, Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Peréz-Martín, R.I., Rey-Mendez, M. and Mackie, I.M. (1998) 'Use of mt DNA Polymerase Chain Reaction (PCR) Sequencing and PCR-Restriction Fragment Length Polymorphism Methodologies in Species Identification of Canned Tuna' in *J. Agric. Food Chem*. 46, 1662–1669
17. Rehbein, H., Mackie, I.M., Pryde, S., Sotelo, C.G., Peréz-Martín, R.I., Quinteiro, J. and Rey-Mendez, M. (1999) 'Fish Species Identification in Canned Tuna by PCR- SSCP: Validation by a Collaborative Study and Investigation of Inter-Species Variability of the DNA Pattems' in *Food Chemistry* 64, 263–268
18. Rehbein, H., Mackie, I.M., Pryde, S., Gonzales-Sotelo, C., Peréz-Martín, R.I., Quinteiro, J. and Rey-Mendez, M. (1998) 'Comparison of Different Methods to Produce Single Strand DNA for Identification of Canned Tuna by Single-Strand Conformation Polymorphism Analysis' in *Electrophoresis* 19, 1381–1384
19. Unseld, M., Beyermann, B., Brandt, P. and Heisel, R. (1995) 'Identification of the Species of Origin of Highly Processed Meat Products by Mitochondrial DNA Sequences' in *PCR Meth. Applic*. 4, 241–243
20. Ram, L.L., Ram, M.L. and Baidoun, F.F. (1996) 'Authentication of Canned Tuna and Bonito Sequence and Restriction Site Analysis of Polymerase Chain Reaction Products of Mitochondrial DNA' in *J. Agric. Food Chem*. 44, 2460–2467
21. Mackie, I.M., Pryde, S.E., Rehbein, H., Quinteiro, J., Rey-Méndez, M., Gonzales-Sotelo, C., Medina, I. and Peréz-Martín, R.I. (1999) Final Report of EC Project, AIR2-CT94-1126 'The Identification of Canned Tuna Species by Characterisation of the Nucleic Acids' in *European Commission, DG XIV, Fisheries*, Brussels, in press
22. FAO (1997) Review of the State of World Fishery Resources: Marine Fisheries C. Special Topics: Global Resources of Tuna and Tuna-like Species. *FAO Fisheries Circular No. 920,* FIRM/C 920