Novel Method for the Discrimination of Tuna (*Thunnus thynnus*) and Bonito (*Sarda sarda*) DNA

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A novel method for the discrimination of bluefin tuna (*Thunnus thynnus*) from Atlantic bonito (*Sarda sarda*) was developed, based on species-specific amplification of a region of the mitochondrial cytochrome *b* gene by Polymerase Chain Reaction (PCR). The method, which uses a one-step amplification reaction, is more rapid to perform than any of the currently described techniques for species determination in fish. The species of origin of the DNA is indicated by the distinctive size of the PCR product on electrophoresis, but the test could readily be adapted to other forms of electrophoresis or fluorescence-based systems for quantification. Given the possibility of intraspecific variability in mitochondrial DNA and the consequent desirability of performing two independent tests, the new method constitutes a valuable addition to the range of tuna speciation methodologies currently available.

Keywords: *Species identification; Thunnus thynnus; Sarda sarda; PCR; mitochondrial cytochrome b gene*

INTRODUCTION

Methods to enable the identification of commercially important fish species are currently receiving considerable attention (Mackie et al., 1999; Bossier, 1999). In recent years, interest has been focused on DNA analysis (Rehbein et al., 1995, 1998, 1999; Unseld et al., 1995; Ram et al., 1996; Quinteiro et al., 1998) rather than conventional protein-based techniques (Osman et al., 1987; Rehbein, 1990; Sotelo et al., 1993; Gallardo et al., 1995). DNA is more thermostable than protein and consequently can be used to analyze processed products such as heat-sterilized canned fish. Furthermore, mitochondrial DNA may present advantages over nuclear DNA, because of its relatively high abundance (Unseld et al., 1995).

Tuna and bonito account for a significant proportion of the global fish market. Different species command different prices; bluefin tuna (*Thunnus thynnus*) is the most expensive, while bonito (*Sarda* sp.) has a much lower market price and a lower import tariff into the European Union. However, in the absence of morphological markers, these species are not easily distinguished because their flesh is similar in both appearance and texture. Consequently, it is possible that bonito could be substituted for, or mixed with, the more expensive species of tuna fish. Considerations such as this have provided an impetus for much of the research described above.

Sequences in the mitochondrial DNA cytochrome *b* gene (mtDNA cyt *b*) have been widely used for species identification (Kocher et al., 1989; Meyer et al., 1990) and such techniques have been widely applied to tuna fish. Early work used the polymerase chain reaction (PCR) to amplify gene fragments which were then sequenced (Bartlett and Davidson, 1991, 1992; Unseld et al., 1995). On the basis of this, several species could be differentiated. Subsequently, secondary methods of DNA analysis, other than direct sequencing, have been developed. Having generated PCR products, these can be analyzed by single-strand conformation polymorphism (SSCP) (Rehbein et al., 1995, 1998, 1999) or restriction fragment length polymorphism (RFLP) (Ram et al., 1996; Quinteiro et al., 1998), both of which use gel-based patterns to facilitate species identification. Because such protocols are easier to perform, they may be more suitable for use in food analytical laboratories than full sequencing.

However, as with all mitochondrial DNA analyses, the possibility of intraspecific variability has to be considered (Moritz et al., 1987; Graves, 1998). The extent of this variation differs according to the DNA fragment under study (Simon et al., 1994). Intraspecific polymorphism could result in individual members of the same species possessing different nucleotides at any position. This could produce differences in SSCP patterns or create or delete a particular restriction site. The precise extent of such variabilty has not been documented. When attention has been focused on this problem, such polymorphism has been found to be relatively limited and has not generally served to confound species identification (Mackie et al., 1999). However, introgression of mtDNA has been found among bluefin tuna and albacore (*T. alalunga*) (Chow and Kishino, 1995). To allow for the possibility of intraspecific mitochondial DNA variation, it has been suggested that two independent DNA analysis techniques should be undertaken in order to improve the degree of confidence that can be placed in species assignation via DNA-based methodologies (Mackie et al., 1999).

Presented here is an additional method of PCR-based analysis of the mitochondrial cytochrome *b* gene that offers a direct (one-step) means of species identification.

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The protocol is more rapid to perform than any of the techniques described above and consequently may prove useful as an adjunct to the same. It relies on species-specific amplification products being generated only in the presence of the correct template nucleic acid and has been used to differentiate between bluefin tuna (*T. thynnus*) and Atlantic bonito *(S. sarda*).

MATERIALS AND METHODS

DNA Extraction. Muscle samples were obtained from authenticated bluefin (*T. thynnus*) and Atlantic bonito (*S. sarda*) specimens. These were kindly supplied by Dr. S. E. Pryde of the Rowett Research Institute in Aberdeen (U.K.). Additional samples (whole fish) were purchased directly from Billingsgate fish market (London, U.K.). The method by which DNA was extracted from these was based on a procedure previously described by Meyer et al. (1994).

To solubilize the tissue, 0.1 g was minced with a sterile surgical blade and transferred to a 1.5 mL Eppendorf tube. Extraction buffer (0.43 mL; 1% [w/v] sodium dodecyl sulfate [SDS], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris-HCl, pH 8.0) was then added to this along with 50 μL 5 M guanidine thiocyanate and 20 μL proteinase K (20 mg/mL in distilled water). The tube contents were mixed by inversion and then incubated at 56 °C for 2 h. After this time, another 20 μL aliquot of proteinase K was added and the incubation was left to proceed overnight (56 °C). Undissolved material was removed by centrifugation for two 5 min periods at 20 000*g*. DNA was isolated from the supernatant using a Wizard DNA Clean Up System (Promega) according to manufacturer's instructions; 1 mL of Wizard DNA Clean Up Resin plus the supernatant were added to a 1.5 mL Eppendorf tube and mixed by inversion. The resin containing the bound DNA was then pipetted into a 3 mL disposable syringe barrel and the syringe plunger enabled the slurry to be pushed into the Wizard minicolumn. The column was washed with 2 mL 80% (v/v) 2-propanol, transferred to a 1.5 mL Eppendorf tube, and centrifuged for 20 s to dry the resin. Finally, the column was transferred to another 1.5 mL Eppendorf tube and prewarmed (65 °C) TE buffer (30 μL, 10 mM Tris-HCl, [pH 8.0], 1 mM EDTA) was pipetted onto it and left for 1 min, after which the DNA was eluted by centrifugation for 20 s. The purified DNA was stored at 4 °C.

Sequence Analysis. The NCBI www. Entrez nucleotide database was searched for mitochondrial cytochrome *b* sequences from bluefin tuna (*T. thynnus*), Atlantic bonito (*S. sarda*), and albacore (*T. alalunga*). (The latter species was included because introgression of mtDNA between bluefin and albacore has been documented (Chow and Kishino, 1995).) These were aligned using the Clustal W multiple sequence alignment program (Thompson et al., 1994) (Figure 1). At one position in the gene (highlighted in bold) a different nucleotide is present in all three species. The possibility that this nucleotide may be phylogenetically informative was therefore given consideration.

Sequencing was undertaken to verify that the polymorphism was present in the bluefin and bonito DNA generated in our laboratory. For one sample from each species, a 255 base pair (bp) amplicon was generated by PCR, using primers designed to flank the target nucleotide (Figure 2). The sequence of the upstream primer was 5′-actggatccctgatgtcgaatcagc-3′ and that of the downstream primer was 5′-acatctgtcctcagggaagaacgta-3′.

The PCR reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each deoxynucleotide 5′-triphosphate (dNTP), 0.1 μM each primer, 1.25 units *Taq* DNA polymerase (Boehringer Mannheim, added during a Hot Start), 250 ng DNA, and sterile distilled water to 50 μL. The thermal cycling regime was 1× (95 °C 1 min; 69 °C 1 min; 72 °C 1 min), 30× (95 °C 30 s; 69 °C 45 s; 72 °C 45 s) and 1× (72 °C 3 min).

Amplification was undertaken using a Hybaid Omnigene thermal cycler and the products were purified directly from the reaction mixtures using Qiagen columns, according to the manufacturer's instructions. These were then sequenced directly, in both directions, using an automated sequencer (ABI Prism 377, PE Biosystems) with fluorescent dye-labeled dideoxynucleotides employed in the chain termination method. The sequences were compared with those already in the nucleotide databases using a FASTA3 homology search (Pearson and Lipman, 1988; Pearson, 1990).

Species-Specific PCR. For direct detection of the polymorphism in a series of bluefin and bonito samples, PCR primers were designed with different 3′ terminal bases. These primers terminate precisely at the point of the single base polymorphism that may be phylogenetically informative. Theoretically, if there is not exact complementarity between the 3′ terminal base of the primer and DNA template, the PCR will be subject to inhibition of product formation (Newton et al., 1989). However this method does not always readily give absolute discrimination between sequences differing only by a single base, because spurious cross reactions may occur (Kwok et al., 1990). Accordingly, Rust et al. (1993) described the technique of mutagenically separated PCR (MS−PCR) in which allele-specific primers differing at their 3′ termini are designed to contain further deliberately introduced mismatches which serve to improve their discriminatory ability. The additional mismatches generate products in the first round of amplification that differ from each other to a greater degree than the original differences in the DNA template. When used as templates in subsequent rounds of PCR, these mutated products ultimately outnumber the original DNA template, thereby enhancing the accuracy of the allelic discrimination. Two allele-specific primers are used in the same reaction, along with a third nonallele-specific primer. The former two primers are of differing length, created by adding extra sequence to the nonextensible 5′ end of one of the primers, which enables different length primer-specific products to be distinguished by agarose gel electrophoresis.

A deliberately introduced mismatch was designed near the 3′ end of the bonito-specific primer, whereas other differences toward the 5′ terminus were a consequence of the interspecific variability of the regions under consideration. Consequently, the sequences of the three primers were 5′-gccgagatgtcaactcggttgactt-3′ (bluefin-specific primer); 5′-cctcagtcgcccacatttcacgagacgttaatttcggctgaata-3′ (bonito-specific primer); and 5′-acatctgtcctcagggaagaacgta-3′ (nonselective primer, identical to the downstream primer described above). The introduced mismatch is shown underlined. When these primers were used (Figure 2), the predicted (theoretical) sizes of the products for bluefin and bonito DNA templates were 207 and 225 bp, respectively.

The PCR reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each dNTP, 0.1 μM nonselective primer, 0.08 μM bonito-specific primer, 0.2 μM bluefin-specific primer, 1.25 units *Taq* DNA polymerase (Boehringer Mannheim, added during a Hot Start), 250 ng DNA, and sterile distilled water to 50 μL.

The cycling regime, undertaken using a Hybaid Omnigene thermal cycler was 1× (95 °C 1 min; 69 °C 45 s; 72 °C 45 s); 30× (95 °C 30 s; 69 °C 45 s; 72 °C 45 s); and finally 1× (72 °C 3 min).

PCR products were analyzed on a 4% MetaPhor agarose minigel (FMC Bioproducts) (1 × TBE). 1 × TBE is 0.09 M Tris-borate, 0.09 M boric acid, 0.002 M EDTA, pH 8.0. The gel was stained with ethidium bromide (0.5 μg/mL solution in distilled water) post-electrophoresis and visualized using a Gel Doc 2000 gel documentation system (BioRad).

RESULTS AND DISCUSSION

Sequence Analysis. Two mitochondrial cytochrome *b* gene sequences for each of bluefin (*T. thynnus*) and Atlantic bonito (*S. sarda*) (Block et al., 1993; Cantatore et al., 1994) and one for albacore (*T. alalunga*) (Block et al., 1993) are shown in Figure 1. Pairwise compari-

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X81563          CTTATTTCTCAGATCCTTACAGGACTATTCCTCGCAATACACTACACCCCTGATGTCGAA 180
L11560          -------------TCCTTACAGGACTATTCCTCGCAATACACTACACCCCTGAYGTCGAA 47
L11556          -------------TCCTTACAGGACTATTCCTCGCAATACACTACACCCCTGATGTCGAA 47
X81562          CTGATCTCCCAAATCCTTACAGGACTATTCCTAGCAATACATTATACCCCTGATGTCGAA 180
L11549          -------------TCCTCACAGGACTATTCCTAGCAATACATTATACCCCTGATGTCGAA 47
                            **** ************** ******** ** ******** ******

X81563          TCAGCCTTCGCCTCAGTAGCCCACATTTGCCGAGATGTCAACTTCGGTTGACTTATCCGG 240
L11560          TCAGCCTTCGCCTCAGTAGCCCACATTTGCCGAGATGTCAACTTCGGTTGACTTATCCGR 107
L11556          TCAGCCTTTGCCTCAGTAGCCCATATCTGCCGAGATGTAAACTTCGGATGACTCATCCGA 107
X81562          TCAGCCTTCGCCTCAGTCGCCCACATTTGCCGGGACGTTAATTTCGGCTGACTAATCCGA 240
L11549          TCAGCCTTCGCCTCAGTCGCCCACATTTGCCGAGACGTTAATTTCGGCTGACTAATCCGA 107
                ******** ******** ***** ** ***** ** ** ** ***** ***** *****

X81563          AACCTCCACGCAAACGGGGCCTCTTTCTTCTTTATCTGTATCTACTTCCACATCGGCCGA 300
L11560          AACCTCCACGCAAACGGAGCCTCTTTCTTCTTTATCTGTATCTACTTCCACATCGGCCGA 167
L11556          AACCTCCACGCAAACGGAGCATCTTTCTTCTTTATCTGCATCTACTTCCACATCGGCCGA 167
X81562          AACCTCCACGCAAACGGGGCCTCCTTCTTCTTCATCTGTATCTACTTCCACATTGGCCGA 300
L11549          AACCTCCACGCAAACGGCGCTTCCTTCTTCTTCATCTGCATCTACTTCCATATTGGTCGA 167
                ****************** ** ** ******** ***** ********** ** ** ***

X81563          GGACTTTACTACGGCTCTTACCTATACAAAGAAACATGAAACATCGGAGTAGTACTCCTA 360
L11560          GGACTTTACTACGGCTCTTACCTATACAAAGAAACATGAAACATCGGAGTAGTACTCCTA 227
L11556          GGCCTTTACTATGGCTCCTACCTATACAAAGAAACATGAAACATTGGAGTAGTTCTCCTA 227
X81562          GGCCTTTACTACGGCTCCTACCTCTACAAAGAAACATGAAACATTGGCGTAGTCCTTCTA 360
L11549          GGCCTTTACTACGGCTCCTACCTCTACAAAGAAACATGAAACATTGGAGTAGTCCTCCTA 227
                ** ******** ***** ***** ******************** ** ***** ** ***

X81563          CTCCTAGTTATGATGACCGCCTTCGTTGGCTACGTCCTTCCCTGAGGACAAATGTCTTTC 420
L11560          CTCCTAGTTATGATGACCGCCTTCGTTGGCTACGTTCTTCCCTGAGGACAAATGTCTTTC 287
L11556          CTTCTNGTTATGATGACCGCCTTCGTTGGCTACGTCCTTCCCTGAGGACAAATGTCCTTC 287
X81562          CTTTTAGTAATGATGACCGCTTTCGTCGGTTACGTTCTTCCCTGAGGACAGATGTCCTTC 420
L11549          CTTTTAGTAATGATGACCGCTTTCGTCGGCTACGTYCTTCCCTGAGGACAGATGTCCTTC 287
                **  * ** *********** ***** ** ***** ************** ***** ***

X81563          TGAGGAGCTACCGTCATTACTAACCTCCTATCCGCAGTCCCATATGTCGGAACTACTCTC 480
L11560          TGAGGAGCTACCGTNATTACTAACCTTCTATCGGCAGTCCCATATGTTGGAACTACTCTC 347
L11556          TGAGGAGCTACCGTCATTACTAACCTCCTATCCGCAGTCCCATATGTTGGAACTACCCTC 347
X81562          TGAGGAGCTACCGTCATTACCAACCTCCTCTCCGCAGTACCCTACGTAGGAACTACCCTC 480
L11549          TGAGGGGCTACCGTCATTACCAACCTCCTCTCCGCAGTACCATACGTNGGAACTACCCTC 347
                ***** ******** ***** ***** ** ** ***** ** ** ** ******** ***

X81563          GTTGAATGAATCTGAGGAGGCTTTTCAGTAGACAATGCCACCCTCACCCGTTTCTTCGCA 540
L11560          GTTGAATGAATCTGAGGAGGCTTTTCAGTAGACAATGCCACCCTCACCCGATTCTTCGCA 407
L11556          GTTGAATGAATCTGAGGGGGCTTTTCAGTAGACAATGCTACCCTTACCCGATTCTTCGCA 407
X81562          GTCGAATGAATCTGAGGGGGCTTCTCTGTAGACAATGCCACCCTCACCCGATTCTTTGCC 540
L11549          GTCGAATGAATCTGAGGGGGCTTCTCTGTAGACAATGCYACCCTTACCCGGTTCTTCGCC 407
                ** ************** ***** ** ********** ***** ***** ***** **

X81563          TTCCACTTCCTATTCCCATTCGTCATCGCAGCTATGACAATTCTTCACCTTCTTTTCCTT 600
L11560          TTCCACTTCCTATTCCCTTTGGTCATCGCAGCTATGACAATTCTTCACCTTCTTTTCCTT 467
L11556          TTCCACTTCCTATTCCCATTCGTCATCGCAGCCATGACAATTCTTCACCTTCTTTTCCTT 467
X81562          TTTCACTTCCTCTTCCCATTCGTTATTTTAGCTATAACAATTCTTCACCTCCTATTCCTA 600
L11549          TTCCACTTCCTCTTCCCGTTCGTTATTTTAGCCATAACAATTCTTCACCTCCTATTCCTA 467
                ** ******** ***** ** ** **   *** ** ************** ** *****

X81563          CACGAAACAGGTTCAAATAATCCAATCGGATTAAACTCAAATGCAGATAAAATCTCATTC 660
L11560          CACGAAACAGGTTCAAATAATCCAATCGGATTAAACTCAAATGCAGATAAAATCTCATTC 527
L11556          CACGAAACAGGTTCAAATAATCCAATCGGATTAAACTCAAATGCAGATAAAATCTCATTC 527
X81562          CACGAAACCGGATCAAACAACCCAATCGGACTAAACTCAAATGCAGACAAAATTTCATTC 660
L11549          CACGAGACCGGATCAAACAACCCAATTGGACTGAACTCAAACGCAGACAAAATTTCATTC 527
                ***** ** ** ***** ** ***** *** * ******** ***** ***** ******

X81563          CACCCATACTTCTCTTACAAAGACCTCCTTGGTTTCGTGATCCTGCTAGTAGCACTCGCC 720
L11560          CACCCATACTTCTCTTACAAAGACCTCCTTGGTTTCGTGATCCTGCTAGTAGCACTCGCC 587
L11556          CACCCATACTTCTCTTACAAAGACCTCCTTGGTTTCGTGATCCTACTAGTGGCACTCGCC 587
X81562          CACCCTTACTTCTCATATAAAGACCTTCTTGGATTCGCAATTCTGCTTGTTGCACTCGCC 720
L11549          CACCCTTACTTCTCATATAAAGACCTTCTTGGATTTGCAATTCTGCTTGTTGCACTAGCG 587
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Figure 1. Alignment of Bluefin (*Thunnus thynnus*), Albacore (*Thunnus alalunga*), and Atlantic Bonito (*Sarda sarda*) mitochondrial cytochrome *b* sequences. The sequences are identified by their accession numbers: X81563 (*T. thynnus,* Cantatore et al., 1994); L11560 (*T. Thynnus,* Block et al., 1993); L11556 (*T. alalunga* Block et al., 1993); X81562 (*S. sarda,* Cantatore et al., 1994); L11549 (*S. sarda,* Block et al., 1993). Bases that are identical in all five sequences are indicated with an asterisk. The base around which the test was designed is highlighted in bold.

sons of the sequences derived from the same species (BLAST 2 analysis) showed the two bluefin sequences to have 98% identity, while the corresponding figure for the bonito was slightly lower at 95%, although this

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                                        I
TCCTTACAGG ACTATTCCTC GCAATACACT ACACCCCTGA YGTCGAATCA GCCTTCGCCT   60

                                  II
CAGTAGCCCA CATTTGCCGA GATGTCAACT TCGGTTGACT TATCCGRAAC CTCCACGCAA  120

ACGGAGCCTC TTTCTTCTTT ATCTGTATCT ACTTCCACAT CGGCCGAGGA CTTTACTACG  180

GCTCTTACCT ATACAAAGAA ACATGAAACA TCGGAGTAGT ACTCCTACTC CTAGTTATGA  240

                                                  III
TGACCGCCTT CGTTGGCTAC GTTCTTCCCT GAGGACAAAT GTCTTTCTGA GGAGCTACCG  300

TNATTACTAA CCTTCTATCG GCAGTCCCAT ATGTTGGAAC TACTCTCGTT GAATGAATCT  360

GAGGAGGCTT TTCAGTAGAC AATGCCACCC TCACCCGATT CTTCGCATTC CACTTCCTAT  420

TCCCTTTGGT CATCGCAGCT ATGACAATTC TTCACCTTCT TTTCCTTCAC GAAACAGGTT  480

CAAATAATCC AATCGGATTA AACTCAAATG CAGATAAAAT CTCATTCCAC CCATACTTCT  540

CTTACAAAGA CCTCCTTGGT TTCGTGATCC TGCTAGTAGC ACTCGCC                 587
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Figure 2. The positions of the primers are shown aligned with the bluefin (*T. thynnus*) sequence (Block et al., 1993). Primer I represents the position of the upstream oligonucleotide as used to generate a product for sequencing. Primer II shows the position of the bluefin-specific primer. Primer III illustrates the position of the downstream primer used to generate products for both the sequencing and species-specific reactions. The bonito-specific primer was in exactly same position as Primer II, but it had an additional 18 bases upstream, taken from the *S. sarda* sequence.

figure was decreased by some ambiguity of base calling in one of the two sequences.

Sequencing of the bluefin and bonito DNA extracted in our laboratory indicated that the target polymorphism in the database also held true for these samples. Complete agreement between the forward and reverse sequences was obtained and a 225 base bonito read exhibited 100% homology with that described by Block et al. (1993). The equivalent bluefin fragment exhibited greater than 99% homology with the bluefin sequence described by Block et al. (1993); at position 125 our laboratory sample had G called, instead of the published A, which is in agreement with Cantatore et al. (1994), and may thus be indicative of intraspecific variation. However, it does not influence the test under development.

The development of a direct method of discriminating between bluefin and Atlantic bonito DNA was accordingly based on the base difference at position 101 identified as a result of the sequence alignments. Base numbering corresponds to that described by Block et al. (1993) so that position 1 of the fish sequences corresponds to position 14 880 of the human mitochondrial genome.

Test Development. The test that was developed reliably differentiated between the two species (Figure 3). Because the discrimination is achieved during the course of the amplification reaction, no secondary step, such as sequencing, SSCP, or RFLP analysis was required. Therefore, the method is less time-consuming than any of the molecular techniques currently in existence.

It is appropriate to consider the general utility of the test described here. Similar PCR-based tests have been developed to reliably discriminate between two samples differing at a specific nucleotide in other food- and agriculture-related contexts, such as the porcine ryano-

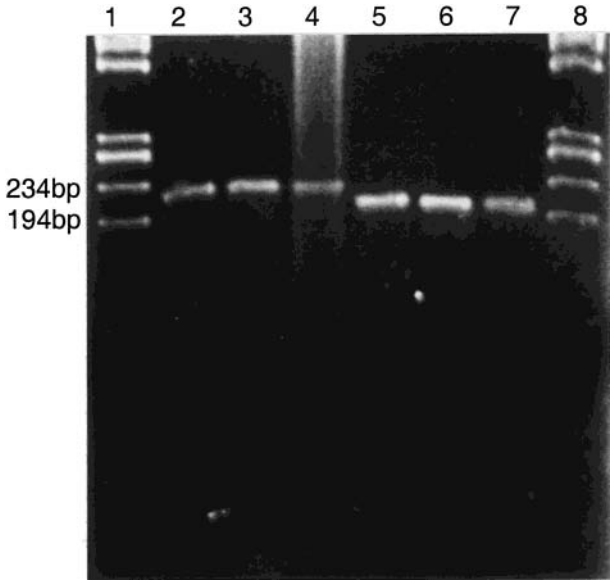

Figure 3. PCR products from bluefin *(T. thynnus*) and bonito (*S. sarda*) DNA subjected to species-specific amplification using primers based on a region of the mitochondrial cytochrome *b* gene. Electrophoresis was performed on a 4% MetaPhor agarose gel (FMC Bioproducts) which was stained with ethidium bromide. Lanes 1 and 8 contain molecular weight markers (*Hae*III digest of ΦX174 DNA). Lanes 2−4 show products of 225 bp resulting from amplification of bonito DNA templates. Lanes 5−7 show the smaller products (207 bp) generated by the amplification of bluefin DNA templates.

dine receptor (*RyR1*) and ovine prion protein (*PrP*) genes, which influence porcine stress syndrome and scrapie, respectively (Lockley et al., 1996, 1997, 2000). However, these templates were genomic rather than mitochondrial DNA in origin. Mitochondrial DNA exhibits some degree of intraspecific variation, and therefore any method that uses a specific sequence for species differentiation could be confounded by this. Consequently, Mackie et al. (1999) recommend that identification be made using two independent DNA analysis techniques. The test described here therefore constitutes a useful addition to those already in existence. Mackie et al. (1999) also stated that the analytical methods should be rapid, easy to perform, and not excessively expensive. Our test fulfils these criteria. It was also predicted that further developments in such testing would focus on species-specific probes for hybridization to target sequences generated by PCR. However, the use of species-specific oligonucleotides as primers in PCR is arguably advantageous over hybridization methods as PCR is easier to perform.

Test Functionality. It is of interest to compare the potential functionality of our test with the available secondary analytical techniques already described in the literature, in the light of potential intraspecific variation. PCR−SSCP patterns are generated on the basis of the product sequence and can be altered by point mutations (Fujita and Silva, 1994). In an analysis of bonito (*S. sarda*), patterns have indeed been reported as showing intraspecific variation in some instances (Rehbein et al., 1999). A polymorphic site at any position in the amplified DNA could have an effect. RFLP analysis will only be affected by polymorphisms in the recognition sequence for the enzyme, generally between four and six bases, depending on the enzyme used. Because this is a relatively small proportion of the amplified DNA, it could be argued that the technique is theoretically less likely to be subject to intraspecific variability than SSCP. In some instances, RFLP patterns have been reported to be altered by intraspecies variation (Quinteiro et al., 1998). A detailed consideration of this problem in red deer (*Cervus elaphus*) found that the use of two appropriate restriction enzymes can enable unambiguous identification (Wolf et al., 1999). Our test will be affected only if the specific base that it has been designed to detect is altered. Because of the degeneracy of the genetic code, the majority of the polymorphisms observed in the intraspecific variation are in the last base of a codon, as is the polymorphism at base 101. If the polymorphisms were one base either side of the last base, it could be argued that the chances of intraspecific variation at this point would have been substantially reduced. However, this situation is no worse than that with regard to the restriction-based tests, because the sites identified here also tend to be a result of degeneracy. Note that the polymorphism at base 101 is not a known restriction site, as shown by analysis of the flanking bases with Webcutter 2.0 software (http://www.ccsi.com/firstmarket/firstmarket/cutter/cut2.html).

To date, intraspecific variation has not been found to present a problem, although this possibility cannot be ruled out. In such an instance, a second site could also be studied. In sequencing a 600 bp fragment of the mitochondrial cytochrome *b* gene in 32 species of Scombroidei and Teleostei, Block et al. (1993) found 218 phylogenetically informative bases. Consequently, it would be expected that appropriate alignment and further confirmatory sequencing would indicate alternative polymorphisms by which bluefin tuna and Atlantic bonito could also be differentiated.

ABBREVIATIONS USED

PCR, Polymerase Chain Reaction; mt, mitochondrial; cyt *b*, cytochrome *b*; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism; dNTP, deoxynucleotide 5′-triphosphate; bp, base pairs; A, adenine: G, guanine; MS, mutagenically separated; RyR, ryanodine receptor; PrP, prion protein.

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