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行政院衛生署九十五年度科技研究計畫

利用基因技術做為台灣重要魚種旗魚及其加工品之種別鑑定研究

95 年 度 成 果 報 告

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媒體發布研究成果應事先徵求本署同意＊

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中文摘要

本研究利用聚合酶連鎖反應-限制片段長度多型性（polymerase chain reaction － restriction fragment length polymorphism; PCR-RFLP）技術，發展出快速鑑定鮪罐魚種之方法，而研究魚種包括黑鮪（*Thunnus thynnus*）、長鰭鮪（*T. alalunga*）、大目鮪（*T. obesus*）、黃鰭鮪（*T. albacares*）、正鰹（*Euthynnus pelamis*; *Katsuwonus pelamis*）、巴鰹（*E. affinis*）、扁花鰹（*Auxis thazard*）及圓花鰹（*Sarda orientalis*）等 8 種常見鮪罐原料魚種。而為了提昇 DNA 粗萃之效率及靈敏度，本研究亦應用了新發展的磁珠吸附技術來抽出樣品之 DNA。研究針對加工品設計之 2 對引子，可分別增幅出 126 bp 及 146 bp 兩段短鏈之粒線體細胞色素 *b* 基因，再搭配 5 組不同的限制酶 *Bsp*1286 I、 *Hinc* II、*Rsa* I、*Sca* I 與 *Mbo* II 作用，於電泳圖上可明確區分各魚種之差異。此發展之 PCR-RFLP 技術，也成功的用來鑑定 18 件市售之鮪罐產品，證實的確為一有效、快速之加工品原料鑑定方法。

關鍵字：聚合酶連鎖反應-限制片段長度多型性、鮪罐、細胞色素 *b* 基因、物種鑑定、磁珠吸附

ABSTRACT

The polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) technique was developed to identify the species of *Thunnus thynnus*, *T. alalunga*, *T. obesus*, *T. albacares*, *Euthynnus pelamis* (*Katsuwonus pelamis*), *E. affinis*, *Auxis thazard* and *Sarda orientalis* in products of canned tuna. To increase the efficiency and sensitivity of DNA extraction, a recent developed method of binding magnetic beads was applied in this study. Two sets of primer were designed to amplify 126 bp and 146 bp of partial mitochondrial cytochrome *b* gene, and 5 restriction enzymes including *Bsp*1286 I, *Hinc* II, *Rsa* I, *Sca* I and *Mbo* II were determined to analyze the short length fragments. The developed PCR-RFLP method was applied to authenticate species of 18 commercial canned tuna successfully, and it really provided a useful and academic technique to identify 8 species in canned tuna.

KEYWORDS: PCR-RFLP, canned tuna, species identification, cytochrome *b* gene, magnetic beads extraction

INTRODUCTION

Canned tuna products are highly important processed food in the world. The fish species of raw materials processed to canned tuna products are mainly *Thunnus*, including light meat bigeye tuna (*T. obesus*) and yellowfin tuna (*T. albacares*), white meat albacore (*T. alalunga*) and high-priced bluefin tuna (*T. thynnus*). Besides *Thunnus*, there are some similar species using to process as canned tuna, such as skipjack (*Euthynnus pelamis* or *Katsuwonus pelamis*), eastern little tuna (*E. affinis*), frigate mackerel (*Auxis thazard*) and oriental bonito (*Sarda orientalis*) (Collette & Nauen, 1983). Different species of canned tuna are quite different in cost. In addition to the price, there are also diverse content of toxicant materials in different species of canned tuna, such as histamine and mercury (Burger & Gochfeld, 2004; Soares & Gloria, 1994). In order to avoid possible fraudulent and vague label of canned tuna, the identification of fish species is becoming a topic of growing concern.

Several methods are used to identify fish species, including traditional morphology, electrophoresis, isoelectric focusing, liquid chromatography, immunoassays, and biological technologies (O'Reilly & Wright, 1995; Osman et al., 1987). In canned tuna, the morphological characteristics of fish are

eliminated during filleting or processing. The protein analyses like electrophoresis, chromatography and immunoassay are also unsuitable for species identification of canned products, because the canning process involves a thermal treatment that irreversibly changes the nature of proteins (Mackie et al, 1999). Comparing with proteins, the DNA extracted from canned products is more stable for species identification. Therefore, the biological technique is more promising and reliable because of its robustness and easy application in routine surveys (Cocolin et al., 2000).

Analysis of mitochondrial DNA (mtDNA) sequences is useful for phylogenetic studies. The mtDNA is a circular nucleotide for inheritance, independent from genomic DNA (Taanman, 1999). The composition of mtDNA is simpler than genomic DNA, which has no complicated intron, pseudogene or repetitive sequence (Gray, 1989). The mtDNA is of maternal inheritance and has no recombination in all vertebrates, so that the sequence of mtDNA is more conservative (Rokas et al., 2003). The rate of base substitution on mtDNA is higher than that on genomic DNA, causing a rapid evolution (Stoneking & soodyall, 1996). Cytochrome *b* gene is a functional gene between $tRNA^{Glu}$ and $tRNA^{Thr}$ in mtDNA (Southern et al., 1988). The combination of cytochrome *b*

gene and other genes in genomic DNA encode the cytochrome c oxidoreductase, which is a complex enzyme in oxidative phosphorylation (Leonard & Schapira, 2000). Many researches that studied about vertebrate cytochrome *b* gene were focused on inheritance and evolution (Antointte & Greg, 2001; Moritz et al., 1987).

The polymerase chain reaction (PCR) technique and direct sequence analysis are useful for species identification. Many techniques derived from PCR were developed to analyze DNA sequences, including random amplified polymorphic DNA (RAPD), PCR single stranded conformational polymorphism (PCR-SSCP), and PCR restriction fragment length polymorphism (PCR-RFLP) (Jones, 1991; Williams et al., 1990). Among them, PCR-RFLP technique was most usual to apply in biology, medicine and food science (Kurihara et al., 1999; Russell et al., 2000; Sato et al., 1998). In our previous papers, the PCR-RFLP technique has been utilized to identify some species of raw fish and processed foods (Hsieh et al., 2005; Hsieh & Hwang, 2004; Hsieh et al., 2002; Hwang et al., 2004; Lin et al., 2005). In this study, we analyzed the stable mitochondrial cytochrome *b* gene and developed a rapid PCR-RFLP method to identify the species of canned

tuna, and then used this method to investigate species of several commercial canned tuna products in Taiwan.

MATERIALS AND METHODS

2.1. Preparation of samples

The raw materials of 4 *Thunnus* species including *T. thynnus, T. alalunga, T. albacares* and *T. obesus* were collected from Singapore, Bangkok (Thailand), Mauritius, Cape Town (South Africa) and Tungkang (Taiwan). The other 4 species suspected to process as canned tuna were including *Euthynnus pelamis* (or *Katsuwonus pelamis*), *E. affinis, Auxis thazard* and *Sarda orientalis*. These 4 specimens were collected from Keelung, Ilan, Tungkang and Kaohsiung in Taiwan. All of 8 species were morphologically identified their external characters by Kwang-Tsao Shao (Research Center for Biodiversity, Academia Sinica), and each species was at least picked 3 individuals to analyze.

To simulate the canned products, about 100 g meats of each species were heated at 121℃ for 15 min in soybean oil or salt water, respectively. Furthermore, eighteen samples of commercial canned tuna were purchased from markets in Taiwan, which were processed with different sauce, including brine, vegetable oil, olive oil, tomato juice and cayenne pepper oil. All samples were stored at −20℃ until use.

2.2. DNA extraction

The traditional DNA extraction method was a modification of the protocol described by Desalle and Birstein (1996). First, about 0.1 g of sample was homogenized with 0.5 ml of digestion buffer (50 mM Tris-HCl, pH 8.0; 1% SDS; 0.2 M NaCl; 0.1 M EDTA) and 50 μl of 5 mg/ml proteinase K (Amresco, Solon, Ohio, U.S.A) was added. The mixture was incubated at 56℃ overnight with shaking. After digestion, each sample was centrifuged at 15,000 x g, 4℃ for 10 min. Afterwards, supernatant was extracted once with phenol, twice with phenol-chloroform-isoamyl alcohol in a 25:24:1 ratio, and once with chloroform. The extract was precipitated twice with ethanol at −20℃ for 20 min and centrifuged at 15,000 x g, 4℃ for 10 min. The dried pellets were resuspended in 100 μl of sterile distilled water.

Because traditional DNA extraction method was inefficient to extract DNA in canned product, a recent developed method of binding magnetic bead was applied in this study. Total DNA was extracted by using magnetic bead technique with the Chemagic DNA Tissue 10 Kit (Chemagen, Baesweiler, Germany) according to the manufacturer's recommendations. About 100 μg of sample was incubated with protease K and lysis buffer at 56℃ until lysis was completed, then magnetic beads were added. After incubation, magnetic beads

binding DNA was separated by a magnet separator. The mixture was washed two times by different wash buffer. Ultimately, the magnetic beads were removed from the solution and the DNA was eluted in 50 μl of elution buffer.

2.3. Design of PCR primers

The primer set of L14841 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and H15149 (5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3') could be used to amplify a 375 bp fragment of partial cytochrome *b* gene in teleostes (Kocher et al., 1989). In this study, primers L14841 and H15149 were used to amplify the specimens of raw material, and the amplified 375 bp fragments were sequencing. To investigate specimens of canned tuna, 3 sets of primers used to amplify shorter fragments were designed, including (1) CbP2L (5'-AAG GAC GTA GCC AAC GAA G-3') and CbP2H (5'-TCA GTA GCC CAC ATT TGC C-3'); (2) Cb126L (5'-GCY TYT ACT ACG GYT CYT AC-3') (Y=C/T) and Cb126H (5'-CCC CTC AGA ATG ATA TTT GTC C-3'); and (3) Cb146L (5'-CCT CGC AAT ACA CTA TAC CCC-3') and Cb146H (5'-CGA TGT GGA AGT AGA TGC AG-3'). The primer sets of CbP2L/H, Cb126L/H and Cb146L/H could be used to amplify respectively 207 bp, 126 bp and 146 bp of cytochrome *b* gene in this study.

2.4. PCR amplification

Each PCR reaction was performed in a total volume of 100 μl, containing 10 μl of template DNA, 2 μM of each primer, 200 μM of dNTP, and 2.5 U of Pro Taq DNA polymerase (Amresco, Solon, Ohio, U.S.A) in a PCR buffer that included 20 mM of Tris-HCl (pH 8.0), 15 mM of $MgCl_2$, 1% Triton X-100, 500 mM of KCl and 0.1% (w/v) gelatin. The PCR amplifications were carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, Calif., U.S.A) programmed to perform a denaturation step at 95℃ for 10 min, followed by 30 cycles consisting of 1 min at 95℃, 1 min at 50℃ and 1 min at 72℃. The final extension step was 10 min or longer.

2.5. DNA electrophoresis and sequence analysis

Three microliter of PCR product and 1 μl of loading dye were mixed and loaded onto a 2% agarose gel containing 1 μg/ml ethidium bromide, then the electrophoresis was running in TBE buffer at 100 V for 40 min. The DNA bands were observed under ultraviolet light and photographed by Image Master VDS (Pharmacia Biotech, Piscataway, New Jersey, U. S. A.).

Purified PCR products were sequencing at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI Prism BigDye Terminator Cycle

Sequencing Ready Reaction Kit (Perkin-Elmer / Applied Biosystems Div., Foster City, Calif., U. S. A.) in an ABI PRISM 377-96 DNA Sequencer (Perkin Elmer / Applied Biosystems Div.). Two complementary DNA sequences obtained from each sample were compared with the database in Genetics Computer Group Wisconsin Package, Version 10.3 (GCG system; Genetics Computer Group, 2002).

2.6. RFLP analysis

After analyzing consensus sequences of partial mitochondrial cytochrome *b* gene of the 8 species, restriction maps were constructed in GCG system. Five restriction enzymes including *Bsp*1286 I, *Hinc* II, *Rsa* I, *Sca* I and *Mbo* II (Promega, Madison, Wisc., U. S. A.) were determined for this study. Each digestion was performed in 10 μl of mixture that contains 100 ng PCR product, 5 U restriction endonuclease, 1:10 dilution of bovine serum albumin and 10X digestion buffer. Digestive reactions were incubated at optimal assay temperature (37 ℃) for 2 hr. The results of RFLP analysis in DNA electrophoresis were read and photographed as described above, except the concentration of agarose gel was rose to 4%.

RESULTS

3.1. PCR amplification and sequence analysis

The 376 bp PCR products of partial mitochondrial cytochrome *b* gene were individually amplified by using primers L14841 and H15149. To exclude the intraspecific diversity, 3 fresh individuals collected from different location were investigated in each species. The simulated specimens and commercial products of canned tuna were also attempted to amplify by primers L14841 and H15149. However, the amplification of 376 bp fragments in canned tuna was all failed, which meant the DNA sequences were degraded after thermal process of canning. Therefore, it was necessary to design new primer sets that could amplify shorter DNA fragments for study.

First, all PCR products of 376 bp fragments amplified from raw specimens were sequencing, and then the interspecific and intraspecific base diversity in 8 species were searched out. Although in most species, the alignment of 376 bp sequences in different individuals were totally the same, there were still 1 intraspecific base diversity in *T. alalunga* and 2 intraspecific base diversities in *T. obesus*. After analyzing consensus 376 bp fragments of total specimens and excluding the intraspecific diversity, primer set of CbP2L and CbP2H was

designed to amplify a 207 bp fragment. However, the outcome of practical PCR amplification was still difficult to operate in canned tuna, and only a few canned specimens could be amplified the 207 bp fragments successfully (data not shown). Therefore, additional 2 sets of primer were constructed to analyze the seriously degraded DNA in canned tuna. Primer set of Cb126L and Cb126H was utilized to amplify a 126 bp fragment, and the 126 bp consensus sequence involved the most diverse bases between 8 species. Furthermore, primer set of Cb146L and Cb146H was designed, that could be used to amplify a 146 bp fragment only in 4 *Thunnus* species. It was successful to amplify the 126 bp and 146 bp fragments in simulated canned tuna of 8 species by using two primer sets of Cb126L/H and Cb146L/H. Figure 1 and Figure 2 show the 126 bp consensus sequences in 8 species, and the 146 bp consensus sequences in 4 *Thunnus* species, respectively.

3.2. Analysis of restriction enzymes

After analyzing 126 bp and 146 bp consensus sequences of partial mitochondrial cytochrome *b* gene, few intraspecific divergences were excluded and restriction maps were constructed by employing GCG system. Five restriction enzymes including *Bsp*1286 I, *Hinc* II, *Rsa* I, *Sca* I and *Mbo* II were

determined to analyze sequences in this study. The 146 bp fragment amplified by Cb146L and Cb146H could only be observed in *Thunnus* species, so that it could preliminarily differentiate 4 tuna species (*Thunnus*) from other 4 species by amplifying the 146 bp fragments.

Due to 4 *Thunnus* species could be distinguished from other species by briefly using primer set of Cb146L and Cb146H, the fragment polymorphism in 4 *Thunnus* species would be discussed above all. First, restriction enzyme *Bsp*1286 I could cleave the 126 bp fragment in *T. thynnus* amplified by Cb126L/H into 75 bp and 51 bp, but there was no cutting site in other 3 *Thunnus* species (Fig. 3-A). Another restriction enzyme reacted in the 126 bp fragment in *Thunnus* was *Rsa* I. By using *Rsa* I, both of *T. obesus* and *T. albacares* could be cut into 74 bp and 52 bp; *T. thynnus* could be cut into 102 bp and 24 bp; and *T. alalunga* could be cut into 74 bp, 28 bp and 24 bp. After practical analysis, the 28 bp and 24 bp fragments in *T. alalunga* would compose a band in electrophoretic gel, and this band could be used to separate *T. alalunga* from other 3 *Thunnus* species (Fig. 3-B). Because there was no suitable restriction enzyme to differentiate *T. obesus* and *T. albacares* in the 126 bp consensus sequence, the 146 bp consensus fragments amplified by Cb146L/H were

analyzed. Restriction enzyme *Hinc* II was chosen to examine the 146 bp consensus fragments. *Hinc* II could cause 2 cutting sites in the 146 bp consensus fragments of *T. thynnus*, *T. alalunga* and *T. albacares*, but there were no cutting site in that of *T. obesus* (Fig. 3-C).

The primer set of Cb146L and Cb146H was designed to detect *Thunnus* species specially, so other species couldn't be amplified the 146 bp fragment. Other potential materials of canned tuna including *E. pelamis, E. affinis, A. thazard* and *S. orientalis* were analyzed by using primer set of Cb126L and Cb126H only. *Sca* I could cleave the 126 bp fragment of *E. affinis* into 74 bp and 52 bp, and there was no cutting site in *E. pelamis, A. thazard* and *S. orientalis* (Fig. 4-A). Equally, the 126 bp fragment in *A. thazard* had a specific cutting site by using restriction enzyme *Mbo* II, and there was no cutting site in *E. pelamis, E. affinis* and *S. orientalis* (Fig. 4-B). Finally, the *Rsa* I could cleave 126 bp fragment in *E. pelamis* into 94 bp and 32 bp, and that in *S. orientalis* into 74 bp and 52 bp. This result could be obviously observed after electrophoretic anlysis, and could readily differentiate *E. pelamis* and *S. orientalis* (Fig. 4-C). The sizes of fragment length cleaved by each restriction enzyme were compiled

in Table 1, and the cutting sites of restriction enzymes in sequences of each species were labeled in Figure 1 and Figure 2.

3.3. Species identification of commercial canned tunas

The PCR-RFLP model was established as above, and 18 specimens of commercial canned tuna purchased in Taiwan were investigated by applying this PCR-RFLP model. All 18 specimens were extracted DNA by using magnetic bead technique. After PCR operated by primers Cb146L and Cb146H, fourteen of 18 specimens were successfully amplified the 146 bp fragments. All of the 14 specimens were supposed to be *Thunnus* species, and other 4 specimens were supposed to be other species. Using *Hinc* II to analyze 146 bp fragments of 14 *Thunnus* species, three samples had no cutting site and other 12 samples were cleaved into 70 bp, 66 bp and 10 bp. The 3 samples with no cutting sites in 146 bp fragments were determined to be *T. obesus*. Other 11 samples were analyzed by restriction enzymes *Bsp*1286 I and *Rsa* I. In these 11 specimens, six specimens were decided to be *T. albacares* and 5 specimens were decided to be *T. alalunga*. In addition, 4 samples that couldn't be amplified by primer set of Cb146L/H were also investigated their 126 bp fragments amplified by primer set of Cb126L/H. After analysis by restriction enzymes *Rsa* I, *Sca* I and *Mbo* II in

their 126 bp sequences, 3 of them were determined to be *E. pelamis* and 1 was *E. affinis*. PCR-RFLP analysis of 18 commercial canned tunas in this study was shown in Table 2.

DISCUSSION

4.1. Extraction and fragment length of DNA in canned tuna

In this study, DNA in raw materials was easy to extract by using traditional phenol-chloroform extraction method described by Desalle and Birstein (1996) and to amplify by PCR reaction. However, the DNA of canned tuna was difficult to extract and observed by PCR amplification, which revealed the DNA degraded severely in canned tunas. The amount and fragment size of DNA sequence in canned products were both less than that in raw tissue (Infante et al., 2004). Therefore, a recent developed method was applied in this study. This method was used by coverage of magnetic beads with nucleic acid-binding matrices, and provided a high potential of sensitivity and automation (Kleines et al., 2003). The practical operation in this study shown that, DNA extracted by magnetic bead technology was more efficient and more sensitive than extracted by traditional phenol-chloroform method.

The nucleic acid molecule is unstable in condition of high temperature over 100℃. In our previous study, many different processed products of seafood have been analyzed the size of DNA fragments. The processed products including cooked fish liver (Hsieh et al., 2002), dressed toasted eel (Hwang et al.,

2004) and dried fried billfish meat (Hsieh et al., 2004) were investigated by PCR amplification, and the fragment sizes of mitochondrial DNA were 376 bp, 362 bp and 436 bp, respectively. Unlike cooked or dried products that were just heated in about 100℃, canned tuna products were heated in a high temperature (over 115℃) for a long time (15－30 min), and it was a challenge to identify species in canned fish. In this study, the 126 bp and 146 bp fragments were easier to amplify than 207 bp fragments in canned tuna. The same results were also discovered in previous study (Infante et al., 2004; Quinteiro et al., 1998; Ram et al., 1996; Rehbein et al., 1999). The fragment sizes of partial mitochondrial cytochrome *b* gene amplified from canned tuna in these researches were, respectively, 120 bp, 123 bp, 126 bp and 146 bp.

4.2. Species identification of canned tuna by PCR-RFLP analysis

The raw tuna species of *Thunnus* have been identified by many different DNA techniques, including PCR-RFLP (Lin et al., 2005), AFLP (amplified fragment length polymorphism) (Han & Ely, 2002) and RFLP (Takeyama et al., 2001). However, among them, PCR-RFLP was the most suitable for differentiation of fish species (Wolf et al., 2000). In 1996, PCR-RFLP was first time to apply in authenticating canned tuna. The degenerated PCR primers were designed to

amplify two short fragments of mitochondrial cytochrome *b* gene that were fewer than 123 bp. Two *Thunnus* and 2 bonito species were examined in this study, but the PCR-RFLP method developed in this study could only distinguish a small number of samples (Ram et al., 1996). Another PCR-RFLP species identification in canned tuna was also developed in 1998. Four *Thunnus* and 2 bonito species from non-commercially available canned samples were identified by restriction patterns of a 126 bp sequence. However, the fragments size digested after restriction enzymes were too small, causing the RFLP patterns need to be observed by complicated SDS electrophoresis and silver stain (Quinteiro et al., 1998). Furthermore, a 276 bp fragment was utilized to authenticate commercial canned tuna in 2004, and it was succeeded to differentiate 5 species by PCR-RFLP (Pardo & Perez-Villareal, 2004). In our study, though DNA fragment size more than 200 bp could be amplified in canned tuna, it was hard to operate smoothly in experiment. In order to advance the efficiency in practical utilization, it is necessary to develop a model of shorter length of DNA fragments on species identification of canned tuna.

It was accurate by using direct DNA sequencing to analyze cytochrome *b* gene of 4 *Thunnus* and 4 bonito species in canned tuna. However, the technique was time-consuming and expensive. The result of PCR-RFLP test in this study

indicated that the 2 primer sets were suitable for PCR amplification, and 5 restriction enzymes were suitable for PCR-RFLP pattern analysis. This developed PCR-RFLP method was applied to authenticate species of commercial canned tuna quickly and easily. Therefore, it really provided a useful and academic technique to identify 8 species in canned tuna.

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TABLES

Table 1

Fragment Length of Digested 126 bp and 146 bp Partial Mitochondrial Cytochrome *b* Gene in 8 Species

Species	Enzyme cuts in 126 bp fragments (bp)				***Hinc* II** cut in 146 bp fragments (bp)
	***Bsp*1286 I**	***Rsa* I**	***Sca* I**	***Mbo* II**	
T. thynnus	75 + 51	102 + 24	126	126	70 + 66 + 10
T. alalunga	126	74 + 28 + 24	126	126	70 + 66 + 10
T. obesus	126	74 + 52	126	126	146
T. albacares	126	74 + 52	126	126	70 + 66 + 10
E. pelamis	126	94 + 32	126	126	–
E. affinis	126	52 + 42 + 32	74 + 52	126	–
A. thazard	126	94 + 32	126	81 + 45	–
S. orientalis	126	74 + 52	126	126	–

Table 2

Species Identification of Commercial Canned Tunas Determined by Using RFLP Analysis of 126 bp and 146 bp PCR Products

Source of PCR products	Enzyme cuts in 126 bp fragments (bp)				***Hinc* II** cut in 146 bp fragments (bp)	Species identification
	***Bsp*1286 I**	***Rsa* I**	***Sca* I**	***Mbo* II**		
Sample 1	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 2	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 3	126	52 + 42 + 32	74 + 52	126	–	*E. affinis*
Sample 4	126	94 + 32	126	126	–	*E. pelamis*
Sample 5	126	74 + 28 + 24	126	126	70 + 66 + 10	*T. alalunga*
Sample 6	126	74 + 52	126	126	146	*T. obesus*
Sample 7	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 8	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 9	126	74 + 28 + 24	126	126	70 + 66 + 10	*T. alalunga*
Sample 10	126	74 + 52	126	126	146	*T. obesus*
Sample 11	126	94 + 32	126	126	–	*E. pelamis*
Sample 12	126	74 + 28 + 24	126	126	70 + 66 + 10	*T. alalunga*
Sample 13	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 14	126	74 + 28 + 24	126	126	70 + 66 + 10	*T. alalunga*
Sample 15	126	74 + 52	126	126	70 + 66 + 10	*T. albacares*
Sample 16	126	74 + 28 + 24	126	126	70 + 66 + 10	*T. alalunga*
Sample 17	126	74 + 52	126	126	146	*T. obesus*
Sample 18	126	94 + 32	126	126	–	*E. pelamis*

```
              1                                        Rsa I            50
   T. thynnus CCCCTCAGAA TGATATTTGT CCTCAGGGAA GGACGTAGCC AACGAAGGCG
  T. alalunga CCCCTCAGAA TGATATTTGT CCTCAGGGAA GGACGTAGCC AACGAAGGCG
    T. obesus CCCCTCAGAA TGATATTTGT CCTCAGGGAA GGACGTAGCC GACGAAGGCG
 T. albacares CCCCTCAGAA TGATATTTGT CCTCAGGGAA GGACGTAGCC AACGAAGGCG
   E. pelamis CCCCTCAGAA TGATATTTGT CCTCAGGGAA GTACGTAACC GACGAAAGCA
   E. affinis CCCCTCAGAA TGATATTTGT CCTCAGGGGA GTACGTAGCC TACGAAGGCA
   A. thazard CCCCTCAGAA TGATATTTGT CCTCATGGAA GTACGTAGCC GACGAATGCA
S. orientalis CCCCTCAGAA TGATATTTGT CCTCAGGGGA GGACGTAACC GACGAAGGCA

              51                    Bsp1286 I Rsa I                     100
   T. thynnus GTCATCATAA CTAGAAGTAG GAGCACTACT CCGATGTTTC ATGTTTCTTT
  T. alalunga GTCATCATAA CTAGAAGTAG GAGTACTACT CCGATGTTTC ATGTTTCTTT
    T. obesus GTCATCATAA CTAGGAGTAG TAGTACTACT CCGATGTTTC ATGTTTCTTT
 T. albacares GTCATCATAA CTAGGAGTAG GAGTACTACT CCGATGTTTC ATGTTTCCTT
   E. pelamis GTCATCATTA CTAGGAGAAG TAGGACTACA CCGATGTTTC ATGTTTCTTT
   E. affinis GTTATCATCA CTAGAAGTAG TAGTACTACA CCGATGTTTC ATGTTTCTTT
   A. thazard GTCATCATGA CTAGCAGTAG AAGAACTACG CCGATGTTTC ATGTTTCTTT
S. orientalis GTTATTATTA CTAAAAGTAG AAGTACAACG CCGATGTTTC ATGTTTCTTT
                                 Mbo II Sca I

            101 Rsa I                126
   T. thynnus GTACAGGTAA GAGCCGTAGT AAAGGC
  T. alalunga GTACAGGTAA GAGCCGTAGT AAAGGC
    T. obesus GTATAGGTAA GAGCCGTAGT AAAGTC
 T. albacares GTATAGGTAA GAGCCGTAGT AAAGTC
   E. pelamis GTAGAGGTAG GAACCGTAGT AGAGAC
   E. affinis GTAGAGGTAA GAGCCGTAGT AAAGAC
   A. thazard GTAGAGGTAG GAGCCGTAGT AAAGGC
S. orientalis GTAGAGGTAG GAGCCGTAGT AAAGGC
```

Fig. 1. The 126 bp partial cytochrome *b* gene of consensus fragments in 8 species amplified with primers Cb126L and Cb126H. Restriction sites of *Bsp*1286 I, *Rsa* I, *Sca* I and *Mbo* II are labeled as box, underline, gray background and double underline, respectively.

```
             1                                                 50
   T. thynnus CGATGTGGAA GTAGATGCAG ATAAAGAAGA AAGAGGCCCC GTTTGCGTGG
  T. alalunga CGATGTGGAA GTAGATGCAG ATAAAGAAGA AAGAGGCCCC GTTTGCGTGG
    T. obesus CGATGTGGAA GTAGATACAG ATAAAGAAGA AAGAGGCCCC GTTTGCGTGG
T. albacares CGATGTGGAA GTAGATGCAG ATAAAGAAGA AAGAGGCCCC GTTTGCGTGG

            51             Hinc II     Hinc II                100
   T. thynnus AGGTTCCGGA TGAGTCAACC GAAGTTGACA TCTCGGCAAA TGTGGGCTAC
  T. alalunga AGGTTCCGGA TGAGTCAACC GAAGTTGACA TCTCGGCAAA TGTGGGCTAC
    T. obesus AGGTTCCGGA TGAGTCACCC GAAATTGACA TCTCGGCAAA TGTGGGCTAC
T. albacares AGGTTCCGGA TGAGTCAACC GAAGTTGACA TCTCGGCAAA TGTGGGCTAC

           101                                           146
   T. thynnus TGAGGCGAAG GCTGATTCGA CATCAGGGGT ATAGTGTATT GCGAGG
  T. alalunga TGAGGCGAAG GCTGATTCGA CATCAGGGGT ATAGTGTATT GCGAGG
    T. obesus TGAGGCGAAG GCTGATTCGA CATCAGGGGT GTAGTGTATT GCGAGG
T. albacares TGAGGCGAAG GCTGATTCGA CATCAGGGGT GTAGTGTATT GCGAGG
```

Fig. 2. The 146 bp partial cytochrome *b* gene of consensus fragments in 4 *Thunnus* species amplified with primers Cb146L and Cb146H. Restriction sites of *Hinc* II are labeled as underline.

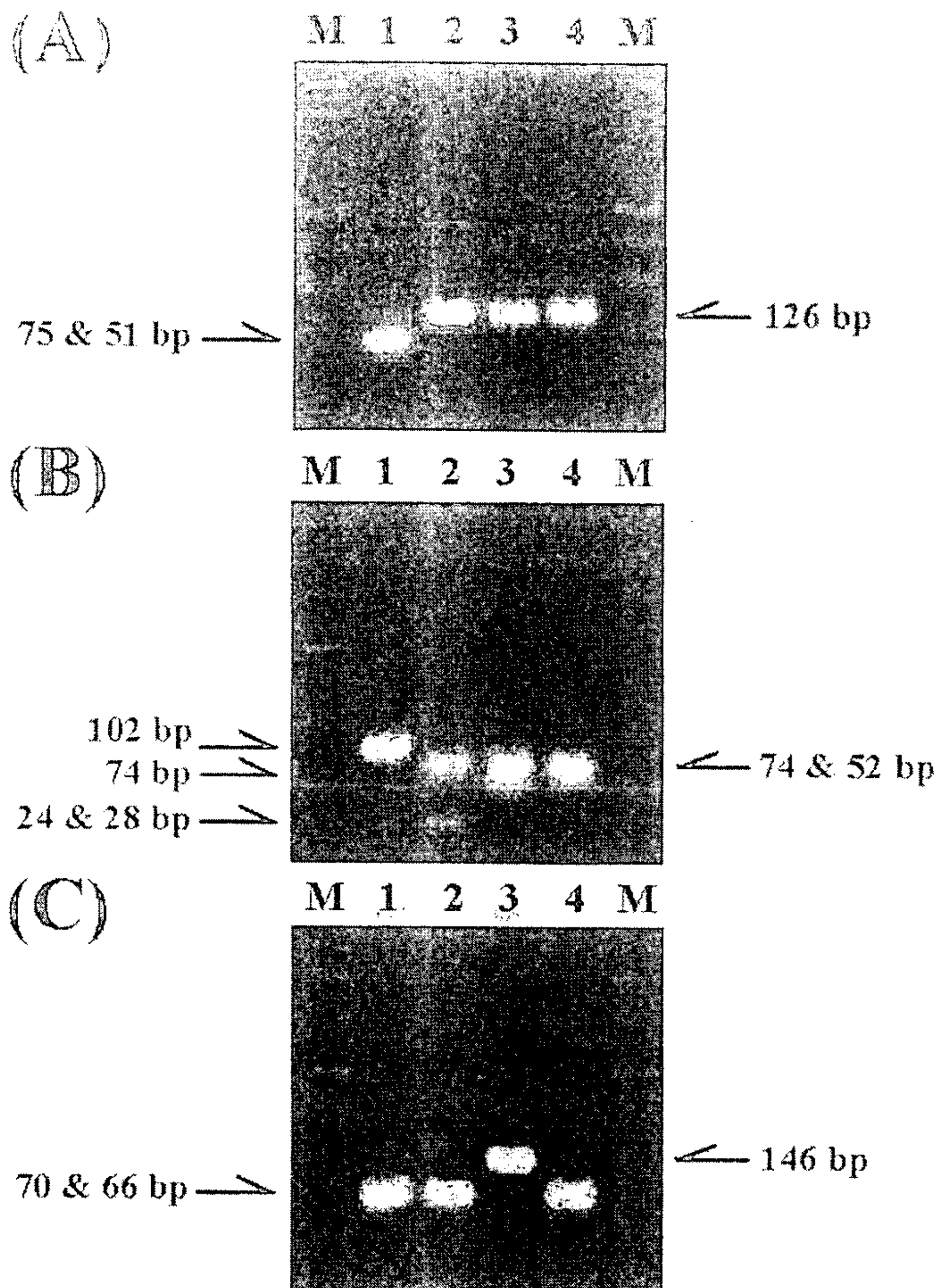

Fig. 3. Electrophoretic analysis of: (A) 126 bp fragments digested with *Bsp*1286 I. (B) 126 bp fragments digested with *Rsa* I. (C) 146 bp fragments digested with *Hinc* II. Samples in lane are as follows: 1. *T. thynnus*; 2. *T. alalunga*; 3. *T. obesus*; 4. *T. albacare*; M = 100 bp ladder.

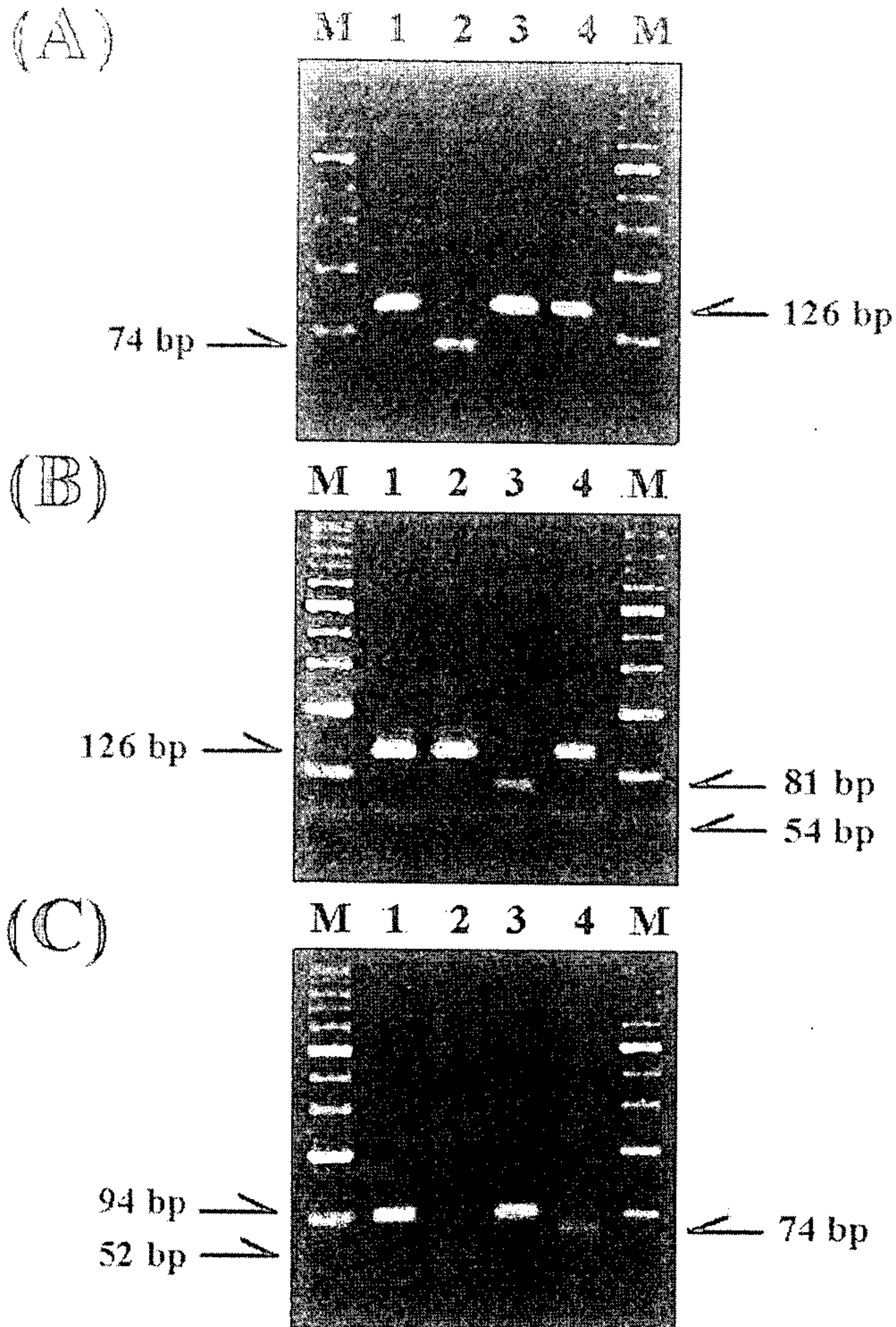

Fig. 4. Electrophoretic analysis of 126 bp fragments digested with (A) *Sca* I, (B) *Mbo* II and (C) *Rsa* I. Samples in lane are as follows: 1. *E. pelamis*; 2. *E. affinis*; 3. *A. thazard*; 4. *S. orientalis*; M = 100 bp ladder.