Journal of Food and Drug Analysis, Vol. 13, No. 4, 2005, Pages 382-387 藥物食品分析 第十三卷 第四期

Identification of Four *Thunnus* Tuna Species Using Mitochondrial Cytochrome *b* Gene Sequence and PCR-RFLP Analysis

WEN-FENG LIN, CHYUAN-YUAN SHIAU AND DENG-FWU HWANG*

Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Rd., Keelung City 202, Taiwan, R.O.C.

(Received: June 21, 2005; Accepted: August 29, 2005)

ABSTRACT

Sequence analysis and polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) technique were used to identify the species of *Thunnus thynnus*, *T. alalunga*, *T. obesus* and *T. albacares*. Genetic variation of 376 bp fragments in mitochondrial cytochrome *b* gene of 4 *Thunnus* species captured from northern Pacific Ocean, northern Atlantic Ocean and Indian Ocean was analyzed. The restriction enzymes and restriction fragment length pattern in each *Thunnus* species were obtained. It was found that the 376 bp fragment of cytochrome *b* gene in *T. obesus* showed the obvious divergence of 4.235% different from other 3 species. The highest interspecific similarity ranged 98.687～99.674% between *T. thynnus* and *T. alalunga*. PCR-RFLP by using 3 specific restriction enzymes *Bsp*1286, *Hinc* II and *Rsa* I could precisely and quickly identify the species of the 4 common *Thunnus* species. Furthermore, this technique successfully detected the species of 12 samples of commercial tuna fillets (sashimi).

Key words: polymerase chain reaction – restriction fragment length polymorphism, PCR-RFLP, tuna, *Thunnus*, species identification, mtDNA, cytochrome *b* gene

INTRODUCTION

Tuna is a high-priced pelagic fish in the world, and the prices differ from each other depending on species. The *Thunnus* fish in the world so far involve 7 species and 1 subspecies of *Thunnus* are found around the world[1]. In the Far East, there are 4 *Thunnus* species, including bluefin tuna (*Thunnus thynnus*), albacore (*T. alalunga*), yellowfin tuna (*T. albacares*) and bigeye tuna (*T. obesus*). However, most customers cannot identify the species of sliced tuna meats in the market. Therefore, it was necessary to develop a scientific technology to identify *Thunnus* species.

Many methods for fish species identification have been reported in the literature, including isoelectric focusing, high performance liquid chromatography, sodium dodecyl sulphate – polyacrylamide gel electrophoresis, enzyme-linked immunosorbent assay, 2-dimentional electrophoresis, and molecular biology techniques[2-5]. Among them, the most promising and reliable method is DNA technique because of its robustness and easy application in routine surveys[6].

Analysis of mitochondrial DNA (mtDNA) sequences is useful for phylogenetic studies. The mtDNA is a circular nucleotide of inheritance, independent from genomic DNA[7,8]. The composition of mtDNA is simpler than genomic DNA, which has no complicated intron, pseudogene or repetitive sequence[9]. The mtDNA is of maternal inheritance and has no recombination in all vertebrates, so that the sequence of mtDNA is more conservative[10,11]. The mtDNA lies in the inner membrane of mitochondria and is easily affected by oxidative phosphorylation. The rate of base substitution on mtDNA is higher than that on genomic DNA, causing faster evolutional speed[12,13]. Cytochrome *b* gene is a functional gene between $tRNA^{Glu}$ and $tRNA^{Thr}$ in mitochondrial genome[14]. 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[15]. Many studies of vertebrate cytochrome *b* gene have focused on inheritance and evolution[16-18].

Many techniques related to polymerase chain reaction (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)[19,20]. Among them, PCR-RFLP technique was often applied in biology, medicine and food science[21-26]. In this study, we analyzed the stable mitochondrial cytochrome *b* gene and developed a rapid PCR-RFLP model to identify the species of 4 *Thunnus* tuna.

MATERIALS AND METHODS

I. *Sampling*

Domestic specimens of *Thunnus thynnus*, *T. alalunga*, *T. albacares* and *T. obesus* were collected from Kaohsiung and Tungkang, whereas foreign specimens were collected from Singapore, Bangkok (Thailand), Mauritius and Cape

* Author for correspondence. Tel: +886-2-2462-2192; Fax: +886-2-2462-6602; E-mail: dfhwang@mail.ntou.edu.tw

Town (South Africa). Total of 42 specimens were gathered and frozen in -20°C. Samples of *T. thynnus* were only gained from northern Pacific Ocean and northern Atlantic Ocean, while samples of other 3 species were obtained from northern Pacific Ocean, northern Atlantic Ocean and Indian Ocean (Table 1). Furthermore, 12 samples of raw fillet (sashimi) were purchased from 6 markets in Taiwan to examine the species by using the PCR-RFLP technique.

II. *DNA Extraction*

The DNA extraction method was a modification of the protocol described by Desalle and Birstein[27]. 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, USA) was added. The mixture was incubated at 55°C overnight with shaking. After digestion, each sample was centrifuged at 15,000 ×g, 4°C 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°C for 20 min and centrifuged at 15,000 ×g, 4°C for 10 min. The dried pellets were resuspended in 100 μL of sterile distilled water, and the concentration of DNA was estimated by absorbance at 260 nm.

III. *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 each dNTP, and 2.5 U of Pro Taq DNA polymerase (Amresco) 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, CA, USA) programmed to perform a denaturation step at 95°C for 10 min, followed by 30 cycles consisting of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C. The final extension step was 10 min or longer. The set of primers used for PCR amplification were coded 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'), respectively. Primer pair was designed to amplify part of cytochrome *b* gene in teleostes[28].

IV. *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, and 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, NJ, USA).

Purified PCR products were sequenced 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, CA, USA) 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).

V. *RFLP Analysis*

After analyzing the consensus sequences of mitochondrial cytochrome *b* gene of the 4 species, restriction maps were constructed in GCG system, and 3 restriction endonuclease including *Bsp*1286 I, *Hinc* II and *Rsa* I were determined. Each digestion was performed in 10 μL of mixture that contains 100 ng PCR product, 5 U restriction

Table 1. Source and fragment length of digested 376 bp cytochrome *b* gene of 4 *Thunnus* species

Species	Number of specimen	Location of collection	Location of capture	PCR-RFLP[a]		
				*Bsp*1286 I	*Hinc* II	*Rsa* I
T. thynnus	3	Kaohsiung, Taiwan	Northern Pacific Ocean			
	3	Tungkang, Taiwan	Northern Pacific Ocean	292 + 84 bp	207 + 150 + 19 bp	265 + 111 bp
	3	Tungkang, Taiwan	Northern Atlantic Ocean			
T. alalunga	3	Tungkang, Taiwan	Northern Pacific Ocean			
	3	Cape Town, South Africa	Northern Atlantic Ocean	376 bp	207 + 150 + 19 bp	265 + 74 + 37 bp
	3	Singapore	Northern Indian Ocean			
	3	Mauritius	Western Indian Ocean			
T. albacares	3	Tungkang, Taiwan	Northern Pacific Ocean			
	3	Bangkok, Thailand	Northern Pacific Ocean	376 bp	376 bp	293 + 83 bp
	3	Tungkang, Taiwan	Northern Atlantic Ocean			
	3	Mauritius	Western Indian Ocean			
T. obesus	3	Tungkang, Taiwan	Northern Pacific Ocean			
	3	Tungkang, Taiwan	Northern Atlantic Ocean	376 bp	207 + 150 + 19 bp	293 + 83 bp
	3	Singapore	Northern Indian Ocean			

[a]Polymerase chain reaction – restriction fragment length polymorphism.

endonuclease (Promega, Madison, WI, USA), 1:10 dilution of bovine serum albumin and 10× digestion buffer. Digestive reactions were incubated at 37°C for 2 hr. The results were read by DNA electrophoresis as described above.

```
1-50
T. thy.  AAACTGCAGC CCCTCAGAAT GATATTTGTC CTCAGGGAAG GACGTAGCCA
T. ala.  AAACTGCAGC CCCTCAGAAT GATATTTGTC CTCAGGGAAG GACGTAGCCA
T. obe.  AAACTGCAGC CCCTCAGAAT GATATTTGTC CTCAGGGAAG GACGTAGCCG
T. alb.  AAACTGCAGC CCCTCAGAAT GATATTTGTC CTCAGGGAAG NACGTAGCCA
51-100                                   Bsp1286 I
T. thy.  ACGAAGGCGG TCATCATAAC TAGAAGTAGG AGCACTACTC CGATGTTTCA
T. ala.  ACGAAGGCGG TCATCATAAC TAGAAGTAGG AGTACTACTC CNATGTTTCA
T. obe.  ACGAAGGCGG TCATCATAAC TAGGAGTAGN AGTACTACTC CGATNTTTCA
T. alb.  ACGAAGGCGG TCATCATAAC TAGGAGTAGG AGTACTACTC CGATGTTTCA
101-150          Rsa I                        Rsa I
T. thy.  TGTTTCTTTG TACAGGTAAG AGCCGTAGTA AAGGCCTCGG CCGATGTGGA
T. ala.  TGTTTCTTTG TACAGGTAAG AGCCGTAGTA AAGNCCTCGG CCGATGTGGA
T. obe.  TGTTTCTTTG TATAGGTAAG AGCCGTAGTA AAGTCCTCGG CCGATGTGGA
T. alb.  TGTTTCCTTG TATAGGTAAG AGCCGTAGTA AAGNCCTCGG CCGATGTGGA
151-200
T. thy.  AGTAGATGCA GATAAAGAAG AAAGAGGCCC CGTTTGCGTG GAGGTTCCGG
T. ala.  AGTAGATGCA GATAAAGAAG AAAGAGGCCC CGTTTGCGTG GAGGTTCCGG
T. obe.  AGTAGATACA GATAAAGAAG AAAGAGGCCC CGTTTGCGTG GAGGTTNCGG
T. alb.  AGTAGATGCA GATAAAGAAG AANGAGGCCC CGTTTGCGTG GAGGTTCCGG
201-250    Hinc II    Hinc II
T. thy.  ATGAGTCAAC CGAAGTTGAC ATCTCGGCAA ATGTGGGCTA CTGAGGCGAA
T. ala.  ATGAGTCAAC CGAAGTTGAC ATCTCGGCAA ATGTGGGCTA CTGAGGCGAA
T. obe.  ATGAGTCACC CGAAATTNAC ATCTCGGCAA ATGTGGGCTA CTGAGGCGAA
T. alb.  ATGAGTCAAC CGAAGTTGAC ATCTCGGCAA ATGTGGGCTA CTGAGGCGAA
251-300
T. thy.  GGCTGATTCG ACATCAGGGG TATAGTGTAT TGCGAGGAAT AGTCCTGTAA
T. ala.  GGCTGATTCG ACATCAGGGG TNTAGTGTAT TGCGAGGAAT AGTCCTGTAA
T. obe.  GGCTGATTCG ACATCAGGGG TGTAGTGTAT TGCGAGGAAT AGTCCTGTAA
T. alb.  GGCTGATTCG ACATCAGGGG TGTAGTGTAT TGCGAGGAAT AGTCCTGTAA
301-350
T. thy.  GGATTTGAGA AATAAGGCAA AGGCCAAGTA GTGAGCCAAA GTTTCATCAT
T. ala.  GGATTTGAGA AATAAGGCAA AGGCCAAGTA GTGAGCCAAA GTTTCATCAT
T. obe.  GGATTTGAGA AATAAGGCAA AGGCCAAGTA GTGAGCCAAA GTTTCATCAT
T. alb.  GGATTTGAGA AATAAGGCAA AGGCCAAGTA GTGAGCCAAA GTTTCATCAT
351-376
T. thy.  GCTGAGATGT TGGATGGAAG CTTTTT
T. ala.  GCTGAGATGT TGGATGGAAG CTTTTT
T. obe.  GCTGAGATGT TGGATGGAAG CTTTTT
T. alb.  GCTGAGATGT TGGATGGAAG CTTTTT
```

Figure 1. The 376 bp consensus fragment of the cytochrome *b* gene of *Thunnus thynnus* (*T. thy.*), *T. alalunga* (*T. ala.*), *T. albacares* (*T. alb.*) and *T. obesus* (*T. obe.*) amplified with primers L14841 and H15149. Restriction sites of *Bsp*1286 I, *Rsa* I and *Hinc* II are underlined and positions of mixed intraspecic bases are labeled with N in sequences (N = G/C/A/T).

Journal of Food and Drug Analysis, Vol. 13, No. 4, 2005

RESULTS AND DISSCUSION

I. *Comparison of 376 bp Mitochondrial Cytochrome b Gene Fragments*

The PCR products were individually amplified by primers L14841 and H15149. Including 2 primers, the length of each consensus mitochondrial cytochrome *b* gene fragment was 376 bp, as shown in Figure 1. To understand whether there has any intraspecific divergence in each species, 3 samples were collected from each place and sequenced. After analyzing a total of 42 specimens, we found that all species had intraspecific divergences except for *T. thynnus*. There were 4 bases of difference in *T. obesus* and 2 bases of difference in both *T. alalunga* and *T. albacares* were observed. In Figure 1, the positions of mixed intraspecies bases were labeled with N in the sequences.

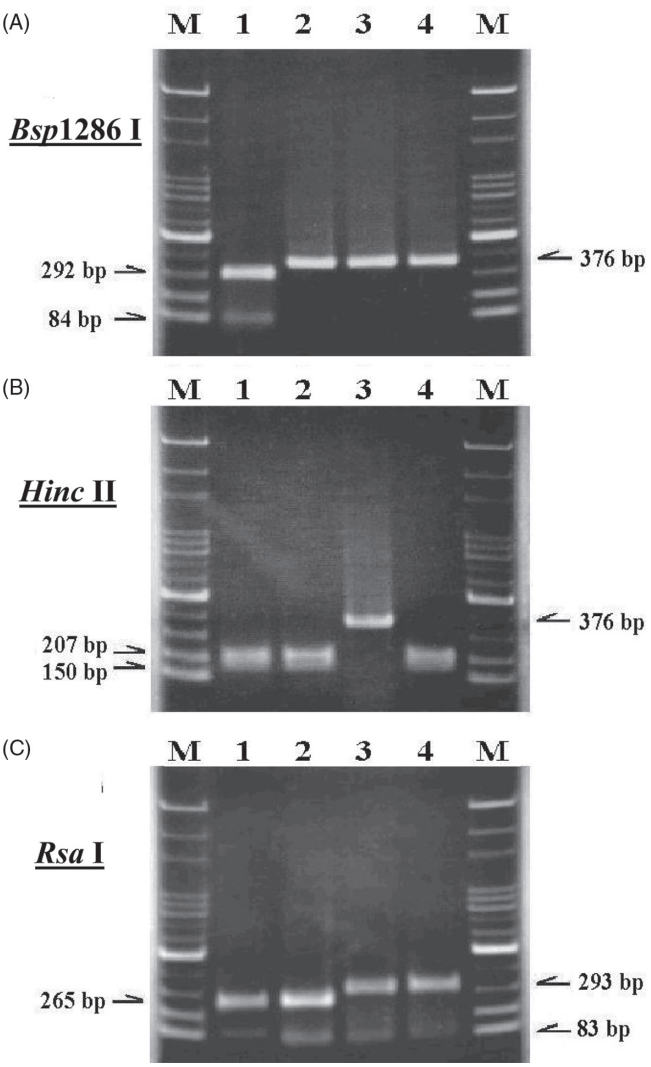

Figure 2. Electrophoretic analysis of 376 bp cytochrome *b* gene PCR products of 4 *Thunnus* species digested with (A) *Bsp*1286 I, (B) *Hinc* II, and (C) *Rsa* I. Samples in lane are as follows: 1. *Thunnus thynnus*, 2. *T. alalunga*, 3. *T. obesus*, 4. *T. albacares*, and M＝100 bp ladder.

Compared with interspecific sequences, the divergence was between 0.325% and 4.235%. The low interspecific divergence indicated that the mitochondrial cytochrome *b* gene was of maternal inheritance and had no recombination[10,11,29]. In mitogenome, the low diversity of cytochrome *b* gene is useful to identify different species, and high diversity of control region could separate different population of the same species[30,31].

II. *Analysis of Restriction Enzyme*

By analysis of the consensus fragment in 4 *Thunnus* species, we excluded the intraspecific divergent bases and then searched the restriction maps on GCG system. Three restriction enzymes with specific cutting sites were chosen, including *Bsp*1286 I, *Hinc* II and *Rsa* I. Restriction enzyme *Bsp*1286 I could cleave the 376 bp fragment into 292 bp and 84 bp in *T. thynnus*, and there was no cutting site in other 3 species. *Hinc* II could cleave the 376 bp fragments of *T. thynnus*, *T. alalunga* and *T. albacares* to separate into 207 bp, 150 bp and 19 bp fragments, but there was no cutting site in *T. obesus*. Furthermore, *Rsa* I cleaved both *T. thynnus* and *T. alalunga* into fragments of 265 bp and 111 bp, different from the fragments of 293 bp and 83 bp in both *T. albacares* and *T. obesus*. Table 1 shows the fragment length of restriction products in this study. Polymorphic patterns of DNA electrophoretic gel obtained

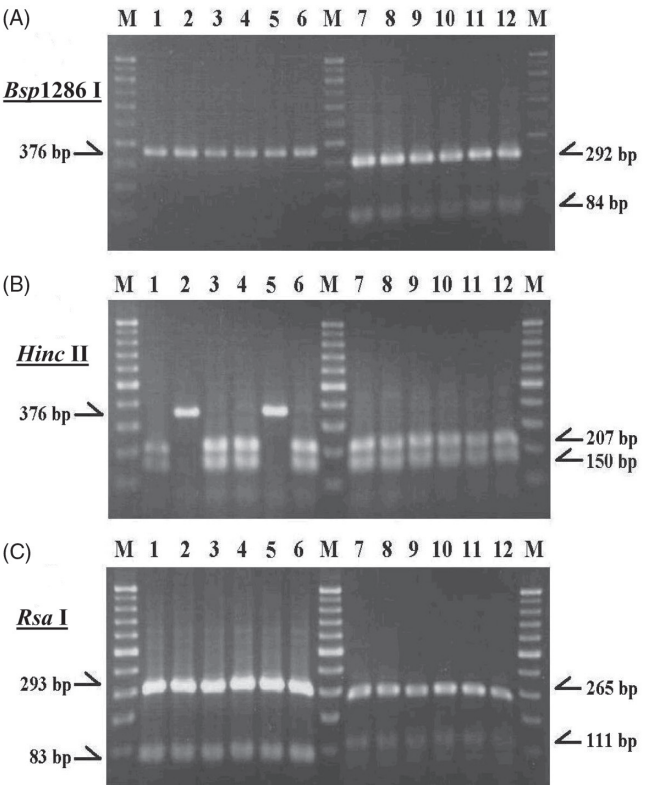

Figure 3. Restriction profiles of the 376 bp cytochrome *b* gene PCR products digested with (A) *Bsp*1286 I, (B) *Hinc* II, and (C) *Rsa* I. Samples in lane are 12 fresh commercial samples obtained from different markets in Taiwan, M＝100 bp ladder.

from the 4 *Thunnus* species are shown in Figure 2.

III. *Species Identification of Commercial Raw Meats*

Lastly, we analyzed 12 samples of commercial raw fish fillets (sashimi products) including 6 pieces of bluefin tuna and cheaper tuna species that were sold in different markets in Taiwan. After testing all samples, 2 out of 6 pieces of the cheaper tuna species were identified as *T. obesus* and the remaining 4 samples were identified as *T. albacares*. In addition, 6 pieces of bluefin tuna were all correcting identified as *T. thynnus* (Figure 3). The result showed the established PCR-RFLP method could identify fresh raw meats of 4 *Thunnus* species.

In this study, the direct DNA sequencing to analyze cytochrome *b* gene of the 4 tuna species was accurate; however, the technique was time-consuming and expensive. In addition, direct sequencing could not detect a large number of samples in a short time. High conservation and inheritable variation of mitochondrial cytochrome *b* gene were recognized to be useful in species classification[17]. Many authors have established reliable PCR-RFLP models to identify different marine fish[22,26,32]. Therefore, we also adopted the PCR-RFLP method and proved it to be a quick, cheap and convenient technique in the identification of tuna species.

CONCLUSIONS

The genetic variation of partial mitochondrial cytochrome *b* gene in 4 *Thunnus* species was employed to develop a PCR-RFLP technique. The result of PCR-RFLP test in this study indicated that the primer set was suitable for PCR amplification and 3 restriction enzymes were suitable for RFLP pattern analysis. This method quickly and easily identifies the species of commercial raw products in market and provides a useful and academic technique to identify four species of *Thunnus*.

ACKNOWLEDGEMENTS

This study was supported by Department of Health (DOH 93-TD-F-113-001), ROC.

REFERENCES

1. Collette, B. B. and Nauen, C. I. 1983. An annotated and illustrated cataloque of tunas, mackerels, bonitos and related species know to date. Scombrids of the world. In "FAO Species Catalogue". Vol. II. Food and Agriculture Organization of USA. New York, U. S. A.
2. Sotelo, C. G., Pineiro, C., Gallardo, J. M. and Perez-Martin, R. I. 1993. Fish species identification in seafood products. Trends Food Sci. Technol. 4: 395-401.
3. Rehbein, H., Etienne, M., Jerome, M., Hattula, T., Knudsen, B., Jessen, F., Luten, J. B., Bouquet, W., Mackie, I. M., Ritchie, A. H., Martin, R. and Mendes, R. 1995. Influence of variation in methodology on reliability of the isoelectric focusing method of fish species identification. Food Chem. 52: 193-197.
4. Mackie, I. M. 1997. Methods of identifying species of raw and processed fish. In "Fish Processing Technology". 2nd ed. pp. 160-199. Blackie Academic and Professional. London, U. K.
5. Cuniff, P. 1998. Official Methods of Analysis. In "Association of Official Analytical Chemists (AOAC)". 16th ed. Vol. II. pp. 24-27. Gaithersburg, Maryland, U. S. A.
6. Cocolin, L., D'Agaro, E., Manzano, M., Lanari, D. and Comi, G. 2000. Rapid PCR-RFLP method for the identification of marine fish fillets (Seabass, Seabream, Umbrine, and Dentex). J. Food Sci. 65: 1315-1317.
7. Curole, J. P. and Kocher, T. D. 1999. Mitogenomes: Digging deeper with complete mitochondrial genomes. Trend Ecol. Evol. 14: 394-398.
8. Taanman, J. W. 1999. The mitochondrial genome: Structure, transcription, translation and replication. Biochim. Biophys. Acta 1410: 103-123.
9. Gray, W. M. 1989. Origin and evolution of mitochondrial DNA. Annu. Rev. Cell Bio. 5: 25-50.
10. Bromham, L., Eyre-Walker, A., Smith, N. H. and Smith, J. M. 2003. Mitochondrial Steve: Paternal inheritance of mitochondrial in humans. Trend Ecol. Evol. 18: 2-4.
11. Rokas, A., Ladoukakis, E. and Zouros, E. 2003. Animal mitochondrial DNA recombination revisited. Trend Ecol. Evol. 18: 411-417.
12. Stoneking, M. and Soodyall, H. 1996. Human evolution and the mitochondrial genome. Curr. Opin. Genetic Dev. 6: 731-736.
13. Mandavilli, B. S., Santos, J. H. and Houten, B. V. 2002. Mitochondrial DNA repair and agong. Mutation Res. 509: 127-151.
14. Southern, S., Peter, J. S. and Andrew, E. D. 1988. Molecular characterization of cloned mitochondrial genome. J. Mol. Evol. 28: 32-42.
15. Leonard, J. V. and Schapira, A. H. V. 2000. Mitochondrial respiratory chain disorders I: Mitochondrial DNA defects. The Lancet 355: 299-304.
16. Moritz, C., Dowling, T. E. and Brown, W. M. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. Annu. Rev. Ecol. Syst. 18: 262-269.
17. Antointte, J. P. and Greg, S. S. 2001. Molecular phylogeny of the Chipmunks inferred from mitochondrial cytochrome *b* and cytochrome oxidase II gene sequences. Mol. Phylog. Evol. 20: 335-350.
18. Elmerot, C., Arnason, U., Gojobori, T. and Janke, A. 2002. The mitochondrial genome of the pufferfish, Fugu rubripes, and ordinal teleostean relationships. Gene 295: 163-172.
19. Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski,

J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 531-535.
20. Jones, J. L. 1991. DNA probes: Application in the food industry. Trends Food Sci. Technol. 2: 28-32.
21. Ishida, K., Zhu, B. L., Sakoda, S., Quan, L., Oritani, S., Fujita, M. Q. and Maeda, H. 2000. Significance of DNA analysis for determination of ABO blood groups from hair and nail of decomposed human remain: A comparison with phenotyping by the absorption-elution method. Legal Med. 2: 212-215.
22. Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Fernandez, A., Asensio, L., Hernandez, P. and Martin, R. 2000. Genetic differentiation between sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR-RFLP analysis of a 12S rRNA gene fragment. J. Sci. Food Agric. 80: 29-32.
23. Sato, T., Matsuyama, J., Takahashi, N., Sato, M., Johnson, J., Schachtele, C. and Hoshino, E. 1998. Differentiation of oral *Actinomyces* species by 16S ribosomal DNA polymerase chain reaction – restriction fragment length polymorphism. Arch. Oral Biol. 43: 247-252.
24. Kurihara, A., Tawata, M., Ikegishi, Y., Aida, K. and Onaya, T. 1999. The procedure of polymerase chain reaction – restriction fragment – single strand conformation polymorphism analysis by *Hha* I / *Hinc* II to detect mitochondrial DNA mutations. Life Sci. 64: 1223-1230.
25. Russell, V. J., Hold, G. L., Pryde, S. E., Rehbean, H., Quinteiro, J., Rey, M. M., Sotelo, C. G., Santos, A. T. and Rosa, C. 2000. Use of restriction fragment length polymorphism to distinguish between salmon species. J. Agric. Food Chem. 48: 2184-2188.
26. Hsieh, Y. W., Shiu, Y. C., Cheng, C. A., Chen, S. K. and Hwang, D. F. 2002. Identification of toxin and fish species in cooked fish liver implicated in food poisoning. J. Food Sci. 67: 948-952.
27. Desalle, R. and Birstein, V. J. 1996. PCR identification of black caviar. Nature 361: 197-198.
28. Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86: 6196-6200.
29. Brown, W. G., Gadaleeta, G., Pepe, G., Saccone, C. and Sbisa, E. 1986. Structural conservation and variation in the D-loop containing region of vertebrate mitochondrial DNA. J. Mol. Biol. 192: 503-511.
30. Alvarado, B. J. R., Naseri, I. and Ely, B. 1997. Orthodox and unorthodox phylogenetic relationships among tunas revealed by the nucleotide sequence analysis of the mitochondrial DNA control region. J. Fish Biol. 50: 540-554.
31. Miya, M. and Nishida, M. 2000. Use of mitogenomic information in teleostean molecular phylogenetics: Atree-based exploration under the maximum-parsimony optimality criterion. Phylog. Evol. 17: 437-455.
32. Sezaki, K., Kuboshima, Y., Mitani, I., Fukui, A. and Watabe, S. 2001 Identification of chub and spotted mackerels with mitochondrial cytochrome *b* gene and its application to respective pelagic eggs fixed with formalin. Nippon Suisan Gakkaishi 67: 17-22.