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ScienceDirect
Food Chemistry 106 (2008) 390–396
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Application of species-specific PCR for the identification of dried bonito product (Katsuobushi)

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Received 7 December 2006; received in revised form 19 May 2007; accepted 28 May 2007

Abstract

The species-specific PCR (polymerase chain reaction) method was developed to identify the species of dried bonito product (Katsuobushi) produced from *Euthynnus pelamis*, *E. affinis*, *Auxis rochei*, *A. thazard*, and *Sarda orientalis*. The 1141 bp complete mitochondrial cytochrome *b* genes of five bonito species and other five related Scombridae species were established, and then five pairs of species-specific primer were designed to amplify short length fragments among bonito species. The developed species-specific PCR method was successfully applied to authenticate species of commercial dried bonito products. Hence, this method really provided a useful and academic technique to identify the sources of bonito product.

Keywords: Species-specific PCR; Dried bonito; Species identification; Cytochrome *b* gene

1. Introduction

Dried bonito product (Katsuobushi) is a traditional and popular seasoned product in both Taiwan and Japan. It is usually pared into slices and cooked as soup stuff. The processes of dried bonito product include boiling, smoking, drying and fermenting (Hiyama et al., 1995). The fish species of skipjack (*Euthynnus pelamis* or *Katsuwonus pelamis*), eastern little tuna (*E. affinis*), frigate mackerel (*Auxis rochei*), frigate tuna (*A. thazard*), and oriental bonito (*Sarda orientalis*) are defined as bonito species, and these five species are all raw materials of dried bonito (Collette & Nauen, 1983). The flavors of dried bonito product are depending on fish species, specially depending on the fat content of fish. The fat content of *E. pelamis* is the highest, so that the taste of its dried bonito product is the best. However, high fat content makes the dried bonito product difficultly preserved. The quality of dried bonito product is according to the species. In order to avoid possible fraudulent and vague label of dried bonito product, the identification of fish species is becoming a topic of growing concern.

Several methods are used to identify fish species, including traditional morphological identification, electrophoresis, isoelectric focusing, liquid chromatography, immunoassay, and biological technologies (O'Reilly & Wright, 1995; Osman, Asoor, & Marsh, 1987). In dried bonito product, morphological character of fish are eliminated by slicing, and potential specific proteins are also unavailable to be detected after complicated processed steps including thermal treatment of boiling, smoking, drying and microbial inoculation. Comparing with proteins, the DNA extracted from processed products is more stable for species identification. Therefore, the molecular assay is more promising and reliable, because of its robustness and easy application in laboratory surveys (Cocolin, D'Agaro, Manzano, Lanari, & Comi, 2000).

Analysis of mitochondrial DNA (mtDNA) sequences is useful for phylogenetic studies. The mtDNA is a circular nucleotide for inheritance and independent from nucleic

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.05.060

DNA (nDNA) (Taanman, 1999). The composition of mtDNA is simpler than nDNA, and 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, Ladoukakis, & Zouros, 2003). The rate of base substitution on mtDNA is higher than that on nDNA, causing a rapid evolution (Stoneking & Soodyall, 1996). Cytochrome *b* gene (Cyt *b*) is a functional gene between $tRNA^{Glu}$ and $tRNA^{Thr}$ in mtDNA, and plays a role in encoding partial cytochrome c oxidoreductase, a complex enzyme in oxidative phosphorylation (Leonard & Schapira, 2000; Southern, Peter, & Andrew, 1988). Many researches that studied about vertebrate cytochrome *b* gene were focused on inheritance and evolution (Antointte & Greg, 2001; Moritz, Dowling, & Brown, 1987).

The polymerase chain reaction (PCR) technique and direct sequence analysis are useful for species identification. In our previous researches, the PCR restriction fragment length polymorphism (PCR-RFLP) technique has been utilized to identify some species of raw fish or processed seafood (Hsieh, Chai, & Hwang, 2005; Hsieh & Hwang, 2004; Hsieh, Shiu, Cheng, Chen, & Hwang, 2002; Hwang, Jen, Hsieh, & Shiau, 2004; Lin & Hwang, 2007; Lin, Shiau, & Hwang, 2005). However, because of the serious DNA degradation of dried bonito product, it is difficult to search out a short consensus DNA fragment containing all specific restriction cutting sites among five bonito species. In this study, a rapid species-specific PCR method was developed to identify the species of dried bonito products (Abdel-Rahman & Ahmed, 2007; Lahiff et al., 2001). The complete sequences of cytochrome *b* gene (1141 bp) from five bonito species and other five related Scombridae species have been established. Then five sets of species-specific primer were designed and used to individually amplify short fragments of cytochrome *b* gene in five species of dried bonito products.

2. Materials and methods

2.1. Preparation of samples

The specimens of five bonito species including *E. pelamis*, *E. affinis*, *A. rochei*, *A. thazard*and *Sarda orientalis* were collected from Keelung (Northern Taiwan) and Tungkang (Southern Taiwan). Other five related Scombridae species including *Acanthocybium solandri*, *Scomberomorus commerson*, *Somber japonicus*, *Thunnus albacares* and *T. thynnus* were also collected from Keelung and Tungkang in Taiwan. All of 10 species were morphologically identified their external characteristics by Dr. Kwang-Tsao Shao (Research Center for Biodiversity, Academia Sinica, Taipei), and each species of bonito was at least picked five individuals to analyze.

Furthermore, fourteen samples of commercial dried bonito product (Katsuobushi) were purchased from markets, supermarkets and convenience stores in Taiwan, and all of them were labeled the materials as bonito. All samples were stored at −20 °C until use.

2.2. DNA extraction

Because traditional phenol–chloroform DNA extraction method was inefficient to extract DNA in processed products (Lin & Hwang, 2007), 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 °C until lysis was completed, then magnetic beads were added. After incubation, magnetic beads binding DNA was separated by a magnetic separator. The mixture was washed two times by different washing buffer. Ultimately, the magnetic beads were removed from the solution and the genomic DNA was eluted in 50 μl of elution buffer.

2.3. Design of PCR primers

Total 12 selected primers from bony fishes were examined, and 8 of them were successfully used to rebuild complete cytochrome *b* genes of 10 species including *E. pelamis*, *E. affinis*, *A. rochei*, *A. thazard*, *S. orientalis*, *A. solandri*, *S. commerson*, *S. japonicus*, *T. albacares* and *T. thynnus*. These sequences of primers are as follows:

L14504-ND6: 5′-GCCAAWGCTGCWGAATAMGCAAA-3′
L14734-Glu: 5′-AACCACCGTTGTTATTCAACT-3′
L15285-CYB: 5′-CCCTAACCCGCTTATTYGC-3′
L15369-CYB: 5′-ACAGGMTCAAAYAACCC-3′
L15411-CYB: 5′-GATAAAATTYCATTCCACCC-3′
H15149-CYB: 5′-GGTGGCKCCTCAGAAGGACATTTGKCCTCA-3′
H15557-CYB: 5′-GGCAAATAGGAARTATCAYTC-3′
H15990-Pro: 5′-AGTTTAATTTAGAATCYTGGCTTTGG-3′

Positions with mixed bases are labeled with their IUB codes: R = A/G; Y = C/T; K = G/T; M = A/C; S = G/C; W = A/T (Inoue, Miya, Tsukamoto, & Nishida, 2000; Inoue, Miya, Tsukamoto, & Nishida, 2001a; Inoue et al., 2001b; Miya & Nishida, 2000). The 1141 bp cytochrome *b* gene of each species could be constructed from three fragments, which were amplified by three sets of primer, respectively. For *E. pelamis*, *E. affinis*, *A. rochei*, *A. solandri* and *S. japonicus*, the three fragments of cytochrome *b* gene could be amplified by primer sets L14504-ND6 / H15149-CYB, L14734-Glu/H15557-CYB and L15369-CYB/H15990-Pro. For *S. orientalis* and *S. commerson*, the three fragments could be amplified by L14734-Glu/H15149-CYB, L14734-Glu/H15557-CYB and L15369-CYB/H15990-Pro. For

Table 1
Species-specific primer sequences and their suitable annealing temperatures of five bonito species

Primers	Sequence (5′ → 3′)	Annealing temperature (°C)	Specific species
AR333-L AR457-H	GCTTGATGTGGGGTGGTGTAAC TCTCCTTGGCTTTGCAATC	60	*Auxis rochei*
AT363-L AT541-H	TGGCGGGTGTAAAATTATCTGG ACACGAGACCGGGTCTAATAAC	64	*A. thazard*
EA642-L EA777-H	CCCCTCAAATTCATTCAACAAG CTAGTGATGATAACTGCCTTCG	60	*Euthynnus affinis*
EP844-L EP976-H	GCCAATATGGGAGTAAATGCAG TACCCCTGACGTAGAATCAGCC	60	*E. pelamis*
SO862-L SO994-H	GCAAATGAAGAAAAAGGAGGCG ATTTCTAGCAATGCACTACACC	62	*Sarda orientalis*

T. albacares and *T. thynnus*, the three fragments were amplified by L14504-ND6/H15149-CYB, L14734-Glu/H15557-CYB and L15285-CYB/H15990-Pro. Finally, L14504-ND6/H15149-CYB, L14734-Glu/H15557-CYB and L15411-CYB/H15990-Pro were needed to amplify the three fragments for *A. thazard* only.

After aligning the complete cytochrome *b* genes from 10 species, five pairs of species-specific primer were designed to amplify short length fragments in dried bonito product. The five original primer sets are listed in Table 1.

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, USA) 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. All primer sets were investigated their suitable annealing temperature sby gradient PCR machine (MyCycler Thermal Cycler, Bio-Rad, Hercules, Calif., USA). Furthermore, the PCR amplifications were carried out in a GeneAmp PCR System 2400 (Perkin–Elmer, Foster City, Calif., USA) programmed to perform a denaturation step at 95 °C for 10 min, followed by 30 cycles consisting of 30 s at 95 °C, 30 s at 50–60 °C and 1 min at 72 °C. 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, 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, Calif., USA) in an ABI PRISM 377-96 DNA Sequencer (Perkin–Elmer/Applied Biosystems Div.). Two complementary DNA sequences obtained from each species were compared with the database and aligned to establish complete cytochrome *b* gene by Genetics Computer Group Wisconsin Package, Version 11.1 (GCG system; Genetics Computer Group, 2006).

3. Results

3.1. Construction of cytochrome b genes

The mitochondrial cytochrome *b* genes of 10 Scombridae species were amplified by established fish-versatile primers (Inoue et al., 2000; Inoue et al., 2001a, Inoue et al., 2001b; Miya & Nishida, 2000). All PCR amplified fragments were overlapped step by step, and 10 sequences of complete cytochrome *b* gene were rebuilt individually. The newly determined sequences are available from DDBJ/EMBL/GenBank databases under accession numbers EF141171 for *A. rochei*, EF141172 for *A. solandri*, EF141173 for *A. thazard*, EF141174 for *E. affinis*, EF141175 for *E. pelamis*, EF141176 for *S. commerson*, EF141177 for *S. japonicus*, EF141178 for *S. orientalis*, EF141179 for *T. albacares* and EF141185 for *T. thynnus*. Besides five species of scombroid fish closely related with bonito species, cytochrome *b* genes of other related fish species were also been searched from GeneBank and compared to five established bonito species.

3.2. Species-specific PCR of short length fragments

After analyzing the 10 consensus sequences of 1141 bp mitochondrial cytochrome *b* gene, five sets of primers were designed and applied to detect five fish species of dried bonito product, respectively. After aligning, the species-specific areas of 10 consensus sequences were searched

out, and potential miss-paired areas were excluded. Five pairs of species-specific primers on five bonito species were designed by employing GCG system, and could be used to amplify short length fragments for five fish species.

Primer set AR333-L/AR457-H could amplify a specific 121 bp fragment for *A. rochei*, and would not have any reaction with other nine Scombridae species. Similarly, primer sets of AT363-L/AT541-H, EA642-L/EA777-H, EP844-L/EP976-H and SO862-L/SO994-H could amplify a 156 bp fragment for *A. thazard*, 113 bp fragment for *E. affinis*, 112 bp fragment for *E. pelamis* and 110 bp fragment for *S. orientalis*, respectively. Every species-specific primer set was routinely investigated by using five intraspecific specimens and nine specimens of other Scombridae species. The results are shown in Fig. 1. The range of PCR annealing temperatures for each primer set is wide. If the annealing temperature set too low, the miss-paired situation would be found in non-specific species. Therefore, the suitable annealing temperature for each primer set was set as high as possible and listed in Table 1.

3.3. Species identification of commercial dried bonito product (katsuobushi)

The species-specific PCR method was established as above and applied to identify the species of 14 samples of commercial dried bonito products purchased in Taiwan. The DNA of each 14 samples was extracted by using magnetic bead technique. After PCR operation by using five sets of species-specific primers individually, 11 out of 14 samples were successfully amplified to obtain species-specific fragments. Five samples of them were amplified to obtain 112 bp fragment by using primer set of EP844-L/EP976-H, and nothing by other four species-specific primer sets. These five samples identified to be produced by bonito species of *E. pelamis*. Furthermore, three samples amplified to obtain 113 bp fragment by using primer set EA642-L/EA777-H and determined to be produced from other bonito species *E. affinis*. Two samples were amplified to obtain 156 bp fragments by using primer set AT363-L/AT541-H and determined to be produced from *A. thazard*.

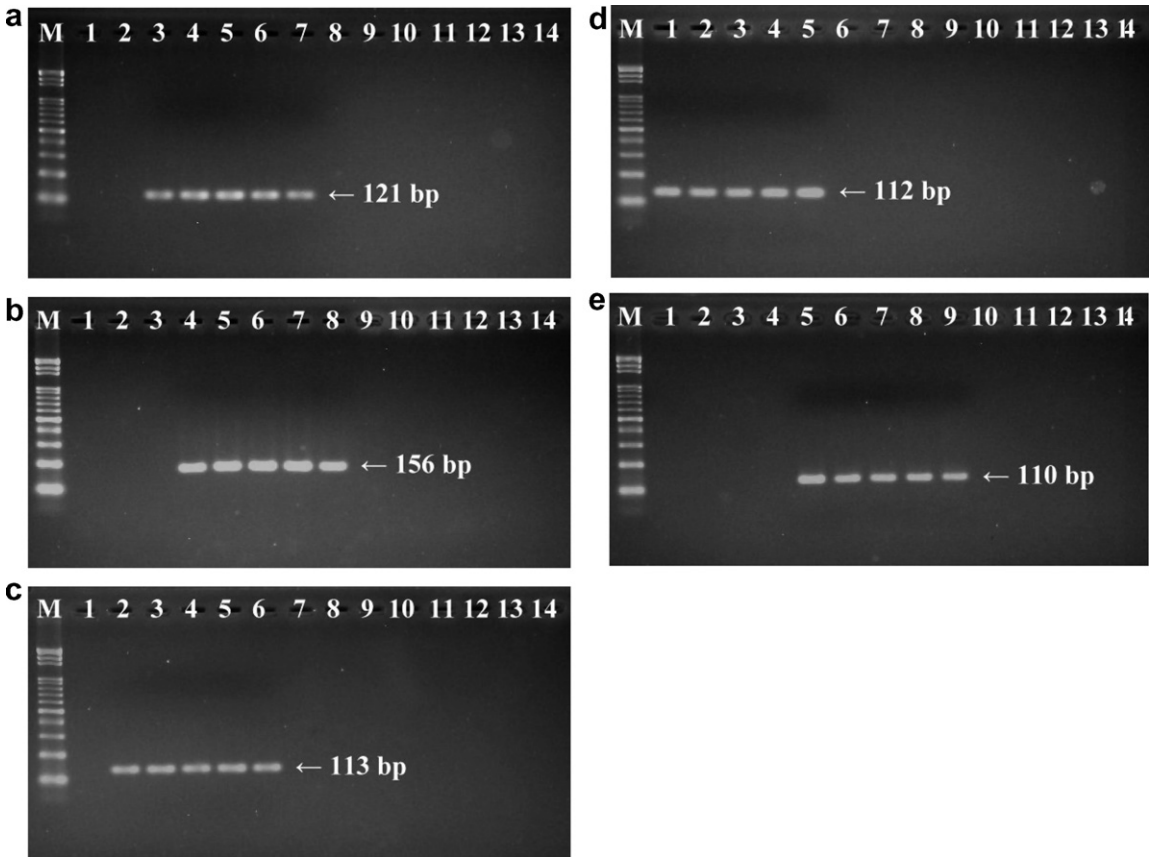

Fig. 1. PCR products amplified by different species-specific primer sets (a) AR333-L / AR457-H, samples in lane are as follows: 1. *Euthynnus pelamis*; 2. *E. affinis*; 3–7. five individuals of *Auxis rochei*; 8. *A. thazard*; 9. *Sarda orientalis*; 10. *Acanthocybium solandri*; 11. *Scomberomorus commerson*; 12. *Somber japonicus*; 13. *Thunnus albacares*; 14. *T. thynnus*. (b) AT363-L/AT541-H, samples in lane are as follows: 1. *E. pelamis*; 2. *E. affinis*; 3. *A. rochei*; 4–8. five individuals of *A. thazard*; 9. *S. orientalis*; 10. *A. solandri*; 11. *S. commerson*; 12. *S. japonicus*; 13. *T. albacares*; 14. *T. thynnus*. (c) EA642-L/EA777-H, samples in lane are as follows: 1. *E. pelamis*; 2–6. five individuals of *E. affinis*; 7. *A. rochei*; 8. *A. thazard*; 9. *S. orientalis*; 10. *A. solandri*; 11. *S. commerson*; 12. *S. japonicus*; 13. *T. albacares*; 14. *T. thynnus*. (d) EP844-L/EP976-H, samples in lane are as follows: 1–5. five individuals of *E. pelamis*; 6. *E. affinis*; 7. *A. rochei*; 8. *A. thazard*; 9. *S. orientalis*; 10. *A. solandri*; 11. *S. commerson*; 12. *S. japonicus*; 13. *T. albacares*; 14. *T. thynnus*. (e) SO862-L/SO994-H, samples in lane are as follows: 1. *E. pelamis*; 2. *E. affinis*; 3. *A. rochei*; 4. *A. thazard*; 5–9. five individuals of *S. orientalis*; 10. *A. solandri*; 11. *S. commerson*; 12. *S. japonicus*; 13. *T. albacares*; 14. *T. thynnus*; $M = 100$ bp ladder.

Table 2
Species identification of commercial dried bonito (Katsuobushi) determined by species-specific PCR analysis

Source of PCR products	PCR amplification of species-specific primer sets					Species identification
	AR333-L/ AR457-H	AT363-L/ AT541-H	EA642-L/ EA777-H	EP844-L/ EP976-H	SO862-L/ SO994-H	
Sample 1	–	–	–	112 bp	–	*Euthynnus pelamis*
Sample 2	–	–	113 bp	–	–	*E. affinis*
Sample 3	–	–	–	–	110 bp	*Sarda orientalis*
Sample 4	–	–	–	–	–	?
Sample 5	–	–	–	112 bp	–	*E. pelamis*
Sample 6	–	–	–	–	–	?
Sample 7	–	156 bp	–	–	–	*Auxis thazard*
Sample 8	–	–	–	112 bp	–	*E. pelamis*
Sample 9	–	–	–	112 bp	–	*E. pelamis*
Sample 10	–	–	113 bp	–	–	*E. affinis*
Sample 11	–	–	–	–	–	?
Sample 12	–	–	113 bp	–	–	*E. affinis*
Sample 13	–	156 bp	–	–	–	*A. thazard*
Sample 14	–	–	–	112 bp	–	*E. pelamis*

And only 1 sample was amplified to obtain 110 bp fragment by using primer set SO862-L/SO994-H and determined to be produced from *S. orientalis*. These identified samples all showed unique fragment corresponding to its species-specific primer and with no confused situation. However, three out of 14 samples could not be amplified by any of five species-specific primer sets, and could not be judged the species of raw materials. The results of species-specific PCR analysis for 14 commercial dried bonito products in this study are shown in Table 2.

4. Discussion

4.1. Sequence diversity of cytochrome b gene

In family Scombridae, the size of bonitos (genera *Euthynnus*, *Auxis* and *Sarda*) was smaller than tunas (mainly genus *Thunnus*) and Spanish mackerels (genera *Acanthocybium*, *Scomberomorus* and others), but bigger than mackerels (genus *Somber*) (Collette & Nauen, 1983). Different species of bonitos have similar morphological characteristics and meat qualities. They are commonly processed as dried bonito products in Taiwan. Previous studies have used mitochondrial cytochrome *b* gene to reconstruct the systematic relationships with family Scombridae, and the sequence length and overall base composition of cytochrome *b* gene in Scombridae are very similar to others previously reported for teleosts (Block, Finnerty, Stewart, & Kidd, 1993; Chow & Kishino, 1995; Manchado, Catanese, & Infante, 2004). In this study, after comparing complete cytochrome *b* genes from five bonito species and other five Scombridae species to each other, the alignments of sequence were totally the same (1141 bp) and the diversities between different genera were obvious. The least diversity of cytochrome *b* gene occurred in the same genus, such as 1.23% diversity between *T. thynnus* and *T. albacares*, and 6.75% diversity between *A. rochei* and *A. thazard*. For each couple between different genera among these 10 tested scombroid species in this study, the diversity of cytochrome *b* gene was from 9.38% to 17.88%. The highest diversity (17.88%) was detected between *A. solandri* and *S. japonicus*. The presence of homoplasy and saturated transitions in cytochrome *b* gene, together with the large variance in the substitution rate and the non-random distribution of substitutions in this gene, imposed by the protein function, may distort the relationships among taxa (Griffiths, 1997; Manchado et al., 2004). Even so, the distinguishable sequences of cytochrome *b* gene were suitable to analyze phylogenic evolution and to develop specific methods for species identification (Antointte & Greg, 2001; Moritz et al., 1987).

4.2. Identification of dried bonito products by species-specific PCR

DNA in raw materials is 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 processed foods is difficult to extract and observed by PCR amplification due to severe DNA degradation. The amount and fragment size of DNA sequence in processed products were both less than those in raw tissue (Infante, Catanese, Ponce, & Manchado, 2004; Quinteiro et al., 1998). Therefore, a recent developed method, which was operated by coverage of magnetic beads with nucleic acid-binding matrices, was applied in this study and provided a high potential of sensitivity and automation (Kleines, Schellenberg, & Ritter, 2003).

PCR-RFLP technique is often applied in many researches of species identification for processed foods (Wolf, Burgener, Hubner, & Luthy, 2000). However, species-specific PCR is simpler than PCR-RFLP, because it does not need to add restriction enzymes for advanced analysis (Abdel-Rahman & Ahmed, 2007; Lahiff et al., 2001). The diversities of cytochrome*b* genes among *E. pelamis*, *E. affinis*, *A. rochei*, *A. thazard* and *S. orientalis* are

high enough. Therefore, the species-specific areas could be found and used to design species-specific primer sets in cytochrome *b* genes of five bonito species. In each of the primer sequence could prevent miss-pairing and ensure the annealing specificity for each species, because there are three species-specific bases at least (Jones, 1991).

The nucleic acid molecule is unstable in condition of high temperature over 100 °C. In our previous study, the size of DNA fragments extracted from canned tuna products has been analyzed as less than 200 bp (Lin & Hwang, 2007). Unlike traditional cooked or only dried products, dried bonito products were processed by including boiling, smoking, drying and fermenting processes. These complicated processed steps made the DNA sequences of bonito products become more fragmental and be hard detected by PCR reaction. Three out of 14 commercial products of dried bonito products could not be identified in this study, and the same situations were also to occur in commercial canned tunas (Quinteiro et al., 1998; Ram, Ram, & Baidoun, 1996). It is accurate by using direct DNA sequencing to analyze cytochrome *b* gene of five bonito species in dried bonito product. However, the technique is time-consuming and expensive. The result of species-specific PCR test in this study indicated that the five primer sets are suitable for PCR amplification and pattern analysis. This developed method is applicable to authenticate species of commercial dried bonito products quickly and easily.

Acknowledgements

This study was supported by Department of Health (DOH 94-TD-F-113-007), Taiwan, ROC. And we sincerely thank Dr. Kwang-Tsao Shao to take time for morphological authentication of fish species in this study.

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