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Food Poisoning Caused by Sunfish *Masturus lanceolatus* in Taiwan

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ABSTRACT

Attempts was made to clarify the toxicity and species of causative sunfish fillet concerning with a food poisoning. The frozen causative sunfish fillet was collected to determine its toxicity after two people eating it found poisoned. Tetrodotoxin (TTX) was not detected, however, the butanol extract of remained causative muscle showed ichthyotoxicity to gold carp and caused human, rat and tilapia red blood cells haemolysis. Patients and rats showed some symptoms similar to those caused by palytoxin. Hence, it is possible that this food poisoning is caused by a palytoxin-like compound. Moreover, fish species of the sunfish fillet was identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A PCR primer set (L14735/H15149) was used to amplify the partial fragment of the cytochrome *b* gene. Endonucleases *Stu* I was used in RFLP. The species of the causative sample was identified as *Masturus lamceolatus*.

Key words: PCR-RFLP, sunfish, *Masturus lamceolatus*, palytoxin, haemolysis

INTRODUCTION

Sunfish distributes in temperate and tropical seas. In Taiwan, sunfish is called as manbo and is one of the most favorable fish species because of its palatability, especially for the intestine part (called as "dragon intestine"). Primarily fresh fish cooked in accordance with consumer's preference is consumed. Although sunfish belongs to the tetrodotoxic fish[(1)], no food poisoning case has been reported.

In June 2005, two victims (mother, 40 and daughter, 16) were found poisoned after eating a sunfish caught off Hwalein County, East Taiwan. The victims complained of an episode of bitter taste of tongue, throat-sore, breast pain, muscle pain, tympanites, vomiting and tachycardia 30 min after fish meat was eaten. They were immediately sent to a local hospital and treated with intravenous fluids. Two to three hours after treatment they were discharged from the hospital. The symptoms of patients were similar to those of palytoxin, such as myalgias, weakness, fever, nausea, vomiting, muscle pain and tenderness[(2,3,4,5)].

Traditional morphological characterization, electrophoresis, liquid chromatography, immunoassay, isoelectric focusing, and biological technologies methods[(6,7)] have been used to identify fish species. However, the most promising and reliable approach is the use of DNA biomarkers because this technique is easily adapted into routine surveys[(8,9)].

Because mitochondrial DNA (mtDNA) is highly conserved[(10)] and is often used to analyze molecular evolution, mtDNA sequences analysis is regarded as a useful technique for phylogenetic studies. In recent years, it has been applied to detect adulteration of market products[(11,12)]. The cytochrome *b* gene (*Cyt b*) is a functional gene between the $tRNA^{Glu}$ and $tRNA^{Thr}$ genes in mtDNA. It plays a role in encoding a component of cytochrome *c* oxidoreductase, a complex enzyme involving in oxidative phosphorylation. Many studies on the vertebrate *Cyt b* gene have been focused on inheritance and evolution[(13)].

At present, DNA-related techniques are utilized to authenticate species in market products, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), multiplex PCR, species-specific PCR, PCR-random amplified polymorphic DNA (RAPD) and PCR-single strand conformation polymorphism (SSCP)[(14,15)]. Due to its high sensitivity, speed and simplicity, PCR-RFLP

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has often been applied to identify the adulteration of food products[16,17,18]. PCR-RFLP also has been applied for the identification of the species in toxic and nontoxic processed puffer fish products[19,20,21]. This method enabled easy and accurate identification of fish species used in the six commercial processed filefish products[22]. In this study, PCR-RFLP method was developed to establish a restriction map for the sunfish species and then this map was used to identify the sunfish species used in the causative sample. Moreover, sunfish is supposed to be a tetrodotoxic fish, so the presence of TTX was determined. On the other hand, the laboratory lacks the authentic palytoxin standard and the residue fish meat was not enough for purifying and identifying palytoxin in this study. But the symptoms of patients were similar to those of palytoxin, so the haemolysis activity, a specific characteristic of palytoxin[1,2,3,4], to red blood cells of human, rat and tilapia in the extract of sunfish meat were examined.

MATERIALS AND METHODS

I. *Samples*

The remained uncooked frozen sunfish fillet (300 g) was immediately collected from the restaurant, after two victims were found poisoned after eating this fillet. This causative sample was kept at -20°C for DNA extraction and toxicity assay. On the other hand, fresh samples of 2 species (*Masturus lanceolatus* and *Ranzania laevis*) of commercial sunfish (3 specimens/per species) were collected from different seafood markets in Miaoli and Hualien in Taiwan. These commercial sunfishes were eviscerated and their meat samples were kept at -20°C for DNA extraction and toxicity assay.

II. *Assay of Toxicity*

One gram of each meat sample was extracted with 4 mL of 0.03 M acetic acid solution and the toxicity of the extract was examined by tetrodotoxin (TTX) bioassay[23]. Furthermore, extract from each sample was partially purified using C18 cartridge column. The methanol eluate was freeze-dried, dissolved in distilled water, and confirmed by liquid chromatography-mass spectroscopy (LC-MS) for TTX[24].

To determine the palytoxin-like toxicity[4], 100 g of each sample was extracted three times with three volumes of 75% ethanol (pH 3.5). The extract was defatted with diethyl ether, and partitioned between aqueous and 1-butanol layers. Butanol layer was concentrated to dryness, made up to 3 mL with water, and then submitted to following tests. At first, 0.5 mL of each butanol layer was duplicately intraperitonealy (i.p.) injected into male mice of ICR strain (18 - 20 g), and the mice were observed for a period of 48 h. On the other hand, small silver carps (body weight 1.80 ± 0.07 g, body length 4.35 ± 0.15 cm) were injected with 0.1 mL of extract by i.p. administration and were orally administrated with 0.1 g of muscle in duplicate.

Since palytoxin possesses haemolytic activity[4], therefore, the haemolytic activity of these extracts was determined by using the red blood cells of human, rat and tilapia as described by a previous report[4]. Briefly, 0.1 mL of each extract was added to 0.9 mL of 0.5% red blood cell suspension in D-PBS containing 0.5 mM boric acid and 1 mM calcium chloride, and incubation for 1 h at 37°C. The cell suspensions were then centrifuged at 900 g for 10 min, and the absorbance of supernatants was measured at 405 nm using an UV-VIS spectrophotometer. Total haemolysis (100%) was achieved by adding the same volume of 1% saponin solution to the red blood cell suspension, and haemolysis (%) of each sample was determined as the ratio of absorbance between each sample and the 1% saponin solution.

III. *DNA Extraction*

DNA extraction was performed using the binding magnetic beads method (Chemagic DNA Tissue 10 Kit, Chemagen, Baesweiler, Germany). Approximately 0.2 g of each meat sample was homogenized with lysis buffer (50 mM of Tris-HCl, pH 8.0, 0.1 M EDTA, 10% SDS, 0.2 M NaCl), followed by incubation at 55°C for 4 h with shaking after addition of 100 μL of 10 mg/mL proteinase K (Amereses, Solon, Ohio, USA). After completion of lysis, the magnetic beads were added. Magnetic beads with bound DNA were recovered using a magnetic separator. The beads were then washed twice with different washing buffer. Finally, the magnetic beads were removed from the solution and the genomic DNA was eluted with 50 μL of elution buffer. The advantages of the automated magnetic particle processing protocol used for DNA purification include the following: (1) particles are collected with magnetic rods covered by disposable tips, (2) magnetic particles are transferred instead of liquids, (3) DNA is bound to the coated particles, (4) for efficient washing, DNA is released, mixed in the well, and re-collected, and (5) purified DNA is released into a small end-volume.

IV. *PCR Primer*

A set of primers (L14735/H15149) described in a previous report[25] was used for PCR amplification of a DNA fragment from each fish species. The sequences of the primers are as follows: L14735: 5'-AAAAACCACCGTTGTTATTCAACTA-3', H15149: 5'-GCNCCTCARAATGAYATTTGTCCTCA-3'.

V. *PCR Amplification*

PCR amplification was performed in a total volume of 50 μL. Each reaction mixture contained 100 ng of extracted template DNA, 0.2 μM of each primer, 200 μM of each dNTP, 2 U of Pro *Taq* DNA polymerase (Promega, Wisconsin, USA), and PCR buffer (20 mM Tris-HCl, 15 mM $MgCl_2$, 10% Triton X-100, 0.1 mM EDTA, 1 mM DDT and 50% glycerol).

The PCR was carried out in a Gene-Amp PCR system 2400 (Perkin-Elmer, Foster, California, USA) programmed

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to perform a denaturation step at 95°C for 5 min, followed by 32 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. The final extension step was 72°C for 10 min. Amplified products were stored at 4v[26].

VI. *PCR Product Analysis and Sequencing*

For confirmation PCR products (5 μL) were loaded onto a 2% agarose gel (containing Healthview DNA dye, 0.02 μL/mL) in TBE buffer and electrophoresed at 100 V for 40 min. DNA bands were detected under ultraviolet light and photographed by Image Master VDS (Pharmacia Biotech, Piscataway, New Jersey, USA). DNA products were sequenced at Mission Biotech (Taipei, Taiwan) using the above primers and the ABI Prism BigDye Terminator B3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, ISA) in an ABI Prism 377 sequencer (Applied Biosystems, Foster City, California, USA).

VII. *Restriction Site Analysis of PCR Products*

For the restriction enzyme analysis of the *Cyt b* gene region, the endonuclease *Stu* I (Promega, Madison, Wisconsin, USA) was searched from the GCG system by uploading our sequences and testing for restriction analysis of the amplified PCR products. The amplified DNA fragments of PCR products were used without purification to obtain the final result. Digestions were performed with 5 μL of amplified DNA, 5 U of enzyme, and a 1 : 10 dilution of the manufacturer's recommended 10 X digestion buffer. Digestion mixtures were incubated at 37°C for 6 h. The results were analyzed by electrophoresis performed on a 3% agarose gel (containing Healthview DNA dye, 0.02 μL/mL) in TBE buffer under 100 V for 45 min.

RESULTS

All samples including causative sunfish fillet and other two well-known species of sunfish were examined by TTX bioassay[23,27] and no toxicity was found. LC-MS analyses did not detect TTX in the meat of the two sunfish and the one that caused food posioning. TTX detection limit of LC-MS method was 1 ng/g[24].

For palytoxin-like toxicity test, bioassay with mice was performed. No lethal potency was found in the extract of the causative sunfish fillet, but the mice showed slight convulsion,

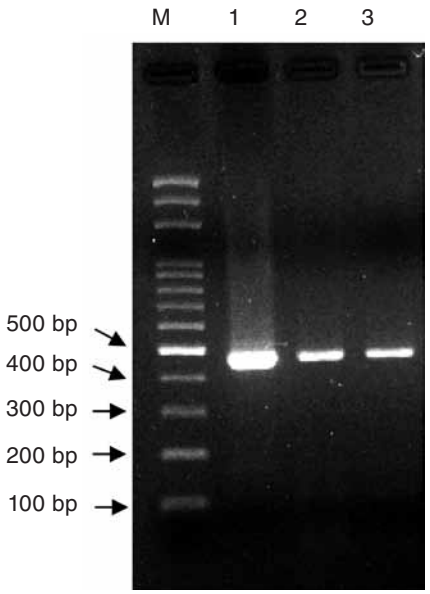

Figure 1. Electrophoretic analysis of the PCR products from fresh sunfish meats and patient's sample using L14735/H15149. Samples in lanes are as follows: M= 100-bp ladder; lane 1: *Masturus lanceolatus*; lane 2: *Ranzania laevis*; lane 3: causative sample.

Table 1. The lethal potency and haemolysis of butanol extract from remained causative sunfish meat and commercial sunfish meats of sunfish *M. lanceolatus* and *R. laevis*

Sunfish meat	Lethal potency*1			Haemolysis to red blood cells*2		
	Mice	Silver carp		Human	Rat	Tilapia
		i.p.	oral			
Causative sample	±	+	+	+	+	+
Commercial samples						
M. lanceolatus						
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	–	–	–	–	–
R. laevis						
1	–	–	–	–	–	–
2	–	–	–	–	–	–
3	–	–	–	–	–	–

*1: "–" no death with no symptoms; "±" no death with symptoms; "+" death; all data are duplicate.
*2: "–" haemolysis less than 1%, "+" haemolysis more than 5%.

drowsiness and collapse symptoms within 4 h after injection of the extract (Table 1). No symptom was observed in those mice injected with the extract of other two species of sunfish. Similarly, the extract of the causative sunfish fillet showed lethal potency to silver carps within 1 h either in i.p. or oral administration, but no lethal potency was observed in the extract of the other two species of sunfish.

The PCR primers L14735/H15149 were determined to be able to specifically amplify the partial mtDNA fragments of the *Cyt b* gene from the 2 fresh sunfish species and the causative sample. The size of *Cyt b* gene fragment amplified from PCR was different in the two sunfish species, a length

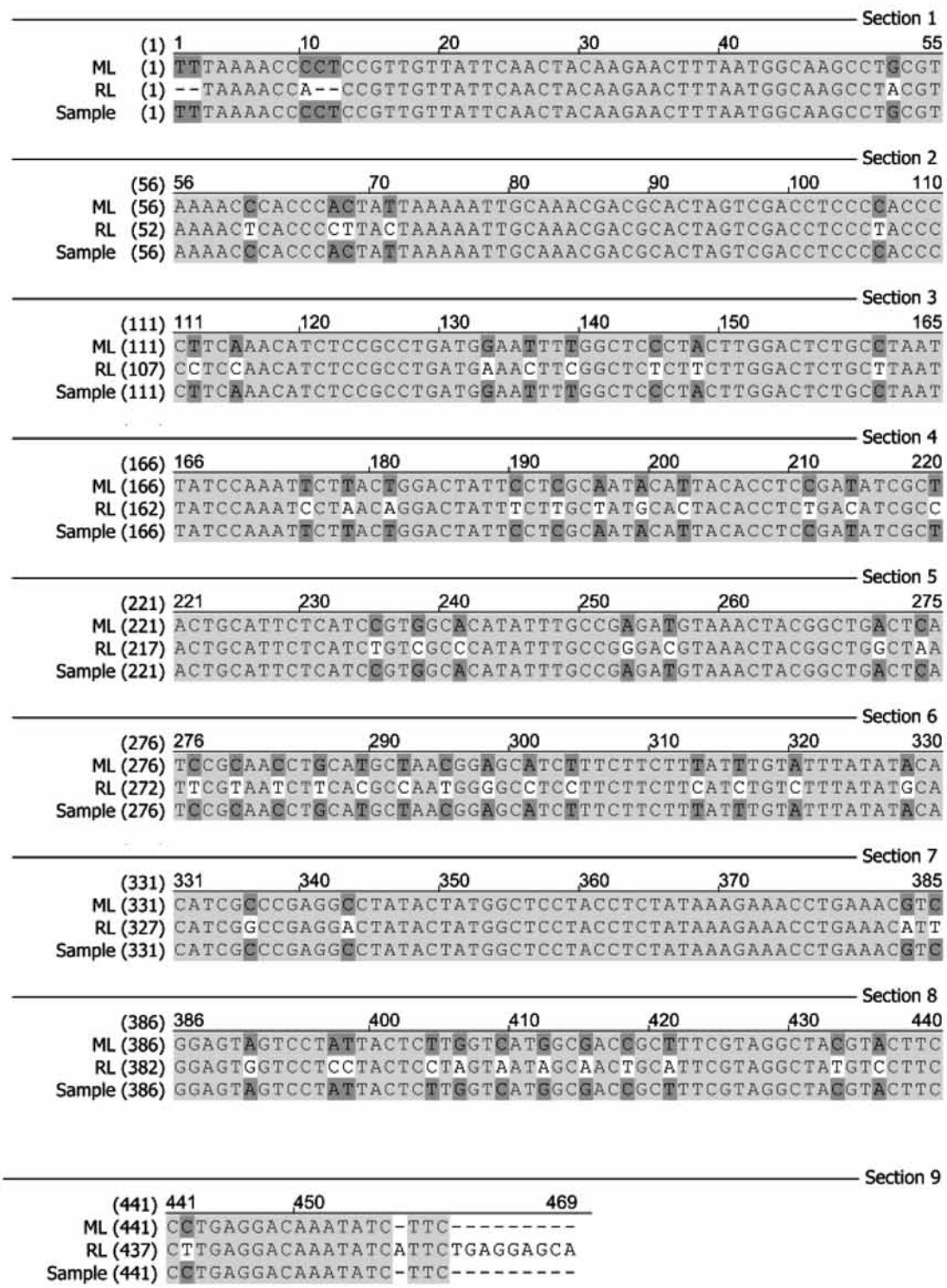

Figure 2. Alignment of partial *Cyt b* sequences of 2 species of fresh sunfishes and causative sample examined in this study. The species are as follows: ML: *Masturus lanceolatus*; RL: *Ranzania laevis*; sample: causative sample.

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of 459 bp for *Masturus lanceolatus* and 465 bp for *Ranzania laevis*. The electrophoretic analyses of the PCR products were shown in Figure 1. Slight difference was found in the sequences of the two sunfish and the causative sunfish fillet (Figure 2). As shown in Figure 3, gel electrophoresis patterns of the PCR products after digestion were different in the two sunfish and the causative sunfish fillet. Partial *Cyt b* gene fragment of *M. lanceolatus* and the causative sunfish fillet had one cutting site, but that of *R. laevis* had no cutting site. Probably the fish species of the causative sunfish fillet is *M. lanceolatus* (Table 2).

Table 2. Species identification of the causative sunfish fillet by fragment length of PCR and the fragment profile of PCR-RFLP

Sample	Fragment length of PCR	Fragment profile of PCR-RFLP
Masturus lanceolatus	459	117 + 342
Ranzania laevis	465	465
Causative sample	459	117 + 342

DISCUSSION

Judging from the data obtained in this study, it is possible that the fish species of the causative sunfish fillet is *M. lamceolatus*. However, the extract from the causative sunfish showed lethal potency to fish and haemolytic activity to the red blood cells of human and animals. The symptoms of victims and the symptoms of rats observed in the bioassay were not similar to those caused by ciguatoxin[28], but similar to those caused by palytoxin[2,3,4,5]. Hence, it is the first report to describe the food poisoning caused by sunfish, and it is suggested that the sunfish *M. lamceolatus* should belong to a species of ciguatera. It is well known that ciguateric fish rarely accumulate the toxin even they are the same species[28]. In this study, the sunfishes from market were found not toxic, so the food safety of sunfish needs to be noted in future.

As reported previously, the *Cyt b* gene is a good molecular marker for examining unknown species of seafood products because (1) this gene is highly conserved, (2) mtDNA in total cell nucleic acid is abundant, and (3) the preparations allow more effective PCR amplification[29,30]. The PCR-RFLP technique is also a quick and effective method for identifying seafood species, especially during a food poisoning incident[6,31,32]. Here, we found that the primers L14735/H15149 were able to successfully amplify the partial *Cyt b* gene from 2 sunfish species that are common and important in the markets of Taiwan. Furthermore, direct sequence analysis and restriction enzyme analysis can be applied to identify species from frozen sunfish fillet.

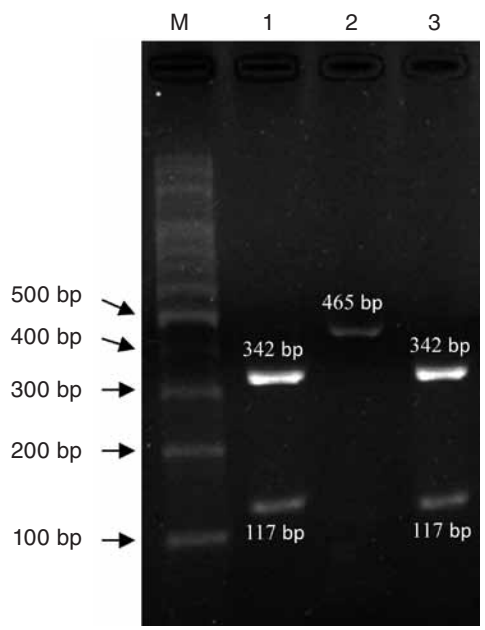

Figure 3. Electrophoretic analysis of the PCR products of 2 fresh sunfish meats and patient's sample digested with *Stu* I on 3% agarose gel. Samples in lanes are as follows: M = 100-bp ladder; lane 1: *Masturus lanceolatus*; lane 2: *Ranzania laevis*; lane 3: causative sample.

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REFERENCES

1. Halstesd, B W. 1988. Poisonous and Venomous Marine Animals of the World. pp. 528-529. The Darwin Press. New Jersey.
2. Noguchi, T., Hwang, D. F., Arakawa, O., Daigo, K., Sato, S., Ozaki, H. and Kawai, N. 1987. Palytoxin as the causative agent in the parrotfish poisoning. In: Gopalakrishnakone, P., Tan, C. K. (eds.). pp. 325-335. Progress in Venom and Toxin Research, National University of Singapore.
3. Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H. and Yasumoto, T. 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37: 55-65.
4. Taniyama, S., Mahmud, Y., Terada, M., Takatani, T., Arakawa, O. and Noguchi, T. 2002. Occurrence of a food poisoning incident by palytoxin from a serranid *Epinephelus* sp. in Japan. J. Nat. Toxins 11: 277-282.
5. Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y. and Noguchi, T. 2003. *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42: 29-33.
6. Lin, W. F. and Hwang, D. F. 2008. Application of

species-specific PCR for the identification of dried bonito product (Katsuobushi). Food Chem. 106: 390-396.
7. O'Reilly, P. and Wright, J. M. 1995. The evolving technology of DNA fingerprinting and its application to fisheries and aquaculture. J. Fish Biol. 47: 29-55.
8. 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.
9. Knowlton, N. 2000. Molecular genetic analyses of species boundaries in the sea. Hydrobiologia 420: 73-90.
10. Rokas, A., Ladoukakis, E. and Zouros, E. 2003. Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol. 18: 411-417.
11. Abdel-Rahman, S. M. and Ahmed, M. M. M. 2007. Rapid and sensitive identification of buffalo's, cattle's and sheep's milk using species-specific PCR and PCR-RFLP techniques. Food Control 18: 1246-1249.
12. Infante, C., Crespo, A., Zuasti, E., Ponce, M., Perez, L., Funes, V., Cananese, G. and Manchado, M. 2006. PCR-based methodology for the authentication of the Atlantic mackerel *Scomber scombrus* in commercial canned products. Food Res. Int. 39: 1023-1028.
13. 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.
14. Pfeiffer, I., Burger, J. and Brenig, B. 2004. Diagnostic polymorphisms in the mitochondrial cytochrome b gene allow discrimination between cattle, sheep, goat, roe buck and deer by PCR-RFLP. BMC Genet. 5: 30-34.
15. Spaniolas, S., May, S. T., Bennett, M. J. and Tucker, G. A. 2006. Authentication of coffee by means of PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis. J. Agric. Food Chem. 54: 7466-7470.
16. Calvo, J. H., Zaragoza, P. and Osta, R. 2001. A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. J. Anim. Sci. 79: 2108-2112.
17. Pascoal, A., Barros-Velázquez, J., Cepeda, A., Gallardo, J. M. and Calo-Mata, P. 2008. Survey of the authenticity of prawn and shrimp species in commercial food products by PCR-RFLP analysis of a 16S rRNA/tRNA^{Val} mitochondrial region. Food Chem. 109: 638-646.
18. Ram, L. J., Ram, M. L. and Baidoun, F. F. 1996. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. J. Agric. Food Chem. 44: 2460-2467.
19. Chen, T. Y., Hsieh, Y. W., Tsai, Y. H., Shiau, C. Y. and Hwang, D. F. 2002. Identification of species and measurement of tetrodotoxin in dried dressed fillets of the puffer fish, *Lagocephalus lunaris*. J. Food Prot. 65: 1670-1673.
20. Hsieh, Y. W., Hwang, P. A., P. A., Pam, H. H., Chen, J. B. and Hwang, D. F. 2003. Identification of tetrodotoxin and fish species in an adulterated dried mullet roe implicated in food poisoning. J. Food Sci. 68: 142-146.
21. Hwang, D. F., Hsieh, Y. W., Shiu, Y. C., Chen, S. K. and Cheng, C. A. 2002. Identification of tetrodotoxin and fish species in dried dressed fish fillet implicated in food poisoning. J. Food Prot. 65: 389-392.
22. Wu, Y. J., Hsieh, C. H., Chen, H. M. and Hwang, D. F. 2008. Identification of six common species of processed filefish using cytochrome b gene sequence and PCR-RFLP analysis. Raffles Bull. Zool. 19: 151-158.
23. Yasumoto, Y. 1991. Tetrodotoxin. In: Environmental Health Bureau, Ministry of Health and Welfare (ed.). pp. 296-300. Standard Methods of Analysis in Food Safety Regulation-Chemistry. Japan Food Hygiene Association. Tokyo.
24. Tsai, Y. H., Hwang, D. F., Cheng, C. A., Hwang, C. C. and Deng, J. F. 2006. Determination of tetrodotoxin in human urine and blood using C18 cartridge column, ultrafiltration and LC-MS. J. Chromat. B 832: 75-80.
25. Wolf, C., Burgener, M. and Lüthy, J. 2000. PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. LWT-Food Sci. Technol. 33: 144-150.
26. Innis, M. A. and Gelfand D. H. 1990. PCR protocols-A guide to methods and applications. pp. 3-12. Academic Press. New York.
27. Hwang, D. F. and Jeng, S. S. 1991. Bioassay of tetrodotoxin by using ICR strain male mice. J. Chin. Biochem. Soc. 20: 80-86.
28. Hashimoto, Y. 1979. Marine Toxins and Other Bioactive Marine Metabolites. pp. 369. Japan Scientific Societies Press. Tokyo.
29. Antointte, J. P. and Grag, S. S. 2001. Molecular phylogeny of the Chipmunks inferred from mitochondrial cytochrome band cytochrome oxidase II gene sequences. Mol. Phylogenet. Evol. 20: 335-350.
30. Unseld, M., Beyermann, B., Brandt, P. and Hiesel, R. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Methods Appl. 4: 241-243.
31. Chen, Y. J., Huang, K. M., Jen, H. C., Su, M. W., Liu, S. M. and Hwang, D. F. 2009. Bighead carp meat adulterated in commercial milkfish ball products. J. Food Drug Anal. 17: 178-182.
32. Hwang, D. F., Jen, H. C., Hsieh, Y. W. and Shiau, C. Y. 2004. Applying DNA techniques to the identification of the species of dressed toasted eel products. J. Agric. Food Chem. 52: 5972-5977.