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Effect of Processing on Sequence of Cytochrome B Gene and Its Restriction Site in the Meat of Puffer *Takifugu rubripes*

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ABSTRACT

The polymerase chain reaction (PCR) and direct sequence analysis were used to determine the amount of genetic variation in a 376-nucleotide region of the mitochondrial cytochrome *b* gene in fresh, frozen and steam sterilized meats of puffer *Takifugu rubripes*. a diversity of sequence between fresh, frozen and steam sterilized meats was not found. The puffer specimens collected from either culture ponds in Taiwan or wild seawaters and culture waters in Japan had the same sequence of 376-nucleotide region. Restriction enzyme *Bst*ZI was found to cut the amplified region of cytochrome *b* gene in fresh, frozen and steam sterilized meats of puffer *T. rubripes*, while restriction enzyme *Aat*II did not. This indicates that the sequence and restriction site analyses might be used to authenticate species of different processed meats of puffer *T. rubripes*.

Key words: processing, cytochrome *b* gene, polymerase chain reaction, puffer, *Takifugu rubripes*

INTRODUCTION

The puffer *Takifugu rubripes* ("torafugu" in Japanese) is a favorite fish species in Japan. Although the wild puffer contains considerable amounts of tetrodotoxin (TTX) in the liver and ovary[(1)], the cultured fish is non-toxic or less toxic[(2,3)]. Due to an increase in the consumption of *T. rubripes* in Japan, the amount of cultured torafugu is increasing in Japan and in countries such as Mainland China and Korea.

Recently, *T. rubripes* has been cultured and sometimes consumed at high-class restaurants in Taiwan[(3)]. The fry and fertilized eggs were provided from Japan. Usually, *T. rubripes* is consumed as raw fish slices ("sashimi" in Japanese) or cooked fish slices with rice. Because *T. rubripes* is a highly valuable economic species and it is very rare in Taiwan, other puffer species may be used as a substitute. Hwang *et al.*[(4)] pointed out that most species of Taiwanese puffer were toxic in the muscles. The use of toxic puffer can produce a problem of serious food poisoning.

Usually, the species of raw fish is easily identified by the use of isoelectric focusing (IEF) of water-soluble proteins, with specific profiles being obtained by IEF. However, when elaboration processes include a certain degree of thermal treatment, such as cooking and frying, the water solubility of these proteins is irreversibly lost, necessitating the use of alternative approaches to identify species in such products[(5,6,7)]. Recently, it has been shown that analysis of mitochondrial DNA was successful in differentiating species of raw and processed fish[(8,9,10,11)]. There have also been several papers describing the polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome *b* gene as useful for identification of fish species in raw and processed fish[(12,13,14,15)]. Therefore, we compared the variations of nucleotide sequence and restriction site of the cytochrome *b* gene in different processed meats of puffer *T. rubripes*.

MATERIALS AND METHODS

I. *Material*

Four specimens of puffer *Takifugu rubripes* were collected from an aquaculture farm in Taipei County and immediately transferred to the laboratory on ice. Two specimens were dissected, and the fresh meat was removed. A portion of fresh meat was used for DNA extraction. Another portion of fresh meat was sterilized at 121˚C for 15 min in autoclave and then used for DNA extraction. The other two specimens were frozen at -20˚C for 1 month, and the meat was then taken out for DNA extraction. For comparison, we also analyzed Japanese specimens obtained from culture ponds, the Japanese Sea, and Japanese areas of the Pacific Ocean.

II. *DNA Extraction*

Total cellular DNA was extracted from the meat of

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puffer *T. rubripes* essentially according to DeSalle and Birstein[9]. Briefly, about 1 g puffer muscle was homogenized with extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 1% SDS, 0.2 M NaCl) and 50 μL of 5 mg/mL proteinase K (Ameresco, Solon, OH, USA) were added. The samples were incubated overnight at 55˚C with shaking. After incubation, tubes were placed on ice for 30 min, centrifuged at 12,000 x g for 10 min and supernatant transferred to a clean tube, DNA was extracted once with phenol, twice with phenol-chloroform-isoamylalcohol in a 25:24:1 ratio and once with chloroform, and then precipitated twice with ethanol at -20˚C. The dried pellets were resuspended in 50-100 μL sterile distilled water and the concentration of DNA was estimated by absorbance at 260 nm.

III. *PCR Amplification of a Fragment of the Cytochrome b Gene*

The PCR amplification reactions were performed in a total volume of 100 μL. Each reaction mixture contained 1 pg of extracted template DNA, 0.4 μM of each primer, 200 μM of each dNTP and 2.5 U of Pro Taq DNA polymerase (Amresco) in a reaction buffer containing 20 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 1% Triton X-100, 0.1 mM DTT and 50% glycerol.

The polymerase chain reaction was carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA) programmed to perform a denaturation step of 95˚C for 10 min, followed by 40 cycles consisting 1 min at 95˚C, 1 min at 50˚C and 2 min at 72˚C. The last extension step was 10 min longer.

The set of primers used for PCR amplification were designated L14841: 5'AAAAAGCTTCCATCCAACCAACATCTCAGCATGATGAAA-3' and H15149: 5'AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'. These primers corresponded with those described by Kocher *et al*[16].

The products of PCR amplification were analyzed by agarose gel electrophoresis. PCR products (5 μL) were mixed with 1 μL gel loading solution and loaded in a 1.2% agarose gel containing 1 μg/mL ethidium bromide in Tris-Borate-EDTA (TBE) buffer. Electrophoretic separation and DNA fragments were operated at 100 V for 60 min.

IV. *Cleanup and Sequencing of the PCR Products*

PCR product (60 μL) was loaded onto a 2% agarose gel containing 1 μg/mL ethidium bromide in TBE buffer and electrophoresed at 50 V for 120 min. The DNA band was excised under UV light and melted in 5 volumes Tris-EDTA (TE) buffer at 65˚C for 5 min. DNA was extracted twice with phenol, once with phenol: chloroform: isoamylalcohol (25:24:1) and once with chloroform. Finally, the DNA was precipitated with 1/10 volume 3 M sodium acetate and two volumes ethanol. The dried pellet was resuspended in 20 μL sterile distilled water. The concentration and quality of the DNA was estimated by agarose gel electrophoresis of a 2 μL sample.

Purified PCR products from the meat of *T. rubripes* 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 Division, Foster City, CA, USA) in a ABI PRISM 377-96 DNA Sequencer (Perkin-Elemer/ Applied Biosystems Division). Two replicate sequences were obtained from each sample. Sequence analysis was performed using the Wisconsin Package, Version 10 [17].

V. *Restriction Site Analysis of PCR Products*

For the restriction site analysis of the cytochrome *b* region, the PCR products were extracted and purified as described in the fourth paragraph of the materials and methods section. The endonucleases *Bst*ZI and *Aat*II (Promega, Madison, WI, USA) were tested for restriction analysis of the amplified PCR products. Digests were performed in 10 μL volumes with 100-200 ng amplified DNA, 5U enzyme and 1:10 dilution of the manufacturer recommended 10x digestion buffer and BSA. Digestions were incubated for 2 h at 37˚C for *Aat*II or 50˚C for *Bst*ZI. The resulting fragments were separated by electrophoresis in a 2.0% agarose gel containing 1 μg/mL ethidium bromide for 1 h at 100 V. The sizes of the resulting DNA fragment were estimated by comparison with a commercial 100 bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS AND DISCUSSION

The pattern of DNA extracted from fresh, frozen and steam sterilized meats of puffer *T. rubripes* is shown in

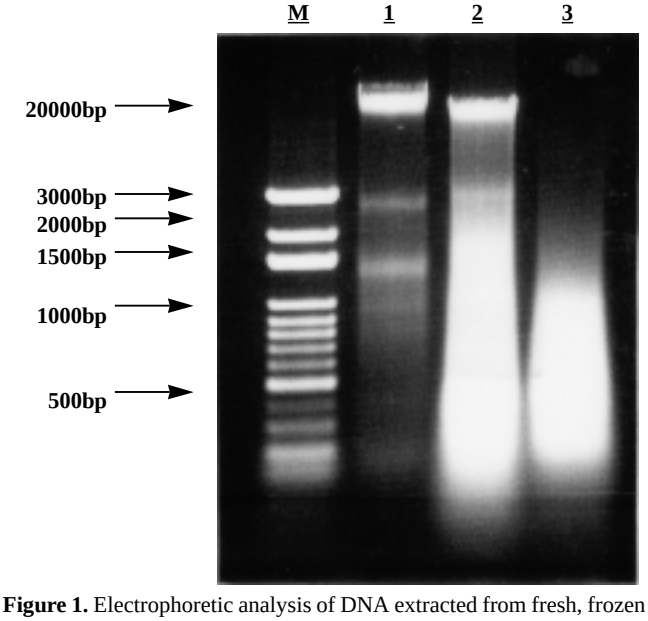

Figure 1. Electrophoretic analysis of DNA extracted from fresh, frozen and steam sterilized meats of puffer *Takifugu rubripes* on 0.8% agarose gel. M, MW marker 100 bp ladder. Samples in lanes are as follows: 1, fresh meat of *T. rubripes*; 2, frozen meat of *T. rubripes* and 3, steam sterilized meat of *T. rubripes*.

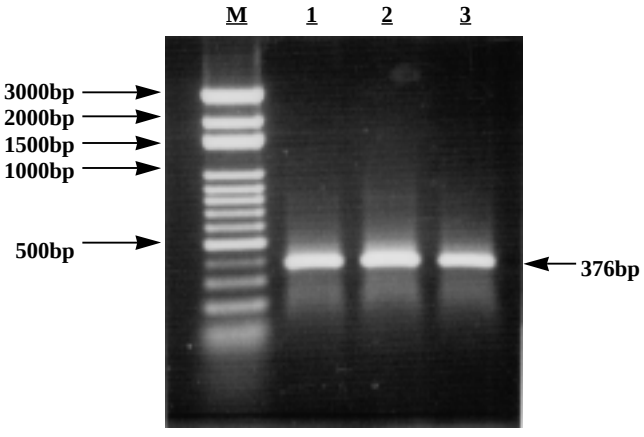

Figure 2. Electrophoreitc analysis of the PCR products from the cytochrome *b* gene of puffer *Takifugu rubripes* on 1.2% agarose gel. M and samples in lanes 1-3: see legend in Figure 1.

Figure 1. One of the main features of DNA extracted from steam sterilized puffer meat is the degradation, produced as a result of thermal treatments employed in the processing of cooked or steam sterilized puffer meat. Frozen sample gave fragments of variable length (20,000-100 bp). In this case, the fragment size of the DNA, which was detected with ethidium bromide, is near 100 bp. The smear of degraded DNA in frozen and steam sterilized puffer meats ranged from 100-3000 bp and 100-1000 bp, respectively.

The DNA extracted from fresh, frozen and steam sterilized puffer meats was tested for amplification using the L1484/H15149 primers, which should produce a 376-bp fragment. The electrophoretic analysis of the PCR products was from fresh, frozen and steam sterilized puffer meats. This means that the proteinase K method appeared to be appropriate for extracting both frozen and thermally steam

```
                1                                                  50
Fresh           AAAAAGCTTCCATCCAACATCTCAGCATGATGAAACTTTGGCTCTCTACT
Frozen          AAAAAGCTTCCATCCAACATCTCAGCATGATGAAACTTTGGCTCTCTACT
Steam sterilized AAAAAGCTTCCATCCAACATCTCAGCATGATGAAACTTTGGCTCTCTACT

               51                                                 100
Fresh           CGGATTATGCCTTATTACACAAATCATCACAGGACTGTTCCTTGCAATAC
Frozen          CGGATTATGCCTTATTACACAAATCATCACAGGACTGTTCCTTGCAATAC
Steam sterilized CGGATTATGCCTTATTACACAAATCATCACAGGACTGTTCCTTGCAATAC

              101                                                 150
Fresh           ACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCACATTTGC
Frozen          ACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCACATTTGC
Steam sterilized ACTACACATCCGACATCTCTACCGCCTTTTCATCCGTAGCCCACATTTGC

              151                                                 200
Fresh           CGAGACGTAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGC
Frozen          CGAGACGTAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGC
Steam sterilized CGAGACGTAAACTACGGCTGACTAATTCGCAATCTACACGCAAACGGTGC

              201                    Bst ZI                       250
Fresh           CTCATTCTTTTTTATTTGCTTATACTCCCACATCGGGCCGAGGTCTTTACT
Frozen          CTCATTCTTTTTTATTTGCTTATACTCCCACATCGGGCCGAGGTCTTTACT
Steam sterilized CTCATTCTTTTTTATTTGCTTATACTCCCACATCGGGCCGAGGTCTTTACT

              251                                                 300
Fresh           ATGGCTCTTACCTAAGTAAAGAAACCTGAAACGTAGGGGTAGTCCTCTTA
Frozen          ATGGCTCTTACCTAAGTAAAGAAACCTGAAACGTAGGGGTAGTCCTCTTA
Steam sterilized ATGGCTCTTACCTAAGTAAAGAAACCTGAAACGTAGGGGTAGTCCTCTTA

              301                                                 350
Fresh           CTTTTAGTAATGGCCACCGCTTTCGTAGGCTACGTTCTCCCATGAGGACA
Frozen          CTTTTAGTAATGGCCACCGCTTTCGTAGGCTACGTTCTCCCATGAGGACA
Steam sterilized CTTTTAGTAATGGCCACCGCTTTCGTAGGCTACGTTCTCCCATGAGGACA

              351                 376
Fresh           AATATCATTCTGAGGGGCTGCAGTTT
Frozen          AATATCATTCTGAGGGGCTGCAGTTT
Steam sterilized AATATCATTCTGAGGGGCTGCAGTTT
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Figure 3. DNA sequences of part of the cytochrome *b* gene from fresh, frozen and steam sterilized meats of puffer *Takifugu rubripes*. *Bst*ZI restriction site is shown with underline. The positions of primers L14841 and H15149 used for PCR amplification are italic.

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sterilized tissues; due to the high lysis power of proteinase K and SDS. Meanwhile, the 376-bp DNA fragment was of adequate size for the amplification of cytochrome *b* gene from fresh, frozen and steam sterilized puffer meats.

The direct PCR sequencing method, using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer, proved to be very useful with frozen and steam sterilized puffer DNA fragments. After comparing these DNA sequences in fresh, frozen and steam sterilize samples, it was observed that all of them were the same (Figure 3). This means that the sequence of 376-bp region of cytochrome *b* gene in the puffer *T. rubripes* was not altered even under freezing and steam sterilizing processes. For comparison, the electrophoretic pattern of extracted DNA and the sequence of 376-bp fragment obtained from each sample of Japanese specimens were the same as those of the Taiwanese samples. Furthermore, the sequence of 376-bp region of cytochrome *b* gene in puffer *T. rubripes* was found to be different from that of other more toxic species, including *T. oblongus*, *T. niphobles* and *Lagocephalus lunaris*[17, 18] which are more toxic puffer species found in Taiwan.

The restriction map of the sequence was searched for selecting endonucleases that could yield two fragments. The restriction enzyme *Bst*ZI was found potentially useful for this purpose (Figure 4). The enzyme *Aat*II might be useful for other puffers *Tetradon fluviatilis* (TFU 2557), *T. nigroviridis* (TNI 248558) and *T. biocellatus* (TBI 248557) based on the sequence of cytochrome *b* gene[19]. Results following digestion of the puffer products showed that band sizes obtained by electrophoresis in 2.0% agarose gel were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis. A single restriction site for *Bst*ZI was found in the sequence of puffer *T. rubripes* products, yielding 2 DNA fragments of 142 and 234 bp. Furthermore, no restriction site for *Bst*ZI was found in the sequences of the other closely related puffer fish species *T. oblongus*, *T. niphobles* and *Lagocephalus lunaris*[17,18]. However, no restriction site for *Aat*II was found in the sequence of puffer *T. rubripes* products, but an undigested PCR product was found. Hence, the utilization of restriction endonuclease

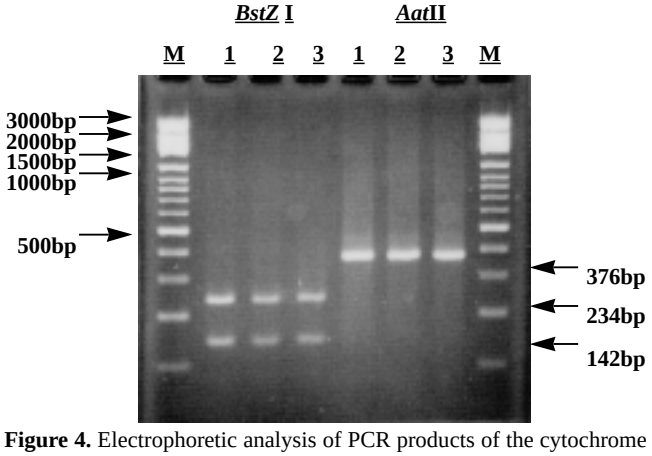

Figure 4. Electrophoretic analysis of PCR products of the cytochrome *b* gene digested with *Bst*ZI and *Aat*II on 2.0% agarose gel. M and samples in lanes 1-3: see legend in Figure 1.

*Bst*ZI in the PCR product of cytochrome *b* gene in the puffer *T. rubripes* may be useful for identifying the puffer *T. rubripes* species.

As described by Unseld *et al.*[11], the cytochrome *b* gene as a molecular marker for investigating phylogenetic relationships within vertebrates is useful for several reasons. First, because of the maternal inheritance of mitochondria, normally only one allele exists per individual and thus no sequence ambiguities are to be expected from the presence of more than one allele. Second, the high abundance of mitochondrial DNA in total cellular nucleic acid preparations allows for more effective PCR amplifications in comparison to the nuclear-encoded, single-copy gene. Third, in vertebrates the mutation rate of mitochondrial genes is nearly 10-fold higher compared to nuclear genes. Thus, point mutations accumulate quickly enough to allow (in most cases) the discrimination of even closely related species. Here, we found that direct sequence analysis and restriction enzyme may be applicable in identifying the same species of Japanese and Taiwanese puffer *T. rubripes*, as well as fresh, frozen and processed samples. The data of gene base in the cytochrome *b* gene of puffer are still few[17,18,19,20,21]. To perfectly apply PCR and direct sequence analysis of cytochrome *b* gene on identification of puffer species, the more puffer species need to be further studied.

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加工對虎河魨肌肉中細胞色素*b*基因序列和限制酶切位之影響

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摘　要

以PCR技術及直接定序分析新鮮、冷凍、蒸煮殺菌之虎河魨中粒線體DNA之cytochrome *b* gene部分片段（376 bp）序列，結果發現新鮮、冷凍及蒸煮殺菌之虎河魨魚肉中，此段序列並無差異。同時台灣產養殖個體和日本產野生或養殖個體之376 bp序列亦無差異度。顯示虎河魨有其獨特之基因型態。經由限制酶*Bst*ZI和*Aat*II作用於新鮮、冷凍、蒸煮殺菌之虎河魨中上述基因序列，發現限制酶*Bst*ZI均有相同切位，可將其切割成相同兩段，而限制酶*Aat*II並無切位。由此顯示限制酶切位和直接定序分析似乎可做為新鮮、冷凍、蒸煮殺菌之虎河魨魚種鑑別方法。

關鍵詞：加工，細胞色素*b*基因，聚合酶連鎖反應，河魨，虎河魨