THE RAFFLES BULLETIN OF ZOOLOGY 2008 Supplement No. **19**: 115–122
Date of Publication: 15 Dec.2008

IDENTIFICATION OF SPECIES AND PARALYTIC SHELLFISH POISONS IN AN UNKNOWN SCALLOP MEAT IMPLICATED IN FOOD POISONING IN TAIWAN

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***ABSTRACT.* –** A paralytic shellfish poisoning caused by meat from an unknown scallop occurred in South Taiwan. Scallop meat residue retained by the victim was assayed for toxicity and mitochondrial DNA. The scallop meat toxin was of low concentration and identified by HPLC-FLD as saxitoxin. The 16S rRNA gene sequence of the unknown scallop meat was determined and PCR-RFLP analyses of the meat were completed. When compared with the well-known scallop species *Atrina pectinata*, *Amusium pleuronectes* and *Mizuhopecten yessoensis*, the 16S rRNA gene sequence and PCR-RFLP analyses showed a match with *Atrina pectinata*. This is the first report in which the scallop *Atrina pectinata* has been found to contain saxitoxin and to have caused a food poisoning incident.

***KEY WORDS.* –** Paralytic shellfish poisons, PCR-RFLP, HPLC-FLD.

INTRODUCTION

Paralytic shellfish poisons (PSP) are responsible for the most severe human seafood poisoning worldwide (Sierra-Beltran et al., 1998; Anderson et al., 1996; Lehane et al., 2001; Hwang et al., 2003; Garcia et al., 2004; Asakawa et al., 2005; Wu et al., 2005; Jen et al., 2007). It is well known that PSP is produced by toxic marine dinoflagellates, including three morphologically distinct genera of saltwater dinoflagellates: *Alexandrium* spp. (Hashimoto et al., 2002), *Pyrodinium* spp. (Harada et al., 1982) and *Gymnodinium* spp. (Anderson et al., 1989). The paralytic shellfish poisoning caused by eating toxin-contaminated bivalves (scallops, mussels, oysters and clams) has been reported (Hallegraeff, 1993; Hwang et al., 1995; Hashimoto et al., 2002; Hwang et al., 2003; Jiang et al., 2003; Yen et al., 2004; Jiang et al., 2006). The contaminated bivalves are commonly sold live and can be identified by morphologically (Garland & Zimmer, 2002) However, it is difficult to identify the bivalve species when they are sold as processed products. Once food poisoning has occurred, high performance liquid chromatography (HPLC) (Hwang et al., 1999; Jen et al., 2007) may be used to detect PSP toxins. In order to prevent further cases of poisoning, the species and source of the toxic bivalves should be determined as soon as possible.

Recently, techniques for the identification of mitochondrial DNA (mtDNA) have proved successful in differentiating species of bivalves for evolutionary investigations. The high number of copies, simplicity of isolation, lack of recombinant events, conservation of sequences and structures across metazoan as well as mutational rates in other regions have all contributed to this success (Fernandez et al., 2000; Smith & Snyder, 2007). Several papers describing RAPD (random amplified polymorphic DNA) (Heipel et al., 1998), PCR (polymerase chain reaction) (Aranishi & Okimoto et al., 2003) and PCR-RFLP (PCR with restriction fragment length polymorphisms) (Lapegue et al., 2002; Zhang et al., 2005) have indicated that the 16S rRNA genes are useful identification tools for bivalve species.

A case of paralytic shellfish poisoning caused by unidentified scallop meat occurred in South Taiwan in Aug.2007. Two victims (a couple, both 59 years old) purchased scallop meat from a traditional market in Tainan City. One and a half hours after consumption of the scallop meat, the couple exhibited symptoms of paralytic shellfish poisoning which included paresthesia of the lips and tongue, temporary dizziness, nausea, vomiting and blurred vision. The victims were discharged from the hospital after one day of treatment and are now healthy.

This study was undertaken to identify toxin components in fresh scallop meats that might be responsible for such a PSP event. Application of PCR amplification and analyses of mtDNA sequence by use of restriction enzyme were used to identify unknown scallop meat implicated in food poisoning with comparison to live scallop specimens.

MATERIALS AND METHODS

***Materials*. –** Eight samples (5.7 ± 0.3 g, mean ± SD) of causative scallop raw meat were obtained from the food poisoning victims in Tainan City, South Taiwan in Aug.2007. To identify the species, 6 specimens each of three scallop species, *Atrina pectinata* (comb pen shell), *Amusium pleuronectes* (Asia moon scallop) and *Mizuhopecten yessoensis* (giant ezo scallop), were purchased from seafood markets in North Taiwan in the summer of 2007. All of three species were morphologically identified based on their external characteristics by Dr. Wen-Lung Wu (Research Center for Biodiversity, Academia Sinica, Taipei). All samples were stored at –20°C until use. Authentic TTX, 4-*epi* TTX and anhydrotetrodotoxin (anh-TTX) were obtained from Wako Pure Chemical Industry (Tokyo, Japan). Authentic PSP components GTX1-4 isolated and purified from the purple clam, *Soletellina diphos*, in Taiwan were used (Hwang et al., 1987). Authentic saxitoxin (STX) and neoSTX obtained from the crab *Zosimus aeneus* in Japan (Daigo et al., 1988) were kindly provided by Professor O. Arakawa, Nagasaki University, Japan. These authentic toxins have their identity verified and purity checked according to the method proposed by Sullivan & Iwaoka (1983). The calibration curves of these toxins have been checked by NRC CRM-STXdiAc agent (National Research Council Canada, Nova Scotia, Canada). The toxins were kept separately in several small black tubes as stock solutions (180 µg/ml), freeze-dried and stored at –70°C. Among them, one stock toxin was dissolved in 1 ml of 0.03 M acetic acid and kept at –20°C until use.

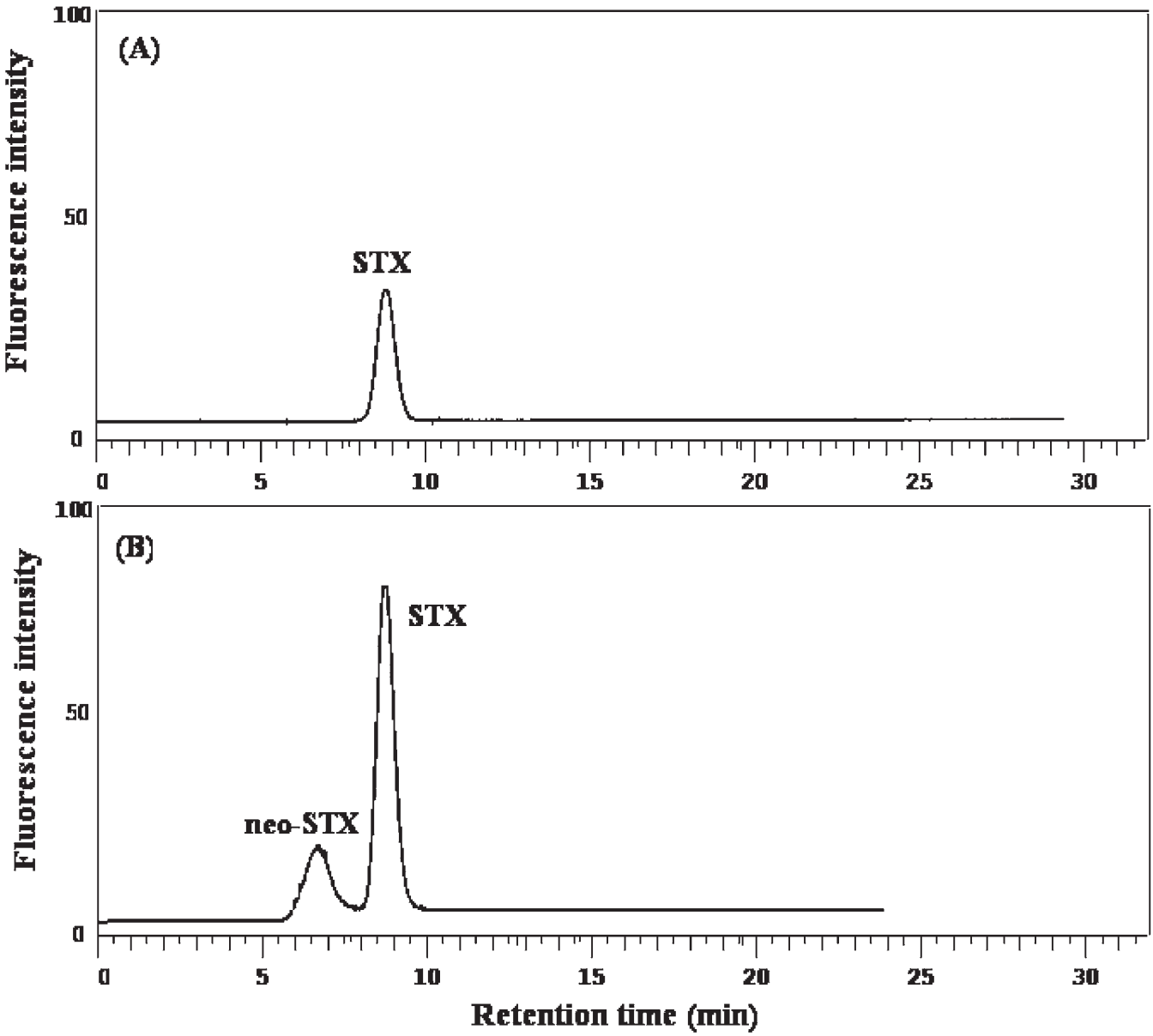

Fig. 1. HPLC chromatography of STX from: A, causative scallop meat; B, authentic STX and neo-STX (B).

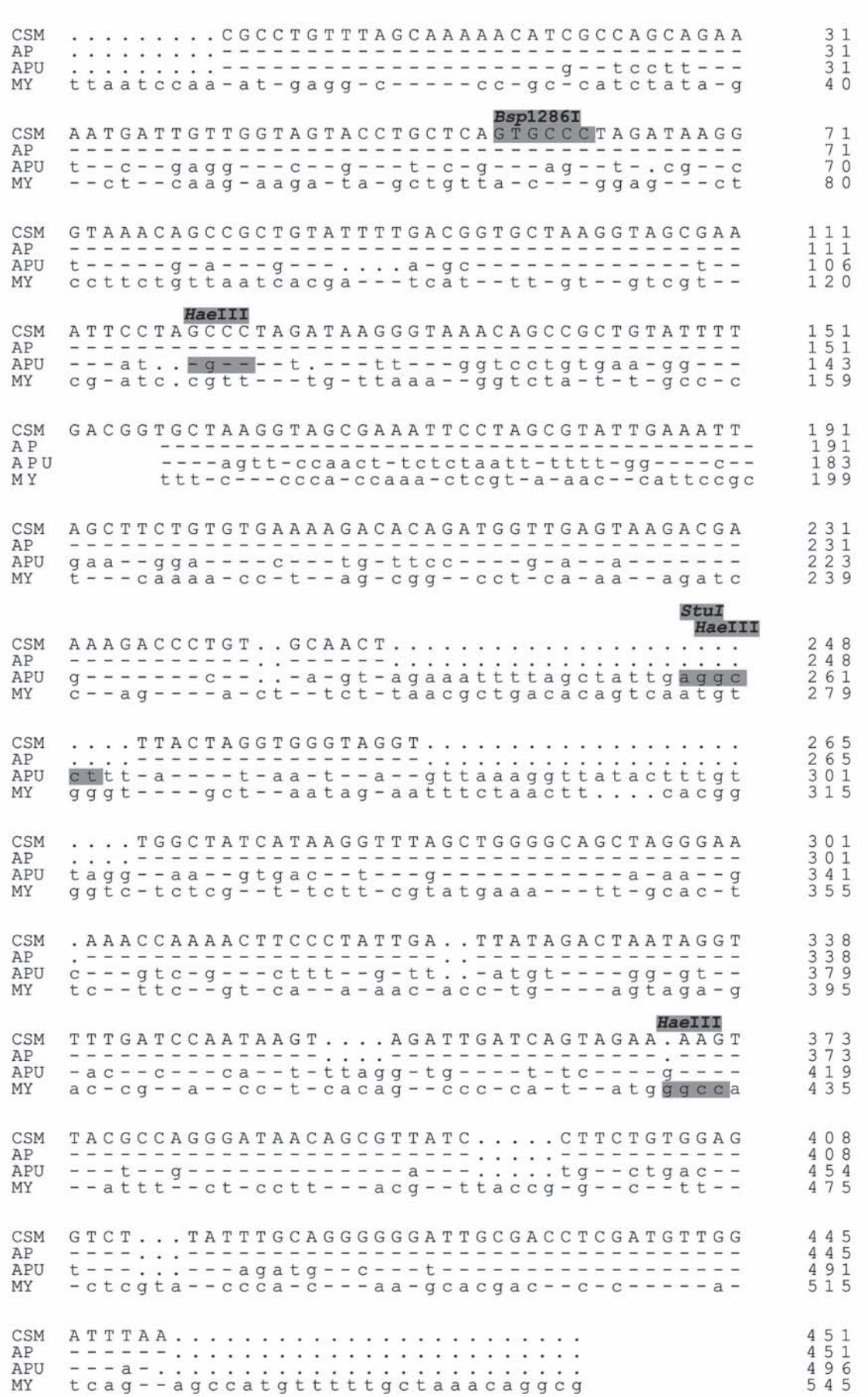

Fig. 2. The partial 16S rRNA of consensus fragment in causatives scallop meat and 3 scallop species amplified with primer set 16SL22/R21. Samples in lane are as follows: CSM, causative scallop meat; AP, *Atrina pectinata*; APU, *Amusium pleuronectes*; MY, *Mizuhopecten yessoensis*; M, 100 bp ladder. Restriction sites of *Bsp*1286 I (5'–GTGCC▼C–3'), *Stu* I (5'–AGG▼CCT–3') and *Hae* III (5'–GG▼CC–3') are labeled as a box, and grey background, respectively.

Extraction and purification of toxin. **–** Scallop meat samples were each mixed and homogenized for 5 min with 3 volumes of 1% acetic acid in methanol and centrifuged (20,000 *g*, 20 min). The operation was repeated twice again. The supernatants were combined, concentrated under reduced pressure at 45°C and examined for toxin as follows: samples were immediately centrifuged at 10,000 *g* for 10 min and the supernatant passed through a cartridge column (C18 Sep-Pack cartridges, Millipore, Waters, MA, USA), previously regenerated with 10 ml methanol and equilibrated with 10 ml water. Toxin absorbed in the column was eluted using 10 ml methanol, resulting in pigments being retained in the column. The eluate was freeze-dried, dissolved in 2 ml of 0.5% acetic acid and filtered through a 3,000 MW cut-off Ultrafree microcentrifuge filters (Micron YM-3, Millipore, Waters, MA, USA). The filtrate was freeze-dried, made to the volume of 1 ml and submitted for subsequent analysis (Andrinolo et al., 1999).

HPLC-FLD analysis for PSP and TTX. **–** All chemicals and solvents used were HPLC or analytical grade. Reversed phase HPLC (L-2100, Hitachi Ltd., Tokyo, Japan) was performed on a reversed-phase column (Merck Lichromsper 100 RP-18, 4 mm I.D. × 20 cm; E. Merck, Darmstadt, Germany) with a fluorescence detector (F-1000, Hitachi Ltd., Tokyo, Japan). For PSP analysis, the mobile phase consisted of sodium 1-heptanesulfonate (2 mM) as an ion-pairing reagent, with 10 mM ammonium phosphate (pH 7.1) for GTXs group and sodium 1-heptanesulfonate (2 mM) in 30 mM phosphoric acid (pH 7.1) containing 5% of acetonitrile for STXs group, pumped at a flow rate of 0.6 ml/min. In all cases the eluate from the column was continuously oxidized with periodic acid (7 mM) in 50 mM potassium phosphate buffer (pH 9.0) while passing through teflon tubing followed by heating at 90°C for 0.5 min and then mixed with an equal volume of acidifying reagent (0.5 M acetic acid) before entering a fluorescence detector. The intensity of fluorescence was measured at 330 nm with 390 nm excitation (Oshima, 1995). The mobile phase for TTX analysis was sodium 1-heptane sulfonate (2 mM) in methanol (1%)-potassium phosphate buffer (0.05 M, pH 7.0). The TTX was detected by mixing the eluate with 3 M NaOH at a ratio of 1:1, followed by heating at 99°C for 0.4 min and monitoring of the fluorescence at 505 nm with 381 nm excitation (Noguchi et al., 1981). Toxin concentration in the scallop extract was determined by comparing the peak area of each of the individual toxins.

DNA extraction. **–** Total DNA was extracted by using the magnetic bead technique with the Chemagic DNA Tissue 10 Kit (Chemagen, Baesweiler, Germany) according to the manufacturer's recommendations. About 100 g of sample meat 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 twice with different washing buffer. The magnetic beads were removed from the solution and the genomic DNA was eluted in 50 µl of elution buffer. The concentration of DNA was estimated by absorbance at 260 nm (Lin & Hwang, 2008).

Partial mitochondrial 16S rRNA gene fragment amplification. **–** The universal primers used were shown as complementary to conserved regions of mitochondrial 16S rRNA gene in molluscs (Jiang et al., 1997). The primer sequences used were as follows: 16SL22 (5'–TTA ATC CAA CAT CGA GGT CGC–3') and 16SR21 (5'–CGC CTG TTT AGC

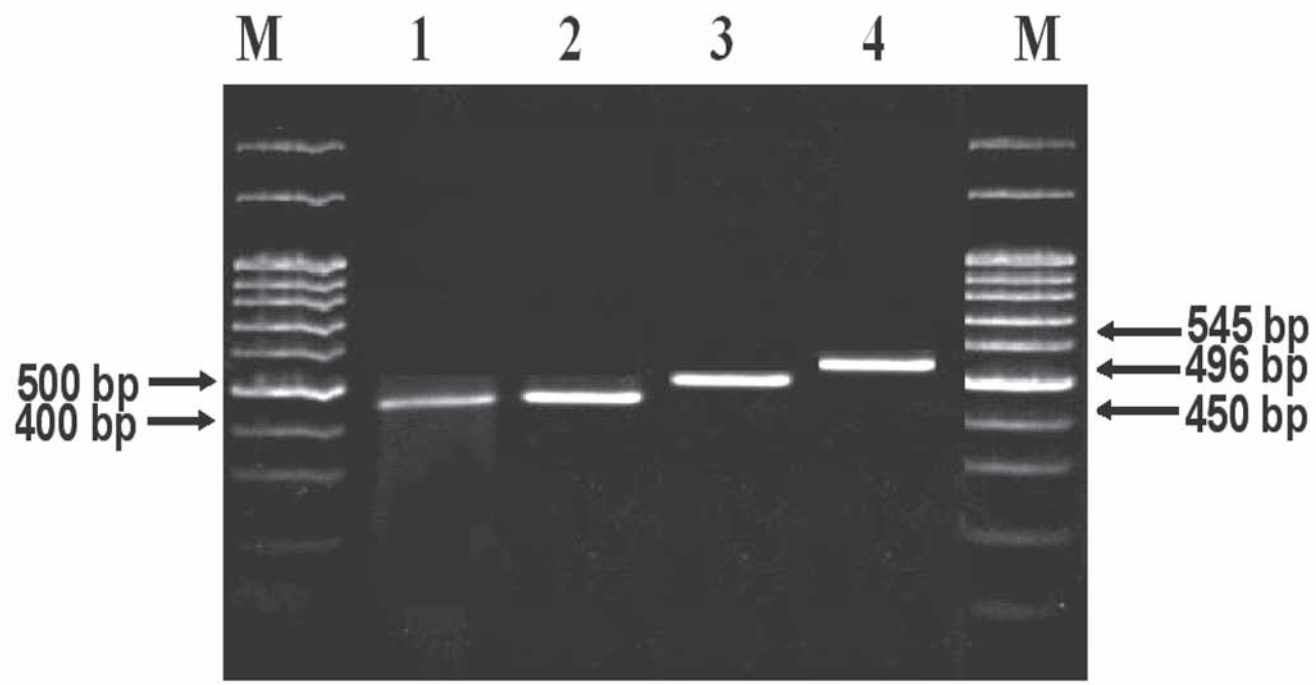

Fig. 3. PCR products from causative scallop meat and three scallop species using the 16SL22/R21 primers. Electrophoresis was performed on 1.2% agarose gels and stained with ethidium bromide. Samples in lane are as follows: 1, causative scallop meat; 2, *Atrina pectinata*; 3, *Amusium pleuronectes*; 4, *M. yessoensis*; M, 100 bp ladder.

AAA AAC AT–3'). The PCR amplification reactions were performed in a total volume of 100 ml. Each reaction mixture contained 100 ng of extracted template DNA, 0.2 mM of each primer, 200 mM of each dNTP and 2.5 U of Pro Taq DNA polymerase (Amresco, Solon, Ohio, USA) in a reaction buffer containing 20 mM Tris–HCl, pH 8.0, 1.5 mM $MgCl_2$, 1% Triton X-100 and 0.1 mM dithiothreitol (DTT). PCR reaction was carried out in a Gene-Amp PCR system 2400 (Perkin Elmer, Foster City, Calif., U.S.A.) programmed to perform a denaturation step of 95°C for 10 min, followed by 30 cycles consisting of 1 min at 95°C, 1 min at 50°C and 2 min at 72°C. The last extension step was extended by an additional 10 min.

***DNA purification and sequencing*. –** Six microliters of PCR product and 1µl of loading dye were mixed and loading dye were mixed and loaded onto a 2% agarose gel containing 1µg/ml ethidium bromide, then electrophoresis was run in TBE buffer (0.089 M Tris; 0.089 M boric acid; 0.002 M EDTA, pH 8.0) 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 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) (Lin & Hwang, 2008).

***Endonuclease digestion and restriction fragment length analysis*. –** After analyzing consensus sequences of partial mitochondrial 16S rRNA gene of three scallop species, restriction maps were constructed in GCG system (Lin & Hwang, 2008). The restriction endonuclease enzyme *Bsp*1286 I, *Stu* I and *Hae* III (Promega, Madison, WI, USA) were searched with the GCG system by inputting out sequences and tested by restriction analysis of the amplified PCR products as determined for this study. Each digestion was performed in 10µl of mixture that contains 100 ng PCR Product, 5 U restriction endonuclease, 1:10 dilution of bovine serum albumin and 10X digestive buffer. Digestive reactions were incubated at optimal assay temperature (37°C) for 2 hr. The resulting fragments were separated by electrophoresis in a 2.0% agarose gel containing 10 µg/ml ethidium bromide for 1 hr at 100 V. The sizes of the resulting DNA fragments were estimated by comparison with a commercial 100 bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS

***Toxin identification*. –** The remaining samples of scallop meat were assayed for toxin by HPLC-FLD. The intensity of fluorescence of the toxic sample in HPLC chromatography was substituted by the amount of toxin according to the method of Sullivan & Iwaoka (1983). Using the mole concentration percentage conversion method to calculate the toxin equivalents, calibration curves of saxitoxin (STX) were used to calculate the respective toxin concentrations in the sample extracts. The chromatography profiles of standard STX and its analogues neo-STX are shown in Fig. 1A. Sample detection limits of STX toxin was determined from calibration data and sample extract volumes from post-column derivation liquid chromatographic method by Oshima (1995), provided the higher sensitivity of detection. The detection limit of STX was 0.02 µg/ml and the calibration curve was linear in the range of 0.2–100.0 µg/ml (Y = 7.38X + 11.24,

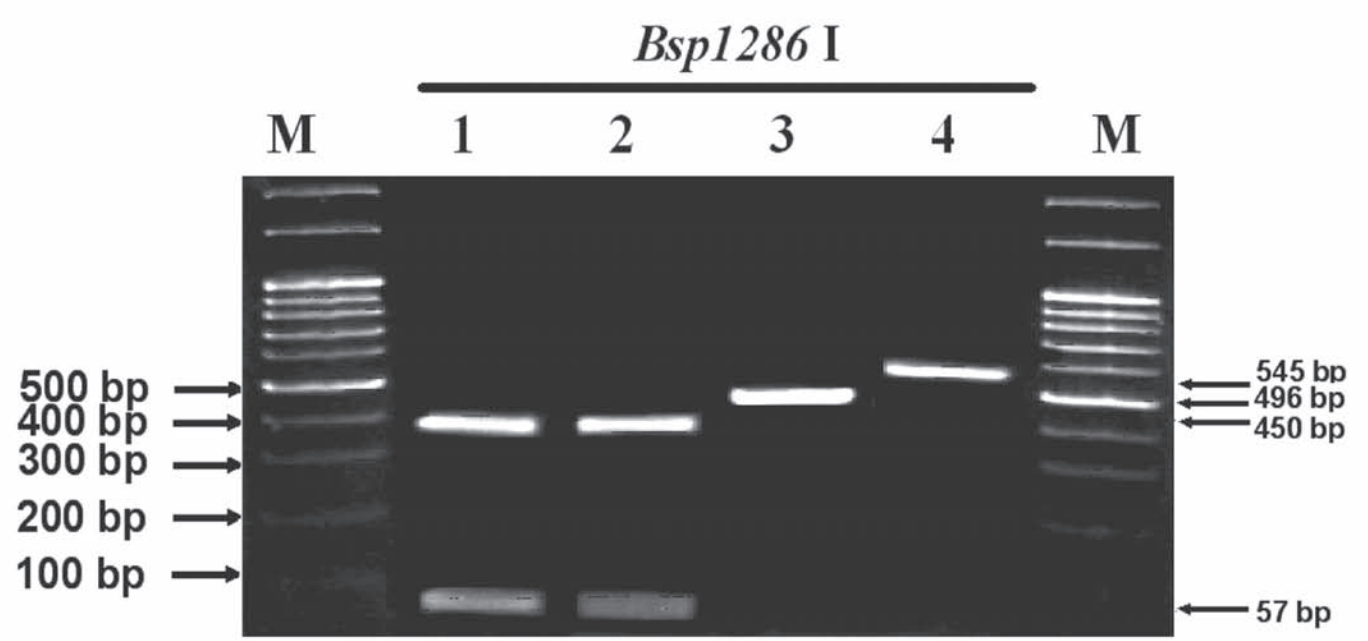

Fig. 4. Electrophoretic analysis of PCR products of the 16S rRNA gene digested with *BSP*1286 I on 2% agarose gel. Samples in lane are as follows: 1, causative scallop meat; 2, *Atrina pectinata*; 3, *Amusium pleuronectes*; 4, *M. yessoensis*; M, 100 bp ladder.

Table 1. Lengths of restriction fragments obtained the PCR products of causative scallop meat and three scallop species by using the *Bsp1286* I, *Stu* I and *Hae* III restriction enzymes.

Species	*Bsp1286* I	*Stu* I	*Hae* III
Atrina pectinata	57+393 bp	450 bp	450 bp
Amusium pleuronectes	496 bp	258+238 bp	237+147+112 bp
Mizuhopecten yessoensis	545 bp	545 bp	433+112 bp
Causative scallop meat	57+393 bp	450 bp	450 bp

r = 0.999). The toxin content of the causative scallop meat from the food poisoning incident was 21.0 ± 1.2 μg STX/g meat. The retention time of one peak coincided with that of STX (8.8 min) (Fig. 1B). The extracted and purified toxin has specific toxicity of 152 μg STX/mg crude toxin. The major toxic compound was identified as STX. Tetrodotoxin and its related compounds were not found.

Species identification and sequencing analysis. **–** For all Archaeogastropoda, Jiang et al. (1997) found that the pair of primers 16SL22/16SR21 yielded amplicon fragments ranging from 400–600 bp. Approximately 400–600 bp fragments were obtained from the DNA extracts obtained from the meat samples of causative scallop meat and other scallop species (*Atrina pectinata*, *Amusium pleuronectes* and *Mizuhopecten yessoensis*). The sequences were analyzed and submitted to Genebank for accession number DQ873919 (*Amusium pleuronectes*) and AB103394 (*Mizuhopecten yessoensis*). Compared with data from Gene Bank (Natl. Center for Biotechnology Information, NCBI), the obtained fragments were encoded on 16S rRNA gene mtDNA (Fig. 2). However, the sequence of the 450 bp region of the 16S rRNA gene in *Atrina pectinata* in this study is the first report for this species. The *Atrina pectinata* gene was more similar to the *Amusium pleuronectes* gene (identity = 62.4%) than to *Mizuhopecten yessoensis* gene (identity = 34.3%). Electrophoretic analysis of the PCR products from causative scallop meat and the scallop species of *Atrina pectinata*, *Amusium pleuronectes* and *Mizuhopecten yessoensis* had 450, 496 and 545 bp fragments, respectively (Fig. 3). The species of causative scallop meat was, according to the size and sequence of PCR product fragments, identified as *Atrina pectinata*.

PCR-RFLP analysis. **–** After digestion of the PCR products by using three restriction endonucleases, different band sizes were obtained from these well-known scallop species. A single restriction site for endonuclease *Bsp*1286 I was found in the sequences of PCR products of *Atrina pectinata*, resulting in 2 fragments of 57 and 393 bp (Fig. 4). However, this enzyme did not cleave *Amusium pleuronectes* and *Mizuhopecten yessoensis* amplicons. Restriction enzyme *Stu* I could cleave 496 bp fragment of *Amusium pleuronectes* into 258 and 238 bp, and there was no cutting site in *Atrina pectinata* and *Mizuhopecten yessoensis*. Finally, the *Hae* III could cleave the 496 bp fragment of *Amusium pleuronectes* into 237, 147 and 112 bp, and the 545 bp fragment from *Mizuhopecten yessoensis* into 433, 112 bp (Fig. 5). There

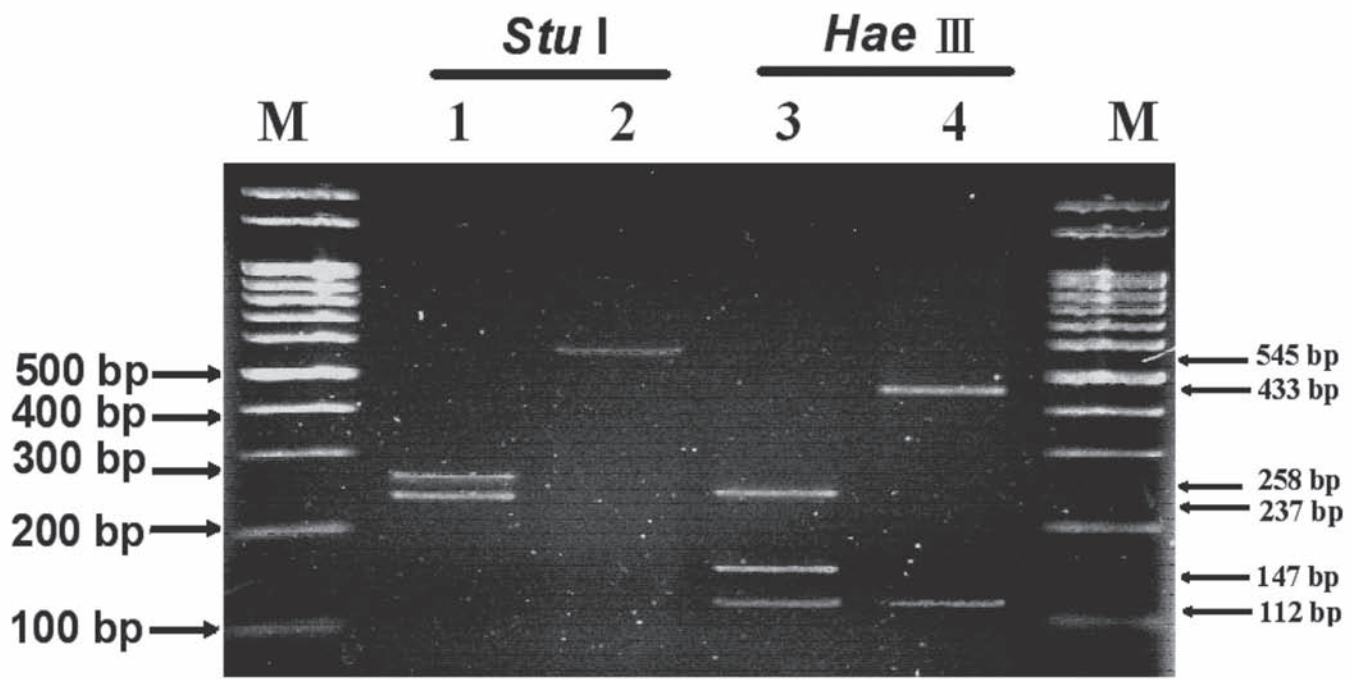

Fig. 5. Restriction profiles of the 16S rRNA gene PCR products from two scallop species digested with *Stu* I and *Hae* III on 2% agarose gel. Samples in lane are as follows:1 & 3, *Amusium* pleuronectes; 2 & 4, *Mizuhopecten yessoensis*; M, 100 bp ladder.

was no cutting site in *Atrina pectinata* (data not shown). The sizes of fragment lengths cleaved by each restriction enzyme are compiled in Table 1 and the cutting sites of restriction enzymes in the sequences for each species are labeled in Fig. 2. The results of the PCR-RFLP analysis indicated that the causative scallop meat was from the species *Atrina pectinata*.

DISCUSSION

The first foodborne case of PSP which aroused Taiwanese attention was caused by the purple clam *Soletellina diphos* in Pingtung County in Jan.1986 (Hwang et al., 1987). One hundred sixteen people were intoxicated and there were two deaths. Later, a bloom of the PSP-producing dinoflagellate *Alexandrium minutum* was found in the inland aquaculture ponds (Hwang et al., 1999). Another food poisoning incident due to ingestion of the same purple clam species, *S. diphos*, occurred in Chaiyi County in Feb.1991. There were 7 patients but no deaths were reported (Hwang et al., 1992).

STX is one of the most potent neurotoxins. The amount of STX (21.0 ± 1.2 μg STX/g meat) in causative scallop meat was detected by HPLC-FLD. The intoxication dose in humans ranges from 200 to 1,000 μg of STX and the lethal dose is at 600–6,000 μg of STX (Morse et al., 1977). In most countries the regulatory limit for the safe consumption of shellfish required is set at 80 μg STX /100 g tissue (Lehane, 2001). Saxitoxin was found to block the action potential of sodium channels in nerve and muscle, resulting in nervous signals that could not be transmitted (Yu & Catterall, 2003). The patients ate more than 10 g scallop meat, immediately appeared the paralytic symptoms in mouth lips and stop to eat. These causative scallops were all collected from southern Taiwan where toxic dinoflagellate has been found. However, toxicity of those live specimens collected from North Taiwan was not detected. Hence, these causative scallops might accumulate STX from toxic dinoflagellate.

Wilding et al. (1997) has applied the PCR-RFLP method of mtDNA variation to the identification of the scallop *Pecten maximus*. The mtDNA has been intensively examined by use of sequencing analysis of the entire mtDNA genomes, but this is a time-consuming process and is currently not practical for evolutionary studies (Fernandez et al., 2000). Hosoi et al. (2004) reported that PCR-RFLP could be used to differentiate 14 bivalve mollusks using one restriction endonuclease. Fernandez et al. (2000) also applied PCR-RFLP analysis to the identification of three clam species and used two restriction endonucleases. In this study, PCR-RFLP analysis using *Bsp*1286 I, *Stu* I and *Hae* III restriction enzymes easily and effectively allowed identification of three species of scallop without any need for sequencing or phylogenetic analysis. In addition, due to its larger size and better conserved structure, 16S rRNA has been demonstrated to be suitable for genomic studies and is optimal for interspecific analysis in the molluscs investigated (Hosoi et al., 2004; Smith & Snyder, 2007). The 16S rRNA gene sequence and PCR-RFLP analyses as applied here have provided reliable identification of *Atrina pectinata* as the species of scallop involved in the above-mentioned case of paralytic shellfish poisoning .

ACKNOWLEDGEMENTS

This study was partly supported by the Center for Marine Bioscience and Biotechnology (Grant No. 97529002I2), National Taiwan Ocean University, Taiwan.

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