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Molecular identification of pufferfish species using PCR amplification and restriction analysis of a segment of the 16S rRNA gene☆

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Abstract

This study amplified the mitochondrial 16S rRNA gene using polymerase chain reaction (PCR) with a template of total DNA from muscle tissues of nine pufferfish species collected from the coastal area of Okinawa Islands in Japan: *Pleuranacanthus sceleratus*, *Triodon macropterus*, *Chelonodon patoca*, *Sphoeroides pachygaster*, *Arothron hispidus*, *A. stellatus*, *A. manilensis*, *A. mappa*, and *A. nigropunctatus*. Then nucleotide sequence encoding a partial region of the 16S rRNA gene was compared among species. The sequenced fragment was also used to select restriction enzymes, yielding species-specific restriction fragment length polymorphisms (RFLP). The sequence of the segment of the 16S rRNA gene consisted of about 615 nucleotides and showed interspecies variations in the targeted region. After calculation of corresponding RFLP-patterns of nine species investigated with suitable restriction enzymes, three restriction enzymes – *Ban*II, *Dde*I, and *Nla*III – were found to be sufficient for identification of all nine species. Successful testing of this methodology in frozen and heated food samples suggests its utility for pufferfish species authentication in food products.

Keywords: Identification; PCR; RFLP; Pufferfish; Mitochondrial DNA; 16S rRNA; Tetraodontidae; Triodontidae; Musculature

1. Introduction

Consumer demand for pufferfish in Japan has increased rapidly in recent years; Japanese imports of them are also increasing. Some pufferfish species are known to be toxic, containing large amounts of tetrodotoxin in muscle tissue (Tani, 1945). Manufacturers and consumers often confuse whether the species are accurate when distinguishing external features of the fish are removed by filleting or processing into flesh portions. Because of high toxicity in the muscle tissue of pufferfish such as *Lagocephalus lunaris*, mislabeling is an important issue for the pufferfish industry. In fact, pufferfish-associated food poisoning incidents sometimes occur in Japan. Therefore, it is crucial to develop analytical methods for identifying species to prevent the consumption of toxic pufferfish species.

Numerous analytical methods have been developed for fish species identification, including isoelectric focusing (IEF) (Rehbein et al., 1995), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Scobbie and Mackie, 1988), liquid chromatography (Osman et al., 1987; Armstrong et al., 1992), immunoassays (Huang et al., 1995; Carrera et al., 1996), and molecular biology techniques (Russell and Carnegie, 1994; Unseld et al., 1995; O'Reilly and Wright, 1995). Among them, protein electrophoreses such as IEF and SDS–PAGE have been performed for pufferfish species identification (Ochiai et al., 1984; Hashimoto et al., 1984; Chen and Hwang, 2002). However,

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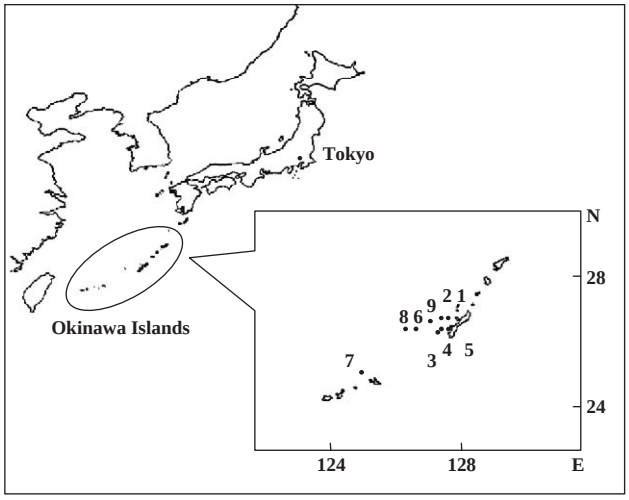

Fig. 1. A map showing sampling sites of the pufferfish species examined.

these methods are not applicable to thermally processed products such as heated, smoked, or dried fish because of severe protein denaturation. As an alternative to protein analysis, a DNA-based method has been published for identification of pufferfish species (Hsieh et al., 2002, 2003). Hsieh et al. (2003) performed analyses based on polymerase chain reaction (PCR) of the nucleotide sequence and analyses of restriction fragment length polymorphism (RFLP) on a DNA fragment flanking the mitochondrial cytochrome *b* gene of six pufferfish including toxic and non-toxic species. They reported that this technique was useful for distinguishing toxic pufferfish species.

The present study examines the use of a partial 16S rRNA sequence for pufferfish species identification. In brief, about 615-bp long fragment amplified with universal primers is digested separately with three restriction enzymes; the resulting fragments are then resolved through electrophoresis in agarose gels. This PCR-RFLP method is useful to identify common pufferfish species. Moreover, it can be applied to degraded samples.

2. Materials and methods

2.1. Samples and DNA extraction

Authenticated 61 sample specimens from nine pufferfish species – *Pleuranacanthus sceleratus*, *Triodon macropterus*, *Chelonodon patoca*, *Sphoeroides pachygaster*, *Arothron hispidus*, *A. stellatus*, *A. manilensis*, *A. mappa*, and *A. nigropunctatus* – were collected from the coastal area of Okinawa Islands in Japan (Fig. 1 and Table 1). The musculature portion was excised and frozen immediately in liquid nitrogen. It was then kept at −80 °C until mitochondrial DNA extraction.

Total cellular DNA was isolated from each individual using TNES–Urea buffer (8M urea, 10 mM Tris–HCl, pH 7.5, 125 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1% SDS) (Asahida et al., 1996) and then extracted with phenol-chloroform method. The obtained pellet was dissolved in 100 μL TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA).

2.2. Mitochondrial 16S rRNA gene fragment amplification

To amplify a partial region of the mitochondrial 16S rRNA gene, PCR was carried out using one pair of primers (16SarL, 5′-CGCCTGTTTATCAAAAACAT-3′ and 16SbrH, 5′-CCGGTCTGAACTCAGATCACGT- 3′) (Palumbi et al., 1991). These primers were used both for PCR amplification and for direct DNA nucleotide sequencing. PCR was performed in 50 μL total volume of reaction buffer containing 0.2 mM dNTPs, 0.4 μM of each primer, two units of Tth DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), and 1 μg of extracted template DNA. We conducted PCR in a Program Temp Control System PC-801 (Astec Co. Ltd., Fukuoka, Japan) under the following conditions: 120 s at 93 °C, followed by 30 alternating cycles of 60 s at 93 °C for denaturation, 90 s at 53 °C for annealing, and 90 s at 72 °C for extension.

2.3. DNA sequencing

After amplification, the PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) to degrade unincorporated primers and dNTP. They were subsequently used as a DNA template for direct sequencing. PCR products that could not be sequenced directly were subcloned into pT7-Blue T-vector (Novagen Inc., Madison WI, USA). The resultant recombinant plasmid DNAs were sequenced. DNA was sequenced with a genetic analyzer (ABI PRISM™ 310 or 3100; Applied Biosystems Japan, Tokyo, Japan) by chain-termination procedure with a Sequencing Ready Reaction kit (BigDye terminator™ Cycle; Applied Biosystems Japan, Tokyo, Japan). The recombinant plasmid was also sequenced with M13 universal primers.

Table 1
Details of pufferfish specimens used in this study

Species	Location of collection*	Date of collection	Number examined	Study code
Arothron hispidus	1	Aug. 2003	10	*A. his*
Arothron stellatus	2	Jul. 2003	8	*A. ste*
Arothron manilensis	3	Apr. 2004	2	*A. man*
Arothron mappa	4	Aug. 2003	1	*A. map*
Arothron nigropunctatus	5	Jan. 2004	8	*A. nig*
Sphoeroides pachygaster	6	Aug. 2003	6	*S. pac*
Plueranacanthus sceleratus	7	Aug. 2003	12	*P. sec*
Triodon macropterus	8	Dec. 2003	6	*T. mac*
Chelonodon patoca	9	Jan. 2004	8	*C. pat*

*Numbers represent sampling locations shown in Fig. 1.