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Applying DNA Techniques to the Identification of the Species of Dressed Toasted Eel Products

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To differentiate the species of processed eel products, the gene identification of four fresh eel species was first established and the species of eel products collected from markets were investigated. Polymerase Chain Reaction (PCR) and sequence analysis were used to determine the genetic variation in a 362-nucleotide region of the mitochondrial cytochrome *b* gene in four fresh eels including *Anguilla japonica*, *Anguilla anguilla*, *Anguilla rostrata*, and *Muraenesox cinereus*. It was found that each eel species had a unique genotype, which was no different among fresh, frozen, and sterilized meats. The restriction enzyme *Hin*fI could differentiate the species of *A. japonica* and *A. rostrata* but could not differentiate *A. anguilla* and *M. cinereus*. Another restriction enzyme, *Sau*96 I, was valuable in the differentiation of *M. cinereus* from the other three species of *Anguilla*. By applying PCR and restriction enzymes, the species of 12 commercial eel products were identified as *A. japonica* (9 samples), *A. anguilla* (2), and *A. rostrata* (1). This indicated that the sequence and restriction enzyme cutting site analyses were very usable to authenticate species of different processed eel products.

KEYWORDS: Eel; processed eel products; species identification; PCR; RFLP; restriction enzyme; cytochrome *b* gene; mitochondria

INTRODUCTION

Dressed toasted eel products (called kabayaki in Japanese) are highly favored in Japan, Taiwan, and other Asian countries. The eel species *Anguilla japonica* is the major fish, which is one of the most important economic cultural species. Due to increased consumption in Asia, the European species *A. anguilla* and the American species *A. rostrata* have been imported and cultured in Taiwan and other Asian countries. The above three eel products are exported to Japan as processed eel products. The processed eel products have no labels indicating species family name. On the other hand, the main conger eel in Taiwan is *Muraensox cinereus*; it is cheaper and is usually made into canned products. It is also of concern that conger eels are used to adulterate any species of *Anguilla*.

There have been several methods reported for the identification of fish species, including electrophoresis, isoelectric focusing (*1*), liquid chromatography (*2*), immunoassays (*3*), and molecular biology techniques (*4*−*6*). Among them, the most promising and reliable technique is a DNA technique due to its easy application in routine surveys and its robustness (*7*).

Mitochondrial sequences are often useful tools for phylogenetic studies, as they are generally maternally inherited and therefore not subject to the diversity generating mechanisms allocated with sexual reassortment (*8*). Furthermore, in many metazoa, including mammals, the mitochondrial genome accumulates at a faster rate than nuclearly encoded sequences, making mitochondrial sequences a useful source of phylogenetic data for differentiating closely related classifications (*9*, *10*). However, the mitochondrial genome is generally not subject to genetic recombination. Mitochondrial gene arrangements therefore tend to be highly conserved across long evolutionary distances (*11*). Because of this, mitochondrial gene order has been used as a phylogenetically informative trait to study evolutionary relationships among distantly related classifications (*12*, *13*).

The mtDNA, which is simply maternally inherited (*14*−*16*), does not have a recombination mechanism as in the nuclear DNA to eliminate error once a mutation occurs. Thus, mtDNA mutations can arise in a population more rapidly than those of nuclear DNA; in addition, a 10-fold higher evolution rate is found in the former than in the latter (*9*). The mtDNA has therefore been recently used as a maker in studies of molecular evolution (*17*, *18*).

The Polymerase Chain Reaction (PCR) technique and sequence analysis are useful for identifying fish species. In our previous papers (*19*−*23*), we have applied the PCR technique and sequence analysis to the identification of the species of toxic and nontoxic puffer fish processed products. In this study, we have applied a pair of PCR primers to the identification of raw, frozen, and sterilized eel products. Then the real species of

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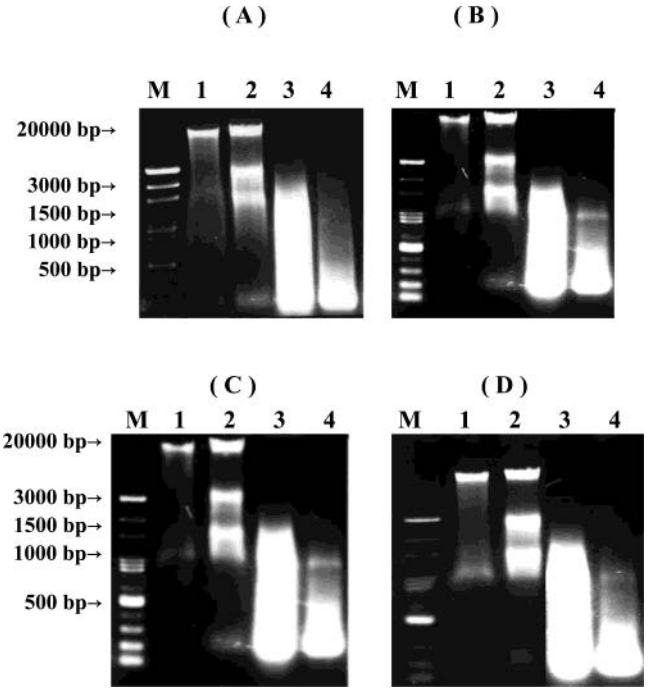

Figure 1. Electrophoretic analysis of DNA extracted from fresh, frozen, and cooked meats of eel on 0.8 agarose gel: (lane M) MW marker Bio 100 bp ladder; (land 1) fresh meat; (lane 2) frozen meat; (lane 3) meated cooked at 100 °C for 10 min; (lane 4) meat steam-sterilized at 121 °C for 15 min; (**A**) *A. japonica*; (**B**) *A. anguilla*; (**C**) *A. rostrata*; (**D**) *M. cinereus*.

commercial dressed toasted eel products collected from markets in Taiwan was analyzed.

MATERIALS AND METHODS

Preparation of Samples. The 12 raw pre-rigor meats of specimens for each eel species including Japanese eel *Anguilla japonica*, European eel *Anguilla anguilla*, and American eel *Anguilla rostrata* were collected from different culture ponds in Pingtung and Ilan counties. Twelve specimens of pre-rigor conger eel *Muraenesox cinereus* were supplied by Tai-rong Co. (Ilan, Taiwan). Each meat specimen was skinned, mixed, and divided into four portions. The pre-rigor meat fillet was kept at 3 °C as fresh meat. A portion of the eel meat was frozen at −20 °C for 2 months as frozen meat materials. Another portion of the eel meat was cooked at 100 °C for 15 min, and a third portion of the eel meat was packed in cans and sterilized for 15 min after the retort reached 121 °C as sterilized materials. Furthermore, 12 dressed toasted eel products were purchased from wholesale markets and supermarkets in Taipei, Keelung, and Kaohsiung, Taiwan, and their fish species were identified.

DNA Extraction. DNA extraction was performed according to the protocol described by DeSalle and Birstein (*24*). Briefly, ∼0.3 g of sample was homogenized with extraction buffer (50 mM of 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) was added. The samples were incubated overnight at 55 °C with shaking. After incubation, the tubes were placed on ice for 30 min and then centrifuged at 12000*g* for 10 min; supernatants were transferred to clean tubes. DNA was extracted once with phenol, twice with phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio, and once with chloroform and then precipitated twice with ethanol at −20 °C. The precipitant was evaporated to dryers using a vacuum pump to free from organic solvent. The dried pellets were resuspended in 50−100 μL of sterilized distilled water, and the concentration of DNA was estimated by absorbance at 260 nm.

PCR Primer. The set of primers (L14841 and H15149) reported by Kocher et al. (*25*) was first used for PCR amplification of each eel species. However, the primers were not valuable for each eel, so new primers were designed as follows: L, 5′-TTCCATCCAACATCTC-

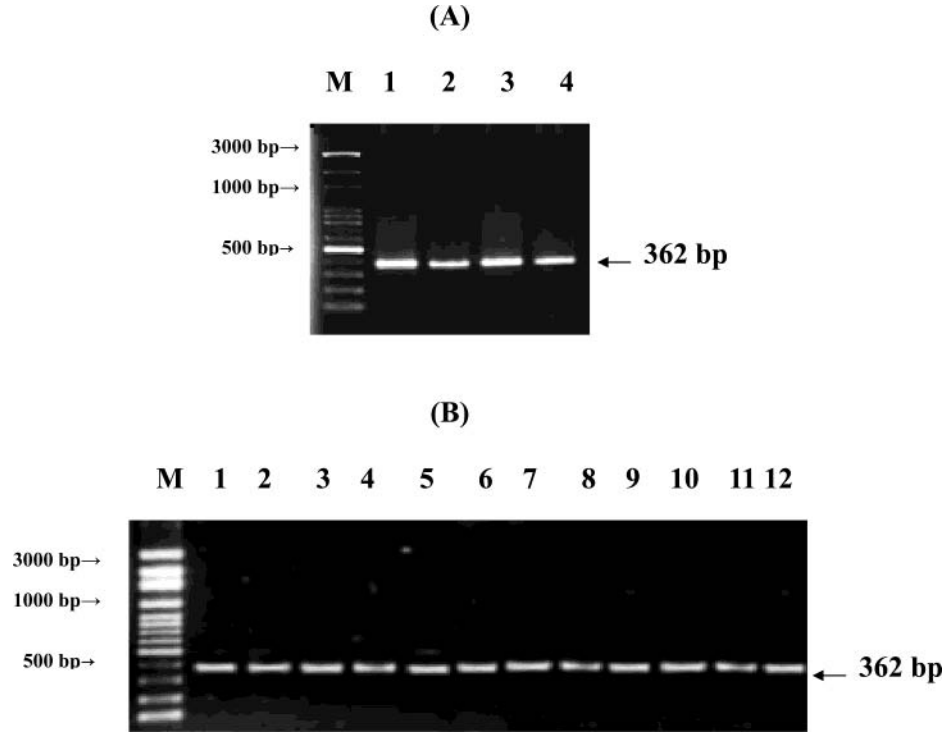

Figure 2. Electrophoretic analysis of the PCR products from the cytochrome *b* gene on 1.2 agarose gel: (**A**) (lane M) MW marker Bio 100 bp ladder; (lane 1) fresh meat of *A. japonica*; (lane 2) fresh meat of *A. anguilla*; (lane 3) fresh meat of *A. rostrata*; (lane 4) fresh meat of *M. cinereus*; (**B**) (lane 1) frozen meat of *A. japonica*; (lane 2) meat of *A. japonica* cooked at 100 °C for 10 min; (lane 3) meat of *A. japonica* steam-sterilized at 121 °C for 15 min; (lane 4) frozen meat of *A. anguilla*; (lane 5) meat of *A. anguilla* cooked at 100 °C for 10 min; (lane 6) meat of *A. anguilla* steam-sterilized at 121 °C for 15 min; (lane 7) frozen meat of *A. rostrata*; (lane 8) meat of *A. rostrata* cooked at 100 °C for 10 min; (lane 9) meat of *A. rostrata* steam-sterilized at 121 °C for 15 min; (lane 10) frozen meat of *M. cinereus*; (lane 11) meat of *M. cinereus* cooked at 100 °C for 10 min; (lane 12) meat of *M. cinereus* steam-sterilized at 121 °C for 15 min.

Figure 3. The 306 bp consensus sequence of the 362 bp segment of mitochondrial cytochrome *b* gene from the fresh meat of *Anguilla* and *Muraenesox* eel species. Primers are shown in italic capital letters.

CGCATGATGAAA-3′, and H, 5′-GTGAGGACAAATATCATTCT-GAGGGGGCT-3′. Both primers were modified from L14841 and H15149 and could obtain a 362 bp fragment from the cytochrome *b* gene of eel mtDNA.

PCR Amplification. The PCR amplification reactions were performed in a total volume of 100 μL. Each reaction mixture contained 1000 ng of extracted template DNA, 0.4 μM of each primer, 200 μM of each dNTP, and 2.5 units 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 PCR was carried out in a Gene-Amp PCR system 2400 (Perkin--Elmer, Foster City, CA) programmed to perform a denaturation step of 95 °C for 10 min, followed by 40 cycles consisting of 1 min at 95 °C, 1 min at 50 °C, and 2 min at 72 °C. The last extension step was 10 min longer.

DNA Purification and Sequencing. 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 detected under UV light and melted in 5 volumes of Tris−EDTA (TE) buffer at 65 °C for 5 min. DNA was extracted twice with phenol and once with a $^1/_{10}$ volume 3 M sodium acetate and 2 volumes of ethanol. The dried pellet was resuspended in 20 μL of sterilized distilled water. The concentration and quality of the DNA were estimated by agarose gel electrophoresis of a 2 μL sample.

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 Division, Foster City, CA) in an ABI Prism 377-96 DNA sequencer (Perkin-Elmer/Applied Biosystems Division). Two replicate sequences were obtained from the Wisconsin Package, version 10 (*26*).

Restriction Site Analysis of PCR Products. For the restriction site analysis of the cytochrome *b* region, the PCR products were extracted and purified the same way as in DNA extraction. Satisfactory results were also obtained without previous purification of the amplified DNA fragments. The endonucleases *Hin*fI, *Ase*I, and *Sap*96 I (Promega, Madison, WI) were searched from the GCG system by inputting our sequences and tested for restriction analysis of the amplified PCR products. Digests were performed in 10 μL volumes with 100−200 ng of amplified DNA, 5 units of enzyme, and a 1:10 dilution of the manufacturer's recommended 10× digestion buffer and bovine serum

Table 1. Species of Eel Product Determined by Using Four Restriction Enzyme Analysis on Cytochrome *b* Gene Fragment of PCR Products

source of PCR product[a]	enzyme cuts (base pairs)			species identified by 362 gene system
	*Hin*fI	*Ase*I	*Sau*96 I	
sample 1	163 + 117 + 82	362	362	*A. japonica*
sample 2	163 + 117 + 82	362	362	*A. japonica*
sample 3	163 + 117 + 82	362	362	*A. japonica*
sample 4	163 + 117 + 82	362	362	*A. japonica*
sample 5	163 + 117 + 82	362	362	*A. japonica*
sample 6	362	362	362	*A. anguilla*
sample 7	163 + 117 + 82	362	362	*A. japonica*
sample 8	163 + 117 + 82	362	362	*A. japonica*
sample 9	280 + 82	198 + 164	362	*A. rostrata*
sample 10	163 + 117 + 82	362	362	*A. japonica*
sample 11	362	362	362	*A. anguilla*
sample 12	362	362	362	*A. anguilla*

[a] Samples 1–6, frozen dressed toasted eel fillets; samples 7–12, frozen dressed toasted eel portions.

albumin (BSA). Digestions were incubated for 2 h at 37 °C (*Hin*fI, *Ase*I, and *Sap*96 I). The resulting fragments were separated by electrophoresis in a 1.5% agarose gel containing 10 μg/mL ethidium bromide for 1 h 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

The results of the mtDNA extraction are shown in **Figure 1**. The elecrophoretic analyses of extracts were obtained from fresh, frozen, cooked, and sterilized eel meat, respectively. In the fresh meat, almost all of the mtDNA was complete. The molecular weight was >15000 bp. However, the mtDNA extract from frozen meat appeared to contain some broken DNA fragments. This result was similar to that reported by Quinteiro et al. (*27*) and Cheng et al. (*20*). The mtDNA from sterilized eel meat was completely broken. The DNA molecule in the electrophoretic pattern was <1000 bp.

The PCR products obtained from fresh, frozen, and sterilized meats of four eel species are shown in **Figure 2**. By using L and H primers, the highly conserved 362 bp segment of mitochondrial cytochrome *b* gene was polymerized for fresh, frozen, and sterilized meats of every eel species. The sequences of 362 bp mtDNA fragment polymerized from four different eel species are shown in **Figure 3**. These sequences were searched from Genbank for accession no. NC_002707 (*A. japonica*), AF006714 (*A. anguilla*), and AF006716 (*A. rostrata*) and submitted to Genbank for accession no. AY295080 (*M. cinereus*). It was found that the 362-bp sequence in fresh, frozen, and sterilized samples was the same for the same eel species. This means that the sequence of the 362-bp region of cytochrome *b* gene in each eel was not altered even under freezing and sterilizing procedures. The sequences of the 362-bp region of the cytochrome *b* gene in all four eels were found to be different from each other, but the same from all tested specimens (12 samples) of the same species. The similarity of the sequence of the 362-bp region of the cytochrome *b* gene in the three *Anguilla* species was 100% compared with those of the same species from Genbank (accession no. NC_002707, AF006716, and AF006714). However, the sequence of the 362-bp region of the cytochrome *b* gene in *M. cinereus* is first reported here, and its similarity is 90.102% when compared with that of another eel, *Muraenesox bagio* (accession no. AB038417). The similarity and divergence of the sequence of the 362-bp region of the cytochrome *b* gene for the above five eel species are shown in **Table 1**. The phylogenic relationship between *A. anguilla* and *A. rostrata* was the nearest. The proposed evolutionary status of *Anguilla* and other eel fishes based on partial mitochondrial cytochrome *b* gene sequence is shown in **Figure 4**.

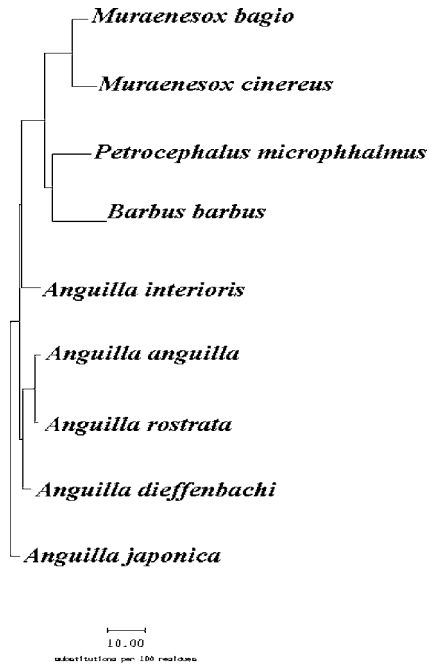

Figure 4. Proposed evolutionary status of *Anguilla*, *Muraenesox*, and other eel fishes based on partial mitochondrial cytochrome *b* gene sequences.

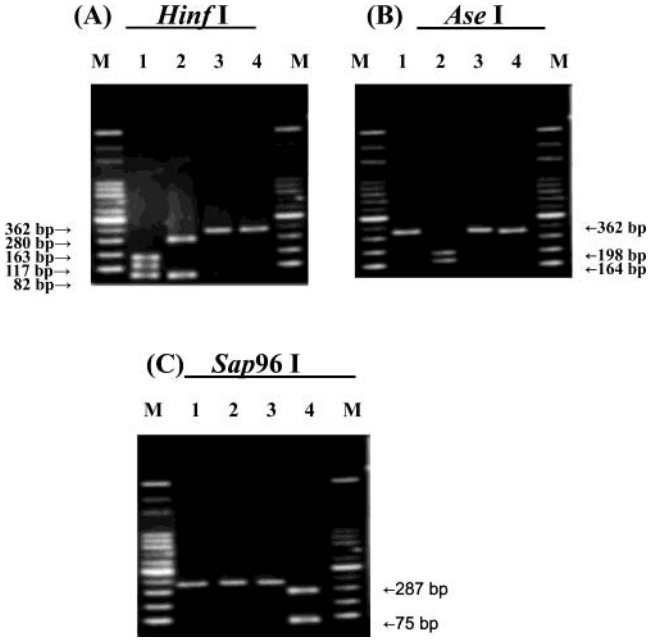

Figure 5. Electrophoretic analysis of the PCR products of the cytochrome *b* gene fragment by using three restriction enzymes digested on 2.0% agarose gel: (**A**) *Hin*fI; (**B**) *Ase*I; (**C**) *Sap*96 I; (lane 1) *A. japonica*; (lane 2) *A. rostrata*; (lane 3) *A. anguilla*; (lane 4) *M. cinereus*.

PCR products of four eels were digested by using three restriction endonucleases. The 362-bp region was amplified and cleaved with restriction enzyme *Hin*fI. The segment from *A. japonica* contained two restriction enzyme cutting sites; however, the segment from *A. rostrata* only had one site, and those from *A. anguilla* and *M. cinereus* did not have any sites (**Figure 5A**). When restriction enzyme *Ase*I cleaved, the segment from *A. rostrata* had one restriction enzyme cutting site, but *A. japonica*, *A. anguilla*, and *M. cinereus* had no cutting sites

(**Figure 5B**). Restriction enzyme *Sau*96 I could differentiate eel species of *M. cinereus* from the other three species of *Anguilla* (**Figure 5C**).

To examine the species of 12 samples of commercial eel products, they were analyzed by the cytochrome *b* gene fragment gene and the difference in restriction enzyme cutting site. The result showed that the species of commercial eel products included *A. japonica*, *A. anguilla*, and *A. rostrata*. Nine of the 12 commercial eel products were *A. japonica*, 1 was *A. rostrata*, and the other 2 were *A. anugilla*. This indicates that the sequence and restriction enzyme cutting site analyses are very usable to authenticate species of different processed eel products (**Table 1**).

DISCUSSION

As described by Unseld et al. (*28*), 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, for invertebrates 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 enzymes may be applicable in identifying the same species of American, European, and Taiwanese eels, as well as fresh, frozen, and processed samples.

In Taiwan, only three species of eel fish, *A. japonica*, *A. auguilla*, and *A. rostrata*, are cultured and processed as dressed toasted eel products. Judging from the data of identifying species of commercial processed eel products in Taiwan, the majority of processed eel products are made of *A. japonica*, with minor amounts from *A. auguilla* and *A. rostrata*. The restriction enzyme and 362-bp region sequence analyses are very usable for authenticating the species of processed eel products.

Furthermore, the data of gene base in the cytochrome *b* gene of eel are still few (*26*). To perfectly apply PCR and direct sequence analysis of the cytochrome *b* gene to the identification of eel species, more eel species also need to be further studied.

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