

IDENTIFICATION OF SIX COMMON SPECIES OF PROCESSED FILEFISH USING CYTOCHROME *b* GENE SEQUENCE AND PCR-RFLP ANALYSIS

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ABSTRACT. –Sequence analysis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique were used to identify the commercial species of *Aluterus monoceros*, *Aluterus scriptus*, *Monacanthus chinensis*, *Thamnaconus hypargyreus*, *Thamnaconus modestus* and *Chaetodermis penicilligerus*. The primers L14735 and H15149ad were designed in the mitochondrial cytochrome *b* gene and the molecular weight of amplified fragment from processed filefish meats was 465 bp. Furthermore, the results obtained from the *Hae*III enzyme digestion could be used to distinguish the six commercial filefish species in fresh and heat-treated meats. Using the PCR-RFLP method, six filefish species could be successfully identified in fresh and heat-treated meats. The PCR-RFLP technique developed in this study was proved to be a rapid, reliable and simple method that enables easy and accurate identification of six commercial filefish processed species.

KEY WORDS. – Filefish, Cytochrome *b* gene, PCR-RFLP, Species identification.

INTRODUCTION

In Taiwan, filefish species are a food delicacy with few bones and are easy to prepare. Every year the filefish are harvested from August to November. Filefish are also used as raw material for dry dressed fish fillets and are now sold as frozen and fried filefish fillets. On the other hand, non-toxic puffers are used as raw material for dried dressed filefish fillets. The toxicities of 23 species of Taiwanese puffers have been studied (Hwang et al., 1992), and only two puffers, *Lagocephalus gloveri* (brown-backed toadfish) and *Lagocephalus wheeleri* (brown-backed toadfish), are nontoxic in all tissues and may be used as materials in Taiwan for producing dried dressed fish fillets. However, there are a few unscrupulous operators using toxic puffers to make dry dressed filefish fillets, inducing food poisoning incidents have occasionally occurred in Taiwan, Japan and mainland China (Hwang & Noguchi, 2007).

To prevent toxic puffer fish being used as raw materials of dried dressed fish fillets, PCR amplification of mitochondrial

DNA sequence and restriction enzyme analysis were used to identify the species of 16 puffer fillets (Hsieh & Hwang, 2004). Species-level identification of most marine fish is typically based on adult morphological characteristics. However, such characteristics are often removed from fish when they are processed for markets or human consumption, making the identification of species based on morphology extremely difficult (McDowell & Graves, 2002). In our previous papers, the PCR-RFLP technique has been utilized by analyzing cytochrome *b* gene to identify some species of raw fish and processed foods (Hsieh & Hwang, 2004; Hsieh et al., 2002; Hsieh et al., 2005; Lin et al., 2005; Lin & Hwang, 2007).

Although gene sequence and PCR-RFLP analyses have been developed for identifying puffer species (Hsieh & Hwang, 2004), the identification method on commercial filefish species is not established. To differentiate the species of dried dressed fish fillets which are made from filefish, primer pairs, H15149ad and L14735, have been used to amplify the cytochrome *b* gene fragments of the mitochondrial

DNA of commercial filefish by PCR. Then, following the specific digestion by restriction enzyme, electrophoresis analysis has been used to enable direct observation of the divergence of the digested DNA fragments. Furthermore, several marketed fried filefish fillets and dried dressed fish fillets were collected and analyzed. The species of marketed fried filefish fillets was identified as *Aluterus monoceros*, indicating that established gene sequence and PCR-RFLP analyses for processed filefish products are useful.

MATERIALS AND METHODS

Preparation of samples. – Fresh iced specimens of raw muscle flesh of six different filefish species including *Aluterus monoceros*, *Aluterus scriptus*, *Monacanthus chinensis*, *Thamnaconus hypargyreus*, *Thamnaconus modestus* and *Chaetodermis penicilligerus* were purchased from seafood markets in Keelung (Northern Taiwan), Taipei (Northern Taiwan), Taichung (Central Taiwan), Changhua (Southern Taiwan), Pingtung (Southern Taiwan), Ilan (Eastern Taiwan) and Penghu (Table 1). For marketed fried filefish fillets and dried dressed fish fillets, material was also collected from Taiwan. From each sample, a 20 g sample was collected from the lateral line and at a distance of 5 cm from the caudal section. Each species was represented by at least 12 individual fish specimens. The filefish specimens were divided into 3 groups. The first group (A) was subjected to DNA extraction of raw fish meat. The second group (B) was cooked at 100°C for 15 min. The third group (C) was cooked at 100°C for 30 min. The second and third groups imitated the processing conditions of dried dressed filefish fillets. Furthermore, 3 fried filefish fillets and 3 dried dressed fish fillets were purchased from Taipei markets and used for following analyses.

DNA extraction. – DNA was extracted according to the protocol described in our previous study (Hsieh et al., 2005). In brief, about 0.3 g of sample was homogenized with the extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 1% SDS, and 0.2 M NaCl) containing 50 µl of 5 mg/ml proteinase K (Amresco, Solon, Ohio, USA). The samples were incubated overnight at 55°C with shaking. After incubation, tubes were placed on ice for 30 min, centrifuged at 12,000 g for 10 min and supernatant was transferred to a clean tube. 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 dried pellets were re-suspended in 50–100 µl sterilized distilled water and concentration of extracted DNA was estimated by absorbance at 260 nm.

PCR primers and amplification. – The PCR amplification reactions were performed in a total volume of 100 µl. Each reaction mixture contained 100 ng of extracted template DNA, 0.2 µM of each primer, 200 µM 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 MgCl₂, 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, CA) programmed to perform a denaturation step at 95°C for 10 min, followed by 40 cycles consisting of 1 min at 95°C, 1 min at 50°C and 5 min at 72°C. The last extension step was extended to 10 min longer. The set of primers used for PCR amplification were coded L-14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3') and H15149ad (5'-GCN CCT CAR AAT GAY ATT TGT CC T CA-3') specific for cytochrome *b* gene of fish were designed in this laboratory and used to amplify a 465 bp fragment in PCR (Sotelo et al., 2001).

DNA purification and sequencing. – PCR product (6 µl) was loaded onto a 2% agarose gel containing 1 µg/ml ethidium bromide in TBE buffer (0.089 M Tris; 0.089 M boric acid; 0.002 M EDTA, pH 8.0) and electrophoresed at 50 V for 120 min. The DNA band was excised under UV light and melted in five volumes of Tris EDTA (TE) buffer at 65°C for 5 min. DNA was extracted twice with ethanol, once with 1/10 volumes of 3 M sodium acetate and two volumes of ethanol. The dried pellet was resuspended in 20 µl sterilized distilled water. The concentration and quality of the DNA was estimated by agarose gel electrophoresis using a 5 µl of 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 a ABI PRISM 377-96 DNA sequencer (Perkin-Elmer/Applied Biosystems Division, Foster City, CA). Two replicate sequences were obtained from the Wisconsin Package, Version 10 (GCG, 2008, San Diego, CA). The amplified PCR products were analyzed by agarose gel electrophoresis (Sotelo et al., 2001; Hsieh et al., 2005, 2007).

RFLP analysis. – As described previously (Hsieh et al., 2005), the endonuclease *Hae*III (Promega, Madison, Wis., USA) was tested for restriction analysis of the amplified PCR products. Digests were performed in a 10 µl volume with 100–200 ng the amplified DNA, 5 U enzyme, and 1:10 diluted manufacturer's 10× digestion buffer, and bovine serum albumin (BSA). Digestion mixtures were incubated for 2 hr at 37°C for endonuclease. The resulting fragments were separated by electrophoresis in a 3.0% agarose gel containing 10 µg/ml ethidium bromide for 1 hr at 100 V. The sizes of the resulting DNA fragments were estimated by comparing with that of a commercial 100 bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS AND DISCUSSION

The PCR primers of L14735 and H15149ad could specifically amplify 465 bp of fragment from different processes of six commercial filefish meats. The electrophoretic analyses of PCR products from different processes of six filefish meats are shown in Fig. 1. Three group (fresh, cooked at 100°C for 15 min and cooked at 100°C for 30 min) were amplified 465 bp in Fig. 1A–C, respectively. A 465 bp fragment band was obtained. After purification and sequencing of PCR products, the sequences of six commercial filefish species were

Table 1. Summary of collection date, collection site and species in Taiwan.

Collection period	Collection site	Species name
May–Nov.2007	Keelung	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Thamnaconus hypargyreus</i>
	Taipei	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Thamnaconus modestus</i>
		<i>Thamnaconus hypargyreus</i>
		<i>Chaetodermis penicilligerus</i>
	Taichung	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Monacanthus chinensis</i>
	Changhua	<i>Aluterus monoceros</i>
	Pingtun	<i>Aluterus scriptus</i>
	Ilan	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Monacanthus chinensis</i>
		<i>hamnaconus hypargyreus</i>
May–Aug.2008	Keelung	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Thamnaconus hypargyreus</i>
	Taipei	<i>Monacanthus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Thamnaconus modestus</i>
		<i>Thamnaconus hypargyreus</i>
		<i>Chaetodermis penicilligerus</i>
	Taichung	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Monacanthus chinensis</i>
	Ilan	<i>Aluterus monoceros</i>
		<i>Aluterus scriptus</i>
		<i>Monacanthus chinensis</i>
		<i>Thamnaconus hypargyreus</i>

determined which had no differentiates between different specimens and processes. In Fig. 2 shows the comparison of DNA sequences in six commercial filefish species. Restriction sites of *HaeIII* are labeled as underlined. Filefish through various processing, including fresh meat (Fig. 2A), cooked at 100°C for 15 min (Fig. 2B) and cooked at 100°C for 30 min (Fig. 2C), the restriction enzyme could be useful. The result showed that the restriction enzyme *HaeIII* (5'–GG[▼]CC–3') could differentiate the species of *A. monoceros* (285 bp+180 bp), *A. scriptus* (180 bp+159 bp+126 bp), *M. chinensis* (424 bp+41 bp), *T. hypargyreus* (180 bp+159 bp+94 bp+32 bp), *T. modestus* (229 bp+180 bp+56 bp), and *C. penicilligerus* (285 bp+139+41 bp). The electrophoretic analyses of restriction site products from different processes of six commercial filefish meats are shown in Fig. 3. However, the 32 bp and 41 bp DNA fragments were difficult to visualize on 3% agarose

gel (Fig. 3). PCR-RFLP analysis of six commercial filefish in this study is shown in Table 2, indicating that filefish can be rapidly identified by restriction enzyme *HaeIII*. The marketed fried filefish fillets and dried dressed fish fillets were amplified by the primer pairs L14735 and H15149ad. As shown in Table 3, the species of 3 fried filefish fillet were identified as *A. monoceros* by judging from the data of gene sequence of PCR product and PCR-RFLP pattern. Another three dried dressed fish fillets were identified as *L. gloveri*. The sequences were compared with data from Gene Bank (Natl. Center for Biotechnology Information, NCBI) and all were the same as that of accession number EU274423 (*L. gloveri*).

In this study, application of direct sequence analysis and restriction enzyme are found to be available for identifying

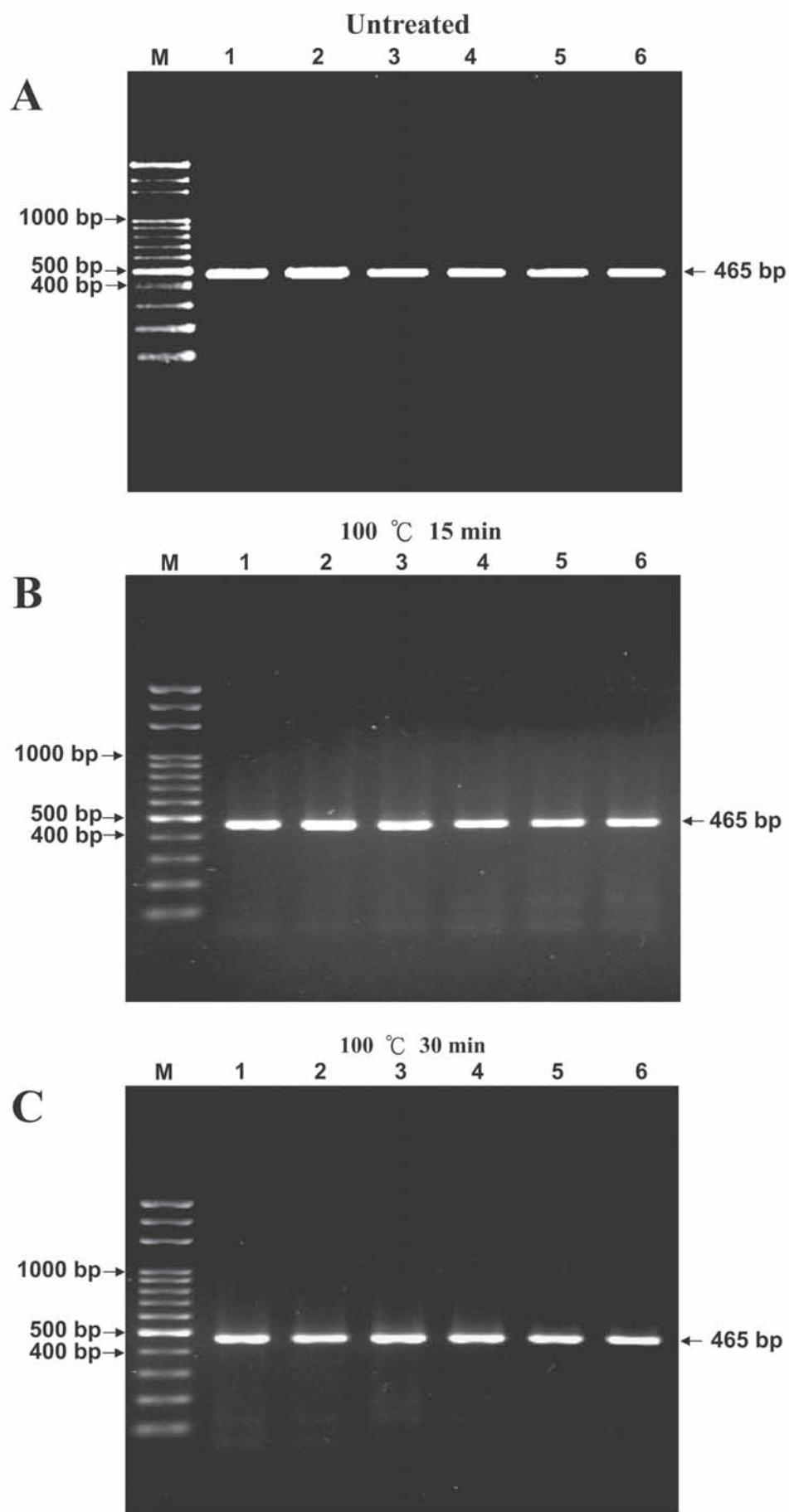


Fig. 1. Electrophoretic analysis of PCR product amplified with primers H15149ad/L14735. Samples in lane are as follows: M, 100-bp ladder; 1, *Aluterus monoceros*; 2, *Aluterus scriptus*; 3, *Monacanthus chinensis*; 4, *Thamnaconus hypargyreus*; 5, *Thamnaconus modestus*; 6, *Chaetodermis penicilligerus*.

<i>A. monoceros</i>	AAAAACCACCGTTGTTATTCAACTACAAGAACTTTTAATG	40
<i>A. scriptus</i>	-----cc-----	40
<i>M. chinensis</i>	-----c-c-----	40
<i>T. hypargyreus</i>	-----c-a-----	40
<i>T. modestus</i>	-----c-a-----	40
<i>C. penicilligerus</i>	-----ccc-----	40
<i>A. monoceros</i>	GCTAGCCTTCGCAAAACCCACCCACTACTAAAAATCGCCA	80
<i>A. scriptus</i>	-----g-c-----	80
<i>M. chinensis</i>	---c-----c-tt-----a-	80
<i>T. hypargyreus</i>	-----c-t-----	80
<i>T. modestus</i>	-----c-t-----	80
<i>C. penicilligerus</i>	---c-----a-----c-tt-g-----a-	80
<i>A. monoceros</i>	ACGACGCACTCGTTGACCTCCCTGCACCTTCTAACATTTC	120
<i>A. scriptus</i>	-----c-t-----	120
<i>M. chinensis</i>	-t-----a-----c-t-c-a-----	120
<i>T. hypargyreus</i>	-t-----c-----c-c-----c-	120
<i>T. modestus</i>	-t-----c-----c-c-----c-	120
<i>C. penicilligerus</i>	-t-----g-g-a-----a-t-----	120
<i>A. monoceros</i>	AGTCTGATGAAACTTCGGCTCCCTACTGGGGATATGCCTA	160
<i>A. scriptus</i>	c-c-----g-a-----g	160
<i>M. chinensis</i>	t-t-----t-a-----a-a-g--t-	160
<i>T. hypargyreus</i>	c-c-at-----t--t--t-a-c-g--t-	160
<i>T. modestus</i>	c-c-at-----t--t--t-a-c-g--t-	160
<i>C. penicilligerus</i>	--t-----t-----a-a-----	160
<i>A. monoceros</i>	ATTATTCAGATCCTCACTGGCCCTCTTCTTAGCAATACATT	200
<i>A. scriptus</i>	-----c-a-----c-----	200
<i>M. chinensis</i>	-----c-a-----a-----c-----	200
<i>T. hypargyreus</i>	-----c-a-----a-----t-c-----c-	200
<i>T. modestus</i>	-----c-a--t-----a-----t-c-c-----c-	200
<i>C. penicilligerus</i>	--c-----a-----t-a-----g--c-----g--c-	200
<i>A. monoceros</i>	ACACCTCCGACATCGCCACTGCTTTTCTTCAGTTGCCCA	240
<i>A. scriptus</i>	-----a-----c-c-----a-----c-----	240
<i>M. chinensis</i>	-----t-----c-c-c-a-c-----c-----	240
<i>T. hypargyreus</i>	-----t-----a-a-c-c-a-t-c-a-----	240
<i>T. modestus</i>	-----t-t-t-a-c-c-a-c-----g--a-----	240
<i>C. penicilligerus</i>	-----t-----a-a-c-c-----g--a-----	240
<i>A. monoceros</i>	TATTTGCCGAGATGTAAATTACGGCTGACTAATCCGGAAT	280
<i>A. scriptus</i>	c--c-----c-----a-----a--c	280
<i>M. chinensis</i>	-----g-----c-----t-----	280
<i>T. hypargyreus</i>	c--c--t-----c-g-c-----t--c	280
<i>T. modestus</i>	c--c--t-----c-----t--c--c	280
<i>C. penicilligerus</i>	c--c-----c-----a-g--t-----c--c	280
<i>A. monoceros</i>	CTCCACGCTAACGGAGCCTCCTTCTTCTTCATCTGCATCT	320
<i>A. scriptus</i>	-----g-----	320
<i>M. chinensis</i>	--t-----c-----	320
<i>T. hypargyreus</i>	-----c-----t-----	320
<i>T. modestus</i>	-----c-----t-----	320
<i>C. penicilligerus</i>	--a-----c-----a-----t--t--t-	320
<i>A. monoceros</i>	ATCTGCACATCGGACGGGGTCTTTACTACGGCTCCTATCT	360
<i>A. scriptus</i>	-c-----a--c-----g-----c-	360
<i>M. chinensis</i>	-c--t-----a-a-----g-----c-	360
<i>T. hypargyreus</i>	-c--a--t-----a--c-----t--c-	360
<i>T. modestus</i>	-c--c--t-----g-----c-----c-	360
<i>A. monoceros</i>	CTATAAAGAAACCTGGAACGTGGGGGTGGTCCTTCTGCTT	400
<i>A. scriptus</i>	a--c-----g-----a-----a-----a-----c	400
<i>M. chinensis</i>	t-----a-----a-a-ca-----c-----a	400
<i>T. hypargyreus</i>	-----g--g--a-----c-----a--t-----c	400
<i>T. modestus</i>	--c-----g-----a-----c-a-a--t-----c	400
<i>C. penicilligerus</i>	g--c-----a--t--t--t--aa-----g-----	400
<i>A. monoceros</i>	CTGGTAATGATAACTGCTTTCGTAGGCTACGTCCTCCCAT	440
<i>A. scriptus</i>	-c--g-----a-----g--t-----t-----	440
<i>M. chinensis</i>	t-a--t-----g-----a--g--t-----	440
<i>T. hypargyreus</i>	-t-----g-----t-----a--c-----	440
<i>T. modestus</i>	-a-----g-----a--c-----	440
<i>C. penicilligerus</i>	--a--t-----g--c--c-----g--c-----	440
<i>A. monoceros</i>	GAGGACAAATGTCAATTCTGAGGAGC	465
<i>A. scriptus</i>	-----	465
<i>M. chinensis</i>	-----	465
<i>T. hypargyreus</i>	-----	465
<i>T. modestus</i>	-----	465
<i>C. penicilligerus</i>	-----	465

Fig. 2. The DNA alignment sequence of a partial mitochondrial cytochrome *b* gene fragment from six common filefish species. Restriction sites of *Hae*III (5'-GG[▼]CC-3') are highlighted in grey.

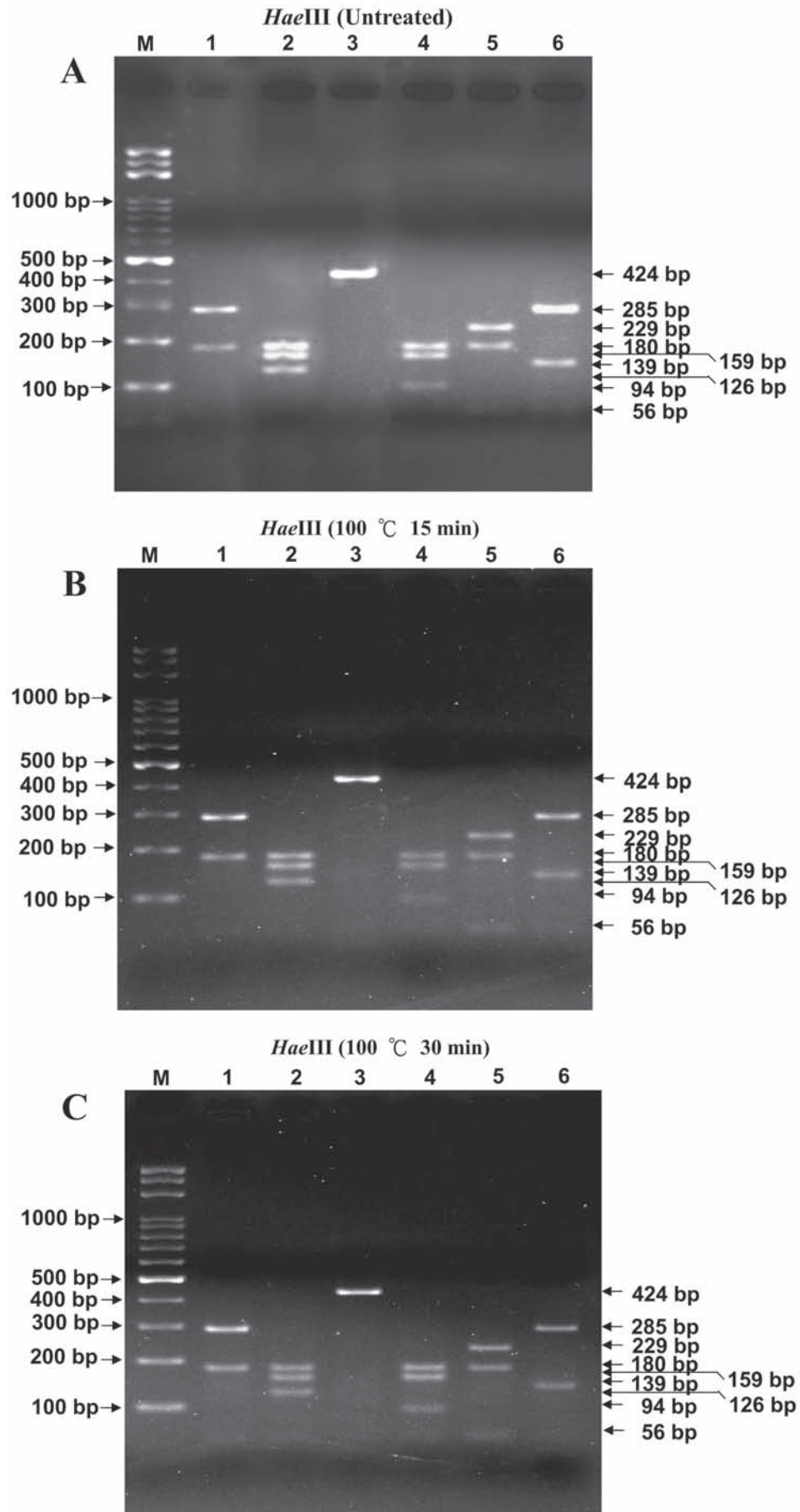


Fig. 3. Electrophoretic analysis of PCR products of the 465 bp cytochrome *b* gene digested with *HaeIII* (5'-GG▼CC-3') on 3.0% agarose gel. M, molecular weight marker, Bio 100-bp DNA ladder; 1, *Aluterus monoceros*; 2, *Aluterus scriptus*; 3, *Monacanthus chinensis*; 4, *Thamnaconus hypargyreus*; 5, *Thamnaconus modestus*; 6, *Chaetodermis penicilligerus*.

Table 2. RFLP product sizes of the partial cytochrome *b* gene of in six filefish with restriction enzyme *Hae*III.

Species	PCR product (bp)	RFLP product sizes (bp)
<i>Aluterus monoceros</i>	465	285+180
<i>Aluterus scriptus</i>	465	180+159+126
<i>Monacanthus chinensis</i>	465	424+41*
<i>Thamnaconus hypargyreus</i>	465	180+159+94+32*
<i>Thamnaconus modestus</i>	465	229+180+56
<i>Chaetodermis penicilligerus</i>	465	285+139+41*

* The low molecular fragment disappeared in Fig. 3 due to this signal being too weak.

Table 3. The data of PCR product amplified with primer pairs L14735 and H15149ad and PCR-RFLP product sizes by using *Hae*III in marketed fried filefish fillets and dried dressed fish fillets.

Species	PCR product (bp)	RFLP product sizes (bp)	Species identified
Fried filefish fillets			
F1	465	285+180	<i>Aluterus monoceros</i>
F2	465	285+180	<i>Aluterus monoceros</i>
F3	465	285+180	<i>Aluterus monoceros</i>
Dried dressed fish fillets			
D1	464	298+126+40	<i>Lagocephalus gloveri</i>
D2	464	298+126+40	<i>Lagocephalus gloveri</i>
D3	464	298+126+40	<i>Lagocephalus gloveri</i>

six common filefish from Taiwanese seawaters. A 465 bp region within the cytochrome *b* gene was successfully amplified from different species of six commercial filefish and could be available for differentiating each species. Although intra-specific genetic variability is present in wild populations (Terol et al., 2002), individual variation of the 465 bp gene from each filefish species collected from Taiwan was not found in this study. Thus, analysis of the partial sequence of cytochrome *b* gene in this study was more efficient and less laborious than complete sequencing of gene, with little loss of information. This result is similar to that of the previous reports (Cocolin et al., 2000; D'Amelio et al., 2000; Hsieh et al., 2005, 2007; Hsieh & Hwang, 2004; Partis et al., 2000).

In the present study, we found that application of direct sequence and restriction enzyme analyses could be useful for raw filefish, processed filefish fillets and dried dressed fish fillets. Furthermore, in many metazoans, 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 taxa (Vawter & Brown, 1986). The phylogenetic data of filefish in Taiwan are progressing to study.

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