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Authentication of Salted-dried Fish Species Using Polymerase Chain Reaction-Single Strand Conformational Polymorphism and Restriction Analysis of Mitochondrial DNA

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Molecular techniques, including restriction fragment length polymorphism (RFLP) and polymerase chain reaction-single strand conformational polymorphisms (PCR-SSCP), were developed to identify salted, dried threadfin (*Eleutheronema tetradactylum*) and white herring (*Ilisha elongata*) fish. Using PCR with universal primers, conserved 367-bp fragments of the cytochrome *b* gene were amplified from fresh fish samples and sequenced. The sequences were then searched for specific restriction sites. The digestion of the PCR products with the endonucleases *Ava*I, *Fok*I, *Mbo*II, and *Msp*I generated RFLP, which was used to identify the commercial products. Similarly, the amplified PCR-SSCP products were developed and the products tested. Overall, similar patterns were found in the majority of the fresh and processed products. Based on the results, both RFLP and PCR-SSCP were useful in determining and validating the authenticity of the fish species used to prepare the commercial salted, dried products. A similar approach can be applied to other species.

Key words: Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP), Restriction fragment length polymorphism (RFLP), Threadfin, White herring

Introduction

Salted, dried fish is one of the most popular traditional foods in southern China. Many fishes can be salt-cured and sun-dried using traditional processes to produce preserved fish with various characteristic qualities on which the market price depends. Threadfin and white herring are popular salted, dried fish that command relatively high prices in Hong Kong. Local fishermen and wholesalers suggest that most of the salted, dried threadfin and white herring are produced from the fish *Eleutheronema tetradactylum* (*E. tetradactylum*) and *Ilisha elongate* (*I. elongate*), respectively (Herklots and Iin, 1961). It is difficult to identify these species of salted, dried fish using morphological characteristics because the apparent fea-tures of the fish are usually distorted after salting, drying, and trimming (Mackie et al., 1999). Compared with identification using morphological features, DNA analysis is a more reliable method for species authentication, particularly with processed foods. To identify the processed fish species, DNA technologies offer advantages over other techniques such as immunological, electrophoretic, and chromatographic methods in terms of sample constraints, technical complexity, costs, and accuracy (Sebastio et al., 2001). The conventional approach of DNA analysis involves amplifying and characterizing the conserved region in a species, parti cularly the mitochondrial cytochrome *b* gene (Kocher et al., 1989; Meyer, 1990). The universal primers designed by Kocher et al. (1989) have been used to amplify and sequence the conserved region of the cytochrome *b* gene in more than 100 animal species, including fish (Ran et al., 1996; Wolf et al., 1999) and invertebrates. The amplified cytochrome *b* fragments can be characterized using the restriction fragment length polymorphism (RFLP) (Ram et al., 1996; Wolf et al., 1999; Sebastio et al., 2001) and single strand

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conformational polymorphism (SSCP) (Rehbein et al., 1997; Asensio et al., 2001) tests.

We amplified and sequenced cytochrome *b* gene fragments of raw *E. tetradactylum* and *I. elongata*. The specific RFLP and SSCP patterns generated from these fragments were used to authenticate salted, dried fish that was labeled as threadfin and white herring in the local market.

Materials and Methods

Samples and DNA extraction

Raw *E. tetradactylum* and *I. elongata* were collected and identified morphologically based on the description keys from the Hong Kong Marine Fish D atabase (AFCD) (http://www.hk-fish.net).

The salted samples of threadfin and white herring were purchased from several wholesalers in Hong Kong during two consecutive years.

The DNA of the raw and salted fishes was extracted from the fish flesh using a FastDNA™ Kit (Bio 101 Qbiogene). The prepared DNA samples were assayed using 1% Tris-borate-EDTA (TBE) agarose gel electrophoresis, and their concentrations were evaluated using the UV absorbance at 260 nm.

PCR amplification of the cytochrome *b* gene fragment and sequence analysis

Conserved fragments of the cytochrome *b* gene were amplified from the raw fish samples using the universal primers L14841 (forward primer: AAAA-AGCTTCCATCCAACATCTCAGCATGATGAAA) and H15149 (reverse primer: AAACTGCAGCCC-CTCAGAATGATATTTGTCCTCA), which were designed by Kocher et al. (1989). The expected length of the specific PCR products was 376 bp. Each 100 μL PCR mixture contained 1×thermophilic DNA polymerase buffer B (Promega), 2.5 mM $MgCl_2$ solution, 4 U of *Taq* DNA polymerase (Promega), 0.2 mM deoxyribonucleotide triphosphate (dNTP), 0.1 μM of each primer, 100 ng of DNA template, and distilled water. The mixture was initially denatured at 94°C for 4 min. This was followed by 35 thermal cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR was finished with a 10-min incubation at 72°C. The PCR products were assayed with 1% SeaKem® LE agarose gel electrophoresis with ethidium bromide staining. The amplified specific PCR products were purified with a QIAquick™ PCR purification kit (QIAGEN) to remove the surplus primers and dNTP. The PCR samples were then concentrated in steps from 100 μL to the final volume of 30 μL. The purified PCR products were sequenced directly in both directions, with the corresponding primers, using an ABI Prism 3300 automatic sequencer (PE Applied Biosystems). In each PCR reaction, 10 μL of solution consisted of 1 μL of purified PCR product, 0.16 pM corresponding primer, 4 μL of dRhodamine Terminator Sequencing Kit (PE Applied Biosystems), and distilled water. Sequencing reactions consisted of 25 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4 min. The sequences were analyzed and edited using the program SeqEd™ version 1.02 (PE Applied Biosystems).

PCR-RFLP analysis

The obtained sequences were searched for specific endonuclease restriction sites of the amplified cytochrome *b* fragment using the software GeneJockey II (Biosoft, UK). The amplified cytochrome *b* fragments of *E. tetradactylum* and *I. elongata* were digested with the appropriate restriction enzymes. In each 20 μL digestion reaction, 3 μL of purified PCR product was digested with 1-2 U of restriction enzymes (Amersham Pharmacia) combined with the corre-sponding amount of specific buffer, bovine serum albumin (BSA), and distilled water at the recom-mended temperature for 12 h. The restricted frag-ments were assayed on 4% MetaPhor® agarose gel, and the sizes of the DNA fragments were estimated in comparison with a 100-bp molecular ladder (Life Technology).

PCR-SSCP analysis

The PCR samples were denatured by mixing in one volume of denaturing solution (98% formamide containing 0.05% bromophenol blue and 0.05% xylene cyanol) and heating to 95°C for 6 min. The denatured PCR products were cooled in an ice-water bath immediately. The treated samples were subsequently loaded in 12.5% polyacrylamide gel (GeneGel Excel 12.5/24 Kit, Amersham Pharmacia Biotech). Low-temperature electrophoresis was carried out using a GenePhor electrophoresis unit (Amersham Pharmacia) with the following conditions: 10°C, 600 V, 25 mA, 15 W, and 120 min. The DNA bands were visualized with silver staining using a Hoefer automated gel stainer and a PulseOne DNA silver staining kit (Amersham Pharmacia).

Testing of the salted, dried fish samples

The PCR-RFLP and PCR-SSCP analyses were used to verify the authenticity of the commercial salted fish samples, which were labeled as either threadfin or white herring. Sixteen samples of each fish species were collected and analyzed.

	10	20	30	40	50
E.tetradactylum	AAAAAGCTTC	CATCCAACAT	CTCAGCATGA	TGAAACTTTG	GCTCGCTCCT
I. elongata	AAAAAGCTTC	CATCCAACAT	CTCAGCATGA	TGAAACTTCG	GATCATTGCT
	60	70	80	↱ *MspI* 90	100
E.tetradactylum	AGGCCTCTGC	CTAGGCATT**C**	AAATCGTTA**C**	**CGG**CCTATTT	CTAGCCATAC
I. elongata	GGGACTATGT	CTTATTTCAC	AAATCCTAAC	AGGACTTTTC	CTGGCTATAC
	110	120	130	140	150
E.tetradactylum	ACTACACCCC	AAACATCACC	TCAGCCTTCG	ACTCTGTCGC	ACACATCTGC
I. elongata	ACTACACCTC	TGACAT**CGCC**	ACTGCATTCT	**CATCC**GTTAC	ACATATCTGC
		↳ *FokI*			
	160	170	180	190	200
E.tetradactylum	CGTGATGTAA	ACTTCGGCTG	ATTGATTCGT	AACATACATG	CCAACGGAGC
I. elongata	CGTGATGTAA	ACTAC**GGATG**	ACTTATCCGA	AATATACATG	CAAACGGTGC
			FokI ↴		*MboII* ↴
	210	220	230	240	250
E.tetradactylum	ATCTTTCTTT	TTTGTCTGCA	TTTACTTTCA	CATCGGCCGA	GGACTATACT
I. elongata	CTCCT**TCTTC**	**TTC**ATCTGCA	TCTACGCACA	CATCG**CTCGA**	**G**GGTTATACT
	↳ *MboII*			↳ *AvaI*	
	260	270	280	290	300
				↱ *MboII*	
E.tetradactylum	ATGGCTCTTA	CCTTTATAAA	GAGACATGGA	ACATCGGAGT	TGT**TCTTC**TT
I. elongata	ATGGCTCCTA	CCTATACAAA	GAAACATGAA	ACATTGGAGT	AGTCCTTCTC
	310	320	330	340	350
E.tetradactylum	TTACTTACAA	TAATAACTGC	TTTCGTAGGT	TATGTGCTTC	CCTGAGGACA
I. elongata	CTTCTAACAA	TAATAACAGC	CTTCGTAGGC	TACGTCCTAC	CTTGAGGACA
	360	370	376		
E.tetradactylum	AATATCATTC	TGAGGGGCTG	CAGTTT		
I. elongata	AATATCATTC	TGAGGGGCTG	CAGTTT		

Fig. 1. Alignment of 376 bp sequences of amplified cytochrome *b* fragment. Specific restriction sites for *Eleutheronema tetradactylum* and *Ilisha elongata* were shown in bold letters with arrow.

Results and Discussion

The concentrations of the DNA samples from raw and salt-preserved fishes were 200-350 ng/μL. The anticipated PCR products with a length of 376 bp, corresponding to the specific fragment of the cytochrome *b* gene, were amplified for both the fresh and salted, dried fish samples using the universal primers L14841 and H15149. No non-specific PCR products were observed in the electrophoresis assays. The amplified cytochrome *b* fragments from *E. tetradactylum* and *I. elongata* were sequenced (Fig. 1). Two diverse sequences, with a difference in 79 bp (21%), were observed. Thirty-seven of the differences were transversions (purines↔pyrimidines). On comparing the two sequences, four endonucleases were found to generate RFLP that allowed differentiation between *E. tetradactylum* and *I. elongata* on electrophoresis. The four endonucleases were *Ava*I ($C\downarrow P_yCGP_uG$), *Fok*I ($CCTACN_{13}\downarrow$), *MboII* ($GAAGAN_8\downarrow$), and *Msp*I ($C\downarrow CGG$; Table 1). In the restriction analysis, *Msp*I could restrict only the cytochrome *b* fragment amplified from *E. tetradactylum*. The *Ava*I and *Fok*I could digest only the cytochrome *b* from *I. elongata*

Table 1. Calculated restriction patterns of the cytochrome *b* gene fragments of *Eleutheronema tetradactylum* and *Ilisha elongata*

Enzyme	Species	Cut position	Fragment size (bp)
*Ava*I	*E. tetradactylum*	No sites found	-
	I. elongata	236	240, 136
*Fok*I	*E. tetradactylum*	No sites found	-
	I. elongata	117 & 179	193, 121, 62
*Msp*I	*E. tetradactylum*	80	296, 80
	I. elongata	No sites found	-
*Mbo*II	*E. tetradactylum*	286	286, 90
	I. elongata	198 & 201	198, 175, 3

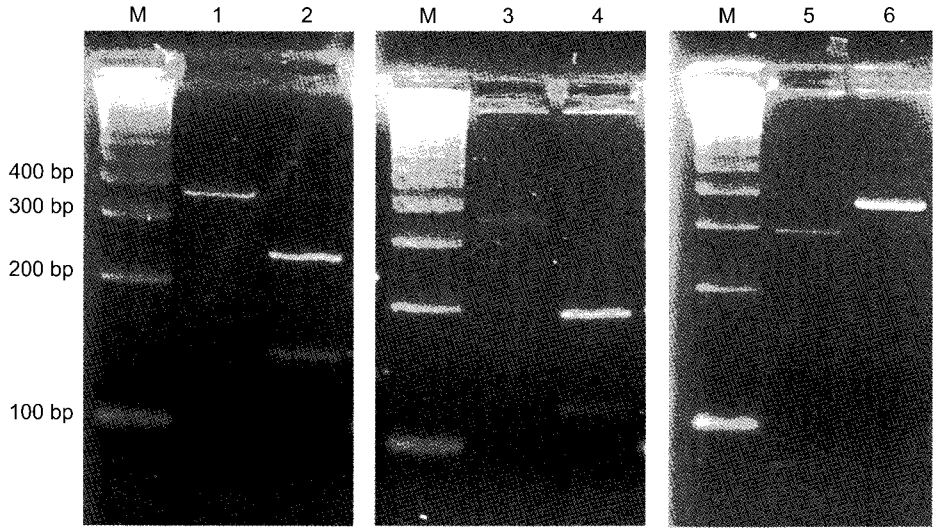

Fig. 2. RFLP patterns of *Eleutheronema tetradactylum* and *I Ilisha elongata* on 4% Metaphor® agarose gel staining with ethidium bromide: 100 bp MW ladder (M); *Ava*I indigestible fragment (376 bp) of *E. tetradactylum* (lane 1); *Ava*I digested fragments of *I. elongata* (lane 2); *Fok*I indigestible fragment of *E. tetradactylum* (lane 3); *Fok*I digested fragments of *I. elongata* (lane 4); *Msp*I digested fragments of *E. tetradactylum* (lane 5) and *Msp*I indigestible fragment of *I. elongata* (lane 6).

(Fig. 2). The *MboII* could digest both amplified fragments to produce two clear RFLP fragments (Fig. 3). The SSCP test of the two amplified cytochrome *b* fragments from the two fish species was evaluated and could differentiate the species (Fig. 4). Similar restriction analyses and SSCP tests were then used to verify the species of the salted, dried fishes that were

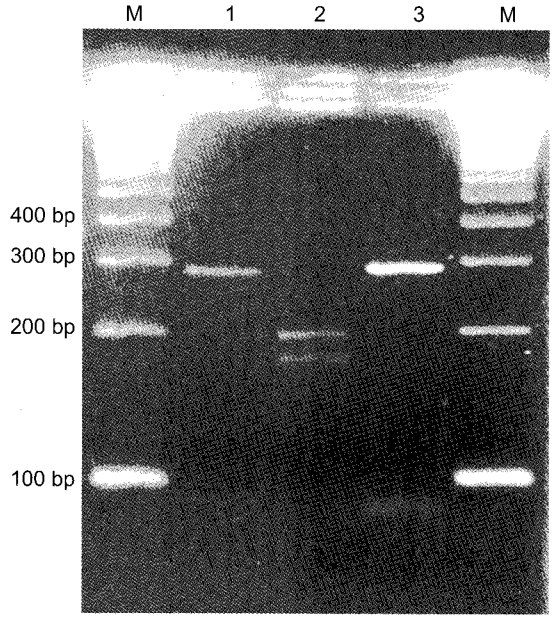

Fig. 3. RFLP patterns of *Eleutheronema tetradactylum*, *Ilisha elongata* and unknown salt-dried white-herring species digested by *Mbo*II on 4% metaphor® agarose gel staining with ethidium bromide: 100 bp MW marker (M); digested fragments of *E. tetradactylum* (lane 1); digested fragments of *I. elongata* (lane 2) and unexpected digested pattern of salt-dried white-herring samples (lane 3). Total nine salt-dried samples resulted with the later RFLP pattern.

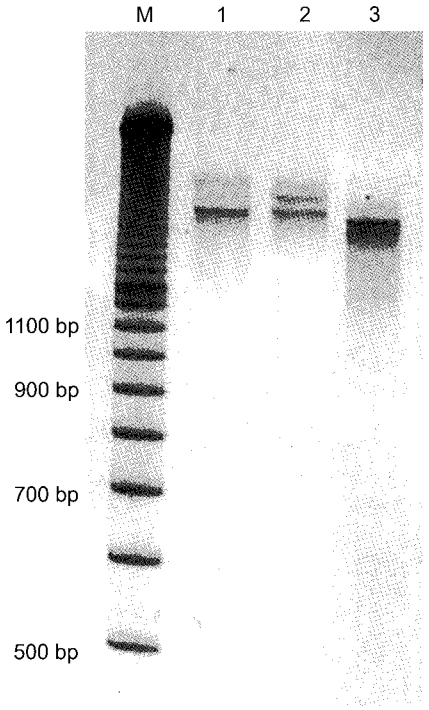

Fig. 4. The PCR-SSCP of *Eleutheronema tetradactylum* (lane 1), *Ilisha elongata* (lane 2) and the unknown salt-dried white-herring species (lane 3). M is 100 bp MW ladder.

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                    10         20         30         40         50
I. elongate         AAAAAGCTTC CATCCAACAT CTCAGCATGA TGAAACTTCG GATCATTGCT
Unknown WH          AAAAAGCTTC CATCCAACAT CTCAGCATGA TGAAACTTCG GATCATTACT

                    60         70         80         90        100
I. elongate         GGGACTATGT CTTATTTCAC AAATCCTAAC AGGACTTTTC CTGGCTATAC
Unknown WH          AGGACTGTGC CTCCTGTCAC AAATCTTAAC AGGACTCTTT TTAGCCATAC

                   110        120        130        140        150
I. elongate         ACTACACCTC TGACATCGCC ACTGCATTCT CATCCGTTAC ACATATCTGC
Unknown WH          ATTACACCTC CGACATTGCT ACTGCATTTT CATCCGTTAC ACATATCTGT

                   160        170        180        190   MboII ↴ 200
I. elongate         CGTGATGTAA ACTACGGATG ACTTATCCGA AATATACATG CAAACGGTGC
Unknown WH          CGTGACGTAA ATTACGGATG ACTTATCCGA AATATACATG CAAACGGCGC

                 ↴ MboII  210        220        230        240        250
I. elongate         CTCCTTCTTC TTCATCTGCA TCTACGCACA CATCGCTCGA GGGTTATACT
Unknown WH          CTCTTTCTTT TTCATTTGCA TCTACGCACA TATCGCCCGA GGACTATACT

                   260        270        280        290        300
I. elongate         ATGGCTCCTA CCTATACAAA GAAACATGAA ACATTGGAGT AGTCCTTCTC
Unknown WH          ATGGTTCCTA TCTATATAAA GAAACATGAA ACATCGGAGT AATCCTTCTT
                                                      ↥ MboII
                   310        320        330        340        350
I. elongate         CTTCTAACAA TAATAACAGC CTTCGTAGGC TACGTCCTAC CTTGAGGACA
Unknown WH          CTCCTAACAA TAATAACAGC CTTCGTAGGC TATGTCTTAC CCTGAGGACA

                   360        370        376
I. elongate         AATATCATTC TGAGGGGCTG CAGTTT
Unknown WH          AATATCATTC TGAGGGGCTG CAGTTT
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Fig. 5. Comparison of specific amplified cytochrome *b* fragment sequences from two different white-herring (WH) samples, one was known as *Ilisha elongata* and the other was suspected as *I. melastoma*. Total 39 bp (10%) differences were observed. The distinguishable *Mbo*II restriction sites between two sequences were indicated with arrows.

labeled as either *E. tetradactylum* (threadfin) or *I. elongata* (white herring) by wholesalers. All of the salted, dried threadfin samples produced consistent RFLP and SSCP patterns when compared to those of the raw *E. tetradactylum* (threadfin). For the salted, dried white herring, seven samples had the same RFLP and SSCP patterns as the raw *I. elongata* (white herring) sample, whereas the remaining samples exhibited a different set of RFLP patterns in the *MboII* restriction test (Fig. 4). The subsequent SSCP test of these samples showed that they had identical SSCP patterns, indicating that they likely belonged to similar species. The fragment amplified from this unknown species was sequenced and compared with that of *I. elongata* (Fig. 5). Distinguishable restriction sites for *MboII* were identified in the two sequences (Table 2).

The cytochrome *b* DNA sequences of *I. elongata* and the unknown white herring species had 39 bp differences, of which 37 were transitions, *i.e.*, interchanges of purines (A↔G) or pyrimidines (C↔T). After translating the DNA sequences into amino acid sequences, only two differences in amino acids were found between the two species (Fig. 6). This indicates that the unknown white herring was a species closely related to *I. elongata* (Kocher et al., 1989; Sebastio et al., 2001). The National Center for Biotechnology Information (NCBI) databases (NCBI, 2003) were searched for the sequence of the unknown white

Table 2. Calculated restriction patterns of the cytochrome *b* gene fragments of *Ilisha elongata* and the unknown white-herring species digested with *Mbo*II

Enzyme	Species	Cut position	Fragment size (bp)
MboII	*I. elongate*	198 & 201	198, 175, 3
	Unknown white-herring species	289	289, 87

	10	20	30	40	50
I. elongate	KKLPSNISAW	WNFGSLLGLC	LISQILTGLF	LAMHYTSDIA	TAFSSVTHIC
Unknown WH	KKLPSNISAW	WNFGSLLGLC	LLSQILTGLF	LAMHYTSDIA	TAFSSVTHIC

	60	70	80	90	100
I. elongate	RDVNYGWLIR	NMHANGASFF	FICIYAHIAR	GLYYGSYLYK	ETWNIGVVLL
Unknown WH	RDVNYGWLIR	NMHANGASFF	FICIYAHIAR	GLYYGSYLYK	ETWNIGVILL

	110	120	125
I. elongate	LLTMMTAFVG	YVLPWGQMSF	WGAAV
Unknown WH	LLTMMTAFVG	YVLPWGQMSF	WGAAV

Fig. 6. Alignment of amino acid sequences translated (1st frame) from the DNA sequences of amplified cytochrome *b* fragment of *Ilisha elongate* and unknown white-herring species.

herring species using blastn and blastx. No perfect match of the sequences was found. Based on the AFCD, the unknown species used to produce salted, dried white herring is likely *Ilisha melastoma* (*I. melastoma*) because it belongs to the same family as *I. elongata* (Pristigasteridae), shares the same local common name with *I. elongata*, and is commonly caught in the East China Sea. The difference in the species of white herring found was not related to either the time or location where they were bought. Therefore, we deduced that the salted, dried white herring sold in the local market was actually produced from at least two fish species. One of them was verified as *I. elongata*, whereas the other one is suspected to be *I. melastoma*. Authentication of the correct species is important for analyses and to prevent misleading the consumer regarding processed fish products.

The two test techniques used are faster and less expensive than sequencing analyses, particularly when a large number of samples are tested (Ram et al., 1996). The SSCP test was more sensitive than the RFLP test at detecting the differences in the white herring species. Although a false negative result is occasionally possible, the SSCP test is a sensitive technique used to detect DNA differences as small as a single base mutation (Hayashi, 1992). Distinguishable SSCP patterns were successfully generated from the 376 bp cytochrome *b* fragment compared to the conventional length limited to a range of 150-300 bp due to the constraints of gel resolution. Longer PCR products could be first digested with suitable restriction enzymes before the application of the SSCP technique (Dean and Gerrard, 1991; Hayashi, 1992). The RFLP test was relatively simple, requiring less equipment and skills, and the results were reliable, as long as adequate restriction tests were done. Consistent results from both SSCP and RFLP tests could increase the credibility of the authentification.

In conclusion, both the RFLP and SSCP test techniques were successfully used to authenticate salted, dried *E. tetradactylum* and *I. elongata* based on cytochrome *b* gene fragments. Whereas the salted, dried threadfin was produced only from *E. tetradactylum*, the salted, dried white herring sold in the local market was likely produced from two closely related *Ilisha* species.

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