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DISCRIMINATION OF PENAEID SHRIMPS WITH PCR-RFLP ANALYSIS

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ABSTRACT A rapid polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis was designed to examine genetic differentiation of some penaeid shrimps. Three hundred and fifty-six bp of cytochrome-b gene, a specific part of mitochondrial genome, was amplified with PCR to figure out differences between shrimp species *Penaeus semisulcatus*, *Penaeus kerathurus*, *Parapenaeus longirostris*, and *Metapenaeus monoceros*. PCR products were digested with different restriction endonucleases (*Dde*I, *Mbo*I, *Mbo*II, and *Mse*I.). None of the tested enzymes, alone, was able to distinguish between the four shrimp species, but with the combination of the results of two digestions, all of the species could be differentiated. It was shown that PCR-RFLP method can be used to expose fraudulent substitutions of processed shrimps prior to marketing.

KEY WORDS: shrimp, PCR, RFLP, cytochrome-b

INTRODUCTION

Considering body size, *Penaeus semisulcatus* and *Penaeus kerathurus* have been more preferred and priced than *Parapenaeus longirostris* and *Metapenaeus monoceros* in Turkish markets (Kumlu 2001). It is known that morphological features can be used successfully to identify fresh shrimps from each other, but not in the case of processed (Céspedes et al. 2000). Therefore, authentication of species is necessary for correct labeling to assure consumers for the quality and safety of food (Ram et al. 1996, Rehbein et al. 1997, Klossa-Kilia et al. 2002).

Methods based on proteins, including electrophoresis, isoelectric focusing, liquid chromatography, and immunoassays have been used to differentiate species in the last decade (Sotelo et al. 1993, Gallardo et al. 1995). However, they were not enough to detect frauds of processed foods because of their small structures or destruction by heating of proteins in heat-treated products (Rehbein 1990). Therefore DNA-based techniques have been used to identify and differentiate closely related or heavily processed foods. (Bourdy et al. 2003, Mackie et al. 1999). Mitochondrial DNA provided some advantages such as having simple genetic structure, exhibiting a straightforward mode of genetic transmission, and easy isolation in the identification of species (Unseld et al. 1995). PCR- RFLP was mostly preferred because of its combining simplicity, speed, resolving power, and low cost compared with the other techniques such as direct sequencing of PCR products and conventional mitochondrial DNA analysis (Céspedes et al. 2000, Wolf et al. 1999). It was aimed to figure out differences between frozen *P. semisulcatus* (Green tiger prawn), *P. kerathurus* (Mediterranean shrimp), *Parapenaeus longirostris* (Pink shrimp), and *Metapenaeus monoceros* by using RFLP in this study.

MATERIALS AND METHODS

Four penaeid shrimp species namely *P. semisulcatus* (Green tiger prawn), *P. kerathurus* (Mediterranean shrimp), *Parapenaeus longirostris* (Pink shrimp), and *Metapenaeus monoceros* were bought from commercial fish markets in Istanbul. All of the fresh specimens were morphologically identified and stored at –20°C until DNA extractions. A total of 40 specimens (10 individuals ($n = 10$) from each one) were tested.

A 50-mg frozen muscle sample was minced with a sterile surgical blade and transferred into a 5-mL sterile tube. Extraction buffer (2 mL, 400mM Tris-HCL [pH 8.0], 150 mM NaCl, 60 mM EDTA, 1% [w/v] sodium dodecyl sulfate [SDS], and 5 μL of 20 mg/mL proteinase K) was added. The mixture was incubated at 50°C for 12 h. After digestion, 2 mL phenol-chloroform-isoamyl alcohol 25:24:1 was added. The samples were vortexed for 5 min and centrifuged at $\times 3{,}000g$ for 5 min at 4°C. Two micro liters of the clear aqueous supernatant was treated with 200 μL sodium acetate and 5 mL of 100% ethanol to allow the precipitation of DNA, which was collected using

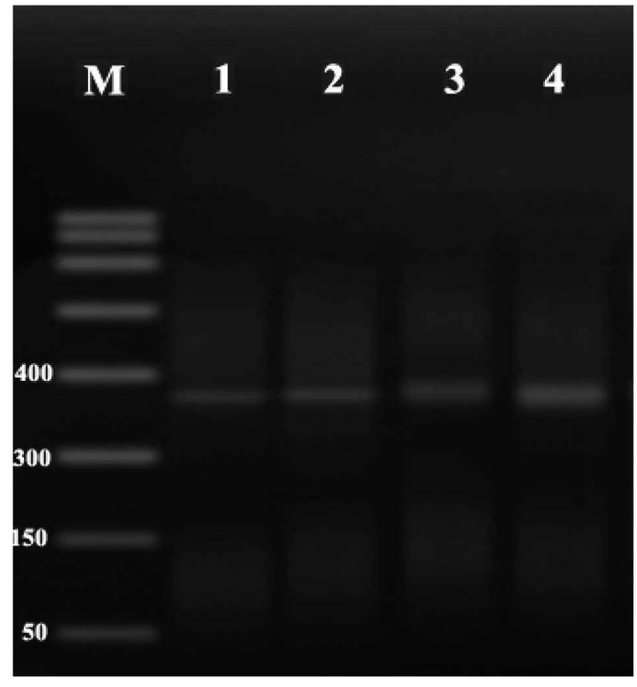


Figure 1. The PCR product sizes of *Penaeus semisulcatus* (Lane 1), *Penaeus kerathurus* (Lane 2), *Parapenaeus longirostris* (Lane 3), and *Metapenaeus monoceros* (Lane 4).

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P. monodon	1 56
	CCAGCTAACATTTCAACTTTGTGAAACTTTGGTTCTCTCCTTGGTTTATGCTTAGT
	57 112
	TATTCAAATCGTTACTGGACTCTTTTTAGCTATACACTAACTGCTCATGTTGACCT
	113 168
	AGCATTCTCTAGTGTAGCACATATTTGTCGAGATGTAAATTAGGATGACTTCTCCG
	169 224
	AACGTTACATGCTAATGGAGCGTCTTTCTTCTTCATTTGTCTGTATATACATACCG
	225 280
	GACGAGGTATTTATTATGGATCGTTTTTATATATACATGCTTGATCAGTAGGTGTC
	281 336
	ATTATTCTTCTACTAGTTATAGCGACTGCCTTCTTAGGGTATGTGTTACCT**TGAGG**
	337 356
	ACAAATATCATTCTGGGGAG

Figure 2. Alignment of the 356 bp cytochrome-b gene amplicon of *Penaeus monodon* (Chaicherdsakul 1999). Priming sites are bold and underlined.

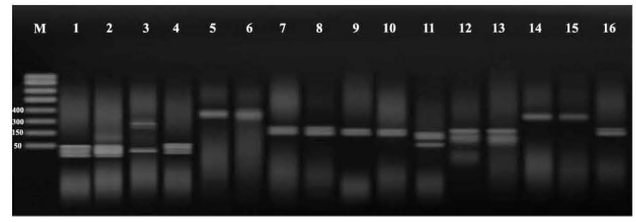


Figure 3. Results of restriction enzyme analysis of cytochrome-b PCR products obtained from the different shrimp species. M, molecular weight marker; Lane 1–4, *Dde*I; Lane 5–8 *Mbo*I; Lane 9–12 *Mbo*II; and Line 13–16 *Mse*I restriction patterns (Lane 1, 5, 9, and 13 *Penaeus semisulcatus*; Lane 2, 6, 10, and 14 *Penaeus kerathurus*; Lane 3, 7, 11, and 15 *Parapenaeus longirostris*; Lane 4, 8, 12, and 16 *Metapenaeus monoceros*).

centrifugation at $\times$ 14,000*g* for 10 min at 4°C. The pellet was washed twice with 70% ethanol and dried under vacuum. The DNA was suspended again in 50-µL sterile distilled water and used directly in the PCR.

DNA amplification was carried out in a final volume 25 µL containing PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin) 2 µL $MgCl_2$, 0.25 µL of each dNTP (0.2 mM), 1 µL of each primer (20 pmol), 0.5 µL Taq-polymerase (5 IU), and 3 µL (200 pg/µL) of the extracted DNA. Primers used for the amplification were designated as cyt-b1 (5′-cca tcc aac atc tca gca tga tga aa-3′) and cyt-b2 (5′-ccc ctc aga atg ata ttt gtc ctc a-3′) (Barlett & Davidson 1991). PCR amplification conditions were as follows: one preliminary denaturation step at 94°C for 3 min followed by 30 PCR cycles. Standard denaturation was made at 94°C for 1 min, annealing at 60.2°C for 0.5 min and primer extension at 72°C for 3 min. A final extension at 72°C for 7 min was performed. PCR products were examined by electrophoresis through a 1% agarose gel (Agarose, Sigma) in 1$\times$ TBE (Tris-Borate-EDTA) buffer (0.89 M Tris borate, pH 8.3, containing 0.02 M EDTA) and stained with ethidium bromide. As size reference, a wide range (50– 1,000 bp) DNA marker (Sigma) was used. The PCR product sizes per species were approximately 350 bp (Fig. 1). DNA sequence analyzes of the studied shrimps have not been determined so far, therefore, considering relationships with the studied species, 356 bp part of cytochrome-b gene of *Penaeus monodon* amplificated by the same primers used in the present study (Chaicherdsakul 1999) was used (Fig. 2).

Four restriction endonucleases were used to differentiate the shrimp species: *Dde*I, *Mbo*I, *Mbo*II, and *Mse*I. It was described that recognition sequence and cut size of each restriction enzymes in Table 1 (Anonymus 2007). Ten micro liters of PCR mixture was digested using 10 units of restriction enzyme at the 37°C for 12 h. Restriction fragments were separated on a 1.5% agarose gel for 1.5 h at 90 V. DNA fragments were observed and photographed under UV illumination. The sizes of DNA bands were compared with DNA marker run on the same gel.

Each species was scored for the sizes of DNA bands and data entered into a proximity matrix. Jaccard coefficients of similarity were calculated; cluster analysis was performed and a dendrogram was produced using the statistical package SPSS Inc. for Windows (1999).

RESULTS

*Mse*I and *Mbo*I restriction enzymes could be used for differentiation of all the species. All enzymes used in RFLP digested the amplification products, but none, alone, was able to differentiate among all four shrimp species. Therefore, the results from two different endonucleases were combined. Result of the RFLP analysis of the shrimp species was shown in Figure 3 and approximate fragment size observed by digesting the cytochrome-b gene of four shrimp species with four different restriction endonucleases in Table 2.

TABLE 1.

Recognition and cutting sizes of *Dde*I, *Mbo*I, *Mbo*II, and *Mse*I enzymes (Anonymus 2007).

Restriction Enzymes	Recognition Sequence	Cutting Size
*Dde*I	C^TNAG	5′ C ↓ T N A G 3′
		3′ G A N T ↑ C 5′
*Mbo*I	^GATC	5′ ↓ G A T C 3′
		3′ C T A G ↑ 5′
*Mbo*II	GAAGA (8/7)	5′ G A A G A N N N N N N N N ↓ 3′
		3′ C T T C T N N N N N N N ↑ N 5′
*Mse*I	T^TAA	5′ T ↓ T A A 3′
		3′ A A T ↑ T 5′

TABLE 2.

Approximate fragment size (bp) observed by digesting the cytochrome-b gene of four shrimp species with four different restriction endonucleases.

	Penaeus Semisulcatus	*Penaeus Kerathurus*	*Parapenaeus Longirostris*	*Metapenaeus Monoceros*
*Dde*I	50	50	280	70
	<50	<50	<50	50
	<50	<50		<50
	<50	<50		<50
*Mbo*I	350	350	200	200
			150	150
*Mbo*II	180	180	150	180
	150	150	130	
			70	
*Mse*I	180	350	350	180
				150

TABLE 3.

Proximity matrix.

Case	Jaccard Measure			
	Penaeus Semisulcatus	*Penaeus Kerathurus*	*Parapenaeus Longirostris*	*Metapenaeus Monoceros*
Penaeus semisulcatus	1.000	0.600	0.000	0.333
Penaeus kerathurus	0.600	1.000	0.143	0.143
Parapenaeus longirostris	0.000	0.143	1.000	0.143
Metapenaeus monoceros	0.333	0.143	0.143	1.000

As for the intraspecific similarity, the highest value was found between *P. semisulcatus* and *P. kerathurus* (60%), whereas the similarity coefficient with resembling value was 14% among *Penaeus kerathurus*, *Parapenaeus longirostris*, and *Metapenaeus monoceros*. *Parapenaeus longirostris* presented coefficient of intraspecific similarity of 0% with *Penaeus semisulcatus* (Table 3, Fig. 4).

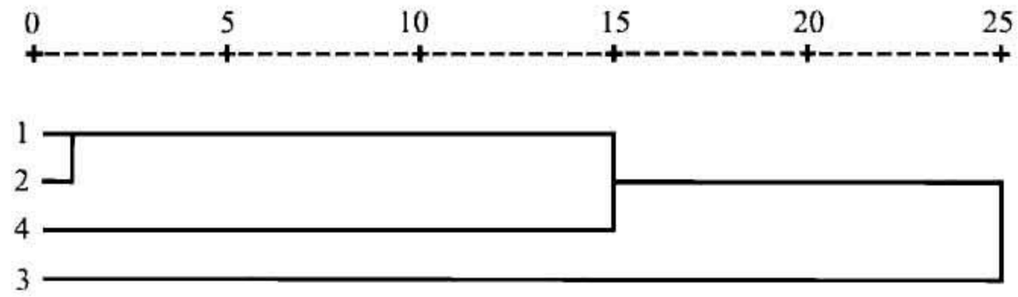


Figure 4. SPSS dendrogram of *Penaeus semisulcatus* (1), *Penaeus kerathurus* (2), *Parapenaeus longirostris* (3), and *Metapenaeus monoceros* (4). The dendrogram was constructed by using conclusions of restriction endonucleases digestible.

DISCUSSION

The penaeid shrimp species used in the present study could be easily distinguished if they were sold as a whole shrimp by the means of traditional taxonomic classification methods. However, in processed products, another technique for identifying species was needed to avoid fraud though species substitution.

The primers used in the present study were previously used by other studies to identify some fish species (Barlett & Davidson 1991, Hisar et al. 2006, Aksakal & Erdogan, 2007) and to distinguish different flatfish species (Céspedes et al. 1998). After purchasing, it was possible to obtain the results necessary to identify the shrimp muscles with the PCR-RFLP including the amplification and the restriction analysis in the first 12 h.

Protocol used in this study was based on a specific amplification of PCR product from the gene encoding the cytochrome-b (a specific part of mitochondrial DNA) and on a restriction enzyme analysis with endonucleases. Mitochondrial DNA (mtDNA) has several advantages over nuclear DNA in the diagnosis of food products. For example, the former has several times higher copy number and, therefore, was abundant in various tissues (Brand et al. 1994, Zhang & Hewitt 2003). Sequence ambiguities as a result of heterozygous genotypes were theoretically avoided because mtDNA was haploid and maternally inherited (Lockley &Bardsley 2000).

In conclusion, this study demonstrated that PCR-RFLP was a sensitive technique for the identification of some penaeid shrimp species and can be used as an accurate, rapid tool to expose commercial fraud in food control laboratories. However, further investigations may be needed to get more data from other aquatic species.

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