Molecular Identification of Commercial Spicy Pollack Roe Products by PCR-RFLP Analysis

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ABSTRACT: Spicy pollack roe products are a popular seafood item made from fish eggs that should be made with salt-cured mature roes of walleye pollack *Theragra chalcogramma*. Because of high demand and poor catch of walleye pollack, however, spicy pollack roe products are often susceptible to substitution with roes of closely related codfish. In this study, a simple method identifying the ingredients of commercial spicy pollack roe products was developed to differentiate walleye pollack from codfish substitutes such as gray cod *Gadus macrocephalus* using PCR-RFLP (Restriction Fragment Length Polymorphism) analysis. PCR amplification of the mitochondrial cytochrome b gene yielded single fragments commonly from pollack and cod. Direct digestion of the PCR products with *Mph*1103I restriction enzyme showed an unique restriction fingerprint only in pollack. This PCR-RFLP analysis enabled the reliable identification of commercial spicy pollack roe products made by only pollack roes from products padded with cod roes. It thus can be useful to expose substitution of pollack roes with lower valued codfish roes in commercial spicy pollack roe products.

Keywords: spicy pollack roe products, walleye pollack, gray cod, PCR-RFLP, cytochrome b

Introduction

We have many kinds of seafood made with fish eggs in Japan. Spicy pollack roe products, which are salt-cured pollack roes smeared with red pepper, stem from Korea and have become one of the most popular commercial seafood items under the name, Mentai. In the legal recipe for commercial spicy pollack roe products, mature roes of walleye pollack must be utilized, because the meaning of Mentai in Korean is pollack. Walleye pollack has been collected by fishery catch in the north Pacific, but its resources have rapidly decreased in recent years (Broadus and Raphael 1994).

Gray cod is closely related to walleye pollack and collected by fishery catch along with walleye pollack in the north Pacific. Like walleye pollack, gray cod has been an important seafood resource in Japan, and cod meats are edible, but cod roes are mostly unused. Because of high demand and poor catch of walleye pollack, spicy pollack roe products are susceptible to substitution with unused roes of gray cod. Even though spicy pollack roe products made only by pollack roes look like fake products padded with cod roes, such substitution is fraudulent, and the identification of the ingredient of commercial spicy pollack roe products is required to enforce labeling regulations.

So far as adult fish are concerned, observation of the morphological features provides a ready means of species identification. In the case of fish roes and eggs, however, the species are no longer recognizable. As an alternative to morphological analysis, protein-based analysis by electrophoretic and immunological methods has been developed for species identification of fish and seafood (Rehbein and others 1995; Hsieh and others 1997). These protein-based methods are, however, inadequate to species identification of commercial spicy pollack roe products, because roes and eggs contain a variety of proteins that prevent detection of species-specific molecules, and further because a high concentration of salt percolated into roes denatures egg proteins.

Alternative methods based on molecular biological analysis of genomic DNA for species identification of fish and seafood are currently preferred over protein-based analysis (Bossier 1999). Genomic DNA is the same in all cell types, while proteins vary in tissues. In addition, DNA is less affected by food-processing technologies than proteins are. These facts suggest that molecular biological analysis may be the best tool to identify the ingredient of commercial spicy pollack roe products. Most molecular analytical methods used to verify species identity are based on PCR amplification of polymorphic portion of genomic DNA due to the simplicity, specificity, and sensitivity (Palumbi 1996). Among several PCR-based methodologies, a simple and promising method is restriction fragment length polymorphism analysis of PCR products (PCR-RFLP). This method constitutes a simpler alternative than costly DNA sequencing technique and has been robustly applied in routine surveys as required in inspection programs of commercial seafood (Ram and others 1996; Davidson 1998; Quinteiro 1998; Cocolin 2000; Hold 2001; Sebastio and others 2001; Sanjuan and Comesana 2002; Hsieh and others 2003).

This article develops a simple and reliable method for the identification of the ingredient of commercial spicy pollack roe products by PCR-RFLP analysis of conserved but polymorphic fragment of the mitochondrial gene encoding cytochrome b. Although this methodology is a 1st report on the application of molecular biological analysis to species identification for salt-cured fish egg products, it can be routinely used to expose fraudulent or unintentional mislabeling of commercial spicy pollack roe products in food-inspection laboratories.

Materials and Methods

Samples

Frozen roes of walleye pollack and gray cod, both of which were caught in Russia, and spicy pollack roe products listed in Table 1

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were obtained from the commercial supplier in Japan. All samples were stored in a laboratory freezer at –40 °C until DNA preparation.

DNA preparation

Total genomic DNA was prepared from frozen roes or commercial spicy pollack roe products by the urea-Chelex-Proteinase K method. Approximately 2.5 to 4.5 mg samples were immersed in extraction buffer containing 4 M urea, 1% Tween 20, 1% Nonidet P-40, 5% Chelex-100 (Sigma, Madrid, Spain), and 10 μg Proteinase K (Sigma), mixed by vortex, and incubated at 55 °C with shaking until complete dissolution of eggs. After boiling for 8 min followed by centrifugation at 15000 × *g* for 5 min, the supernatant was collected, and pH was adjusted to 8.0 by addition of one-hundredth volume of 100TE (1 M Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0).

PCR-RFLP analysis

PCR amplification of the 59 segment of the cytochrome b gene was performed in 10 mL of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 0.005% Brij35, 10 mM DTT) containing 200 mM each dNTP, 0.5 unit *Taq* DNA polymerase (Sigma), 0.5 mM each primer, TARA-14F (59-GGAAA ACCCA TCCAA TCCTA-39) and TARA-571R (59-CAGCA ACAAC AAAGG GGAAT-39), and 1 mL of template DNA. PCR reaction was performed in a Techgene thermal cycler (Techne, Albany, N.Y., U.S.A.) programmed as 2 min at 94 °C, 30 cycles of 10 s at 94 °C, 10 s at 58 °C, and 40 s at 72 °C, and finally 5 min at 72 °C. RFLP analysis was carried out in 10 mL of R+ buffer (Fermentas, Hanover, Md., U.S.A.) containing 5 unit *Mph*1103I (Fermentas) and 5 mL of PCR amplicon at 37 °C for 2 h. A 10 mL portion of reactant was run at 15 V/cm for 40 min on 2.0% agarose gels and visualized under ultraviolet illumination in an EDAS290 Gel Documentation System (Invitrogen, Chuo-ku, Tokyo, Japan).

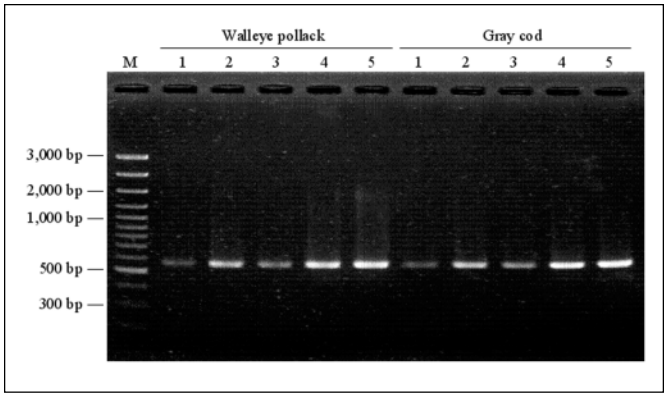

Figure 1—PCR amplification of the mitochondrial cytochrome b gene using codfish universal primers from roe samples of walleye pollack and gray cod. Numbers indicate individual samples. Lane M, 100 bp ladders.

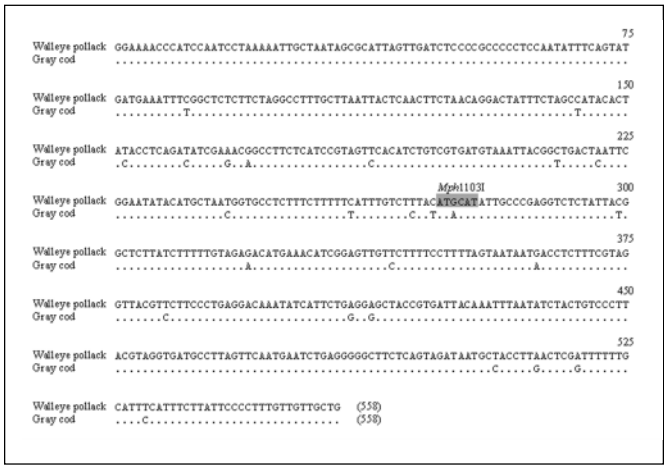

Figure 2—Aligned nucleotide sequences of the 5′ segment of the mitochondrial cytochrome b gene amplified using codfish universal primers from walleye pollack and gray cod. *Mph*1103I restriction site is with shadow.

Results and Discussion

Mitochondrial DNA sequences, so far, have been extensively used for species identification of animals (Avise 1994; Kocher and Stepien 1997) because of the design of various universal primers for PCR amplification of specific sequences, a great abundance in nuclear acid extracts, and a high copy number (Albert and others 1994). Because the mitochondrial genome is maternally inherited, sequence ambiguities from heterozygous genotypes are avoided (Lockley and Bardsley 2000). The cytochrome b gene of the mitochondrial genome has been shown to be of ancient origin (Esposti and others 1993) and among the most extensively sequenced genes in vertebrates (Johns and Avise 1998), including fish (Hsieh and others 1997). For the identification of the species origin of fish and seafood products, a relatively high mutation rate and sufficient point mutations of the cytochrome b gene allow us to discriminate even closely related fish species (Ram and others 1996; Quinteiro 1998; Cocolin 2000; Hold 2001; Sebastio and others 2001; Bartlett and Davidson 1991; Chow and others 1993; Cespedes and others 1998; Lindstrom 1999; Russell and others 2000; Jerome and others 2003).

PCR amplifications of single 558 bp fragments at the 5′ segment between positions 14 and 571 on the H strand of the mitochondrial gene encoding cytochrome b were successfully carried out for roe samples of walleye pollack and gray cod using codfish universal primers (Figure 1). These primers were designed based on the conserved region found from the alignment of DNA sequences of walleye pollack [GenBank AB094061] and gray cod [GenBank AB078152]. For 5 individual specimens per each species, both strands of the PCR products were sequenced, and no intraspecies nucleotide polymorphism in the partial cytochrome b gene was verified. A comparison of the sequences indicated that a unique recognition site of *Mph*1103I restriction enzyme was present only in walleye pollack (Figure 2). As expected, *Mph*1103I digestion yielded 2 fragments at a size of 277 bp and 281 bp in walleye pollack, apparently a single 280 bp fragment due to a 4-bp difference, but a single 558-bp fragment in gray cod (Figure 3). These electrophoretic patterns showed no intraspecies polymorphism for all samples tested. Results indicate that PCR-RFLP analysis using *Mph*1103I restriction enzyme enables the differentiation of walleye pollack from gray cod.

Table 1 summarizes the details of 7 different products that were sold at the market. The products numbered 5 and 6 were labeled "spicy codfish roes" and indicated as a mixture of roes of pollack and cod. All other products were labeled "spicy pollack roes," indicating they contained only roes of pollack. PCR amplifications using codfish universal primers were successfully carried out for all samples to obtain single 558 bp fragments (Figure 4, Panel A), and digestion of them with *Mph*1103I restriction enzyme yielded 2 different restriction profiles (Figure 4, Panel B). Profile-1 was defined to show the apparent single 280 bp fragment, the same as in case of pollack roe samples after *Mph*1103I digestion (Figure 3, Lane 3),

Table 1—List of commercial spicy pollack roe products analyzed in this study

Product	Production country	Label	Labeled ingredient
1	China	Spicy pollack roe	Walleye pollack
2	Korea	Spicy pollack roe	Walleye pollack
3	Japan	Spicy pollack roe	Walleye pollack
4	Japan	Spicy pollack roe	Walleye pollack
5	Japan	Spicy codfish roe	Walleye pollack and Gray cod
6	Japan	Spicy codfish roe	Walleye pollack and Gray cod
7	Japan	Spicy pollack roe	Walleye pollack

and obtained from the products numbered 1, 2, and 7. Profile-2 was also defined to show at least 2 fragments at a size of 558 bp and around 280 bp and obtained from the products numbered 3 to 6. The larger and smaller fragments were observed in cases of cod roe sample (Figure 3, Lane 3) and pollack roe sample (Figure 3, Lane 4), respectively, after *Mph*1103I digestion. These findings indicate that the profile-2 is derived from the mixture of pollack and cod. It is noteworthy that not only the products numbered 5 and 6, labeled as the mixture of pollack and cod, but also the products numbered 3 and 4, labeled as genuine pollack, have the profile-2.

The extraction method employed in this study allowed us to obtain sufficient amounts of total genomic DNA, which were enough for over 100 PCR amplifications, from small clusters comprising 5 eggs of all samples tested. Based on our previously reported urea-SDS-Proteinase K method (Aranishi and Okimoto 2004), the improved method saved both time and procedure and could be easily performed in food inspection laboratories. A high concentration of urea denatured egg proteins under conditions where a Chelex 100 chelating resin prevented the breakdown of DNA. In addition, this procedure not only dispensed with the homogenization procedure of eggs but also promoted the disruption of organelle membranes by Proteinase K and detergents. All procedures could be completed in 30 to 60 min, depending on the number of eggs sacrificed. The quality of DNA obtained was not affected by the sample conditions, such as fresh, frozen, or processed roes.

Our results indicate that PCR-RFLP analysis of the partial mitochondrial cytochrome b gene is a sensitive technique for the differentiation of walleye pollack roe from gray cod roe in commercial spicy pollack roe products. It can be used as an accurate and rapid tool to expose commercial fraud, as demonstrated in Figure 3. In addition, with any products sold at market, we can obtain the results necessary to identify the ingredient roes, because all procedures from DNA preparation to RFLP analysis are completed in 5 h. Compared with alternative methods, including direct nucleotide sequencing of PCR products, PCR-RFLP analysis has the advantage to be simpler, cheaper, and, especially, more useful for routine analysis of many samples in food inspection programs.

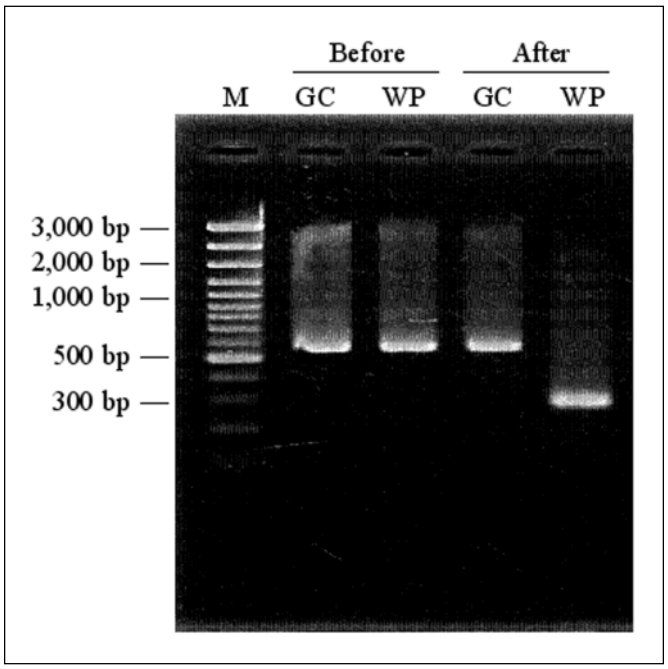

Figure 3—PCR-RFLP analysis of the mitochondrial cytochrome b gene amplified using codfish universal primers from roe samples of walleye pollack (WP) and gray cod (GC) before and after *Mph*1103I digestion. Lane M, 100 bp ladders.

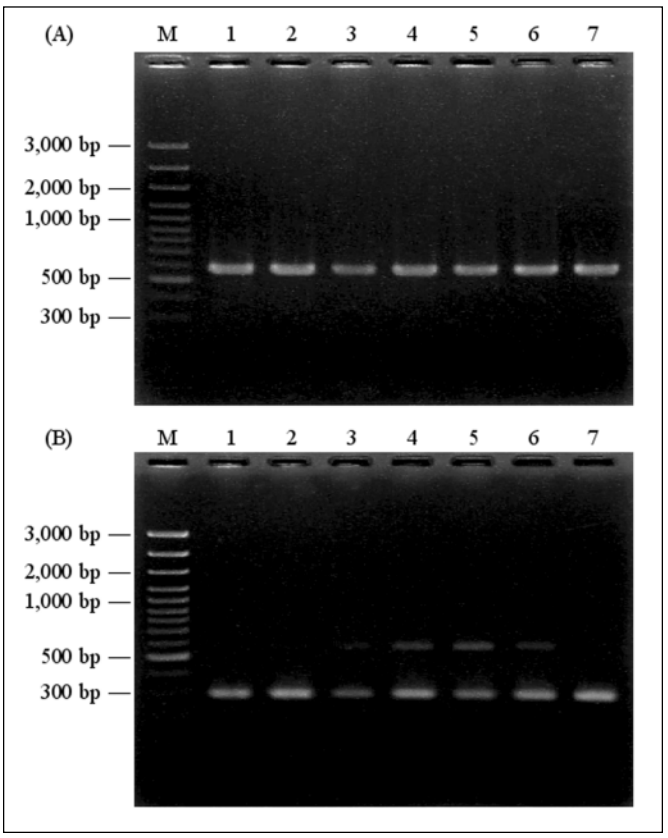

Figure 4—PCR-RFLP analysis of the mitochondrial cytochrome b gene amplified using codfish universal primers from commercial spicy pollack roe products listed in Table 1. Panels (A) and (B) indicate the PCR products and those after *Mph*1103I digestion, respectively. Lane M, 100 bp ladders.

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References

Albert B, Lewis D, Raff J, Roberts K, Watson JD. 1994. Molecular biology of the cell. New York: Garland Publishing.

Aranishi F, Okimoto T. 2004. PCR-based detection of allergenic mackerel ingredients in seafood. J Genet 83:193–5.

Avise JC. 1994. Molecular markers, natural history and evolution. New York: Chapman & Hall.

Bartlett SE, Davidson WS. 1991. Identification of Thunnus tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. Can J Fish Aquat Sci 48:309–17.

Bossier P. 1999. Authentication of seafood products by DNA patterns. J Food Sci 64:189–93.

Broadus JM, Raphael VV. 1994. Living resource problems. In: Broadus JM, Raphael VV, editors. The oceans and environmental security. Covelo, Calif.: Island Press. p 50–85.

Cespedes A, Garcia T, Carrera E, Gonzalez I, Sanz B, Hernandez PE, Martin R. 1998. Identification of flatfish species using polymerase chain reaction (PCR) and restriction analysis of the cytochrome b gene. J Food Sci 63:206–9.

Chow S, Clarke ME, Walsh PJ. 1993. PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): A simple method for species and stock identification. Fish Bull 30:207–24.

Cocolin L, D'Agaro E, Manzano M, Lanari D, Comi G. 2000. Rapid PCR-RFLP method for the identification of marine fish fillets (seabass, seabream, umbrine, and dentex). J Food Sci 65:1315–7.

Davidson WS. 1998. DNA/PCR techniques. In: Ashurst PR, Dennis MJ, editors. Analytical methods of food authentication. London: Blackie Academic and Professional/Thomson Science. p 182–203.

Esposti MD, De Vries S, Crimi M, Ghelli A, Patarnello T, Meyer A. 1993. Mitochondrial cytochrome b: evolution and structure of the protein. Biochim Biophys Acta 1143:243–71.

Hold GL, Russell VJ, Pryde SE, Rehbein H, Quinteiro J, Vidal R, Rey-Mendez M, Sotelo CG, Perez-Martin RI, Santos AT, Rosa C. 2001. Development of a DNA-based method aimed at identifying the fish species present in food products. J Agric Food Chem 49:1175–9.

Hsieh Y-HP, Chen F, Nur M. 1997. Rapid identification of cooked red snapper using isoelectric focusing. J Food Sci 62:15–9.

Hsieh YW, Hwang PA, Pan HH, Chen JB, Hwang DF. 2003. Identification of tetrodotoxin and fish species in an adulterated dried mullet roe implicated in food poisoning. J Food Sci 68:142–6.

Jerome M, Lemaire C, Bautista JM, Fleurence J, Etienne M. 2003. Molecular phylogeny and species identification of sardines. J Agric Food Chem 51:43–50.

Johns GC, Avise JC. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome b gene. Mol Biol Evol 15:1481–90.

Kocher TD, Stepien CA. 1997. Molecular systematics of fishes. New York: Academic Press.

Lindstrom DP. 1999. Molecular species identification of newly hatched Hawaiian amphidromous gobioid larvae. Mar Biotechnol 1:167–74.

Lockley AK, Bardsley RG. 2000. DNA-based methods for food authentication. Trends Food Sci Technol 11:67–77.

Palumbi SR. 1996. Nucleic acid II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK, editors. Molecular systematics. Sunderland, Mass.: Sinauer Associates. p 205–47.

Quinteiro J, Sotelo CG, Rehbein H, Pryde SE, Perez-Martin RI, Rey-Mendez M, Mackie IM. 1998. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. J Agric Food Chem 46:1662–9.

Ram JL, Ram ML, Baidoun FF. 1996. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. J Agric Food Chem 44:2460–7.

Rehbein H, Etienne M, Jerome M, Hattula T, Knudsen LB, Jessen F, Luten J, Bouquet W, Mackie IM, Richie AH, Perez-Martin R and Mendes R. 1995. Influence of variation in technology on the reliability of the isoelectric focusing method of fish species identification. Food Chem 52:193–7.

Russell VJ, Hold GL, Pryde SE, Rehbein H, Quinteiro J, Rey-Mendez M, Sotelo CG, Perez-Martin RI, Santos AT, Rosa C. 2000. Use of restriction fragment length polymorphism to distinguish between salmon species. J Agric Food Chem 48:2184–8.

Sanjuan A, Comesana AS. 2002. Molecular identification of nine commercial flaffish species by polymerase chain reaction-restriction fragment length polymorphism analysis of a segment of the cytochrome b region. J Food Prot 65:1016–23.

Sebastio P, Zanelli P, Neri TM. 2001. Identification of anchovy (*Engraulis encrasicholus* L.) and gilt sardine (*Sardinella aurita*) by polymerase chain reaction, sequence of their mitochondrial cytochrome b gene, and restriction analysis of polymerase chain reaction products in semipreserves. J Agric Food Chem 49:1194–9.