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Specific Detection by the Polymerase Chain Reaction of Potentially Allergenic Salmonid Fish Residues in Processed Foods

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Salmonid fish is one of the allergenic items that are recommended to be labeled in the Japanese allergen-labeling system. This study develops a salmonid-specific polymerase chain reaction (PCR) method. A new primer pair, SKE-F/SKE-R, was designed to specifically detect the salmonid fish gene encoding mitochondrial DNA cytochrome *b*. Genomic DNAs extracted from 58 kinds of seafood and 11 kinds of processed food were individually subjected to PCR by using the primer pair, and a salmonid-specific fragment of 212 bp was only amplified in the salmonid samples and salmonid-containing processed foods. The detection limit of the PCR method was as low as 0.02 fg/μL of salmonid fish DNA (corresponding to 10 copies). There is no ELISA method for salmonid fish, making our PCR method the only reliable measure for detecting salmonid fish in processed foods.

Key words: food allergy; seafood; salmonid; PCR; cytochrome *b*

Food allergy, an example of type I allergies mediated by immunoglobulin E antibodies, is a serious problem to human health in industrialized countries.[1–4] Of a variety of allergenic foods, fish is recognized as one of the most common causes of food allergy, especially in such coastal countries as Scandinavia and Japan where fish is consumed daily as an important source of proteins and nutrients.[4–6] Immediately after ingesting fish, a sensitized subject with a high level of fish-specific immunoglobulin E develops such allergic reactions as urticaria, diarrhea, and anaphylactic shock; even fatal cases have been recorded.[7–9] The sole effective treatment to prevent possible life-threatening reactions in food allergy, including fish allergy, is strict avoidance of the consumption of allergenic foods. Adequate information regarding potentially allergenic ingredients in processed food products is herefore essential to patients. The Ministry of Health, Labour, and Welfare in Japan has enforced a labeling system for allergenic ingredients in processed foods since April 2002; labeling of allergenic ingredients is classified into two levels, mandatory (seven ingredients) and recommended (18 ingredients), on the basis of the number of cases of actual illnesses and the respective degree of seriousness.[4] Salmon and mackerel, as examples of fish, are recommended to be labeled by the allergen-labeling system because they are considered to be most frequently responsible for incidents of fish allergy in Japan.

Methods for analyzing the allergenic ingredients in processed foods are indispensable to ensure the value of the allergen-labeling system. Sensitive and specific methods for analyzing allergenic ingredients in processed foods are generally based on detecting species-specific proteins by an enzyme-linked immunosorbent assay (ELISA) and for species-specific DNA molecules by the polymerase chain reaction (PCR). Quantitative ELISA methods have already been developed for egg,[10] milk,[10] wheat,[10] buckwheat,[10] peanut,[10] crustacean,[11,12] soybean,[13] and walnut,[14] qualitative (conventional) PCR methods for wheat,[15] buckwheat,[16] peanut,[17] shrimp,[18] crab,[18] soybean,[19] walnut,[20] kiwifruit,[21] banana,[22] and pork,[23] and semi-quantitative real-time PCR methods for banana[22] and meat.[24] At present, however, no analytical methods for salmon or mackerel are available. A sandwich ELISA method using a polyclonal anti-cod parvalbumin antibody has recently been developed for determining fish in foods, but unfortunately cannot discriminate between fish species.[25] Parvalbumin, the well-known major fish allergen,[5,6] is extremely thermostable, being a desirable target protein to be analyzed in processed foods by ELISA. However, it seems to be difficult to obtain a monoclonal antibody specific to either salmon parvalbumin or mackerel parvalbumin, because of the high amino acid sequence similarity among fish parvalbumins.

In view of these circumstances, analytical methods for salmon or mackerel in processed foods need to be developed based not on ELISA, but on conventional PCR. Salmon is often included in such highly processed foods as chazuke (dried ingredients that are added to boiled rice with tea) and furikake (rice seasoning powder), rather than mackerel. This study was therefore applied to develop a conventional PCR method for specifically detecting salmonid species in foods. We designed two primer sets for PCR amplification; one is

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Table 1. Primers Used in This Study

Name		Nucleotide sequence	Specificity	Amplicon
Specific primer for salmonid fish	SKE-F	5′-AACAGCTTTTTCCTCTGT(C/T)TG-3′	Cytchrome *b*/sense	212 bp
	SKE-R	5′-AGCCTACGAATGCAGTTATTATAGTG-3′	Cytchrome *b*/antisense	
Universal primer for animals	AN1-5′	5′-TGACCGTGCGAAGGTAGC-3′	16S rRNA/sense 1	350–470 bp
	AN2-5′	5′-TAACTGTGCTAAGGTAGC-3′	16S rRNA/sense 2	
	AN-3′	5′-CTTAATTCAACATCGAGGTC-3′	16S rRNA/antisense	
Universal primer for plants	CP 03-5′	5′-CGGACGAGAATAAAGATAGAGT-3′	Chloroplast/sense	123 bp
	CP 03-3′	5′-TTTTGGGGATAGAGGGACTTGA-3′	Chloroplast/antisense	

for detecting the salmonid-specific mitochondrial cytochrome *b* gene and the other for the mitochondrial 16S ribosomal RNA (rRNA) gene that is universal in animals. The latter primer set was used to examine whether the DNAs extracted from animal samples or food samples containing animals are valid for PCR quality. We provide in this report evidence for the established PCR method being highly specific and sensitive for use in detecting salmonid fish DNA even in highly processed foods.

Materials and Methods

Samples. A total of 69 food samples, including 58 kinds of seafood (refer to Table 2 for details of these samples) and 11 kinds of highly processed food (refer to "*Application of the PCR method to various processed foods*" in the Results and Discussion section for the details of these samples) were all purchased at retail stores. The whole body of sakura shrimp, soft parts of four kinds of bivalves (Japanese oyster, short-neck clam, common orient clam and brackishwater clam), and muscles of the remaining seafoods were used for DNA extraction. The highly processed foods were directly subjected to DNA extraction.

Extraction of genomic DNA. Genomic DNA was extracted from each sample by using a silica-gel membrane-type kit (DNeasy Blood & Tissue kit; Qiagen, Hilden, Germany) as recommended by the manufacturer. The extracted DNA was diluted with distilled water to a final concentration of 20 ng/μL and stored at −20 °C until being used. An undiluted DNA extract was used for subsequent PCR analyses when the concentration of the extracted DNA was less than 20 ng/μL.

Oligonucleotide primers. The primers used in this study are listed in Table 1. Based on the reported cytochrome *b* DNA sequences of the four salmonid species, chum salmon *Oncorhynchus keta*, king salmon *O. tshawytscha*, pink salmon *O. gorbuscha*, and rainbow trout *O. mykiss*, the SKE-F/SKE-R primer pair was designed for PCR amplification of the salmonid-specific gene (Fig. 1). In addition, the AN1-5′/AN2-5′/AN-3′ primer mixture was designed from the 16S rRNA gene of mitochondrial DNA and used for universal detection of DNA derived from animals. The CP 03-5′/CP 03-3′ primer pair, for universal detection of DNA derived from plants was the same as that previously reported.[17] Each primer was synthesized and purified in a reversed-phase column by Operon Biotechnology (Tokyo, Japan), diluted with distilled water to a final concentration of 50 μM and stored at −20 °C until being used.

PCR amplification and agarose gel electrophoresis. The reaction mixture for PCR was prepared in a PCR reaction tube. In the case of the SKE-F/SKE-R primer pair, the 25 μL volume contained 50 ng of DNA, 0.2 mM dNTP, 2.0 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of EX Taq DNA polymerase (Takara Bio, Otsu, Japan). An undiluted DNA extract was directly used when the concentration of the extracted DNA was less than 20 ng/μL. PCR amplification was performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 62 °C for 0.5 min, and extension at 72 °C for 0.5 min, and final extension at 72 °C for 7 min. PCR using the AN1-5′/AN2-5′/AN-3′ universal primer mixture was similarly carried out, except that the annealing temperature and $MgCl_2$ concentration were respectively changed to 50 °C and 3.0 mM. The amplified products after PCR were analyzed by agarose gel electrophoresis on 1.5% gel. DNA was stained with ethidium bromide.

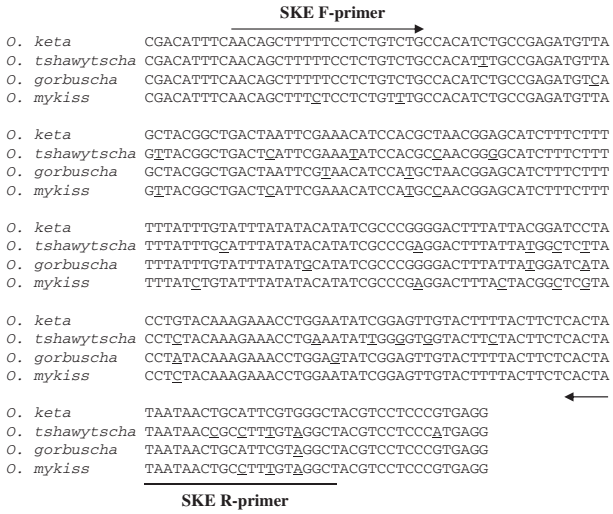

Fig. 1. Comparison of the Nucleotide Sequences of the Target Region on the Cytochrome *b* Gene among Four Species of Salmonid Fish (chum salmon *Oncorhynchus keta*, king salmon *O. tshawytscha*, pink salmon *O. gorbuscha*, and rainbow trout *O. mykiss*).

The positions of the SKE-F and SKE-R primers are respectively indicated by right facing and left facing arrows. Underlined nucleotides indicate the disparity with the DNA sequence of *O. keta*. GenBank accession nos.: *O. keta*, AF125212; *O. tshawytscha*, AF392054; *O. gorbuscha*, AJ314562; and *O. mykiss*, AB120717.

Cloning and sequencing of the amplified fragments. Each PCR product was investigated for its nucleotide sequence by the TA cloning technique. The PCR product was cloned into the pCR® II-TOPO® vector (TOPO TA cloning® kit; Invitrogen, Tokyo, Japan) by standard procedures, and the recombinant plasmid was transformed into the *Escherichia coli* strain JM109 competent cell. The sequence of the clones was determined with a BigDye® Terminators v1.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 genetic analyzer (Applied Biosystems).

Sensitivity of the PCR method. Seven concentration levels (0, 0.02 fg/μL, 0.2 fg/μL, 2.0 fg/μL, 20 fg/μL, 0.2 pg/μL, and 2.0 pg/μL) of the chum salmon plasmid DNA sample were prepared and subjected to PCR with the SKE-F/SKE-R primer pair. The amplified products were analyzed by agarose gel electrophoresis.

Results

Specificity of the PCR method

PCR with the SKE-F/SKE-R primer pair was evaluated for specificity by using the genomic DNAs

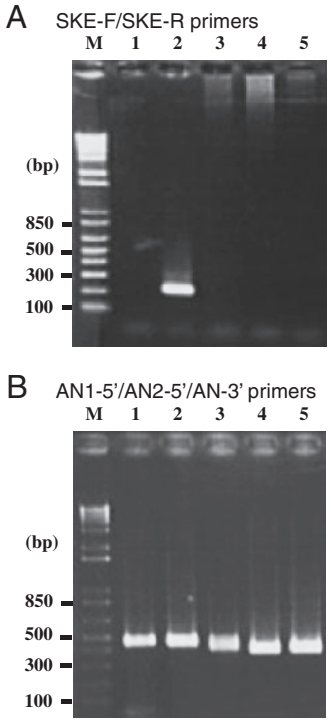

Fig. 2. Specific Detection by PCR of Salmonid Fish in Various Seafoods.

PCR products amplified by using the SKE-F/SKE-R primer pair (A) and the AN1-5′/AN2-5′/AN-3′ primer mixture (B) were analyzed by agarose gel electrophoresis. Lane M, 100-bp ladder size standard; lane 1, Pacific mackerel *Scomber japonicus*; lane 2, chum salmon *Oncorhynchus keta*; lane 3, spear squid *Loligo bleekeri*; lane 4, black tiger prawn *Penaeus monodon*; lane 5, red snow crab *Chionoecetes japonicus*.

extracted from the following five kinds of seafood: raw samples of chum salmon, Pacific mackerel *Scomber japonicus*, black tiger prawn *Penaeus monodon* and spear squid *Loligo bleekeri*, and a boiled sample of red snow crab *Chionoecetes japonicus*. When the PCR products were subjected to agarose gel electrophoresis, a salmonid-specific fragment of 212 bp was detected in chum salmon, but not in the other four kinds of seafood (Fig. 2A). In contrast, the AN1-5′/AN2-5′/AN-3′ primer set generated a fragment of about 400 bp from all the five samples (Fig. 2B). Nucleotide sequencing and a subsequent homology search of the GenBank database revealed the amplified fragments from chum salmon (449 bp), Pacific mackerel (454 bp), black tiger prawn (398 bp), and red snow crab (398 bp) to individually correspond to a part of the species-specific 16S rRNA gene (GeneBank accession nos.: AF125512 for chum salmon, AB032521 for Pacific mackerel, AY744272 for black tiger prawn, and AB188107 for red snow crab). It is very likely that the 406 bp fragment from spear squid also encoded a part of the spear squid 16S rRNA gene whose sequence has not been registered in the GenBank database. The results obtained with the AN1-5′/AN2-5′/AN-3′ primer set confirmed the DNAs extracted from the five samples to certainly be valid for PCR quality. The result that PCR using the SKE-F/SKE-R primer pair generated no amplified fragment from the four samples other than chum salmon was also reliable, not being false-negative.

The SKE-F/SKE-R primer pair and AN1-5′/AN2-5′/AN-3′ primer mixture were respectively assumed to be specific to salmonid fish and universal to animals as already described. The applicability of the PCR method was further examined by using four kinds of raw salmonid fish sample, king salmon *Oncorhynchus tshawytscha*, red salmon *O. nerka*, steelhead trout (anadromous species of *O. mykiss*) and rainbow trout (landlocked species of *O. mykiss*), and a number of other seafood samples (Table 2). The DNAs extracted from these seafood samples were all confirmed to be of PCR quality by using the AN1-5′/AN2-5′/AN-3′ universal primer set. In contrast, PCR with the SKE-F/SKE-R primer pair only produced a salmonid-specific fragment of 212 bp from the DNAs extracted from the four kinds of salmonid fish sample, supporting the results of our PCR method being highly specific to salmonid fish. These results, together with those with the five kinds of seafood shown in Fig. 2, are summarized in Table 2. Overall, of the 58 kinds of seafood sample examined, five salmonid samples were positive; neither false-positive nor false-negative results were obtained for any sample. Although not distinguished in Table 2, we observed a positive detection in such heat-sterilized salmonid foods as canned products in which the DNAs were degraded.

Detection limit for the PCR method

The PCR method was tested for its sensitivity by using various concentrations (0.02 fg/μL–2 pg/μL) of the chum salmon plasmid DNA. Figure 3 shows that PCR with the SKE-F/SKE-R primer pair clearly afforded a specific fragment of 212 bp, even at a DNA concentration as low as 0.02 fg/μL (equivalent to 10 copies). This suggests that the proposed PCR method was highly sensitive, its detection limit being 0.02 fg/μL or less.

Application of the PCR method to various processed foods

In the next step, the PCR method was applied to the following 11 kinds of processed food: breakfast cereal, kezuribushi (flakes of dried mackerel, horse mackerel, and sardine), katsuobushi (flakes of dried skipjack tuna), yogurt including aloe, yogurt including aloe and kiwi-fruit, salmon zosui (dried ingredients for rice porridge), chicken zosui, crab zosui, salmon chazuke (dried ingredients for adding to boiled rice with tea), salmon furikake (rice seasoning powder), and apple cookie. Of these processed food products, salmon zosui, salmon chazuke, and salmon furikake were respectively labeled to contain salmon, salmon flakes and salmon extract, and salmon extract. When the DNAs extracted from these processed foods were individually subjected to PCR by using the SKE-F/SKE-R primer pair, amplification of a salmonid-specific fragment of 212 bp was found in salmon zosui, salmon chazuke, and salmon furikake, but not in the remaining processed foods (Fig. 4A). Nucleotide sequencing of the amplified products revealed that chum salmon was contained in salmon zosui and salmon chazuke, and pink salmon in salmon furikake. In contrast, PCR using the AN1-5′/AN2-5′/AN-3′ primer set for animal DNA produced a fragment (350–470 bp) in nine processed foods, while non-specific fragments were apparent in breakfast cereal and no fragment in apple cookie (Fig. 4B). PCR using

Table 2. PCR Analysis of Salmonid Fish DNA in Various Seafoods with the SKE-F/SKE-R Primer Pair

Seafood sample*		PCR
Raw sample (5 species)	Fish (5 species): chum salmon (*Oncorhynchus keta*, Sirozake), king salmon (*Oncorhynchus tshawytscha*, Masunosuke), red salmon (*Oncorhynchus nerka*, Benizake), steelhead trout (*Oncorhynchus mykiss*, Steelhead), rainbow trout (*Oncorhynchus mykiss*, Nijimasu)	+
Raw sample (45 species)	Fish (29 species): sweetfish (*Plecoglossus altivelis*, Ayu), round greeneye (*Chlorophthalmus borealis*, Maruaomeeso), Pacific saury (*Cololabis saira*, Sanma), Pacific cod (*Gadua macrocephalus*, Madara), monkfish (*Lophius litulon*, Kianko), channel rockfish (*Sebastolobus macrochir*, Kichiji), brown rockfish (*Sebastes inermis*, Mebaru), red baraccuda (*Sphyraena pinguis*, Akakamasu), Japanese whiting (*Sillago japonica*, Sirogisu), Japanese amberjack (*Seriola quinqueradiata*, Buri), horse mackerel (*Trachurus japonicus*, Maaji), striped jack (*Pseudocaranx dentex*, Shimaaji), red seabream (*Chrysophrys major*, Madai), striped marlin (*Tetrapturus audax*, Makajiki), black marlin (*Makaira mazara*, Kurokajiki), swordfish (*Xiphias gladius*, Mekajiki), Pacific mackerel (*Scomber japonicus*, Masaba), spotted mackerel (*Scomber australasicus*, Gomasaba), Japanese Spanish mackerel (*Scomberomorus niphonius*, Sawara), skipjack tuna (*Katsuwonus pelamis*, Katsuo), bigeye tuna (*Thunnus obesus*, Mebachi), Japanese butterfish (*Psenopsis anomala*, Ibodai), Japanese flounder (*Paralichthys olivaceus*, Hirame), Pacific halibut (*Hippoglossus stenolepis*, Ohyo), willowy flounder (*Tanakius kitaharai*, Yanagimushigarei), slime founder (*Microstomus achne*, Babagareri), thread-sail filefish (*Stephanolepis cirrhifer*, Kawahagi), black scraper (*Thamnaconus modestus*, Umazurahagi), green rough-backed puffer (*Lagocephalus wheeleri*, Shirosabafugu) Ascidiacea (1 species): sea squirt (*Halocynthia roretzi*, Maboya) Crustacean (3 species): black tiger prawn (*Penaeus monodon*, Ushiebi), Pacific white shrimp (*Penaeus vannami*, Banameiebi), pink shrimp (*Pandalus eous*, Hokkokuakaebi) Mollusk (12 species): golden cuttlefish (*Sepia esculenta*, Kouika), kisslip cuttlefish (*Sepia lycidas*, Kaminariika), spear squid (*Loligo bleekeri*, Yariika), Japanese flying squid (*Todarodes pacificus*, Surumeika), diamond squid (*Thysanoteuthis rhombus*, Sodeika), horned turban (*Turbo cornutus*, Sazae), abalone (*Haliotis* sp., Awabi), common scallop (*Patinopecten yessoensis*, Hotategai), Japanese oyster (*Crassostrea gigas*, Magaki), short-neck clam (*Tapes japonica*, Asari), common orient clam (*Meretrix lusoria*, Hamaguri), brackishwater clam (*Corbicula* sp., Sijimi)	−
Dried sample (6 species)	Fish (5 species): round herring (*Etrumeus teres*, Urumeiwashi), Japanese anchovy (*Engraulis japonicus*, Katakuchiiwashi), Pacific cod (*Gadus macrocephalus*, Madara), Arabesque greenling (*Pleurogrammus azonus*, Hokke), Atlantic mackerel (*Scomber scombrus*, Taiseiyomasaba) Crustacean (1 species): sakura shrimp (*Sergia lucens*, Sakuraebi)	−
Boiled sample (2 species)	Crustacean (1 species): red snow crab (*Chionoecetes japonicus*, Benizuwaigani) Mollusk (1 species): common octopus (*Octopus vulgalis*, Madako)	−

*Each sample is listed by the common name, followed by the scientific and Japanese names in parentheses.

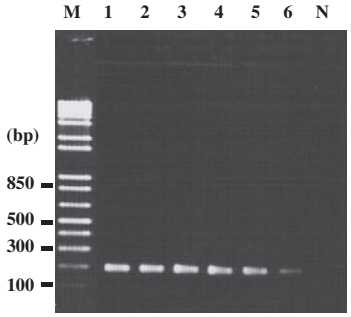

Fig. 3. Sensitivity of the PCR Method Using the SKE-F/SKE-R Primer Pair.

Plasmid DNA was used as the template DNA. The PCR products were analyzed by agarose gel electrophoresis. Lane M, 100-bp ladder size standard. The DNA concentration was as follows: lane 1, 2 pg/μL; lane 2, 0.2 pg/μL; lane 3, 20 fg/μL; lane 4, 2 fg/μL; lane 5, 0.2 fg/μL; lane 6, 0.02 fg/μL; N, no template control.

the CP 03-5′/CP 03-3′ primer pair for plant DNA produced a plant-specific fragment of 123 bp in nine processed foods, excepting kezuribushi and katsuobushi containing no plant ingredients (Fig. 4C). These results supported the clain that the DNAs extracted from all the processed foods examined were of PCR quality.

Discussion

A conventional PCR method with the new SKE-F/SKE-R primer pair was developed in this study for the specific detection of salmonid fish, one of the items that are recommended to be labeled with the Japanese allergen-labeling system. It is sometimes required to identify salmonid species in processed foods to check false labeling or false descriptions. Our PCR method also satisfies this requirement. Indeed, species identification of salmonid fish in the three processed foods (salmon zosui, salmon chazuke, and salmon furikake) was achieved by sequencing the PCR products.

The choice of the target gene and the design of primers are known to strongly affect the specificity and sensitivity of a PCR detection system. The target region for PCR amplification has usually been chosen from the mitochondrial DNA in previous species identification studies on seafood products. This is not only because the mitochondrial DNA shows an adequate degree of intraspecific and interspecific variations, but also because it has numerous copies per cell.[26] To detect salmon residues in processed foods by using a PCR technique, therefore, we also searched for salmonid-specific regions in the mitochondrial DNA and eventually chose the cytochrome *b* region as a desirable target for amplification. The useful SKE-F/SKE-R primer pair could therefore be designed from the DNA sequence of the salmonid fish cytochrome *b* region. Furthermore, the AN1-5′/AN2-5′/AN-3′ primer set universal to animal DNAs could also be designed from the DNA sequence of the mitochondrial 16S rRNA gene.

The PCR method developed in this study was highly specific to salmonid fish. Although a number of food samples were examined by the PCR method when using

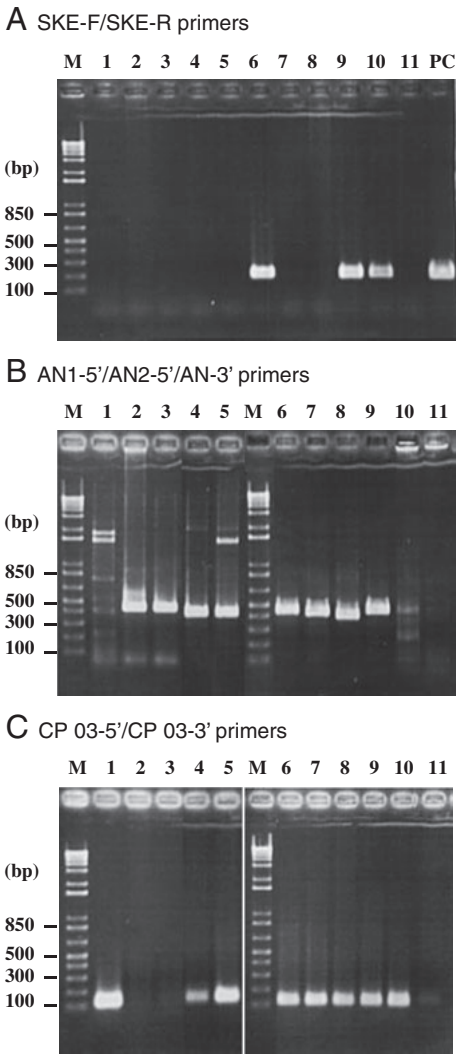

Fig. 4. Specific Detection by PCR of Salmonid Fish in Various Processed Foods.

PCR products amplified by using the SKE-F/SKE-R primer pair (A), the AN1-5′/AN2-5′/AN-3′ primer mixture (B), and the CP 03-5′/CP 03-3′ primer pair (C) were analyzed by agarose gel electrophoresis. Lane M, 100-bp ladder size standard; lane 1, breakfast cereal; lane 2, kezuribushi (flakes of dried mackerel, horse mackerel and sardine); lane 3, katsuobushi (flakes of dried skipjack tuna); lane 4, yogurt including aloe; lane 5, yogurt including aloe and kiwifruit; lane 6, salmom zosui (dried ingredients for rice porridge); lane 7, chicken zosui; lane 8, crab zosuicrab; lane 9, salmon chazuke (dried ingredients for adding to boiled rice with tea); lane 10, salmon furikake (rice seasoning powder); lane 11, apple cookie; lane PC, positive control (chum salmon DNA).

the SKE-F/SKE-R primer pair, positive results were only obtained with the salmonid samples and salmonid-containing processed foods. The genomic DNAs from the remaining foods were evaluated to be of PCR quality by amplification with the AN1-5′/AN2-5′/AN-3′ primer mixture for animal DNA or the CP 03-5′/CP 03-3′ primer pair for plant DNA, but none of them afforded a salmonid-specific fragment. Salmon zosui, salmon chazuke, and salmon furikake are highly processed foods in which DNA is likely to have been degraded to a large extent. Nevertheless, they were confirmed to contain salmon residues by the PCR method in accordance with their ingredient lists, implying that the PCR method would be useful for specifically detecting salmonid fish even in highly processed foods. In addition to the high specificity to salmonid fish, the PCR method proved very sensitive, experiments with the chum salmon plasmid DNA showing the detection limit to be as low as 0.02 fg/μL (corresponding to 10 copies). No protein-based detection method has previously been developed for salmonid species, so our PCR method stands as the only reliable way to detect trace amounts of salmonid fish in processed foods.

Our conventional PCR method is not quantitative, but only qualitative. Allergenic ingredients with the Japanese allergen-labeling system are required or recommended to be labeled if they are contained at more than several μg/g (or μg/mL) in processed foods. Accordingly, it is important to develop quantitative methods for evaluating salmonid fish in processed foods to verify the labeling of salmon. Semi-quantitative real-time PCR methods have already been established for banana[22] and meat.[24] It seems to be possible to develop a semi-quantitative real-time PCR method for salmon by using the same SKE-F/SKE-R primer pair designed in this study, although an expensive real-time PCR instrument would be needed. An alternative quantitative method is protein-based ELISA. Sandwich ELISA using a polyclonal anti-cod parvalbumin antibody has recently been developed for determining fish in processed foods.[25] No specific species of fish (*e.g.*, salmon and mackerel) but fish in general is listed as one of allergenic ingredients in the labeling systems of EU and USA. The recently developed sandwich ELISA method might therefore be utilized to verify the labeling of fish. However, no salmonid-specific protein has so far been found and no region specific only for salmonid fish has been recognized in salmon parvalbumin. It is thus assumed to be difficult to develop a salmonid-specific ELISA method. The quantification of salmonid fish in processed foods may only be possible by semi-quantitative real-time PCR based on our conventional PCR method.

It should finally be noted that, besides salmon, the Japanese allergen-labeling system also recommends mackerel to be labeled. Further study is needed to develop a conventional PCR method for specifically detecting mackerel in processed foods.

Acknowledgment

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