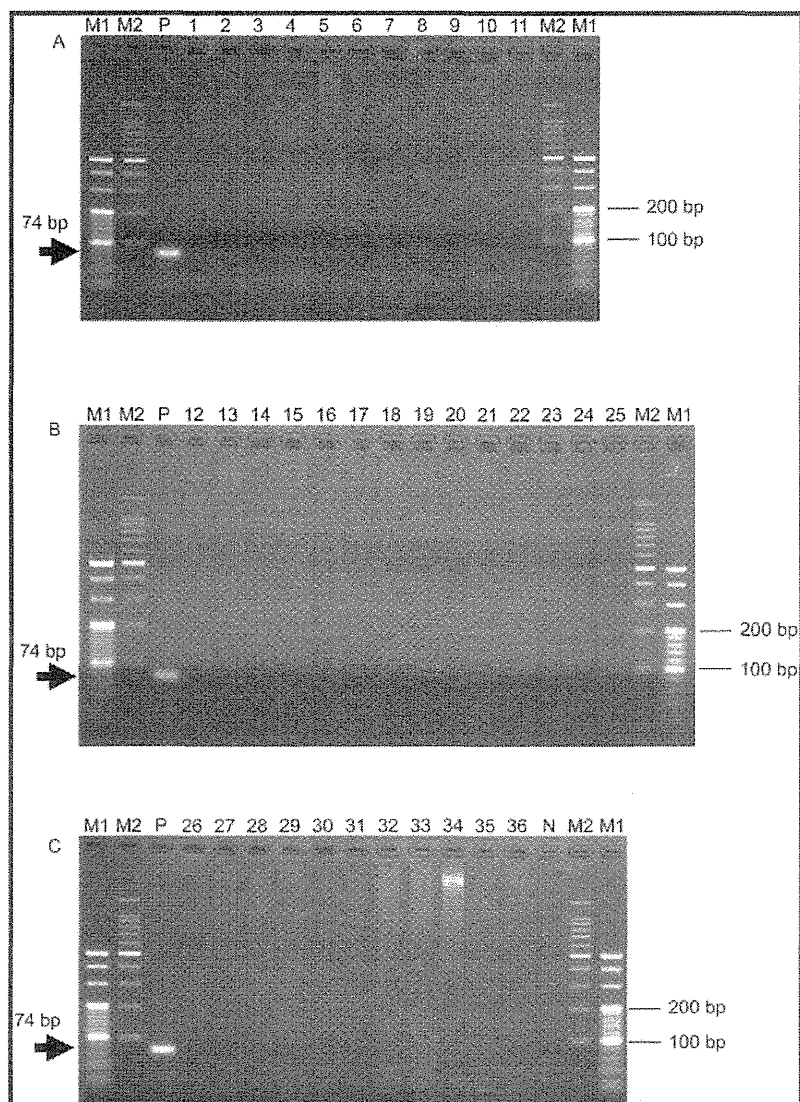

Figure 2. Amplification of the plant DNAs by the peach PCR method: amplification from genomic DNAs of various Rosaceae fruits (A), other fruits (B), and other plant foods (C). The arrowheads indicate the expected size of PCR product. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is a 20 bp DNA ladder DNA marker (Takara Bio Inc.). Lanes: P, amplification of 500 fg of genomic DNA extracted from *P. persica* cv. Hakuho; 1–36, amplification of 50 ng of genomic DNA extracted from Japanese plum (1), apricot (2), cherry (3), Japanese apricot (4), almond (5), prune (6), apple cv. Fuji (7), pear (8), Japanese pear (9), strawberry (10), raspberry (11), aloe vera (12), pineapple (13), papaya (14), orange (15), satsuma orange (16), melon (17), Japanese persimmon (18), fig (19), mango (20), banana (21), avocado (22), blueberry (23), grape (24), kiwifruit (25), rice (26), soybean (27), maize (28), wheat (29), potato (30), carrot (31), onion (32), Chinese cabbage (33), spinach (34), cucumber (35), and tomato (36); N, negative control (no template).

also confirmed through PCR simulations and experiments, but with some exceptions. Amplification products of the target sizes were predicted from *P. mira* in the peach PCR simulation and from *M. sieversii*, *M. sylvestris*, *M. asiatica*, *M. prunifolia*, and *M. niedzwetzkyana* in the apple PCR simulation. Because these wild species were unavailable, PCR experiments were not conducted.

P. mira is one of the phylogenetically closest relative of peach,[30] growing wild in the western Himalayan region. It bears inedible fruit and is used as rootstock for peach and almond.[31] To our knowledge, allergenicity of *P. mira* to patients with peach allergies has not been reported. However, in view of its growing region and limited use, unintentional commingling of its fruit in food products is considered to be unlikely. If *P. mira* turns out to be nonallergenic, its detection by our method gives rise to false positives. Such incidence, however, is considered to be low, because, again, in view of its growing region and limited use, frequent unintentional commingling of its fruit in food products would be unlikely.

M. sieversii from Central Asia and *M. sylvestris* from eastern Europe are the two most likely progenitors of domesticated apple.[13,14,32,33] In addition, *M. asiatica* in East Asia, *M. niedzwetzkyana* in eastern Europe, and *M. prunifolia* in eastern China[13] are among the species phylogenetically most closely related to *M. domestica*.[14,32] Whereas these species are used mainly as rootstock or ornamental plants, there are places where fruits of

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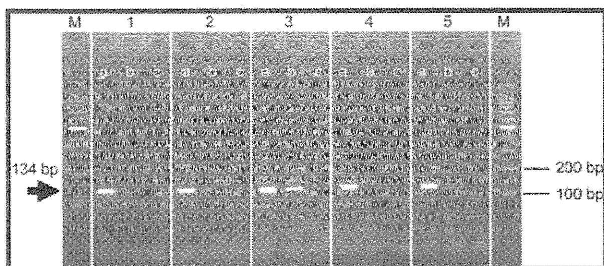

Figure 3. Amplification of the apple cultivar DNAs by the apple PCR method. The arrowheads indicate the expected size of PCR product. Samples 1−5 are amplifications of genomic DNA extracted from *M. domestica* cv. Fuji (1), Ohrin (2), Jonagold (3), Jonathan (4), and Mutsu (5). Lanes a−c represent amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA. Lane M is a 100 bp DNA ladder DNA marker (Takara Bio Inc.).

M. asiatica, *M. sieversii*, and *M. sylvestris* are consumed fresh or processed into preserves.[13] Although our search revealed no documented information as to the allergenicity of these wild species, the phylogenetically close relationship with domesticated apple strongly suggests that these species could be allergenic as well. Detection of these wild species by our method may therefore be warranted for the extra safety of apple allergic patients.

The sensitivities of the methods were confirmed by analyzing two incurred foods containing approximately the threshold level (10 ppm) of total soluble proteins from peach and apple. Because amplification products could be obtained from as low as 5 ng, $^{1}/_{10}$ of the usual amount of DNA in a reaction tube, we considered that the peach and apple detection PCRs were sensitive enough for use as confirmatory tests.

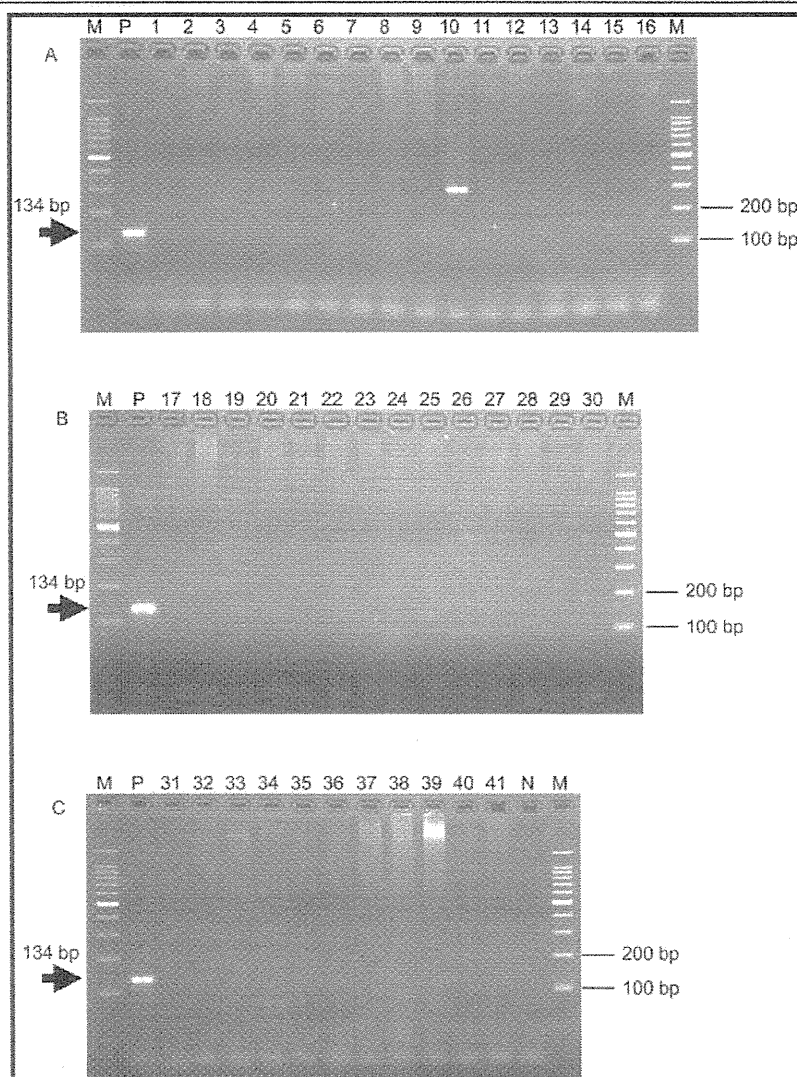

Figure 4. Amplification of the plant DNAs by the apple PCR method: amplification from genomic DNAs of various Rosaceae fruits (A), other fruits (B), and other plant foods (C). The arrowheads indicate the expected size of PCR product. Lane M is a 100 bp DNA ladder DNA marker (Takara Bio Inc.). Lanes: P, amplification of 500 fg of genomic DNA extracted from *M. domestica* cv. Fuji; 1−41, amplification of 50 ng of genomic DNA extracted from strawberry (1), raspberry (2), Japanese plum (3), apricot (4), cherry (5), Japanese apricot (6), almond (7), prune (8), peach cv. Hakuho (9), pear (10), Japanese pear (11), loquat (12), hawthorn (13), juneberry (14), Chinese quince (15), quince (16), aloe vera (17), pineapple (18), papaya (19), orange (20), satsuma orange (21), melon (22), Japanese persimmon (23), fig (24), mango (25), banana (26), avocado (27), blueberry (28), grape (29), kiwifruit (30), rice (31), soybean (32), maize (33), wheat (34), potato (35), carrot (36), onion (37), Chinese cabbage (38), spinach (39), cucumber (40), and tomato (41); N, negative control (no template).

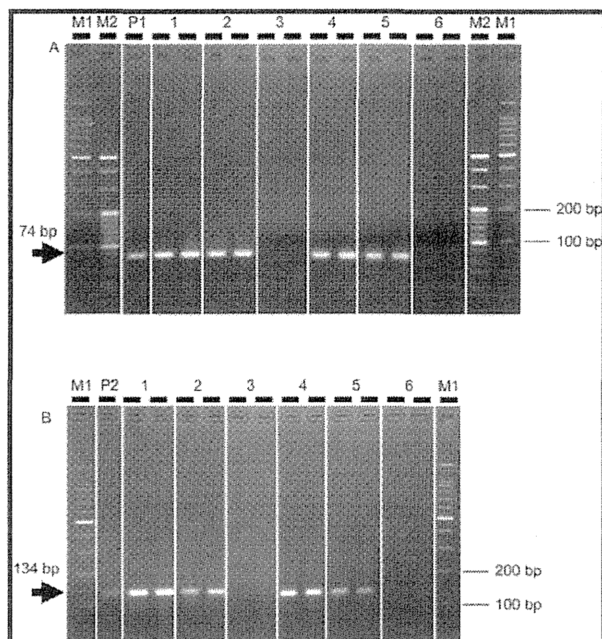

Figure 5. Sensitivity of two PCR methods (A, peach PCR method; B, apple PCR method) determined using incurred foods. The arrowheads indicate the expected size of PCR product. Samples 1−6 are amplifications of model incurred food samples of 50 ng of DNA extracted from incurred jam (1), 5 ng of DNA extracted from incurred jam (2), 50 ng of DNA extracted from control (unspiked) jam (3), 50 ng of DNA extracted from incurred cookies (4), 5 ng of DNA extracted from incurred cookies (5), and 50 ng of DNA extracted from control (unspiked) cookies (6). Lanes: P1, amplification of 50 fg of genomic DNA extracted from Hakuho; P2, amplification of 50 fg of genomic DNA extracted from Fuji. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is a 20 bp DNA ladder DNA marker (Takara Bio Inc.).

As Pettersson et al.[28] reported that a mismatch introduced at the second base from the 3′ end of a primer dramatically improved allele specificity in their molecular haplotype determination using allele-specific PCR, we adopted the technique in our primer design for allergen detection PCR. The deliberate mismatch used in the reverse primer for peach PCR (ppersica-R) was found to be effective in improving the specificity of the primer pairs. When it was not used, the primer pair amplified the target size product from nontarget cherry (*P. avium*) in addition to the target *P. persica* (data not shown). Despite the mismatch in the primer, our PCR experiments using incurred foods demonstrated that the target PCR product was amplifiable with sufficient sensitivity. The use of deliberate mismatch was not necessary in the apple PCR primer pair, because the second base from the 3′ end of the reverse primer originally designed on the ITS sequence of *M. domestica* did not match the corresponding base of nontarget *Malus* spp.

Although a proper and accurate label is an effective means of helping allergic patients avoid being exposed to the allergens, the presence of undeclared allergens cannot be ruled out entirely. Therefore, allergen detection methods are indispensable for further controlling the risk of unexpected exposure of patients to allergens. It should be kept in mind that PCR methods target a specific DNA sequence, not allergenic proteins, to detect the presence of an allergenic food. Because DNAs are generally less susceptible to degradation than proteins are to denaturation and because PCR methods are highly sensitive, they may detect very low levels of a contaminant that may be clinically insignificant. Thus, PCR methods are particularly useful as confirmatory tests after positive results from ELISA determine the levels of contaminating proteins.

In conclusion, we developed two qualitative PCRs, one for peach detection and the other for apple detection. These methods' specificity and sensitivity were considered to be sufficient for the detection of peach or apple contamination at the threshold level for declaration of allergens established by the Japanese food allergen labeling regulation. These methods are expected to be useful for monitoring and controlling possible contamination of foods by peach and apple and, consequently, for preventing unexpected exposure of allergic patients to these fruits.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; ITS, internal transcribed spacer; IGS, intergenic spacer; rbcL, large subunit of ribulose-1,5-bisphosphate carboxylase; trn (L, S, G, and H), transfer ribonucleic acid (leucine, serine, glycine, and histidine); matK, maturase K; psbA, 32 kDa quinone-binding protein of photosystem II reaction center.

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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.

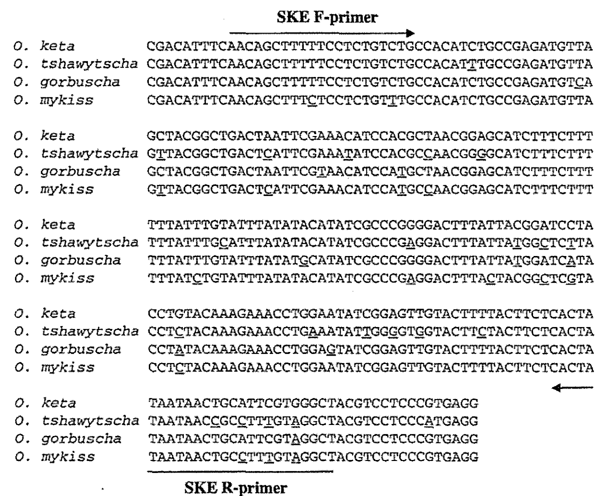

Fig. 1. Comparison of the Nucleotide Sequences of the Target Region on the Cytochrome *b* Gene among Four Species of Salmonid Fish (chum salmon *Oncorhynchus keta*, king salmon *O. tshawytscha*, pink salmon *O. gorbuscha*, and rainbow trout *O. mykiss*).

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

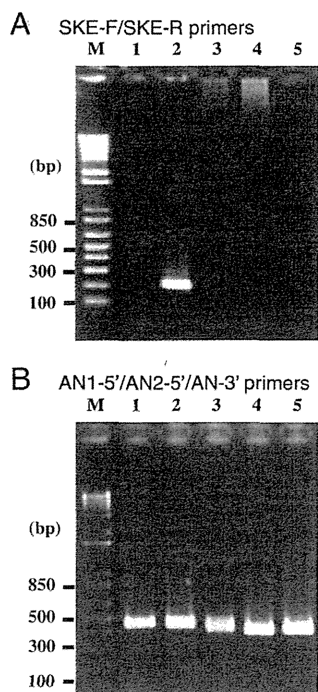

Fig. 2. Specific Detection by PCR of Salmonid Fish in Various Seafoods.

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.

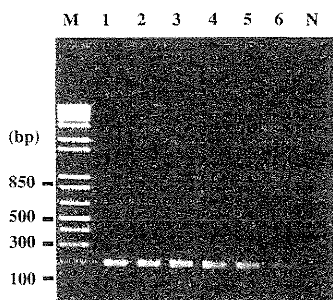

Fig. 3. Sensitivity of the PCR Method Using the SKE-F/SKE-R Primer Pair.

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

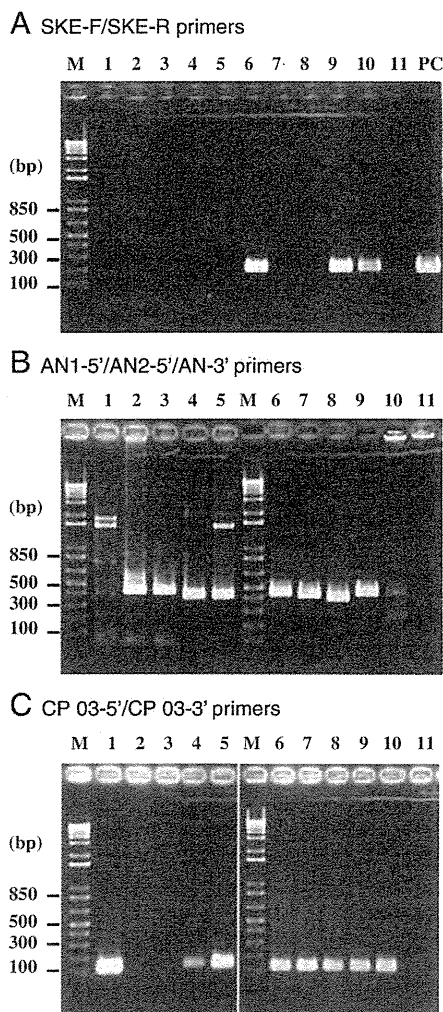

Fig. 4. Specific Detection by PCR of Salmonid Fish in Various Processed Foods.

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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Multiplex Real-Time PCR Assay for Simultaneous Detection of *Omphalotus guepiniformis* and *Lentinula edodes*

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A rapid multiplex real-time PCR assay was developed to achieve highly specific, simultaneous detection of two kinds of mushrooms, *Omphalotus guepiniformis* and *Lentinula edodes*. Primers and TaqMan minor groove binder probes were designed according to the internal transcribed spacers 1-5.8S region of rDNA and evaluated by the specificity for fruiting bodies of 17 *O. guepiniformis*, 16 *L. edodes* and samples from 57 other species. DNA extracts of all the target species had positive signals with no cross-reaction, the limit of detection being 0.00025 ng of DNA. Threshold cycle (Ct) values for raw and processed fruiting bodies and for fruiting bodies (1% (w/w)) mixed with foodstuffs or artificial gastric juice contents ranged from 17.16 to 26.60 for both examined species. This new assay proved specific to the target species, highly sensitive, and applicable to processed food samples and gastric juice contents, making it useful for rapidly identifying *O. guepiniformis* and *L. edodes*.

Key words: species identification; multiplex real-time PCR; *Omphalotus guepiniformis*; *Lentinula edodes*; poisonous mushroom

Many species of wild mushroom are consumed worldwide, serving as both nutritional and medicinal resources. *Lentinula edodes*, commonly known as shiitake in Japan, is one of the most popular edible mushrooms native to Japan, Southeast Asia, Papua New Guinea, and New Zealand.[1)] The collection and consumption of wild mushrooms such as *L. edodes* has become increasingly popular in Japan and other countries; however, mushroom poisoning has become more prevalent due to this increasing popularity and the subsequent increase in incidences of species misidentification. Poisoning by *Omphalotus guepiniformis* (Tsukiyotake), which contains the toxic compound illudin S,[2,3)] accounted for the largest percentage (31.6%) of mushroom poisoning cases in Japan between 1996 and 2005 (584 total cases).[4)] Fruiting bodies of *O. guepiniformis* are frequently found on the debris of beech species in cool temperate forests in Japan, their morphology being similar to that of the fruiting bodies of *L. edodes* and other edible mushrooms. A major cause of food poisoning by *O. guepiniformis* is its misidentification as an edible species such as *L. edodes*.

Food poisoning by *O. guepiniformis* is associated with gastrointestinal symptoms such as stomachache, diarrhea, and vomiting that can last anywhere from 30 min to 3 h after consumption.[3)] Rapid identification of the source of food poisoning is necessary for proper medical treatment. Although mushrooms are mainly identified by their morphological characteristics, their morphology can be influenced by environmental factors and by alterations during processing. In cases of mushroom poisoning thought to be caused by *O. guepiniformis*, identification of *O. guepiniformis* has previously been achieved by determining the presence of illudin S, the main toxic component of *O. guepiniformis*, using liquid chromatography,[5)] liquid chromatography with tandem mass spectrometry[6)] or gas chromatography-mass spectrometry.[7)] An illudin S reference standard is necessary to identify *O. guepiniformis* by these chromatographic methods; however, such a reference standard has not yet been made commercially available. Alternatively, a real-time PCR assay using SYBR Green I has been used in order to identify *O. guepiniformis*.[8)] Although DNA amplification-based real-time PCR using SYBR Green I is not dependent on the specimen morphology and can accurately and rapidly identify target mushroom species, a dye like SYBR Green I sometimes provides false positive results due to non-specific double-stranded DNA binding, as well as to dimerization of primers and other non-specific reaction products.[9,10)]

The TaqMan assay, using such a hybridization probe as the TaqMan minor groove binder (MGB) probe, allows for very specific species identification.[9–11)] MGB increases the melting temperature of the probe, allowing the use of a shorter probe.[11)] Real-time PCR using TaqMan MGB probes has been used to identify species of mushroom[12)] and other ingredients in foods.[13–16)] The TaqMan assay can simultaneously detect distinct fluorescent signals emitted by different reporter dyes attached at the 5′-ends of nucleic acid strands.[17)] In order to determine the cause of food poisoning when mushroom is suspected, the ability to rapidly distinguish between *O. guepiniformis* and *L. edodes* is required, as *O. guepiniformis* is frequently misidentified as *L. edodes*.

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Abbreviations: R^2, square regression correlation; MGB, minor groove binder; ITS1, internal transcribed spacers 1; FAM, 6-carboxyfluorescein; VIC, 6-carboxyrhodamine; NFQ, non-fluorescent quencher; Ct value, threshold cycle value; Rn, reporter signal normalized to the fluorescence signal of the reference dye (ROX); ΔRn, Rn minus the baseline; LOD, limit of detection; SD, standard deviation; CV, coefficient of variation

Table 1. *O. guepiniformis* and *L. edodes* Used in This Study

Species	Name	Locality	Number	Date of collection[a]	Collection number[b]	Remarks
O. guepiniformis	OG1-5	Mt. Haguro, Yamagata, Japan	5	2009/Oct.	—	Fruiting body isolated from the field
	OG6-13	Mt. Yudono, Yamagata, Japan	8	2009/Sept.	—	Fruiting body isolated from the field
	OG14	Mt. Gassan, Yamagata, Japan	1	2009/Oct.	—	Fruiting body isolated from the field
	OG15	Nagano, Japan	1	2005/Oct.	CBM-FB-36045	Freeze-dried fruiting body isolated from the field
	OG16	Nagano, Japan	1	2009/Oct.	CBM-FB-37900	Freeze-dried fruiting body isolated from the field
	OG17	Ibaraki, Japan	1	2002/Oct.	CBM-FB-31805	Freeze-dried fruiting body isolated from the field
L. edodes	LE1-2	Nagasaki, Japan	2	—	—	Commercial cultivar
	LE3-4	Oita, Japan	2	—	—	Commercial cultivar
	LE5-6	Miyazaki, Japan	2	—	—	Commercial cultivar
	LE7	Fukuoka, Japan	1	—	—	Commercial cultivar
	LE8	Gunma, Japan	1	—	—	Commercial cultivar
	LE9-16	China	8	—	—	Commercial cultivar (dried fruiting body)

[a]—: Date of collection unknown
[b]—: No number, CBM: Natural History Museum and Institute, Chiba

This study presents a rapid and specific identification method, using multiplex real-time PCR with a TaqMan MGB probe, for the simultaneous detection of *O. guepiniformis* and *L. edodes*.

Materials and Methods

Materials. α-Amylase from human saliva and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St. Louis, MO, USA). A TE buffer (pH 8.0) was purchased from Nippon Gene (Tokyo, Japan). The primer pairs and TaqMan MGB probes were synthesized by Life Technologies (Carlsbad, CA, USA) and dissolved in the TE buffer. A DNeasy Plant mini kit was purchased from Qiagen (Hilden, Germany), and a Universal Master mix was purchased from Life Technologies.

Samples. Fruiting bodies from 17 samples of *O. guepiniformis* (OG1-17) and 16 samples of *L. edodes* (LE1-16) were used in this study (Table 1). The 14 fruiting bodies of *O. guepiniformis* (OG1-14) collected in Yamagata, Japan (Mt. Haguro, Mt. Yudono, and Mt. Gassan) were provided by Dr. Yoshimasa Kasahara (Yamagata Prefectural Institute of Public Health), and the other three specimens (OG15-17), collected in both Nagano and Ibaraki prefectures, Japan, were provided by Dr. Toshimitsu Fukiharu (Natural History Museum and Institute, Chiba). The fruiting bodies of *L. edodes* used in the current study were commercial cultivars produced in Japan and China, and were obtained from markets in Fukuoka, Japan. In addition to the specimens of *O. guepiniformis* and *L. edodes*, 57 other species of mushroom (Table 2) were used in order to test for cross-reactivity; among these 57 species, 52 were purchased from markets in Fukuoka, Japan. The *A. pantherina*, *C. acromelalga*, *E. rhodopolium*, and *T. ustale* specimens were provided by Dr. Toshimitsu Fukiharu (Natural History Museum and Institute, Chiba) and Mr. Toshiyasu Ishii (Chiba Prefectural Institute of Public Health). *Omphalotus olearius* (NBRC8533) was purchased from the NITE Biological Resource Center (Chiba, Japan).

Processing of the samples. The fruiting bodies of *O. guepiniformis* (OG6) and *L. edodes* (LE8) were cut into 5-mm-thick pieces measuring approximately 30 mm × 30 mm. These pieces were processed either by baking, stir-frying, deep-frying, boiling or digesting. The pieces processed by baking were baked on both sides at 240 °C for a total time of 2 min on a hotplate; the pieces processed by stir-frying were stir-fried on both sides at 240 °C for 2 min total time on a hotplate greased with canola oil; the pieces processed by deep-frying were deep-fried at 180 °C for 2 min in a beaker filled with canola oil; the pieces processed by boiling were boiled for 30, 60, 120, or 180 min in a beaker filled with water; and artificially digested samples were prepared according to the procedure derived from *Chanvrier et al.*[18–20] To each 500 mg of a powdered sample ground with a mortar and pestle after freezing with liquid nitrogen, 1 mL of artificial saliva containing α-amylase (250 unit/mL of distilled water) was added. After vortexing for 15–20 s, the mixture was incubated with 5 mL of pepsin (1 mg/mL of 0.02 M HCl) at 37 °C for 30 min in an aluminum block bath (ALB-221, Iwaki, Japan). Each sample was inactivated by adding an equal volume of 95% ethanol and then centrifuged for 10 min at 2,000 *g* with a 6200 high-speed refrigerated centrifuge (Kubota Co., Tokyo, Japan). The supernatant was discarded, and the resulting residue was washed twice, first with a 0.2 M acetate buffer (pH 6.0) and then by a second wash with water.

Miso soup replicates containing either an *O. guepiniformis* or *L. edodes* sample were prepared from instant vegetable miso soup (Nagatanien, Tokyo, Japan). Approximately 1% (w/w) of the fruiting body of *O. guepiniformis* or *L. edodes* was added to the raw miso and dried vegetables supplied in the instant vegetable miso soup package, after which 180 mL of boiling water was added to the mixture. The miso soup replicates were then centrifuged for 10 min at 2,000 *g*, the residue obtained from each centrifugation being washed twice with distilled water and ground by a mixer.

Oligonucleotide primers and probes for multiplex real-time PCR. Two primer pairs and TaqMan MGB probes were designed from internal transcribed spacers 1 (ITS1)-5.8S rDNA by using Primer Express software v. 3.0 (Life Technologies) for detecting *O. guepiniformis* and *L. edodes*. Sequence data for the ITS1-5.8S rDNA region of *O. guepiniformis* (accession no. AY313286) and *L. edodes* (AF079572) were obtained from NCBI GenBank. The sequences of the primers and probes are listed in Table 3. The *O. guepiniformis* detection system (OMGU-F and OMGU-R with OMGU-Taq) and *L. edodes* detection system (LEED-F and LEED-R with LEED-Taq) were used in the multiplex real-time PCR assay. OMGU-Taq was labeled with the 6-carboxyfluorescein (FAM) reporter at the 5′ end, and LEED-Taq was labeled with the 6-carboxyrhodamine (VIC) reporter at the 5′ end. Both Taq-Man MGB probes were conjugated to a non-fluorescent quencher (NFQ) and MGB at the 3′ end.

DNA extraction. Each sample was frozen by using liquid nitrogen and ground to a fine powder with a mortar and pestle. Genomic DNA was extracted from 300 mg (wet weight) or 50 mg (dry weight) of the finely ground powder of each individual specimen by using the DNeasy Plant mini kit. DNA extraction was carried out according to

Table 2. Species Tested for Specificity by Multiplex Real-Time PCR

Species
Agaricus bisporus[a]
Auricularia auricular[a]
Flammulina velutipes[a]
Grifola frondosa[a]
Hypsizigus marmoreus[a]
Lyophyllum decastes[a]
Panellus serotinus[a]
Pholiota nameko[a]
Pleurotus eryngii[a]
Pleurotus ostreatus[a]
Amanita pantherina[b]
Clitocybe acromelalga[b] (CBM-FB-37920)[c]
Entoloma rhodopolium[b] (CBM-FB-35976)[c]
Omphalotus olearius[b] (NBRC 8533)[d]
Tricholoma ustale[b] (CBM-FB-37159)[c]
Allium cepa
Allium fistulosum
Allium sativum
Allium tuberosum
Amorphophallus konjac
Apium graveolens
Arctium lappa
Asparagus officinalis
Brassica oleracea var. *capitata*
Brassica oleracea var. *italica*
Brassica rapa var. *chinensis*
Brassica rapa var. *peruviridis*
Capsicum annuum
Chrysanthemum coronarium
Colocasia esculenta
Cucumis sativus
Cucurbita maxima
Daucus carota
Ipomoea batatas
Lactuca sativa
Momordica charantia
Nelumbo nucifera
Persea Americana
Petroselium crispum
Phaseolus vulgaris
Phyllostachys pubescens
Pisum sativum
Raphanus sativus
Sargassum fusiforme
Solanum lycopersicum
Solanum melongena
Solanum tuberosum
Spinacia oleracea
Undaria pinnatifida
Zingiber mioga
Zingiber officinale
Oryza sativa subsp. *japonica*
Triticum aestivum
Glycine max
Bos Taurus
Gallus gallus domesticus
Sus scrofa domesticus

[a]Edible mushrooms
[b]Poisonous mushrooms
[c]Collection number, CBM: Natural History Museum and Institute, Chiba
[d]Collection number, NBRC: National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan

manufacturer's protocol with the following modifications to the fluid volume: 600 µL of buffer AP1, 6 µL of RNase A (100 mg/mL concentration) and 195 µL of buffer AP2. The concentrations of the obtained DNA solutions were evaluated by the absorbance at 260 nm, using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Each DNA solution was diluted to a final concentration of 1 ng/µL in the TE buffer.

Multiplex real-time PCR conditions. In order to simultaneously detect *O. guepiniformis* and *L. edodes*, multiplex real-time PCR was performed with an ABI 7900HT real-time PCR system (Life Technologies). The reaction volume of 25 µL contained 2.5 µL of a DNA solution (1 ng/µL), 12.5 µL of the Universal Master mix, 0.3 µL of OMGU-F and R (25 µM), 0.25 µL of OMGU-Taq (10 µM), 0.3 µL of LEED-F and R (25 µM), and 0.25 µL of LEED-Taq (10 µM). Real-time PCR amplification in the reaction plate was performed in triplicate for each sample, using a no-template control as the negative control. The PCR conditions comprised 2 min at 50 °C (initial activation of uracil-*N*-glycosylase) and 10 min at 95 °C, this being followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Analysis of the multiplex real-time PCR data. The real-time PCR data was analyzed by using SDS software v. 2.3 (Life Technologies). The baseline was set to cycles 3 to 15. The threshold line was manually set to 0.2, this being in the region of exponential amplification across all amplification plots of the positive samples (genomic DNA extracted from *O. guepiniformis* or *L. edodes*) for each detection system. The threshold cycle value (Ct value) was automatically calculated as the cycle number at which logarithmic PCR plots crossed the threshold line. The Ct value for each sample was determined by using the mean of triplicate wells. Reactions with a Ct value of less than 38 were scored positive for the presence of *O. guepiniformis* and *L. edodes*, since three ΔRn values of the cycle number (35–37) before the Ct value and of the cycle number (38–40) after the Ct value around the detection limit were required to verify the exponential amplification in a 40-cycle run.[21] If a Ct value could not be obtained, the reaction was scored negative for the presence of *O. guepiniformis* and *L. edodes*.

Results

Primer and probe design

The ITS1-5.8S region of rDNA within the genomic DNA was selected as the target gene for detecting *O. guepiniformis* and *L. edodes*. The ITS1-5.8S region of rDNA has already been investigated in many fungi, including *O. guepiniformis* and *L. edodes*.[22–24] The ITS1 region is a non-coding region intervening between 5.8S rDNA and 18S rDNA and has areas of high conservation and high variability, making it suitable for PCR primer design to identify fungal species.[25] Some PCR methods using the ITS1 region have already been developed for identifying fungal species.[8,26,27] The primer pairs and probes were based on the ITS1-5.8S rDNA region for a multiplex real-time PCR assay in order to simultaneously detect the presence of *O. guepiniformis* and *L. edodes*. It has also been reported that, in certain species of the genus *Omphalotus* (*O. guepiniformis*, *O. olearius*, *O. illudens*, *O. nidiformis*, *O. olivascens*, *O. subilludens*, and *O. mexicanus*), the ITS regions have highly homologous sequences.[22,28] The TaqMan MGB probe was therefore used for the assay as it would provide higher sequence specificity than the SYBR Green and standard DNA probes.[9–11] The sequences of the designed TaqMan MGB probes had no significant homology with other known sequences, this being confirmed by a BLAST search. The sequences used for the designed primers and TaqMan MGB probes are listed in Table 3.

Conditions for multiplex real-time PCR

Multiplex real-time PCR assays were conducted with *O. guepiniformis* or *L. edodes* DNA (2.5 ng) in order to determine the optimal concentrations of the designed

Table 3. Primers and Probes Used in This Study

Targeted species	Name[a]	Sequence of oligonucleotide (5′-3′)[b]	Amplicon length
O. guepiniformis	OMGU-F	5′-TCTGGGCTTCTATGTCTTACAAACTC-3′	101 bp
	OMGU-R	5′-CCGTTGCTGAAAGTTGTATAAGTTTT-3′	
	OMGU-Taq	5′-(FAM)-CTCTTTATTGGTACTTAATTG-(NFQ)-(MGB)-3′	
L. edodes	LEED-F	5′-CATCCACCTGTGCACTTTTTGTAG-3′	93 bp
	LEED-R	5′-GAAGCCTTGTCAACTAGTCTTTTCAA-3′	
	LEED-Taq	5′-(VIC)-AGGTGCTCATTATGAGTTA-(NFQ)-(MGB)-3′	

[a]F: Forward primer, R: Reverse primer, Taq: TaqMan MGB probe
[b]FAM: 6-carboxyfluorescein, VIC: 6-carboxyrhodamine, NFQ: non-fluorescent quencher, MGB: minor groove binder

primer pairs and TaqMan MGB probes. The concentrations of both primer pairs were tested in the range of 100–400 nM (100, 200, 300 and 400 nM) and no other parameters were varied, including the concentrations of both probes (200 nM). Almost no difference was apparent in both the *O. guepiniformis* and *L. edodes* detection systems between the Ct values at primer concentrations of 300 and 400 nM, although these values were lower at 300 and 400 nM than those at 100 and 200 nM. The optimal concentration of both primer pairs was therefore determined to be 300 nM.

Setting the concentration of both primer pairs at 300 nM, the concentrations of both probes were then tested in the range of 50–200 nM (50, 100 and 200 nM). Almost no difference was apparent between the Ct values obtained at probe concentrations of 100 and 200 nM with both detection systems, although the Ct values were lower at 100 and 200 nM than at 50 nM. The optimal reaction conditions were therefore determined to be 300 nM for the primer pairs and 100 nM for the probes.

Specificity

The designed primer pairs and TaqMan MGB probes were tested for their specificity toward the target species and for their cross-reactivity with other species of mushroom and with common food items. Multiplex real-time PCR was performed by using DNA extracted from the fruiting bodies of 17 *O. guepiniformis*, 16 *L. edodes* and 57 other species (Tables 1 and 2). The *O. guepiniformis* samples gave Ct values in the range of 17.50–20.28, and the *L. edodes* samples gave Ct values in the range of 16.51–19.21 (data not shown). Ct values were obtained from all the samples of *O. guepiniformis* and *L. edodes*, and no cross-reaction was apparent for any non-target species in either detection system. These results suggest that the designed primer pairs and probes were highly specific and selective for their target species and exhibited no cross-reactivity with other species.

Sensitivity and linearity

The limit of detection (LOD) and linearity of the standard curves for both the *O. guepiniformis* and *L. edodes* detection systems were determined by using a 10-fold dilution series of *O. guepiniformis* and *L. edodes* DNA ranging from 2.5 to 0.00025 ng. LOD was 0.00025 ng of DNA for both systems. Ct values obtained from the 10-fold dilution series were plotted against the logarithm of the absolute amount of DNA (ng) to obtain the standard curves. The linearity of both detection systems ranged from 2.5 ng to 0.00025 ng of DNA with a square regression correlation (R^2) of >0.999 (Fig. 1). PCR efficiency of 100% is achieved when the slope is close to the theoretical value of −3.32. The observed slopes (−3.58 for *O. guepiniformis* and −3.58 for *L. edodes*) shown in Fig. 1 are close to this theoretical value. Based on this data, a PCR efficiency of 90% for the *O. guepiniformis* and *L. edodes* detection systems was calculated by using the equation PCR efficiency $= (10^{-1/\text{slope}} - 1) \times 100$. In order to mimic the conditions present during the extraction of *O. guepiniformis* and *L. edodes* DNA from different foods, genomic DNA of each species was mixed with genomic DNA of *Panellus serotinus* (Mukitake) at five mixing levels (0.01, 0.1, 1, 10 and 100%). The amount of total template DNA (*Panellus serotinus* DNA plus *O. guepiniformis* or *L. edodes* DNA (0.00025, 0.0025, 0.025, 0.25 or 2.5 ng)) was adjusted to 2.5 ng. The LOD value was 0.01% (0.00025 ng of DNA of *O. guepiniformis* or *L. edodes*), and the linearity of both detection systems was in the range of 0.01–100% with a square regression correlation (R^2) of >0.999 (data not shown). The PCR efficiency of the *O. guepiniformis* and *L. edodes* detection systems was greater than 80%, as calculated by the foregoing equation (a slope of −3.85 for *O. guepiniformis* and −3.82 for *L. edodes*). These results suggest that both detection systems were highly sensitive and gave good linearity for the standard curves, as well as high PCR efficiency for detecting *O. guepiniformis* and *L. edodes*.

Application of the assay to samples of processed foods containing other food items and artificial gastric juice

The applicability of the assay to processed mushrooms was investigated by analyzing samples of raw and processed (baked, stir-fried, deep-fried, boiled, and digested) fruiting bodies of *O. guepiniformis* and *L. edodes*. DNA extracted from these samples was tested by using the multiplex real-time PCR assay described in this current study. The Ct values obtained for the processed samples ranged from 20.48 to 26.55 (18.55 for the raw *O. guepiniformis* sample) with the *O. guepiniformis* detection system and from 17.79 to 22.73 (17.16 for the raw *L. edodes* sample) with the *L. edodes* detection system (Table 4). Although all processed samples of *O. guepiniformis* and *L. edodes* had higher Ct values than the corresponding raw samples, Ct values with sufficient sensitivity for detection were still obtained. The applicability of the assay for processed mushrooms containing other food items and gastric juice was investigated by using miso soup samples containing vegetables with approximately 1% (w/w) *O. guepini-*

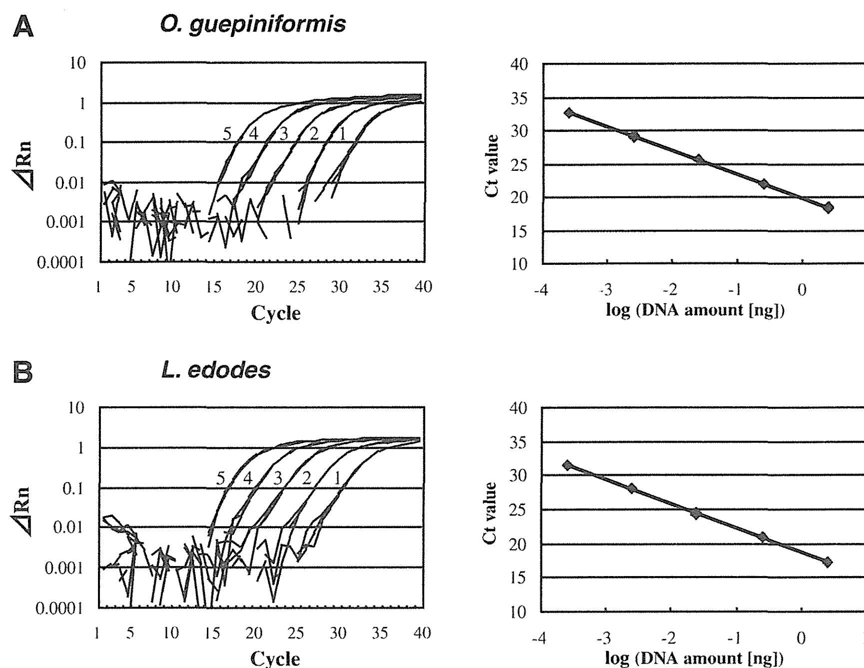

Fig. 1. Amplification Plots and Standard Curves for Multiplex Real-Time PCR.
The amplification plots were generated by 10-fold serial DNA dilutions of *O. guepiniformis* (A) and *L. edodes* (B) DNA (1, 0.00025 ng; 2, 0.0025 ng; 3, 0.025 ng; 4, 0.25 ng; 5, 2.5 ng). The standard curves were generated from the amplification data. The relationships between threshold cycle (Ct) and log DNA amount are $y = -3.58x + 19.83$ ($R^2 = 1.000$) (A) and $y = -3.58x + 18.68$ ($R^2 = 1.000$) (B).

Table 4. Ct Values of Processed Mushrooms by Multiplex Real-Time PCR

Processing method	*O. guepiniformis* Mean Ct value ± SD[a]	*L. edodes* Mean Ct value ± SD[a]
None	18.55 ± 0.03	17.16 ± 0.01
Baking at 240°C for 2 min on hot plate	20.48 ± 0.02	18.34 ± 0.04
Stir-frying at 240°C for 2 min on hot plate	26.55 ± 0.02	22.73 ± 0.06
Deep-frying at 180°C for 2 min in oil	21.14 ± 0.02	21.53 ± 0.04
Boiling for 30 min in water	21.71 ± 0.05	18.94 ± 0.04
Boiling for 60 min in water	21.16 ± 0.02	17.79 ± 0.02
Boiling for 120 min in water	24.92 ± 0.03	17.79 ± 0.02
Boiling for 180 min in water	23.08 ± 0.05	17.90 ± 0.04
Digesting raw mushroom	19.00 ± 0.02	17.99 ± 0.01
Miso soup spiked with 1% mushroom[b]	25.71 ± 0.09	25.18 ± 0.02
Digested miso soup spiked with 1% mushroom[b]	26.60 ± 0.07	25.93 ± 0.07

[a]Mean Ct value and SD of triplicate wells for each sample
[b]*O. guepiniformis* or *L. edodes*

formis or *L. edodes* and artificially digested samples of *O. guepiniformis* and *L. edodes*. The Ct values obtained from these samples ranged from 25.18 to 26.60 (Table 4) with the *O. guepiniformis* and *L. edodes* detection systems. These results indicate that the multiplex real-time PCR assay described in this current study was applicable for use in the identification of processed and digested fruiting bodies of *O. guepiniformis* and *L. edodes*, and of fruiting bodies of *O. guepiniformis* and *L. edodes* in processed foods containing other food items and gastric juice.

Repeatability and reproducibility of multiplex real-time PCR

The repeatability and reproducibility were evaluated by using a 10-fold dilution series of both *O. guepiniformis* and *L. edodes* DNA ranging from 2.5 to 0.00025 ng. The mean, standard deviation (SD), and coefficient of variation (CV) of the Ct values for repeatability were calculated from the mean values obtained from three replicates performed by one researcher on the same day. The same variables for the Ct values of reproducibility were calculated from the mean values obtained from replicates performed over three separate days of trials. In respect of the repeatability, CV of the Ct values for *O. guepiniformis* DNA ranged from 0.04 to 0.38%, whereas CV of the Ct values for *L. edodes* DNA ranged from 0.05 to 0.30%. In respect of the reproducibility, CV of the Ct values for *O. guepiniformis* DNA ranged from 0.20 to 1.23%, whereas CV of the Ct values for *L. edodes* DNA ranged from 0.21 to 0.85% (Table 5). These results for the repeatability and reproducibility tests indicate that the multiplex real-time PCR assay was reliable in its detection of *O. guepiniformis* and *L. edodes* DNA.

Table 5. Repeatability and Reproducibility of Multiplex Real-Time PCR

DNA dilution (ng)		*O. guepiniformis*				*L. edodes*			
		Same day (n = 3)			3 days	Same day (n = 3)			3 days
		1	2	3	(n = 9)	1	2	3	(n = 9)
2.5	mean Ct	18.40	18.45	18.38	18.41	17.24	17.24	17.19	17.22
	SD	0.02	0.01	0.04	0.04	0.02	0.02	0.05	0.04
	CV	0.08	0.05	0.24	0.20	0.13	0.13	0.28	0.21
0.25	mean Ct	21.97	22.24	22.13	22.11	20.84	21.21	21.18	21.08
	SD	0.01	0.01	0.05	0.12	0.06	0.02	0.03	0.18
	CV	0.05	0.05	0.21	0.54	0.30	0.11	0.13	0.85
0.025	mean Ct	25.55	26.03	26.27	25.95	24.39	24.58	24.57	24.51
	SD	0.02	0.03	0.03	0.32	0.02	0.07	0.05	0.10
	CV	0.08	0.10	0.10	1.23	0.09	0.29	0.21	0.43
0.0025	mean Ct	29.21	29.54	29.97	29.57	28.08	28.27	28.32	28.23
	SD	0.06	0.10	0.01	0.34	0.02	0.02	0.01	0.11
	CV	0.21	0.35	0.04	1.13	0.06	0.08	0.05	0.40
0.00025	mean Ct	32.67	33.09	33.42	33.06	31.54	31.85	31.93	31.77
	SD	0.12	0.05	0.13	0.34	0.03	0.09	0.03	0.19
	CV	0.35	0.16	0.38	1.02	0.09	0.27	0.09	0.58

Discussion

Wild fruiting bodies of the poisonous mushroom *O. guepiniformis* are frequently misidentified as those of such an edible mushroom as *L. edodes*, resulting in cases of food poisoning throughout Japan. In cases of suspected mushroom poisoning, species identification is commonly achieved through an examination of the morphological characteristics; however, this is often impossible when mushroom samples have been processed, or when only residual amounts of the food items and vomit remain. If a morphological examination is inconclusive, an assay without depending on morphology may be required to verify the cause of mushroom poisoning. Real-time PCR assays have proved useful for identifying mushroom species independently of morphology.[8,12,27] A multiplex real-time PCR assay using DNA probes has been able to distinguish such different species as *O. guepiniformis* and *L. edodes* in one run, allowing for rapid detection, reduced costs and less sample waste when compared to similar assays using simplex PCR.

O. guepiniformis has been identified in the past with a real-time PCR assay using SYBR Green I;[8] however, the detection of highly homologous target sequences such as those in the ITS regions of *Omphalotus* DNA by using SYBR Green I is difficult, and this method is not applicable to multiplex real-time PCR. Moreover, a real-time PCR assay using SYBR Green I has not yet been applied to clinical samples such as those that have been exposed to gastric juices, or to processed food samples containing other food items. Identifying *O. guepiniformis* in samples such as these may be required in order to determine the cause of food poisoning.

A multiplex real-time PCR assay using TaqMan MGB probes was developed in the present study for simultaneously detecting *O. guepiniformis* and *L. edodes* with high specificity. The detection of target sequences using TaqMan MGB probes is more specific than detection using SYBR Green and standard DNA probes.[9–11] No cross-reaction was apparent with any non-target species listed in Tables 1 or 2 in a cross-reactivity test. Among the 57 species listed in Table 2, 52 are frequently used in cooking; the other five species (*Amanita pantherina*, *Clitocybe acromelalga*, *Entoloma rhodopolium*, *Omphalotus olearius* and *Tricholoma ustale*) are poisonous and are often the cause of mushroom poisoning in Japan.[4] In addition, all the target species used for the test (OG1-17 and LE1-16) were detected by the assay. Although some species of genus *Omphalotus* were not available, the sequences of the TaqMan MGB probes were confirmed to have no significant homology with the known sequences of other species of genus *Omphalotus* as determined by a BLAST search.

The assay described in the current study proved effective for detecting *O. guepiniformis* and *L. edodes* in processed and digested samples, as well as in processed food samples containing other food items and artificial gastric juice. Ct values were obtained from all the processed and digested fruiting bodies, as well as from miso soup samples containing 1% fruiting bodies and artificially digested samples (Table 4). The Ct values obtained from all the processed and digested fruiting bodies were higher than those obtained from the raw fruiting bodies, suggesting the presence of some genomic DNA degradation in the processed and digested samples caused by high temperature and the presence of acid in the artificial gastric juice.[29–33]

This assay proved to have high sensitivity and good linearity at low DNA concentrations (0.00025–2.5 ng) (Fig. 1) due to the ITS region chosen as the target sequence possessing multiple copies.[25] The assay also provided sufficient accuracy with good repeatability (CV of Ct values = 0.04–0.38%) and reproducibility (CV of Ct values = 0.20–1.23%) (Table 5).

The proposed method can detect 0.01% *O. guepiniformis* DNA in the mixture of genomic DNAs extracted from *Panellus serotinus* and *O. guepiniformis* DNA, as well as 0.01% *L. edodes* DNA in the mixture of genomic DNAs extracted from *Panellus serotinus* and *L. edodes* DNA. The method can also detect 1% *O. guepiniformis* DNA and 1% *L. edodes* DNA in the mixture of *O. guepiniformis* DNA and *L. edodes* DNA (data not shown). This method would therefore be applicable to a processed sample involving food poisoning which would contain more than 1% *O. guepiniformis* in a practical case of accidental poisoning.

In conclusion, the current study presents a rapid and target-specific multiplex real-time PCR assay able to

simultaneously detect the presence of *O. guepiniformis* and *L. edodes*. This novel PCR assay has high specificity for the target species, high sensitivity, good linearity and applicability to processed samples. These characteristics would make the assay useful for rapidly identifying *O. guepiniformis* and *L. edodes*.

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Original Paper

Quantification and Identification of Genetically Modified Maize Events in Non-Identity Preserved Maize Samples in 2009 Using an Individual Kernel Detection System

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We investigated the GM maize grain content of non-identity preserved (non-IP) maize samples produced in 2009 in the USA using our individual kernel detection system, involving two multiplex qualitative PCR methods coupled to microchip electrophoresis and partially real-time PCR array analysis, to clarify how many GM event maize grains were present in the samples and which GM events frequently appeared in 2009. The average percentage and standard deviation of GM maize grains on a kernel basis in five non-IP sample lots were 81.9%±2.8%, the average percentage of single GM event grains was 46.9%, and the average percentage of stacked GM event grains was 35.0%. MON88017 grains and NK603 grains were the most frequently observed as single GM event grains. The most frequent stacked GM event grains were MON88017×MON810 grains. This study shows that our method can provide information about GM maize events present in imported maize samples on a kernel basis.

Key words: genetically modified maize; event; multiplex qualitative PCR; microchip electrophoresis

Introduction

Genetically modified (GM) crops are currently cultivated widely as sources of food and feed in many countries[1)]. GM crops generally have been assessed and authorized for food use by administrative authorities. In some countries, the labeling of grains, feed and foodstuffs is mandatory if the GM crop content exceeds a certain level of the approved GM varieties. For instance, the European Union, Japan and Korea have set threshold values of 0.9%, 5%, and 3%, respectively, of GM organism material in a non-GM background as the basis for labeling[*1–*7].

In Japan, non-GM crops are segregated as non-GM material and imported from the United States using an identity preserved (IP) handling system that requires documentary certification from US farms to Japanese processing traders. Recently, the production of stacked GM maize grains, in which two or more characteristic

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[*1] Regulation (EC) No. 258/97 of the European Parliament and of the Council of Europe, 27 January 1997, *Official J. Eur. Communities* L 043, pp. 1–7.
[*2] Commission Regulation (EC) No. 49/2000 of 10 January 2000, *Official J. Eur. Communities* L 6, pp. 13–14.
[*3] Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of Europe, 22 September 2003. *Official J. Eur. Communities*. Available from URL: http://europa.eu.int/eurlex/pri/en/oj/dat/2003/l_268/l_26820031018en00010023.pdf
[*4] Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of Europe, 22 September 2003, *Official J. Eur. Communities*. Available from URL: http://eurlex.europa.eu/LexUriServ/site/en/oj/2003/l_268/l_26820031018en00240028.pdf
[*5] Notification No. 517 of 31 March 2000, Ministry of Agriculture, Forestry and Fisheries of Japan.
[*6] Notification No. 79 of 15 March 2000, Department of Food Safety, Ministry of Health, Labour and Welfare of Japan.
[*7] Notification No. 2000–31 of 22 April 2000, Ministry of Agriculture and Forestry of Korea.

events have been inserted, has increased in the United States due to enhanced production efficiency[1]. Although the levels of adventitious commingling of GM maize in non-GM maize according to the labeling system refer to GM maize as a weight per weight (w/w) percentage, conventional applicable detection methods, such as quantitative real-time PCR, do not directly measure the w/w percentage of GM maize, but rather provide relative copy numbers between a specific DNA sequence and a taxon-specific DNA sequence, and these values are converted into a w/w percentage using appropriate reference materials. The GM maize content in a maize sample containing stacked GM maize grains, as determined by current quantitative real-time PCR methods, is likely to be overestimated compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis. To solve this problem, we have developed an individual kernel detection system that consists of grinding individual maize kernels, DNA extraction from each individual ground maize kernel, multiplex real-time PCR using the extracted DNA samples from individual ground maize kernels for GM detection, and multiplex qualitative PCR using the extracted DNA for GM event detection to analyze the precise commingling level and varieties of GM maize[2)-4)]. The detection system has already been implemented in Japan as an official GM maize detection method*8.

It is important to investigate the content of GM maize commingled in actual maize samples that contain many GM maize grains, such as non-IP maize samples, in order to determine the main current GM maize and stacked GM maize events and to predict which events are likely to be commingled with IP maize samples. However, there has been little information on the determination of stacked GM maize in non-IP maize samples, because no method is available, except for detection in individual kernels. We previously investigated GM maize content on a kernel basis and determined the varieties of the GM kernels in non-IP maize samples imported from the USA in 2005[5)] using an individual kernel analysis system including a multiplex real-time PCR method[2),3)], and coupled it to a multiplex qualitative PCR method[4)] followed by analysis using multi-channel capillary gel electrophoresis[5)].

The present study was designed to clarify the GM maize content of non-IP maize samples that contain GM maize produced in 2009, to investigate the content of GM maize grains, and to determine how many stacked GM maize grains are contained therein and which GM maize and stacked GM maize events frequently appeared in 2009 by using the multiplex real-time PCR method[2),3)], two multiplex qualitative PCR detection methods[4),6)] both coupled to the microchip electrophoresis and partially real-time PCR array analysis[7)].

*8 Department of Food Safety, Ministry of Health, Labour and Welfare of Japan. Notice No. 0803, Article 8; Tokyo, 2009.

Experimental

Maize samples

The non-IP maize samples produced in 2009 were purchased from a trading company in Japan. Bt11, GA21 and MIR604 seeds were kindly provided by Syngenta. TC1507 and DAS 59122 seeds were kindly provided by Pioneer Hi-Bred International, Inc. Seeds of MON88017, MON810, MON863, NK603 and stacked maize were kindly provided by Monsanto Co. T25 maize seeds were imported directly from the USA as positive controls of GM maize.

Oligonucleotide primers and probes for multiplex real-time PCR method

Sets of primer pairs and TaqMan® probes for construct-specific and universal GM quantification were described in our previous papers[2),3)]. The SSIIb-3 system (SSIIb 3–5′ and SSIIb 3–3′ with SSIIb-TaqV) was used for the primers and probe for the detection of the taxon specific gene encoding the maize starch synthase IIb (SSIIb) in the multiplex real-time PCR method, while the p35S-1 system (P35S 1–5′ and P35S 1–3′ with P35S-Taq) and GA21–3 system (GA21 3–5′ and GA21 3–3′ with GA21-Taq) were used in the multiplex real-time PCR method. All sets of primer pairs and Taq-Man® probe p35S-Taq for the detection of the cauliflower mosaic virus (CaMV) 35S promoter sequence (p35S) and GA21-Taq GA21 for the detection of specific sequence were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). SSIIb-TaqV, which is labeled with VIC® at the 5′ and TAMRA™ at the 3′ ends, was synthesized by Life Technologies (Carlsbad, CA, USA) and used as a probe for the detection of SSIIb. The target sequence used by the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in the recombinant DNA of almost all GM events with the exception of GA21. The GA21–3 system was designed to detect the construct specific sequence of GM maize event GA21[2),3)].

Grinding and DNA extraction of individual maize kernels

The grinding of individual maize kernels were performed according to previous reports[2),3)]. DNA extraction and purification were carried out with the GM quicker 96 kit (Nippon Gene Co., Ltd., Tokyo, Japan) using the method described here; genomic DNA extraction from the ground powder of individual kernels was performed according to the kit procedure. GE1 buffer and RNase A solution (100 mg/mL) were mixed to make a working solution at respective volumes of 1.5 mL and 5 μL. A 1.5 mL aliquot of the working solution was added to each sample tube containing the ground maize powder and metal corn. Twenty-four sample tubes were arrayed in the tube holder. The maize powder and working solution were mixed by vigorously shaking the tubes and the metal corn in a multi-bead shocker at 2,000 rpm for 15 s and incubated for 10 min at room temperature. A 180 μL aliquot of GE2-K buffer solution was then added to each

solution. The sample tube was capped to avoid leakage, and vigorously shaken for 15 s in the multi-bead shocker. The tube holder was centrifuged for 10 min at 1,400 ×g using a Metalfuge centrifuge (MBG100; Yasui Kikai Co., Ltd., Osaka, Japan). A 400 μL aliquot of each supernatant was carefully transferred to a 96-well plate. A 250 μL aliquot of GB3 buffer–isopropanol (1 : 1, v/v) was added. A 650 μL aliquot of each sample was then carefully transferred to the 96-well column plate, which was centrifuged for 20 min at 1,400×g. After removal of the filtrate, 650 μL of GW buffer was added to each well. The 96-well column plate was centrifuged for 10 min at 1,400 ×g. After removal of the filtrate, the 96-well column plate was recentrifuged for 20 min at 1,400×g. The plate was placed in a collection plate and 50 μL of DW was added to each well. The plate was incubated for 3 min at room temperature, and then centrifuged for 10 min at 1,400×g. For DNA extraction from individual maize kernels, we used a glass-fiber silica-plate base sheet (EPM 2000; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) for the 96-well column plate.

Multiplex real-time PCR conditions

To simultaneously detect the genomic DNA from individual GM maize kernels and to confirm the validity of PCR amplification of the extracted genomic DNA, multiplex real-time PCR analyses were performed according to previous papers[2),3),5)]. The amplification curves of the target sequence were monitored using a fluorescent dye, which was used to label the designed oligonucleotide probes, using an ABI PRISM® 7900HT sequence detection system (Life Technologies). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA (10 ng/μL), 12.5 μL of Universal Master Mix® (Life Technologies), 0.5 μM each primer pair, and 0.2 μM probe (except 0.1 μM for the p35S probe). The PCR step-cycle program was as follows: 2 min at 50℃, and 95℃ for 10 min followed by 45 cycles of 30 s at 95℃ and 1 min 30 s at 59℃.

If an amplification curve indicating GMO detection could be clearly observed after 15 cycles, we considered the sample as positive for GMOs; otherwise, it was considered negative, because we adopted exponential character of the amplification curve after 15 cycles of real-time PCR as the threshold for discrimination of GM and non-GM maize kernels in previous studies[2),3),5)]. In this study, the GM Maize Detection Plasmid Set–ColE1/TE– (Nippon Gene Co.) was used as the positive control. This plasmid set contains six concentrations of the reference plasmid pMul5, into which has been inserted the amplification products of p35S, GA21 and SSIIb, diluted with TE buffer (pH 8.0) including 5 ng/μL of the ColE1 plasmid[3),5)]. The ColE1 plasmid contained none of the amplification GM products, and was used as the negative control. The positive controls were prepared using two concentrations of the plasmid, set at 250,000 and 1,500 copies per plate. In the reaction plate, real-time PCR was performed in duplicate (each two wells) for the negative control, and for one positive control (250,000 copies) and for the other positive control (1,500 copies). The other 90 reaction wells were used for genomic DNA samples extracted from individual maize kernels.

Two multiple qualitative PCR methods

To identify which GM event grains are contained in genomic DNA extracted from individual kernels, two multiple qualitative PCR detections were performed according to our previously reported methods[4)]. The first method was performed for the detection of MON810, NK603, T25, GA21, TC1507, Event176, Bt11, and MON863 (construct specific)[4)]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 1.25 units AmpliTaq Gold® DNA polymerase (Life Technologies), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1–5′, NK603 1–5′ M863 1–5′, M863 1–3′, Bt11 1–5′, and CryIA 1–3′; 0.1 μmol/L for T25 2–5′, T25 2–3′, GA21 1–5′, GA21 1–3′, TC1507 1–5′ and TC1507 1–3′; 0.05 μmol/L for Event 176 1–5′; and 0.045 μmol/L for SSIIb 1–5′ and SSIIb 1–3′. The reactions were buffered with PCR buffer II (Life Technologies) and amplified in a Silver 96-well GeneAmp PCR System 9700 (Life Technologies) thermal cycler in max mode, according to the following PCR step-cycle program: pre-incubation at 95℃ for 10 min, 10 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 65℃ for 1 min, and extension at 72℃ for 1 min; 27 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min; followed by a final extension at 72℃ for 7 min.

The second method was performed for the detection of DAS-59122-7, MIR604, MON863 (event-specific) and MON88017[6)]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 0.625 units AmpliTaq Gold® DNA polymerase (Life Technologies) and nine sets of primers at the following concentrations: 0.50 μmol/L for MON88017-mF and MON88017-mR; 0.40 μmol/L for MON863-mF and MON863-mR, 0.30 μmol/L for DAS59122-7-rb1R; 0.25 μmol/L for DAS59122-7-rb1F; 0.15 μmol/L for MIR604-mF; 0.06 μmol/L for SSIIb 3–5′ and SSIIb 3–3′. The PCR conditions were the same as for the first method described above. Both methods were followed by microchip electrophoresis analysis.

Microchip electrophoresis analysis

The PCR products of multiple samples were analyzed using an MCE-202 MultiNA™ microchip electrophoresis system (Shimadzu, Kyoto, Japan). This system uses microchip technology for automated electrophoretic separation at high sample throughput using a 96-well PCR plate and high-sensitivity fluorescence detection. The analysis was run essentially according to the manufacturer's instruction manual using a DNA-1000 reagent kit (Shimadzu), which consists of Separation Buffer,