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Differential Detection of Shrimp and Crab for Food Labeling Using Polymerase Chain Reaction

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ABSTRACT: Shrimp and crab are well-known as allergenic ingredients. According to Japanese food allergy labeling regulations, shrimp species (including prawns, crayfishes, and lobsters) and crab species must be differentially declared when ≥10 ppm (total protein) of an allergenic ingredient is present. However, the commercial ELISA tests for the detection of crustacean proteins cannot differentiate between shrimp and crab. Therefore, two methods were developed to discriminate shrimp and crab: a shrimp-PCR method with postamplification digestion and a crab-PCR method that specifically amplifies a fragment of the 16S rRNA gene. The sensitivity and specificity of both PCR methods were verified by experiments using DNA extracted from 15 shrimp species, 13 crab species, krill, mysid, mantis shrimp, other food samples (cephalopod, shellfish, and fish), incurred foods, and commercial food products. Both PCR methods could detect 5 pg of DNA extracted from target species and 50 ng of genomic DNA extracted from incurred foods containing 10 ppm (μg/g) total protein of shrimp or crab. The two PCR methods were considered to be specific enough to separately detect species belonging to shrimp and crab. Although false-positive and false-negative results were obtained from some nontarget crustacean species, the proposed PCR methods, when used in conjunction with ELISA tests, would be a useful tool for confirmation of the validity of food allergy labeling and management of processed food safety for allergic patients.

KEYWORDS: food allergy, shrimp, crab, PCR, differential detection, crustaceans

INTRODUCTION

Crustaceans are consumed in many coastal countries. In Japan, large amounts of shrimp, lobster, spiny lobster, and crab are imported from Asian countries and many other regions, and are processed as materials for commercial foods. Crustaceans are well-known allergens, and several clinical cases have been reported.[1,2] It is known that crustacean allergy generally presents as skin (urticaria, flushing, and itching) and respiratory tract symptoms (dyspnea, wheezing, and cough). Furthermore, anaphylaxis can also be induced in sensitive patients by the intake of trace amounts of crustacean.[2,3]

In many countries, it is recommended that allergenic ingredients, such as wheat, peanuts, and crustaceans, be declared on food labels to alert allergic consumers to their presence. In Japan, the Ministry of Health, Labor and Welfare (MHLW) has enforced food labeling regulations for allergenic food materials since April 2002.[4] Under this system, it was mandatory that five food items (eggs, milk, wheat, buckwheat, and peanuts) be declared on food labels, and it was recommended that 19 others, including shrimp and crab, be declared when possible. Epidemiological investigations in Japan, from 2004 to 2005, have shown that the number of patients with a crustacean allergy are large after the five food items and approximately 65% of shrimp-allergic patients cross-react with crab.[3] In other words, approximately 35% of patients allergic to shrimp have no reaction to crab, although cross-reactivity and cross-sensitization among crustaceans have been reported.[1,2] Taking these results into consideration, in 2008, the MHLW added shrimp/prawn (including crayfish and lobster) and crab as two separate groups to the list of mandatory food items to be declared.[5] Taxonomically, the species belonging to shrimp/prawn group (shrimp species, for brevity) includes those belonging to suborder Dendrobranchiata, and infraorder Caridea, Astacidea, and Achelata of suborder Pleocyemata. The species belonging to crab group (crab species) includes those belonging to infraorder Brachyura and family Lithodidae of suborder Anomura (Figure 1).

In Japanese regulation, PCR is used as a method for confirming positive ELISA screening tests and excluding false positives.[6−9] These methods are complementary and are important for accurate allergenic ingredient testing. In Japan, two commercially available ELISA kits for determining soluble crustacean protein content have already been developed as the official testing method.[10,11] The performance of both ELISA kits satisfies the validation criteria described in the official guidelines published by the Japanese government.[12] However, these ELISA kits, which target tropomyosin, cannot differentiate between shrimp and crab species due to their high amino acid homology. As Japanese labeling regulations recommend separate declaration of shrimp and crab species in processed foods, novel methods discriminating shrimp and crab species would be required to confirm the validity of the labeling.

In this study, we therefore developed two novel PCR methods for differential detection of shrimp and crab species for confirmation of the ELISA results. The sensitivity and specificity of

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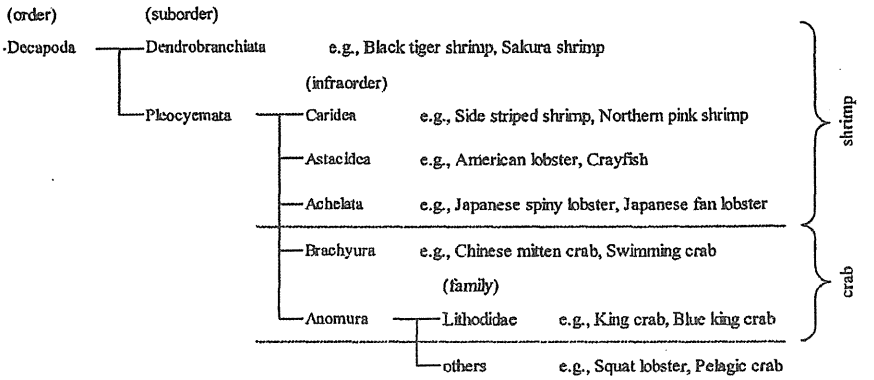

Figure 1. Target species of two PCR methods for detecting shrimp and crab species. Shrimp indicates the species which belong to the suborder Dendrobranchiata and the infraorder Caridea, Astacidea, and Achelata. Crab indicates the species which belong to the infraorder Brachyura and the family Lithoudidae.

the developed methods are examined and the application to commercially processed food analysis is also discussed.

MATERIALS AND METHODS

Samples. Fifteen shrimp species [Kuruma shrimp (*Marsupenaeus japonicus*), Whiskered velvet shrimp (*Metapenaeopsis barbata*), Black tiger shrimp (*Penaeus monodon*), Shiba shrimp (*Metapenaeus joyneri*), Sakura shrimp (*Sergia lucens*), Side striped shrimp (*Pandalopsis japonica*), Northern pink shrimp (*Pandalus eous*), Botan shrimp (*Pandalus nipponensis*), Japanese lobster (*Metanephrops japonicus*), Crayfish (*Procambarus clarkii*), American lobster (*Homarus americanus*), Japanese spiny lobster (*Panulirus japonicus*), Japanese fan lobster (*Ibacus ciliatus*), Cuba lobster (*Panulirus* spp.), and Akiami paste shrimp (*Acetes japonicus*)], thirteen crab species [Snow crab (*Chionoecetes opilio*), Red snow crab (*Chionoecetes japonicus*), Giant spider crab (*Macrocheira kaempferi*), Hair crab (*Erimacrus isenbeckii*), Dungeness crab (*Metacarcinus magister*), Deep sea red crab (*Chaceon granulatus*), Swimming crab (*Portunus trituberculatus*), Chinese mitten crab (*Eriocheir sinensis*), Spanner crab (*Ranina ranina*), King crab (*Paralithodes camtschaticus*), Blue king crab (*Paralithodes platypus*), Spiny king crab (*Paralithodes brevipes*), and Golden king crab (*Lithodes turritus*)], and three other crustaceans species that do not belong to either of the groups [Pacific krill (*Euphausia pacifica*), Mysid (*Neomysis intermedia*), and Mantis shrimp (*Oratosquilla oratoria*)] were purchased at markets in Tokyo and Chiba, Japan, or were provided by Maruha Nichiro Holdings, Inc. (Tokyo, Japan). The other samples, such as cephalopod, shellfish, fish, cereal grain, fruits, and vegetables, were purchased at local stores in Chiba.

Preparation of Incurred Foods. Six incurred foods were prepared to validate the sensitivity of each PCR detection method. Freeze-dried muscle powder of Black tiger shrimp/King crab, which was kindly provided by Maruha Nichiro Holdings, Inc., was added to materials prior to processing for a final concentration of soluble shrimp/crab protein at 10 μg/g of food sample. The muscle powder protein concentration was determined using a 2-D Quant Protein assay kit (GE Healthcare UK, Ltd., Little Chalfont, U.K.). The recipes for making each incurred sample are described below. The freeze-dried soup was made of broccoli, carrot, onion, chicken consommé, starch, and sweet cooking rice wine. Consommé, starch, sweet cooking rice wine, water, and the spiking shrimp/crab powder were stirred over medium heat until thickened. Boiled broccoli and carrot were added to the mixture, and cooled to −80 °C in a freezer, and then freeze-dried at −50 °C. The miso soup paste was made of miso, dry gluten, and dry welsh onion. Miso paste and water were thoroughly mixed with the spiking shrimp/crab powder. The mixture was packaged in a retort pouch, treated in a constant-temperature oven at 86 °C for 5 min, cooled in flowing water for 5 min, and then mixed with dry gluten and dry welsh onion. The soup powder was made of dry konjac noodles and commercial powdered flavoring, and was thoroughly mixed with the spiking shrimp powder. The dry condiment sprinkled on rice was made of fried rice cake, dried bonito, grated sesame seeds, and salt. These were mixed with the spiking crab powder and dried at 105 °C for 5 min. The rice gruel was made of rice and water. Rice and water were mixed with the spiking shrimp/crab powder and cooked in a rice cooker. The cream croquette, provided by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan), was made of milk, butter, flour, cornstarch, salt, sugar, pepper, and bread crumbs. Flour was sautéed in butter for 4 min, hot milk was added and stirred until the sauce became homogeneous, and then starch, salt, sugar, pepper, and the spiking shrimp/crab powder were added. The mixture was breaded and cooled in the freezer at −20 °C. The chicken meatball, containing shrimp/crab powder, was provided by Maruha Nichiro Holdings, Inc.[10]

Commercial Food Products. Twenty-seven commercial food products, 11 products with declaration of shrimp, 6 products with declaration of crab, 2 products with declaration of shrimp and crab, and 8 products without declaration of shrimp or crab in the list of ingredients, were purchased from local stores.

Preparation of DNA Templates. Genomic DNA was extracted from 0.2 g of sample and 2 g of homogenized incurred food or commercial food product with 2 and 20 mL of buffer G2 (Qiagen, Hilden, Germany), respectively, and purified using Genomic-tip 20/G (Qiagen) according to the manufacturer's instructions with slight modifications. DNA concentrations were determined by measuring UV absorption at 260 nm. All DNA samples were adjusted to a concentration of 20 ng/μL with TE buffer (pH 8.0) or distilled water, and used for PCR template. For the sensitivity studies, the shrimp/crab DNA samples were further diluted with 20 ng/μL of salmon testis DNA (Sigma Chemical Co., St. Louis, MO) solution. All of the DNA samples used in the specificity and sensitivity studies gave the expected PCR products with the following primer pairs: AN-5′ and AN-3′, designed to amplify a partial region of mitochondrial DNA; or CP03-F and CP03-R, designed to amplify a partial region of plant chloroplast DNA and used for the quality validation of the DNA.[13]

Primer Design. About 400 crustacean 16S rRNA gene sequences (shrimp, lobster, crab, krill, mysid, and mantis shrimp) were obtained

Table 1. Primer Sequences

detection method for	name	sequence 5′→3′ (with IUPAC mixed base codes)	blend ratio	length of the PCR product
shrimp				
F[a]	ShH12-05′-1,2	TTATATAAAGTCTRGCCTGCC	0.3	185−194 bp
R[b]	ShH 13-03′-1	GTCCCTCTAGAACATTTAAGCCTTTTC	0.1	
	ShH 13-03′-2	GTCCCTTTATACTATTTAAGCCTTTTC	0.1	
	ShH 13-03′-3	GTCCCCCCAAATTATTTAAGCCTTTTC	0.1	
crab				
F	CrH16-05′-1,2	GCGTTATTTTTTTTGAGAGTTCWTATCGTA	0.10	62 bp
	CrH16-05′-3	GCGTAATTTTTTCTGAGAGTTCTTATCATA	0.01	
	CrH16-05′-4,5	GCGTTATTTTTTTTAAGAGTACWTATCGTA	0.06	
	CrH16-05′-6	GCGTTATTTCTTTTGAGAGCTCATATCGTA	0.03	
R	CrH 11-03′	TTTAATTCAACATCGAGGTCGCAAAGT	0.2	
akiami paste shrimp				
F	AsH 11-05′	GGTTGTACAAAAAGAAAGCTGTCTCA	0.3	82 bp
R	ShH13-03′-1,2,3[c]		0.3	
mantis shrimp				
F	StH12-05′-1,2	TTGTATGAATGGTCSGACAAGAT	0.2	95 bp
R	StH12-03′-1,2	ATCGTCCCTCCATATYATTTAAGCTTTTTT	0.2	

[a] Forward primer. [b] Reverse primer. [c] A mixture of primers of equal parts of ShH13-03′-1, -2, -3.

from GenBank. In addition, 16S rRNA gene sequences of shrimp and crab species purchased for this study were determined by a direct sequencing analysis of the amplified products using the sequencing primer pairs, namely, SPP1 (forward primer 5′-CAA ATA TTG TTT CTG CCT GTT TAT C-3′ and reverse primer 5′-AAG ATT TAT AGG GTC TTA TCG TC-3′) for the upstream region and SPP2 (forward primer 5′-TTA AAG GGA CGA TAA GAC CCT ATA A-3′ and reverse primer 5′-TAG ATA GAA ACC AAC CTG GCT-3′) for the downstream region. Two sets of primer pairs based on the highly conserved sequence among the target species, ShH12-05′ and ShH13-03′ for detecting shrimp and CrH16-05′ and CrH11-03′ for detecting crab, were designed to amplify the upstream and downstream regions of the 16S rRNA gene, respectively. PCR simulations were performed with Amplify 1.0 software (Bill Engels, University of Wisconsin, Madison, WI) to predict whether PCR products of the target size would be obtained from the 16S rRNA gene sequences of crustaceans used for food in Japan, such as shrimp, lobster, crab, krill, mysid, and mantis shrimp, and some representative sequences of other crustaceans belonging to the classes Maxillopoda and Branchiopoda, which are not used for food. Two sets of primer pairs, AsH11-05′ and ShH13-03′ for detecting akiami paste shrimp and StH12-05′ and StH12-03′ for detecting mantis shrimp, were also designed in the same manner as above.

Shrimp-PCR and Akiami Paste Shrimp-PCR. For the detection of shrimp, the PCR reaction was carried out in a 25 μL reaction volume containing 0.2 mM of each dNTP, 1× buffer (PCR buffer II), 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.3 μM of the ShH12-05′ and ShH13-03′ primers, and 5 pg to 50 ng of template DNA. The GeneAmp PCR System 9700 (Applied Biosystems) was set at a ramping speed of 1 °C/s (9600 emulation mode). The amplifications were performed as follows: preincubation at 95 °C for 10 min; 45 cycles consisting of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; and final extension at 72 °C for 7 min. For the detection of akiami paste shrimp species, the PCR reaction was carried out as for the shrimp-PCR, with 0.3 μM of the AsH11-05′ and ShH13-03′ primers. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 45 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 56 °C for 0.5 min, and extension at 72 °C for 0.5 min; and final extension at 72 °C for 7 min. The PCR products (7.5 μL) were electrophoresed on a 2% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator (Bio-Rad Laboratories, Inc., Hercules, CA).

Restriction Enzyme Digestion of Shrimp-PCR Products. Seventeen microliters of shrimp-PCR product was digested with the restriction enzyme *Hae*III (Takara Bio, Inc., Shiga, Japan) in a final volume of 20 μL for 16 h at 37 °C, according to the manufacturer's instructions. The entire reaction volume was assayed on a 2% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator.

Crab-PCR and Mantis Shrimp-PCR. For the detection of crab, the PCR reaction was carried out as for shrimp PCR with 0.2 μM of the CrH16-05′ and CrH11-03′ primers. The GeneAmp PCR System 9700 was run with 9600 emulation mode. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min. For the detection of mantis shrimp species, the PCR reaction was carried out as for shrimp PCR, with 0.3 μM of the StH12-05′ and StH12-03′ primers. The amplifications were performed as follows: preincubation at 95 °C for 10 min; 34 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 54 °C for 0.5 min, and extension at 72 °C for 0.5 min. The PCR products (7.5 μL) were electrophoresed on a 3.5% agarose gel containing 0.3 μg/mL ethidium bromide and analyzed with a ChemiDoc XRS illuminator.

ELISA Procedure for Detection of Crustaceans. ELISA was performed for the determination of crustacean protein using two commercial kits: the Food Allergen Test EIA Crustacean "Nissui" (Nissui Pharmaceutical Co., Ltd.) and the crustacean kit "Maruha" (Maruha Nichiro Holdings, Inc.). One gram of the food product was extracted using 19 mL of extraction buffer. The mixture was shaken horizontally overnight at room temperature, and centrifuged at 3000*g* for 20 min after adjusting the pH to 6.0−8.0. The supernatant was filtered if necessary, diluted 20 times using each kit dilution buffer, and subjected to ELISA according to the manufacturer's instructions. Each sample extract was analyzed in duplicate (2 wells/product extraction), and the average absorbance of 2 wells was calculated.

RESULTS AND DISCUSSION

Primer Design for Shrimp-PCR and Crab-PCR. In this study, we designed PCR primers for the 16S rRNA gene of

Table 2. Predicted Specificity of Primer Pairs with PCR Simulation Software

crustaceans used for food	(A) ShH12-05′ and ShH13-03′ GenBank accession no.	match with 3′-end nucleotide of primers[a]	wt no.[b]
suborder Dendrobranchiata		40/40[c]	7/8[d]
e.g., *Metapenaeus affinis*	AY264904	+	5
Metapenaeus ensis	AF279810	+	5
Metapenaeus joyneri	FJ435636	+	5
Penaeus monodon	EU105471	+	/
Penaeus semisulcatus	EU024679	+	5
infraorder Caridea		20/20	15/15
e.g., *Palaemon debilis*	FM986647	+	4
Macrobrachium nipponense	FM986632	+	4
Exopalaemon modestus	EU493144	+	4
Pandalus latirostris	AB244633	+	4
Plesionika ensis	AY612883	+	4
infraorder Astacidea		21/21	7/7
e.g., *Homarus americanus*	DQ666843	+	4
Cherax tenuimanus	AF492809	+	/
Nephropsis stewarti	AY583891	+	4
Paranephrops zealandicus	EF060258	+	5
Procambarus clarkii	DQ666844	+	4
infraorder Achelata		45/45	8/8
e.g., *Palinurus delagoae*	EF546312	+	/
Palinurus mauritanicus	DQ062208	+	4
Jasus edwardsii	AF337979	+	3
Jasus lalandii	EU221225	+	/
Scyllarides latus	DQ377974	+	/
infraorder Brachyura		2/34	2/2
e.g., *Eriocheir sinensis*	AJ250642	+	4
Metacarcinus magister	AY789473	+	4
Callinectes sapidus	AJ130813	−	−
Chaceon affinis	AF100914	−	−
Telmessus cheiragonus	AB220027	−	−
infraorder Anomura		1/10	1/1
family Lithodidae			
e.g., *Lithodes aequispinus*	AF425329	−	−
Lithodes maja	AF425330	−	−
Paralithodes brevipes	AF425337	−	−
Paralithodes camtschaticus	AF425338	−	−
Paralomis granulosa	AF425339	−	−
Paralithodes sp.	AY789472	+	3
family Galatheidae			
e.g., *Munida gregaria*	EF428963	−	−
Cervimunida johni	AY351244	−	−
Pleuroncodes monodon	AY351259	−	−
family Euphausiidae		0/38	
e.g., *Euphausia longirostris*	AF281273	−	−
order Mysida		0/19	
e.g., *Mesopodopsis slabberi*	AJ966898	−	−
suborder Stomatopoda		0/15	
e.g., *Squilla mantis*	AY639936	−	−
family Balanidae		0/13	
e.g., *Semibalanus balanoides*	AY520728	−	−
order Pedunculata		0/7	
e.g., *Calantica spinosa*	AY428051	−	−

Table 2. Continued

crustaceans used for food	(B) CrH16-05′ and CrH11-03′ GenBank accession no.	match with 3′-end nucleotide of primers[a]	wt no.[b]
infraorder Brachyura		49/53[c]	46/53[d]
e.g., *Atelecyclus undecimentatus*	AM946018	+	4
Callinectes sapidus	AJ298189	+	4
Cancer irroratus	AJ130812	+	4
Chaceon affinis	AF100914	+	4
Chionoecetes opilio	AY227445	+	4
Erimacrus isenbeckii	AB197677	+	4
Eriocheir sinensis	AJ250642	+	4
Loxorhynchus crispatus	EU682798	+	4
Maja brachydactyla	EU000850	−	−
Maja squinado	DQ079723	−	−
Portunus trituberculatus	AM410527	+	4
Scylla serrata	AF109318	+	4
Telmessus cheiragonus	AB220027	+	4
infraorder Anomura		12/12	12/12
family Lithodidae			
e.g., *Lithodes aequispinus*	AF425329	+	4
Lithodes maja	AF425330	+	4
Paralithodes brevipes	AF425337	+	4
Paralithodes camtschaticus	AF425338	+	4
Paralomis granulosa	AF425339	+	4
family Galatheidae			
e.g., *Munida gregaria*	EF428963	+	4
Cervimunida johni	AY351244	+	4
Pleuroncodes monodon	AY351259	+	4
suborder Dendrobranchiata		0/69	
e.g., *Metapenaeus affinis*	AY264904	−	−
infraorder Caridea		0/49	
e.g., *Palaemon debilis*	FM986647	−	−
infraorder Astacidea		0/35	
e.g., *Homarus americanus*	DQ666843	−	−
infraorder Achelata		6/60	6/6
family Palinuridae			
e.g., *Palinurus delagoae*	EF546312	−	−
Palinurus mauritanicus	DQ062208	−	−
Jasus edwardsii	AF337979	−	−
Jasus lalandii	EU221225	−	−
family Scyllaridae			
e.g., *Scyllarides herklotsii*	FJ174906	+	2
Scyllarides latus	DQ377974	+	2
Thenus orientalis	FJ174914	+	2
Thenus unimaculatus	FJ174915	+	2
family Euphausiidae		0/39	
e.g.,*Euphausia longirostris*	AF281273	−	−
order Mysida		0/19	
e.g.,*Mesopodopsis slabberi*	AJ966898	−	−
suborder Stomatopoda		15/15	15/15
e.g., *Squilla mantis*	AY639936	+	4
family Balanidae		0/13	
e.g., *Semibalanus balanoides*	AY520728	−	−
order Pedunculata		0/7	
e.g., *Calantica spinosa*	AY428051	−	−

[a] Whether the sequence of the primer's target region matches the 3′ end nucleotides of both primers in the pair is shown + (match) or − (mismatch). [b] An approximate guide to the quality of the matches and the strength of the amplifications. The larger the weight number (1 to 6), the higher the probability of amplification. [−] indicates no PCR product was predicted. [/] indicates sequence for PCR simulation was not available. [c] The number of sequences matched with the 3′ end nucleotide of the primers per the number of representative sequences that have enough length to check the nucleotides at the position corresponding to the 3′ end of the primers. [d] The number of sequences that give a PCR product of the target size per number of representative sequences that have enough length to simulate primer hybridization.

mitochondrial DNA, because multicopy genes that can be used for congeneric or conspecific classification are useful targets for constructing sensitive and specific PCR methods.[14−16] By aligning the 16S rRNA gene sequences of shrimp species, crab species, and the other crustaceans (krill, mysid, and mantis shrimp) obtained from GenBank, two sets of primer pairs for detecting shrimp (ShH12-05′ and ShH13-03′) and crab species (CrH16-05′ and CrH11-03′) were designed (Table 1). The target species for each primer pair are shown in Figure 1. Each primer was designed so that the nucleotides at the position corresponding to the 3′ end of the primer would be the same in the sequences of the target species and would differ from those of the nontarget species. In addition, the primers for detecting crab species were designed to contain a mismatch base at the second position from the 3′ end of primers in all the sequences of the target species to prevent amplification of PCR products from nontarget species.[17] Because of the diversity in 16S rRNA gene sequences within each target group and similarity between the groups to be segregated, it is difficult to design differential PCR methods for varieties of shrimp and crab species. Therefore, we employed mixed primer PCR for both shrimp and crab species to increase the sensitivity and restriction fragment length polymorphisms (RFLP) for the shrimp-PCR method to reduce the chance of false positives originating from some crab.

Specificity Analysis of Both Primer Pairs Predicted Using PCR Simulation Software. The specificity of the primer pairs was predicted with PCR simulation software. With each primer pair, PCR products of the expected sizes (≈190 bp for shrimp ShH12-05′ and ShH13-03′ and 62 bp for crab CrH16-05′ and CrH11-03′) were predicted to be produced from most of the 16S rRNA gene sequences of target species in Table 2. With the ShH12-05′ and ShH13-03′ primer pair, products of the target size were also predicted from nontarget species, such as Chinese mitten crab (*Eriocheir sinensis*) and Dungeness crab (*Metacarcinus magister*), as shown in Table 2A, and from other crustaceans such as hermit crab and some species belonging to the class Branchiopoda and the superorder Syncarida (data not shown). With the CrH16-05′ and CrH11-03′ primer pair, products of the target size were also predicted from nontarget species such as mantis shrimp (*Squilla mantis*), as shown in Table 2B, and from other crustaceans such as hermit crab (data not shown). The PCR products predicted from other crustaceans, including hermit crab, and some species not used for food would not appear to cause significant problems in developing detection methods, because those nontarget species were presumed not to be used as material for processed foods and were thus unlikely to be mixed in processed foods.

Specificity and Sensitivity of the Shrimp Detection Method (Shrimp-PCR and Akiami Paste Shrimp-PCR). The specificity and sensitivity of the proposed shrimp-PCR method combined with restriction enzyme digestion were confirmed experimentally by using food sample DNA. As shown in Figure 2, PCR products of the target size (approximately 190 bp) were amplified from 5 pg of genomic DNA of 14 target species (Figure 2A), and restriction fragments of the target size (approximately 150 bp) were also obtained by restriction enzyme digestion of these PCR products (Figure 2B), but not from the genomic DNA of akiami paste shrimp (Figure 2C). The results from shrimp-PCR of nontarget crab species and the restriction enzyme digestion of the amplified products are presented in Figure 3, panels A and B, respectively. Although a PCR product of the target size was amplified from 50 ng of the genomic DNA of some crab species, such as Red snow crab, Giant spider crab, Dungeness crab, Deep sea red crab, Swimming crab, and Chinese mitten crab (Figure 3A), a restriction fragment of approximately 150 bp was not detected from the PCR products of these crab species, except for Chinese mitten crab (Figure 3B). Thus, the restriction enzyme digestion with *Hae*III can be used for differentiating shrimp species from most of the crab species that would give false positive products in shrimp-PCR. Nonspecific products often amplified from some of the other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables) were clearly different in size from the target products (data not shown).

Dried akiami paste shrimp (*Acetes japonicus*), which gave a false-negative result in shrimp-PCR, could be used in *okonomiyaki* (a Japanese pancake dish). We considered the solution of this problem to be very important in ensuring appropriate labeling. Therefore, we developed a PCR method for detecting akiami paste shrimp with the AkH11-05′ and ShH13-03′ primer pair (Table 1) for use in combination with shrimp-PCR. As the akiami paste shrimp sequence was not available from GenBank, we determined the sequence of the upstream region of 16S rRNA gene of akiami paste shrimp by direct sequencing analysis (GenBank accession number AB583753, AB583754, AB583755). Analysis of the sequence data revealed that the sequence of the forward primer (ShH12-05′ primer) binding region was different from that of Sakura shrimp (*Sergia lucens*), which belongs to the same family (Sergestidae) as akiami paste shrimp. Thus, we designed a new forward primer (AkH11-05′) for the 16S rRNA gene based on a highly conserved sequence among the akiami paste shrimp species. The proposed akiami paste shrimp-PCR method gave PCR products with a target size of 82 bp from 5 pg of genomic DNA from akiami paste shrimp and some other shrimp samples but not from 50 ng of genomic DNA from other crustaceans such as crab, krill, and mysid and other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables) (data not shown).

Specificity and Sensitivity of the Crab Detection Method (Crab-PCR and Mantis Shrimp-PCR). The specificity and sensitivity of the proposed crab-PCR method was confirmed using food sample DNA. As shown in Figure 4A, a PCR product of the target size (62 bp) was amplified from 5 pg of the genomic DNA from 13 target species. As shown in Figure 4B, PCR products of the target size were amplified from 5 pg to 50 ng of mantis shrimp (squilla) genomic DNA and sporadically amplified from 50 ng of Japanese lobster and American lobster genomic DNA. Although nonspecific products were often amplified from the genomic DNA extracted from other food samples (cephalopod, shellfish, fish, cereal grain, fruits, and vegetables), all of them were different in size from the target (data not shown).

As trace mantis shrimp DNA (5 pg) also produced a product matching the target size in the crab-PCR method, commercial foods contaminated by mantis shrimp without any crab contamination could be falsely positive. Therefore, we developed the mantis shrimp-PCR method to check for the presence of mantis shrimp in a crab-PCR positive commercial food. The PCR reaction was performed for 34 cycles to detect 5 pg of mantis shrimp DNA with detection sensitivity approximately equal to that of crab-PCR. The proposed PCR method for detecting mantis shrimp with the StH12-05′ and StH12-03′ primer pair (Table 1) amplified the PCR product from 5 pg of genomic DNA from mantis shrimp but not from 50 ng of genomic DNA from other crustaceans such as shrimp and crab species (data not shown).

Specificity and Sensitivity of Shrimp and Crab Detection Methods. Both PCR methods for detecting shrimp and crab

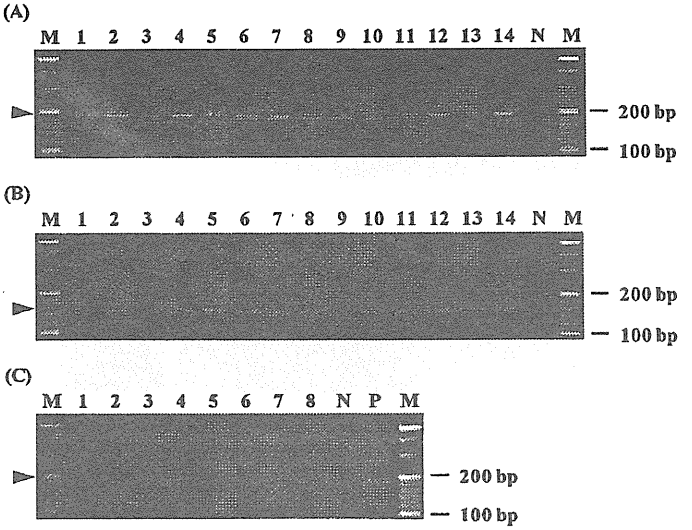

Figure 2. Specificity of the shrimp-PCR method for target species. The arrowheads indicate the expected size of PCR (A, C) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−14 of panels A and B, amplification of 5 pg of genomic DNA and restriction digestion of PCR products of genomic DNA, respectively, from Kuruma shrimp (1), Whiskered velvet shrimp (2), Black tiger shrimp (3), Shiba shrimp (4), Sakura shrimp (5), Side striped shrimp (6), Northern pink shrimp (7), Botan shrimp (8), Japanese lobster (9), American lobster (10), Crayfish (11), Japanese spiny lobster (12), Japanese fan lobster (13), and Cuba lobster (14). Lanes N, no template (A) and nonrestriction digestion of PCR products of Kuruma shrimp as a negative control (B). Lanes 1−8 of panel C, amplification of 50 pg (1, 2, 3, 4) and 5 pg (5, 6, 7, 8) genomic DNA from akiami paste shrimp. N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control.

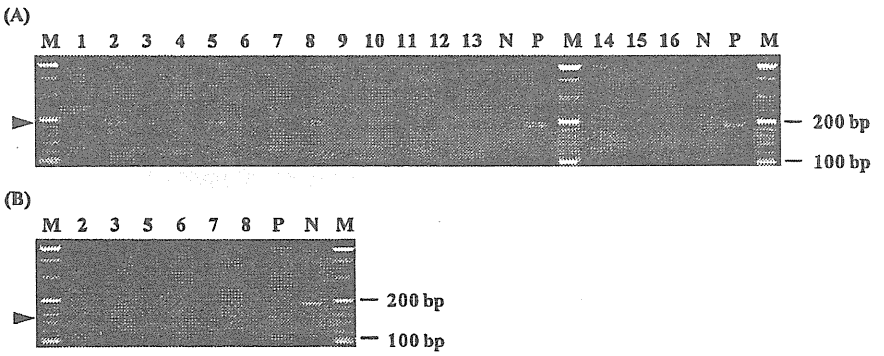

Figure 3. Specificity of the shrimp-PCR method for nontarget species. The arrowheads indicate the expected size of PCR (A) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 50 ng of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair crab (4), Dungeness crab (5), Deep-sea red crab (6), Swimming crab (7), Chinese mitten crab (8), Spanner crab (9), King crab (10), Blue king crab (11), Spiny king crab (12), Golden king crab (13), Pacific krill (14), Mysid (15), and Mantis shrimp (16). N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control. Lanes 2, 3, and 5−8 of panel B, restriction digestion of PCR products of genomic DNA from crab species corresponding to lane numbers of panel A. Lanes P and N, restriction digestion (P) and nonrestriction digestion (N) of PCR product of Kuruma shrimp genomic DNA.

species gave positive results from almost all target species used for food and negative results from nontarget species, including those selected from a variety of food items. Although some false-positives and false-negatives are identified, these results show that the proposed methods can differentially detect shrimp and crab species with reasonable sensitivity and specificity. In addition, using the akiami paste shrimp- and mantis shrimp-PCR methods in combination with the shrimp- and crab-PCR methods, the specificity and accuracy of the analytical results would be improved. Sakai et al.[18] reported that marine samples such as

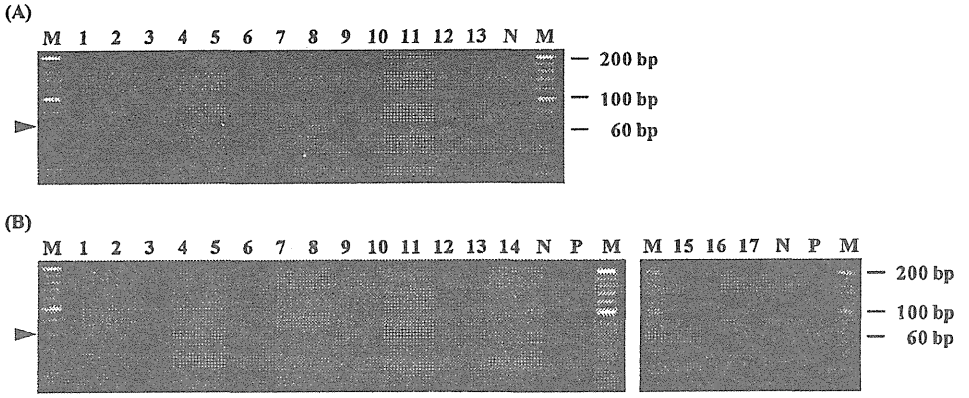

Figure 4. Specificity of the crab-PCR method. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 5 pg of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair crab (4), Dungeness crab (5), Deep-sea red crab (6), Swimming crab (7), Chinese mitten crab (8), Spanner crab (9), King crab (10), Blue king crab (11), Spiny king crab (12), and Golden king crab (13). Lanes 1−17 of panel B, amplification of 50 ng of genomic DNA from Kuruma shrimp (1), Whiskered velvet shrimp (2), Black tiger shrimp (3), Shiba shrimp (4), Sakura shrimp (5), Side striped shrimp (6), Northern pink shrimp (7), Botan shrimp (8), Japanese lobster (9), American lobster (10), Crayfish (11), Japanese spiny lobster (12), Japanese fan lobster (13), Cuba lobster (14), Pacific krill (15), Mysid (16), and Mantis shrimp (17). N, negative control (no template). P, amplification of 5 pg of King crab genomic DNA as a positive control.

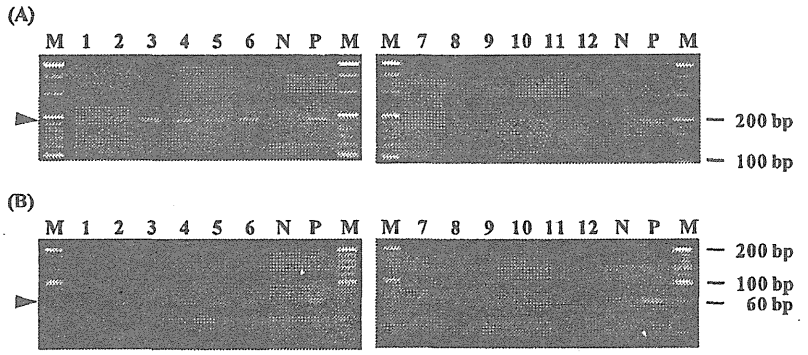

Figure 5. Sensitivity of two PCR methods (A, ShH12-05′ and ShH13-03′; B, CrH16-05′ and CrH11-03′) in incurred foods. The arrowheads indicate the expected PCR product for each primer pair. M, DNA marker (20 bp ladder). Lanes 1−6 of panel A, amplification of positive model samples, using the shrimp detection method, of 50 ng of DNA extracted from freeze-dried soup (1), paste of miso soup (2), powder of soup (3), rice gruel (4), cream croquette (5), and chicken meatball (6). Lanes 7−12 of panel A, amplification of negative model samples, using the shrimp detection method, of 50 ng of DNA extracted from freeze-dried soup (7), paste of miso soup (8), powder of soup (9), rice gruel (10), cream croquette (11), and chicken meatball (12). N, negative control (no template). P, amplification of 5 pg of Kuruma shrimp genomic DNA as a positive control. Lanes 1−6 of panel B, amplification of positive model samples, using the crab detection method, of 50 ng of DNA extracted from freeze-dried soup (1), paste of miso soup (2), sprinkling powder (3), rice gruel (4), cream croquette (5), and chicken meatball (6). Lanes 7−12 of panel B, amplification of negative model samples, using the crab detection method, of 50 ng of DNA extracted from freeze-dried soup (7), paste of miso soup (8), sprinkling powder (9), rice gruel (10), cream croquette (11), and chicken meatball (12). N, negative control (no template). P, amplification of 5 pg of King crab genomic DNA as a positive control.

laver, dried young sardine, and minced fish were frequently contaminated with crustacean. As the contamination is caused by bycatch and their feeding, those samples may be contaminated by variety of shrimp, crab, and other crustaceans including unknown falsely positive species with shrimp- or crab-PCR. Therefore, one should be careful when these commercial foods are examined and the presence of shrimp and crab species is to be comprehensively evaluated not only with the PCR and ELISA results but also the data relating to the manufacturing processes, materials, and recipes to ensure the accuracy of the list of ingredients of processed food.

Analysis of Incurred Foods. As described above, two PCR methods for shrimp and crab species have sufficiently high sensitivity to detect 5 pg of DNA from target species. Under Japanese food allergy labeling regulations, specified allergenic ingredients must be declared on the food label when 10 ppm - (μg/g or μg/mL) or more of its total protein is present in the food. Since processed commercial foods are made from many kinds of materials and processed by various methods, it is thought that the sensitivity of the detection methods could be affected by PCR inhibition, DNA degradation, and differences in DNA

extraction efficiency between target species and other matrices. Therefore, we confirmed the sensitivity of each detection method using various incurred foods containing 10 μg/g (shrimp or crab soluble protein weight/sample weight). As shown in Figure 5, the target size PCR products were detected from the DNA of all positive model foods and were not detected from all negative model foods, although there is a difference in the signal strength of these PCR products. Therefore, we concluded that the two PCR methods would be sensitive enough to detect trace amounts of shrimp and crab species in processed commercial foods and as a confirmation method for positive ELISA screening tests.

Analysis of Commercial Food Products. To compare the sensitivity of PCR and ELISA and to validate the specificity of shrimp- and crab-PCR, we tested 27 commercial products for the presence of shrimp and crab using two ELISA kits and two PCR methods as shown in Table 3. In 15 of 27 samples, the results of PCR amplification were consistent with the declaration in the list of ingredients and the content of crustacean protein measured using ELISA. Sample 14 tested positive with the shrimp-PCR, although shrimp was not declared in the list of ingredients. As the sequence of the amplification product from sample 14 matched with that of Western Australian rock lobster (Panulirus cygnus) in GenBank, sample 14 was thought to be contaminated with a trace amount of it. In Japanese regulation, shrimp and crab must be differentially declared when ≥10 ppm (total protein) of an allergenic ingredient is present. Four samples (no. 3, 8, 10, and 18) with declaration contained protein levels of less than 10 ppm with ELISA, but were positive with either of the PCR methods. The other seven samples (no. 4, 6, 7, 9, and 11−13) with declaration contained levels of less than 10 ppm with ELISA, and were also negative with the PCR methods. During processing of foods, proteins and DNA are subject to denaturation and fragmentation, which may render them undetectable by ELISA or PCR methods. It should be kept in mind that, since DNAs are generally less susceptible to degradation than proteins are to denaturation, and PCR methods are highly sensitive, they may detect very low levels of a contaminant that may not be clinically relevant. Therefore, PCR methods are to be used in conjunction with ELISA tests to determine the levels of the contaminating proteins. The shrimp- and crab-PCR methods detected shrimp in samples 1, 2, and 5, and crab in samples 15, 16, and 17, correctly as declared in the respective list of ingredients. Hence, they are particularly useful as confirmatory tests for differential detection of shrimp and crab species after positive ELISA results.

Conclusion. We newly developed the shrimp- and crab-PCR methods for final and differential detection of shrimp and crab species. The PCR methods were sensitive enough to detect 5 pg of DNA extracted from target species and 50 ng of genomic DNA extracted from incurred foods containing 10 ppm (μg/g) total protein of either Black tiger shrimp or King crab, and were considered to be specific enough to detect shrimp and crab separately, although some false-positive and false-negative results occurred. PCR technique targets a specific DNA sequence, not allergenic protein, to detect the presence of an allergenic food, and is particularly suitable for confirmation of positive results from ELISA tests that determine the levels of the contaminating proteins. Both PCR methods developed here met the specifications for, and were included in the notification by Japanese regulatory agency in 2009 as the methods for final identification of the presence of shrimp and crab species after two ELISA assays. They are useful for confirming the validity of food labeling and giving allergic consumers a wider range of food options. The akiami paste shrimp-PCR and mantis shrimp-PCR, developed in this study, complement the shrimp- and crab-PCR in case false-negative or false-positive results are suspected.

Table 3. Analysis of 27 Commercial Food Products for the Presence of Shrimp and Crab

sample		D[a]		ELISA (ppm)		PCR	
no.	description	shrimp	crab	N kit[b]	M kit[c]	shrimp[d]	crab
1	candy I	+	−	>50	23.3	pos	neg
2	instant noodle I	+	−	>50	23.0	pos	neg
3	clam chowder	+	−	<0.78	<0.78	pos	neg
4	curry sauce I	+	−	3.5	3.1	neg	neg
5	bouillabaisse sauce	+	−	25.4	22.3	pos	neg
6	pasta sauce I	+	−	<0.78	<0.78	neg	neg
7	pasta sauce II	+	−	<0.78	<0.78	neg	neg
8	pasta sauce III	+	−	<0.78	<0.78	pos	neg
9	curry sauce II	+	−	<0.78	<0.78	neg	neg
10	stew roux block	+	−	<0.78	0.8	pos	neg
11	chowder roux block	+	−	<0.78	<0.78	neg	neg
12	instant noodle II	−	+	<0.78	<0.78	neg	neg
13	dehydrated soup I	−	+	<0.78	<0.78	neg	neg
14	seasoning	−	+	8.7	7.5	pos	pos
15	soup	−	+	>50	24.8	neg	pos
16	pasta sauce IV	−	+	>50	24.5	neg	pos
17	rice gruel I	−	+	>50	25.7	neg	pos
18	seasoning paste	+	+	<0.78	<0.78	neg	pos
19	rice gruel II	+	+	54.8	27.9	pos	pos
20	candy II	−	−	<0.78	<0.78	neg	neg
21	dehydrated soup II	−	−	<0.78	<0.78	neg	neg
22	curry sauce II	−	−	<0.78	<0.78	neg	neg
23	pasta sauce V	−	−	<0.78	<0.78	neg	neg
24	pasta sauce VI	−	−	<0.78	<0.78	neg	neg
25	pasta sauce VII	−	−	<0.78	<0.78	neg	neg
26	curry sauce III	−	−	<0.78	<0.78	neg	neg
27	curry roux block	−	−	<0.78	<0.78	neg	neg

[a] Labeling of shrimp and crab components: +, declaration of shrimp or crab; −, without declaration. [b] N kit is Food Allergen Test EIA Crustacean "Nissui" (the Nissui Pharmaceutical Co., Ltd.). [c] M kit is crustacean kit "Maruha" (the Maruha Nichiro Holdings, Inc.). Range of quantitation of both ELISA kits is 0.78−50 ppm. [d] The results of shrimp- and akiami paste shrimp-PCR; pos indicates that PCR product with target size was detected with at least shrimp- or akiami paste shrimp-PCR; neg indicates that no PCR product with target size was detected with both shrimp- and akiami paste shrimp-PCR.

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Quantification of Pork, Chicken and Beef by Using a Novel Reference Molecule

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A standard plasmid was constructed as a novel reference molecule for use in real-time quantitative PCR assays to verify the identity of beef, pork, chicken, mutton, and horseflesh. The plasmid contained a target domain of the cytochrome b (cyt b) gene and an artificial DNA sequence. Primers CO-F and CO-R, and probe CO-P were specifically designed to detect the artificial sequence. The calculated R^2 values of the standard curves (10^3–10^7 copies per reaction) for the five species ranged between 0.998 and 0.999 in the quantification analysis. The constructed plasmid provides a universal method for measuring the copy number of cyt b DNA in minced meat. This method would be a useful procedure for verifying food labels.

Key words: real-time PCR; reference molecule; cytochrome b; meat

Food labeling is an important information source for the consumer, especially for patients with food allergies. Several methods to verify food labeling, using the polymerase chain reaction (PCR), have been developed to confirm the identity of plant and animal ingredients in food products.[1–6]

Minced meat is commonly used as a raw material in modern processed foods. Since the 2007 meat-mislabeling incident that occurred in Japan, confirmation of the composition of minced meat at the ingredient stage has become important for assuring the quality of processed foods produced by manufacturers. It is necessary to develop a rapid and useful method to quantitatively detect meat ingredients in processed foods. We have previously developed a real-time semi-quantitative PCR (semi-qPCR) method for identifying beef, pork, chicken, mutton and horseflesh.[7] This semi-qPCR method enables more sensitive and rapid analysis than conventional PCR methods; however, it lacks universal applicability because the extracted DNAs are used as reference materials with these methods. It is therefore necessary to establish a reference material that enables the copy number of target DNA sequences in meat to be determined.

Many qPCR systems have been developed to identify genetically modified (GM) maize, GM soybeans and GM examples of other agricultural commodities.[8–13] A plasmid is used in these methods as a reference material to determine the relative copy number of the target sequences in GM crops.[14,15] However, reference plasmids risk becoming a source of contamination in the PCR systems because they contain the target sequences. It is important to avoid false positive results when using a PCR analysis to confirm the validity of the food labeling system. Hence, it is also important to develop a method for confirming the presence of contamination caused by the reference plasmid.

We constructed in this study a reference plasmid for qPCR detection with beef, pork, chicken, mutton and horseflesh. The reference plasmid contains an artificial DNA target sequence with primer pairs and a probe specifically designed to detect these sequences in the plasmid. We evaluated the performance of the plasmid by using minced meat samples. The established plasmid was universally useful as a positive control and enabled independent laboratory results to be compared.

Materials and Methods

Samples. All meat samples were kindly provided by Marudai Food Co., Ltd. (Osaka, Japan). Deoxyribonucleic acid sodium salt from *Escherichia coli* strain B was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deoxyribonucleic acid sodium salt from salmon testes was purchased from Nacalai Tesque (Kyoto, Japan), and ColE1 DNA was purchased from Nippon Gene (Tokyo, Japan). We minced the pork, chicken and beef, as well as a sample containing a mixture of beef, pork and chicken at 1:1:1 (w/w) for a quantitative analysis of the major meats.

DNA extraction. DNA was prepared by using the Genomic-tip 20/G anion-exchange kit (Qiagen, Hilden, Germany) as previously described.[1] A 1.0-g amount of a milled food sample was used for DNA extraction. The DNA concentration was determined by measuring the UV absorption at 260 nm with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted DNA was diluted with an appropriate volume of distilled water to a final concentration of 10 ng/μL and stored at −20 °C until needed. These DNA samples were used for the subsequent PCR analysis.

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Table 1. Primers and Fluorescent Probes Used in This Work

Serial no.	Name	Sequence (5′–3′)	Amplicon Target	(bp)	Reference
1	18S-F	CGG AGA GGG AGC CTG AGA A	18S ribosomal RNA	251	This work
2	18S-R	CTA CGA GCT TTT TAA CTG CAG CAA			
3	IN-1	TTA AGT GAC GTA AAA TCG TGT TGA GGC CAA CGC CCA TAA TGC GGG CTG TTG CCC GGC GC	*E. coli* Iso-IS1		This work
4	IN-2	GGC CGC GCC GGG CAA CAG CCC GCA TTA TGG GCG TTG GCC TCA ACA CGA TTT TAC GTC ACT TAA			
5	PO-XhoI-F	CCC GCT CGA GCT TGC AAA TCC TAA CAG GCC TG	Porcine Cytochrome b	151	This work
6	PO-XbaI-R	CTA GTC TAG ACG TTT GCA TGT AGA TAG CGA ATA AC			
7	SH-XbaI-F	CTA GTC TAG ACC TTA TTA CAC CAT TAA AGA CAT CCT AGG T	Sheep Cytochrome b	115	This work
8	SH-EcoRI-R	CCC GGA ATT CGG GTC TCC GAG TAA GTC AGG C			
9	CH-EcoRI-F	CCC GGA ATT CTC TGG GCT TAA CTC TCA TAC TCA CC	Chicken Cytochrome b	126	This work
10	CH-HindIII-R	CCC CAA GCT TGG TTA CTA GTG GGT TTG CTG GG			
11	BO-HindIII-F	CCC CAA GCT TCC CGA TTC TTC GCT TTC CAT	Bovine Cytochrome b	139	This work
12	BO-KpnI-R	CGG GGT ACC CTA CGT CTG AGG AAA TTC CTG TTG			
13	HO-KpnI-F	CGG GGT ACC CAG CCA ATG CGT ATT CTG ACT CT	Horse Cytochrome b	95	This work
14	HO-ApaI-R	TTC CGG GCC CGT GTT CCA CTG GCT GTC CG			
15	CO-F	GCT CGT AGA AGG GCG AAT TCT	Conche	64	This work
16	CO-R	ATG GGC GTT GGC CTC AA			
17	CO-P	(VIC)-AGT GAC GTA AAA TCG-(NFQ)(MGB)			
18	PO-F	CTT GCA AAT CCT AAC AGG CCT G	Porcine Cytochrome b	131	7
19	PO-R	CGT TTG CAT GTA GAT AGC GAA TAA C			
20	PO-P	(FAM)-ACA GCT TTC TCA TCA GTT AC-(NFQ)(MGB)			
21	SH-F	CCT TAT TAC ACC ATT AAA GAC ATC CTA GGT	Sheep Cytochrome b	95	7
22	SH-R	GGG TCT CCG AGT AAG TCA GGC			
23	SH-P	(FAM)-ACT AAT CCT CAT CCT CAT GC-(NFQ)(MGB)			
24	CH-F	TCT GGG CTT AAC TCT CAT ACT CAC C	Chicken Cytochrome b	106	7
25	CH-R	GGT TAC TAG TGG GTT TGC TGG G			
26	CH-P	(FAM)-CAT TCC TAA CAC TAG CCC TA-(NFQ)(MGB)			
27	BO-F	CCC GAT TCT TCG CTT TCC AT	Bovine Cytochrome b	120	7
28	BO-R	CTA CGT CTG AGG AAA TTC CTG TTG			
29	BO-P	(FAM)-CAT CAT AGC AAT TGC C-(NFQ)(MGB)			
30	HO-F	CAG CCA ATG CGT ATT CTG ACT CT	Horse Cytochrome b	76	7
31	HO-R	GTG TTC CAC TGG CTG TCC G			
32	HO-P	(FAM)-AGT GGC AGA CTT ACT G-(NFQ)(MGB)			

FAM, 6-carboxy-fluorescein; MGB, minor groove binder; NFQ, non-fluorescent quencher
The primers and oligonucleotides used for constructing pCON are in the upper section (nos. 1–14), and the primers and probes used for meat detection are in the lower section (nos. 15–32).

Oligonucleotide primers and probes. The sequences of oligonucleotide primers and TaqMan fluorescent probes employed in this study are listed in Table 1. The oligonucleotide primers and probes were designed by using Primer Express v3.0 software (Life Technologies, Foster City, CA, USA). The Conche probe, which specifically detects the constructed reference molecule, was labeled with the VIC fluorescent reporter dye at the 5′ end. The primers were synthesized and purified with an oligonucleotide purification cartridge by Invitrogen (Tokyo, Japan), and the fluorescent probe was synthesized and purified by Life Technologies Japan (Tokyo, Japan).

Construction of a standard plasmid as the reference molecule. The standard plasmid shown in Fig. 1 was constructed in three steps to quantitatively detect the target DNA in beef, pork, chicken, mutton, and horseflesh. The first step involved inserting a 251-bp fragment of the horse 18S rRNA gene (GenBank accession no. AJ311673). This 251-bp DNA fragment was amplified by PCR with primers 18S-F and 18S-R, using horseflesh genomic DNA as the template. The PCR product was inserted into the pCR 2.1 vector and then cloned by using a TA cloning kit according to the standard procedure (Invitrogen, San Diego, CA, USA). The second step involved inserting a 57-bp fragment of the *E. coli* iso-IS1 gene (GenBank accession no. D63569). Two complementary oligonucleotides, IN-1 and IN-2, were synthesized for preparing the iso-IS1 fragment which had a *Not* I site at the 3′ end. The oligonucleotides were incubated at 95 °C, and double-stranded DNA was synthesized by gradually cooling the oligonucleotides to 15 °C. The fragment was ligated into the *Eco* RV-*Not* I site of the pCR 2.1-r18S vector, and pCR 2.1-18S-E was generated. The third step involved inserting pork, sheep, chicken, bovine and horse cyt b DNA fragments which contained restriction enzyme sites at both ends. The porcine cyt b DNA fragment was amplified by PCR with a primer set of PO-XhoI-F and PO-XbaI-R, using pork genomic DNA as the template. The sheep, chicken, bovine and horse cyt b DNA fragments were also amplified by PCR with the primer sets shown in Table 1, using the respective DNA from each type of meat. Each PCR product was designed to contain restriction enzyme sites at each end, being

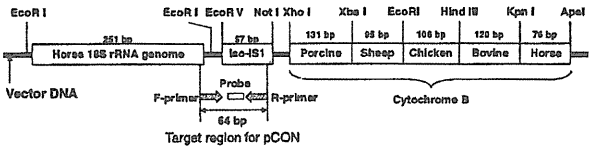

Fig. 1. Structure of pCON.
Construction details are described in the Materials and Methods section. The inserted genes are shown as boxes. Arrows indicate the location and direction of each primer and bars indicate the location of the probe.

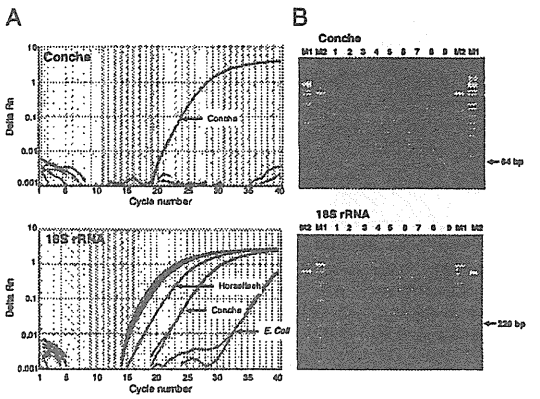

Fig. 2. Specificity of the qPCR Method When Using the Conche Primer/Probe Set (CO-F/CO-R and CO-P).
Genomic DNA (25 ng) or 10^5 copies of pCON were used as the template. All reactions were performed in duplicate. A, Amplification curve generated from 8 kinds of DNA with the Conche primers and probe set (top) or the 18S rRNA primers and probe set (bottom). B, 2% (w/v) agarose gel electrophoresis of PCR products amplified with the Conche primers and probe set (top) or 18S rRNA primers and probe set (bottom) shown in A. Lane 1, no template control (NTC); lane 2, beef; lane 3, pork; lane 4, chicken; lane 5, horseflesh; lane 6, mutton; lane 7, *E. coli* strain B; lane 8, salmon; lane 9, pCON; M1, 1-kb DNA Plus Ladder (Invitrogen); M2, 50-bp DNA Step Ladder (Promega). The arrow indicates the expected size of the PCR product.

treated with restriction enzymes (Takara, Shiga, Japan) and then ligated into pCR 2.1-18S-E. The plasmid obtained as a result of these ligations is designated as the Conche plasmid (pCON). Direct sequencing was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan) to confirm the sequence of the inserted fragments. The constructed plasmid from the *E. coli* transformant was isolated and purified by using a plasmid midi kit (Qiagen). The extracted DNA was diluted with the appropriate volume of a TE buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA) to a final concentration of 500 ng/μL, and stored at −20 °C until needed. The concentration of pCON was measured by UV absorption at 260 nm.

qPCR conditions. Quantitative PCR was performed by an ABI Prism 7500 instrument (Life Technologies). Each PCR reaction mixture was made up in a 25-μL final volume containing 2.5 μL of the template DNA, 12.5 μL of TaqMan Universal PCR Master Mix (Life Technologies), 0.75 μL of primer pairs (10 μM of each primer), and 0.5 μL of the TaqMan probes (10 μM) listed in Table 1. Fluorescence was monitored during every PCR cycle at the annealing step. The reaction conditions were set for 2 min at 50 °C and 10 min at 95 °C, and then for 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The 18S rRNA genes were detected as a control according to the instruction manual with the TaqMan ribosomal RNA control reagents VIC probe (Life Technologies).

Construction of the standard curves. Five serially diluted concentrations (10^7, 10^6, 10^5, 10^4, and 10^3 copies) of the standard plasmid DNA were diluted in a ColE1 plasmid DNA solution (5 ng/μL) and used as reference molecules for constructing a standard curve. ColE1 plasmid DNA (5 ng/μL) was used as the no template control (NTC). The copy number of the plasmid was calculated from the concentration and number of base pairs in pCON (4767 bp), using the formula $X\,ng/\mu L \times 10^{-9} \times 6.02 \times 10^{23}/[\text{plasmid length} \times 660] = Y$ copies/μL.

Results and Discussion

Construction of a reference molecule

A novel standard plasmid was constructed for use in the specific qPCR method for detecting pig, chicken, cattle, sheep, and horse DNA. We named this standard plasmid pCON, as shown in Fig. 1. Cyt b gene fragments for pig, chicken, cattle, sheep, and horse were targeted for this meat detection method. These gene fragments were tandemly arrayed in pCON. A universal portion of the sequence of the 18S rRNA gene and a unique chimeric sequence were also inserted into pCON. This unique chimeric sequence was generated by inserting the *E. coli* iso-IS1 gene fragment between the 18S rRNA and cyt b sequences. A specific primer pair and probe (CO-F, CO-R and CO-P) were designed according to the chimeric sequence to confirm the presence of pCON.

The specificity of CO-F/CO-R and CO-P was evaluated by using pCON (10^5 copies per reaction) and genomic DNA extracted from beef, pork, chicken, mutton, horseflesh, salmon and *E. coli* (Fig. 2). Only the amplification from pCON was detected. Amplification from the 18S primers and probe was observed in each

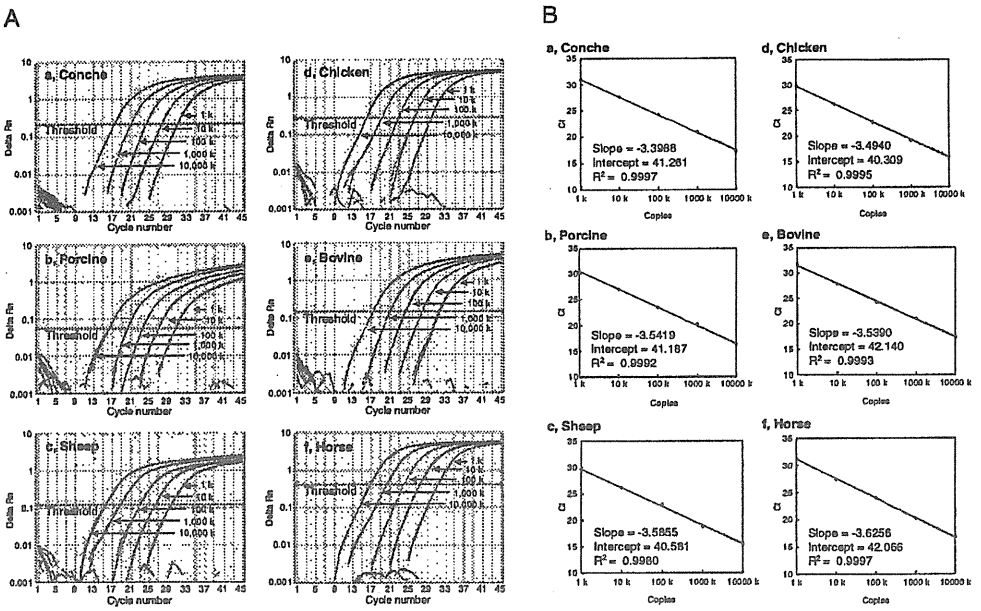

Fig. 3. Sensitivity of the qPCR Methods.

A, Amplification plots generated by five serial dilutions of pCON corresponding to 10^7, 10^6, 10^5, 10^4, and 10^3 copies. a, Conche primer/probe (CO-F/R/P); b, porcine primer/probe (PO-F/R/P); c, sheep primer/probe (SH-F/R/P); d, chicken primer/probe (CH-F/R/P); e, bovine primer/probe (BO-F/R/P); f, horse primer/probe (HO-F/R/P). All reactions were run in duplicate in 96-well plates. The horizontal line indicates the threshold as determined for the standard curve. B, Standard curve for pCON generated from the amplification data given in A.

DNA sample, confirming that the quality of the genomic DNA was appropriate for the qPCR analysis. These results suggest that using CO-F/CO-R and CO-P enabled the specific detection of pCON due to the artificial nature of the DNA sequence and its absence in natural products.

Quantification of pCON

We evaluated the quantitative performance of pCON. The standard curves were produced with pCON used in the range of 10^3–10^7 copies per reaction. The calculated R^2 values for the standard curves were 0.9997, 0.9992, 0.9980, 0.9995, 0.9993, and 0.9997 for the respective pCON, porcine, sheep, chicken, bovine, and horse detection system (cyt b) (Fig. 3). These results suggest that each detection system had outstanding linearity. We employed a pCON recovery test, using minced porcine meat DNA spiked with 5000 copies of pCON, to assess the ability of the Conche detection system to specifically detect pCON during preparation of the PCR mixture. We confirmed that the recovery of pCON was 80.3% (data not shown).

Verification of pCON as a quantitative method using test samples

We mixed minced beef, pork, and chicken at a ratio of 1:1:1 and determined the yield and cyt b copy number in the extracted DNA to assess the performance of pCON as a positive control for a quantitative analysis (Table 2). The respective yields of extracted DNA were 44.32, 35.54, 39.72, and 36.77 μg/100 μL for minced beef, pork, chicken, and mixed meat. We then measured the copy number of the bovine, porcine, and chicken cyt b genes by using 10 ng of each DNA.

Table 2 shows that the respective copy numbers of cyt b in 25 ng of extracted DNA from minced beef, pork and chicken were 19.3×10^6, 37.2×10^6, and 49.0×10^6. The respective copy numbers for the bovine, porcine, and chicken cyt b genes in mixed meat were 7.19×10^6, 9.91×10^6, and 16.1×10^6. We evaluated whether the copy number of the cyt b gene for each type of meat differed among animals for the amount of mitochondrial DNA per weight by using the developed methods. The percentages of individual meats in the mixed meat sample were calculated, where the copy number of cyt b in the individual samples was taken to be 100%. We therefore determined that the respective cyt b copy number for each meat constituted 37.3%, 26.6%, and 32.9% for beef, pork, and chicken.

We attempted to determine the proportions of minced beef, pork and chicken as the critical first step in the developed detection system. The proportions of individual meats in the mixed sample were calculated by using a conversion coefficient defined as the ratio of the copy number, in which the copy number of cyt b in 25 ng of extracted DNA was assumed to be 100%. The results suggest that rough measurement of the amount of each meat and the proportion of each meat species in a mixed minced meat sample can be determined by using the developed method.

We developed in the present study a standard plasmid as a reference material and verified the practical

Table 2. Verification of the qPCR Assay Using a Standard Plasmid and Minced Meat Samples

Sample		Total DNA (μg/100 μL)	Real-Time PCR					
			Bovin Cyto b		Porcine Cyto b		Chicken Cyto b	
			Ct value	Copy no. ($\times 10^6$)	Ct value	Copy no. ($\times 10^6$)	Ct value	Copy no. ($\times 10^6$)
Beef	Mean	44.32	17.54	19.3				
minced meat	SD	8.41	0.97	8.41				
Pork	Mean	35.54			14.53	37.2		
minced meat	SD	8.72			0.30	7.34		
Chicken	Mean	39.72					15.32	49.0
minced meat	SD	13.93					0.20	6.65
Combination	Mean	36.77	18.98	7.19	16.44	9.91	16.97	16.1
minced meat	SD	14.28	0.86	2.95	0.28	1.87	0.13	1.40

SD, standard deviation; Ct, cycle of threshold
DNA extraction was performed five times. All reactions were run in duplicate in 96-well plates, with 25 ng of genomic DNA used as the template.

application of the meat detection method developed in a previous paper.[7] The previous study showed that the method could detect the mitochondrial cyt b gene of individual types of meat in food samples, demonstrating high-sensitivity and no cross-reactivity. However, the results of the present study suggest that there is a limit to the quantification approach by cyt b copy number because it is difficult to determine the copy number of mitochondrial genes in each cell. We also evaluated the cyt b copy number of heated and non-heated porcine minced meat and such beef parts as short plate, gooseneck round, sirloin, chuck and liver. The present results show that the cyt b copy number of non-heated minced meat was 2.4 times higher than that of heated minced meat, and there was a small difference in the cyt b copy number in the muscle samples ($17.1–22.4 \times 10^6$ copies per 25 ng of DNA), whereas the cyt b copy number of liver was 3.3 times greater than that of the muscle samples (data not shown).

These results suggest that the individual meat proportions remain to be precisely determined with suitable accuracy, due to differences in the amount of the extracted mitochondrial DNA from each meat species, and to such differences as the extraction efficiency, food preparation method, age of the domestic animals, meat cuts, and the proportion of fat in the sample materials.

Applying this system to a more general sample requires finding an appropriate universal gene for various meat species so that precise meat amounts can be quantified in mixed minced meat samples. Further studies are underway to develop a quantitative detection method for meat species by using a combination of the meat-specific gene used in this study and an appropriate universal gene.

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Identification and Detection Method for Genetically Modified Papaya Resistant to Papaya Ringspot Virus YK Strain

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Unauthorized genetically modified (GM) papaya (*Carica papaya* LINNAEUS) was detected in a commercially processed product, which included papaya as a major ingredient, in Japan. We identified the transgenic vector construct generated based on resistance to infection with the papaya ringspot virus (PRSV) YK strain. A specific detection method to qualitatively monitor papaya products for contamination with the GM papaya was developed using the real-time polymerase chain reaction.

Key words genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* LINNAEUS) is an important fruit crop in tropical and subtropical areas.[1] Infection with the papaya ringspot virus (PRSV) causes disastrous damage to papaya harvests.[2] In response to this problem, genetically modified (GM) papayas have been developed in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, Philippines and Vietnam.[2]

Japan has announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients, and the importation of any unauthorized GM foods to Japan has been prohibited since April 1, 2001. Therefore, the establishment of qualitative detection methods for unauthorized GM foods was required for monitoring purposes. We previously developed and reported qualitative detection methods for various GM crops, such as potato,[3,4] maize,[5—8] rice,[9] and flax,[10] using polymerase chain reaction (PCR) methods. In the case of papaya, we established a qualitative detection method for GM papaya (Line 55-1), which was the first commercialized PRSV-resistant GM papaya developed in Hawaii, using a PCR test and a histochemical assay.[11—13] A safety assessment for Line 55-1 by the Food Safety Commission of Japan was finished in 2009.[14] Since Japan imports many papayas from Southeast Asia, we are required, in Japan, to monitor commercially processed products that include papaya as a major ingredient for contamination with other unauthorized GM papayas generated in the region.

GM papayas carry the transgenic vector construct generated based on resistance to PRSV infection by expressing the PRSV's coat protein (CP) gene. Since the other unauthorized GM papayas developed may differ in the transgenic vector construct of the authorized GM papaya (Line 55-1), we developed a method for detecting contamination with unauthorized GM papaya. In the present study, we found the unauthorized GM papaya, PRSV-YK, in processed products containing papaya as a major ingredient, papaya-leaf-tea, pickles and jam, and developed a method for the detection of PRSV-YK using the real-time PCR.

MATERIALS AND METHODS

Papaya Samples Papaya products were purchased through the internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

Purification of DNA Dried papaya leaves in papaya-leaf-tea, papayas in pickles and Sunset sarcocarp were ground using a mixing mill. Papaya jam was used for purification of DNA without grinding. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; QIAGEN, Hilden, Germany) as follows: 30 ml Buffer G2 (QIAGEN), 20 μl 100 mg/ml RNase (QIAGEN) and 500 μl cellulase (Sigma-Aldrich, St. Louis, MO, U.S.A.) were added to the sample and vortexed thoroughly, then incubated at 50 °C for 1 h. The mixture was incubated at 50 °C for another 1 h after the addition of 200 μl Proteinase K (QIAGEN). During the incubation, the samples were mixed several times by inverting the tubes. The samples were then centrifuged at 3000×*g* at 4 °C for 20 min. The supernatant was applied to a Genomic-tip 100/G column (QIAGEN), which was pre-equilibrated with 4 ml Buffer QBT (QIAGEN). The tip was washed three times with 7.5 ml Buffer QC (QIAGEN) and transferred to a fresh centrifuge tube, and 3 ml pre-warmed Buffer QF (QIAGEN) (50 °C) was added to elute the DNA. The DNA sample was transferred to a centrifuge tube, an equal volume of isopropyl alcohol was added, and the sample was mixed thoroughly. DNA was collected by centrifugation at 12000×*g* for 15 min. The pellet was rinsed with 1 ml 70% (v/v) ethanol and centrifuged at 12000×*g* for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 20 μl water for use in analyses. The DNA was quantified by measuring UV absorption at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (25 μl) contained 2.5 μl 10×PCR buffer II (Life Technologies, Carlsbad, CA, U.S.A.), 0.16 mM of each deoxyribonucleotide triphosphate (dNTP) (Life Technologies), 1.5 mM $MgCl_2$, 1.2 μM forward and reverse primers, 0.8 U AmpliTaq Gold (Life Technologies) and 25 ng template DNA. The PCR conditions were as follows: 95 °C for 10 min, followed by 50 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 60 s and terminal elongation at 72 °C for 7 min. PCRs were carried out using the GeneAmp PCR System 9700 (Life Technologies). To determine the nucleotide sequence of the transgenic vector construct harbored in GM

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papaya, DNA fragments were amplified by PCR using the following primer set. Forward primer: 5′-GACATCTCCACTGACGTAAGGG-3′ (p324). Reverse primer: 5′-CTATCRCTCTCTCCAGTTTTTG-3′ (p323).

DNA Sequencing The PCR-amplified DNA fragments were extracted from the agarose-gel and purified using a QIAquick PCR purification kit (QIAGEN). The DNA fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide sequences were analyzed using Lasergene version 7.2 software (DNASTAR Inc., Madison, WI, U.S.A.).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM™ 7900 Sequence Detection System (Life Technologies). The 25 μl reaction mixture consisted of 2.5 μl sample DNA solution (25 ng), 12.5 μl Universal Master Mix® (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. GM papaya was detected using the following primers and probe. Forward primer: 5′-GATCCCCGGGTGGTCAGT-3′ (YK-1F). Reverse primer: 5′-CCGGTATCCACAGCTTCATTTT-3′ (YK-1R). Probe: 5′-FAM-AGACGCCATGGAAGG-MGB-3′ (YK-P).

For detecting the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession No.: AY803756), we designed the following primers and probe referring to published report.[15] Forward primer: 5′-CCATGCGATCCTCCCA-3′ (Q-Chy-1F2). Reverse primer: 5′-CATCGTAGCCATTGTAACACTAGCTAA-3′ (Q-Chy-2R). Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCATTCCCACTCTTGAGA-3′ (Q-Chy-P). Black-hole quencher 1 (BHQ1) was labeled for Q-Chy-P at the underlined thymidine in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water, and stored at −20 °C until use. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for ABI PRISM™ 7900 Sequence Detection System.

Real-Time PCR Data Analysis Typically, the baseline was set to cycles 3 through 15. The ΔRn threshold for plotting the cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions with Ct values of less than 48 and exponential amplification plots were scored as positive. If the Ct value could not be obtained, the reaction was

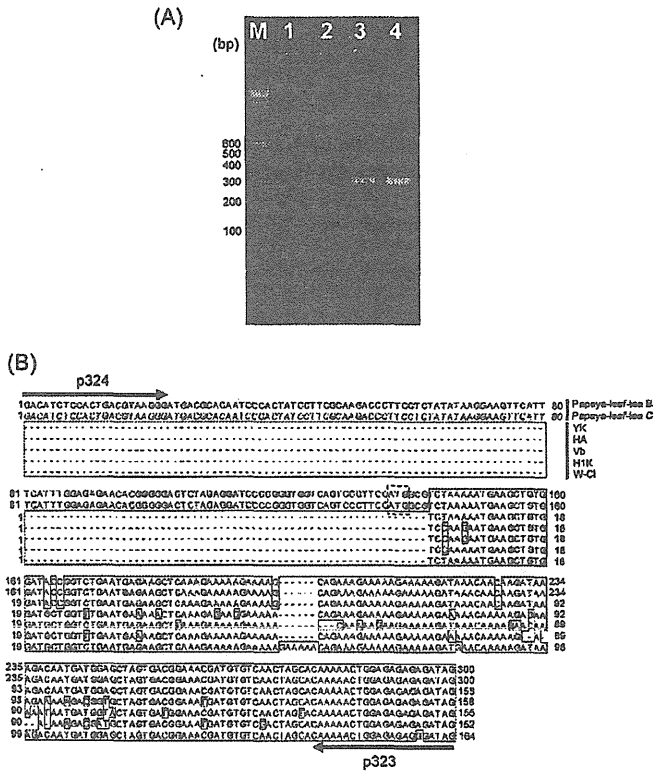

Fig. 1. PCR Targeting Construct Specific Sequence of PRSV-Resistant GM Papaya

(A) DNA templates (lane 1, non-GM papaya (Sunset); lane 2, papaya-leaf-tea A; lane 3, papaya-leaf-tea B; lane 4, papaya-leaf-tea C) were used for the PCR test using the p324 and p323 primer set. The PCR-amplified products were run on a 3% (w/v) agarose-gel. Lane M, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from YK, HA, Vb, H1K, W-CI strains and the PCR products obtained using papaya-leaf-tea B and C. Homologous sequences were boxed. The initiation codon for the GM papaya was boxed with a dashed line. Numerals beside the sequence indicate the numbers of nucleotides from the 5′ terminus.

scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

RESULTS AND DISCUSSION

Detection of Unauthorized GM Papaya To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-Cl, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

Development of a Construct-Specific Detection Method for PRSV-YK In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

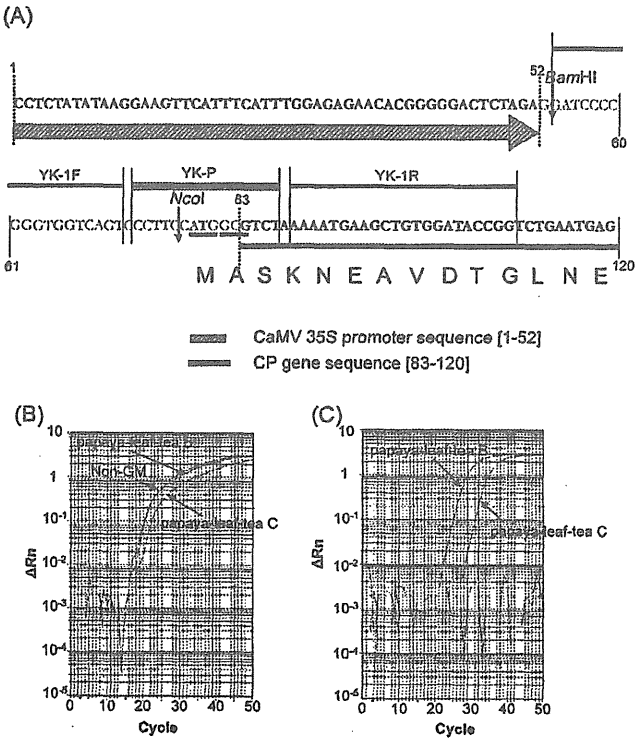

Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

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