JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

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

Received: October 5, 2010
Accepted: February 14, 2011
Revised: February 8, 2011
Published: March 11, 2011

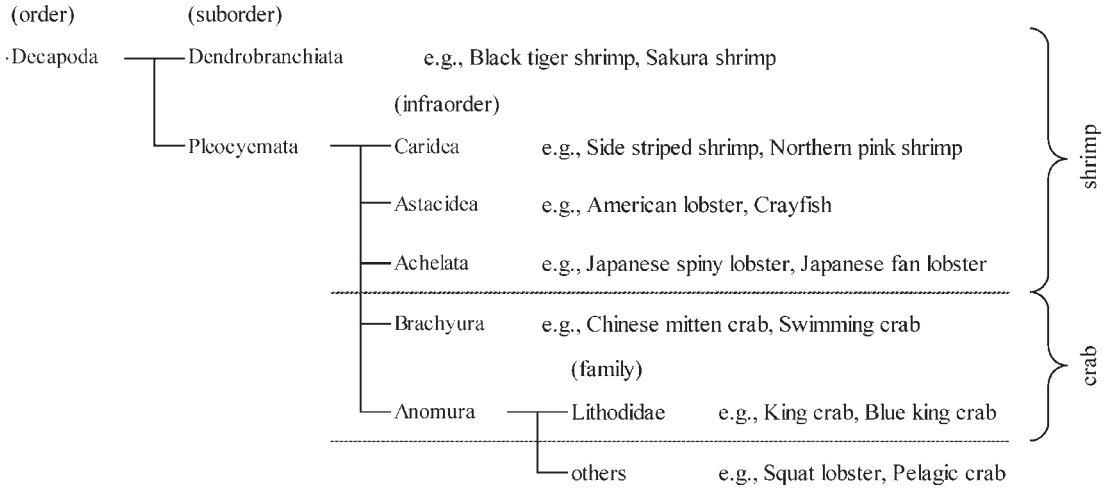

Figure 1. Target species of two PCR methods for detecting shrimp and crab species. Shrimp indicates the species which belong to the suborder Dendrobranchiata and the infraorder Caridea, Astacidea, and Achelata. Crab indicates the species which belong to the infraorder Brachyura and the family Lithoudidae.

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′-l,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′-l,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′-l,2,3[c]		0.3	
mantis shrimp				
F	StH12-05′-l,2	TTGTATGAATGGTCSGACAAGAT	0.2	95 bp
R	StH12-03′-l,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

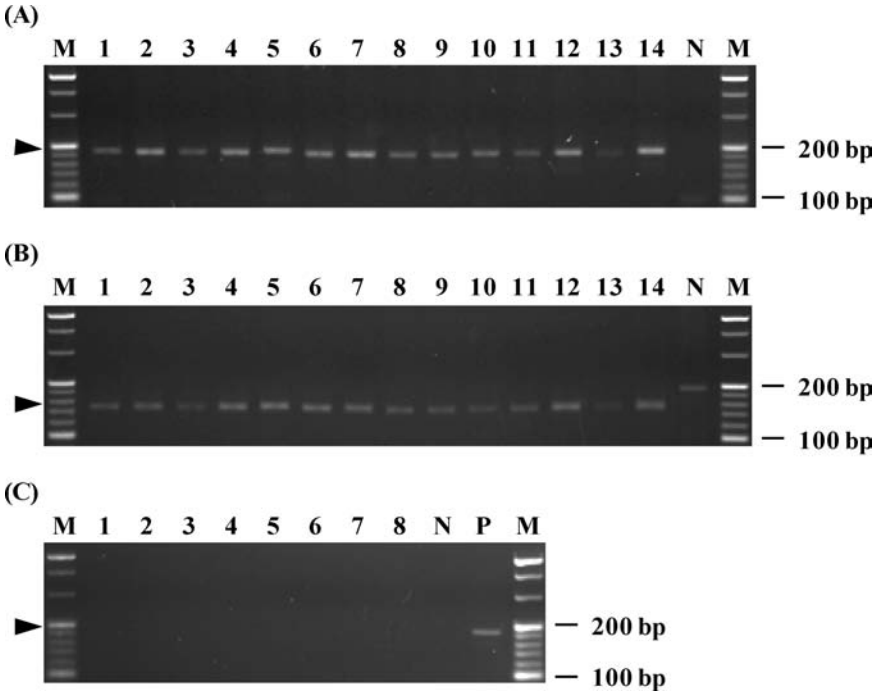

Figure 2. Specificity of the shrimp-PCR method for target species. The arrowheads indicate the expected size of PCR (A, C) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−14 of panels A and B, amplification of 5 pg of genomic DNA and restriction digestion of PCR products of genomic DNA, 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.

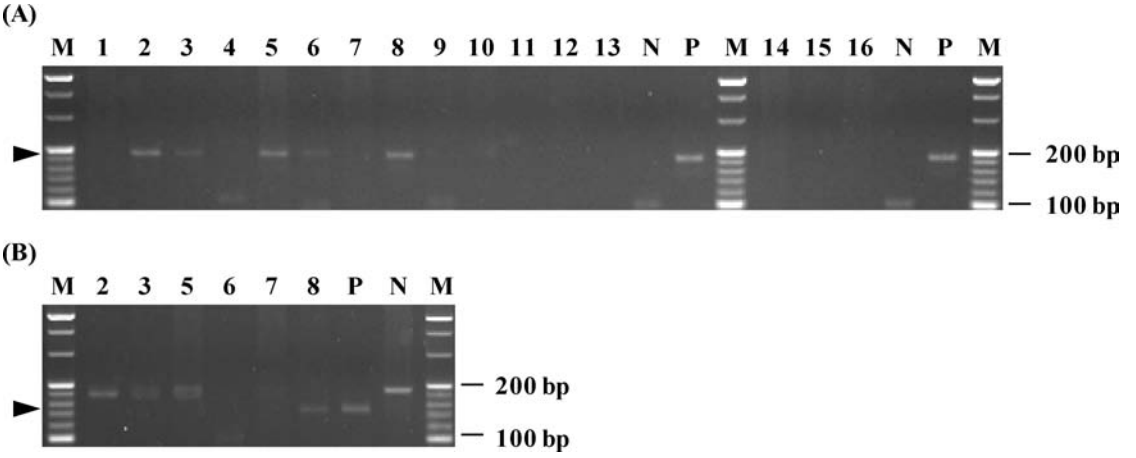

Figure 3. Specificity of the shrimp-PCR method for nontarget species. The arrowheads indicate the expected size of PCR (A) and RFLP products (B). M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 50 ng of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair 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

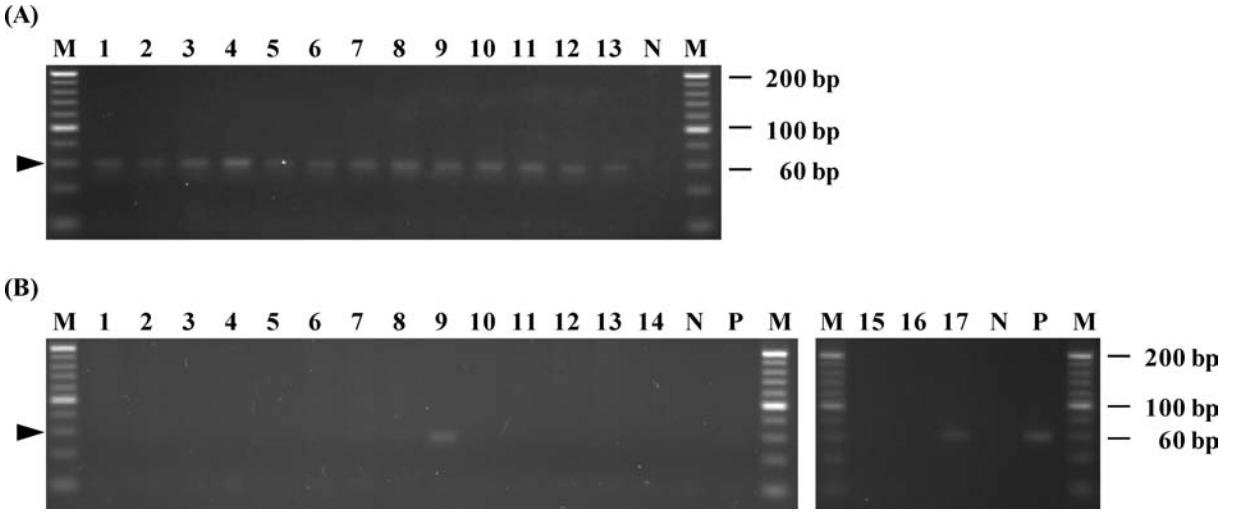

Figure 4. Specificity of the crab-PCR method. The arrowhead indicates the expected PCR product. M, DNA marker (20 bp ladder). Lanes 1−13 of panel A, amplification of 5 pg of genomic DNA from Snow crab (1), Red snow crab (2), Giant spider crab (3), Hair crab (4), Dungeness crab (5), Deep-sea red crab (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.

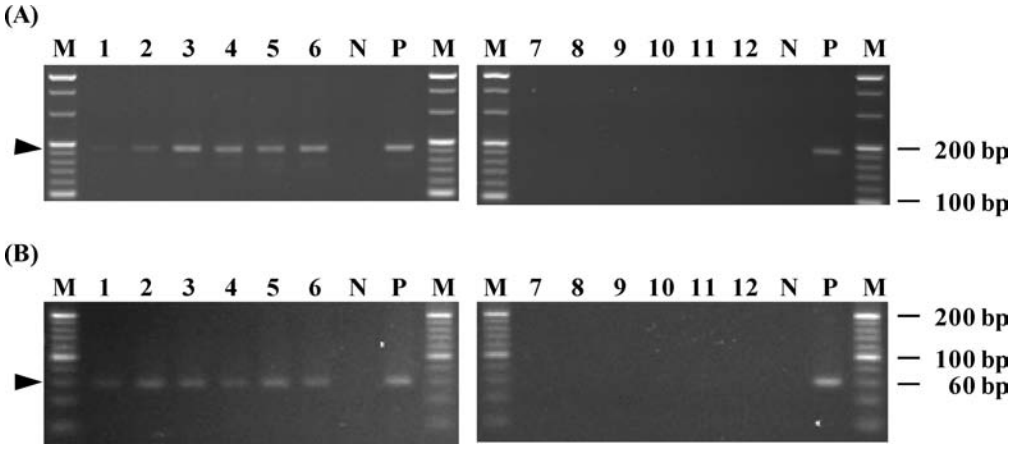

Figure 5. Sensitivity of two PCR methods (A, ShH12-05′ and ShH13-03′; B, CrH16-05′ and CrH11-03′) in incurred foods. The arrowheads indicate the expected PCR product for each primer pair. M, DNA marker (20 bp ladder). Lanes 1−6 of panel A, amplification of positive model samples, using the shrimp detection 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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Funding Sources

This study was supported by Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan.

■ ACKNOWLEDGMENT

We would like to thank Mr. K. Seiki and Dr. K. Oda of Maruha Nichiro Holdings, Inc., and Mr. Shibahara of Nippon Suisan Kaisha, Ltd., for kindly providing the crustacean samples.

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