

A multiplex PCR assay combined with capillary electrophoresis for the simultaneous detection of tropomyosin allergens from oyster, mussel, abalone, and clam mollusk species

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ABSTRACT

Tropomyosin present in mollusk species is the most common allergen in humans and causes excessive immune responses. To simultaneously detect tropomyosin allergens in mollusk species, a multiplex PCR assay combined with capillary electrophoresis was developed for the detection of tropomyosin genes of oyster, mussel, abalone, and clam, and the 18S rRNA gene of eukaryotes. The developed multiplex PCR revealed specific amplicons of four mollusk species [oyster (*Crassostrea gigas*), 150 bp; mussel (*Mytilus edulis*), 119 bp; abalone (*Haliotis discus hannai*), 98 bp; clam (*Ruditapes philippinarum*), 76 bp] and an amplicon of universal eukaryotic primer (eukaryotes, 190 bp); the detection limit of DNA was confirmed to be 16 pg. This multiplex PCR assay was applied for monitoring commercially processed seafood products, achieving successful detection of tropomyosin genes in 19 processed seafood products in Korea. The developed assay is an efficient and useful method for detecting tropomyosin allergens from mollusk species in seafoods.

1. Introduction

Food allergies are common disorders impacting the quality of human life and health costs, occurring in approximately 1–3% in adults 4–6% in children of the general population (Jablonski, Fu, Jackson, & Gendel, 2010). Food allergy symptoms can cause health risks, such as urticaria, asthma, diarrhea, and life-threatening anaphylaxis (Wild & Lehrer, 2005). Among the proteinaceous food allergens, tropomyosin (myofibrillar protein), which was first described as a crustacean allergen in shrimp in 1981, is a major crustacean shellfish allergen. It exists in muscles, where it regulates muscle contraction in both vertebrates and invertebrates. Owing to its functional importance, tropomyosin levels in muscles are higher than other allergens in crustacean and mollusk shellfish (Kamath, Abdel Rahman, Komoda, & Lopata, 2013). Moreover, it is reported as an allergen in mollusks [abalone (*Haliotis midae*), clam (*Pseudocardium sachalinensis*), and oyster (*Crassostrea gigas*)]; crustaceans [shrimp (*Litopenaeus vannamei*), crab (*Portunus trituberculatus*)]; and cephalopods [squid (*Todarodes pacificus*), octopus (*Octopus vulgaris*)] (Emoto, Ishizaki, & Shiomi, 2009; Fu, Wang, Zhu, & Wang, 2019; Lehrer, Ayuso, & Reese, 2003). Tropomyosin is degraded by intense boiling and high-pressure treatment, although it has been shown to be heat stable (Yu et al., 2011). It has been classified

as a food allergen of crab, shrimp, oyster and abalone in WHO/IUIS allergen database (<http://www.allergen.org>). The amino acid homology of tropomyosin among mollusk species ranges from 68% to 88% (Faber et al., 2017; Pedrosa, Boyano-Martínez, García-Ara, & Quirce, 2015). Previous studies convinced researchers that this high homology of tropomyosin among crustacean and mollusk shellfish species has a strong potential to cause IgE-mediated immunological crosslinked reactions across these species; this renders the specific distinction of these shellfish species for food labeling very difficult (Faber et al., 2017; Jeong, Hong, & Yong, 2006; Kamath et al., 2013).

Nowadays, the type and number of processed seafood products are steadily increasing due to their increased consumption, which has resulted in more frequent reports of associated allergies (Lopata & Lehrer, 2009; Lopata, O'Hehir, & Lehrer, 2010). The unintentional mixing of allergens in food processing can also cause allergy-related incidents (Herrero, Vieites, & Espiñeira, 2012; Lopata et al., 2010; Lopata & Kamath, 2012).

Therefore, labeling of allergens in food products is essential, and it is required by law in developed countries (Jeong, Park, Lee, & Park, 2017). Currently, the WHO/FAO Codex Alimentarius Commission requires the labeling of 8 common food allergens such as cereals containing gluten, eggs, peanuts, soybean, milk, tree nuts, crustaceans, and

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fish worldwide, and the European Commission list includes 12 allergens of celery, mustard, sesame seeds, and sulfite at concentrations of 10 mg/kg or more, besides these 8 allergenic foods described above (Soon, 2018). In addition, the labeling of allergens on processed food product packages and non-prepacked food, including in restaurants and cafes is proposed in the EU legislation (Köppel et al., 2010; Popping & Diaz-Amigo, 2018). Among Asian countries, Japan that enforces allergen labelling as the first country has the mandatory labelling of 7 allergens, and Hong Kong enforces the 9 allergen labeling (Fu et al., 2019). China has just recommended to label allergens and Korean Ministry of Food and Drug Safety (MFDS) has established food labeling for the following 21 foods: egg, milk, buckwheat, peanut, soybean, wheat, mackerel, crab, shrimp, pork, peach, tomato, sulfite, walnut, chicken, beef, squid and mollusk shellfish (clam, mussel, abalone, and oyster) (Suh, Park, Kim, & Kim, 2019).

However, studies and regulations based on epidemiology, diagnostics, and labeling of food products have focused on tropomyosin from crustaceans, while tropomyosin from mollusks has been relatively overlooked (Lopata & Lehrer, 2009). The main detection methods for investigating food allergens include enzyme-linked immunosorbent assays (ELISA) of proteins and PCR amplification of DNA (Van Hengel, 2007). Protein-based techniques, such as ELISA, biosensor, lateral flow immunoassay, etc. that react directly allergens have been reported to detect invertebrate tropomyosin (Wang et al., 2019; Zhang, Lu, Ushio, & Shiomi, 2014; Zhou et al., 2020). However, regarding the comparison of these two methods in terms tropomyosin detection, previous reports have stated some advantages of the DNA-based method and noted that DNA is a good surrogate for allergen proteins due to the inherently higher DNA level and its greater heat-stability than that of proteins during food processing (Eischeid, Kim, & Kasko, 2013). In addition, commercial food products generally consist of many ingredients that can cause allergic reactions. To control hidden ingredients and reduce cost and time associated with detection, multiplex PCR combined with capillary electrophoresis was developed (Cheng et al., 2016). Gel electrophoresis has low resolution and is associated with difficulties in comparing the degree of fluorescence at similar concentrations. In contrast, capillary electrophoresis has the advantages of effectively separating amplified DNA fragments and can represent DNA concentrations in complex mixtures (Zhang, Zhu, Zhang, He, & Wang, 2018).

Given the importance of tropomyosin as a food allergen in mollusk species, the development of a method for simultaneously detecting tropomyosin present in four mollusk species (clam, abalone, mussel, and oyster) is essential for the monitoring and labeling of food allergens in processed food to prevent allergy-related problems and to enhance public health.

2. Methods

2.1. Sample collection

All of 8 raw seafood species used as the reference samples, namely white leg shrimp (*Litopenaeus vannamei*), swimming crab (*Portunus trituberculatus*), squid (*Todarodes pacificus*), blue mussel (*Mytilus edulis*), abalone (*Haliotis discus hannai*), oyster (*Crassostrea gigas*), clam (*Ruditapes philippinarum*), and mackerel (*Scomber japonicus*), were obtained from local markets in the Republic of Korea (Table 1). A total of 19 commercial fishery products including raw, frozen and dried seafood as well as seasoned, porridge and canned seafood products were purchased from local markets in the Republic of Korea. All of the samples were ground in liquid nitrogen using a mortar and immediately stored at -20°C until analysis.

2.2. DNA extraction

Genomic DNA was extracted using DNeasy® Blood & Tissue Kit

Table 1

List of eight crustacean and mollusk seafood species used in this study.

Taxonomic name of target seafood	Common name ^a
<i>Litopenaeus vannamei</i>	White leg shrimp
<i>Portunus trituberculatus</i>	Swimming crab
<i>Todarodes pacificus</i>	Squid
<i>Ruditapes philippinarum</i>	Clam
<i>Mytilus edulis</i>	Blue mussel
<i>Haliotis discus hannai</i>	Abalone
<i>Crassostrea gigas</i>	Giant Pacific oyster
<i>Scomber japonicus</i>	Mackerel

^a Only one of the possible common names is shown for each species.

(Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The purity and concentration of extracted DNA were measured using a UV spectrophotometer (UV-1700; Shimadzu, Kyoto, Japan) to confirm the integrity and quality of extracted DNA. Genomic DNA from samples with an A_{260}/A_{280} ratio within the range of 1.8–2.0 was used as the PCR template.

2.3. Primers

Genes used for PCR are listed in Table 2, including the target mollusk species, primer sequence, and expected size of PCR products. The specific primer sets were designed based on tropomyosin-coding sequences of oyster, mussel, abalone, clam, and 18S rRNA for eukaryotes retrieved from GenBank of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>). To find specific sequence of each species, the tropomyosin-coding sequences of eight seafood species including target species were aligned using ClustalW2 (<ftp://ebi.ac.uk/Tools/msa/clustalw2/>).

Primers were designed using the Primer Designer Program (Version 3.0; Scientific and Educational Software, Durham, NC, USA). The five primer sets used in this study were synthesized by the Bionics Company (Seoul, Republic of Korea).

2.4. Simplex PCR amplification

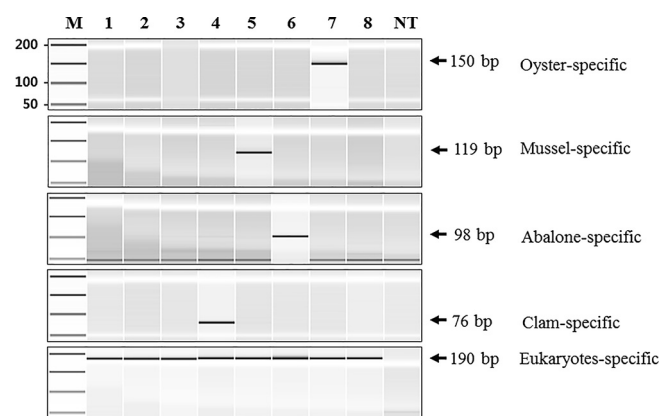
Simplex PCR amplification on each target allergen was implemented to confirm the primer specificity and sequence of amplified DNA. Each simplex PCR was performed in a 25 μL mixture containing 2.5 μL of $10\times$ buffer, 200 μM dNTPs, 0.5 U Ex-Taq polymerase (Takara, Shiga, Japan), 400 nM each primer, and 10 ng of DNA template. Amplification was using a thermal cycler (PC-808; ASTEC, Kyoto, Japan) with the following program: pre-incubation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 5 min. PCR products were confirmed by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.5. DNA sequencing of amplified target DNA

Each amplified PCR product was purified from 1% agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Purified PCR product was inserted into the pGEM-T easy vector (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Each plasmid DNA containing amplified PCR product was extracted using the DNA-spin™ Plasmid DNA Purification Kit (Intron Biotechnology, Seongnam, Republic of Korea). Sequencing was bilaterally implemented using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) by GenoTech (Daejeon, Republic of Korea). The sequences of PCR products were confirmed using the Basic Local Alignment Search Tool (BLAST) of the NCBI database (Zhang, Schwartz, Wagner, & Miller, 2000).

Table 2
Primer pairs for multiplex PCR designed and used in this study as well as their sources.

Target organism (taxonomic nomenclature)	Target gene	Primer name ^a	Sequence (5' → 3')	Product size (bp)	Concentration (nM)	Accession no. ^b
Oyster (<i>Crassostrea gigas</i>)	Tropomyosin (Cra g 1)	OysT-F OysT-R	CCC TAA ACA GCC ATC CAT AC GCC ACA ACT GAG AGT TGT TC	150	800	NM_001308906
Mussel (<i>Mytilus edulis</i>)	Tropomyosin (Myt e 1)	MusT-F MusT-R	GAT TAG TCA AGC CAA TTC CC CCA TTT TCA TGG CAA CCA TC	119	3200	U40035
Abalone (<i>Haliotis discus hannai</i>)	Tropomyosin (Hal d 1)	AbalT-F AbalT-R	GAA CAA GAG GTG AGT GGA AC TCG AGT CTC TCT GTG GCA GT	98	400	AB444939
Clam (<i>Ruditapes philippinarum</i>)	Tropomyosin (Rud p 1)	ClaT-F ClaT-R	CTT GCT GAA AAG GAG AAG TAC CTC ACA TGC CAG CTA ACT CA	76	800	AB444948
Eukaryotes	18S rRNA	Euk-F Euk-R	TAA AGG AAT TGA CGG AAG GG TCG TTA TCG GAA TCA ACC AG	190	200	AY557510.1

^a -F: forward primer, -R: reverse primer.^b Accession numbers were collected from GenBank of the NCBI (<http://www.ncbi.nlm.nih.gov>).**Fig. 1.** Simplex PCR results with each primer set designed for the detection of the tropomyosin allergen gene present in each target mollusk species using genomic DNA of various seafood species. Lane M: 100-bp DNA ladder, lane 1: white leg shrimp, lane 2: crab, lane 3: squid, lane 4: clam, lane 5: mussel, lane 6: abalone, lane 7: oyster, lane 8: mackerel, lane NT: non-template control.

2.6. Multiplex PCR amplification

The amplification of multiplex PCR was carried out using the same conditions and instrument as the simplex PCR. Optimized multiplex PCR was performed with the final selected five primer sets at set concentrations, as shown in Table 2. PCR products were confirmed by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

3. Results and discussion

3.1. Specificity of designed PCR primers

For the development of a multiplex PCR method for tropomyosin genes of target mollusk species, the specificity of each primer set was preferentially confirmed through simplex PCR with genomic DNA of seafood species, contains tropomyosin as food allergen in WHO/IUIS or is classified as allergen in MFDS. Each targeted region of the tropomyosin-coding gene was specifically amplified with the predicted size of PCR product by each specific primer set, as shown in Fig. 1 (oyster, 150 bp; mussel, 119 bp; abalone, 98 bp; clam, 76 bp). Each primer set in simplex PCR revealed no cross-reactions within the other eight seafood species. Each target species-specific primer set amplified only target species, but not other related species, which showed that the newly designed primer set was species specific. The universal eukaryote-specific primer targeting the 18S rRNA gene amplified a PCR product of 190 bp for all of the samples used, as expected. In addition, sequencing analysis was performed using amplicons of each PCR product to validate specific amplification of the target region of the tropomyosin gene and the 18S rRNA gene. The amplicon sequencing results were verified as each target region of tropomyosin allergen genes or the 18S rRNA gene by a BLAST search in the NCBI nucleotide database (data not shown). The obtained results demonstrated the specificity of the designed primer sets on tropomyosin-coding genes of target species of mollusk and the 18S rRNA gene of eukaryotes.

3.2. Optimization and specificity of the multiplex PCR assay

During the development and optimization of multiplex PCR, PCR-related factors such as *Taq* DNA polymerase concentrations, PCR buffer, dNTP, and primer set were adjusted to minimize non-specific interactions (Elnifro, Ashshi, Cooper, & Klapper, 2000). Through this optimization process, the concentrations of each primer set were adjusted, as shown in Table 2, and the optimized annealing temperature was set to 62 °C, which is nearly the same as that of simplex PCR. Finally, a

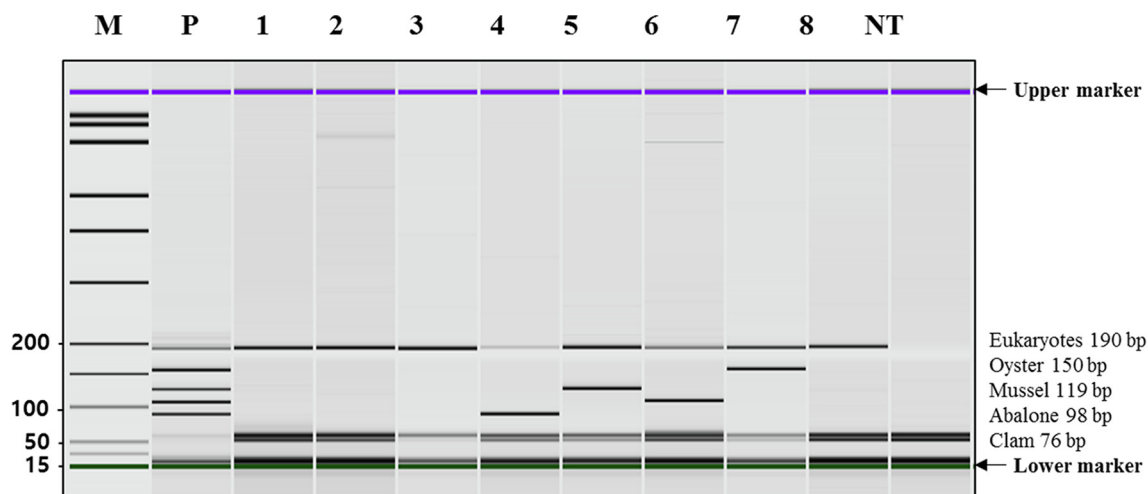


Fig. 2. Simultaneous detection performance and specificity of multiplex PCR for the detection of tropomyosin allergen genes in mollusk species with genomic DNA of various seafood species. Lane M: 100-bp DNA ladder, lane P: mixture of oyster, mussel, abalone, and clam, lane 1: white leg shrimp, lane 2: crab, lane 3: squid, lane 4: clam, lane 5: mussel, lane 6: abalone, lane 7: oyster, lane 8: mackerel, lane NT: non-template control.

multiplex PCR assay that simultaneously detects tropomyosin allergens in four mollusk species was successfully established. In addition, the specificity of multiplex PCR was confirmed for the four target mollusk species relative to eight seafood species, including shrimp, crab, squid, clam, mussel, abalone, oyster, and mackerel, as shown in Fig. 2. No false-positive amplicons or non-specific bands for other non-target species (shrimp, crab, squid, and mackerel) were observed. In this study, the use of capillary electrophoresis based on Lab-on-a-chip technology leads to discriminate effectively similar size of bands amplified simultaneously with five primer sets in a single reaction tube, however, typical error range in the assignments of the bands length has been reported (Garino et al., 2014). Thus, in order to prevent this drawback, the actual size of each fragment was considered with the upper and lower markers (1500 and 15 bp, respectively) in all well. Capillary electrophoresis used to verify all five amplification products was successfully applied to multiplex PCR (Cheng et al., 2016). Meanwhile, the universal primer targeting the 18S rRNA gene achieved amplification with DNA of all seafood samples used.

3.3. Sensitivity of the multiplex PCR assay

The genomic DNA extracted from clam, abalone, mussel, and oyster was measured by UV spectrometer, 50 ng of each DNA was mixed and then fivefold serially diluted ranging from 50 ng to 0.0032 ng. The diluted DNA mixtures were then applied to evaluate the limit of detection (LOD) of our developed multiplex PCR for the detection of tropomyosin allergen genes in mollusk species. The LOD test was independently conducted in triplicate. As shown in Fig. 3A, all five amplified PCR products were shown clearly down to a level of each genomic DNA of 0.016 ng and the peak of five amplicons were confirmed same level (Fig. 3B). At least 0.016 ng of target DNA was detected in all PCR reactions (Fig. S1). LOD was determined when the band of PCR products were observed in all 3 replicates, (i.e. the amplification of at least 95% of the replicates). Thus, LOD of this assay was 0.016 ng of the DNA mixture. The sensitivity of our developed multiplex PCR is comparable to that previously reported for similar detection methods, although to the best of our knowledge, no reports have described this for mollusk species. Matsunaga et al. (1999) have reported a detection limit of 0.25 ng for meat (from cattle, pig, chicken, sheep, goat, and horse). In addition, Farrokhi and Joozani (2011) have reported a detection limit of 0.1 ng for pork, while Cheng, He, Huang, Huang, and Zhou (2014) have reported a detection limit of 0.15 ng for duck, pig, and chicken. The LOD of the developed multiplex PCR thus

revealed improvements in sensitivity and efficiency over previous approaches. This confirmed the adequate sensitivity and accuracy of the developed multiplex PCR and its potential for application for the detection of allergens in processed foods.

3.4. Application of multiplex PCR assay in commercial seafood products

The applicability of the developed multiplex PCR for the tropomyosin allergen gene in mollusks was evaluated with 19 commercially available processed seafood products, including 4 fresh seafood products, 2 canned seafood products, 3 seasonings, 3 porridges, 1 frozen oyster, 3 dried seafood products, 2 instant seafood products, and 1 seafood soup. The application test was independently conducted in triplicate. Except for fresh seafood, all samples were heated, pressurized and frozen, especially canned seafood products and seasonings were extremely processed. DNA extracted from these 19 products was applied as a PCR template for the multiplex PCR. To increase the efficiency of recovery of genomic DNA from processed foods, inhibitor such as salt was washed off with water prior to DNA extraction and the double amount of lysis buffer was used for sufficient lysis of sample. Furthermore, multiplex PCR was developed having small fragments (> 200 bp) to successfully detect DNA degraded during processing steps. As shown in Table 3, the results of multiplex PCR of all processed seafood products were in agreement with the allergenic seafood products declared on the product labeling (Fig. S2).

Along with the PCR product for the tropomyosin gene, the universal primer set for the 18S rRNA gene positively amplified a 190-bp PCR product for all DNA samples extracted from the 19 processed seafood products. These results demonstrated that the developed multiplex PCR could be an adequate detection tool for the monitoring of tropomyosin allergens in processed seafood products.

CRediT authorship contribution statement

Seung-Man Suh: Conceptualization, Methodology, Data curation, Writing - original draft. **Mi-Ju Kim:** Data curation, Methodology, Writing - review & editing. **Hee-In Kim:** Validation, Methodology. **Hyun-Joong Kim:** Writing - review & editing. **Hae-Yeong Kim:** Supervision, Validation, Writing - review & editing, Funding acquisition, Project administration.

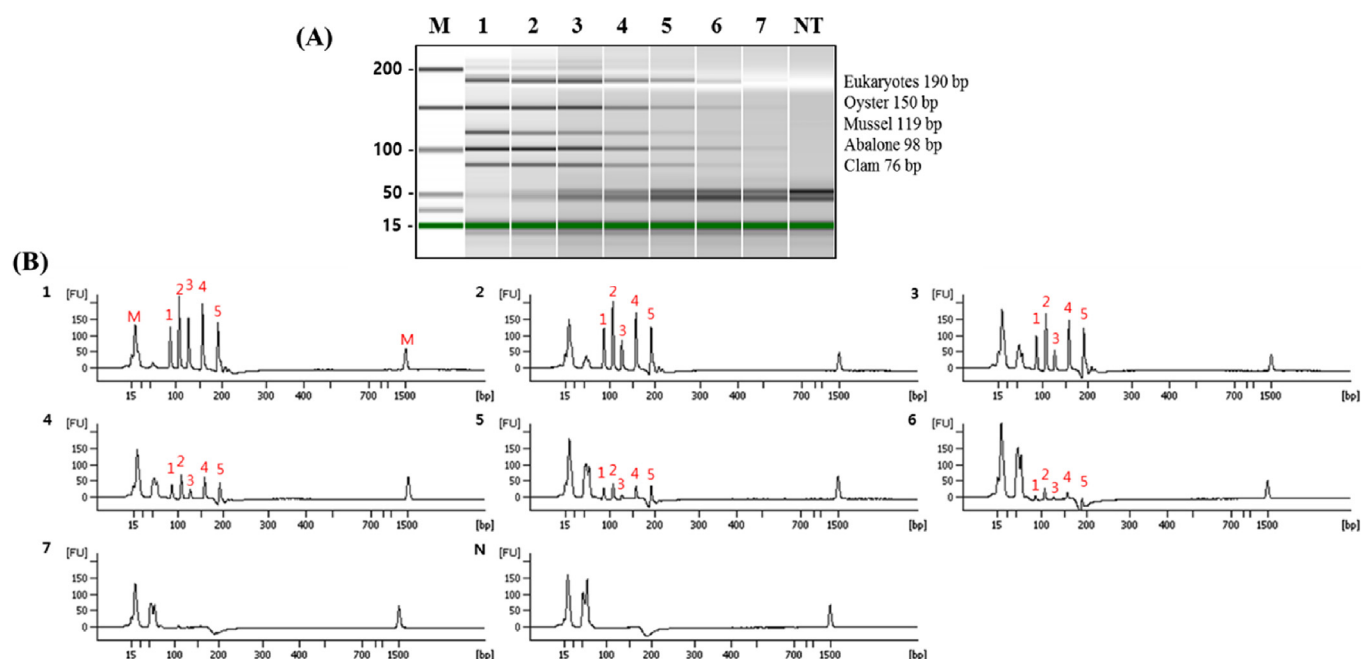


Fig. 3. Limit of detection (LOD) of the multiplex PCR for the detection of the tropomyosin allergen gene of mollusk species. (A) gel, Lane M: 100-bp DNA ladder, lanes 1–7: 50, 10, 2, 0.4, 0.08, 0.016, and 0.0032 ng of each genomic DNA present in the DNA mixture of four mollusk species (oyster, mussel, abalone, and clam), lane NT: non-template control. (B) peak, 1: clam, 2: abalone, 3: mussel, 4: oyster, 5: eukaryotes.

Table 3

Various commercially available processed seafood products and their diagnostic results using multiplex PCR for the detection of the tropomyosin allergen gene in mollusk species.

Sample no.	Seafood products	Declared allergenic seafood	Multiplex PCR results			
			Oyster	Mussel	Abalone	Clam
1	Fresh A	Mussel		+	+	+
2	Fresh B	Clam				+
3	Fresh C	Oyster	+	+		+
4	Fresh D	Abalone			+	+
5	Canned	Clam				+
6	product A	Abalone			+	+
7	Seasoning A	Mussel		+	+	+
8	Seasoning B	Mussel		+	+	+
9	Seasoning C	Mussel		+	+	+
10	Porridge A	Clam				+
11	Porridge B	Oyster	+	+		+
12	Porridge C	Abalone			+	+
13	Dried	Mussel		+	+	+
14	product A	Clam				+
15	Dried	Oyster	+	+		+
16	product C	Oyster	+	+		+
17	Instant A	Mussel, Clam		+	+	+
18	Instant B	Mussel		+	+	+
19	Instant C	Mussel		+	+	+

4. Conclusions

For simultaneous detection of the allergenic tropomyosin gene in processed mollusk shellfish products, especially in four mollusk species, specific primer sets for clam, abalone, mussel, and oyster were designed and their specificity was confirmed. Multiplex PCR analysis combined with capillary electrophoresis was developed using the four designed

primer sets combined with one primer set targeting the 18S rRNA gene of eukaryotes. The multiplex PCR assays were successfully applied for practical, efficient, and simultaneous detection of allergenic mollusk species in seafood products. We anticipate that the developed multiplex PCR will provide benefits for patients with allergies, seafood manufacturers, and governments in terms of the prevention, labeling, monitoring, and regulation of allergenic tropomyosin in processed mollusk shellfish. We believe that this study is the first to achieve the simultaneous analysis of allergenic mollusk species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.126451>.

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Supplementary Fig. 1 Limit of detection (LOD) of the multiplex PCR for the detection of the tropomyosin allergen gene of mollusk species. The multiplex PCR reactions were repeated in triplicates on different days. Lane M: 100-bp DNA ladder, lanes 1–7: 50, 10, 2, 0.4, 0.08, 0.016, and 0.0032 ng of each genomic DNA present in the DNA mixture of four mollusk species (oyster, mussel, abalone, and clam), lane NT: non-template control.

Supplementary Fig. 2 Multiplex PCR results with extracted DNAs from various commercially processed seafood products purchased from local markets in Korea.

Lane M: 100 bp DNA ladder, lane P: positive control, lane 1: fresh A, lane 2: fresh B, lane 3: fresh C, lane 4: fresh D, lane 5: canned product A, lane 6: canned product B, lane 7: seasoning A, lane 8: seasoning B, lane 9: seasoning C, lane 10: Porridge A, lane 11: Porridge B, lane 12: Porridge C, lane 13: dried product A, lane 14: dried product B, lane 15: dried product C, lane 16: frozen product A, lane 17: instant A, lane 18: instant B, lane 19: instant C, and lane NT: non-template control.

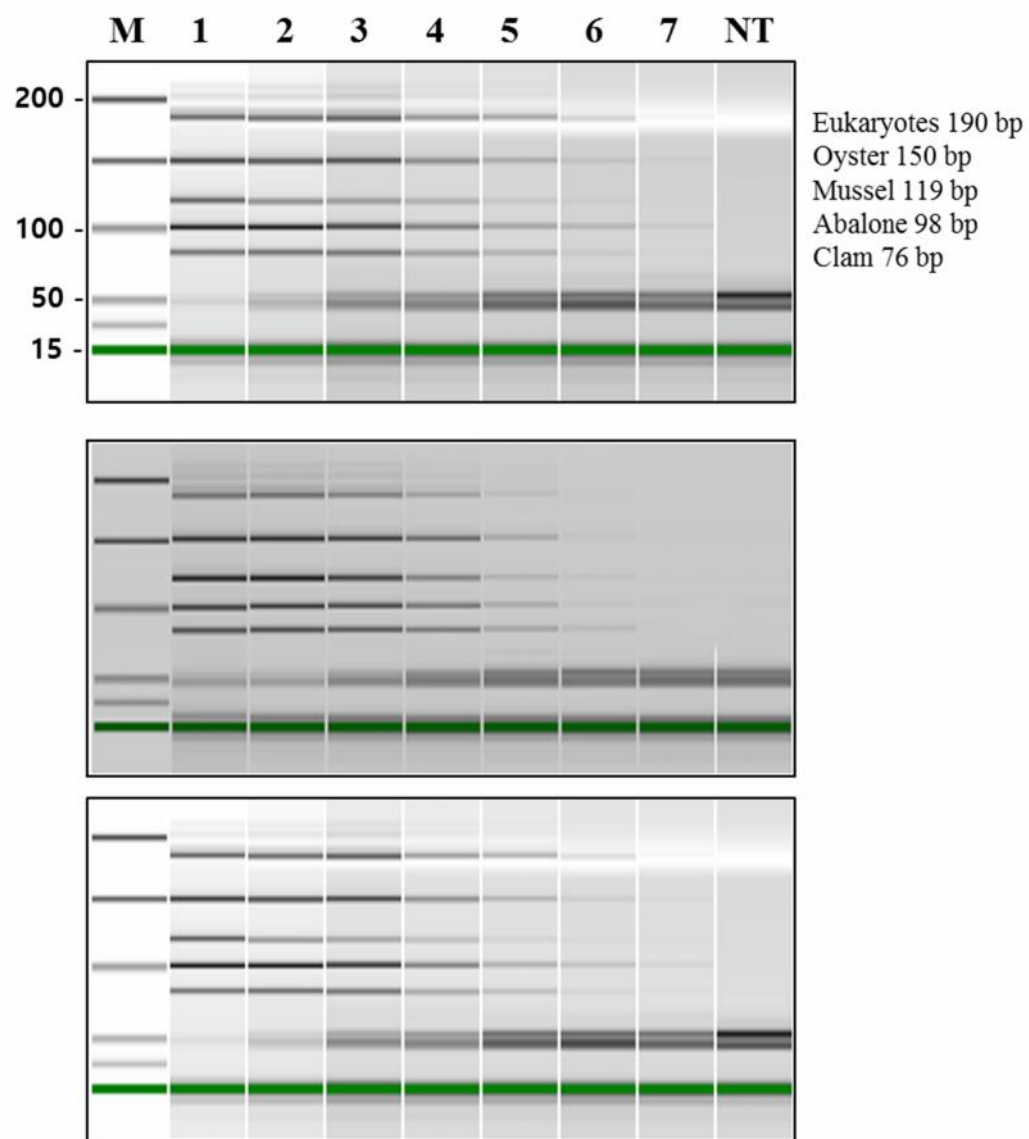


Figure S1.

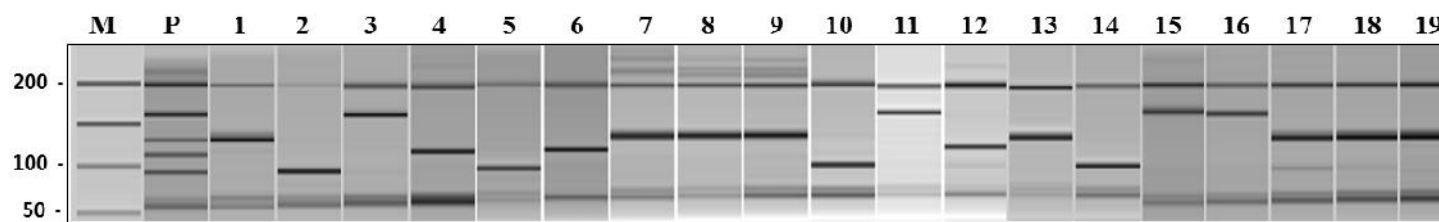


Figure S2.