

# Simultaneous Detection and Prevalence of Allergens in *Anisakis* Species Isolated from Marine Fishes

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## ABSTRACT

This study was conducted to develop a multiplex reverse transcription (RT) PCR for the detection of *Anisakis* allergens and to investigate the relationship between allergen profiles and anisakid larvae isolated from *Scomber japonicus*, *Trichiurus lepturus*, and *Conger myriaster* in Korea. The species of *Anisakis* was determined using *Anisakis pegreffii*-specific PCR and restriction fragment length polymorphism analysis. The prevalence and profiles of five Ani s allergens were examined by multiplex RT-PCR. *A. pegreffii* and *Anisakis typica* accounted for 97.1 and 2.9%, respectively, of the 140 larvae examined. In *A. pegreffii*, allergen prevalence was 41.2% for Ani s 1, 72.1% for Ani s 2, 69.9% for Ani s 3, 86.7% for Ani s 4, and 93.4% for Ani s 5. Most *A. pegreffii* larvae had multiple allergen profiles, and 80.7% of *A. pegreffii* carried both Ani s 4 and Ani s 5, which are heat-resistant allergens. Fifty-two to 65% of *A. pegreffii* isolated from *S. japonicus* and *C. myriaster* carried all five Ani s allergens.

Key words: Allergen; *Anisakis pegreffii*; *Anisakis typica*; Multiplex reverse transcription PCR; Marine fish

Since human infection by the nematode *Anisakis simplex* was first reported in The Netherlands in 1960, over 20,000 cases of anisakiasis have been reported worldwide (20). Most of the clinical cases of anisakiasis in Spain and Japan were closely associated with the consumption of raw fish (10, 12). *A. simplex* larvae have often been found in the whole body cavity, viscera, and muscle tissues of marine fish (7). In Korea, 107 cases of human anisakiasis were reported in 1995 (11). Acute abdominal pain is a typical clinical sign of anisakiasis in Korean patients who consumed raw marine fish. Edema, erosion, ulcer, and hemorrhage of the gastric wall have been revealed by gastrofibroscopy (11). Symptoms of anaphylaxis, such as rash, angioedema, and high levels of immunoglobulin E (IgE), have been reported in anisakiasis patients (24).

Since the allergenic potential of *A. simplex* was first addressed in Japanese urticaria patients who consumed mackerel (12), urticaria has been recognized as a common allergic symptom in *Anisakis* allergy cases. Thirty percent of allergic patients who were sensitized to *A. simplex* had abdominal pain, angioedema, and anaphylaxis. Allergic conjunctivitis, dermatitis, and asthma also have been described, depending on the occupational exposure of *Anisakis* allergens via inhalation or skin contact, and the effects of *Anisakis* allergens is a great concern in public health (20).

Traditionally, morphologic identification of anisakid third stage larvae was used to differentiate between among

*Anisakis* species (11). The genus is classified into two groups of species based on morphological and genetic characteristics (16, 18). Based on results of PCR restriction fragment length polymorphism (RFLP) analysis, *A. simplex* complex has been differentiated into *A. simplex* C, *A. simplex sensu stricto*, and *Anisakis pegreffii* (18). Other *Anisakis* species (*A. typica*, *A. ziphidarum*, *A. physeteris*, and *A. brevispiculata*) have been identified based on results of multilocus enzyme electrophoresis (16). *A. simplex sensu stricto*, *A. pegreffii*, *A. simplex* C, *A. typica*, and hybrid genotypes were identified among anisakid larvae from mackerel (*Scomber japonicus*), squid (*Todarodes pacificus*), yellow corvina, sea eel, rock cod, and Pacific cod (*Gadus macrocephalus*), which are widely consumed in Korea and Japan (2, 11, 15).

To date, 14 Ani s allergens have been reported in *A. simplex* (26). Nine of these allergens have been well characterized (20, 26). Ani s allergens are categorized into three groups: (i) excretory-secretory (ES) allergens, which are proteases and protease inhibitors, (ii) somatic allergens, and (iii) cuticular allergens. Ani s 1, 2, 3, and 7 are recognized as major allergens. Ani s 4, 5, 6, 8, 9, and 10 are considered minor allergens (20). Ani s 1, 4, and 5 are heat resistant (5, 17, 20). Ani s 2 and Ani s 3 are somatic allergens that encode paramyosin and tropomyosin, respectively, in *A. simplex* (20). The cross-reaction of Ani s 2 or Ani s 3 is due to high homology with other myosins. Because Ani s 3 widely cross-reacts with dust mites, crustaceans, mollusks, cockroaches, and other insects, the consumption of *Anisakis*-contaminated food could cause hypersensitivity in patients who are sensitive to mites (4, 19,

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TABLE 1. Biochemical function, GenBank number, molecular mass, and references for allergens of the *Anisakis simplex* complex

| Allergen | Type                  | Name or function              | GenBank no. | Mass (kDa) | Reference |
|----------|-----------------------|-------------------------------|-------------|------------|-----------|
| Ani s 1  | Major, heat resistant | Kunitz-type trypsin inhibitor | AB100095    | 21         | 24        |
| Ani s 2  | Major, panallergen    | Paramyosin                    | AF173004    | 100        | 19        |
| Ani s 3  | Panallergen           | Tropomyosin                   | Y19221      | 41         | 4         |
| Ani s 4  | Heat resistant        | Cystatin                      | AM279414    | 9          | 17        |
| Ani s 5  | Heat resistant        | SXS/RAL-2 family protein      | AB274998    | 15         | 14        |

20). Five well-characterized Ani s allergens (Ani s 1 through 5) were chosen as target allergens to develop a multiplex reverse transcription (RT) PCR assay for detecting Ani s allergens simultaneously (Table 1).

Although most of the previous studies have focused on the identification and characterization of individual Ani s allergens in *A. simplex* larvae (5, 6, 10, 14, 20), the prevalence and profiles of Ani s allergens have not yet been examined in other anisakid larvae. In this study, a multiplex RT-PCR was used to investigate the correlation between Ani s allergens and *Anisakis* larvae collected from marine fishes.

## MATERIALS AND METHODS

**Anisakid larvae.** The visceral organs of 49 mackerel (*S. japonicus*), 45 large head hairtail (*Trichiurus lepturus*), and 46 whitespotted conger (*Conger myriaster*) were collected from seafood markets and retailers. Nematode larvae were collected and examined by macroscopically. Each larva was washed in sterile phosphate-buffered saline (PBS, pH 7.0) and dissected into two parts using a sterilized knife. The pieces were used to extract DNA and RNA for genotyping and detecting Ani s allergens, respectively.

**DNA extraction.** DNA was extracted from each nematode larva by using the modified phenol-chloroform method (15). Each larva isolated from *S. japonicus*, *T. lepturus*, or *C. myriaster* was added to a sample tube with 500  $\mu$ l of 0.1 N sodium hydroxide and boiled at 100°C for 10 min until the larva dissolved, at which time 500  $\mu$ l of phenol was added. Each tube was vortexed vigorously and then centrifuged at  $5,157 \times g$  for 10 min. The aqueous phase of each tube was transferred to another sterile tube and mixed with 500  $\mu$ l of phenol-chloroform (1:1). These tubes were vortexed vigorously and centrifuged at  $5,157 \times g$  for 10 min. The aqueous phase of each tube was transferred to a sterile tube and mixed with 500  $\mu$ l of cold ethanol and 50  $\mu$ l of 3 M sodium acetate. The tubes were kept at -20°C overnight then centrifuged for 30 min at  $17,968 \times g$ , rinsed with cold 70% ethanol, and centrifuged again at  $17,968 \times g$  for 30 min. After the supernatant was removed, the DNA pellet was air dried for 30 min and resuspended with 30  $\mu$ l of diethylpyrocarbonate (DEPC)-treated deionized water. The concentration of DNA in each sample was measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT) and the Gen 5 data analysis software program (BioTek).

**Genotyping of anisakid larvae.** For determining the genotype of anisakid larvae, PCR-RFLP analysis was conducted as described previously (15). The primers NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') and NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT-3') were used to amplify the entire internal transcribed spacer region (9). The PCR was performed in an MJ mini thermal cycler, with a total volume of 20  $\mu$ l consisting of 2  $\mu$ l of extracted DNA product, 1 $\times$  PCR buffer, 3 mM MgCl<sub>2</sub>, 250  $\mu$ M

concentrations of deoxynucleoside triphosphates (dNTPs), 0.5  $\mu$ l of each primer, and 1  $\mu$ l of *Taq* DNA polymerase. Initial denaturation was performed at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and then a final extension at 72°C for 10 min. PCR-RFLP analysis with 10 U of *Hinf*I (Bioneer, Daejeon, Korea) or *Hha*I (Bioneer) determined the genotype of the *Anisakis* larvae (15). Reference DNA for PCR-RFLP were the same as those used in a previous study (15). To confirm the genotype of the anisakid larvae, an *A. pegreffii*-specific PCR was also performed with primers ASF1 (5'-CAG CTT AAG GCA GAG TC-3') and AS2 (5'-TAT CAT TTT TGA TCA CAT AGA C-3') developed in a previous study (1).

**RNA extraction.** The RNA of the anisakid larvae was extracted by using a modified TRIzol method (22). The homogenate of anisakid larvae in 500  $\mu$ l of PBS was mixed vigorously with 500  $\mu$ l of TRIzol (Invitrogen, Carlsbad, CA) in a sample tube at room temperature for 10 min, 300  $\mu$ l of chloroform was added, and the tube was vortexed for 10 min and then centrifuged at  $5,157 \times g$  for 15 min. The aqueous phase of each tube was transferred to another sterile tube and mixed with 500  $\mu$ l of cold isopropanol. The tubes were kept at -20°C overnight, then rinsed with cold 70% ethanol, and centrifuged again at  $17,968 \times g$  for 30 min. After the supernatant was removed, the RNA pellet was air dried and resuspended with 30  $\mu$ l of DEPC-treated deionized water. The concentration of RNA in each sample was measured using the Epoch microplate spectrophotometer and the Gen 5 data analysis software program.

**Multiplex RT-PCR for Ani s allergens.** Sequences for Ani s 1 through 5 were obtained from GenBank (4, 13, 14, 17, 19, 22, 24). For the multiplex RT-PCR, all primer sets for Ani s allergens were newly designed for this study (Table 2). To eliminate DNA contamination, 1  $\mu$ g of the total RNA of each sample was treated with DNase I (Invitrogen) following the manufacturer's guidelines. A mixture of 5  $\mu$ l of DNA-free RNA, 1.5  $\mu$ l of DEPC-treated distilled water, 1  $\mu$ l of dNTP, 1  $\mu$ l of 10 $\times$  PCR buffer, random hexamers (50 ng/ $\mu$ l), 0.5  $\mu$ l of RNase inhibitor (20 U/ $\mu$ l), and 0.5  $\mu$ l of murine leukemia virus reverse transcriptase (50 U/ $\mu$ l) was prepared to synthesize cDNA. Reverse transcription was performed at 45°C for 15 min and then terminated at 99°C for 5 min. Three microliters of cDNA, 1 $\times$  PCR buffer, 3 mM MgCl<sub>2</sub>, 250  $\mu$ M concentrations of dNTPs, 0.5  $\mu$ l of each primer (25 mM), and 1  $\mu$ l of 1.25 U *Taq* DNA polymerase were mixed. The multiplex RT-PCR was performed as follows: denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s; and final extension at 72°C for 10 min. The multiplex RT-PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Bands were visualized with a UV transilluminator (UVP, Upland, CA) and analyzed using the Launch DocIT LS software (UVP). Because the profile of the Ani s allergens was determined by multiplex RT-PCR, RT-PCR was used to confirmed the presence of mRNA for individual Ani s allergens.

TABLE 2. Oligonucleotides designed for the detection of *Ani s* allergens by multiplex RT-PCR

| Target  | GenBank no. | Primer type | Oligonucleotide sequence (5' to 3') | $T_m$ (°C) | Product size (bp) |
|---------|-------------|-------------|-------------------------------------|------------|-------------------|
| Ani s 1 | AB100095    | Forward     | GAC AAA TGC CCT AAC GGC TA          | 57         | 192               |
|         |             | Reverse     | AGG GCA GAA TTG ATC GTC AC          |            |                   |
| Ani s 2 | AF173004    | Forward     | TTG ATC GCT CTC ACT GAT CG          | 57         | 316               |
|         |             | Reverse     | TTT TCT CAG CGA CGT GTT TG          |            |                   |
| Ani s 3 | Y19221      | Forward     | AAC ATG AAG AGC GTG CGA AT          | 57         | 413               |
|         |             | Reverse     | TTC CTC GGA AAT GCT CTT GT          |            |                   |
| Ani s 4 | AM279414    | Forward     | TGC AAT CCA GAA TCG TCG TA          | 57         | 493               |
|         |             | Reverse     | AGC AAA CAA GCC AGT GT CAT          |            |                   |
| Ani s 5 | AB274998    | Forward     | GCT TGG TAG TTT TGA AGG CAA         | 57         | 607               |
|         |             | Reverse     | ACG ATC GTA ACG ACG GAA AA          |            |                   |

**Statistical analysis.** Genotyping of anisakid larvae and the multiplex RT-PCR for *Ani s* allergens were conducted twice. The SAS 12.0 software (SAS Institute Inc., Cary, NC) was used for statistical analysis. The relationship between the fish species and the *Ani s* allergens was evaluated using chi-square analysis and the Mann-Whitney test. Statistical significance was determined at  $P < 0.05$ .

## RESULTS

**Genotyping of anisakid nematode larvae.** A PCR-RFLP assay was used to determine the genotype of anisakid larvae isolated from 49 *S. japonicus*, 45 *T. lepturus*, and 46 *C. myriaster* samples. An *A. pegreffii*-specific PCR assay confirmed the presence of *A. pegreffii* (Table 3). Among the 140 larvae isolated from these marine fishes, 136 (97.1%) were identified as *A. pegreffii* and 4 (2.9%) were identified as *A. typica* (Fig. 1). Only *A. pegreffii* was isolated from the 49 *S. japonicus* and 45 *T. lepturus* samples. Other *Anisakis* species were not identified in *S. japonicus* and *T. lepturus*. Among the 46 *C. myriaster* samples, in 42 (91.3%) the anisakid larvae were identified as *A. pegreffii*, and in 4 (8.7%) the larvae were identified as *A. typica*. *A. simplex* sensu stricto was not found in *C. myriaster* (Table 3).

**Prevalence of individual *Ani s* allergens.** The multiplex RT-PCR analysis was used to simultaneously detected *Ani s* allergens 1 through 5 in the anisakid larvae collected from *S. japonicus*, *T. lepturus*, and *C. myriaster* (Fig. 2). The detection rates for each *Ani s* allergen were 93.4% for *Ani s* 5, 86.7% for *Ani s* 4, 69.9% for *Ani s* 3, 72.1% for *Ani s* 2, and 41.2% for *Ani s* 1. Among the 49 *A. pegreffii* isolated from *S. japonicus*, *Ani s* 1 was detected in 26 larvae (63.3%), *Ani s* 2 was detected in 41 larvae (83.7%), *Ani s* 3 was detected in 43 larvae (87.8%), *Ani s* 4

was detected in 46 larvae (93.9%), and *Ani s* 5 was detected in 47 larvae (95.9%). Among the 45 *A. pegreffii* from *T. lepturus*, *Ani s* 1 was detected in 3 larvae (6.7%), *Ani s* 2 was detected in 19 larvae (42.2%), *Ani s* 3 was detected in 14 larvae (31.1%), *Ani s* 4 was detected in 32 larvae (71.1%), and *Ani s* 5 was detected in 40 larvae (88.9%). Of the 42 *A. pegreffii* larvae isolated from *C. myriaster*, *Ani s* 1 was detected in 22 (52.4%), *Ani s* 2 was detected in 38 (90.5%), *Ani s* 3 was detected in 38 (90.5%), *Ani s* 4 was detected in 40 (95.2%), and *Ani s* 5 was detected in 40 (95.2%); however, 4 *A. typica* larvae collected from *C. myriaster* did not carry any of the *Ani s* allergens (Table 4). The detection rate of *Ani s* 1, *Ani s* 2, and *Ani s* 3 in *T. lepturus* was significantly lower than that in *S. japonicus* and *C. myriaster* ( $P < 0.05$ ).

**Profile of *Ani s* allergens in anisakid larvae.** The multiplex RT-PCR analysis revealed that 127 (93.4%) of 136 *A. pegreffii* carried at least one of the *Ani s* allergens (Table 5). Fifty-seven (41.7%) of 136 *A. pegreffii* carried all five of the tested *Ani s* allergens. Other *Ani s* allergen

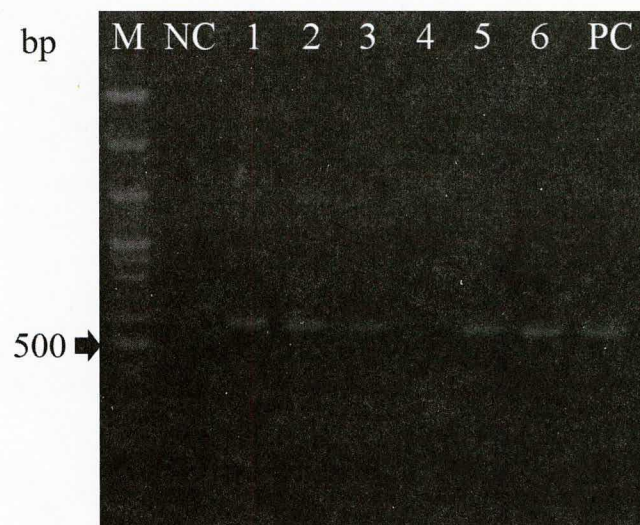


FIGURE 1. Species-specific PCR of *Anisakis pegreffii* collected from *Scomber japonicus* and *Conger myriaster*. Lane M, 100-bp DNA marker; lane NC, negative control; lane 1, *S. japonicus* S11001; lane 2, *S. japonicus* S11002; lane 3, *S. japonicus* S11003; lane 4, *C. myriaster* C12012; lane 5, *C. myriaster* sample C12014; lane 6, *C. myriaster* C12015; lane PC, *A. pegreffii* positive control.

TABLE 3. Genotypic prevalence of *Anisakis* species determined by PCR-RFLP analysis

| Host fish                  | No. of samples | No. (%) of positive samples |                                 |                  |
|----------------------------|----------------|-----------------------------|---------------------------------|------------------|
|                            |                | <i>A. pegreffii</i>         | <i>A. simplex</i> sensu stricto | <i>A. typica</i> |
| <i>Scomber japonicus</i>   | 49             | 49 (100)                    | 0                               | 0                |
| <i>Trichiurus lepturus</i> | 45             | 45 (100)                    | 0                               | 0                |
| <i>Conger myriaster</i>    | 46             | 42 (91.3)                   | 0                               | 4 (8.7)          |

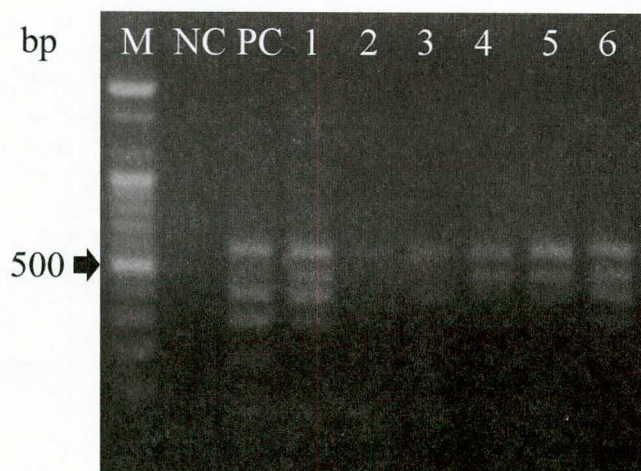


FIGURE 2. Multiplex RT-PCR profile of *Anisakis* allergens in *Anisakis pegreffii* larvae from *Scomber japonicus*. Lane M, 100-bp DNA marker; lane NC, negative control; lane PC, positive control for *Anisakis* allergens; lane 1, *S. japonicus* sample S11002; lane 2, *S. japonicus* sample S11003; lane 3, *S. japonicus* sample S11004; lane 4, *S. japonicus* sample S11005; lane 5, *S. japonicus* sample S11006; lane 6, *S. japonicus* sample S11007. Product sizes of *Anisakis* 1, 2, 3, 4, and 5 were 192, 316, 413, 493, and 607 bp, respectively.

profiles were *Anisakis* 2 + 3 + 4 + 5 (24.3%), *Anisakis* 4 + 5 (14.7%), *Anisakis* 5 alone (4.4%), *Anisakis* 2 + 4 + 5 (3.7%), *Anisakis* 3 + 4 + 5 (2.2%), and *Anisakis* 2 + 3 + 5 (1.5%). Nine (6.6%) of 136 *A. pegreffii* did not carry any of the *Anisakis* allergens. *Anisakis* allergens were not detected in all *A. typica* collected from *C. myriaster*.

In *S. japonicus* samples, 65.3% of the larvae were positive for all five types of *Anisakis* allergens with the following profiles: *Anisakis* 2 + 3 + 4 + 5 (16.3%), *Anisakis* 3 + 4 + 5 (6.1%), *Anisakis* 4 + 5 (4.1%), *Anisakis* 2 + 4 + 5 (2.0%), and *Anisakis* 5 alone (2.0%). In *T. lepturus*, the profiles were *Anisakis* 4 + 5 (35.5%), *Anisakis* 2 + 3 + 4 + 5 (20.0%), *Anisakis* 5 alone (11.1%), *Anisakis* 2 + 4 + 5 (8.9%), *Anisakis* 1 + 2 + 3 + 4 + 5 (6.7%), and *Anisakis* 2 + 5 (2.2%). Five (11.1%) of 45 larvae collected from *T. lepturus* were negative for all allergens. In *C. myriaster*, the profiles were *Anisakis* 1 + 2 + 3 + 4 + 5 (52.4%), *Anisakis* 2 + 3 + 4 + 5 (38.0%), and *Anisakis* 4 + 5 (4.8%). In 4.8% of the larvae collected from *C. myriaster*, none of the five *Anisakis* allergens tested were detected.

## DISCUSSION

Anisakiasis has been reported often in Japanese and Spanish individuals who consumed raw fish (10, 12, 20). Im

et al. (11) reported 107 cases of gastric anisakiasis in Cheju-do residents of Korea. Squid and yellow corvina, sea eel, ling, yellowtail, and other marine fishes have been identified as sources of *A. simplex* infection. A high prevalence of *A. simplex* was found in flatfish, conger, squid, beltfish, and mackerel, which are frequently consumed in Korea (8, 15). *S. japonicus*, *T. lepturus*, *G. macrocephalus*, *Lophiomus setigerus*, *Pleurogrammus azonus*, *Scomberomorus niphonius*, and *Platycephalus indicus* were especially associated with a high infection rate at all times of the year (7). However, 10 of 15 species of marine fishes in Korea appear to have a higher *A. simplex* infection in February or April (7). In another study, in which this phenomenon was termed the "spring rise," the prevalence and abundance of *Anisakis* larvae was higher in March and April in *Gadus morhua* and *Pollachius virens* from Norwegian waters (25). In the present study, the high abundance and prevalence of *A. pegreffii* in *S. japonicus*, *T. lepturus*, and *C. myriaster* was consistent with these previous studies.

In the present study, the *A. pegreffii* genotype was only anisakid larval genotype identified from *S. japonicus* and *T. lepturus* samples. Although 91.3% of anisakid larvae from *C. myriaster* were *A. pegreffii*, 8.7% were *A. typica*. In a previous study, the researchers also found only *A. pegreffii* in *S. japonicus* (15). Although *A. simplex* C, *A. simplex* sensu stricto, and *A. pegreffii* belong to the *A. simplex* complex, only one incidence of *A. simplex* sensu stricto was reported in *T. pacificus* in a previous study, and *A. simplex* C has not yet been reported in Korea (15). However, *A. simplex* sensu stricto was the predominant species in Pacific cod caught in Japan, whereas *A. pegreffii* was first found in Pacific cod (2). Based on these data, the diversity of *Anisakis* species appears to be low in three fish species popularly consumed in Korea. To clarify the correlation between *Anisakis* species and fish species, a more extensive investigation is needed.

Although the expression and characteristics of *Anisakis* allergens have been investigated previously, a reliable antibody to detect *Anisakis* allergens has not been available commercially. Thus, the multiplex RT-PCR developed in this study was used instead of a Western blot (immunoblot) to detect expression of *Anisakis* allergens. One limitation of this approach is that the multiplex RT-PCR detects the mRNA of the *Anisakis* allergen rather than the allergen itself.

The mRNA of each *Anisakis* allergen was detected in 41.2 to 93.4% of samples by the multiplex RT-PCR assay. *Anisakis* 5 and *Anisakis* 4 were detected more often than *Anisakis* 2, *Anisakis* 3, and *Anisakis* 1 in *A. pegreffii*. Because *Anisakis* 1, 2, 3, and 7 are considered major allergens and *Anisakis* 4, 5, 6, 8, 9, and 10 are considered minor allergens (20), the actual potency of the

TABLE 4. Prevalence of *Anisakis* species and *Anisakis* allergens in *Scomber japonicus*, *Trichiurus lepturus*, and *Conger myriaster*

| Host fish                  | <i>Anisakis</i> species | No. of samples | No. (%) of positive samples |                   |                   |                   |                   |
|----------------------------|-------------------------|----------------|-----------------------------|-------------------|-------------------|-------------------|-------------------|
|                            |                         |                | <i>Anisakis</i> 1           | <i>Anisakis</i> 2 | <i>Anisakis</i> 3 | <i>Anisakis</i> 4 | <i>Anisakis</i> 5 |
| <i>Scomber japonicus</i>   | <i>A. pegreffii</i>     | 49             | 31 (63.3)                   | 41 (83.7)         | 43 (87.8)         | 46 (93.9)         | 47 (95.9)         |
| <i>Trichiurus lepturus</i> | <i>A. pegreffii</i>     | 45             | 3 (6.7)                     | 19 (42.2)         | 14 (31.1)         | 32 (71.1)         | 40 (88.9)         |
| <i>Conger myriaster</i>    | <i>A. pegreffii</i>     | 42             | 22 (52.4)                   | 38 (90.5)         | 38 (90.5)         | 40 (95.2)         | 40 (95.2)         |
|                            | <i>A. typica</i>        | 4              | 0                           | 0                 | 0                 | 0                 | 0                 |

TABLE 5. Relationship between *Anisakis* species and profiles of Ani s allergens in anisakid larvae as determined by multiplex RT-PCR

| Host fish                  | Anisakis species    | No. of samples | No. (%) of positive samples |                     |                 |                 |                 |             |             |          |          |  |
|----------------------------|---------------------|----------------|-----------------------------|---------------------|-----------------|-----------------|-----------------|-------------|-------------|----------|----------|--|
|                            |                     |                | Ani s 1 + 2 + 3 + 4 + 5     | Ani s 2 + 3 + 4 + 5 | Ani s 3 + 4 + 5 | Ani s 2 + 3 + 5 | Ani s 2 + 4 + 5 | Ani s 4 + 5 | Ani s 2 + 5 | Ani s 5  | None     |  |
| <i>Scomber japonicus</i>   | <i>A. pegreffii</i> | 49             | 32 (65.3)                   | 8 (16.3)            | 3 (6.1)         | 0               | 1 (2.0)         | 2 (4.1)     | 0           | 1 (2.0)  | 2 (4.1)  |  |
| <i>Trichiurus lepturus</i> | <i>A. pegreffii</i> | 45             | 3 (6.7)                     | 9 (20.0)            | 0               | 2 (4.4)         | 4 (8.9)         | 16 (35.5)   | 1 (2.2)     | 5 (11.1) | 5 (11.1) |  |
| <i>Conger myriaster</i>    | <i>A. pegreffii</i> | 42             | 22 (52.4)                   | 16 (38.0)           | 0               | 0               | 0               | 2 (4.8)     | 0           | 0        | 2 (4.8)  |  |
|                            | <i>A. typica</i>    | 4              | 0                           | 0                   | 0               | 0               | 0               | 0           | 0           | 0        | 4 (100)  |  |

Ani s allergen may be different; the multiplex RT-PCR assay could not indicate quantitative expression of each allergen nor its allergenicity. For food or feed applications, further study to compare the detection of Ani s allergen mRNA and protein in anisakid larvae or a food matrix is needed.

Most (95.6%) of *A. pegreffii* larvae carried at least one Ani s allergen. the prevalent profiles were Ani s 1 + 2 + 3 + 4 + 5 (41.9%), Ani s 2 + 3 + 4 + 5 (24.3%), and Ani s 4 + 5 (14.7%). Surprisingly, 80.7% of *A. pegreffii* carried both Ani s 4 and Ani s 5, which are heat-resistant allergens, and only 4.4% of *A. pegreffii* did not carry any of the five Ani s allergens tested. In contrast, none of the *A. typica* larvae carried any of the Ani s allergens tested. Because the primer set for Ani s allergens was designed from the sequence of *A. simplex*, the detection rate of Ani s allergens for *A. pegreffii* and *A. typica* may be different. However, the allergenic profiles of *A. pegreffii* isolated from *T. lepturus* was significantly different than those for *A. pegreffii* isolated from *S. japonicus* and *C. myriaster*.

Heat-resistant Ani s allergens became a public concern after some patients had allergy responses after the consumption of cooked or canned fish (6). The high concentrations and prevalence of Ani s 1, Ani s 4, and Ani s 5 could be a potential risk to allergic patients because these allergens are highly resistant to boiling for 30 min and to pepsin digestion (5, 20). In contrast, in a double-blind, placebo-controlled clinical study lyophilized *A. simplex* larvae did not cause allergic reactions in *Anisakis*-allergic patients (23). This discrepancy between results of clinical studies may be explained by the difference in the antigenicity of *A. simplex* larvae depending on the exact *Anisakis* species. The allergenic profile of *A. pegreffii* from *T. lepturus* was significantly different from the profile of *A. pegreffii* from *S. japonicus* and *C. myriaster* in this study; thus, the allergenicity of crude or lyophilized allergens prepared from anisakid larvae could be different depending on host fish species. However, comparative analysis of four Ani s allergens between *A. simplex sensu stricto* and *A. pegreffii* isolates revealed well-conserved amino acid sequences of IgE-binding epitope in Ani s 1 and a binding motif of human leukocyte antigen in Ani s 2 and Ani s 9 (21). Because of the similarity of amino acid sequences between *A. pegreffii* and *A. simplex sensu stricto* from *S. japonicus*, the allergens of these two *Anisakis* species could have similar potential for causing an allergic reaction. Thus, in future clinical or experimental studies the Ani s allergen profile and host fish species of *Anisakis* larvae should be taken into account.

Since Kasuya et al. (12) reported in 1990 that a food allergy induced by mackerel was not associated with the mackerel itself but with *A. simplex* L3 larval contamination in the mackerel, many allergens associated with *A. simplex* larvae have been studied in marine fishes (4–6, 12, 14, 19, 20, 24). Because it has been generally believed that cooking or freezing would reduce the risk of *Anisakis* infection and allergic reaction, the general guideline for *A. simplex*-allergic patients has been to avoid eating raw or uncooked fish that could be contaminated with *A. simplex* (3). However, recent uncommon cases of allergic reaction have been reported after consumption of chicken meat by some *A.*

*simplex*-sensitive patients who were restricted from eating fish or seafood. When chicken feed is formulated with a high proportion of fishmeal to boost nutrient values, a large amount (60 to 70%) of the protein in the fishmeal can survive the heating process intact, and heat-resistant Ani s 1 has been found in meat from chickens fed with fishmeal (3). Because heat-stable *A. simplex* allergens are not limited to whole fish but can also be found in processed fish, further study is needed to analyze these allergens in meat from animals fed fishmeal.

In conclusion, *A. pegreffii* was the most prevalent *Anisakis* species in the 49 samples of *S. japonicus*, 45 samples of *T. lepturus*, and 42 samples of *C. myriaster*. *A. typica* was identified in only four samples of *C. myriaster*. Multiplex RT-PCR analysis for Ani s allergens revealed that most of the *A. pegreffii* samples were positive for multiple allergens, and 41.9% of the *A. pegreffii* samples contained all five allergens tested. Among the heat-stable allergens, the Ani s 1, Ani s 4, and Ani s 5 were found in 41.2, 86.7, and 93.4% of samples, respectively. These results clearly indicate the high prevalence and multiple profiles of Ani s allergens in *A. pegreffii* and *A. typica* larvae from marine fishes consumed in Korea.

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