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Running head
The infection states of *Anisakis* in Sekisaba.
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Summary

Anisakidosis is developed by ingesting *Anisakis* in marine fish including the chub mackerel, *Scomber japonicus* without proper pre-treatment such as cooking or freezing. Two sibling species of *Anisakis* are found in *S. japonicus* from Japanese waters and the prevalence and species of *Anisakis* in the fish depend on sea area. For example, *Anisakis simplex* sensu stricto is found in the Pacific stock of *S. japonicus*, while *Anisakis pegreffii* is found in the Tsushima Warm Current stock. *S. japonicus* caught in the Bungo Channel, off the coast of Saganoseki in Oita Prefecture, which is branded as Sekisaba, inhabits a very limited area; however, the infection states of *Anisakis* found in Sekisaba remain unclear. In this study, we compared the infection states of *Anisakis* in Sekisaba with those in *S. japonicus* caught in South Oita area and Nagasaki Prefecture. All of *Anisakis* from Nagasaki Prefecture were *A. pegreffii*, while most of them found in Sekisaba and fish from South Oita area were *A. simplex s.s.*. Interestingly, the prevalence of *Anisakis* in Sekisaba was significantly lower than those of two other areas. This may be reflected that Sekisaba might belong to a distinct stock of *S. japonicus* varying from other stocks.
Introduction

Anisakidosis is a fish-borne parasitic zoonosis caused by the accidental ingestion of larval nematodes including *Anisakis* and *Pseudoterranova* found in raw fish such as chub mackerel (*Scomber japonicus*) (1, 2). *Anisakis* Type I larvae penetrate the gastric mucosa resulting in acute epigastric pain, occasionally accompanied by nausea and vomiting in patients (3). At present, the most effective treatment is an endoscopic removal of the nematode, though a recent report has shown that an over-the-counter medicine containing wood creosote (Seirogan) ameliorates the symptoms (4).

*Anisakis simplex* sensu lato (s.l.) is highly associated with amisakidosis and presently comprises three sibling species, *Anisakis simplex* sensu stricto (s.s.), *Anisakis pegreffii* and *Anisakis berlandi*. It has become possible to identify the species of *A. simplex* s.l. using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) (5). PCR-RFLP analysis of the ribosomal DNA internal transcribed spacer (rDNA ITS) provides a useful approach for the specific identification of both distantly and closely related ascaridoid species including *Anisakis* Type I larvae since these spacers show high levels of interspecific sequence differences with low-level intraspecific variation (5-7).

*Scomber japonicus* is one of the most common fish in Japanese coastal waters and forms two stocks, the Tsushima Warm Current (TWC) stock (found in the East China Sea and the Sea of Japan) and the Pacific stock (found off the Pacific coast of Japan). *S. japonicus* from Nagasaki Prefecture on the Sea of Japan coast and the East China Sea coast belong to the TWC stock, while those from the southern area of Oita Prefecture on...
the Pacific Ocean coast (South Oita area) belong to the Pacific stock (8). As previously reported, the species of *Anisakis* Type I larvae found in *S. japonicus* differed between these two stocks. Umehara *et al.* revealed that *A. simplex* s.s. was found in the Pacific stock of *S. japonicus*, while *A. pegreffii* was predominantly found in the TWC stock (9).

*Scomber japonicus* caught in the Bungo Channel, off the coast of Saganoseki in Oita Prefecture is called Sekisaba, a brand of chub mackerel which is frequently eaten raw in Japan. Sekisaba inhabits the Bungo Channel which is a geomorphologically unique strait separating the Kyushu and Shikoku islands and connecting the western part of the Seto Inland Sea to the Pacific Ocean (Fig. 1A). Thus far, the prevalence (the proportion of fish infected), mean intensity (the average number of parasites per infected fish) and the species of *Anisakis* Type I larvae in Sekisaba have not been investigated.

In this study, we compared the prevalence of *Anisakis* Type I larvae found in *S. japonicus* captured from three different areas of Kyushu Island including Nagasaki Prefecture (representative of the TWC stock), South Oita area (representative of the Pacific stock) and Saganoseki (Sekisaba). We found that the prevalence of *Anisakis* Type I larvae in Sekisaba was significantly lower than that seen in *S. japonicus* from the other two areas. A possible explanation for the fact of a low prevalence is that Sekisaba may form a unique stock which is distinguishable from the other stocks of *S. japonicus* in Japanese waters.
Materials and Methods

Fish samples and method for detecting anisakid nematodes

*Scomber japonicus* were obtained in batches from fish markets in Oita, Japan from October 2014 and April 2016. We purchased 40 fish (in a pool of n=4-13 whole-bodies of fish) caught in coastal waters off South Oita area and Nagasaki Prefecture. For *S. japonicus* from Saganoseki (Sekisaba), 10 whole bodies of fish and the viscera from 64 fish were provided by the Oita fishermen's cooperative association (Saganoseki branch) (Fig. 1A). Each sample of fish was weighed and measured. *S. japonicus* from Nagasaki Prefecture (n = 40, mean fork length: 36.7 cm, range: 33- 41 cm, mean body weight 582.6g range: 416-835g), South Oita area (n = 40, mean fork length: 37.7 cm, range: 34- 42 cm, mean body weight 582.1g range: 435-775g) and Saganoseki (n = 10, mean fork length: 38.9 cm, range: 32- 46 cm, mean body weight 604.8g range: 528-693g) were used for examination. The fish samples were examined within 24 hours of purchase. After separating the viscera and muscles from the fish body, anisakid larvae were detected by visual inspection, removed using forceps and the morphology of the isolated larvae was observed by using a stereomicroscope. Particularly, anisakid larvae in the muscle were detected by gently pressing the muscle between two glass plates. The Anisakis type I larvae were used for further investigation.

Epidemiological data of *Anisakis* Type I larvae in *S. japonicus*

Epidemiological data including the prevalence and mean intensity of Anisakis Type I larvae in *S. japonicus* were analyzed by method described by Bush et al. (1997) (10).
The prevalence was defined as the proportion of fish hosts infected with *Anisakis* species. The mean intensity was determined as the average number of parasites found in the infected hosts. Differences in the prevalence and mean intensity values of *Anisakis* Type I larvae infection between the *S. japonicus* from three areas were assessed respectively with the Fisher’s exact test using the GraphPad Prism software ver.8.42 (GraphPad Software Inc. San Diego, CA) and the bootstrap t-test using the QPweb software (11).

**PCR-RFLP**

Genomic DNA from *Anisakis* Type I larvae was prepared by alkaline extraction method. The larva was boiled in 180 µl of 50 mM NaOH for 60 min and then 20 µl of 1 M Tris-HCl (pH 8.0) was added. PCR-RFLP analysis was performed to identify the species of *A. simplex* as previously described (5). Briefly, PCR amplification of rDNA ITS region (ITS-1 and ITS-2 plus their spanning regions) was performed in a 25 µl reaction volume containing 10 ng template DNA, 0.25 units of KOD FX neo (TOYOBO, Osaka, Japan), 12.5 µl of 2 x buffer (provided with the polymerase by the manufacturer), 5 µl of dNTPs (2 mM), 0.75 µl of forward primer (5’-GTAGGTGAACCTGCGGAAGGATCATT-3’, 10 µM), 0.75 µl of reverse primer (5’-TTAGTTTCTTTTCCTCCGC-3’, 10 µM). The PCR condition was as follows: 95 ºC for 2 min for 1 cycle; 95 ºC for 15 sec, 60 ºC for 30 sec and 68 ºC for 1min for 30 cycles and a final extension of 5 min at 68 ºC. The PCR products were then digested by restriction endonuclease, Hha I (Takara, Shiga, Japan) or Hinf I (Takara) according to
manufacturer’s recommendations for RFLP analysis of rDNA ITS. The digested products were resolved in 2% agarose gel electrophoresis, stained with ethidium bromide, visualized by illumination with short wave UV light and photographed.
Results

In this study, all anisakid nematodes were detected from the viscera, and no nematodes were found in muscle tissues. *Anisakis* Type I larvae were counted among the detected anisakid nematodes. The prevalence of *Anisakis* Type I larvae in *S. japonicus* from Nagasaki Prefecture was 75% (30/40) (Fig. 1B). Conversely, the prevalence of *Anisakis* Type I larvae in *S. japonicus* from South Oita area was 38% (15/40). Surprisingly, the prevalence of *Anisakis* Type I larvae in Sekisaba was only 6.8% (5/74). This difference in prevalence was statistically significant between the groups; Nagasaki versus South Oita area: \( P<0.005 \), South Oita area versus Saganoseki: \( P<0.0001 \) and Nagasaki versus Saganoseki: \( P<0.0001 \).

Next, we compared the average numbers of *Anisakis* Type I larvae found in an infected fish from the three groups. Although Sekisaba showed a relatively low intensity, there was no significant difference in mean intensity between the groups (Nagasaki Prefecture: 16.2±5.97 (n=30), South Oita area: 5.9±1.54 (n=15), Saganoseki: 2.8±1.61 (n=5) [larvae per fish]) (Fig. 1C).

To identify the species of *Anisakis* Type I larvae found in *S. japonicus* from the three areas, we performed PCR-RFLP analysis. The amplification of the rDNA ITS resulted in approximately 1,000 bp of fragment (Fig. 2A) which was followed by digestion with either Hha I (Fig. 2B) or Hinf I (Fig. 2C) to enable identification between the seven closely related *Anisakis* species.

Hha I digestion of a PCR product amplified from the ITS region of rDNA of *A. simplex s.s.* resulted in two fragments of 530 and 420 bp (Fig. 2B left) and Hinf I
digestion yielded 620, 250 and 80 bp fragments, though the 80 bp band was too faint to be seen in the photograph (Fig. 2C left). Hha I digestion of *A. pegreffii* showed 530 and 420 bp fragments (Fig. 2B right) and 370, 300 and 250 bp fragments by Hinf I digestion (Fig. 2C right). As shown in Fig. 2D, all of the *Anisakis* Type I larvae collected from Nagasaki Prefecture were identified as *A. pegreffii*, while 93% of the larvae collected from the South Oita area were *A. simplex* s.s.. Identification of *Anisakis* Type I larvae in Sekisaba resulted in a similar trend with that of South Oita area. Namely, 13 larvae out of 14 analyzed (93%) were *A. simplex* s.s. and the remaining one was *A. pegreffii*. Recently Umehara *et al.* reported that hybrid genotypes possibly generated by natural interspecific hybridization between *A. simplex* s.s. and *A. pegreffii* have been identified in *S. japonicus* (6). However, no hybrid genotype of larvae was identified in this study.
Discussion

According to the annual report of the National Institute of Infectious Diseases in Japan, it is estimated that more than 7,000 cases of anisakidosis occur annually in Japan due to the Japanese food habit of eating fish raw such as sushi and sashimi (12). Particularly, chub mackerel is frequently eaten raw in Kyushu Island, further adding to the importance of investigating the prevalence of Anisakis Type I larvae in S. japonicus. In Kyushu, there is easy access to fish from both the Pacific and TWC stocks of S. japonicus. In this study, we sought to investigate the prevalence and species of Anisakis Type I larvae in Sekisaba, a brand of chub mackerel from Oita Prefecture in Kyushu.

First of all, Anisakis Type I larvae that found in this study were most prevalently detected in the viscera of the S. japonicus we inspected; however, they were never found in the fish muscle in this surveillance study. In this study, we examined Anisakis Type I larvae by means of visual inspection of the viscera under a stereomicroscope and of the muscle between two glass plates, respectively. However, the inspection method had a limitation to detect anisakid larvae, due to a low sensitivity. To more accurately detect Anisakis Type I larvae in S. japonicus, it is necessary to employ more sensitive methods, such as the artificial peptic digestion method and the UV-press method. To achieve more accurate results, we need to use the above methods in future study. Next, we found that most of the Anisakis Type I larvae found in Sekisaba was A. simplex s.s., while all of the Anisakis Type I larvae collected from Nagasaki Prefecture were A. pegreffii suggesting that Sekisaba does not belong to the TWC stock of S. japonicus. This was also supported by the finding that no hybrid genotypes were detected in
Sekisaba. Consistent with a previous report, the prevalence of *Anisakis* Type I larvae in *S. japonicus* from South Oita area was lower than that from Nagasaki Prefecture (8). Notably, the prevalence of *Anisakis* Type I larvae in Sekisaba (6.8%) was even lower than that of South Oita area (38%), which may be due to the different habitats of the two populations of *S. japonicus*. Indeed, the main feeding grounds of chub mackerel of the Pacific stock are thought to be located in the waters off of northeastern Japan, and their spawning grounds are in the coastal waters around the Izu Islands and off of southwestern Japan (13). However, Sekisaba is thought to use the waters around the Bungo Channel for both feeding and spawning (14). In the Bungo Channel there are two depressions in the seafloor of the strait and the tides induce strong upwelling followed by the blooming of phytoplankton with a high concentration of nutrients (15). Thus, the geomorphological factors of the Bungo Channel provide a unique marine ecological environment that may in turn create a unique local chub mackerel population; and this may partially explain the low prevalence of *Anisakis* Type I larvae in fish from that area.

The habitat of the intermediate hosts as well as the final hosts is a critical factor influencing the prevalence of *Anisakis* Type I larvae in the paratenic hosts. The most important first intermediate hosts in the life cycle of *Anisakis* Type I larvae species are Euphausiids (krill) (16) that transmit the infective larvae along the food chain into paratenic hosts such as *S. japonicus*. The prevalence of infection in Euphausiids is generally low (17), but it is uncertain whether the prevalence of *Anisakis* Type I larvae in the first intermediate hosts is relatively low in the Bungo Channel. On the other hand,
the adult stages of *Anisakis* Type I larvae reside in the stomach of marine mammals such as dolphins and whales (16). It is known that dolphins (*Delphinus capensis*, *Tursiops truncatus*), finless porpoises (*Neophocaena phocaenoides*), and whales (*Megaptera novaeangliae*) inhabit the Bungo Channel (18), however, the prevalence of *Anisakis* Type I larvae in the final hosts in that area is still unknown. Thus, the distribution and abundance of the intermediate and final hosts infected with *Anisakis* Type I larvae in the Bungo Channel should be investigated. To fully elucidate this complex relationship, it is necessary to integrate the knowledge of various fields including parasitology, gastroenterology, ecology, oceanography and fisheries science.

“In conclusion, we revealed that the prevalence of *Anisakis* Type I larvae in Sekisaba was significantly lower than that seen in *S. japonicus* from the other two areas. Our findings provide a possibility that Sekisaba forms a unique stock that is distinguishable from the other stocks of *S. japonicus* in Japanese waters.”.
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Conflict of interest

None to declare.
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**Figure legend**

Fig. 1. (A) A map showing the collection sites of *S. japonicus* in Japanese waters around Kyushu Island. (B) Bar graph shows prevalence (%) of *Anisakis* Type I larvae in each group of *S. japonicus*. Statistical significance of differences between experimental groups of *S. japonicus* was determined using the Fisher's exact test. (C) Mean Intensity of *Anisakis* Type I larvae in *S. japonicus* from the three different areas in Kyushu, Japan. Data are presented as mean ±SE. Statistical significance of differences between groups was determined using the bootstrap t-test. *: *P* < 0.005. **: *P* < 0.0001. NS: not significant.

Fig. 2. PCR-RFLP analysis for the identification of *Anisakis* Type I larvae species in *S. japonicus*. (A) The ITS region of rDNA of either *A. simplex* s.s. or *A. pegreffii* was amplified by PCR and the products were separated by agarose gel electrophoresis. M indicates DNA size maker (GeneRuler DNA Ladder, Thermo Fisher Scientific). (B) PCR products in A were digested with Hha I and separated by agarose gel electrophoresis. (C) PCR products in A were digested with Hinf I and separated by agarose gel electrophoresis. (D) Percentage of genotypes of *Anisakis* Type I larvae species determined by PCR-RFLP analysis.
