Identification of Larval Anisakis spp. (Nematoda: Anisakidae) in Alaska Pollock (Theragra chalcogramma) in Northern Japan Using Morphological and Molecular Markers

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ABSTRACT: The Alaska pollock, Theragra chalcogramma (Pallas), is an important raw source for surimi and other food products in Japan. However, Alaska pollock caught in the Atlantic and Mediterranean regions has been reported to harbor Anisakis species that pose considerable food safety problems. Here, we identified the third-stage (L3) Anisakis spp. sample from Alaska pollock caught in northern Japan using a combination of morphological and molecular analyses which included PCR-RFLP and sequencing of the ITS (ITS1-5.8S rDNA-ITS2) region and mtDNA cox2 gene markers. Four Anisakis spp. were confirmed, namely Anisakis simplex (sensu stricto [s.s.]), A. pegreffii, A. brevispiculata, and Anisakis sp. (Anisakis Type II) belonging to the Anisakis Type II group. The identification of different Anisakis spp. occurring in Alaska Pollock, and the identification of A. brevispiculata and Anisakis sp. (Anisakis Type II) in the northwest Pacific region are, first reports. Anisakis simplex (s.s.) composed the majority of Anisakis spp. in Alaska pollock at 91.0%, followed by A. pegreffii (5.2%), Anisakis sp. (Anisakis Type II) (2.4%), and A. brevispiculata (1.4%).

Third-stage (L3) larvae of Anisakis Dujardin, 1845 species infesting marine fishes have been initially categorized morphologically into 2 type groupings, namely Anisakis Type I and Anisakis Type II, with the former having a longer ventriculus and a mucron at the posterior tip while the latter having a shorter ventriculus and a non-existent mucron (Berland, 1961; Koyama et al., 1969). Recent results, using different morphological and molecular combinations, revealed that Anisakis Type I group is actually composed of A. simplex complex, A. simplex (sensu stricto [s.s.]), A. pegreffii, and A. simplex C. The Anisakis Type II group is composed of A. peggaeae, A. brevispiculata, and A. physyeterus (Mattucci et al., 1998, 2002, 2005; Paggi et al., 1998; Mattiucci and Nasceetti, 2006; Valentinii et al., 2006). Morphologically, members of both larval types (I and II) have been reported from marine fishes in the northwest Pacific region (Koyama et al., 1969; Moravec et al., 1985; Moravec and Nagasawa, 1989). Similarly, to date, molecular analyses using the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2) have shown the presence only of A. simplex (s.s.) and A. pegreffii (Anisakis Type I), and A. physyeterus (Anisakis Type II), in the Pacific region (Mattucci et al., 1998; Abe et al., 2005; Mattiucci and Nasceetti, 2006; Umehara et al., 2006, 2008; Yoshinaga et al., 2006; Quiazon et al., 2008). Alaska pollock, Theragra chalcogramma (Pallas), caught in the northwestern Pacific and particularly in northern Japan, is a major raw material in the country for the production of surimi or fish paste, from which various food products such as imitation crab meat, fish balls, and fish cakes are made. Anisakis simplex, Anisakis sp., and Anisakis Type I larvae have been reported, not only in the body cavity but also in the body muscle of pollock, thus posing a considerable food safety problem (Koyama et al., 1969; Arthur et al., 1982). For example, potential allergens were found in A. simplex (which were not further identified to sibling species level) and in A. pegreffii that cause allergic reactions in humans when consumed together with fish as fresh, frozen, cooked, or processed food (Armentia et al., 1998; del Pozo et al., 1999; Daschner et al., 2000; Alonso-Gómez et al., 2004; Nieuwenhuizen et al., 2006; Kobayashi et al., 2008). Thus, from the perspective of food safety, it is important to be precise in identifying the different Anisakis spp. found in Alaska pollock that are caught in the northwestern Pacific Ocean. In the present study, we collected Anisakis L3 larvae from Alaska pollock caught in northern Japan and identified them using morphological and molecular markers.

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region were determined and analyzed. The following restriction pattern representative groups were sequenced: pattern 1 (A. simplex [s.s.] group) = 4 samples; pattern 2 (A. pegreffii group) = 4 samples; pattern 3 = 3 samples; and pattern 4 = 4 samples. Identical sequences under each grouping were found, such that only 1 sequence per group was used for subsequent analysis. The MP tree derived from the nucleotide sequences of the ITS region from different individuals confirmed the taxonomic identity of the specimens, with 4 different patterns (Fig. 3). Specimens with pattern 1, as represented by EU624342, clustered with A. simplex (s.s.) while specimens with pattern 2, as represented by EU624343, grouped with A. pegreffii. Specimens with pattern 3, as represented by EU624344, were exhibited to be A. brevispiculata because it grouped (at 99% bootstrap value) with the deposited ITS sequence of A. brevispiculata (AY826719) in the GenBank database. Specimens with pattern 4, as represented by EU624345, grouped with the A. physeteris (AB201789) sequence. However, strangely, EU624345 and AB201789 did not group with another clade consisting of 5 reported A. physeteris sequences (AY826721, AB277821, EU327691, AY603530, and EU327690), which made the identity of the worm numbered GenBank AB201789 suspect. Alignment and pairwise similarity further supported the MP tree, particularly on the specimens with patterns 3 and 4 (Fig. 4). Specimens with pattern 3 (EU624344) were highly similar to the reported A. brevispiculata (AY826719) at 99.5%, but not to specimens with pattern 4 (EU624345) (at 92.9% similarity) or to the 5 A. physeteris sequences (94.3–95.4%). Specimens with pattern 4 (EU624345) were identical (at 99.9% similarity) to the suspected A. physeteris (AB201789), but exhibited lower similarity (at 90.9–91.9% similarity) to 5 other A. physeteris sequences. Because of this, we putatively identified EU624345 as an Anisakis sp. belonging to the Anisakis Type II group (as will be further described later in the text).

The mtDNA cox2 gene was also analyzed; these results supported those obtained in the PCR-RFLP analysis and in sequencing the ITS region. The same restriction pattern representative groups used in sequencing the ITS region were also used for the mtDNA cox2 gene sequencing: pattern 1 (A. simplex [s.s.] group) = 4 samples; pattern 2 (A. pegreffii group) = 4 samples; pattern 3 = 3 samples; and pattern 4 = 4 samples. The sequences under each grouping were identical, such that only 1 sequence per group was used for subsequent analysis. There were 2 slightly different sequences (EU560907 and EU560911) derived from specimens with pattern 1 (A. simplex [s.s.] group from Iwate Prefecture). Because there are only 11 different base pairs (at 97.6% similarity), compared to only 94.9–95.7% similarities with specimens having pattern 2 (A. pegreffii group), these 2 slightly different sequences were treated as similar species.

The MP tree derived from nucleotide sequences of the mtDNA cox2 gene from different individuals further supported the taxonomic results of the 4 restriction pattern groups, as revealed in the ITS marker (Fig. 5). Specimens with pattern 1, as represented by EU560907 and EU560911, clustered with A. simplex (s.s.) while specimens with pattern 2, as represented by EU560907, grouped with A. pegreffii. Specimens with pattern 3, as represented by EU560907 and EU560911, were determined to be A. brevispiculata, as it grouped (at 99% bootstrap value) with the previously reported A. brevispiculata (DQ116433) by Valentini et al. (2006). Specimens with pattern 4, as represented by EU560910, clustered significantly with published A. paggiae (DQ116434) at a 99% bootstrap value. Alignment and pairwise similarity of mtDNA cox2 gene sequences further supported the MP tree, particularly the specimens with patterns 3 and 4 (Fig. 6). The putative Anisakis sp. specimens (EU560910) with pattern 4 were confirmed to be close to A. paggiae (at 96.3% similarity), as
it clustered to the reported *A. paggiae* (DQ116434) but not with the reported *A. brevispiculata* (at 89.1% similarity), or to *A. physeteris* (at 88.8% similarity). However, due to a 96.3% similarity value, we cannot confirm if these are really *A. paggiae*. Therefore, in the present study, these specimens were referred to as an *Anisakis* sp. belonging to the *Anisakis* Type II group. In contrast, the putative *A. brevispiculata* (EU560909) was 98.8% identical to the reported *A. brevispiculata* (DQ116433), but with only 89.2% similarity to *A. paggiae* and 90.7% similarity to *A. physeteris*, thereby confirming its identity as *A. brevispiculata*.

The combining all of our results, e.g., morphology, PCR-RFLP, ITS region, and the mtDNA *cox2* gene, conclusively supported the identification of 3 *Anisakis* spp. taken from Alaska pollock caught from northern Japan, namely *A. simplex* (s.s.), *A. pegreffii*, and *A. brevispiculata*, while the remaining specimens can only be identified as an *Anisakis* sp. belonging to the *Anisakis* Type II group.

The ITS sequence (AB201789), reported to have been taken from *A. physeteris*, should be tentatively identified as an *Anisakis* sp. belonging to the *Anisakis* Type II group, based on its high homology and close phylogenetic relationship with those specimens with pattern 4 that were established in this study.

The identification of *A. brevispiculata* and *Anisakis* sp. belonging to *Anisakis* Type II group in Alaska pollock caught from northern Japan, and in general from the northwest Pacific region, represents novel information on the geographical distribution of the species, particularly of *A. brevispiculata*, which has so far only been reported in the Atlantic (Valentini et al., 2006). Alaska pollock from off Miyako in Iwate Prefecture were found to be infected with 4 different *Anisakis* species, 2 of which, i.e., *A. simplex* (s.s.) and *A. pegreffii*, could be identified morphologically based on the difference on their ventriculus lengths. Mixed infections of L3 larvae have also been reported from different host fishes and marine mammals in the Atlantic Ocean and the Mediterranean Sea (Paggi et al., 1998; Mattiucci et al., 2002).

Because of the confirmation of the 4 *Anisakis* species, we were able to clearly describe the species composition harbored by Alaska pollock from northern Japan, i.e., *A. simplex* (s.s.) (91.0%), *A. pegreffii* (5.2%), *A. brevispiculata* (1.4%), and an *Anisakis* sp. belonging to the *Anisakis* Type II group (2.4%) (Fig. 1). Moreover, both *A. simplex* (s.s.)
and *A. pegreffii* were found to be in both sampling sites, i.e., Hokkaido and Iwate Prefectures, whereas *A. brevispiculata* and *Anisakis* sp. (*Anisakis* Type II) were only observed in Iwate Prefecture. This apparent location specificity is not understood at this point and warrants further investigation.

In conclusion, the confirmation of the presence of the 4 *Anisakis* species in Alaska pollock is a first report. Precise identification of *Anisakis* species, using morphological and molecular markers, has important implications to the safety of the Alaska pollock-based surimi and other fish food production in Japan. In light of this discovery, stricter measures are now needed in ensuring the use of *Anisakis* spp.-free Alaska pollock. Since the 2 major *Anisakis* species present in Alaska pollock in Japan, i.e., *A. simplex* (s.s.) and *A. pegreffii*, are commonly reported to cause allergic reactions to sensitive consumers, rapid on-site identification prior to fish processing is possible and can be accomplished through morphological examination, which can be further confirmed using different molecular markers. The molecular techniques employed in the present study are applicable for efforts such as monitoring and quality control for safety in human consumption.

![Multiple alignment of the ITS region of the presently and previously studied *Anisakis* spp.](http://www.ebi.ac.uk/Tools/clustalw2/) was used. Similarity was computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

**Figure 4.** Multiple alignment of the ITS region of the presently and previously studied *Anisakis* spp. ClustalW alignment employed at (http://www.ebi.ac.uk/Tools/clustalw2/) was used. Similarity was computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).
**FIGURE 5.** Phylogenetic analyses of the mtDNA cox2 region of the presently and previously studied *Anisakis* spp. Maximum parsimony (MP) trees were generated using MEGA version 4 (Tamura et al., 2007), drawn using the close neighbor interchange (CNI) routine, with 10 random trees added at 1,000 bootstrap values with complete deletion.

**FIGURE 6.** Multiple alignment of the mtDNA cox2 region of the presently and previously studied *Anisakis* spp. ClustalW alignment employed at (http://www.ebi.ac.uk/Tools/clustalw2/) was used. Similarity was computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

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