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

K. M. A. Quiazon, T. Yoshinaga*†, M. D. Santos‡, and K. Ogawa*, Freshwater Aquaculture Center and College of Fisheries, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines; *Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan; ‡Marine Fisheries Research and Development Division, National Fisheries Research and Development Institute, 940 Kayumanggi Bldg., Quezon Ave., Quezon City 1103, Philippines.; ††To whom correspondence should be addressed. *e-mail: atyoshi@mail.ecc.u-tokyo.ac.jp*

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. sampled 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 *mt*DNA *cox*2 gene markers. Four *Anisakis* spp. were confirmed, namely *Anisakis simplex* (sensu stricto [s.s.]), *A. pegreffii*, *A. brevispiculata*, and an *Anisakis* sp. belonging to the *Anisakis* Type II group. The identification of 4 different *Anisakis* spp. occurring in Alaska Pollock, and the identification of *A. brevispiculata* and an *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 infecting 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. ziphidarum*, *A. typica*, and 3 sibling species of the *A. simplex* complex, i.e., *A. simplex* (sensu stricto [s.s.]), *A. pegreffii*, and *A. simplex* C. The *Anisakis* Type II group is composed of *A. paggiae*, *A. brevispiculata*, and *A. physeteris* (Mattiucci et al., 1998, 2002, 2005; Paggi et al., 1998; Mattiucci and Nascetti, 2006; Valentini 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) has shown the presence only of *A. simplex* (s.s.) and *A. pegreffii* (*Anisakis* Type I), and *A. physeteris* (*Anisakis* Type II), in the Pacific region (Mattiucci et al., 1998; Abe et al., 2005; Mattiucci and Nascetti, 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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Fifty Alaska pollock caught from 2 sites off northern Japan (Fig. 1) were examined for *Anisakis* spp. infection. A total of 210 L3 larvae was collected from the body cavity of all fish samples, removed from their sheaths, cleaned in phosphate-buffered saline, and fixed in 70% ethanol. After measurement of total body length, the worms were cut into 3 pieces, i.e., anterior, middle, and posterior. The anterior and posterior parts were cleared in glycerin and morphologically identified using light microscopy following previous reports (Berland, 1961; Koyama et al., 1969; Quiazon et al., 2008). The middle portion was stored in 100% ethanol for the extraction of the genomic DNA used for subsequent molecular characterization. Initial species identification was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS region (ITS1-5.8S rDNA-ITS2) using the digestion enzymes *Taq*I, *Hinf*I, and *Hha*I. Species were identified with the aid of the RFLP keys previously reported (D'Amelio et al., 2000; Pontes et al., 2005). Species identities were then confirmed by sequencing the ITS region and *mt*DNA *cox*2 gene of RFLP-selected samples (Abe et al., 2005; Umehara et al., 2006; Valentini et al., 2006; Quiazon et al., 2008).

Reported ITS and *mt*DNA *cox*2 nucleotide sequences for all *Anisakis* species were collected from the GenBank database. The nucleotide sequences were aligned using ClustalW2 employed at EMBL-EBI server (http://www.ebi.ac.uk/Tools/clustalw2/), while pairwise percentage similarities were computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Maximum parsimony (MP) trees were constructed using the Kimura 2-Parameter (K2P), and bootstrap values were calculated (1,000 replicates) with the aid of the MEGA version 4 program (Tamura et al., 2007). The ITS and *mt*DNA *cox*2 sequences were deposited in GenBank under the accession numbers indicated (ITS: EU624342 to EU624345; *mt*DNA *cox*2: EU560907 to EU560911).

Based on non-overlapping ventriculus lengths of the 210 L3 larvae examined, 191 (91.0%) were initially and putatively identified as *A. simplex* (s.s.) (at 0.90–1.50 mm ventriculus length) and 11 (5.2%) as *A. pegreffii* (at 0.52–0.70 mm ventriculus length), which coincides with a recent report (Quiazon et al., 2008). However, there were difficulties in identifying the remaining 8 samples because of their overlapping ventriculus lengths (data not shown).

To confirm the initial morphological classification, as well as to determine the unknown 8 samples, all 210 L3 samples were subjected to PCR-RFLP analysis. Results revealed 4 groups of PCR-RFLP restriction patterns (patterns 1 and 2—*Anisakis* Type I group; patterns 3 and 4—*Anisakis* Type II group) (Fig. 2). Based on the *Anisakis* spp. RFLP keys previously reported, specimens with pattern 1 corresponded to *A. simplex* (s.s.), obtaining 2 major DNA fragment lengths after *Taq*I (430 and 400 base pairs), *Hinf*I (620 and 240 bp), and *Hha*I (550 and 440 bp) digestion, whereas specimens with pattern 2 corresponded to *A. pegreffii*, obtaining 3 major DNA fragment lengths after *Taq*I (390, 310, and 140 bp) and *Hinf*I (370, 320, and 260 bp), and 2 major DNA fragment lengths after *Hha*I (550 and 440 bp) digestion (D'Amelio et al., 2000; Pontes et al., 2005). These results confirmed the identifications based on morphological examination. In addition, specimens with pattern 3 produced 3 (280, 260, and 150 bp), 1 (900 bp), and 3 (400, 330, and 190 bp) major DNA fragment lengths after *Taq*I, *Hinf*I, and *Hha*I digestion, respectively, whereas specimens with pattern 4 yielded 3 (290, 270, and 135 bp), 1 (900 bp), and 2 (530 and 430 bp) major DNA fragment lengths after *Taq*I, *Hinf*I, and *Hha*I digestions, respectively. Restriction patterns 3 and 4 have not been previously observed, and their identities remain unknown at this point.

To further identify the taxonomy of the larvae, particularly that of specimens with patterns 3 and 4, the nucleotide sequences of the ITS

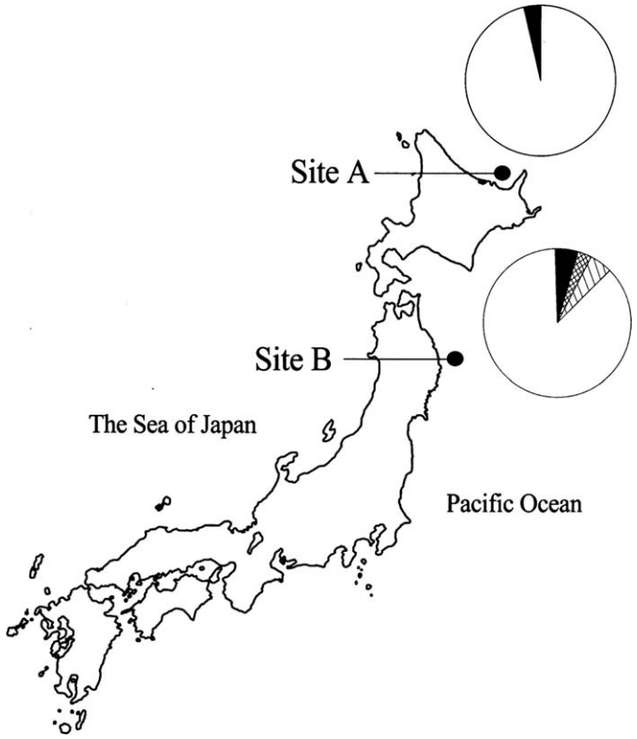

FIGURE 1. Geographic location and relative proportion of *Anisakis* spp. in the examined Alaska pollock (*Theragra chalcogramma*) in northern Japan. Site A: off Rausu, Hokkaido Prefecture; Site B: off Miyako, Iwate Prefecture. ○—*A. simplex* (s.s.); ●—*A. pegreffii*; ⊗—*A. paggiae*; ⦶—*A. brevispiculata.*

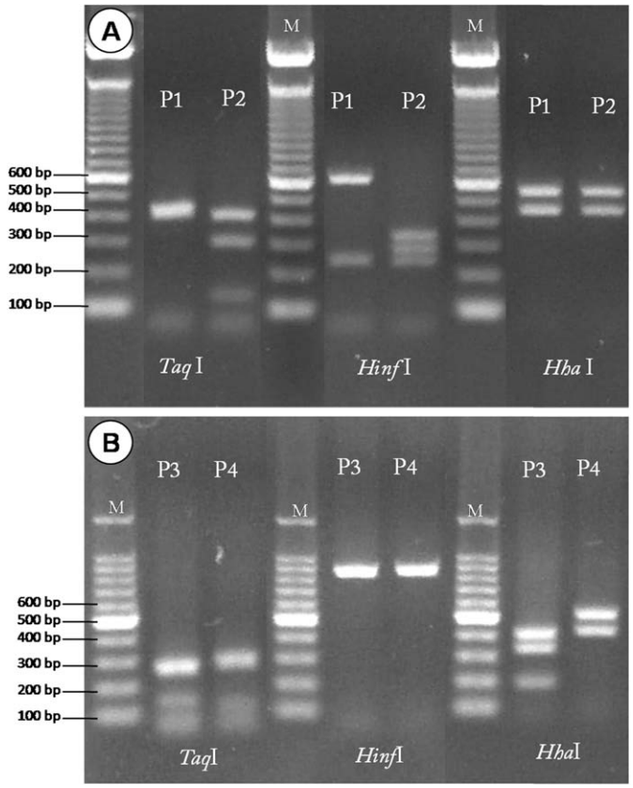

FIGURE 2. PCR-RFLP fragment patterns of 2 *Anisakis* spp. belonging to *Anisakis* Type I (**A**) and *Anisakis* Type II (**B**) groupings after digestion with restriction enzymes. Ap—*Anisakis pegreffii*; As—*Anisakis simplex* (s.s.); Ab—*Anisakis brevispiculata*; AII—*Anisakis* sp. belonging to *Anisakis* Type II groupings; M—100 bp marker.

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 *mt*DNA *cox*2 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 *mt*DNA *cox*2 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 *mt*DNA *cox*2 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 EU560908, grouped with *A. pegreffii*. Specimens with pattern 3, as represented by EU560909, 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 *mt*DNA *cox*2 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

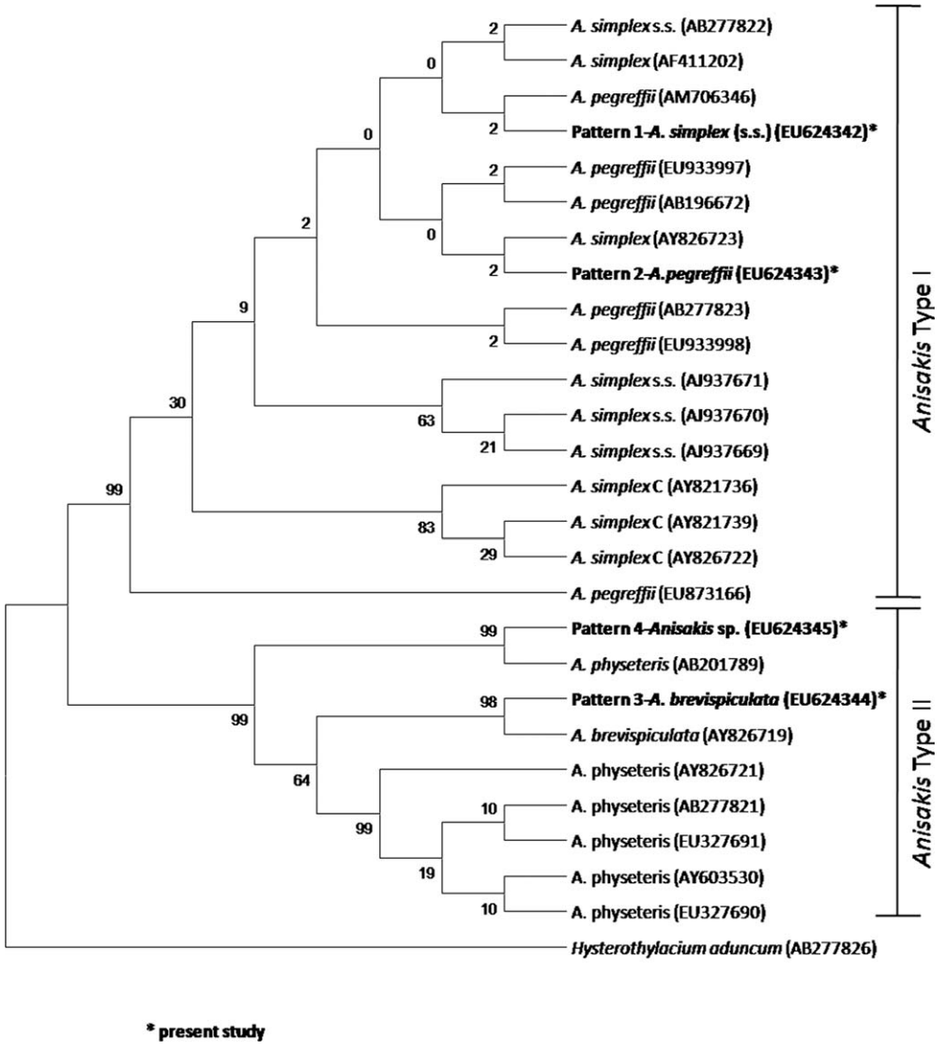

FIGURE 3. Phylogenetic analyses of the ITS region of the presently and previously studied *Anisakis* spp. Maximum parsimony (MP) trees were generated using MEGA version 4 (Tamura et al., 2007), drawn using the close neighbor interchange (CNI) routine, with 10 random trees added at 1,000 bootstrap values with complete deletion.

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 *mt*DNA *cox*2 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.)

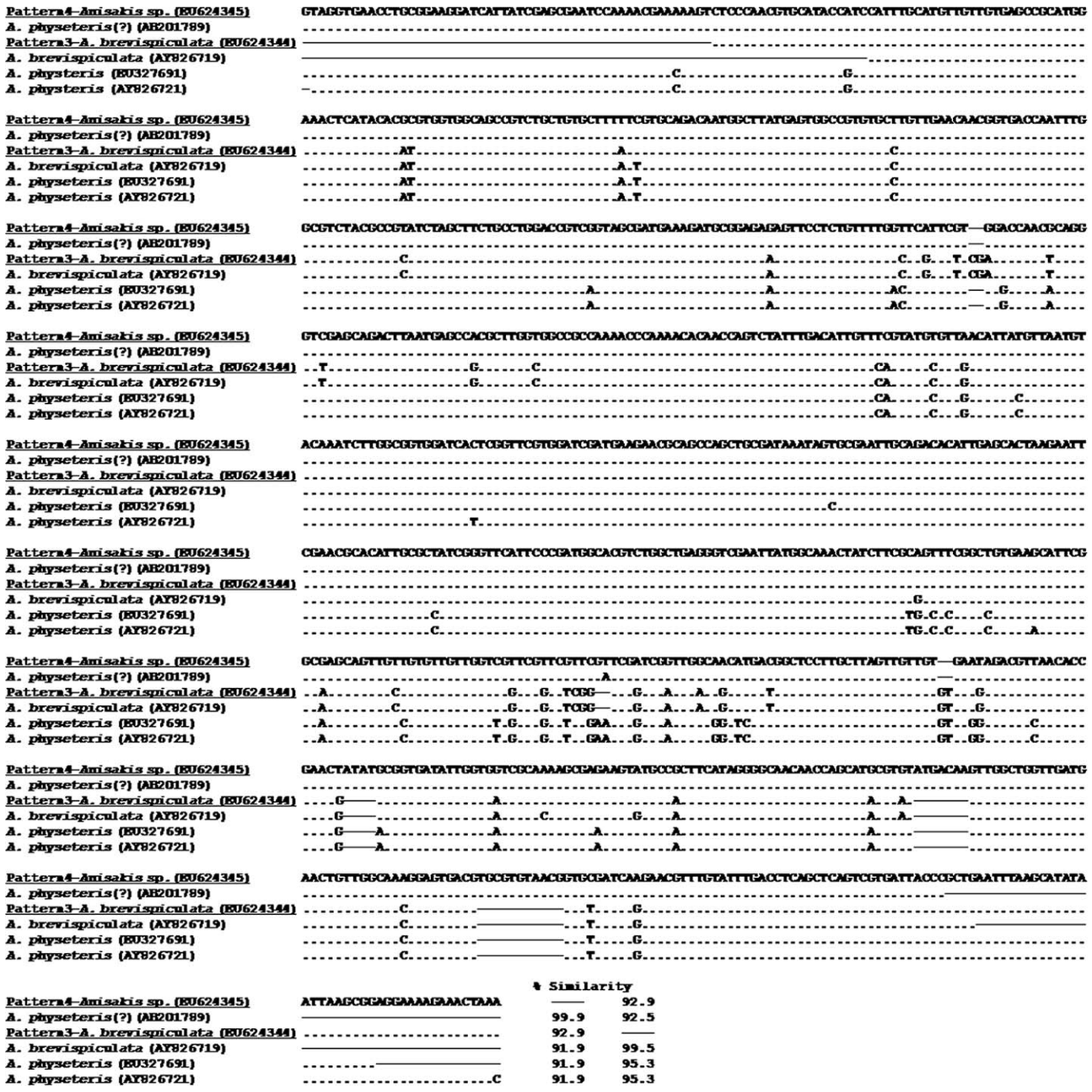

FIGURE 4. Multiple alignment of the ITS region of the presently and previously studied *Anisakis* spp. ClustalW alignment employed at (http://www.ebi.ac.uk/Tools/clustalw2/) was used. Similarity was computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

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.

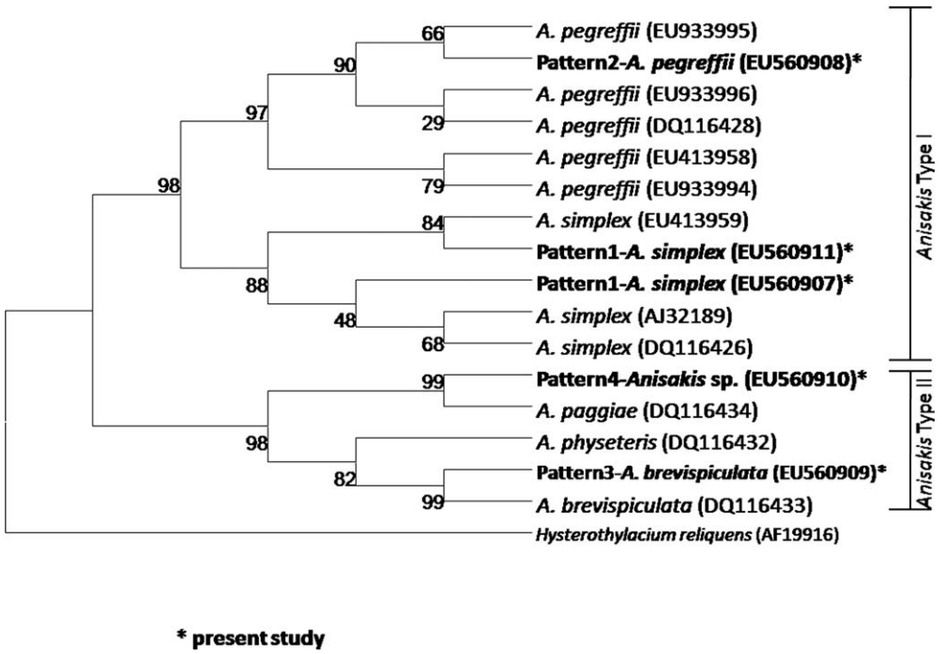

FIGURE 5. Phylogenetic analyses of the *mt*DNA *cox*2 region of the presently and previously studied *Anisakis* spp. Maximum parsimony (MP) trees were generated using MEGA version 4 (Tamura et al., 2007), drawn using the close neighbor interchange (CNI) routine, with 10 random trees added at 1,000 bootstrap values with complete deletion.

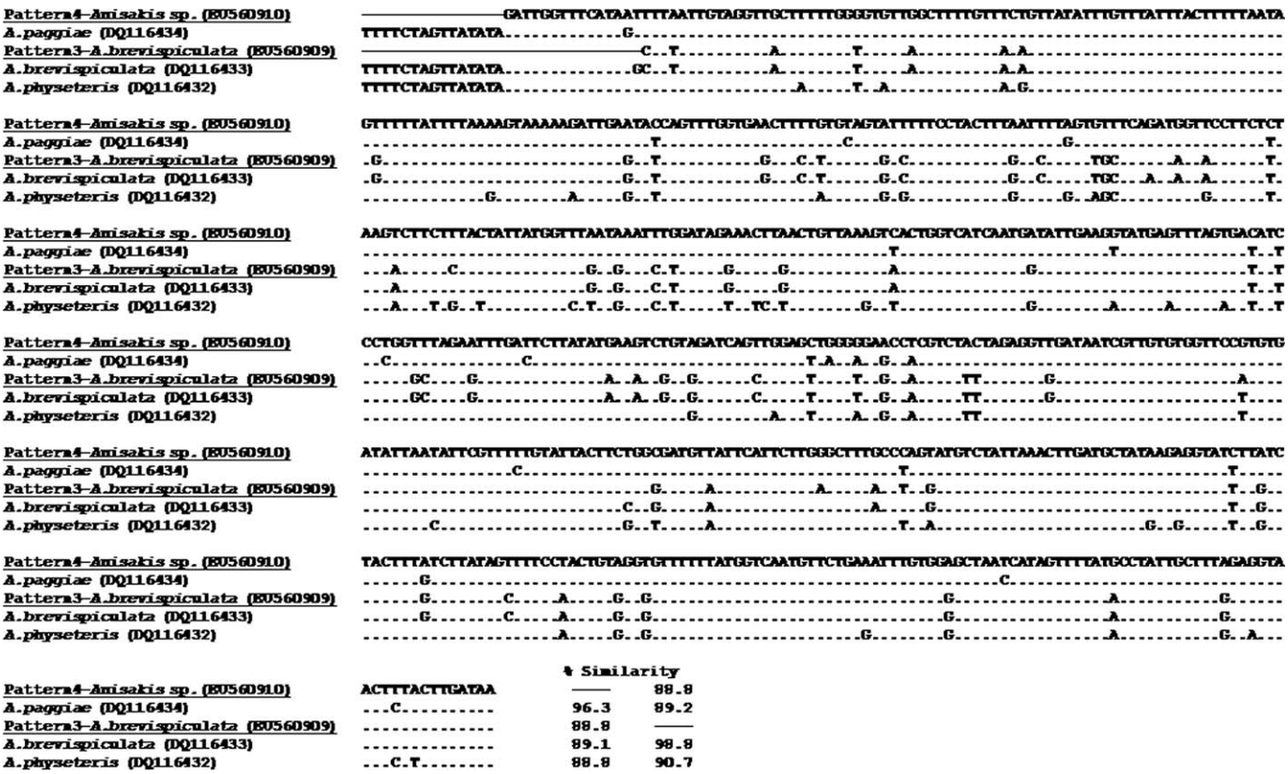

FIGURE 6. Multiple alignment of the *mt*DNA *cox*2 region of the presently and previously studied *Anisakis* spp. ClustalW alignment employed at (http://www.ebi.ac.uk/Tools/clustalw2/) was used. Similarity was computed using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

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