



Morphological and molecular characterization of *Anisakis* larvae (Nematoda: Anisakidae) in *Beryx splendens* from Japanese waters

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ARTICLE INFO

Article history:

Received 13 October 2010

Received in revised form 28 February 2011

Accepted 28 February 2011

Available online 3 March 2011

Keywords:

Anisakis physeteris

Anisakis brevispiculata

Anisakis paggiae

Morphological characteristics

Genetic analysis

Beryx splendens

ABSTRACT

The third-stage (L3) larvae of *Anisakis*, which are the etiological agents of human anisakiasis, have been categorized morphologically into *Anisakis* Type I larvae and *Anisakis* Type II larvae. Genetic analysis has allowed easy identification of these larvae: *Anisakis* Type I larvae include the species *Anisakis simplex* sensu stricto, *Anisakis pegreffii*, *Anisakis simplex* C, *Anisakis typica*, *Anisakis ziphidarum*, and *Anisakis nascettii*, whereas *Anisakis* Type II larvae include the species *Anisakis physeteris*, *Anisakis brevispiculata*, and *Anisakis paggiae*. Since human consumption of raw fish and squid is common in Japan, we investigated *Anisakis* L3 larvae in 44 specimens of *Beryx splendens* from Japanese waters. A total of 730 *Anisakis* L3 larvae collected from *B. splendens* were divided morphologically into 4 types: Type I, Type II, and 2 other types that were similar to *Anisakis* Type III and Type IV described by Shiraki (1974). *Anisakis* Type II, Type III, and Type IV larvae all had a short ventriculus, but their tails were morphologically different. In addition, data from genetic analysis indicated that *Anisakis* Type II, Type III, and Type IV larvae could be identified as *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, respectively. Therefore, *A. physeteris*, *A. brevispiculata*, and *A. paggiae* can be readily differentiated not only by genetic analysis but also by morphological characteristics of L3 larvae.

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1. Introduction

The genus *Anisakis* Dujardin, 1845, consists of common parasites of marine mammals, which were previously considered to be only 3 valid species: *Anisakis simplex*, *Anisakis typica*, and *Anisakis physeteris* [1]. In addition to morphological characterization, recent data from molecular techniques, such as allozyme analysis, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP), and sequencing of the internal transcribed spacer (ITS) region (ITS1–5.8S rRNA–ITS2) of ribosomal DNA, revealed that the *Anisakis* genus consists of 9 valid species: *Anisakis simplex* sensu stricto, *Anisakis pegreffii*, *Anisakis simplex* C (3 sibling species of the *A. simplex* complex), *A. typica*, *Anisakis ziphidarum*, *Anisakis nascettii*, *A. physeteris*, *Anisakis brevispiculata*, and *Anisakis paggiae* [1–6]. Furthermore, the third-stage (L3) larvae of *Anisakis*, which are the etiological agents of human anisakiasis, were categorized morphologically into *Anisakis* Type I larvae and *Anisakis* Type II larvae on the basis of their ventriculus length and the presence or absence of a mucron [7,8]. Shiraki described 2 more types of L3 larvae (Type III and Type IV) [9], but his findings have been overlooked, and these types of larvae have been confused with Type II larvae. Therefore, through a combination of morphological and molecular analyses, *Anisakis* Type I larvae were found to include the species *A. simplex* sensu stricto, *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum*, and *A.*

nascettii, whereas *Anisakis* Type II larvae include the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae* [3,6,10,11].

Since eating raw fish and squid is common in Japan, we have previously described the prevalence of *Anisakis* larvae in fish such as young Pacific bluefin tuna (*Thunnus orientalis*) and chub mackerel (*Scomber japonicus*), which are frequently eaten raw [12,13]. In this study, we investigated the presence of *Anisakis* L3 larvae in splendid alfonso (*Beryx splendens*) from Japanese waters, which is nowadays eaten raw. Type I, Type II, and 2 other types of *Anisakis* L3 larvae that were similar to Type III and IV larvae [9] were collected from *B. splendens*. We evaluated the morphological characteristics of L3 larvae and identified their species using genetic analysis.

2. Materials and methods

2.1. Parasite materials

Forty-four raw specimens of *B. splendens* were obtained from fish markets in Japan from May to November 2007 and from August to November 2009. Among these, 8 (fork length of 36–43 cm) were from Tokyo, 9 (fork length of 28–38 cm) were from Chiba Prefecture, and 19 (fork length of 37–48 cm) were from Shizuoka Prefecture. The fishing grounds of Tokyo, Chiba, and Shizuoka prefectures are along the Pacific coast from Chiba Prefecture to Shizuoka Prefecture and around the Izu Islands. Eight specimens (fork length of 35–44 cm) were from Kochi Prefecture, the fishing grounds of which are off Shikoku Island. After separation of the muscle and internal organs of

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the fish, *Anisakis* larvae were collected from the fish and were preserved either at -20°C or in 70% ethanol until use.

2.2. Morphological examination

The body length of larvae was measured directly. For morphological identification, the larvae were cleared and mounted in Gater's solution and were observed under a light microscope [7–9]. The following morphological characteristics were measured: body width, esophagus length, ventriculus length, tail length, body length/body width, body length/esophagus length, body length/ventriculus length, and body length/tail length. The larvae were washed in distilled water to remove Gater's solution and were preserved in 70% ethanol until DNA extraction was performed.

2.3. DNA extraction

The larvae were cut into 3 pieces, and the middle portion of individual larvae was used to identify the species by genetic analysis. DNA was extracted using QIAamp DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions.

2.4. PCR amplification

The ITS region and mitochondrial cytochrome oxidase 2 (mtDNA *cox2*) gene were targeted for PCR amplification. PCR amplification was performed in a reaction mixture (50 μL) containing 5 μL of DNA template, 1.5 U of TaKaRa EX Taq (Takara Bio Inc., Japan), 0.4 μM of each primer, and 0.2 mM of each dNTP. The ITS region was amplified using primers NC5 (forward: 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse: 5'-TTAGTTTCTTTCTCCGCT-3') [14]. The following cycling parameters were used: an initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 40 s, 58°C for 40 s, and 72°C for 1 min; a final extension at 72°C for 5 min. The mtDNA *cox2* gene was amplified using primers 210 (forward: 5'-CACCAACTCTAAATTATC-3') and 211 (reverse: 5'-TTTCTAGTTATATAGATTGRTTYAT-3') [15]. The following cycling parameters were used: an initial denaturation at 94°C for 3 min; 34 cycles of 94°C for 30 s, 46°C for 1 min, and 72°C for 90 s; a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 2.0% Seakem ME agarose gel (Lonza Inc., USA) in Tris–Borate–EDTA buffer and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining.

2.5. Sequence analysis

PCR products of the ITS region and mtDNA *cox2* gene were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequenc-

ing Ready Reaction kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, USA).

2.6. Phylogenetic analysis

Multiple alignment and phylogenetic analysis of the mtDNA *cox2* gene sequences of the *Anisakis* species were performed using Clustal W [16], maximum likelihood (ML) method by PHYML version 2.4.5 software [17], and Bayesian (MrBayes version 3.1.2) method [18]. In Bayesian analysis (BI), we ran 4 simultaneous chains (nchain = 4), 2,000,000 generations, and an initial burn-in of 5000, at which point the likelihood values were stabilized. A general time-reversible (GTR) model was used with a proportion of invariant bases and 4 categories of among-site rate variation, and trees were sampled every 100 generations. The ML tree was derived with a GTR model by using estimates of the proportion of invariable site parameters of 0.361 and gamma distribution parameters of 0.459, and the statistical support was evaluated using bootstrapping of 1000 replicates. The BI and ML tree data files were visualized using MEGA version 4.0.2 [19]. The GenBank accession numbers of the *Anisakis* species used in the phylogenetic analysis were as follows: *A. simplex* sensu stricto (no. DQ116426), *A. pegreffii* (nos. DQ116428 and EU413958), *A. simplex* C (no. DQ116429), *A. typica* (no. DQ116427), *A. ziphidarum* (no. DQ116430), *A. nascettii* (no. FJ685642), *A. physeteris* (no. DQ116432), *A. brevispiculata* (no. DQ116433), *A. paggiae* (no. DQ116434), and *Pseudoterranova decipiens* (no. AF179920).

2.7. PCR–RFLP

The PCR products of the ITS region were digested with the restriction enzymes *HinfI* and *HhaI* (Toyobo, Japan) according to the manufacturer's recommendations. The digested products were analyzed by electrophoresis on a 2.0% Seakem ME agarose gel and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining. The larvae were distinguished on the basis of the combination of RFLP patterns [4,20,21].

3. Results

3.1. Morphology of *Anisakis* L3 larvae detected

Anisakis L3 larvae collected from *B. splendens* were divided morphologically into 4 types: Type I, II, and 2 different types that were similar to Type III and IV (Table 1). Type II, III, and IV larvae had a short ventriculus, and the junction between the ventriculus and intestine was horizontal (Fig. 1; 1B–3B). These 3 types of larvae had a boring tooth at the anterior end: Type II larvae had a slightly tilted or

Table 1
Measurement of third-stage larvae of *Anisakis* Types II, III, and IV.

Type of larvae (no. measured)	Type II (90)		Type III (6)		Type IV (27)	
	Range	(Mean $\pm$ SD)	Range	(Mean $\pm$ SD)	Range	(Mean $\pm$ SD)
Body length (mm) ^a	22.00–34.50	(29.14 $\pm$ 2.29)	27.00–35.00	(32.17 $\pm$ 3.13)	14.00–23.00	(18.22 $\pm$ 2.28)
	21.60–31.90	(25.70 $\pm$ 2.94)	23.80–38.40	(28.90 $\pm$ 3.73)	14.80–26.40	(20.10 $\pm$ 3.75)
Body width (mm) ^a	0.51–0.75	(0.65 $\pm$ 0.05)	0.75–0.95	(0.86 $\pm$ 0.09)	0.40–0.60	(0.50 $\pm$ 0.05)
	0.53–0.71	(0.61 $\pm$ 0.06)	0.65–0.97	(0.84 $\pm$ 0.08)	0.41–0.67	(0.54 $\pm$ 0.07)
Esophagus length (mm) ^a	1.70–2.40	(2.11 $\pm$ 0.17)	1.70–2.35	(1.96 $\pm$ 0.27)	1.25–1.85	(1.46 $\pm$ 0.19)
	1.80–2.51	(2.15 $\pm$ 0.21)	1.46–2.33	(1.83 $\pm$ 0.24)	1.24–2.18	(1.59 $\pm$ 0.28)
Ventriculus length (mm) ^a	0.50–0.72	(0.59 $\pm$ 0.06)	0.45–0.56	(0.51 $\pm$ 0.05)	0.22–0.40	(0.31 $\pm$ 0.04)
	0.47–0.71	(0.58 $\pm$ 0.06)	0.47–0.73	(0.57 $\pm$ 0.07)	0.30–0.59	(0.38 $\pm$ 0.09)
Tail length (mm) ^a	0.16–0.38	(0.26 $\pm$ 0.05)	0.11–0.15	(0.12 $\pm$ 0.01)	0.07–0.17	(0.11 $\pm$ 0.03)
	0.21–0.38	(0.28 $\pm$ 0.05)	0.10–0.20	(0.16 $\pm$ 0.03)	0.11–0.19	(0.15 $\pm$ 0.03)
Body length/body width	37.7–50.8	(45.2 $\pm$ 3.0)	28.4–46.7	(38.0 $\pm$ 6.4)	30.0–46.0	(36.6 $\pm$ 4.2)
Body length/esophagus length	11.1–17.4	(13.9 $\pm$ 1.2)	11.5–20.6	(16.8 $\pm$ 3.3)	10.3–15.0	(12.6 $\pm$ 1.6)
Body length/ventriculus length	39.0–62.0	(49.7 $\pm$ 4.9)	49.1–77.8	(63.7 $\pm$ 9.5)	46.8–81.8	(59.7 $\pm$ 8.5)
Body length/tail length	84.0–171.0	(115.0 $\pm$ 23.1)	206.7–291.7	(261.2 $\pm$ 33.4)	124.1–242.9	(170.9 $\pm$ 35.5)

^a Measurements in the second column of body length and width, esophagus, ventriculus, and tail length are taken from Shiraki [9].

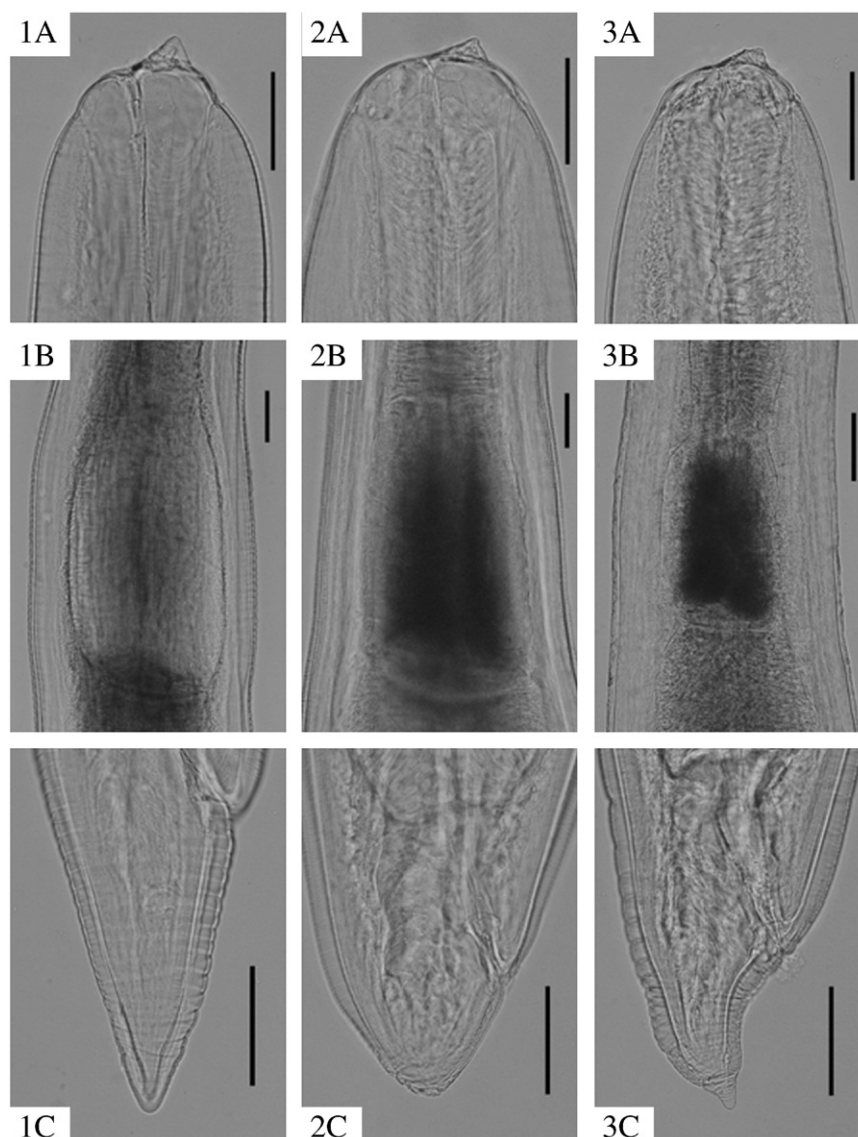


Fig. 1. Morphology of third-stage larvae of *Anisakis* Types II, III, and IV. Row 1, Type II; row 2, Type III; row 3, Type IV. Line A, cephalic end; line B, ventricular part; line C, caudal end. Bar: 100 μ m.

parallel tooth, while Types III and IV larvae had only a slightly tilted tooth (Fig. 1; 1A–3A). However, the tails of these 3 types of larvae were morphologically different: Type II larvae had a long (tail length, 0.16–0.38 mm; body length/tail length, 115.0), conical, and tapering tail without a mucron (Fig. 1; 1C). Type III larvae were characterized by a short (tail length, 0.11–0.15 mm; body length/tail length, 261.2) and rounded tail (Fig. 1; 2C). At least 2 Type III larvae possessed a tail with a tiny spine-like mucron (data not shown), but the others lacked it. On the other hand, Type IV larvae were characterized by a short (tail length 0.07–0.17 mm, body length/tail length 170.9), conical, and pointed tail without a mucron (Fig. 1; 3C).

3.2. Prevalence and intensity of *Anisakis* L3 larvae

The 730 *Anisakis* L3 larvae were collected from the visceral surface and body cavity of 44 specimens of *B. splendens*. These larvae were morphologically characterized as *Anisakis* Types I–IV larvae. Larvae were not collected from the muscle of the fish. A total of 48 Type I larvae, 624 Type II larvae, 10 Type III larvae, and 48 Type IV larvae were collected from 26 (59.1%), 41 (93.2%), 9 (20.5%), and 14 (31.8%) fish, respectively (Table 2). All 4 types of larvae were collected from

each landing district. On the basis of the landing district, the highest average number of *Anisakis* L3 larvae per fish was 25.2 larvae from Shizuoka Prefecture, whereas the lowest average number of larvae per fish was 4.1 larvae from Chiba Prefecture.

3.3. Genetic analysis

Three types of *Anisakis* L3 larvae with a short ventriculus (4 Type II larvae [Aph1–Aph4], 10 Type III larvae [Ab1–Ab10], and 16 Type IV larvae [Apg1–Apg16]) were analyzed by sequencing the ITS region (Table 3). The sequences of the ITS region from the 4 Type II larvae (Aph1 [AB592792]–Aph4) were 100% identical to the sequence of *A. physeteris* (AB277821) from mackerel and had only 2 base substitutions (C instead of T at position 21 from the 5' end of 5.8S rRNA and G instead of A at position 34 from the 5' end of ITS2) compared with the sequence of *A. physeteris* (AY826721) from *Physeter catodon*. The sequences of the ITS region from the 9 Type III larvae (Ab1 [AB592793], Ab2, and Ab4–Ab10) were 100% identical. Compared to the sequence of *A. brevispiculata* (EU624344) from *Theragra chalcogramma*, the sequences of these 9 larvae exhibited a high level of similarity with only 1 base substitution (G instead of A

Table 2
Number of third-stage larvae of *Anisakis* spp. in *Beryx splendens*.

Landing district ^a	No. of fish examined	No. of fish infected	Total no. of <i>Anisakis</i> larvae	Type I		Type II		Type III		Type IV	
				Positive samples	No. of larvae	Positive samples	No. of larvae	Positive samples	No. of larvae	Positive samples	No. of larvae
Tokyo	8	8	123	6	12	8	107	1	2	1	2
Chiba	9	9	37	3	4	8	31	1	1	1	1
Shizuoka	19	19	479	12	26	17	403	6	6	11	44
Kochi	8	8	91	5	6	8	83	1	1	1	1
Total	44	44	730	26	48	41	624	9	10	14	48

^a The fishing grounds of Tokyo, Chiba, and Shizuoka prefectures are along the Pacific coast from Chiba Prefecture to Shizuoka Prefecture and around the Izu Islands. The fishing grounds of Kochi Prefecture are off Shikoku Island.

at position 19 from the 5' end of ITS2). When compared to the sequence of *A. brevispiculata* (AY826719) from *Kogia breviceps*, the sequences of these 9 larvae exhibited a high level of similarity with only 3 base substitutions: A instead of C and A instead of G at positions 165 and 177, respectively, from the 5' end of ITS2, and C instead of T at position 259 from the 3' end of ITS1. The sequence of Ab3 (AB592794) had T instead of C at position 259 from the 3' end of ITS1, compared with the sequences of Ab1, Ab2, and Ab4–Ab10. The sequences of the ITS region from the 14 Type IV larvae (Apg1 [AB592795]–Apg7, Apg9–Apg14, and Apg16) were 100% identical to the sequence of *A. paggiae* (EU624345) from *T. chalcogramma*. The sequences of Apg8 (AB592796) and Apg15 (AB592797) had A instead of T at position 79 from the 5' end of ITS2 and G instead of A at position 330 from the 3' end of ITS1, respectively.

Sequence analysis of the mtDNA *cox2* gene indicated that the sequences of the 4 Type II larvae (Aph1–Aph4 [AB592798–AB592801]), compared to the reference *A. physeteris* strain (DQ116432), had 7, 6, 17, and 17 base substitutions at the nucleotide level and 1, 2, 2, and 2 amino acid substitutions, respectively. The sequences of the 4 Type III larvae (Ab1, Ab3, Ab5, and Ab10 [AB592802–AB592805]) compared to the reference *A. brevispiculata* strain (DQ116433) had 9, 9, 11, and 10 base substitutions at the nucleotide level and 5, 6, 5, and 5 amino acid substitutions, respectively. The sequences of the 5 Type IV larvae (Apg1, Apg2, Apg8, Apg15, and Apg16 [AB592806–AB592810]) compared to the reference *A. paggiae* strain (DQ116434) had 18, 26, 17, 19, and 22 base substitutions at the nucleotide level, respectively. However, the amino acid sequences of these 5 larvae were identical and had only a single substitution compared to the amino acid sequence of the reference strain. In the phylogenetic trees of the mtDNA *cox2* gene constructed using BI and ML, the adult reference strains of *A. physeteris* and *A. brevispiculata* were sister taxa that formed a clade with that of *A. paggiae*, and the bootstrap values at the two branch points of three reference strains were 100% (BI) and 97% (ML). Type II larvae (Aph1–

Aph4) clustered with *A. physeteris*, but Aph1–2 and Aph3–4 had 2 major lineages and the bootstrap values in BI and ML trees were 100% and 98%, respectively. Type III larvae (Ab1, Ab3, Ab5, and Ab10) and Type IV larvae (Apg1, Apg2, Apg8, Apg15, and Apg16) clustered with *A. brevispiculata* and *A. paggiae*, respectively (Fig. 2; Table 3).

PCR–RFLP analysis of the ITS region with *HinfI* and *HhaI* was performed on 48 Type I larvae, 128 Type II larvae, 10 Type III larvae, and 48 Type IV larvae. On the basis of the combination of RFLP patterns, within the 48 Type I larvae, 1, 5, and 42 larvae corresponded to *A. ziphidarum*, *A. simplex* C, and *A. simplex* sensu stricto, respectively. Types II, III, and IV larvae exhibited the following RFLP patterns: digestion of the ITS region of the Type II larvae with *HinfI* produced 3 bands (ca. 360, 270, and 250 bp), while *HhaI* digestion produced 2 bands (ca. 520 and 390 bp). Digestion of the ITS region of the Type III larvae with *HinfI* produced 1 band (ca. 900 bp), while *HhaI* digestion produced 3 bands (ca. 390, 330, and 190 bp). Digestion of the ITS region of the Type IV larvae with *HinfI* produced 1 band (ca. 920 bp), while the *HhaI* digestion produced 2 bands (ca. 520 and 410 bp) (Fig. 3).

4. Discussion

On the basis of the BI and ML trees derived from the mtDNA *cox2* gene nucleotide sequence, Types II, III, and IV larvae belonged to the clades of *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, respectively (Fig. 2). These trees showed a topology congruent with the previously

Table 3
Specimen information and GenBank accession numbers.

Type of larvae	Specimen code	<i>Anisakis</i> species ^a	Landing district	Date of collection	GenBank accession number	
					ITS	<i>cox2</i>
Type II	Aph1	<i>A. phy.</i>	Tokyo	Sep. 2007	AB592792	AB592798
	Aph2	<i>A. phy.</i>	Chiba	Sep. 2009	AB592799	AB592799
	Aph3	<i>A. phy.</i>	Shizuoka	Nov. 2009	AB592800	AB592800
	Aph4	<i>A. phy.</i>	Kochi	Nov. 2009	AB592801	AB592801
Type III	Ab1	<i>A. b.</i>	Tokyo	Nov. 2007	AB592793	AB592802
	Ab3	<i>A. b.</i>	Chiba	Sep. 2009	AB592794	AB592803
	Ab5	<i>A. b.</i>	Shizuoka	Sep. 2009	AB592804	AB592804
	Ab10	<i>A. b.</i>	Kochi	Jul. 2007	AB592805	AB592805
	Apg1	<i>A. pag.</i>	Tokyo	Sep. 2007	AB592795	AB592806
Type IV	Apg2	<i>A. pag.</i>	Chiba	Aug. 2009	AB592807	AB592807
	Apg8	<i>A. pag.</i>	Shizuoka	Oct. 2009	AB592796	AB592808
	Apg15	<i>A. pag.</i>	Shizuoka	Nov. 2009	AB592797	AB592809
	Apg16	<i>A. pag.</i>	Kochi	Nov. 2009	AB592810	AB592810

^a *A. phy.*, *Anisakis physeteris*; *A. b.*, *Anisakis brevispiculata*; *A. pag.*, *Anisakis paggiae*.

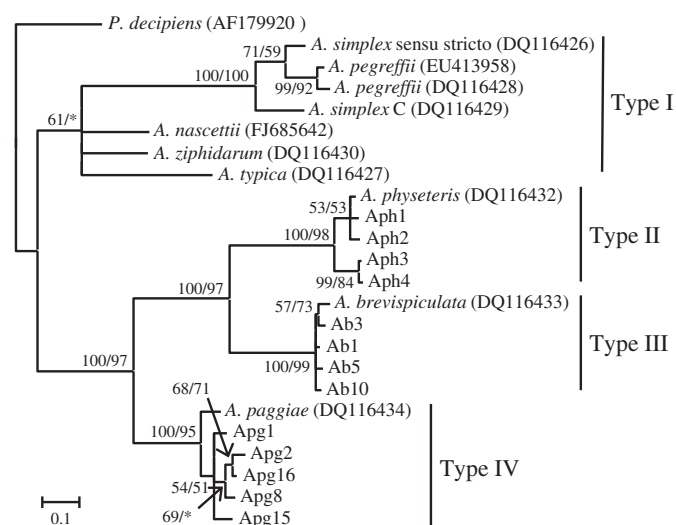


Fig. 2. Phylogenetic tree based on mtDNA *cox2* gene sequences exploring the relationships among *Anisakis* species. The Bayesian analysis (BI) tree derived using a general time-reversible model is shown. Significant bootstrap supports (>50%) from BI and 1000 replicates of maximum likelihood (ML) are shown at each node in the order BI/ML and asterisks indicate bootstrap values less than 50%. The scale bar represents the distance in substitutions per nucleotide. Specimen codes are referenced in Table 3.

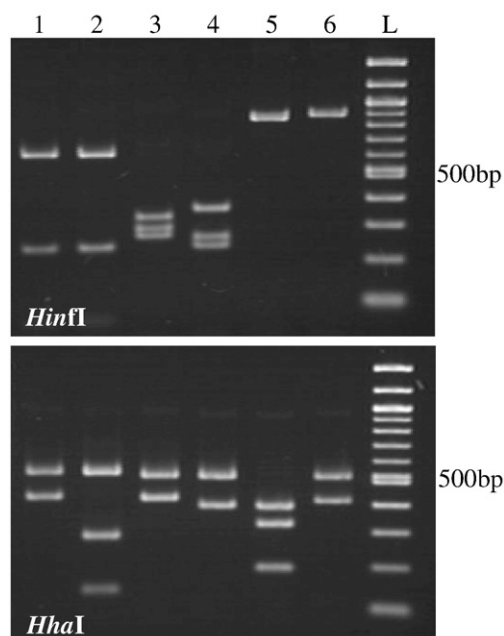


Fig. 3. Restriction fragment length polymorphism patterns obtained by digestion of the internal transcribed spacer (ITS) region (ITS1–5.8S rRNA–ITS2) of rDNA with the restriction enzymes *HinfI* and *HhaI*. Lane 1, Type I (*Anisakis simplex sensu stricto*); Lane 2, Type I (*Anisakis simplex C*); Lane 3, Type I (*Anisakis ziphidarum*); Lane 4, Type II (*Anisakis physeteris*); Lane 5, Type III (*Anisakis brevispiculata*); Lane 6, Type IV (*Anisakis paggiae*); L. 100 bp ladder.

reported trees based on the same gene of *Anisakis* spp. [6,15,21]. Moreover, the sequences of the ITS region in Types II, III, and IV larvae were 100% identical or had only 1–3 base substitutions compared with sequences of reference adult or larval strains of *A. physeteris*, *A. brevispiculata*, and *A. paggiae*. The combination of PCR–RFLP patterns of the ITS region with *HinfI* and *HhaI* indicated the same results. These results clearly indicate that Types II, III, and IV larvae can be identified as *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, respectively.

B. splendens is a demersal fish that inhabits tropical and temperate water zones of 25–1300 m in depth along continental slopes and sea mountains all over the world. In Japan, *B. splendens* lives along the Pacific coast, south of ca. 37° N. *B. splendens* consumes shrimp, fish, squid, and crustacean zooplankton [22,23]. On the other hand, adult worms of *A. physeteris* are found in sperm whales (*P. macrocephalus*), which have a wide distribution throughout the world [24], including Japanese waters [25]. The sperm whale is known to dive deeper and to consume squid and benthic fish [1,25]. Therefore, *A. physeteris* (Type II larvae) exhibited the highest prevalence value (41/44 fish) and were the most prevalent *Anisakis* species (624/730 larvae) in *B. splendens*, probably because *B. splendens* and *P. macrocephalus* inhabit the same geographical areas. The prevalence of *A. brevispiculata* (Type III) and *A. paggiae* (Type IV) was 20.5% and 31.8%, respectively. According to Shiraki [9], the prevalence of Types III and IV larvae in *T. chalcogramma* captured in northern Japanese waters was much lower than our data, which is thought to depend on the collection area. Forty-eight Type I larvae were detected, and *A. simplex sensu stricto* was the most prevalent species (42/48 larvae). As previously reported, in *S. japonicus* from Japanese waters, *A. simplex sensu stricto* was the most prevalent species from the Pacific coast, whereas *A. pegreffii* was the most prevalent species from the Sea of Japan [13]. Therefore, it follows that *A. simplex sensu stricto* was the most prevalent species of the Type I larvae in *B. splendens* living in the Pacific coastal waters of Japan.

Morphologically, *Anisakis* L3 larvae were previously categorized into 2 types (Type I and Type II) [7,8], with the exception of the description by Shiraki [9]. Consequently, L3 larvae of *A. physeteris*, *A. brevispiculata*,

and *A. paggiae* have been categorized into Type II morphotypes, although the adult males of the above 3 species were distinguished morphologically by their spicule length and ventriculus length [3]. In this study, we detected 2 morphologically different types of *Anisakis* L3 larvae from *B. splendens* that had a short ventriculus, which were similar to Type III and Type IV larvae described by Shiraki [9]. Some Type II larvae and all Types III and IV larvae had a slightly tilted boring tooth, which was parallel to the body axis compared to that in Type I. It was reported that the boring tooth in Types II, III, and IV larvae was almost straight and parallel to the body axis [9]. The boring tooth in Type II larvae depicted in figures of several reports seems to show slight tilting, although the boring tooth was not described in detail [7,8]. While the cause of boring tooth tilt differences is unknown, the difference in methods (glycerol–alcohol or lactophenol in previous studies versus Gater's solution in this study for clearing of larvae) may have caused the morphological changes.

Type III larvae were characterized by a short rounded tail as observed in Type I larvae and a stout body, but the body width and ventriculus length differentiated Type III larvae from Type I larvae. In 1974, Shiraki [9] described that Type III larvae lacked a mucron at the tail end. However, when Type III larvae were first reported at a society meeting in 1969, they were designated to have a tiny mucron (see Oshima, 1972 [26]). In this study, at least 2 of 10 larvae possessed a tail with a tiny spine-like mucron but the others lacked it. Hence, the tail mucron is an unstable characteristic of Type III larvae. In larvae with a tapering tail, each measurement of tail length and ventriculus length in Type IV larvae was clearly shorter than that of Type II larvae. In a previous report of Type IV larvae [9], measurement of 1 larva was apparently larger than that of the others, and it is likely that this larva could be categorized into Type II. Except for this larva, the range of each structure in Type IV larvae described by Shiraki [9] (body length, 14.80–23.20 mm; body width, 0.41–0.61 mm; esophagus length, 1.24–1.69 mm; ventriculus length, 0.30–0.40 mm; tail length, 0.11–0.19 mm) was similar to that in Type IV larvae from *B. splendens* (Table 1). According to the results of genetic analysis of the ITS region and the mtDNA *cox2* gene, Types II, III, and IV larvae were identified as *A. physeteris*, *A. brevispiculata*, and *A. paggiae*, respectively. Therefore, we concluded that *A. physeteris*, *A. brevispiculata*, and *A. paggiae* are readily differentiated not only by genetic analysis but also by morphological characteristics of L3 larvae.

Acknowledgments

We are very grateful to Mr. Shouju Sakai, Tokyo Metropolitan Institute of Public Health, Tama Branch Institute, for providing the fish samples. We would like to sincerely thank Dr. Jun Araki, Meguro Parasitological Museum, Tokyo, for valuable advice. This study was supported in part by a grant for Research on Food Safety from the Ministry of Health, Labour and Welfare of Japan (H22-Shokuhin-ippan-009).

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