



Morphological differences between larvae and *in vitro*-cultured adults of *Anisakis simplex* (sensu stricto) and *Anisakis pegreffii* (Nematoda: Anisakidae)[☆]

Karl Marx A. Quiazon^{a,b}, Tomoyoshi Yoshinaga^{a,*}, Kazuo Ogawa^a, Ryuji Yukami^c

^a Department of Aquatic Biosciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

^b Freshwater Aquaculture Center and College of Fisheries, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120, Philippines

^c Seikai National Fisheries Research Institute, Fisheries Agency, Taira-machi, Nagasaki-shi, Nagasaki 851-2213, Japan

ARTICLE INFO

Article history:

Received 6 February 2008

Received in revised form 6 June 2008

Accepted 18 June 2008

Available online 1 July 2008

Keywords:

Anisakis simplex (s.s.)

Anisakis pegreffii

Japan

Morphological characters

In vitro-culture

ABSTRACT

Proper identification of *Anisakis* species infecting host fishes is very important to both human health and fish disease diagnosis. The foremost problem in the identification of *Anisakis* larvae in fishes is that L3 larvae cannot be easily differentiated morphologically, especially between *A. simplex* (sensu stricto) (s.s.) (Rudolphi, 1809) and *A. pegreffii* Campana-Rouget et Biocca, 1955. Instead, molecular means such as allozyme, mitochondrial DNA (mtDNA) *cox2* region and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses had been successfully used. In this study, morphological differences of L3 larvae collected from fishes and *in vitro*-cultured L4 larvae and adult *A. simplex* (s.s.) and *A. pegreffii* were evaluated. *Anisakis* larvae were collected from 7 different host fishes within Japan. Undamaged *A. simplex* (s.s.) and *A. pegreffii* collected from *Oncorhynchus keta* (Walbaum) and *Scomber japonicus* Houttuyn, respectively, were used for *in vitro*-culture in order to obtain L4 and adult stages. Species identification was confirmed by PCR-RFLP analysis of the ITS region (ITS1-5.8S-ITS2) of ribosomal DNA and by mtDNA *cox2* gene sequencing. Results revealed that L3, L4 and adult stages of *A. simplex* (s.s.) and *A. pegreffii* are morphologically distinguishable based on ventriculus length, wherein the former has longer ventriculus (0.90–1.50 mm) than the latter (0.50–0.78 mm). For oesophagus/ventriculus ratio, these two species are distinguishable only during L4 and adult stages. Also, adult male *A. simplex* (s.s.) and *A. pegreffii* were found to be distinguishable by differences in the distribution pattern of the caudal papillae, particularly the 3rd pair of distal papillae.

© 2008 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Anisakis Dujardin, 1845 are parasitic nematodes in the family Anisakidae Railliet et Henry, 1912 that have a global distribution. Until early 80's when allozyme analysis was used in identifying sibling species, only 3 valid species had been recognized, namely *A. simplex*, *A. typica* and *A. physeteris* [1–3]. The third-stage larvae (L3 larvae) of *Anisakis* are found not only in the body cavities but also as well in the muscles of selected host species [4–9]. *Anisakis simplex* (Rudolphi, 1809, det. Krabbe, 1878) is the most frequently reported species, which continuously attracts human health concerns relating to anisakiasis. Proper species identification of L3 larvae taken from

patients with anisakiasis and from wild hosts is very important in implementing guidelines for future reduction and prevention of this disease. Morphological diagnosis using L3 larvae can only determine its type groupings, *Anisakis* Type I larvae or *Anisakis* Type II larvae, wherein the former has longer ventriculus and has mucro while the latter has short ventriculus and has no mucro [10,11]. Precise identification up to species level is difficult with the current unavailability of distinguishing morphological keys in L3 larvae, especially in differentiating *A. simplex* from *A. pegreffii*.

On the other hand, recent molecular studies on *Anisakis*, which launched from a series of studies using allozyme and mitochondrial DNA (mtDNA) *cox2* gene analyses, revealed that the *Anisakis* species consisted of 8 valid species, 5 species [*A. ziphidarum*, *A. typica* and three sibling species of *A. simplex* complex, namely *A. simplex* (sensu stricto) (s.s.), *A. pegreffii* and *A. simplex* C] and 3 species [*A. paggiae*, *A. brevispiculata* and *A. physeteris*] of which are in the *Anisakis* Type I and *Anisakis* Type II groupings, respectively [2,12–19]. In Japan, *Anisakis* species identified through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [20,21] and multiplex PCR [22] analyses of the ITS region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) are *A. simplex* (s.s.), *A. pegreffii* and *A. physeteris*. The *A. simplex* (s.s.), which is abundantly distributed worldwide, can be found mostly

Abbreviations: s.s., Sensu stricto; L3 larvae, Third-stage larvae; L4 larvae, Fourth-stage larvae; rDNA, Ribosomal DNA; mtDNA, Mitochondrial DNA; ITS, Internal transcribed spacer; *cox2*, Cytochrome oxidase subunit 2; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; SEM, Scanning electron microscopy.

[☆] Nucleotide sequence data reported in this paper are available in the GenBank under the accession numbers EU413958 and EU413959.

* Corresponding author. Tel.: +81 3 5841 5283.

E-mail address: atyoshi@mail.ecc.u-tokyo.ac.jp (T. Yoshinaga).

in northern Japan while the *A. pegreffii*, which was the dominant species in the genus *Anisakis* in the Mediterranean [18] and which was first described from the digestive tract of a Mediterranean monk seal, *Monachus monachus*, captured on eastern Sardinia [23], can be found mostly in southern Japan [20,21]. Even with the success in the development of different molecular protocols, costs, time duration needed for examination and availability of technical expertise limit its widespread application. This is also impractical and impossible during field surveys needing rapid on-site identification of *Anisakis* species.

In contrast to larvae, species identification of adults is morphologically possible in some species. However, no obvious morphological differences have been reported between *A. simplex* (s.s.) and *A. pegreffii*. Among the morphological keys used in species identification, the number and arrangement of caudal papillae in the males of adult nematodes are used by systematists as valid criteria [3,24]. The caudal papillae of *A. pegreffii* have not been examined. Those of *A. simplex*, which was described when the presence of sibling species has not yet been recognized, was reported [3] while those of *A. simplex* (s.s.) are not available. In addition, the spicule length, which is also frequently used in distinguishing one species from another overlapped between *A. simplex* and *A. pegreffii* [3,23].

This study was conducted due to the current difficulties with the morphological differentiation not only on L3 larvae but also in adults of *A. simplex* (s.s.) and *A. pegreffii*. The aim of the present paper was to closely examine any major morphological differences to find keys for identification of L3 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii*. Such identification keys would be useful for immediate identification of *Anisakis* recovered from human patients and from infected fishes and other hosts, thereby providing precautionary measures in preventing and reducing anisakiasis. We applied an *in vitro*-culture technique [25,26] to obtain adult worms as collection of adults from infected cetacean hosts is difficult.

2. Materials and methods

2.1. Parasite collection and fixation

L3 larvae were collected from the musculature of chum salmon (*Oncorhynchus keta* (Walbaum)) caught in the Chitose River in Hokkaido and from the visceral surface of 5 different fish species, namely Pacific cod (*Gadus macrocephalus* Tilesius), Alaska pollock (*Theragra chalcogramma* (Pallas)), blue mackerel (*Scomber australasicus* Cuvier), chub mackerel (*Scomber japonicus* Houttuyn) and Japanese Spanish mackerel (*Scomberomorus niphonius* (Cuvier)) caught in Japanese waters. Also, L3 larvae were collected from the

body cavity of juvenile greater amberjack (*Seriola dumerili* (Risso)) imported from China (Table 1, Fig. 1).

L3 larvae collected from fishes and L4 larvae and adults obtained by *in vitro*-culture were fixed in 70% ethanol. After measurement of total length, they were cut into three pieces: the anterior, middle and posterior parts. Anterior and posterior parts were used for light microscopic and scanning electron microscopic observations. The middle part was stored in 100% ethanol to extract genomic DNA for PCR-RFLP analysis of rDNA and for mtDNA *cox2* gene sequencing.

2.2. *In vitro*-culture

L3 larvae collected from *O. keta* caught in Chitose River and *S. japonicus* caught in off Kumamoto Prefecture were used. *In vitro*-culture from L3 larvae to adult was performed following a previous report [26]. Briefly, undamaged worms were washed and immersed in an antibiotic-antimycotic solution [25] for 30 min for sterilization before *in vitro*-culture. Subsequently, worms were placed individually in a single well of 24-well plates containing culture media (i.e., RPMI-1640 supplemented with 20% heat-inactivated fetal bovine serum and 1% commercial pepsin and adjusted to pH 4.0). *In vitro*-culture was performed at 37 °C in an air atmosphere with 5% CO₂. Medium was replaced twice a week during L3 to L4 stage, and was replaced more frequently during the adult stage, according to the amount of debris and color change observed in the medium. Worms were transferred to bigger 12-well plates after 2 weeks and finally, to 6-well plates after moulting from L4 to adult stage. L4 larvae and adults were sampled during 3 to 26 days and 27 to 60 days of culture, respectively.

2.3. DNA extraction and data analyses

2.3.1. DNA extraction and PCR

The genomic DNA was extracted from 100% ethanol-fixed worms using DNeasy™ Tissue Kit from Qiagen Inc. (protocol for animal tissues). The ITS region (ITS1-5.8S-ITS2) of rDNA was amplified using primers A (5'-GTCCGAATTCGTAGGTGAACCTGCGGAAGGA TCA-3') and B (5'-GCCGGATCCGAATCCTGGTTAGTTCTTTCCT-3') [21]. The PCR assay was performed with 1 µL sample DNA as template in a total volume of 20 µL containing 0.6 µL forward and reverse primer, 14.1 µL double distilled water and 3.7 µL Taq mix (containing 0.1 µL TAKARA Ex Taq™ HS; 2 µL [10×] Ex Taq Buffer and; 48 µL dNTP mixture). Amplification was carried out using iCycler™ (BIO-RAD, Japan). PCR mixture was denaturized at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 49 °C for 30 s, 72 °C for 75 s, followed by post-amplification at 72 °C for 7 min following previous reports [21,27].

Table 1
Identification of *A. simplex* (s.s.) and *A. pegreffii* from different host fishes in Japanese waters

Fish species	Locality ^a	Date of collection	No. of worms collected	PCR-RFLP identified <i>Anisakis</i> species		Hybrid genotype ^b
				<i>A. simplex</i> (s.s.)	<i>A. pegreffii</i>	
<i>Oncorhynchus keta</i> ^c	Chitose river, Hokkaido Prefecture (1)	October 2005 and October 2006	101	99	0	2
<i>Theragra chalcogramma</i>	Hokkaido Prefecture (2)	March 2007	10	10	0	0
<i>Theragra chalcogramma</i>	Between Aomori-Hokkaido (3) ^d	September 2006	20	18	0	2
<i>Theragra chalcogramma</i>	Off Iwate Prefecture (4)	April 2007	22	10	7	5
<i>Scomberomorus niphonius</i>	Off Kyoto Prefecture (5)	January 2005	6	0	6	0
<i>Scomber australasicus</i>	Off Kanagawa Prefecture (6)	June 2006	8	8	0	0
<i>Scomber australasicus</i>	Off Miyazaki Prefecture (7)	June 2006	5	5	0	0
<i>Scomber japonicus</i>	Off Kumamoto Prefecture (8)	October–November 2006	67	0	66	1
<i>Seriola dumerili</i> ^e	Off Kagoshima Prefecture (9) ^d	June 2005	10	0	9	1
<i>Gadus macrocephalus</i>	Between Aomori-Hokkaido (3) ^d	April 2007	19	8	8	3
<i>Gadus macrocephalus</i>	Off Iwate Prefecture (4)	May 2006	6	6	0	0

^a Number coding (in parenthesis) after each locality is shown in Fig. 1.

^b Hybrid genotype showed the same fragment pattern after Hinf I digestion as previously reported [21,30,31].

^c Wild salmon migrating upstream to spawn were examined.

^d A detailed locality was not specified.

^e *Anisakis*-infected juveniles imported from China and reared in net cages were examined.

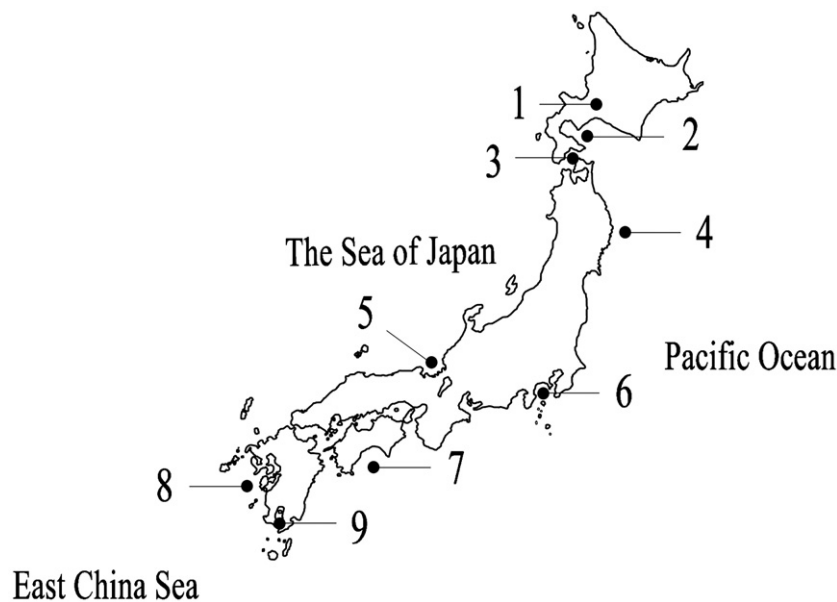


Fig. 1. Geographical locations of examined host fishes. The numbers correspond to the locality numbers in Table 1.

2.3.2. Digestion of PCR products with restriction enzymes

PCR-RFLP analysis was carried out in all specimens for species identification according to the reported genetic keys [27,28]. The restriction enzymes used in the RFLP analysis of ITS rDNA were Taq I, Hinf I and Hha I (Takara Bio Inc., Japan). Manufacturer's recommendations were followed with regard to the digestion of PCR products. Finally, digested products were analyzed by electrophoresis in a 1.5% agarose gel containing ethidium bromide and visualized by illumination with shortwave ultraviolet light.

2.3.3. DNA amplification and sequencing

For further confirmation of the identified *A. simplex* (s.s.) and *A. pegreffii* using PCR-RFLP analysis, the *mtDNA* *cox2* region of three specimens representing each species was individually sequenced. The *mtDNA* *cox2* gene was amplified using the primers 210 (5'-CACC AACTCTTAAAATTATC-3') and 211 (5'-TTTCTAGTTATATAGATTGRT-TYAT-3') [29]. The PCR mixture was denatured at 94 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 46 °C for 1 min, 72 °C for 1 min and 30 s, followed by post-amplification at 72 °C for 10 min following a previous report [19]. Nucleotide bases were sequenced using a DNA automatic sequencer (ABI Prism® 310 Genetic Analyzer, Applied Biosystems) after purification of the *mtDNA* *cox2* gene. Nucleotide sequences were translated into amino acid sequences, compared and aligned with the reported *mtDNA* *cox2* sequences of all previously studied *Anisakis* species [19] using the blastn and blastp programs

(<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>) for nucleotide and translated amino acid sequences, respectively. Nucleotide sequences were deposited and made available in the GenBank under accession numbers EU413958 and EU413959.

2.4. Morphological examination

For light microscopic observation, excised anterior and posterior parts were cleared in glycerin and individually mounted on slides. The following morphological characters were measured and examined: total body length, maximum body width, distance of nerve ring to anterior end, oesophagus length, ventriculus length, ventriculus width, ratio between oesophagus and ventriculus length, tail length and mucro length. Relations between the morphological measurements and total body length were plotted on graphs for detailed comparisons between different species detected by PCR-RFLP analysis. All measurements were in millimeters.

SEM examination was performed in order to study other morphological structures of the anterior and posterior portion in detail, which were difficult to examine using light microscopy. For SEM, 57 specimens (i.e., 36 *A. simplex* (s.s.) and 21 *A. pegreffii*) used for light microscopic observation were placed in 70% ethanol to remove glycerin, post-fixed in 1.25–1.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and dehydrated through a series of ascending ethanol concentrations. Samples were subjected to three changes of

Table 2

Estimates of evolutionary divergence on *mtDNA* *cox2* region showing percentage similarities of the nucleotide sequences^a and translated amino acid sequences^b (in parenthesis) of *Anisakis* species presently and previously studied

Species	1	2	3	4	5	6	7	8	9	10	Authors	GenBank acc. no.
1. <i>A. simplex</i> (s.s.)	–										Present study	EU413959
2. <i>A. pegreffii</i>	95.7 (100)	–									Present study	EU413958
3. <i>A. simplex</i> (s.s.)	99.0 (100)	95.7 (100)	–								Valentini et al. (2006)	DQ116426
4. <i>A. pegreffii</i>	96.1 (100)	98.2 (100)	96.5 (100)	–							Valentini et al. (2006)	DQ116428
5. <i>A. simplex</i> C	95.1 (100)	93.9 (100)	94.2 (100)	93.2 (100)	–						Valentini et al. (2006)	DQ116429
6. <i>A. ziphidarum</i>	89.6 (97.6)	87.4 (97.6)	89.2 (98.1)	89.3 (98.1)	88.3 (98.1)	–					Valentini et al. (2006)	DQ116430
7. <i>A. typica</i>	87.6 (97.1)	87.6 (97.1)	88.0 (97.6)	88.3 (97.6)	88.3 (97.6)	87.2 (99.5)	–				Valentini et al. (2006)	DQ116427
8. <i>A. brevispiculata</i>	85.9 (95.9)	85.2 (95.9)	86.6 (94.7)	86.3 (94.7)	86.1 (94.7)	87.8 (96.6)	84.9 (96.2)	–			Valentini et al. (2006)	DQ116433
9. <i>A. paggiae</i>	88.3 (96.5)	87.6 (96.5)	88.2 (96.2)	88.2 (96.2)	88.0 (96.2)	89.8 (98.1)	86.4 (97.6)	88.8 (97.1)	–		Valentini et al. (2006)	DQ116434
10. <i>A. physeteris</i>	88.7 (95.9)	88.1 (95.9)	88.6 (96.2)	88.5 (96.2)	88.5 (96.2)	87.6 (98.1)	86.9 (97.6)	90.3 (98.6)	88.9 (98.1)	–	Valentini et al. (2006)	DQ116432

^a Percentage similarities computed using blastn program.

^b Percentage similarities computed using blastp program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

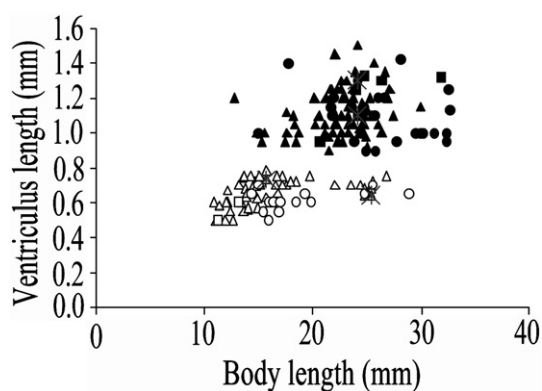


Fig. 2. Relationship between the ventriculus and total body length in L3 larvae and *in vitro*-cultured L4 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii* infecting fishes in Japanese waters ($n=274$). Legend: $\triangle$: *A. pegreffii* (L3 larvae); $\square$: *A. pegreffii* (L4 larvae); $\circ$: *A. pegreffii* (adult); $\blacktriangle$: *A. simplex* (s.s.) (L3 larvae); $\blacksquare$: *A. simplex* (s.s.) (L4 larvae); $\bullet$: *A. simplex* (s.s.) (adult); $*$: Hybrid genotype.

absolute butyl alcohol and freeze dried. Freeze dried samples were subsequently spat-coated and observed under a scanning electron microscope (SEM S-4000 Hitachi, Japan).

3. Results

3.1. PCR-RFLP of rDNA

A total of 274 individual worms were examined. Species of all examined L3 larvae, L4 larvae and adults were identified using PCR-RFLP analysis. Based on the genetic keys [27,28], 76.5% of the examined worms from host fishes caught from the Pacific side of Japan, which were represented by locality nos. 2, 4, 6 and 7 in Fig. 1, were *A. simplex* (s.s.), while 98.6% of the examined worms from host fishes caught from the Sea of Japan and East China Sea regions, which were represented by locality nos. 5 and 8, respectively, were *A. pegreffii* (Table 1). Currently, since the number of examined fishes and worms in this study was relatively small, the species distribution between these two major *Anisakis* species is still not conclusive. Aside from these two major *Anisakis* species, there were some specimens observed to have different PCR-RFLP fragment pattern after Hinf I digestion that was not included in the reported genetic keys [27,28]. This fragment pattern was found similar to that of the previously reported hybrid genotype between *A. simplex* (s.s.) and *A. pegreffii* [21,30,31].

3.2. mtDNA *cox2* analysis

The translated amino acid sequences of mtDNA *cox2* gene showed 100% similarities among the three sibling species of *A. simplex*

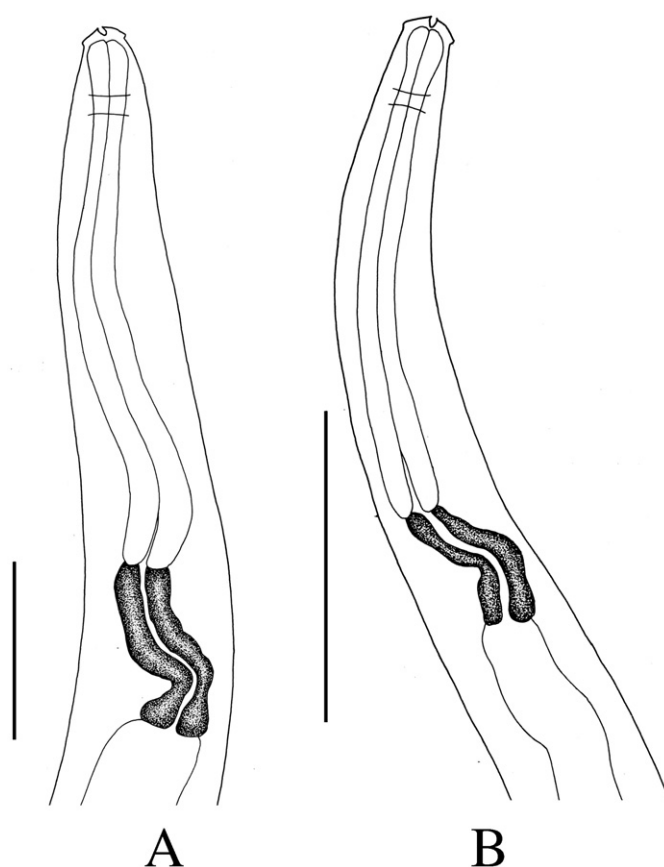


Fig. 3. Morphological identification between *A. simplex* (s.s.) (A) and *A. pegreffii* (B) through differences on the ventriculus length. Ventriculus length at different life-history stages of *A. simplex* (s.s.) ranges from 0.90 to 1.50 mm while that of *A. pegreffii* ranges from 0.50 to 0.78 mm. Scale bar = 1 mm.

complex (*A. simplex* (s.s.), *A. pegreffii* and *A. simplex* C), while using nucleotide sequences showed 99.0% and 98.2% similarities with the previously reported sequences of *A. simplex* (s.s.) (DQ116426) and *A. pegreffii* (DQ116428) [19], respectively (Table 2). Comparison among other reported mtDNA *cox2* sequences of *Anisakis* species showed 4.9–14.8% inter-species variations, which thereby confirms the species level of the examined worms. In the present study, no intra-species variation was found among the 3 specimens of *A. simplex* (s.s.) and *A. pegreffii*, while inter-species variation observed was 4.3%. The small sequence difference of the species examined in this study from those previously reported [19] might be due to differences on geographical sources of the host fishes.

Table 3

Measurements of some morphological characters in *A. simplex* (s.s.) and *A. pegreffii* at different life-history stages

Morphological characters	Life-history stages							
	L3 larvae		L4 larvae		Adult			
					<i>A. simplex</i> (s.s.)		<i>A. pegreffii</i>	
	<i>A. simplex</i> (s.s.)	<i>A. pegreffii</i>	<i>A. simplex</i> (s.s.)	<i>A. pegreffii</i>	Male	Female	Male	Female
No. of specimens examined	112	71	16	4	8	28	11	10
Total body length	12.75–29.94	11.10–26.78	12.07–33.72	11.20–17.76	17.68–29.65	14.95–32.65	14.30–20.55	15.90–29.01
Maximum Body width	0.45–0.75	0.38–0.60	0.56–1.00	0.38–0.79	0.74–1.10	0.80–1.33	0.68–1.03	0.72–1.15
Distance of nerve ring to anterior end	0.21–0.35	0.20–0.31	0.29–0.48	0.27–0.29	0.40–0.42	0.37–0.47	0.27–0.34	0.34–0.36
Length of oesophagus	1.18–2.58	1.04–2.11	2.23–3.18	1.55–1.85	2.33–2.95	2.75–3.40	2.00–2.95	2.27–3.07
Ventriculus length	0.90–1.50	0.50–0.78	0.95–1.33	0.50–0.60	1.10–1.40	0.90–1.42	0.50–0.70	0.60–0.70
Ventriculus width	0.13–0.31	0.12–0.27	0.18–0.32	0.18–0.22	0.24–0.32	0.20–0.34	0.21–0.24	0.25–0.27
Ratio between oesophagus and ventriculus length	1:0.9–1:2.3	1:1.5–1:3.1	1:2.0–1:2.5	1:3.08–1:3.1	1:2.1–1:2.6	1:1.9–1:2.8	1:3.1–1:4.2	1:3.5–1:4.7
Tail length	0.04–0.14	0.05–0.12	0.10–0.25	0.12–0.14	0.18–0.22	0.18–0.28	0.14	0.17–0.21
Mucro length	0.02–0.03	0.02–0.03	Absent	Absent	Absent	Absent	Absent	Absent

3.3. In vitro-culture

Anisakis simplex (s.s.) and *A. pegreffii* successfully reached the adult stage *in vitro*. Both parasites moulted from L3 to L4 stage between 3 and 4 days of culture. Moulted from L4 to adult stage was observed between 27 and 41 days for both species. Moulted from L3 to L4 and from L4 to adult stage occurred not only on the outer cuticle but also on the oesophageal linings. During the *in vitro*-culture period of 60 days, spicules of adult males were not observed.

3.4. Morphological differences

One distinguishing morphological character that can differentiate *A. simplex* (s.s.) from *A. pegreffii* was found during the examination of the different morphological characters plotted in a graph in relation to the total body length of the worms. Among the morphological measurements examined from L3 to adult stages, clear difference was found in the ventriculus length (Fig. 2). As shown in Table 3 and Fig. 2, the ventriculus length did not increase as the worms grew in length

and developed from L3 larvae to adults. In any life-history stages examined, the ventriculus length was shorter in *A. pegreffii* than in *A. simplex* (s.s.), which ranged from 0.50 to 0.78 mm and 0.90–1.50 mm, respectively (Fig. 3). The shorter ventriculus in *A. pegreffii* reflected the ratio between oesophagus and ventriculus length, wherein *A. pegreffii* has higher oesophagus/ventriculus ratio (1:3.08–1:4.7) than those of *A. simplex* (s.s.) (1:1.9–1:2.8), with no overlapping ratios observed particularly during L4 and adult stages.

SEM examination revealed no morphological differences between *A. simplex* (s.s.) and *A. pegreffii* in L3 larvae collected from fishes and *in vitro*-cultured L4 larvae. Difference was only observed in adult males. Out of 36 specimens of *A. simplex* (s.s.) and 21 specimens of *A. pegreffii* examined under SEM, 8 and 11 specimens of the former and the latter were males, wherein out of these males, only 5 and 6 specimens of the former and the latter were clearly analyzed. Following the nomenclature of papillae from previous reports [17,24], the observed caudal papillae in all adult males examined are a median papilla, a pair of single proximal papilla, a pair of double procloacal papillae and four pairs of single distal papillae from the anus to the posterior end (Fig. 4B). No caudal

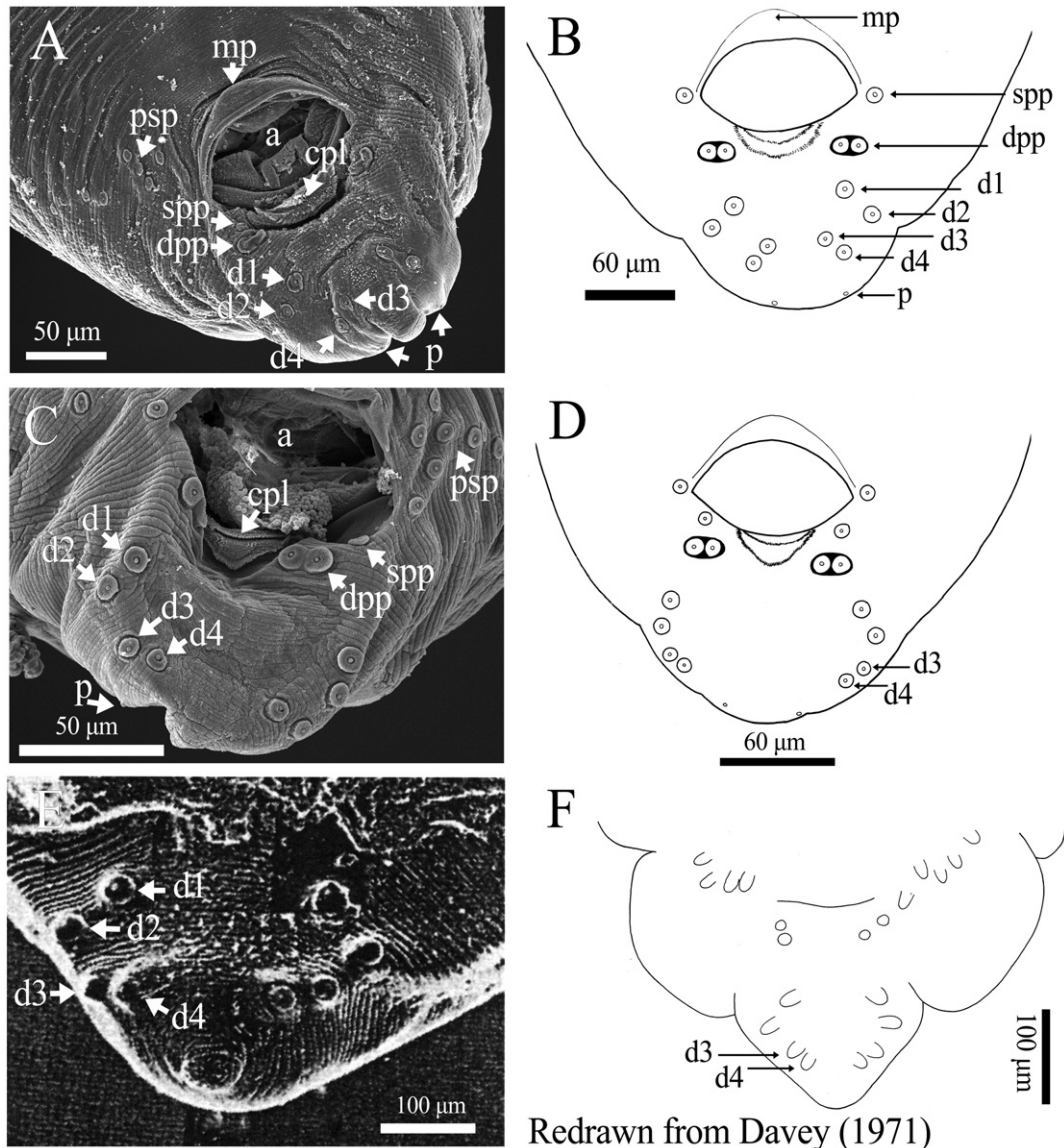


Fig. 4. Distribution patterns of caudal papillae in the *in vitro*-cultured adult males of *A. simplex* (s.s.) (A and B) and *A. pegreffii* (C and D) and that of *A. simplex* (E and F) reported by Davey (1971). Left and right rows are SEM micrographs and drawings based on SEM micrographs, respectively. Abbreviations: a, anus; cpl, caudal plates; d1, distal papilla no.1; d2, distal papilla no.2; d3, distal papilla no.3; d4, distal papilla no.4; dpp, double procloacal papillae; mp, median papilla; p, phasmid; psp, proximal subventral papillae; spp, single proximal papilla.

papillae were observed in females, but a pair of phasmids was observed on both male and female at the posterior most ends. Difference was observed in the distribution pattern of the caudal papillae, particularly in the location of the 3rd pair of distal papillae. In *A. simplex* (s.s.), the 3rd pair of distal papillae were located inside the 4th pair (Fig. 4A,B), while in *A. pegreffii*, the 3rd pair were located outside the 4th pair (Fig. 4C,D).

4. Discussion

The ventriculus length, oesophagus/ventriculus ratio and distribution pattern of caudal papillae, together with the body length and spicule length, are among the morphological characters in adults that were considered important in the diagnosis of anisakid nematodes and had been used specifically for species of *Anisakis* [3,17,24]. Based on the morphological examination of *A. simplex* (s.s.) and *A. pegreffii*, which were molecularly identified using PCR-RFLP and *mtDNA* *cox2* analyses, a clear difference was observed in the ventriculus length. As the difference was constant from L3 larvae to adults, the difference could be used as a major identification key for these two species. The ventriculus length had also been used as one criterion in differentiating *Anisakis* Type I larvae from *Anisakis* Type II larvae wherein the former has longer ventriculus length (0.65–1.5 mm) than the latter (0.52–0.75 mm) [10,11]. Based on these ranges of ventriculus length in *Anisakis* Type I larvae, it is expected that these are mixed specimens of *A. simplex* (s.s.) and *A. pegreffii*, especially if examined closely based on the observed ventriculus length of the former (0.90–1.50 mm) and the latter (0.50–0.78 mm) in the present study. However, such ranges of ventriculus length in the reported *Anisakis* Type II larvae also falls within the range of ventriculus length in *A. pegreffii*, but both could be differentiated on the absence or presence of mucro. Apart from ventriculus length, morphological difference was also observed in the oesophagus/ventriculus ratio. Since no increase in ventriculus length was observed as the total body length increased slightly during *in vitro*-culture, major difference on the oesophagus/ventriculus ratio, without any overlapping ratios, was observed only from L4 to adult stages. The ventriculus length and oesophagus/ventriculus ratio has been reported to differentiate *A. brevispiculata* from *A. paggiae* [17] and the present study shows that such morphological characters also applies in differentiating *A. simplex* (s.s.) from *A. pegreffii*.

In addition to ventriculus length and oesophagus/ventriculus ratio, morphological difference was also found in the distribution pattern of the caudal papillae. SEM examination revealed a remarkable difference between adult male *A. simplex* (s.s.) and *A. pegreffii*, particularly in the position of the 3rd pair of distal papillae. The position of the 3rd pair of distal papillae in *A. simplex* (s.s.) was similar to that of *A. ziphidarum* [14], while that of *A. pegreffii* was similar with that of *A. paggiae* [17]. However, *A. simplex* (s.s.) and *A. pegreffii* can be differentiated with *A. ziphidarum* and *A. paggiae* base on the difference on the locations of the 1st and 2nd pairs of distal papillae. There has been no reported distribution pattern of the caudal papillae in *A. pegreffii*. Even the original description also failed to describe it, since the specimen sent by Professor G. Pegreff for examination was preserved in lacto-phenol, which resulted in a deterioration of the entire cuticle and complete destruction of the papillae [23]. The distribution pattern of the caudal papillae in *A. simplex* was previously reported, in which the number and origin of the specimens examined were not described though [3] (Fig. 4E,F). This previously reported distribution pattern in *A. simplex* was almost identical to that of *A. pegreffii* observed in the present study. The current findings created confusion as to whether the reported distribution pattern of the distal papillae of *A. simplex* [3] was really that of *A. simplex* (s.s.) or rather that of *A. pegreffii* which was mistakenly identified as *A. simplex*.

In contrast to the above morphological characters that can be used to differentiate *A. simplex* (s.s.) from *A. pegreffii*, the spicules were not observed in adult males in the present study, which were obtained by *in vitro*-culture. In this regard, the reported overlapping spicule length

between *A. simplex* and *A. pegreffii* [3] was not confirmed. Matured adult females of *A. simplex*, which had begun ovoposition 97–143 days after *in vitro*-culture, have been previously reported [26], but any presence of spicules in adult males were not mentioned. It is possible that the worms in the present study were still young adults and required more time for their spicules to develop.

Based on the present results, specimens of *A. pegreffii* from fish and cetaceans in the Mediterranean should be re-examined. By doing so, *A. pegreffii* can be redescribed and characterized.

In conclusion, morphological characteristics distinguishing L3 larvae and adults of *A. simplex* (s.s.) and *A. pegreffii* were found for the first time in this study. The finding makes the distinction between the two species much easier and will contribute in the epidemiological studies on human and fish anisakiasis.

Acknowledgements

We would like to thank Dr. S. Urawa and Mr. A. Nakamura of the National Salmon Resources Center and Miyazaki Prefectural Fisheries Experimental Station, respectively, for collection of fish and parasite samples.

References

- Nascetti G, Paggi L, Orecchia P, Mattiucci S, Bullini L. Two sibling species within *Anisakis simplex* (Ascaridida: Anisakidae). *Parassitologia* 1983;25:306–7.
- Nascetti G, Paggi L, Orecchia P, Smith JW, Mattiucci S, Bullini L. Electrophoretic studies on the *Anisakis simplex* complex (Ascaridida: Anisakidae) from the Mediterranean and North East Atlantic. *Int J Parasitol* 1986;16:633–40.
- Davey JT. A revision of the genus *Anisakis* Dujardin, 1845 (Nematoda: Ascaridata). *J Helminthol* 1971;45:51–72.
- Templeman W, Squires HJ, Fleming AM. Nematodes in the fillets of cod and other fishes in Newfoundland and Neighbouring Areas. *J Fish Res Board Can* 1957;14:831–97.
- Scott DM, Martin WR. The incidence of nematodes in fillets of small cod from Lockeport, Nova Scotia, and the Southwestern Gulf of St. Lawrence. *J Fish Res Board Can* 1959;16:213–21.
- Novotny AJ, Uzman JR. A statistical analysis of the distribution of a larval nematode (*Anisakis* sp.) in musculature of chum salmon (*Oncorhynchus keta* Walbaum). *Exp Parasitol* 1960;10:245–62.
- Smith JW. The abundance of *Anisakis simplex* L3 in the body-cavity and flesh of marine teleosts. *Int J Parasitol* 1984;14:491–5.
- Wootton R, Waddell IF. Studies on the biology of larval nematodes from the musculature of cod and whiting in Scottish waters. *J Cons Int Explor Mer* 1977;37:266–73.
- Strømnes E, Andersen K. Distribution of whale worm (*Anisakis simplex*, Nematoda, Ascaridoidea) L3 larvae in three species of marine fish; saithe (*Pollachius virens* (L.)), cod (*Gadus morhua* L.) and red fish (*Sebastes marinus* (L.)) from Norwegian waters. *Parasitol Res* 1998;84:281–5.
- Berland B. Nematodes from some Norwegian marine fishes. *Sarsia* 1961;2:1–50.
- Koyama T, Kobayashi A, Kumada M, Komiya Y. Morphological and taxonomical studies on *Anisakinae* larvae found in marine fishes and squids. *Jpn J Parasitol* 1969;18:466–87 (In Japanese with English abstract).
- Mattiucci S, Nascetti G, Cianchi R, Paggi L, Arduino P, Margolis L, et al. Genetic and ecological data on the *Anisakis simplex* complex with evidence for a new species (Nematoda, Ascaridoidea, Anisakidae). *J Parasitol* 1997;83:401–16.
- Mattiucci S, Paggi L, Nascetti G, Ishikura H, Kikuchi K, Sato N, et al. Allozyme and morphological identification of *Anisakis*, *Contracaecum* and *Pseudoterranova* from Japanese waters (Nematoda, Ascaridoidea). *Syst Parasitol* 1998;40:81–92.
- Paggi L, Nascetti G, Webb SC, Mattiucci S, Cianchi R, Bullini L. A new species of *Anisakis* Dujardin, 1845 (Nematoda, Anisakidae) from beaked whales (Ziphiidae): allozyme and morphological evidence. *Syst Parasitol* 1998;40:161–74.
- Mattiucci S, Paggi L, Nascetti G, Abollo E, Webb SC, Pascual S, et al. Genetic divergence and reproductive isolation between *Anisakis brevispiculata* and *Anisakis physeteris* (Nematoda: Anisakidae). *Int J Parasitol* 2001;31:9–14.
- Mattiucci S, Paggi L, Nascetti G, Santos CP, Costa G, Di Benedetto AP, et al. Genetic markers in the study of *Anisakis typica* (Diesing, 1860): larval identification and genetic relationships with other species of *Anisakis* Dujardin, 1845 (Nematoda: Anisakidae). *Syst Parasitol* 2002;51:159–70.
- Mattiucci S, Nascetti G, Dailey M, Webb SC, Barros NB, Cianchi R, et al. Evidence for a new species of *Anisakis* Dujardin, 1845: morphological description and genetic relationships between congeners (Nematoda: Anisakidae). *Syst Parasitol* 2005;61:157–71.
- Mattiucci S, Nascetti G. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. *Parasite* 2006;13:99–113.
- Valentini A, Mattiucci S, Bondanelli P, Webb SC, Mignucci-Giannone AA, Colom-Llavina MM, et al. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial *cox2* sequences, and comparison with allozyme data. *J Parasitol* 2006;92:156–66.

- [20] Abe N, Ohya N, Yanagiguchi R. Molecular characterization of *Anisakis pegreffii* larvae in Pacific cod in Japan. *J Helminthol* 2005;79:303–6.
- [21] Umehara A, Kawakami Y, Matsui T, Araki J, Uchida A. Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. *Parasitol Int* 2006;55:267–71.
- [22] Umehara A, Kawakami Y, Araki J, Uchida A. Multiplex PCR for the identification of *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and other anisakid nematodes. *Parasitol Int* 2008;57:49–53.
- [23] Campana-Rouget Y, Biocca E. Une Nouvelle Espèce D'*Anisakis* Chez un Phoque Méditerranéen. *Ann Parasitol* 1955;30:5–6 (In French).
- [24] Fagerholm HP. Patterns of caudal papillae in *Contracaecum osculatatum* (Nematoda) and some related species from different regions of the world. *Int J Parasitol* 1988;18:1039–51.
- [25] Iglesias L, Valero A, Adroher FJ. Some factors which influence the *in vitro* maintenance of *Anisakis simplex* (Nematoda). *Folia Parasitol* 1997;44:297–301.
- [26] Iglesias L, Valero A, Benitez R, Adroher FJ. *In vitro* cultivation of *Anisakis simplex*: pepsin increases survival and moulting from fourth larval to adult stage. *Parasitology* 2001;123:285–91.
- [27] D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanço M, et al. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. *Int J Parasitol* 2000;30:223–6.
- [28] Pontes T, D'Amelio S, Costa G, Paggi L. Molecular characterization of larval anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence for a new species. *J Parasitol* 2005;91:1430–4.
- [29] Nadler SA, Hudspeth DSS. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *J Parasitol* 2000;86:380–93.
- [30] Abollo E, Paggi L, Pascual S, D'Amelio S. Occurrence of recombinant genotypes of *Anisakis simplex* s.s. and *A. pegreffii* (Nematoda: Anisakidae) in an area of sympatry. *Infect Genet Evol* 2003;3:175–81.
- [31] Martín-Sánchez J, Artacho-Reinoso ME, Díaz-Gavilán M, Valero-López A. Structure of *Anisakis simplex* s.l. populations in a region sympatric for *A. pegreffii* and *A. simplex* s.s. Absence of reproductive isolation between both species. *Mol Biochem Parasitol* 2005;141:155–62.