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Research article

Anisakis simplex (Nematoda: Anisakidae) third-stage larval infections of marine cage cultured cobia, *Rachycentron canadum* L., in Taiwan

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ABSTRACT

The first confirmed case of *Anisakis simplex* infection of the marine cage cobia, *Rachycentron canadum* (L.), was recorded in Taiwan. The case investigation revealed the presence of third-stage larvae (L3) in either the stomach lumen or abdominal cavity of the cobia but never within the musculatures. Larvae were mainly encapsulated in the peritoneal mesentery on the outer surface of the stomach wall and occasionally on the liver surface. Part of the diet fed to the cobia includes chopped raw fish, and of these, seven species were found to harbor these larvae (as paratenic hosts), indicating that these particular fish might be the larval sources for this infection. To illustrate the course of infection and distribution of this parasite inside cobia, both juvenile and adult cobia were experimentally infected with live L3 by oral transmission. The prevalence of infection reached 100% at the end of all trials. The course of the infection was assessed after necropsy by histological and ultrastructural observations. *A. simplex* L3 recovered from various locations within juvenile cobia at different post-infection (p.i.) times were at the L3 stage and did not grow significantly. The L3 either adhered to or penetrated into the gastric mucosa of cobia by 2 h p.i. By 25 d p.i., many were trapped within the submucosa and encapsulated by fibroconnective tissue. This phenomenon was more apparent in adult cobia, such that 37.5–86.0% of the injected L3 were primarily found encapsulated within the gastric submucosa. Based upon a PCR-RFLP assay, the larvae encountered in this study were identified as having a recombinant genotype of *A. simplex* sensu stricto and *A. pegreffii*. Based upon the results of this study, strategies to ensure the safety of seafood manufactured from cobia and to prevent the potential risks of anisakiasis or allergies risk to consumers were suggested.

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1. Introduction

When a given fish species is reported as one of the paratenic hosts in the life history of *Anisakis simplex*, two aspects of the situation become important to humans: esthetics and public health (Schmidt and Roberts, 2000). The first aspect relates to the disgust experienced by per-

sons who find worms in the fish meals that they are preparing or eating. The latter and more-important aspect concerns the pathological conditions caused by penetration or migration of the *A. simplex* larvae, which is referred to as anisakiasis in humans. Acute gastric anisakiasis, which is the most frequent clinical entity, is accompanied by symptoms of epigastric pain, nausea, and vomiting. In addition, the allergenic potential of *A. simplex* has recently been recognized (Moreno-Ancillo et al., 1997). Although patients typically have recurrent hypersensitivity symptoms after the intake of various fish, they have neither

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skin prick test (SPT) responses nor specific IgE for the implicated fish. Since the involvement of an IgE-mediated response that is specific for *A. simplex* has been proven, an *A. simplex* allergy has been described as a distinct clinical entity that is independent of parasitism (Daschner et al., 2000).

Cobia, *Rachycentron canadum* (Linnaeus), is a highly prized, commercially important fish found worldwide from the Indo-Pacific waters to the southern Atlantic Ocean (Bunkley-Williams and Williams, 2006). It has been farmed in Taiwan since the early 1990s, and currently, nascent cobia aquaculture operations can be found throughout Southeastern and Eastern Asia as well as in the Gulf of Mexico, the Caribbean Sea, and the United States (McLean et al., 2008). Many other nations are presently considering adopting cobia as a new species for aquaculture. The interest in the species has increased due in large part to its many excellent characteristics, including good growth, with production 6-kg live weight fish being possible over a year-long production cycle (Liao et al., 2004; McLean et al., 2008). Additionally, cobia is regarded as having the highest potential for net cage aquaculture, and around 80% of marine cages are presently devoted to cobia culture in Taiwan (Liao et al., 2004).

A dormant threat of anisakid nematode infection to the cage farming industry in Taiwan had been pronounced, as heavy infections with *Hysterothylacium aduncum* and many other nematodes, such as *A. simplex*, *Porrocaecum decipiens*, and *Raphidascaris trichiuri*, had been commonly discovered in cutlass fish and other wild fish around Taiwanese waters (Shih and Jeng, 2002; Shih, 2004). Furthermore, as early as the 1960s, *Anisakis*-type larvae were recorded from both chondrosts, including *Squalus megalops*, *Galeorhinus japonicus*, and *Raja hollandi*, and a few teleosts, including *Caranx (Selaroides) leptolepis*, *Rastrelliger kanagurta*, *Scomber japonicus*, *Mene maculata*, *Sparus macrocephalus*, *S. sarba*, and *Priacanthus cruentatus*, taken around Taiwan and its offshore islands (Myers and Kuntz, 1967). These concerns became a reality when troubles with the nematode infection occurred in cage-farmed cobia in the Penghu archipelagos, which are located in the Taiwan Strait, where cage cultures are centralized.

This study investigated several aspects: (1) the first case of *A. simplex* infestation in cobia from Taiwan; (2) the possible larval sources for this infection; and (3) the distribution of *A. simplex* larvae inside juvenile and adult cobia by means of experimental infections.

2. Materials and methods

2.1. Field investigations of cobia infection and *A. simplex* larvae sources

Interviews with the cobia cage farm where the nematode infections occurred revealed the practice of occasionally feeding cobia with chopped fish before harvest. From March to May 2004, six specimens of *R. canadum* (body length 60 cm; body weight 3 kg) from the cage farm where chopped raw fish were frequently fed to cobia, along with specimens of the local raw fish, were collected and brought to the laboratory for careful examination. The body

cavity, gastrointestinal tract, belly flaps, and musculature of the cobia were examined for larval *A. simplex* and other metazoan parasites under a stereomicroscope. The raw fish were identified, measured (body length), and examined the same manner. Nematode larvae were fixed, stored in 70% alcohol, and cleared in glycerin before examination. Third-stage larvae (L3) of *A. simplex* complex were first identified by the following morphological characters: (1) the shape and the presence of the boring tooth; (2) the shape of the tail and the presence of the mucron; (3) the presence of a long ventriculus and the absence of either intestinal cecum or ventricular appendage; (4) the position of excretory pore (Smith, 1983; Anderson, 2000; Shih, 2004).

2.2. Experimental infection of cobia

Two groups of cobia at different stages were used for experimental infection trials. Juvenile cobia were used to monitor the early migration of the L3 inside this host and the time course of the short-term migration. Adults were used to reveal the larval distributions over a long-term time course. For the first trial, twelve juvenile cobia (each weighing approximately 140 g) bred by the fish farm at the National Taiwan Ocean University in Keelung, Taiwan and never fed with raw fish were transferred to the laboratory. The fish were acclimated for a week in a 250-L tank. The tank was aerated and supplied with seawater, and the temperature was maintained at $26 \pm 1^\circ\text{C}$ using a heating device throughout the trial. The fish were fed a mixture of commercial white fishmeal and water that was freshly prepared to be small enough to fit the mouth of the fish. They were fed twice a day at a daily feeding rate of 3% of total body weight. After acclimation, nine fish were orally administered live *A. simplex* L3 through their diet, and the three remaining fish were used as controls. Each fish was infested with a total number of 30 L3. The L3 used in this trial were collected from the gastrointestinal tract lumen of the cutlass fish, *Trichiurus lepturus*, from commercial catches. All of the cobia were successfully fed, with no observed regurgitation. At 2 h, 24 h, and 25 d after exposure to the larvae, three fish were randomly sampled, and their gastrointestinal tracts, body cavities, and muscles were examined. Larvae were collected, identified, and measured for body length by using a calibrated ocular micrometer. The data were analyzed using Duncan's new multiple range tests to identify the statistical difference.

The second group included eight cobia adults (each weighing approximately 6 kg) from a cage farm in Penghu, Taiwan where raw fish were never fed to cobia. After acclimatization, six of them were fed as described above over three consecutive days, so that each fish was infested with a total number of 200 L3. The remaining two fish served as controls. Cobia were sacrificed and examined for larval *Anisakis* distributions 102 days post-infection (d p.i.). Each fish was filleted and candled. The muscle surrounding the body cavity was carefully examined by simultaneously employing two tweezers and tearing the muscle into individual fibers. The viscera were examined under a dissecting microscope. The stomach walls were cut into pieces and

then torn apart to collect any existing capsules. The number and location of larvae were recorded.

2.3. Histological observation

Stomach specimens from the juvenile cobia in the first trial were fixed in 10% buffered formalin and processed for paraffin sectioning. The sections were stained with hematoxylin and eosin (H&E).

2.4. Ultrastructural observation

For scanning electron microscopy (SEM), larvae were fixed with 1% glutaraldehyde for 12 h and post-fixed with OsO_4 for 1 h. They were then dehydrated, critical point-dried, and ion sputter-coated. Mounted larvae were observed under a Hitachi S2500 scanning electron microscope.

2.5. Molecular delineation of *Anisakis simplex* third-stage larvae (L3)

The species identity of the encapsulated *A. simplex* L3 that were collected from fish body cavities were identified to species by a PCR-linked restriction fragment length polymorphism (RFLP) analysis, as previously described (D'Amelio et al., 2000; Abollo et al., 2003). Larvae from three groups of fish samples were assayed, including the confirmed cobia case, the cutlass fish used to feed cobia, and the experimentally infected juvenile cobia. Ten individual larvae from each group were randomly picked and assayed as described.

The same protocols and conditions for the PCR-RFLP analysis of ribosomal DNA (rDNA) internal transcribed spacers (ITS-1 and ITS-2) were followed in the present study. Two endonucleases, *Hinf*I and *Hha*I, were used for delineation of the three sibling species of the *A. simplex* complex, which are *A. simplex sensu stricto*, *A. pegreffii*, and *A. simplex* C.

3. Results

3.1. Field investigations of cobia infection

Based upon the outer and inner morphology, these cobia samples looked healthy and active at the time of collection. All specimens were infected, and *A. simplex* complex L3 (Fig. 1) were identified to be the most abundant parasitic form, although a few adult digeneans belonging to the Hemiuiridae family were also observed (data not shown). The larvae were found either in the stomach or abdominal cavity of the cobia but never in their musculatures. Larvae inside the stomach of a given specimen were either free or penetrating into the fish mucosa (Fig. 2a). After invasion of the body cavity, L3 were mainly encapsulated in the peritoneal mesentery on the outer surface of the stomach wall (Fig. 2b) and occasionally on the liver surface (Fig. 2c) of the cobia. The intensity of infection, excluding the entrapped larvae inside the stomach walls ranged from approximately 16 to 45 larvae per host.

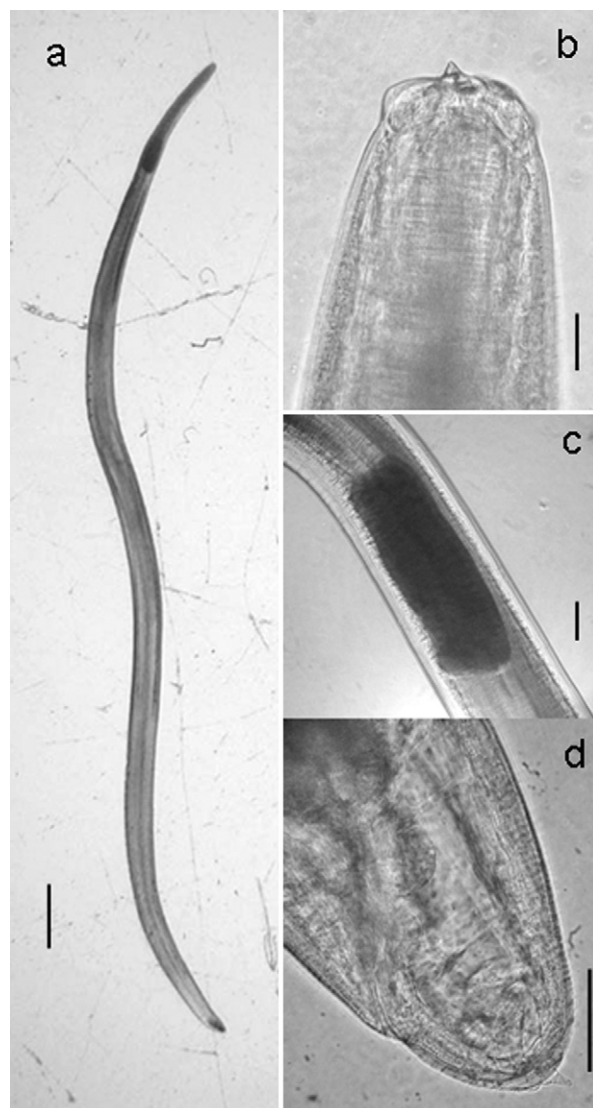


Fig. 1. Morphology of *Anisakis simplex* third-stage larvae collected from farmed cobia. (a) A complete larva. Scale bar = 0.6 mm. (b) Cephalic end. (c) Ventricular part. (d) Caudal end. Scale bars = 50 μm in (b–d).

3.2. Field investigations of *A. simplex* larvae sources in cobia infection

Among the chopped raw fish that were fed to the cobia, seven species, including *Egraulis japonica*, *Etrumeus teres*, *Decapterus maruadsi*, *Megalaspis cordyla*, *Rastrelliger faughni*, *T. lepturus*, and *Trachurus japonicus*, were identified as the reservoirs or paratenic hosts of the *A. simplex* larvae (Table 1). Third-stage larvae were found either free in host gastrointestinal (GI) tracts or encapsulated on the external surfaces of the host digestive tract and other visceral organs. The prevalence of infection varied, with the lowest prevalence being 25.0% in *T. japonicus* and the highest being 92.8% in *T. lepturus*. Mean intensities ranged from 1.0 in *T. japonicus* to 25.92 ± 7.58 in *T. lepturus* (Table 1). Because the cutlass fish, *T. lepturus*, was the most heavily

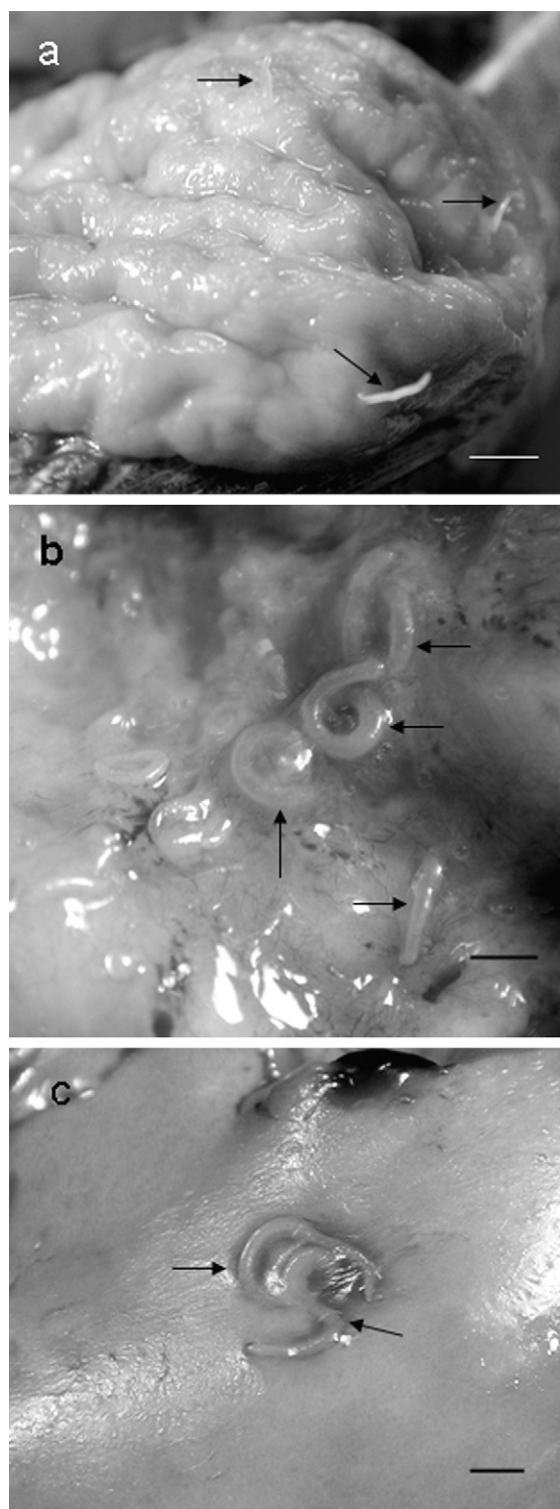


Fig. 2. *Anisakis simplex* third-stage larvae infection of the cobia, *Rachycentron canadum*. (a) Larvae (arrows) inside the stomach of cobia penetrating the fish mucosa. Scale bars = 5 mm. For proper visualization, the stomach was turned inside out. Encysted larvae (arrows) were located mainly in mesentery tissue (b) and occasionally in the liver (c). Scale bars = 2 mm in (b and c).

infected fish species in this investigation, they were used as the larvae source for the experimental infection of cobia.

3.3. Experimental infection of juvenile cobia and the distribution of *A. simplex* larvae

In the first trial, the prevalence of infection reached 100% in experiment group, but no L3 were recovered from the control group. After ingestion by juvenile cobia, some *A. simplex* L3 successfully penetrated the stomach walls and reached into the body cavities. They did not grow significantly and never developed further (Table 2). Third-stage larvae were recovered from various locations at different p.i. times (Table 2). Although most larvae resided in the GI tract 2 h p.i., the anterior ends of some larvae were attached to or embedded in the gastric mucosa, which was the likely entry point (Fig. 3a). Larvae invaded the body cavity after successful penetration through the stomach wall. Within the coelom, they were frequently found free but occasionally bored into the hypaxial muscle by 24 h p.i. (Fig. 3b). No boring larvae were observed to have embedded themselves entirely within the muscle tissue. Larvae encysted both just under the peritoneal membrane and in the mesenteries were observed 25 d p.i. (Fig. 3c).

Many larvae were trapped and failed to penetrate the stomach wall of the juvenile cobia. All these larvae were encapsulated within the stomach submucosa, but not the mucosa or muscularis, by granulomatous reactive tissue 25 d p.i. (Fig. 4a). There was a translucent space between the parasite cuticle and host tissue in all infected stomachs (Fig. 4a and b). Capsules were often distributed densely, and sometimes as many as nine capsules appeared within a 5 mm × 7 mm section (partial section, Fig. 4b).

All larvae recovered from various sites of juvenile cobia at different p.i. time intervals morphologically resembled third-stage larvae (Fig. 5), indicating that molting did not occur inside this fish. A conspicuous boring tooth was always present at the anterior extremity of each worm. Young L3 characterized by poorly developed lips or only low bulges with papillae present on their heads were harvested either from the GI tract at 2 h p.i. (Fig. 5a and b) or the body cavity at 24 h p.i. (Fig. 5c and d). Late L3 with more developed lips were only found from those encapsulated on the mesenteries at 25 d p.i. (Fig. 5e and f).

3.4. Experimental infection of adult cobia and the distribution of *A. simplex* larvae

After 102 days of exposure of adult cobia to the L3, the prevalence of infection reached 100% in the experimental groups, but no L3 were recovered from the two control fish. Infection intensities ranged from 16 to 86 (Table 3). The L3 were primarily found encapsulated within the gastric submucosa, ranging from 37.5% to 86.0%. The secondary site of residence was encapsulation on the mesenteries, ranging from 4.0% to 45.0%.

3.5. Molecular delineation of *Anisakis simplex* larvae

The results of the PCR-RFLP analysis were the same for all thirty randomly sampled larvae, with an example shown

Table 1Investigation of *Anisakis simplex* third-stage larvae infections in raw fish fed to cage cultured cobia.

Fish species	Samples	Body length (cm)	Prevalence (%)	Mean intensity ^a
<i>Egraulis japonica</i>	22	10.0–15.9	68.2	21.60 ± 7.46
<i>Etrumeus teres</i>	70	7.0–18.0	25.7	4.78 ± 2.34
<i>Decapterus maruadsi</i>	28	13.0–16.5	53.6	7.80 ± 4.57
<i>Megalaspis cordyla</i>	11	20.0–23.5	72.7	10.25 ± 3.41
<i>Rastrelliger faughni</i>	3	16.5–18.0	66.7	3.50 ± 0.71
<i>Trichiurus lepturus</i>	14	45.0–58.0	92.8	25.92 ± 7.58
<i>Trachurus japonicus</i>	4	20.5–21.5	25.0	1.00

^a Values are mean and standard deviations.**Table 2**Distribution and larval growth of *Anisakis simplex* third-stage larvae (L3) in juvenile cobia at different time intervals post-infection (p.i.) and larvae growth.

Time p.i.	Locality of L3 recovered	L3 body length (mm) ^a
2 h	Free in the gastrointestinal (GI) tract	10.07 ± 1.85
24 h	Free in the GI tract or the body cavity	12.63 ± 0.51
25 d	Encapsulated within the stomach submucosa or on the mesenteries	13.68 ± 1.16

^a Values are mean and standard deviations for four L3 from each group. They are not significantly different from each other.

in Fig. 6. Amplification of the rDNA region, spanning the ITS-1, ITS-2, and the 5.8S subunit, produced a fragment of ~900 base pairs (bp) (Fig. 6, lane 1). Restriction digests with *HinfI* produced four fragments of approximately 620, 370, 300, and 250 bp (lane 2), and those with *HhaI* produced two major fragments of 550 and 430 bp (lane 3), respectively. According to the taxonomic key based upon these two restriction enzymes for the identification of species and sibling species of the genus *Anisakis* (D'Amelio et al., 2000; Abollo et al., 2003), the larvae collected and used herein were identified to have a recombinant genotype of *A. simplex* sensu stricto and *A. pegreffii*.

4. Discussion

This is the first confirmed report of *A. simplex* larvae infestation in cage-farmed cobia in Taiwan. Recently, parasites infected cobia were reviewed by McLean et al. (2008), who observed that there were only a few nematodes that were recovered or reported in cobia. These included *Iheringascaris inquires* from the stomach of cobia caught in Australian waters (Bruce and Cannon, 1989), which may be synonymous with *Contracaecum megacephalum* from the stomach of a cobia taken from the South China Sea, *Mabiarara prevesiculata* derived from the stomach of cobia taken in Brazil (Bunkley-Williams and Williams, 2006), and *Anisakis* sp. and *Philometroides* sp. from cobia in Vietnamese waters (Arthur and Te, 2006). In addition, a new species, *Philometroides mariuns*, was described from the body cavity (surface of gonads) of wild cobia captured off the Atlantic coast of South Carolina, U.S. (Moravec and de Buron, 2009). Currently, information on *A. simplex* larvae infections in captive cobia is only described in the present study.

The possible sources of this parasite inside cage-farmed cobia were the chopped mixture meals that were prepared from unfrozen, fresh raw fish or residuals, which were occasionally fed to the cobia to provide additional nutrients. The fact that these local raw fish showed both a high infection prevalence and intensity of *A. simplex* larvae indicated a current potential threat to sea farmed cobia and related aquatic products in Taiwan as well as other nations presently culturing cobia or considering adopting cobia as a new species for aquaculture. Raw-fish dishes made from cobia, like sushi and sashimi, have recently become popular among consumers. On a positive note, the muscle encapsulation of these larvae was neither observed from the present cobia case nor the experimentally infected cobia used in this study. Unfortunately, removing parasites adds appreciable costs to packaging, which reduces the value of the product and represents a financial loss in marketing values (Abollo et al., 2001).

The results of the present study demonstrate that *A. simplex* L3 can infect cobia if ingested by the fish. For the first time, the cobia is added to the teleost paratenic host list of *A. simplex* based upon observations that L3 larvae that are transmitted orally to cobia remained at the same developmental stage regardless of the location of their migration. Ontogenetic migration, a process accompanied by growth and development of the nematode, did not occur. In addition, these observations consist with the life cycle of *A. simplex* during which seafish and cephalopods usually play the role of the paratenic host of this nematode (Audicana et al., 2002).

The gut wall as a site of penetration by *Anisakis* sp. larvae in the brown trout, *Salmo trutta*, at different time intervals after experimental infection has been reported (Wootton and Smith, 1975). From 2 to 4 h p.i., penetrating larvae were observed in the region of the stomach and cecum. After 6–10 h, penetration also occurred in the post-cecal intestine. At 12 and 24 h p.i., penetration occurred only in this part of the gut. In addition, a number of larvae could successfully penetrate the body cavity of the trout by 2 h p.i., although most larvae penetrated within 24 h. *A. simplex* L3 were not able to penetrate the gut wall of cobia as quickly as they did in brown trout. Most larvae orally infected in the cobia remained inside the GI tract, especially the stomach, with only a few beginning to penetrate the stomach mucosa by 2 h p.i., which was the only site of penetration observed herein. Penetration of the gut wall in cobia was generally almost completed within 24 h. Most larvae recovered from the body cavity of cobia were located on the surface of the stomach and the surrounding mesentery. Distribution of

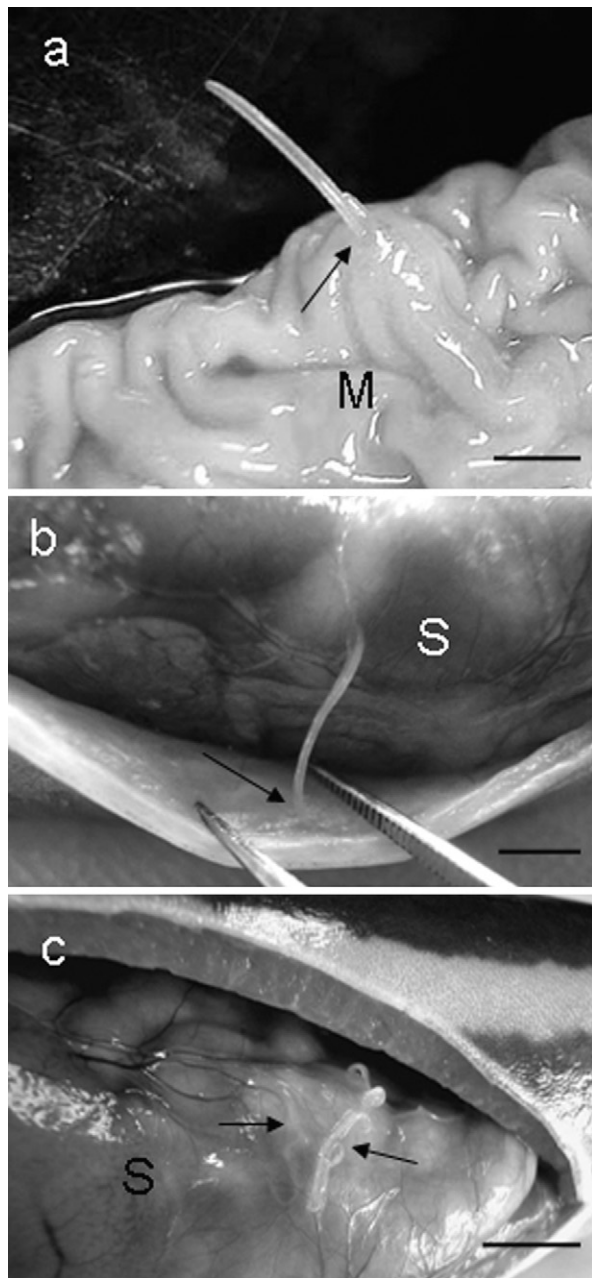


Fig. 3. Experimental infection of *Anisakis simplex* third-stage larvae in juvenile cobia. (a) Example of a larva (arrow) boring its anterior end into the stomach mucosa 2 h post-infection (p.i.). The stomach was cut open and turned inside out to show the mucosa. Scale bar = 1 mm. (b) Example of a larva (arrow) boring into the hypaxial muscle 24 h p.i. Scale bar = 1 mm. (c) Larvae encapsulated under the peritoneal membrane together with the mesenteries (arrows) 25 d p.i. Part of the fish's abdominal muscle was cut away (asterisk). Scale bar = 5 mm. M, mucosa; S, stomach.

the larvae in cobia likely reflects the site of penetration. In the cobia, the destination of the *A. simplex* L3 appeared to be encapsulated within the submucosa of stomach or on the mesenteries, which occurred by 25 d p.i.

A model that describes *A. simplex* L3 distribution in fish based on the biochemical environment within host species

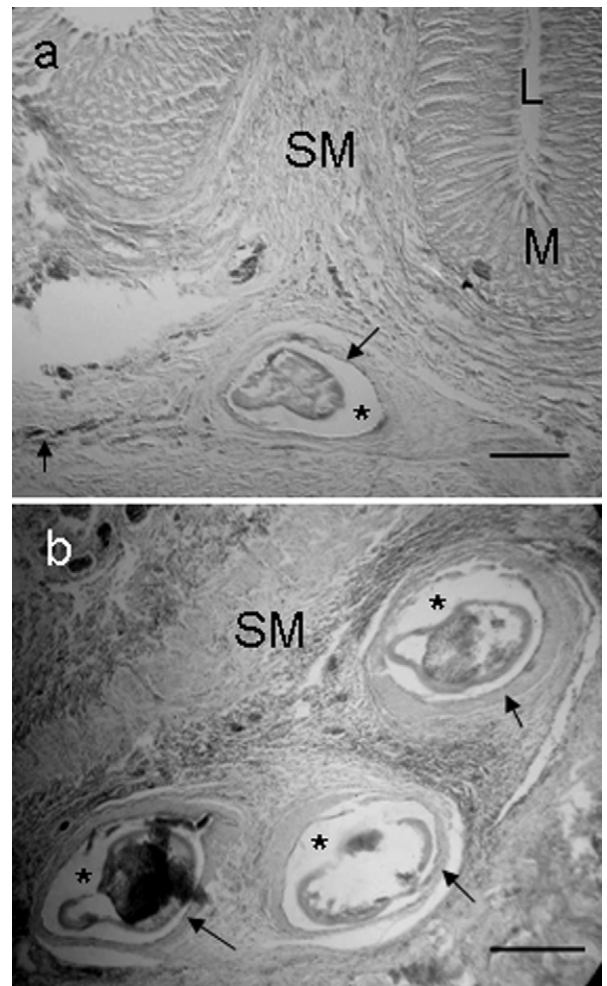


Fig. 4. *Anisakis simplex* third-stage larvae encapsulated in the stomach submucosa of experimentally infected juvenile cobia 25 d p.i. (a) Example of an encapsulated larva (arrow) located within the submucosa adjacent to the mucosa and the lumen of the stomach. Scale bar = 0.2 mm. (b) Three encapsulated larvae (arrows) located closely within submucosa. Scale bar = 0.2 mm. In both (a and b), there are white spaces around each larva (asterisks). L, lumen; M, mucosa; SM, submucosa.

has been suggested (Stromnes and Andersen, 1998). In this model, the distribution patterns between muscle and viscera were not significantly affected by host size but instead were governed by the conditions encountered within host tissues, which was possibly related to the availability of nutrients. This model does not support an earlier hypothesis that stated that *A. simplex* L3 distributions were determined by an optimal pre-encapsulation migratory distance within host tissues (Young, 1972). Instead, the more recent model was supported by a study of *A. simplex* L3 distribution among three marine fish. Results showed that L3 occurred more frequently in the muscle of redfish, *Sebastes marinus*, than in the muscle of either cod, *Gadus morhua*, or saithe, *Pollachius virens*. This observation was attributed to the greater fat reserves in the muscle tissue of the redfish (Stromnes and Andersen, 1998).

The distribution of *A. simplex* L3 observed in both juvenile and adult cobia hosts is likely dependent on the

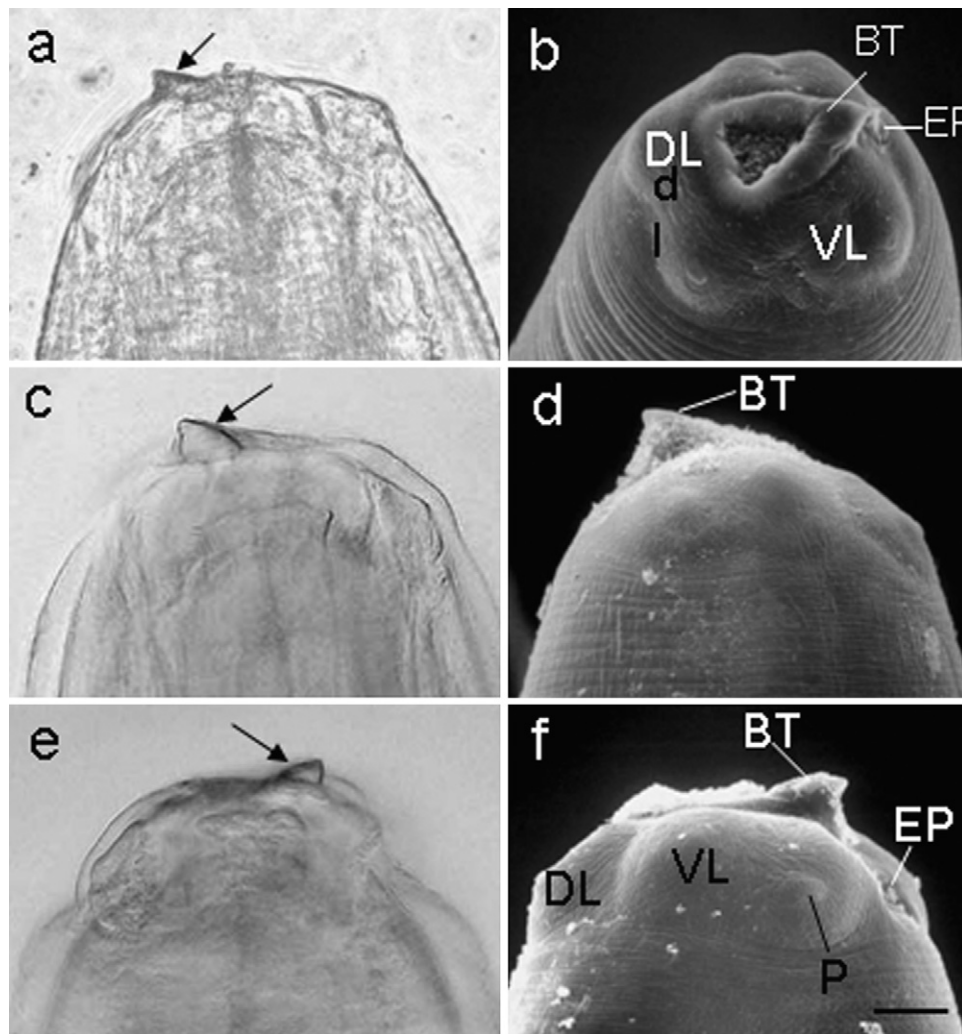


Fig. 5. Morphology of the anterior extremities of *Anisakis simplex* L3 harvested from different locations at various time points over the course of the infection. (a and b) Larva within the GI tract 2 h p.i. (c and d) Larva freely distributed in the abdominal cavity 24 h p.i. (e and f) Larva encapsulated in the mesentery with more developed lips 25 d p.i. Micrographs taken from a phase contrast microscope (a, c, e) or SEM (b, d, f). A conspicuous boring tooth of each larva is indicated with an arrow. Scale bar = 0.01 mm. BT, boring tooth; DL, dorsal lip; EP, excretory pore; P, papilla; VL, ventrolateral lip.

random migration of larvae radiating from the stomach. The cobia is likely not an ideal paratenic host of *A. simplex* L3 based upon observations that relatively large proportions of larvae failed to penetrate the stomach wall of cobia, instead being trapped within thick capsules localized to the

fish gastric submucosa. Larger cobia might hold a greater percentage of their fat reserves in their muscle tissue due to consumption of high fat feed (16–18% fat in total weight) that is supplied to adult cobia weighing over 2 kg in Taiwan. Since L3 occurred in the muscle of cobia was not ever

Table 3
Distribution of *Anisakis simplex* third-stage larvae (L3) in adult cobia 102 d p.i.

Cobia no.	Total no. of L3 recovered	Distribution of L3 (%)			
		Body cavity ^a	Gastric submucosa ^b	Mesentery ^b	Liver ^b
1	16	12.5	37.5	4.0	0
2	80	0	55.0	45.0	0
3	86	0	86.0	14.0	0
4	30	13.3	46.7	33.3	6.7
5	46	0	52.2	43.5	4.3
6	68	8.8	73.5	14.7	2.9

^a Free.

^b Encapsulated.

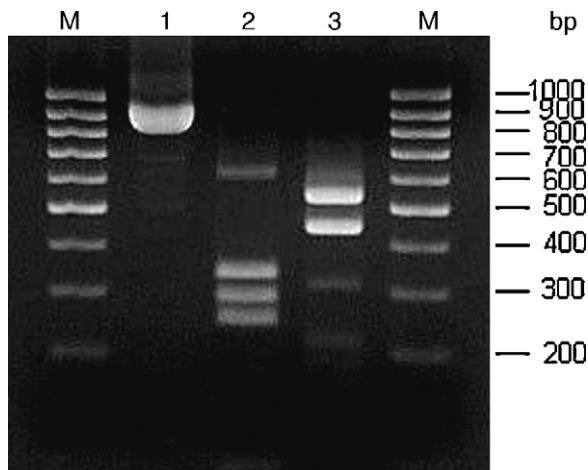


Fig. 6. Molecular delineation of the *Anisakis simplex* species used in the present study by PCR-linked restriction fragment length polymorphism analysis. The undigested PCR product amplified from the genomic DNA of *A. simplex* L3 representing the rDNA region spanning the ITS-1, the 5.8S gene and the ITS-2 (lane 1); RFLP patterns of the product using the endonucleases *Hinf*I (lane 2) or *Hha*I (lane 3). M: DNA marker (100-bp ladder).

observed in this study, the likelihood that entrance into the fish muscle by these larvae does occur and the potential factor(s) that would induce such a situation or affect the distribution inside cobia require further investigation.

Polymerase chain reaction-based restriction fragment length polymorphism analysis (PCR-RFLP) was performed to establish genetic markers of rDNA, in order to identify the three sibling species of the *A. simplex* complex as well as other morphologically differentiated *Anisakis* species, such as *A. physeteris*, *A. schupakovi*, *A. typica*, and *A. ziphidarum* (D'Amelio et al., 2000). The morphospecies of *A. simplex* L3 collected from cultured cobia and the cutlass fish caught from Taiwan Strait were identified herein to have a recombinant genotype of *A. simplex sensu stricto* and *A. pegreffii* according to the PCR-RFLP pattern that corresponded to the banding pattern of these two sibling species (Abollo et al., 2003). *A. simplex sensu stricto* is widespread between 30°N and the Arctic polar circle, and *A. pegreffii* is widely distributed in the Australian region and the Mediterranean Sea (Mattiucci et al., 1997). This recombinant was first described from anisakid populations collected from fish and stranded cetaceans along Iberian Peninsula waters and was suggested to be a product of natural interspecific hybridization (Abollo et al., 2003). Its occurrence from the Taiwanese coastal waters of the Northwest Pacific is reported for the first time in the present study. Since the taxonomy of these sibling species of the *A. simplex* complex based on genetic markers is still operational, worms encountered in the present study are described as *A. simplex*.

PCR-RFLP analysis of rDNA (ITS1 and ITS2) and the 5.8 subunit rRNA gene had been successfully employed for the identification of these *Anisakis* species (Abollo et al., 2001, 2003; Abe et al., 2006). Furthermore, polymorphism obtained by RFLP analysis of mtDNA *cox1* was also proved the usefulness as new genetic markers to distinguish *A.*

simplex s. s. and *A. pegreffii* (Umehara et al., 2006). Since these assays are carried out on individual worms, the question about how many samples or with what proportion of population, especially for long-term investigations which frequently deal with thousands of samples, are sufficient enough to reveal the exact infrastructure of the population and/or community is emerged and need to be clarified.

In contrast to the dominance of this recombinant genotype in Taiwanese waters, distribution and abundance of it and two species which it hybridized from, namely *A. simplex s.s.* and *A. pegreffii*, were quite different as reported from a geographical proximity, the Japanese waters (Umehara et al., 2006). The recombinant was so rare that only 6% adult worm from northern Japanese coastal waters and 3% larva from southern Japan were identified to be this species. On the contrary, 100% larvae and 94% adults collected from northern Japan were identified as *A. simplex s.s.* and 97% larvae from southern Japan were identified as *A. pegreffii* (Umehara et al., 2006). The conflicted results reported from Taiwanese and Japanese waters might due to the differences of fish species and/or stock sampled, sampling locality, and season. The distribution of *Anisakis* species around these regions seems still obscure and further studies to reveal that are eagerly needed.

Although there was no host fish mortality recorded and no musculature encapsulation observed in either the present case or experimentally infected cobia, attentions concerning the sea net cultivation of cobia and aquatic products production could be suggested based upon the results of this study. First, cultured cobia should not be fed with fresh, unfrozen raw fish to evade any sources of *A. simplex* larvae. In the case that feeding with fresh fish meals in cobia cultivation was firmly believed to be essential by the farmers, deep-frozen treatment at –20 °C or below for 7 d or –30 °C or below for 15 h, as suggested by the US FDA, of these fish must be performed beforehand. Second, visual examination along with early evisceration during preparations of cobia and their products should thus be carried out to avoid possible contaminations of visceral larval capsules to fish flesh. This precaution is based upon the fact that the wild fish caught from the same waters around the cobia net were also found to be the paratenic hosts of *A. simplex* L3, indicating that this parasite may be already endemic within these sea regions. Finally, cobia stomachs and livers, which are local delicacies in Taiwan, should be examined before preparation and ingestion, since the gastric submucosa was the primary location where abundant, and often live, L3 were usually encapsulated. Overall, the suggestion from this study is that *A. simplex* infections should be carefully monitored while cultivation or processing sea net farmed cobia to ensure the safety of this seafood and to prevent the potential risks of anisakiasis or allergies risk to consumers.

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The authors declare that all the experiments comply with the current laws of Taiwan.

References

- Abe, N., Tominaga, K., Kimata, I., 2006. Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of rDNA for identification of *Anisakis simplex* complex. Jpn. J. Infect. Dis. 59, 60–62.
- Abollo, E., Gestal, C., Pascual, S., 2001. *Anisakis* infestation in marine fish and cephalopods from Galician waters: an updated perspective. Parasitol. Res. 87, 492–499.
- Abollo, E., Paggi, L., Pascual, S., D'Amelio, S., 2003. Occurrence of recombinant Genotypes of *Anisakis simplex* s.s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. Infect. Genet. Evol. 3, 175–181.
- Anderson, R.C., 2000. Nematode Parasites of Vertebrates. Their Development and Transmission, 2nd ed. CABI Publishing, Wallingford, p. 650.
- Arthur, J.R., Te, B.Q., 2006. Checklist of the parasites of fishes of Viet Nam. FAO Fish Tech. Pap. 369/2, Rome, Italy, p. 133.
- Audicana, M.T., Ansotegui, I.J., de Corres, L.F., Kennedy, M.W., 2002. *Anisakis simplex*: dangerous-dead and alive? Trends Parasitol. 18, 20–25.
- Bruce, N.L., Cannon, L.R.G., 1989. *Hysterothylacium lheringascaris* and *Maricostula* new genus nematodes (Ascaridoidea) from Australian pelagic marine fishes. J. Nat. Hist. 23, 1397–1441.
- Bunkley-Williams, L., Williams, E.H., 2006. New records of parasites for culture cobia, *Rachycentron canadum* (Perciformes: Rachycentridae) in Puerto Rico. Rev. Biol. Trop. 54, 1–7.
- D'Amelio, S., Mathiopoulos, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanco, M., Paggi, L., 2000. 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. 30, 223–226.
- Daschner, A., Alonso-Gomez, A., Cananas, R., Suarez-de-Parga, J.M., Lopez-Serrano, M.C., 2000. Gastroallergic anisakiasis: borderline between food allergy and parasitic disease-clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by *Anisakis simplex*. J. Allergy Clin. Immunol. 105, 176–181.
- Liao, I.C., Huang, T.S., Tsai, W.S., Hsueh, C.M., Chang, S.L., Leaño, E.M., 2004. Cobia culture in Taiwan: current status and problems. Aquaculture 237, 155–165.
- Mattiucci, S., Nascetti, G., Cianchi, R., Paggi, L., Arduino, P., Margolis, L., Brattey, J., Webb, S., D'Amelio, S., Orecchia, P., Bullini, L., 1997. Genetic and ecological data on the *Anisakis simplex* complex, with evidence for a new species (Nematoda Ascaridoidea, Anisakidae). J. Parasitol. 83, 401–416.
- McLean, E., Salze, G., Craig, S.R., 2008. Parasites, diseases and deformities of cobia. Ribarstvo 66, 1–16.
- Moravec, F., de Buron, I., 2009. Two new species of philometrids (Nematoda: Philometridae) from marine fishes off South Carolina. J. Parasitol. 95, 722–727.
- Moreno-Ancillo, A., Caballero, M., Cabanas, R., Contreras, J., Martin-Barroso, J.A., Barranco, P., 1997. Allergic reactions to parasitizing seafood. Ann. Allergy Asthma Immunol. 79, 246–250.
- Myers, B.J., Kuntz, R.E., 1967. Nematode parasites of fishes taken on Taiwan (Formosa) and its offshore islands. Can. J. Zool. 45, 237–241.
- Schmidt, G.D., Roberts, L.S., 2000. Nematodes: Ascaridida, intestinal large roundworms. In: Schmidt, G.D., Roberts, L.S. (Eds.), Foundations of Parasitology, 6th ed. McGraw-Hill, Singapore, pp. 427–428.
- Shih, H.H., 2004. Parasitic helminth fauna of the cutlass fish, *Trichiurus lepturus* L., and the differentiation of four anisakid nematode third-stage larvae by nuclear ribosomal DNA sequences. Parasitol. Res. 93, 188–195.
- Shih, H.H., Jeng, M.S., 2002. *Hysterothylacium aduncum* (Nematoda: Anisakidae) infecting a herbivorous fish, *Siganus fuscus*, off the Taiwanese coast of the Northwest Pacific. Zool. Stud. 41, 208–215.
- Smith, J.W., 1983. *Anisakis simplex*, (Rudolphi, 1809, det. Krabbe, 1878) (Nematoda: Ascaridoidea): morphology and morphometry of larvae from euphausiids and fish, and a review of the life-history and ecology. J. Helminthol. 57, 205–224.
- Stromnes, E., Andersen, K., 1998. Distribution of whaleworm (*Anisakis simplex*, Nematoda, Ascaridoidea) L3 larvae in three species of marine fish; saithe (*Pollachius virens* (L.)), cod (*Gadus morhua* (L.)) and redfish (*Sebastes marinus* (L.)) from Norwegian waters. Parasitol. Res. 84, 281–285.
- Umehara, A., Kawakami, Y., Matsui, T., Araki, J., Uchida, A., 2006. Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. Parasitol. Int. 55, 267–271.
- Wootton, R., Smith, J.W., 1975. Observation and experimental studies on the acquisition of *Anisakis* sp. larvae (Nematoda: Ascaridida) by trout in fresh water. Int. J. Parasitol. 5, 373–378.
- Young, P.C., 1972. The relationship between the presence of larval anisakine nematodes in cod and marine mammals in British home waters. J. Appl. Ecol. 9, 459–485.