1 Discovery of intermediate hosts for two species of blood flukes *Cardicola orientalis* and *Cardicola*
2 *forsteri* (Trematoda: Aporocotylidae) infecting Pacific bluefin tuna in Japan

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Abstract

Fish blood flukes (Aporocotylidae) are important pathogens of farmed finfish around the world. Among them, *Cardicola* spp. infecting farmed tuna are considered to be serious threats to tuna farming and have received tremendous attention. We conducted periodical samplings at a tuna farming site in Japan between January and May, 2015 to determine the lifecycle of *Cardicola* spp.. We collected over 4,700 terebellid polychaetes from ropes, floats and frames of tuna culture cages and found nearly 400 infected worms. Sporocysts and cercariae found in *Nicolea gracilibranchis* were genetically identified as *Cardicola orientalis* by 28S and ITS2 ribosomal DNA sequences. This was the first discovery of the intermediate host for this parasite species. Infection prevalence and the abundance of *N. gracilibranchis* significantly varied between sampling points and the highest number of infected terebellids were collected from ropes. We also demonstrated morphologically and molecularly that asexual stages found in a single *Amphitrite* sp. (Terebellidae) and adult worms isolated from farmed juvenile tuna were *Cardicola forsteri*. This is the first report of *C. forsteri* in Pacific bluefin tuna (PBT) *Thunnus orientalis* in Japan. Our results demonstrated that all three species of *C. orientalis*, *C. forsteri* and *C. opisthorchis* exist in Japanese farmed PBTs and that they all use terebellid polychaetes as the intermediate hosts.

Keywords

cercariae, sporocyst, polychaete, *Nicolea gracilibranchis*, *Amphitrite*, *Thunnus orientalis*

1. Introduction

Aporocotylid trematodes, also known as fish blood flukes, presently comprise over 130 known species from 35 genera [1]. They are one of the few trematode groups that significantly affect aquaculture industries, and research on their taxonomy, ecology, and control, is increasing [2-6]. However, aporocotylids are still one of the most poorly understood trematode families [7], especially in the case of marine fish blood flukes despite the fact that more than 70% of described aporocotylids are from marine fish [1]. Among marine aporocotylids, those infecting bluefin tuna are the most well studied species owing to their high impact on tuna farming/ranching industries.

To date, three species of blood flukes have been described from three bluefin tuna species each with different host-parasite combinations: *Cardicola forsteri* from southern bluefin tuna *Thunnus maccoyii* (SBT) and Atlantic bluefin tuna *T. thynnus* (ABT), *C. opisthorchis* from ABT and Pacific bluefin tuna *T. orientalis* (PBT), and *C. orientalis* from ABT, PBT and SBT [8-14]. Furthermore, existence of yet another species of *Cardicola* blood fluke has recently been suggested based on the molecular information derived from eggs from the gills of ABT [13]. Infections with these blood flukes result in mortality in farmed and ranched tuna, causing significant economic losses in Japan and Australia [15-18]. Studies on *Cardicola* spp. infecting farmed tuna over the last several years in Japan, Australia, and Europe have increased our knowledge regarding their taxonomy, distribution, control measures, and lifecycles.

Aporocotylids have two-host lifecycles with a definitive fish host and an invertebrate intermediate host. All freshwater and marine aporocotylids, whose lifecycles are known, use either gastropods or terebellid polychaetes as the intermediate host, respectively. In addition, larval stages of aporocotylids have been reported from marine bivalves, but their identities remain unknown [1, 19]. To date, lifecycles of marine aporocotylids have been revealed for only three species, two of which are of tuna blood flukes. *Aporocotyle simplex*, which infects flatfishes in the North Sea, uses terebellid polychaete

Artacama proboscidea and possibly *Lanassa nordenskioeldi* as the intermediate hosts [20, 21]. This has long been the only marine fish blood fluke with a known lifecycle. Recently, the intermediate hosts for two tuna blood flukes have been discovered. Cribb et al. [19] found the larval stages of *C. forsteri* in a single individual of terebellid polychaete *Longicarpus modestus*. A few years later, Sugihara et al. [22] revealed that *C. opisthorchis* uses yet another terebellid polychaete, *Terebella* sp. as the intermediate host.

During our research to determine the lifecycles of *Cardicola* spp. in Japan, we found two species of terebellid polychaetes with sporocysts in their body cavity. Furthermore, we found a couple of unfamiliar adult flukes in the heart of farmed juvenile PBTs. In the present study, we aim to study the sporocysts and identify the adult flukes in order to determine the lifecycles of *Cardicola* blood flukes in Japan.

2. Materials and Methods

2.1. Sample collections

Monthly samplings were conducted between January and May, 2015 at a tuna farming site in Wakayama prefecture, where the infections of both *C. orientalis* and *C. opisthorchis* have been detected. We specifically searched for terebellid polychaetes as they are the known intermediate hosts for two tuna blood flukes and also the most abundant polychaete family in the sampling area. To obtain terebellids, we collected fouling and sessile periphytons from the floats, ropes and frames of farming cages (Fig. 1). In addition, sediments and oyster shells were collected from the sea bottom (approximately 15 m deep). These samples were collected by divers or by using hand nets. Sampled materials from each sampling points were checked separately, except for samples collected in January, wherein some samples were mixed.

Collected samples were immediately brought back to the laboratory and polychaetes were isolated.

Terebellids were often found in sessile organisms such as sponges, sea squirts, bivalves, and barnacles. Worms were carefully removed from their tube nests with forceps and checked individually for parasite infection. Each worm was flattened between two glass plates (10 x 12 cm) and observed under a stereomicroscope with transmitted light to detect sporocysts in the body cavity. When sporocysts were detected, the infected polychaete was transferred into a petri dish with clean seawater to isolate the sporocysts. A portion of sporocysts were fixed in 70% ethanol for molecular analyses and others were kept alive in seawater for morphological observation. Infected polychaetes were fixed in 10% seawater formalin and later identified based on their morphological characteristics.

For morphological observation of the larval parasites, we used both fresh sporocysts and ones fixed in AFA (mixture of alcohol, formalin and acetic acid), stained with Heidenhain's iron hematoxylin or alum carmine, dehydrated in ethanol series and mounted in Canada balsam. Fresh cercariae were obtained by pushing sporocysts between a coverslip and a glass slide and observed live under differential interference contrast microscopy. Some cercariae were stained with neutral red and observed microscopically. Measurements of sporocysts and cercariae using digital photo equipment (DS-Fi1 and DS-L2, Nikon) are given as ranges followed by means in parentheses. The number of cercariae in five live sporocysts collected from each of six polychaetes (one on April 15, 2015 and five on May 8, 2015) was counted. Cercariae were pushed out from live sporocysts and photographed under differential interference contrast microscopy at low magnifications. The number of daughter sporocysts in mother sporocysts collected from a single polychaete on April 15, 2015 was counted based on stained mother sporocyst specimens.

During our routine check of farmed juvenile PBTs, we found a two unfamiliar adult flukes in the hearts. These individuals were clearly different from *C. opisthorchis* in shape and noticeably larger than *C. orientalis*. One specimen was from a frozen fish sample and not intact, thus it was fixed in 70% ethanol and used for molecular analysis. Another specimen was from a fresh fish and still alive at the

time of collection, thus we made a whole mount specimen stained with Heidenhain's iron hematoxylin as described above for morphological observation and deposited at Meguro Parasitological Museum, Tokyo, MPM Collection No. 21011.

2.2. Molecular analyses

DNA was extracted from the sporocysts obtained from six polychaete samples (5 from the same species and another one from different species) and an adult fluke from the frozen fish sample using QIAamp DNA Mini Kit (QIAGEN Inc., Germany) following the manufacturer's instructions. A part of the 28S and internal transcribed spacer 2 (ITS2) of the ribosomal DNA was amplified by PCR using the forward primer U178 (5-GCA CCC GCT GAA YTT AAG-3), the reverse primer L1642 (5-CCA GCG CCA TCC ATT TTC A-3) [23], the forward primer 3S (5-GGT ACC GGT GGA TCA CGT GGC TAG TG-3) [24] and the reverse primer ITS2.2 (5-CCT GGT TAG TTT CTT TTC CTC CGC-3) [25], respectively. The PCR was conducted with the following cycling program: initial denaturation of 95 °C for 2 min followed by 30 cycles of 95 °C for 50 s, 56 °C for 50 s, 72 °C for 2 min and a final extension at 72 °C for 4 min. PCR products were treated with QIAquick Gel Extraction Kit (QIAGEN) to remove excess primers and dNTPs and directly sequenced with BigDye™ Terminator v3.1 in an ABI 3730xl Sequencer.

The new ITS2 sequences obtained in the present study were aligned by Clustal X 2.0 [26] with the homologous sequences of other *Cardicola* species available on the GenBank database. Bayesian inference (BI) was used to reconstruct the phylogenetic tree from datasets containing 19 sequences of the ITS2 sequences with *Paradeontacylix ibericus* as an outgroup. For BI analysis, nucleotide substitution models were selected using Akaike information criterion (AIC) as implemented in jModeltest 2.1.7 [27, 28] and the transition model 1 with estimates of gamma distributed among-site rate variation (TIM1 + G) was chosen as the best-fit nucleotide substitution model. The

metropolis-coupled Markov chain Monte Carlo (MCMC) algorithm implemented in MrBayes 3.2.4 [29] was run for a sufficient number of generations until the average standard deviation of the split frequencies was < 0.01 . The sampling frequency was set for every 100 generations for 1,000,000 samples. The first 100,000 samples from each run were discarded as burn-in, and the remaining were analyzed using the “sumt” command in MrBayes. Gaps were treated as missing data. A consensus tree was created using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

3. Results

3.1. Field samplings

We checked a total of 4,729 terebellid polychaetes and found a total of 394 infected worms. The abundance of terebellids and the infection prevalence varied considerably between the samplings and sampling points (Table 1). Terebellid worms were highly abundant on cage floats and ropes, and while they were present on cage frames, their abundance was considerably less than on floats and ropes. Only a few worms were collected from the bottom substrates. The abundance and infection prevalence of terebellids were high in the case of cage ropes (up to 29.7%), but the prevalence in the case of cage floats tended to be low (up to 2.0%), despite their high abundance. The overall infection prevalence increased from 0.96% in January to 16.8% in May, though the values may not reflect actual prevalence due to unsystematic sampling (Table 1). Although we do not have accurate quantitative data on the temporal changes in sporocyst development, they appeared to develop as the season progressed.

For all samples collected in January, all infected terebellids except for one harbored developing sporocysts filled with germinal masses (i.e. without cercariae or daughter sporocysts). These terebellids with developing sporocysts were morphologically identified as *Nicolea gracilibranchis* (Grube, 1878) (Fig. 2). This species was the most common polychaete in the sampling area and found

in the majority of collected samples. The body cavity of infected *N. gracilibranchis* was filled with sporocysts, though the severity of infection varied considerably between individuals (Fig. 3). All other infected terebellids, except a single specimen collected in January, were morphologically indistinguishable from each other and presumably identified as *N. gracilibranchis*. We did not conduct precise identification of all infected polychaetes owing to their high number.

Developed sporocysts containing mature cercariae were detected after February. Among the many infected polychaetes found in January through May, there was only one individual with sporocystogenous sporocysts, which was collected in April (Fig 4A). This individual was also identified as *N. gracilibranchis*. All other sporocysts were either developing (Fig 4B) or cercariogenous (Fig 4C). Cercariae obtained from *N. gracilibranchis* were clearly different from those of *C. forsteri* and *C. opisthorchis* owing to the presence of a minute round tail (described below). The molecular analysis (below) indicated that they are *C. orientalis* (hereafter referred as such). We did not confirm the identity of all the sporocysts because of high numbers, but all observed cercariae from *N. gracilibranchis* possessed the same morphological characteristics.

Among 13 infected terebellids collected in January, one individual collected from floats/ropes harbored sporocysts with developed cercariae. This specimen was apparently different from *N. gracilibranchis* and identified as *Amphitrite* sp. Further identification was not possible because the specimen was damaged and some parts were missing. The cercariae in the *Amphitrite* sp. possessed a simple long tail and were morphologically similar to the cercariae of *C. forsteri* (Fig. 5). The molecular analysis (below) also indicated that they were *C. forsteri* (hereafter referred as such). We found only a single *Amphitrite* sp. throughout our sampling period, and *C. forsteri* cercariae were detected only in this specimen.

We confirmed *Cardicola* infections in the juvenile PBT farmed in the sampling area. The mean intensity of *C. opisthorchis* in the heart was 6.3 ± 6.7 (n=13; prevalence 13/24 = 54.2 %) in January.

We also found a few adult *C. orientalis* in the gills but could not determine their intensity as accurate enumeration of adult *C. orientalis* was extremely difficult. There were also eggs from both blood fluke species in the gill lamellae. Furthermore in April, we found an unfamiliar adult worm along with *C. opisthorchis* in the heart of juvenile PBT. Close observation of a whole mount specimen revealed that this worm possessed morphological characteristics similar to those of *C. forsteri*. In the single stained adult specimen, some body parts were not clearly observed; however, we were able to determine that the body was 4.17 mm long and 1.18 mm wide, that the esophagus was 1.45 mm long or 34.8% of body length, and that the testes occupied the intra- and extra-caecal field, extending from somewhere behind the nerve commissure anteriorly to the end of the ovary posteriorly. These morphological characteristics were not seen in *C. orientalis* or *C. opisthorchis*, but were almost consistent with those of *C. forsteri* [10, 14]. Also, molecular analysis (below) of another adult fluke specimen from a frozen fish sample collected in 2014 confirmed the existence of *C. forsteri* in farmed PBTs in Japan.

3.2. Morphological observations

Cardicola orientalis Ogawa, Tanaka, Sugihara et Takami, 2010

1) Sporocysts:

All infected polychaetes contained cercariogenous or undeveloped sporocysts, except one which contained sporocystogenous sporocysts only. Measurements were based on 30 cercariogenous sporocysts collected in February and April, 2015 and 18 sporocystogenous sporocysts collected in April, 2015.

Body fusiform, 512-1272 μm (874.7 μm) long, 107-284 μm (201.9 μm) wide for cercariogenous sporocysts, and 391-1012 μm (624.3 μm) long, 98-255 μm (168.8 μm) wide for sporocystogenous sporocysts. Cercariogenous sporocysts containing different maturation stages of cercariae, from

undifferentiated germinal masses to mature cercariae. The number of cercariae, including mature and immature ones with a tail, 59-193 (mean 106.6; n=30). Sporocysts containing both cercariae and undifferentiated germinal masses, the latter being as many as or more numerous than cercariae (accurate determination not possible). Sporocystogenous sporocysts (= mother sporocysts) containing about 20-50 daughter sporocysts including germinal masses, the size in each mother sporocyst, 10-20 µm in diameter for germinal masses, 69-218 µm (142.3 µm) long and 39-79 µm (58.1 µm) wide for developing sporocysts.

Material: Meguro Parasitological Museum, Tokyo, MPM Collection No. 21012-21013.

Host: *Nicolea gracilibranchis* (Terebellidae).

Site: body cavity.

Locality and dates of sampling: Kushimoto, Wakayama Prefecture, Japan (33°28'N, 135°47'E), 26th February 2015, 15th, 16th, and 18th April, 2015 for cercariogenous sporocysts, 15th April, 2015 for sporocystogenous sporocysts.

Taxonomical data on the intermediate host

Type locality: Philippines (Grube, 1878)

Specific characters: with 17 thoracic chaetigers; without lateral lobes; tentacular membrane with eye-spots; 2 rows of teeth observable on MF (main fang) of anterior thoracic uncini in side view; dental formula of thoracic uncini dominantly MF: 3:2 abdominal neuropodial lobes well developed; body greenish in color. The characters listed here depend on the actual observations of the specimens from Kushimoto, Wakayama prefecture, Japan.

Synonyms: *N. gracilibranchis* sensu Marenzeller, 1884 (Enoshima, Japan), Fauvel, 1936 (Tanabe in Wakayama, Japan), *N. venustula* sensu Day, 1967 (South Africa), and Day, 1973 (India).

? nec *N. gracilibranchis* sensu Ehlers, 1907 (New Zealand), Zachs, 1933 (northern part of the

Sea of Japan), Annenkova, 1938 (the Sea of Japan), Okuda & Yamada, 1954 (northern part of the Main Land of Japan), Uschakov, 1955 (the Sea of Okhotsk), Buzhinskaya, 1967 (Posjet Bay).

Distribution: middle to south Japan, Philippines, Vietnam, Gulf of Siam, India, Arabian Gulf, South Africa, New Caledonia, & Hawaii.

The species is the most common terebellid species at the mariculture farm in Kushimoto.

2) Cercaria (based on 31 live specimens, Fig. 6):

Body cylindrical and elongated, 78-163 μm (118.5 μm ; n=27) long including tail, body proper, 70-151 μm (107.9 μm ; n=27) long, 17-30 μm (24.3 μm ; n=31) wide. Tail ellipsoidal, 7-15 μm (10.7 μm ; n=31) long, 7-12 μm (9.0 μm ; n=31) wide. Head, armed with 6-7 rows of concentric spines (Fig. 7), marked off from the rest of the body by occasional constrictions. Mouth opening as a transverse slit immediately behind the spines (Fig. 6, Fig. 8A). Oesophagus long and winding (Fig. 8A), 45-99 μm (66.4 μm ; n=28) long in a straight line, the posterior end positioned 54-72% (63.4%; n=28) from the anterior extremity. The caeca with two anterior and two posterior branches, measuring 3-9 μm (5.6 μm ; n=31) long and 3-7 μm (4.6 μm ; n=31) long, respectively. The lumen of the caeca stained with neutral red (Fig. 8B). The head gland cells located posterior to the pyriform gland reservoir, containing granular substances.

Three groups of large penetration gland cells located in the anterior, middle and posterior parts of body (Fig. 9A). The four anterior cells in two pairs. The middle cells consisting of five cells, a single cell in the mid-line, followed by two pairs of cells laterally. The posterior cells, six in number arranged in three pairs lengthwise laterally behind the caeca, and the anterior pair largest. Their ducts running anteriorly along the lateral side of body, opening at the anterior end. Besides, two granular cells with unknown function arranged in the mid-line longitudinally, one in the middle of body proper and the

other at caecal level (Fig. 9B). Openings of their secretions not traced.

The excretory vesicle located at the end of the body proper, a pair of excretory ducts extending anteriorly along the right and left side of the body and another pair present in the tail. The ducts in the main body traced up to about one-fourth of the body proper. A set of flame cells near the anterior end of the observed ducts. Flame cells of each pair, only 3-8 μm apart ($n=5$), the right and left pair arranged symmetrically or subsymmetrically to each other. Connection of these flame cells with the ducts not clear. The flame cell formula, $2[1+1]=4$. Each duct extending into the tail forming an excretory vesicle, opening at the posterior end through a narrow duct.

Cardicola forsteri Cribb, Daintith et Munday, 2000

1) Sporocysts (based on 7 specimens, Fig. 10):

A single infected polychaete containing cercariogenous sporocysts. Body fusiform, 530-698 μm (625.9 μm) long, 182-278 μm (228.7 μm) wide. Sporocysts containing different maturation stages of cercariae, from undifferentiated germinal masses to mature cercariae.

Locality and dates of sampling: Kushimoto, Wakayama Prefecture, Japan (33°28'N, 135°47'E), 27 January, 2015.

Site: Body cavity.

Material: Meguro Parasitological Museum, MPM Collection No. 21014.

Host: *Amphitrite* sp. (Terebellidae).

Taxonomical data on the intermediate host

Specific characters : 3 pairs of branchiae not bushy but ramified; notosetae with denticulate tips, and the tips not strongly geniculate; with small lateral lobes on 2nd and 3th segments; tentacular membrane without eye-spots; each thoracic uncinus with ca. 6 rows of small teeth on

a large MF, and dental formula as follows; MF: 5: 4: ∞.

Remarks: Only a single damaged specimen was obtained. Species identification was impossible, for the number of thoracic chaetigers could not be determined.

2) Cercaria (based on 10 live specimens):

Body cylindrical and elongated, 111-136 µm (121.7 µm) long including tail, body proper 71-95 µm (82.9 µm) long, 12-21 µm (17.6 µm) wide. Tail straight without bifurcation, tapering distally, 29-48 µm (38.9 µm) long, 6-10 µm (7.4 µm) wide.

3.2. Molecular analyses

The results of sequence comparison were summarized in Table 2. The 28S and ITS2 rDNA sequences obtained from sporocysts isolated from five *N. gracilibranchis* showed 99-100% similarity with each other and we obtained 1475bp of 28S (KT119355) and 569bp of ITS2 (KR818840) rDNA sequences. These sequences showed 100% identity with those from adult *C. orientalis* isolated from PBT in Japan (28S, HQ324225, 1475/1475bp; ITS2, HQ324226, 524/524bp). We also obtained 1585 bp of 28S (KT119354) and 620 bp of ITS2 (KR818839) rDNA sequences from sporocysts found in *Amphitrite* sp. and they revealed 100% (1583/1583bp) and 99.8% (557/558bp) similarity with the adult *C. forsteri* from SBT in Australia (28S, AB742426; ITS2, AB742428). Additionally, 1475bp (KT119353) of 28S and 576bp of ITS2 (KR818838) sequences were obtained from adult worm isolated from juvenile PBT, and they also revealed 100% and 99.8% similarity with the adult *C. forsteri* from SBT in Australia (28S, 1475/1475bp; ITS2, 566/568bp), respectively. These sequences of the adult worm from PBT showed 100% (28S, 1475/1475bp) and 99.8% (ITS2, 563/564bp) similarity with those derived from sporocysts in *Amphitrite* sp.

In the BI phylogenetic analyses, *Cardicola* spp. were divided into two groups except for *C. aurata*,

the position of which was poorly resolved. *C. orientalis* clustered with *Cardicola* spp. that infect fish species other than tuna, such as *C. chaetodontis*, *C. milleri*, *C. coeptus*, *C. bartolii*, *C. watsonensis*, *C. covacinae*, *C. tantabiddii*, *C. lafii* and *C. parilus*, whereas *C. forsteri* clustered with *C. opisthorchis* which share *Thunnus* spp. as the common host (Fig. 11).

4. Discussion

The high genetic identity between the adult flukes from PBT and the sporocysts from the terebellid polychaetes showed that *C. orientalis* uses *N. gracilibranchis* as the intermediate host. This is the fourth species among marine aporocotylids and the third species among *Cardicola* spp. for which the intermediate host has been identified. The infection prevalence of *C. orientalis* in *N. gracilibranchis* can be as high as 29.8%, but the prevalence varied considerably between the sampling points. This value was relatively higher than the prevalence of other marine aporocotylids in their terebellid hosts. The infection prevalence of *A. simplex* in *A. proboscidea* ranged from 3.9 to 13.6% [20], and those for *C. forsteri* and *C. opisthorchis* were reported to be only 1/734 (0.1%) and 5/139 (3.6%), respectively [19, 22]. However, the actual prevalence of the host polychaete in our study and these studies may be higher as not all collected terebellids were identified at the species level.

In the present study, the infection level in terebellids varied both temporally and spatially. The overall infection of *C. orientalis* in *N. gracilibranchis* tended to increase between January and May. Also, the sporocysts seemed to become more developed, from germinal masses to cercariae, during this period. In the study area, juvenile PBTs were transferred from a land-based hatchery to sea cages in late July/ early August and they become infected by *Cardicola* blood flukes shortly thereafter. Therefore, a large number of cercariae must be present around farming cages at the time of transfer. In the past years, we detected *C. orientalis* eggs in fish gills around late August and September (Shirakashi, unpublished data). Miracidia hatched from the eggs likely invade *N. gracilibranchis* after

September, undergo development within the intermediate host during winter and spring and then are released as cercariae during the summer. However, the precise duration of sporocyst development and time period of infection persistence in *N. gracilibranchis* remain unknown. Continuous monitoring of the sporocysts development in the intermediate host and laboratory experiment, including experimental infection, will provide more information on the biology of *C. orientalis* in the intermediate host.

It was interesting to find such significant differences in the infection prevalence of *N. gracilibranchis* between the sampling points. It may be partly due to the microhabitat selection of the intermediate host, but there must be other important factors determining the infection in terebellids. We found a high number of *N. gracilibranchis* on the cage ropes and their infection prevalence was also considerably higher (up to nearly 30%) than most other sampling points (Table 1). In contrast, despite the high abundance of *N. gracilibranchis* on the cage floats their infection prevalence was significantly lower than that for ropes, typically less than one percent. We sampled polychaetes from the ropes and floats of the same culture cage and these sampling points were in very close proximity (within 10 m apart at most).

The abundance of *N. gracilibranchis* tended to be lower in deeper water: relatively small numbers were found on the cage frames (6 m deep) and in bottom substrate (15 m deep). Although factors influencing *N. gracilibranchis* abundance are unknown, terebellid polychaetes seem to have strong microhabitat preferences and there may be “hot spots” for each species. This is also suggested by the fact that we found only a single *Amphitrite* sp. during the study period (out of over 4,700 worms). Also, we have not yet found even a single *Terebella* sp., the intermediate host for *C. opisthorchis*, in our study area in spite of the high intensity of adult worms in tuna farmed in the area. These terebellids species must exist in significant population sizes, and they likely populate in close proximity to tuna farming cages as the locomotive ability of *Cardicola* cercariae appears to be poor.

The cercariae of *C. forsteri* possessed simple straight tails but they only wriggled in the water and did not exhibit any swimming behavior. The cercariae of *C. opisthorchis* have tails similar to those of *C. forsteri*, and thus it is suspected to have similar behavior [22]. *C. orientalis* cercariae possessed only a minute round tail and likely have almost no ability to swim. Therefore, these cercariae may rely on the water current for dispersion and to reach PBTs. Identifying the factor(s) affecting the distribution of the terebellids and their infection level may be the key to understanding the lifecycle of the parasites as well as to develop a new biological control measure against *Cardicola*. We plan to continue the monthly samplings at different sampling points to obtain more information on the temporal and spatial changes in the polychaete abundance and their infection prevalence.

The discovery of adult *C. forsteri* from PBT and its intermediate host in Japanese waters provides important information about the global distribution of tuna blood flukes. We found that *C. forsteri* uses different terebellid intermediate hosts in Australia (*L. modestus*) and in Japan (*Amphitrite* sp.), though these two species are taxonomically close to each other. In general, aporocotylids show high host specificity, but some species are known to use multiple intermediate hosts. For instance, three species of *Lymnaea* freshwater snail can be the intermediate hosts for *Sanguinicola inermis* [30]. The cercariae of *A. simplex* were first found in *A. proboscidea*, but morphologically indistinguishable cercariae have also been found in *Lanassa nordenskiöldi* [20, 21]. Therefore, it is possible that an aporocotylid species may use different intermediate hosts in different geographical regions. This is important as blood flukes are capable of establishing their lifecycles in new geographical areas and extending their distribution using different invertebrate hosts. Nevertheless, with our findings, the number of terebellid species known to host aporocotylids is now eight, including two species harboring cercariae of unknown species. This supports the idea by Cribb et al. [19] that terebellid polychaetes are important hosts for marine aporocotylid blood flukes.

To date, *C. forsteri* has been reported in SBTs in Australia [10] and ABTs in the Mediterranean

and Atlantic [8, 9, 13]. Its existence in PBTs in Japan indicates that *C. forsteri* is a cosmopolitan species distributed widely in the Pacific, Atlantic and Mediterranean. It is unclear whether it is native to Japan or recently expanded its distribution. To the best of our knowledge, there is no record of importing live tuna or large foreign vessels entering the study area. It may be that all three species of tuna blood flukes, *C. forsteri*, *C. orientalis* and *C. opisthorchis*, always parasitize wild PBTs, probably at a low level, and have recently become prominent with increased tuna farming. In fact, all three *Cardicola* spp. are cosmopolitan species sharing the same bluefin tuna species as their host. They have been found in PBTs, SBTs, and ABTs in both the northern and southern hemisphere of the Pacific Ocean, Mediterranean and northern Atlantic, except for *C. opisthorchis*, which has not yet been detected in SBTs [8, 13, 14]. Palacios-Abella et al. [13] proposed the existence of a fourth species of *Cardicola* in Mediterranean ABTs based on molecular evidence. Further investigation with more molecular data will clarify the distribution and host range of *Cardicola* spp. in tunas.

In summary, our study has provided insights into new hosts and the geographical range for *C. forsteri* and has revealed the lifecycle of *C. forsteri* and *C. orientalis* in Japan. With this information, the intermediate hosts for all three *Cardicola* spp. infecting PBT in Japan have been identified. Since the three *Cardicola* spp. may co-infect the same individual PBT, the pathological effects of each species need to be understood. Also, investigations on the factors influencing the abundance and *Cardicola* infection of each terebellid intermediate host species are important to understand the biology of the parasite as well as to develop control measures against these pathological blood flukes in tuna farming.

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Fig. 1. A schematic diagram of the sampling points in a tuna farming cage.

Fig. 2. *Nicolea gracilibranchis*, the intermediate host of *Cardicola orientalis*.

Fig. 3. Infected *Nicolea gracilibranchis* filled with *Cardicola orientalis* sporocysts in the body cavity. Scale bars: A, 10 mm; B, 5 mm.

Fig. 4. *Cardicola orientalis* sporocysts, containing (A) daughter sporocysts, (B) underdeveloped cercariae and (C) mature and developing cercariae,. Scale bars: A, C, 100 µm; B, 500 µm.

Fig. 5. *Cardicola forsteri* cercariae released from sporocysts found in *Amphitrite* sp.

Fig. 6. *Cardicola orientalis* cercaria (semidiagrammatic). Scale bar, 10 µm.

Fig. 7. *Cardicola orientalis* cercariae, showing 6-7 concentric rows of head spines (differential interference contrast microscopy). Scale bar, 10 µm.

Fig. 8. Immature *Cardicola orientalis* cercaria (A, differential interference contrast microscopy) showing mouth (arrow) followed by a long, winding oesophagus and cercaria stained with neutral red showing caeca (B, arrow). Scale bars, 10 µm.

Fig. 9. Live *Cardicola orientalis* cercaria showing sets of gland cells in the body proper at two different foci (differential interference contrast microscopy). Three groups of cells (2, 5 and 6 cells) in anterior, middle and posterior part of body, respectively (A, arrows) and remaining 2 anterior cells (B, arrows) and another group of 2 cells in middle part (B, arrowheads) are shown. Scale bar, 10 µm.

Fig. 10. *Cardicola forsteri* sporocyst, containing mature and developing cercariae. Scale bar, 100 µm.

Fig. 11. Phylogenetic tree of *Cardicola* spp. generated by Bayesian Inference analysis of ITS2 rDNA sequences. Host species is indicated for *C. orientalis*, *C. forsteri*, *C. opisthorchis* from terebellid polychaete and tuna species in Japan, Australia and Spain. The values at each node are posterior probabilities and *Paradeontacylix ibericus* was used as outgroup.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Table 1.

Number of infected terebellids (prevalence in parentheses) from different sampling points found during the monthly samplings in 2015.

	25 Jan		25 Feb		25 Mar		15 Apr		7 May		Total
Floats & ropes	1/871	Floats	10/500	4/430	1/326	2/315					
	(0.1%)		(2.0%)	(0.9%)	(0.3%)	(0.6%)					
		Ropes	22/398	62/276	120/438	119/400					
			(5.5%)	(22.5%)	(27.4%)	(29.8%)					
Cage frame & bottom	12/484	Cage frame	0/34	0/12	40/214	0/5					
	(2.5%)		(0%)	(0%)	(18.7%)	(0%)					
		Bottom			1/17						
					(5.9%)						
Total	13/1355		32/932	66/718	162/995	121/729	394/4729				
	(1.0%)		(3.4%)	(9.2%)	(16.3%)	(16.8%)	(8.3%)				

Table 2.

Comparison of identities among 28S (shaded) and ITS2 (unshaded) sequences between present isolates and *Cardicola* spp. registered in NCBI.

	<i>C. forsteri</i> KT119354	<i>C. forsteri</i> KT119353	<i>C. orientalis</i> KT119355	<i>C. forsteri</i> AB742426	<i>C. orientalis</i> HQ324225	<i>C. opisthorchis</i> HQ324227
<i>C. forsteri</i> KR818839 (<i>Amphitrite</i> sp.)		1475/1475 (100%)	1413/1472 (95%)	1583/1583 (100%)	1515/1580 (95%)	1568/1585 (98%)
<i>C. forsteri</i> KR818838 (<i>Thunnus orientalis</i>)	563/564 (99%)		1413/1472 (95%)	1475/1475 (100%)	1413/1472 (95%)	1458/1475 (98%)
<i>C. orientalis</i> KR818840 (<i>Nicolea gracilibranchis</i>)	501/563 (88%)	507/572 (88%)		1413/1472 (95%)	1475/1475 (100%)	1416/1472 (96%)
<i>C. forsteri</i> AB742428	557/558 (99%)	566/568 (99%)	500/563 (88%)		1513/1578 (95%)	1569/1586 (98%)
<i>C. orientalis</i> HQ324226	466/528 (88%)	465/527 (88%)	524/524 (100%)	465/528 (88%)		1524/1586 (96%)
<i>C. opisthorchis</i> HQ324228	499/521 (95%)	499/521 (95%)	456/526 (86%)	500/521 (95%)	455/525 (86%)	

Life cycle of *Cardicola orientalis*

Nicolea gracilibranchis