1 Developmental stages of fish blood flukes, *Cardicola forsteri* and *Cardicola opisthorchis* (Trematoda:
2 Aporocotylidae), in their polychaete intermediate hosts collected at Pacific bluefin tuna culture sites in
3 Japan

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ABSTRACT

Farming of Pacific bluefin tuna (PBT), *Thunnus orientalis*, is a rapidly growing industry in Japan. Aporocotylid blood flukes of the genus *Cardicola* comprising *C. orientalis*, *C. opisthorchis* and *C. forsteri* are parasites of economic importance for PBT farming. Recently, terebellid polychaetes have been identified as the intermediate hosts for all these parasites. We collected infected polychaetes, *Terebella* sp., the intermediate host of *C. opisthorchis*, from ropes and floats attached to tuna cages in Tsushima, Nagasaki Prefecture, Japan. Also, *Neoamphitrite vigintipes* (formerly as *Amphitrite* sp. sensu Shirakashi et al., 2016), the intermediate host of *C. forsteri*, were collected from culture cages in Kushimoto, Wakayama Prefecture, Japan. The terebellid intermediate hosts harbored the sporocysts and cercariae in their body cavity. Developmental stages of these blood flukes were molecularly identified using species specific PCR primers. In this paper, we described the cercaria and sporocyst stages of *C. opisthorchis* and *C. forsteri* and compared their morphological characteristics among three *Cardicola* blood flukes infecting PBT. We also discussed phylogenetic relations of the six genera of the terebellid intermediate hosts (*Artacama*, *Lanassa*, *Longicarpus*, *Terebella*, *Nicolea* and *Neoamphitrite*) of blood flukes infecting marine fishes, based on their morphological characters.

Keywords

Aporocotylidae; intermediate host; cercaria; sporocyst; *Thunnus orientalis*

1. Introduction

In Australia, farmed southern bluefin tuna, *Thunnus maccoyii*, is known to be infected with aporocotylid blood flukes, *Cardicola forsteri* and *Cardicola orientalis* [1,2]. Later, Cribb et al. [3] found the asexual stages of *C. forsteri* from a single specimen of *Longicarpus modestus* (Terebellidae) collected from the sea bottom also in Australia. In Japan, two species of blood flukes, *C. orientalis* and *C. opisthorchis*, have been described from cultured Pacific bluefin tuna (PBT), *Thunnus orientalis* [4,5]. Recently, *C. forsteri* was identified as a third blood fluke of cultured PBT in a survey conducted in 2015 [6]. Over the last few years, the intermediate hosts of all these blood flukes infecting PBT have been identified in Japan. Sugihara et al. [7] demonstrated molecularly that the larval stages (cercaria and sporocyst) collected from *Terebella* sp. (Terebellidae) were those of *C. opisthorchis*. Further, Shirakashi et al. [6] specified *Nicolea gracilibranchis* (Grube, 1878) and *Amphitrite* sp. as the intermediate hosts of *C. orientalis* and *C. forsteri*, respectively and described morphology of the cercaria and sporocyst stages of *C. orientalis*. In this paper, we made detailed descriptions of the developmental stages of the other species of PBT blood flukes, *C. opisthorchis* and *C. forsteri*, based on newly collected specimens from their respective intermediate hosts. The identity of *Amphitrite* sp., the intermediate host of *C. forsteri* infecting PBT, was provisional, because only a single, damaged infected polychaete was available at the time [6]. Accurate identification of the polychaete was made, based on the newly collected specimens in this paper.

2. Material and methods

2.1. Field sampling

A total of 64 terebellid polychaetes, including 39 *Terebella* sp. were collected from ropes and floats attached to tuna cages at a PBT culture farm in Tsushima, Nagasaki Prefecture, Japan on February 15, 2016, the same site as Sugihara et al. [7] collected infected polychaetes. Terebellid polychaetes were also sampled at a PBT culture farm in Kushimoto, Wakayama Prefecture, Japan on June 23, 2015 as a part of monthly samplings [6]. We were unable to find *Amphitrite* sp. sensu Shirakashi et al., 2016 between February and May, even though a large number of *C. orientalis*-infected *N. gracilibranchis* (400 individuals) were found from among more than 3,500 terebellids. On June 23, in addition to the regular sampling points (floats, ropes and frames of farming cages) [6], we tried a new point, the bottom floor of a net cage, approximately 6 m deep, in which 2 year-old PBT were reared. The cage had been used for over two years, with occasional cleanings, and there were deposited materials on the cage floor. These materials including muddy sediments, periphytons and some sessile organisms were collected by a diver. The terebellids were isolated and checked for infection as previously described [6].

Each polychaete was flattened and examined for sporocysts in the body cavity by light microscopy in Tsushima as in Sugihara et al. [8] or by stereomicroscopy in Kushimoto as in Shirakashi et al. [6]. When found infected, sporocysts were isolated in clean sea water and later used for morphological and molecular analyses. For sporocysts collected in Kushimoto, the 28S ribosomal DNA sequences were determined as

described in Shirakashi et al. [6]. For those collected in Tsushima, *C. opisthorchis* was confirmed by the sequences of the PCR products targeting the internal transcribed spacer 2 and 28S of the ribosomal DNA sequences, as described in Sugihara et al. [7]. The infected terebellids were fixed in 10% seawater formalin for traditional identification.

2.2. Description of larval stages of parasites

Sporocysts were flattened between a coverslip and a glass slide, fixed in AFA (mixture of alcohol, formalin and acetic acid) and subsequently stained with Heidenhain's iron hematoxylin or alum carmine; dehydrated in an ethanol series, cleared in creosote and mounted in Canada balsam. Additionally, the sporocysts of *C. forsteri* were killed in hot saline, transferred to 70% ethanol and stained with alum carmine.

Fresh cercariae were obtained by putting pressure on sporocysts that were held between a coverslip and a glass slide. Sporocysts and cercariae were photographed and measured (in micrometer) using digital photo equipment (DS-Fi1 and DS-L2, Nikon). Measurements are given as ranges followed by arithmetic means in parentheses. For *C. forsteri* sporocysts, the measurements of the AFA and hot saline specimens were indicated as "(AFA)" and "(HS)" after each set of measurements, respectively. The cercarial body parts were observed live using well-flattened specimens under differential interference contrast microscopy and photos were taken with a 100× objective lens. For semidiagrammatic figures, a total of 34 photos of fresh cercariae of *C. opisthorchis* (n=7) and *C. forsteri* (n=3) were traced and body parts were combined to make the figures of the cercarial whole body.

For both *C. opisthorchis* and *C. forsteri*, the number of cercariae in five live sporocysts collected from a single polychaete specimen was counted. For *C. forsteri*, the number of daughter sporocysts in mother sporocysts collected from six polychaetes was also counted based on the stained mother sporocyst specimens.

3. Results

3.1. Field sampling

In Tsushima, three out of 39 *Terebella* sp. specimens collected from ropes and floats attached to tuna cages were found infected with sporocysts in the body cavity (prevalence of infection: 7.7%). Other 25 unidentified terebellid polychaetes were not infected.

In Kushimoto, a total of 638 terebellid polychaetes were collected from all sampling points, and a total of 72 *Nicolea gracilibranchis* were found infected with *C. orientalis* sporocysts. We found six specimens of infected specimens of

Neoamphitrite vigintipes (Grube, 1870) (= *Amphitrite* sp. in Shirakashi et al. [6]) (Fig. 1), all from the materials collected from the bottom of the net cage. A total of 77 terebellids was collected from the bottom of the cage, of which 25 individuals of *N. gracilibranchis* were infected with *C. orientalis* (32.5%) and six *N. vigintipes* were infected with *C. forsteri* (7.8%). The precise identification of all the collected terebellids was unclear due to the large number of samples. *N. vigintipes* appeared to be associated with muddy

sediments accumulated between algae and bivalves. No *N. vigintipes* were noticed in the samples collected from other sampling points such as cage floats, ropes or frames.

3.2. Molecular identification of sporocysts

In Kushimoto, we obtained 1,519–1,520 bp of the 28S rDNA sequences from the sporocysts obtained from six *N. vigintipes*. They were 100% identical with each other and with the sequences of *C. forsteri* sporocyst we obtained in January 2015 (KT119354) and the adult specimen from PBT (KT119353). The sequence also showed 100% identity with that of *C. forsteri* asexual stages from *L. modestus* in Australia (JF800668). In Tsushima, we sequenced 28S rDNA (1609 bp) and ITS2 region (579–582 bp) extracted from *C. opisthorchi* sporocysts collected from the three infected *Terebella* sp. The three sets of sequences were 100% identical to the registered sequences (GenBank Nos. AB829900, HQ324227, AB830082, KP988306, KP217044, KP217045, KP217047), except one ITS2 sequence which was different by 1 bp from HQ324228, but this difference was a result from misreading of one bp in HQ324228 (data not shown).

3.3. Morphological observations

3.3.1. *Cardicola opisthorchi* Ogawa, Ishimaru, Shirakashi, Takami et Grabner, 2011

1) Sporocyst

The infected *Terebella* sp. contained cercariae and sporocysts.

Body fusiform, 384–995 μ m (699 μ m) long ($n=30$), 152–357 μ m (258 μ m) wide ($n=30$), containing different maturation stages of cercariae, from undifferentiated germinal masses to mature ones. The number of mature and immature cercariae and underdeveloped embryos, 150–500 ($n=5$).

2) Cercaria (measurements based on 16 live mature specimens (the number of specimens measured given in parentheses when all were not measurable); observation of the excretory system based on 66 live mature specimens; Figs. 2&3):

Body cylindrical and elongated, 151–225 μ m (186 μ m) long including tail; body proper 115–173 μ m (141 μ m) long, 18–35 μ m (26 μ m) wide (Fig. 2a). Tail straight without bifurcation, 36–64 μ m (45 μ m) long, 7–14 μ m (9 μ m) wide (Fig. 2a). Head, 25–35 μ m (29 μ m) long, 15.5–23.3% (20.7%) of body proper length, armed with five rows of concentric spines (Fig. 3a), those on anteriormost row being largest. Demarcation of head and the rest of body not distinct. Mouth and oesophagus unclear. Intestinal caeca, 79–124 μ m (96 μ m) from anterior end of body or 63.6–72.5% (68.9%) of body proper length ($n=14$), with two short anterior and two short posterior branches, each elliptical in shape, 2–4 μ m long ($n=45$) (Fig. 3a). Head gland reservoir pyriform, containing granular substances secreted by mass of cells located posterior to gland reservoir (Fig. 2a).

Three groups of penetration gland cells, located in the anterior, middle and posterior parts of body; a total of 15 cells present (Fig. 2a); four anterior cells in two pairs, one pair slightly behind the other; middle cells consisting of five cells, one pair of cells laterally, followed by a single cell in mid-line and another

pair of cells laterally; six posterior cells arranged in three pairs lengthwise laterally behind caeca. Secretion of these penetration glands directed anteriorly along lateral side of body, opening at anterior end of body(Fig. 3b).

Excretory vesicle located at posterior end of body proper. A pair of ducts extending anteriorly from excretory vesicle along right and left side of body and another duct running posteriorly to end of tail (Fig.2b).A pair of ducts in main body behind intestinal caeca extending asymmetrically; one being about twice as long as the other.Two pairs of flame cells present; flame cells connecting with shorter excretory ductpositioned posterior to those connecting with longer duct; cells of each pair located close to each other (Fig. 3b). Flame cell formula, $2[1+1]=4$. Duct extending into tail opening at end of tail without forming an excretory vesicle.

3) Immature cercaria (measurements based on 5live specimens; Fig.4):

Body stout and elongated, 89–152 μ m (109 μ m) long including tail; body proper 66–117 μ m (83 μ m) long,20–33 μ m (28 μ m) wide. Tail short, thick and straight, 20–35 μ m (26 μ m) long,11–15 μ m (13 μ m) wide(Fig. 4b). Head, 16–25 μ m (20 μ m) long, 21.4–28.8% (24.4%) of body proper length, spines not formed. Mouth funnel-shaped, opening10–14 μ m (12 μ m) from anterior end, positioned in the middle to posterior part of head.Oesophagusmeandering (Fig.4b)or almost straight(Fig. 4c), 31–70 μ m (44 μ m) long, measured in a straight line.Intestinal caecanot clearly branched, 41–84 μ m (57 μ m) from anterior end of body or 46.1–55.3% (51.6%) of body proper length.

4) Remarks

Sugihara et al. [7]was the first to describe the larval stages of *C. opisthorchis* in the intermediate host. They described the cercaria was 125.2 μ m long, while in this paper it was 186 μ m long. This wide difference is apparently caused by the methods of preparing specimens; in the former, they were fixed in AFA, while in the latter, they were well-flattened before photographed and measured live in order to describe detailed structures. We found cercariogenoussporocysts only, while Sugihara et al. [8]reported both sporocystogenous and cercariogenoussporocysts. This may simply be the matter of chance; we found only three infected terebellids, while they conducted a monthly survey for one year and found nine *Terebellasp.* containing sporocystogenoussporocysts out of 54 infected terebellids.

The digestive system (mouth, oesophagus and intestinal caeca) was observed in immature cercariae. However, it became difficult to trace as the cercaria matured. The same tendency was also noticed in *C. forsteri* (described below). In contrast, the digestive system was more clearly observed in *C. orientalis*, which has larger intestinal caeca, each caecum with an arithmetic mean of 5.6 μ m long[6].

5) Taxonomic summary

Host: *Terebellasp.* (Terebellidae).

Locality and dates of sampling: off Tsushima, Nagasaki Pref., Japan (34°18'14"N, 129°13'33"E), 15 February 2016.

Site: Body cavity.

192 Material: Meguro Parasitological Museum, Tokyo (MPM), MPM Collection No.XXXXXX.

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194 3.3.2. *Cardicolaforsteri*Cribb, Daintith et Munday, 2000

195 1) Sporocysts:

196 The infected *N. vigintipes* contained both sporocystogenous sporocysts and cercariogenous sporocysts.

197 Sporocysts containing cercariae (Fig. 5a): Body fusiform, 435–767 µm (606 µm) long ($n=17$), 154–248
198 µm (196 µm) wide ($n=17$) (AFA); 431–596 µm (501 µm) long ($n=6$), 159–217 µm (186 µm) wide ($n=6$) (HS),
199 containing different maturation stages of cercariae, from undifferentiated germinal masses to mature
200 ones. The number of cercariae, including mature and immature ones with a tail, 66–116 (mean 83; $n=5$).

201 Mother sporocysts (Fig. 5b): Body fusiform, 414–774 µm (mean 561 µm) long ($n=19$), 126–250 µm
202 (185 µm) wide ($n=19$) (AFA); 420–702 µm (522 µm) long ($n=13$), 126–214 µm (171 µm) wide ($n=13$) (HS).
203 The number of sporocysts per mother sporocyst, 30–60 ($n=18$) (AFA); 20–60 ($n=13$) (HS), containing
204 undifferentiated germinal masses, smallest being 6–19 µm (13 µm) long ($n=18$), 5–15 µm (9 µm) wide ($n=18$)
205 (AFA); 10–15 µm (12 µm) long ($n=13$), 7–12 µm (10 µm) wide ($n=13$) (HS); largest being 52–247 µm
206 (141 µm) long ($n=19$), 33–93 µm (63 µm) wide ($n=19$) (AFA), 83–197 µm (135 µm) long ($n=12$), 42–104 µm
207 (75 µm) wide ($n=12$) (HS).

208

209 2) Cercaria (measurements based on 21 live specimens (the number of specimens measured given in
210 parentheses when all were not measurable); the excretory system based on 64 live specimens; Figs.
211 6&7):

212 Body cylindrical and elongated, 127–177 µm (155 µm) long including tail; body proper 97–131 µm
213 (116 µm) long, 20–26 µm (22 µm) wide (Fig. 6a). Tail straight without bifurcation, 30–49 µm (39 µm)
214 long, 6–11 µm (8 µm) wide (Fig. 6a). Head, 26–31 µm (28 µm) long, 21.8–27.8% (24.%) of body proper
215 length, armed with five rows of concentric spines (Fig. 6a), those on anteriormost row being largest.
216 Demarcation of head and the rest of body not distinct. Mouth and oesophagus unclear. Intestinal caeca,
217 71–89 µm (81 µm) from anterior end of body or 68.5–73.2% (70.5%) of body proper length ($n=14$), with
218 two short anterior and two short posterior branches, each elliptical in shape, 1–4 µm long ($n=41$) (Fig. 6a).
219 Head gland reservoir pyriform, containing granular substances secreted by mass of cells located posterior
220 to gland reservoir (Fig. 6a). Three groups of penetration gland cells, the same arrangement as in *C.*
221 *opisthorchis* (Fig. 6a). Excretory vesicle located at posterior end of body proper. A pair of ducts in main
222 body, one slightly shorter than the other (Fig. 6b–d). Two pairs of flame cells as in *C. opisthorchis*. Flame
223 cell formula, $2[1+1]=4$. Duct extending into tail opening at end of tail.

224

225 3) Immature cercaria (measurements based on 3 live specimens):

226 Body stout and elongated, size variable depending on degree of development, 134–183 µm long
227 including tail; body proper 104–154 µm long, 27–31 µm wide. Tail short, thick and straight, 27–30 µm
228 long, 14–16 µm wide. Head, 22–27 µm long, 17.5–23.1% of body proper length, spines not formed. Mouth
229 opening 10–19 µm from anterior end, positioned in the middle to posterior part of

230 head. Oesophagus meandering or almost straight, 55–89 µm long. Intestinal caeca not clearly branched,
231 67–108 µm from anterior end of body or 64.4–70.1% of body proper length.

232

233 4) Remarks

234 There are no large differences in the measurements of the AFA-fixed cercariogenoussporocysts of *C.*
235 *forsteri* between Shirakashi et al. [6] and the present study: 626 µm long ($n=7$) vs. 606 µm long ($n=17$) as
236 the mean value of the body proper.

237 In terms of morphology of the sporocyst and cercaria, *C. forsteri* is very similar to *C. opisthorchis*.
238 The *C. forsteri* cercaria has a slightly smaller body proper and tail than *C. opisthorchis* cercaria, but these
239 measurements overlapped. A pair of the excretory ducts in the main body is of much the same length in
240 the former, while one is about twice as long as the other in the latter. However, the excretory system in
241 these cercariae is difficult to trace. Consequently, it is practically very difficult to distinguish these larval
242 stages of *C. forsteri* from those of *C. opisthorchis* by morphology.

243

244 5) Taxonomic summary

245 Host: *Neoamphitrite vigintipes* (Grube, 1870) (Terebellidae).

246 Synonym: *Terebellavigintipes* Grube, 1870 (Type locality: Red Sea); nec *Amphitrite*
247 *vigintipes* sensu Marenzeller, 1884 (Enoshima & Kagoshima, Japan) (= *Amphitrite rubra* (Risso, 1826));
248 nec? *Neoamphitrite vigintipes* Hesse, 1917 (Sagami Bay).

249 Specific characters: Branchiae not bushy but ramified; with three branchiae; notosetae with
250 denticulate tips, and the tips not strongly geniculate; with 20 (rarely 21) thoracic chaetigers; with
251 small lateral lobes on 2nd to 4th segments; tentacular membrane without eye-spots; nephridial
252 papillae in six pairs on 3rd to 8th, but last three pairs indistinctly small; each thoracic uncus with ca.
253 six rows of small teeth on a large main fang, and dental formula MF: 5: 4:

254 Remarks on host polychaete species: *N. vigintipes* with 20 (rarely 21) thoracic chaetigers, but *A.*
255 *rubra* with 22–24 thoracic chaetigers [9]. Branchiae bushy in the genus *Amphitrite*, but ramified in
256 *Neoamphitrite*.

257 Locality and dates of sampling: Kushimoto, Wakayama Prefecture, Japan (33°28'N, 135°47'E), 23 June
258 2015.

259 Site: Body cavity.

260 Material: Meguro Parasitological Museum, Tokyo (MPM), MPM Collection No. XXXXX.

261

262 4. Discussion

263 With the description of the cercariae of the two species in this paper, the larval stages of all three
264 species of blood flukes infecting Pacific bluefin tuna (*C. orientalis*, *C. opisthorchis* and *C. forsteri*) have
265 been described. Among them, the cercaria of *C. orientalis* is easily distinguished by a short and
266 ellipsoidal tail (Shirakashi et al., 2016), whereas the other two have a longer and straight tail.
267 Besides, there were 15 gland cells divided into three groups in *C. opisthorchis* and *C. forsteri*. In these two

species, the additional two cells in the middle part of the body observed in *C. orientalis* were not confirmed. Differences in the tail morphology and the number of gland cells suggest that the former two species are more closely related to each other than to *C. orientalis*. On the other hand, morphologically distinguishing sporocysts and cercariae of *C. opisthorchis* from those of *C. forsteri* is difficult. In this study, we identified sporocysts as *C. forsteri* by the sequence of the 28S rDNA and as *C. opisthorchis* by the sequences of the ITS2 and 28S rDNA. For more practical use, we need to develop a multiplex species-specific PCR which can differentiate the three species simultaneously.

In comparison with the shorter tail of *C. orientalis*, the tail is straight and longer in *C. opisthorchis* and *C. forsteri*. This indicates that the latter two cercariae may have greater locomotive ability than that of *C. orientalis*, though their swimming ability appears rather limited. Similar structures in the head spines, head gland and penetration gland suggest that, upon encounter of the final host, the mode of invasion into the host tissue is basically the same among the three parasites.

Prior to our study, Cribb et al. [3] identified the intermediate host of *C. forsteri* in Australia as *Longicarpus modestus* (Terebellidae). The result of our present study reveals that the intermediate hosts of *C. forsteri* infecting southern bluefin tuna and Pacific bluefin tuna are different terebellid species. Although Cribb et al. [3] did not give a detailed description of the developmental stages, there are some differences in the measurements of sporocysts and cercariae between theirs and the present study. First, sporocysts (killed in hot saline) in Australia were 251–426 µm long, which is considerably smaller than those killed in hot saline of 431–596 µm long in the present study. Second, although the methods were different (hot saline-killed cercariae vs. flattened live ones), the cercariae in Australia had a body proper of 66–75 µm long and a tail of 40–55 µm long, considerably smaller than those of the Japanese counterparts, which were 97–131 µm long and 30–49 µm long, respectively. The Japanese cercariae were well flattened upon observation, which explains these dramatic size differences.

It is assumed that the developmental stages from *L. modestus* in Australia and those from *N. vigintipes* in Japan are the same species, *C. forsteri*, as their 28S rDNA sequences are 100% identical. The differences in the size of the sporocyst and cercaria from *L. modestus* as compared with those from *N. vigintipes* may reflect how the blood fluke has evolved and adapted to different intermediate and final hosts in two different geographical localities. However, little is known about the biology and ecology of the asexual stages of *Cardicola* spp. of bluefin tunas, except seasonality in the *C. opisthorchis* infection of the intermediate host, *Terebella* sp. [8]. Aside from differences in the cercarial morphology, possible differences in the seasonal occurrence and reproductivity in the intermediate host and infectivity to the final host between Japanese and Australian *C. forsteri* remain to be studied.

The Terebellidae is an exceptionally rich group among polychaetes [10], but aporocotylids have been recorded from only a handful of them. Here, we summarize the records of the terebellid intermediate hosts of blood flukes infecting marine teleosts (Table 1) and present a phylogenetic tree of the eight genera of terebellids (Fig. 7), based on the analysis of morphological characters modified from Nogueira et al. [10]. Table 1 shows that four out of eight known intermediate hosts are those of *Cardicola* spp. infecting farmed bluefin tunas of the genus *Thunnus*. Each *Cardicola* species infects two to

three bluefin tunas from different geographical localities. Considering that *C. forsteri* uses two different terebellids, as its intermediate hosts, *L. modestus* in Australia and *N. vigintipes* in Japan, it is possible that *C. orientalis* and *C. opisthorchis* also use more than one species of intermediate hosts in different localities.

According to the ITS2 phylogenetic tree of *Cardicola* and its related genera (Fig. 1 in Ogawa et al. [5]), *C. orientalis* belongs to a different cluster from that of *C. forsteri* and *C. opisthorchis*. It is interesting to note that the phylogenetic relation of the intermediate hosts of *C. forsteri* and *C. opisthorchis*, both producing cercariae with a straight tail, is also distant to the intermediate host of *C. orientalis*, producing cercariae with a short, elliptical tail (Fig. 7). Given the fork-tailed cercaria is the plesiomorphic form [14], the morphological change from the forked tail to the short elliptical one in *C. orientalis* may have required more than one event. Alternatively, *C. orientalis* infection of *N. gracilibranchis* may have resulted from host switch. Aporocotylidae gen. sp. of *Reteterebella* *aloba* (not *Reterebella* *aloba* as cited [3]) is very likely to be a species of *Cardicola*, as the molecular analysis showed that it was included in the *Cardicola* cluster [3]. Then, the known cercariae of *Cardicola* including the Aporocotylidae gen. sp. are rather poor swimmers with either a straight or a short and ellipsoidal tail. They may have evolved into these forms which have abandoned direct pursuit of the host [14]. The relationship between the terebellid phylogeny and the cercarial types of the aporocotylids is not very clear (Fig. 7). However, it will become more informative, as the increasing number of aporocotylid life cycles are demonstrated, which will give an important key to understand evolution and phylogeny of teleost blood flukes.

Many infected *N. gracilibranchis* and *Terebella* sp. specimens have been collected at Pacific bluefin farm sites in Japan, providing good opportunities to study biology and ecology of this rarely known group of parasites. It appears that *N. vigintipes* is only distributed in a specific microhabitat. We examined over 4,800 terebellids between January and June, 2015, and only seven infected *N. vigintipes* were found. One was collected in January 2015 but the precise sampling point was unclear because some samples were mixed at the time [6]. In the present study, we found six additional infected *N. vigintipes*, all from the bottom floor of a net cage. This result indicates that this species prefers specific environmental conditions, or at least environments different from those of *N. gracilibranchis*, the intermediate host of *C. orientalis*; the latter terebellid is abundant among fouling and sessile periphytons attaching to the ropes and floats of farming cages [6]. The actual abundance or population size of *N. vigintipes* at our study site is unclear. *C. forsteri* infection in cultured PBT is not common in Japan. In fact, only a few adults have been found from the juvenile PBT cultured at the study site. Estimating the population size of the intermediate hosts, as well as their microhabitats or preferred environmental conditions is important to assess the infection risk in farmed tuna.

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390 **Figure legends**

391

392 **Fig. 1.** Live *Neoamphitrite vigintipes*, the intermediate host of *Cardicola forsteri* infecting Pacific bluefin
393 tuna in Japan. Scale bar: 1 cm

394

395 **Fig. 2.** *Cardicola opisthorchis* cercariae. **a** Whole body (semidiagrammatic), **b** Posterior body showing a pair
396 of excretory ducts with four flame cells in photomicrograph under differential interference contrast
397 microscopy (top) and its diagrammatic drawing (bottom). Scale bars: 10 μ m

398

399 **Fig. 3.** Anterior part of *Cardicola opisthorchis* showing five rows of head spines (**a**) and secretion from
400 penetration gland cells (**b**; arrow) (differential interference contrast microscopy). Scale bars: 10 μ m

401

402 **Fig. 4.** Immature cercariae of *Cardicola opisthorchis*. Arrowheads indicate the mouth, followed by a long
403 winding (**a**) or almost straight (**b**) oesophagus leading to the intestinal caeca (arrows) (differential
404 interference contrast microscopy). Scale bars: 10 μ m

405

406 **Fig. 5.** *Cardicola forsteri* sporocysts, containing mature and developing cercariae (**a**) and daughter
407 sporocysts (**b**), fixed in AFA and stained with Heidenhain's iron hematoxylin (**a**) or alum carmine (**b**).
408 Scale bars: 100 μ m

409

410 **Fig. 6.** *Cardicola forsteri* cercariae. **a** Whole body (semidiagrammatic), **b–d** Posterior body, showing the
411 excretory system, viewed from frontal side (**b**) and lateral side (**c** & **d**). Scale bars: 10 μ m

412

413 **Fig. 7.** Phylogenetic relationships of the eight terebellid genera containing intermediate hosts of blood
414 flukes of marine teleosts, based on the analysis of morphological characters (modified from Nogueira et
415 al. [10]). As illustrated, the blood flukes are categorized into three groups by the tail structure. Scientific
416 names and localities of the blood flukes are shown in parentheses.

FIG 1

a

b

FIG 2

FIG 3

a

b

FIG 4

FIGURE 5

a

b

FIGURE 6

FIGURE 7

Table1. List of intermediate hosts of blood flukes infecting marine teleosts

Blood flukes	Intermediate hosts (no. of infected specimens found)	Developmental stages in intermediate hosts	Final hosts	Localities	References
<i>Aprocotyle simplex</i>	<i>Artacama proboscidea</i> (48) ^a	Redia	many pleuronectids	North Sea	[11] ^a
<i>Aprocotyle</i> sp.	<i>Lanassa nordenskiöldi</i> (1) ^b	Redia	unknown	East of Iceland	[12] ^b
<i>Cardicola forsteri</i>	<i>Longicarpus modestus</i> (1) ^c	Sporocyst	<i>Thunus maccoyii</i>	Australia	[3] ^c
	<i>Neoamphitrite vigintipes</i> (7) ^{d,e}	Sporocyst	<i>Thunus orientalis</i>	Japan	[6] ^d ; this study ^e
<i>Cardicola orientalis</i>	<i>Nicolea gracilibranchis</i> (466) ^{d,e}	Sporocyst	<i>Thunus orientalis</i>	Japan	[6] ^d ; this study ^e
<i>Cardicola opisthorchis</i>	<i>Terebella</i> sp. (62) ^{e,f,g}	Sporocyst	<i>Thunus orientalis</i>	Japan	[7] ^f , [8] ^g , this study ^e
Aporocotylidae gen. sp.	<i>Reteterebella aloba</i> (1) ^c	Sporocyst	unknown	Australia	[3] ^c
<i>Cercaria hartimanae</i>	<i>Lanicides vayssierei</i> (1) ^h	Redia	unknown	Ross Island, Antarctica	[13] ^h

Phylogeny of the eight polychaete genera containing intermediate hosts of blood flukes of marine teleosts