Gobioecetes biwaensis n. g., n. sp. (Monogenea: Dactylogyridae) from the gills of a freshwater gobiid fish, *Rhinogobius* sp. BW Takahashi & Okazaki, 2002, with a redescription of *Parancyrocephaloides daicoci* Yamaguti, 1938

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Keywords: *Gobioecetes biwaensis*, *Parancyrocephaloides daicoci*, *Rhinogobius* sp. BW, new genus, Monogenea

Abstract—A description is given of *Gobioecetes biwaensis* n. g., n. sp. (Monogenea: Dactylogyridae) on the gills of a freshwater gobiid fish, *Rhinogobius* sp. BW Takahashi & Okazaki, 2002 from Lake Biwa, Shiga Prefecture and *Rhinogobius* sp. from Sento Imperial Palace, Kyoto Prefecture, Japan. This monogenean is most similar to *Parancyrocephaloides rhinogobius* Ling, 1973, proposed here as *Gobioecetes rhinogobius* n. comb., collected from the gills of *Rhinogobius giurinus* (Rutter, 1897) in Hupei Province, China, but can be differentiated from it in that ventral hamuli of *G. biwaensis* are larger than those of *G. rhinogobius* and in that in *G. biwaensis*, ventral hamuli are larger than dorsal ones, but in *G. rhinogobius*, ventral and dorsal hamuli are almost equal in size. The new genus is characterized by the absence of a bar supporting dorsal hamuli. *Parancyrocephaloides daicoci* Yamaguti, 1938, the type species of the genus, is redescribed based on newly collected specimens from a marine dactylopterid, *Dactyloptena peterseni* (Nyström, 1887) and the type specimens, revealing that it has a pair of vestigial bars supporting dorsal hamuli. The generic diagnoses of *Gobioecetes* n. g. and *Parancyrocephaloides* are given and systematic position of these genera in Dactylogyridae is discussed in this paper.

1. Introduction

We had a chance to examine parasites of a gobiid fish in a pond at Sento Imperial Palace, Kyoto Prefecture and found a species of monogenean closely related to *Parancyrocephaloides rhinogobius* Ling, 1973 on the gills, *Rhinogobius giurinus* (Rutter, 1897) (now *Rhinogobius similis* Gill, 1859 [1]) in Hupei Province, China [2]. The genus *Parancyrocephaloides* was created by Yamaguti [3] based on a new species *Parancyrocephaloides daicoci* collected from the gills of starry flying gurnard, *Daicocus peterseni* (Nyström, 1887) (now *Dactyloptena peterseni* (Nyström, 1887) [4]) at Tosa, Kochi Prefecture, Japan. According to their original descriptions, both *P. rhinogobius* and *P. daicoci* have morphological features in common in the opisthohaptoral sclerites: two pairs of hamuli, of which the ventral pair is supported by a connecting bar, while the dorsal one has none. However, the hosts of these monogeneans are very different from each other: the former from a freshwater gobiid (order Perciformes) and the latter from a marine dactylopterid (order Scorpaeniformes). *P. daicoci* has not been examined since the creation of the genus in 1938. An attempt was made to reexamine the parasite morphologically and molecularly based on newly collected specimens, together with the type specimens of *P. daicoci* and its closely related *Parancyrocephaloides dactylopteri* Yamaguti, 1968 collected from Oriental flying gurnard, *Dactylopterus orientalis* Cuvier, 1829 (now *Dactyloptena orientalis* (Cuvier, 1829) [4]) in Hawaii [5].

As for the monogenean of the goby from Sento Imperial Palace, the host fish was initially thought to be *Rhinogobius* sp. BW Takahashi & Okazaki, 2002 (in which BW stands for Lake Biwa and Japanese name is Biwa-yoshinobori [6]), as the pond of the Palace, now isolated from outside water systems, had once been connected with Lake Biwa through water channels (Ms. T. Sako, personal communication). Subsequent ichthyological and molecular analyses have revealed that the host fish was morphologically similar to, but molecularly distinguishable from, *Rhinogobius* sp. BW, tentatively designated here as *Rhinogobius* sp. (Biological Laboratory, Imperial Palace, personal communication). Further attempts were then made to collect monogeneans

from *Rhinogobius* sp. BW in Lake Biwa.

Here, we describe the monogenean from *Rhinogobius* sp. BW in Lake Biwa and *Rhinogobius* sp. at Sento Imperial Palace, Kyoto as a new genus and a new species and redescribe *P. daicoci*, together with the diagnoses of the new genus and *Parancyrocephaloides*. We also discuss their phylogenetic positions based on the morphological and molecular analyses.

2. Materials and methods

2.1. Collection and preparation of parasite specimens

Rhinogobius sp. was caught in South Pond, at Sento Imperial Palace, Kyoto Prefecture in June 28 and July 7, 2013 and *Rhinogobius* sp. BW were caught off Hikone, Lake Biwa in July 23, 2013 and off Takashima, Lake Biwa in June 19, 2014. They were transported to Meguro Parasitological Museum, Tokyo and examined for monogeneans on the gills under a dissecting microscope. Live monogeneans (one from a *Rhinogobius* sp. in Sento Imperial Palace and five from *Rhinogobius* sp. BW off Takashima, Lake Biwa) were flattened and fixed in AFA (mixture of 70% ethanol, 10% formalin and acetic acid) and stained with Heidenhain's iron hematoxylin or alum carmine. A total of 15 (six from *Rhinogobius* sp. at Sento Imperial Palace and nine from *Rhinogobius* sp. BW off Hikone, Lake Biwa) formalin-fixed monogenean specimens and two live specimens from *Rhinogobius* sp. at Sento Imperial Palace were mounted in ammonium picrate-glycerin (AP-G) medium between a cover slip and glass slide, later dehydrated, cleared in xylol and mounted in Canada balsam. Stained specimens and AP-G specimens were used for observation and measurements of general anatomy and sclerotized hard parts, respectively. Sizes of the body and male copulatory organ (penis and its accessory piece) were measured, using both stained and AP-G specimens.

Two individuals of *Dactyloptena peterseni* were caught by a commercial dragnet off Sakitsu, Amakusa, Kumamoto Prefecture, Japan and transported live to Kumamoto Prefectural Fisheries Research Center, Kumamoto Prefecture, where parasitological examination was conducted in February 19, 2014. Live specimens of

Parancyrocephaloides daicoci were collected from the gills and prepared for permanent mount hematoxylin/carmin and AP-G slides as in gobiid monogeneans described above. Measurements were made as in gobiid monogeneans, except for some sclerotized parts, which were measured, using both stained and AP-G specimens.

All figures except for *P. dactylopteri* were drawn using a drawing tube. As for *P. dactylopteri*, images of the holotype and paratypes were printed and traced. Measurements, using a calibrated ocular micrometer or digital photo equipment (DS-Fi1 and DS-L2, Nikon), were given in micrometers as the range and the number of specimens in parentheses. Fish scientific names follow Echmeyer et al. [4].

Other parasite specimens used in this study are: *P. daicoci* holotype and four paratypes, Meguro Parasitological Museum, MPM Coll. No. 23719; *P. dactylopteri* holotype and two paratypes, US National Museum, USNM 1359362.

2.2. DNA extraction, PCR amplification and DNA sequencing

Two individuals of monogeneans on the gills of *Rhinogobius* sp. BW from Lake Biwa and two individuals of *Parancyrocephaloides daicoci* on the gills of *Dactyloptena peterseni* from Sakitsu, Amakusa were stored in 99% EtOH. Total DNA was extracted using DNeasy Mini Kit (QIAGEN) and partial 18S rRNA and entire ITS1 region were amplified by S1 primer (5'-ATT CCG ATA ACG AAC GAG ACT-3') [7] and IR8 primer (5'-GCT AGC TGC GTT CTT CAT CA-3') [8]. Amplification reaction was carried out in 20 µL volume containing 0.1 µL of Takara Ex Taq DNA polymerase (Takara Bio), 2.0 µL of PCR buffer (Takara Bio), 1.6 µL of dNTP mixture (2.5 mM each dNTP) (Takara Bio), 0.6 µL of 10 µM each primer, 1.0 µL of extracted DNA and 14.1 µL of DNase-RNase free distilled water. PCR temperature condition was first-denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55.0 °C for 1 min and extension at 72 °C for 1.5 min, and final extension step at 72 °C for 10 min. After running on 1.5% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen), PCR product with approximately 1200 bp were extracted using FastGene Ge/PCR Extraction Kit (Nippon Genetics). Purified PCR products were sent to Eurofins

Genomics for direct sequencing with the PCR primers plus one reverse primer IR5 (5'-TAC GGA AAC CTT GTT ACG AC-3') [7] and one newly designed forward primer IF (5'-AAG GGT TTC CGT AGG TGA AC-3').

In order to closely examine the phylogenetic position of *P. daicoci* in marine ancyrocephalines, partial 28S rRNA region was amplified by C1 primer (5'-ACC CGC TGA ATT TAA GCA T-3') and D2 primer (5'-TGG TCC GTG TTT CAA GAC-3') [9]. Amplification reaction was carried out in 20 µL volume containing 0.1 µL of Takara Ex Taq DNA polymerase (Takara Bio), 2.0 µL of PCR buffer (Takara Bio), 1.6 µL of dNTP mixture (2.5 mM each dNTP) (Takara Bio), 0.6 µL of 20 µM each primer, 1.0 µL of extracted DNA and 14.1 µL of DNase-RNase free distilled water. PCR temperature condition was first-denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 53.0 °C for 1 min and extension at 72 °C for 1 min, and final extension step at 72 °C for 5 min. After running on 1.5% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen), PCR product with approximately 900 bp were extracted using QIAquick Gel Extraction Kit (QIAGEN). Purified PCR products were sent to Macrogen Japan for direct sequencing with the PCR primers.

2.3. Molecular analyses

According to the previous phylogenetic analyses of Dactylogyridea [8], 18S rRNA data of 36 monogenean species belonging to Monopisthocotylea were retrieved from GenBank database (Table 1) and aligned with the two sequences identified above using Clustal W of MEGA 6.06-mac package [10]. The default setting was used for the alignment. Gaps were treated as missing data, and 387 aligned positions were used for the following analyses.

Phylogenetic trees were constructed from both Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses using MEGA 6.06-mac package [10]. ML analyses followed the discrete gamma model (5 categories) and the general time reversible (GTR) model in which the initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms. The tree with the

highest log likelihood was selected as the best tree and bootstrap (BS) values for internal nodes were calculated with 1000 replicates. The MP tree was obtained using Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Bootstrap method with 1000 replicates was used for the phylogeny test.

For phylogenetic analyses of *P. daicoci* in marine ancyrocephalines, according to Wu et al. [11], 28S rRNA data of 48 marine ancyrocephalines and four ancyrodiscoidines as outgroups were retrieved from GenBank database (Table 2) and aligned with the identified sequences using the same setting described above. Gaps were treated as missing data, and 565 aligned positions were used for MP and ML analyses using the exactly same settings for analyses of 18S rRNA region as above.

3. Results

3.1. Morphological description

3.1.1. *Parancyrocephaloides Yamaguti, 1938*

Monogenea (Van Beneden, 1858) Bychowsky, 1937

Dactylogyridea Bychowsky, 1937

Dactylogyridae Bychowsky, 1933

Parancyrocephaloides Yamaguti, 1938

Emended diagnosis: Body small. Opisthohaptor with 14 marginal hooks and two pairs (ventral and dorsal) of hamuli containing canal in point, ventral bar and a pair of vestigial dorsal bars. Head organs and two pairs of eyes present. Intestine bifurcate, confluent posteriorly. Testis large, folded upon itself. Vas deferens looping left intestinal crus. Seminal vesicle formed by mere dilatation of vas deferens. Two prostatic reservoirs. Male copulatory organ consisting of cylindrical base and sclerotized penis without accessory piece. Ovary compact, slightly dextral, pretesticular, not looping right intestinal crus. Vagina opening dextral. Seminal receptacle at the end of vaginal duct. Vitellarium co-extensive with intestine. Parasites of marine teleosts.

***Parancyrocephaloides daicoci* Yamaguti, 1938**

Redescription based on 5 whole mount specimens (Figs. 1–3): Body stout, 1308–1710 long (n=3), 669–702 wide (n=3) at level of testis. Head in three lobes, central one largest, with three to four pairs of head organs. Head gland cells on both sides of body at level of intestinal bifurcation. Two pairs of eyes anterodorsal to pharynx. Pharynx 117–134 long (n=3), 88–106 wide (n=3). Postpharyngeal gland cells on both sides of pharynx. Oesophagus short. Intestine bifurcate, confluent behind testis. Opisthohaptor short, transversely wide, 119–178 long (n=3), 352–373 wide (n=3). Ventral hamuli thick and massive, 88–103 long (n=5); hamulus base 51–60 long (n=2), point 17–18 long (n=2), ratio of hamulus length to base length 1.70–1.98 (n=2); external process, thick and uniform width along its length, 48–52 (n=2) long, 23–27 wide (n=2); internal process, shorter and narrower than external one, tapering distally, 25–31 long (n=2), 14–21 wide (n=2). A bar supporting ventral hamuli, V-shaped, thickened and lumpy centrally, 46–57 long (n=5). Dorsal hamuli thick and massive, 90–99 long (n=3), almost equal size as ventral ones; hamulus base 57–67 long (n=2), point 20–21 long (n=2), ratio of hamulus length to base length 1.48–1.60 (n=2); external process, thick and uniform width along its length, 26–41 (n=2) long, 17–28 wide (n=2); internal process, longer and narrower than external one, 29–45 long (n=2), 19–24 wide (n=2). A pair of weakly sclerotized bars supporting dorsal hamuli, almost straight, widely apart from each other, 27–29 long (n=2), 6–8 wide (n=2). Marginal hooks in 7 pairs, 14–15 long (n=5).

Testis 492–624 long (n=3), 396–411 wide (n=3), oval, folded back on itself, occupying central area of posterior body proper inside intestinal loop. Vas deference emerging at apical end of testis, looping around left intestinal crus, forming seminal vesicle inside intestinal loop on left side of body, bulging posteriorly, 124–208 long (n=3), 61–70 wide (n=3), then forming another seminal vesicle behind intestinal bifurcation, smaller, sausage-shaped, 48–74 long (n=3), 19–25 wide (n=3) before leading into base of penis. Two prostatic reservoirs lying side by side on left side of second seminal vesicle; sausage-shaped one, 37–67 long in a straight line (n=3), 15–18

wide (n=3) and round, thick-walled one, 32–41 long (n=3), 17–24 wide (n=3). Prostatic cells of the former, extending outside of intestinal loop on both sides of body at level of intestinal bifurcation to level of first seminal vesicle and those of the latter, extending posteriorly to level of first seminal vesicle. Male copulatory organ, 96–105 long (n=5), consisting of sclerotized, pincer-shaped penis, 39–43 long (n=5), 10–11 wide at base (n=5) and cylindrical solid base, 56–68 long (n=5), 13–16 wide (n=5). No accessory piece of penis. Genital pore immediately behind intestinal bifurcation.

Ovary 175–204 long (n=3), 99–114 wide (n=3), tapering proximally, slightly right to midline, immediately anterior to testis, directing obliquely left. Germiduct arising at apical end of ovary, directing posteriad, then anteriad to join seminal receptacle, leading to oötype and short uterus. Mehlis gland cells widely distributed inside of intestinal loop on both sides of germiduct immediately posterior to oötype. Vagina opening ventrally on right side of body behind mass of prostatic cells, surrounded by gland cells, narrow vaginal duct directing obliquely posteriad to mid-body, leading into seminal receptacle. Vitellarium co-extensive to intestine.

Remarks: Yamaguti's original description of *P. daicoci* [3] shows several differences from the present redescription. However, reexamination of his specimens confirmed that a pair of dorsal bars are present and that vas deferens does loop around left intestinal crus. The two bars are weakly sclerotized and widely apart from each other, suggesting that they are vestigial and not functional. *P. daicoci* is most similar to *Parancyrocephaloides dactylopteri* from the gills of *Dactyloprerus orientalis* in Hawaii [5]. However, *P. daicoci* is distinguished from *P. dactylopteri* by the larger size of several sclerotized parts: ventral hamulus 88–103 long in this study and 90 long in the Yamaguti's original description vs. 46–58 long in *P. dactylopteri*; dorsal hamulus 90–99 long and 78 long vs. 56–68 long; male copulatory organ 96–105 long and 93 long vs. 60–75 long. Close to the dorsal hamuli, one pair of marginal hooks were drawn [5], the shape of which is quite different from those of *P. daicoci* in the thick handle part. Reexamination of his type specimens (USNM 1359362) in this study confirmed that they

are not marginal hooks but a pair of dorsal bars (Fig. 4).

Three more species of *Parancyrocephaloides* were reported from Asian fishes: *P. leiocassis* Reichenbach-Klinke, 1959 on Asian bumblebee catfish, *Leiocassis siamensis* Regan, 1913 (now *Pseudomystus siamensis* (Regan, 1913) [4]) (Bagridae) imported from Siam (=Thailand) to Germany [12], *P. indicus* Kurkarni, 1969 on *Clupea rarengus* from Ramappa Lake, Hyderabad, India [13] and *P. putitorai* Singh, Arya et Anuradha, 2002 on Putitor mahseer, *Tor putitora* (Hamilton, 1822) (Cyprinidae) from Indus River System, India [14]. All these monogeneans are removed here from *Parancyrocephaloides*, as they lack a dorsal bar. Their taxonomic status is further discussed later in this paper [see Remarks in 3.1.2]

Taxonomic summary

Specimens used: 5 newly collected specimens (3 stained specimens, deposited in the Meguro Parasitological Museum, Tokyo, MPM Coll. No. 21025; 2 ammonium picrate-glycerine-fixed specimens, MPM Coll. No. 21026), caught off Sakitsu, Amakusa, Kumamoto Prefecture, Japan; Holotype and 4 paratypes, MPM Coll. No. 23719.

Type species, host and localities: *Parancyrocephaloides daicoci* Yamaguti, 1938 on gills of *Daicocus peterseni* (Nyström, 1887) (now *Dactyloptena peterseni* (Nyström, 1887) [4]); Tosa, Kochi Prefecture, Japan

Other species:

P. dactylopteri Yamaguti, 1968 on the gills of *Dactyloprerus orientalis* Cuvier, 1829 (now *Dactyloptena orientalis* (Cuvier, 1829) [4]); Hawaii

3.1.2. *Gobioecetes* n. g.

Diagnosis: Dactylogyridae Bychowsky, 1933. Body small. Opisthohaptor with 14 marginal hooks and two pairs (ventral and dorsal) of hamuli, of which ventral hamuli have a connecting bar and dorsal ones have none. Three pairs of head organs and two pairs of eyes present. Intestine bifurcate, confluent posteriorly. Testis elliptical, in

posterior body proper. Vas deferens looping left intestinal crus. Seminal vesicle formed by mere dilatation of vas deferens. Single prostatic reservoir. Male copulatory organ sclerotized, consisting of tubular penis and accessory piece. Ovary compact, median, pretesticular, not looping right intestinal crus. Vagina opening dextral, unarmed. Seminal receptacle at the end of vaginal duct. Vitellarium co-extensive with intestine. Parasites of teleosts.

Type species, host and localities: *Gobioecetes biwaensis* n. sp. on gills of *Rhinogobius* sp. BW in Lake Biwa.

Etymology: The genus name is a combination of Latin *gobius*, the gobiid host and Greek *oikētēs*, meaning an inhabitant.

***Gobioecetes biwaensis* n. sp.**

Description: based on 23 whole mount specimens (Figs. 5–7): Body slender, 273–622 long (n=18), 111–200 wide (n=18) in mid-body. Head lobes indistinct, with three pairs of head organs. Head gland cells on both sides of body at level of posterior pharynx. Two pairs of eyes dorsal to pharynx. Pharynx 50–75 long (n=5), 44–65 wide (n=5). Postpharyngeal gland cells at base of pharynx. Oesophagus short. Intestine bifurcate, confluent behind testis. Opisthohaptor indistinctly marked off from body proper, 130–181 wide (n=6). Cement gland well developed.

Ventral hamuli 40–48 long (n=15); hamulus base 28–35 long (n=15), point 10–14 long (n=14), ratio of hamulus length to base length 1.26–1.55 (n=15); external process, short and straight, 9–12 (n=15) long, 5–6 wide (n=15); internal process, thick and long, slightly tapering distally, 20–27 long (n=15), 7–10 wide at base (n=15). Ventral bar straight, thickened at both ends, 27–37 long (n=14). Dorsal hamuli 34–43 long (n=15), shorter than ventral ones; hamulus base 29–37 long (n=15), point 10–12 long (n=14), ratio of hamulus length to base length 1.03–1.26 (n=15); external process, thick and short, sometimes widened basally and triangular in shape, 4–9 (n=15) long, 4–8 wide at base (n=15); internal process, thick and straight, slightly tapering distally, 11–17 long (n=15), 8–12 wide at base (n=15). No dorsal bar. Marginal hooks in 7 pairs, 14–17 long

(n=15).

Testis, variable in size, 42–116 long (n=6), 19–69 wide (n=6), in posterior body proper inside intestinal loop. Vas deference emerging at apical end of testis, directing anteriad, looping around left intestinal crus, forming seminal vesicle in mid-body, 19–30 long (n=6), 7–16 wide (n=6), before leading into base of penis. Prostatic reservoir, curved, just behind seminal vesicle, 10–28 long in a straight line (n=5), 5–9 wide (n=5). Male copulatory organ, sclerotized, consisting of penis and accessory piece. Penis, long, coiled and tapering tube, 1–2 wide (n=19), 4–9 wide at base (n=17), with unsclerotized, non-muscular bulb, 7–11 in diameter (n=17). Accessory piece, 18–28 long (n=16), 4–7 wide at base (n=16), with rod-shaped proximal half and widened distal half with bifurcate claw-like ends. Genital pore behind intestinal bifurcation.

Ovary, variable in size, 61–110 long (n=5), 29–63 wide (n=5), same size or slightly larger than and immediately anterior to testis. Germiduct arising at apical end of ovary, directing anteriad, leading to oötype and uterus. Mehlis gland opening at base of oötype. Vagina opening on right lateral side of body anterior to oötype; narrow vaginal duct directing obliquely posteriad to mid-body, leading into seminal receptacle immediately anterior to ovary. Vitellarium co-extensive to intestine.

Remarks: The specimens used in this study consist of two groups, one from *Rhinogobius* sp. at Sento Imperial Palace, Kyoto and the other from *Rhinogobius* sp. BW in Lake Biwa. There are slight morphological differences in the sclerotized body parts (male copulatory organ and haptoral armatures) between the two (see Figs. 7 and 8). However, similar differences were also noticed within the groups, and we consider them intraspecific morphological variations.

G. biwaensis n. sp. is most similar to *Parancyrocephaloides rhinogobius* Ling, 1973 collected from the gills of *Rhinogobius giurinus* in Hupei Province, China [1]. The present new species is distinguished from *P. rhinogobius* by the shape and size of the ventral and dorsal hamuli; 1) ventral hamuli of *G. biwaensis* n. sp. are larger than those of *P. rhinogobius* (40–48 μm vs. 37–40 μm), and 2) in *G. biwaensis*, ventral hamuli are

larger than dorsal ones and their internal and external process are more slender than those of dorsal ones, but in *P. rhinogobius*, ventral and dorsal hamuli are almost equal in size and shape. Hence, *Gobioecetes rhinogobius* n. comb. is proposed here for *P. rhinogobius*.

Three more species of *Parancyrocephaloides* are known; *P. leiocassis* Reichenbach-Klinke, 1959 on *Leiocassis siamensis* Regan, 1913 (now *Pseudomystus siamensis* (Regan, 1913) [4]), *P. indicus* Kulkarni, 1969 on *Clupea rarengus* and *P. putitorai* Singh, Arya et Anuradha, 2002 on *Tor putitora* (F. Hamilton, 1822) [12,13,14]. They all lack a dorsal bar, the same characteristic as that of *Gobioecetes biwaensis*. However, the description of *P. leiocassis* was too simple to justify its assignment to *Gobioecetes*. Unfortunately, the type specimens of *P. leiocassis* have been lost (D. Grabner, personal communication), and it was not possible to examine them in this study. *P. indicus* has different characters from those of *G. biwaensis*; dorsal hamuli with a bar, ventral hamuli without a bar, only three pairs of marginal hooks and no head organs. This monogenean will not be assigned to *Gobioecetes*, if its dorso-ventral orientation was as described. Besides, the scientific name of the host fish, *Clupea rarengus*, may not be correct, as it is not listed in Catalog of Fishes [4], and it is possibly a misspelling for *C. harengus* Linnaeus, 1758, Atlantic herring, which is not distributed in freshwater India. *P. putitorai* can be excluded from *Gobioecetes* by its very peculiar features: having a pair of ventral bars and five pairs of marginal hooks. Now it has made clear that these three monogeneans no longer belong to *Parancyrocephaloides*, and their taxonomic statuses are required to be reexamined.

Taxonomic summary

Type-host and localities: *Rhinogobius* sp. BW Takahashi & Okazaki, 2002 [Japanese name: Biwa-yoshinobori]; off Takashima, Lake Biwa, Shiga Prefecture (35°19'N, 136°03'E) (type-locality) on June 19, 2014, off Hikone, Lake Biwa (35°17'N, 136°12'E) on July 23, 2013.

Other-host and locality: *Rhinogobius* sp.; South Pond, Sento Imperial Palace, Kyoto

Prefecture (35°01'N, 135°46'E) on June 28 & July 7, 2013.

Site on host: gill filaments

Type material: Holotype deposited at Meguro Parasitological Museum, Tokyo, MPM Collection Number 251027 and 11 paratypes, collected from *Rhinogobius* sp. BW in Lake Biwa, MPM Coll. No. 21028; nine paratypes collected from *Rhinogobius* sp. at Sento Imperial Palace, MPM Coll. No. 21029.

Etymology: The species is named after Lake Biwa, the type locality of the host fish.

Other species: *Gobioecetes rhinogobius* n. comb. on the gills of *Rhinogobius giurinus* (Rutter, 1897) (now *Rhinogobius similis* Gill, 1859 [1]) in Hupei Province, China.

Synonym: *Parancyrocephaloides rhinogobius* Ling, 1973.

3.2. Molecular analyses

3.2.1. DNA sequencing

Totally, 1069 bp and 1190 bp 18S rRNA sequences were identified from *G. biwaensis* and *P. daicoci*, respectively. Additionally, 828 bp of 28S rRNA sequences were identified from *P. daicoci*. Intraspecific variation in the sequence data was not found for both species. BLAST searches confirmed that any identical DNA sequence to them was deposited in GenBank. Identified DNA sequences of 18S rRNA from *G. biwaensis* and *P. daicoci*, and 28S rRNA from *P. daicoci* were registered in GenBank under the accession number, GenBank under the accession numbers, LC176446, LC176447 and LC190513, respectively.

3.2.2. Phylogenetic trees based on ML and MP analyses

In the phylogenetic tree constructed with ML analyses using 18S rRNA data set (Fig. 9), Gyrodactylidea Bychowsky, 1937, Capsalidea Lebedev, 1988, Monocotylidea Lebedev, 1988 and Dactylogyridea were clearly distinguished with high BS values, and two suborders, Dactylogyrynea Bychowsky, 1937 and Tetraonchinea Bychowsky, 1937, were recognized in the order Dactylogyridea. In the family Dactylogyridae, four subfamilies, Dactylogyryinae Bychowsky, 1933, Pseudodactylogyryinae Ogawa & Egusa, 1986, Ancyrocephalinae Bychowsky, 1937 and Ancylo-discoidinae Bychowsky, 1937,

were included, while Ancyrocephalinae appeared polyphyletic with two distinct clades; one is formed with *Haliotrema* spp., *Thylacicleidus* sp. and *Pseudohaliotrema sphincteropus* Yamaguti, 1953, and the other with *Cleidodiscus pricei* Mueller, 1936, *Ancyrocephalus percae* Ergens, 1966, *Urocleidus similis* (Mueller, 1936) and *Thaparocleidus* spp.

Although the phylogenetic position of *Pseudomurraytrema ardens* sensu Littlewood, Rohde & Clough, 1998 (treated as a nomen nudum by Boeger et al. [15]; now *Pseudomurraytrema* sp.) and interrelationships among Monocotylidea and Ancyrocephalinae were different from the ML tree, the topology of the MP tree was mostly identical to the ML tree (Fig. 10).

Gobioecetes biwaensis was closely related to *Pseudodactylogyroides apogonis* Ogawa & Egusa, 1986 and *Pseudodactylogyrus* spp. and placed in the subfamily Pseudodactylogyrinae in both ML and MP trees. However, this relationship was not well supported by bootstrap tests in both analyses.

Based on 18S rRNA region, *P. daicoci* formed a clade with a group of marine Ancyrocephalinae containing *Haliotrema* spp., *Thylacicleidus* sp. and *Pseudohaliotrema sphincteropus*, and the branch from the Ancyrocephalinae group was strongly supported in both ML and MP analyses (Fig. 9 and Fig. 10, respectively). The phylogenetic position of *P. daicoci* in marine ancyrocephalines based on 28S rRNA region was shown (Fig. 11). *P. daicoci* formed a clade with *H. epinepheli* and *H. cromileptis*, both from Serranidae, with moderate BS values in both MP and ML analyses, and this clade was placed to the clade consisting of ancyrocephaline species from Pomadasyidae (now Haemulidae [4]) (*Bravohollisia* spp. and *Caballeria intermedius* Lim, 1995), while this relationship was not supported with confident BS values.

4. Discussion

Bychowsky and Nagibina [16] raised the Ancyrocephalinae to family status, but Kritsky and Boeger [17] suggested that the Ancyrocephalidae Bychowsky, 1937 return to subfamily status within the Dactylogyridae, because the former contains both

para- and polyphyletic components. Šimková et al. [8,18] showed Anacyrocephalinae is polyphyletic within the Dactylogyridae based on both small and large subunit rDNA analyses. Their phylogenetic trees imply that anacyrocephaline species consist of two different clades, one from freshwater fishes and the other from marine fishes.

Burn [19] proposed *Protancyrocephaloides liopsettae* gen. et sp. n. from smooth flounder, *Liopsetta putnami* (Gill, 1864) collected in New Hampshire, USA, which is similar to species of *Gobioecetes* in that both genera have no dorsal bar. However, *Protancyrocephaloides* Burn, 1978 is different from *Gobioecetes* in that the penis is a simple tube without an accessory piece. Molecular comparison of *Protancyrocephaloides liopsettae* Burn, 1978 with *Gobioecetes* spp. was not possible, as no DNA data is available for *P. liopsettae*. Both *Linguadactyla* Brinkmann, 1940 (Linguadactylinae Bychowsky, 1957) and *Liguadactylodes* Thatcher & Kritsky, 1983 (Linguadactylodinae Thatcher & Kritsky, 1983) lack a dorsal bar [20,21], but they can be separated from *Gobioecetes* by having multiple testes. The phylogenetic tree including *G. biwaensis* was based on 18S rDNA sequences, as only 18S rDNA data were available for pseudodactylogyrids including *P. apogonis* and *P. haze* Ogawa, 1984. In both ML and MP analyses, *Gobioecetes* was included within the Pseudodactylogyridae clades, though the nodes were supported by low BS values. Morphologically, *Gobioecetes* shares many characteristics with Pseudodactylogyridae. Particularly, *Pseudodactylogyroides* Ogawa & Egusa, 1986, more ancestral than *Pseudodactylogyrus* Gusev, 1965 within the subfamily, has a pair of vestigial dorsal hamuli with no supporting bar, a long, tubular penis accompanied by an accessory piece and a single prostatic reservoir attached to the base of the penis [22,23]. However, in *Gobioecetes*, dorsal hamuli are functional, though not supported by a bar, and the prostatic reservoir is adjacent, not attached, to the penis. Instead, an unsclerotized bulb is attached to the base of the penis. Among the existing subfamilies in Dactylogyridae, *Gobioecetes* is closest to Pseudodactylogyridae, but its subfamily status remains to be determined until molecular analysis based on sequence data of 28S rDNA become available for *P. apogonis* and *P. haze*.

Pseudodactylogyrinae consists of two genera: *Pseudodactylogyrus* and *Pseudodactylogyroides*. *Pseudodactylogyrus* has eight species, of which two are parasites of Gobiidae: *P. haze* on yellowfin goby, *Acanthogobius flavimanus* (Temminck & Schlegel, 1845) and *P. bialatus* (Wu, Wang & Jian, 1988) on Asian freshwater goby, *Synechogobius ommaturus* (Richardson, 1845), and *Pseudodactylogyroides* has four species, of which three are from Gobioidae fish (two from Eleotridae, one from Gobiidae): *P. marmorata* Lim, 1995 on marble goby, *Oxyelotris marmorata* (Bleeker, 1952), *P. butisensis* Lim 1995 on duckbill sleeper, *Butis butis* (Hamilton-Buchanan, 1822) and *P. papuensis* Li, 2004 on frogface goby, *Oxyurichthys papuensis* (Valenciennes, 1837) [23-26]. This suggests that Gobioidae fish have played an important role in the evolution of Pseudodactylogyrinae and *Gobioecetes*. It is to be noted that these five pseudodactylogyrids are parasites of freshwater gobies, except for *P. haze* on *A. flavimanus* which was collected from both fresh and brackish water [23-26].

Systematic position of *Parancyrocephaloides* has not been examined since Yamaguti [5,27] placed *P. dactylopteri* and *P. daicoci* in Ancyrocephalinae, Dactylogyridae. Morphologically, haptor sclerites of *P. daicoci* are similar to those of *Pseudomurraytrema copulatum* (Mueller, 1938) Bychowsky, 1957 in that both species have ventral hamuli with a bar and dorsal hamuli with a pair of bars. However, *P. daicoci* differs from *P. copulatum* in that in the former species the oviduct leads to oötype without looping the intestine, whereas in the latter it loops left intestinal crus [18]. This study, as in Šimková et al. [8,18], clearly showed that *Pseudomurraytrema* sp. from *Catostomus ardens* Jordan & Gilbert representing the Pseudomurraytrematidae formed a sister clade to the Dactylogyridae. The cylindrical, solid base of the male copulatory organ, a pair of vestigial dorsal bars and a folded testis of *Parancyrocephaloides* are unique in the Ancyrocephalinae and its related genera (see also Table 3 of [11]). In the ML and MP analyses of 18S rDNA, *P. daicoci* was included in marine ancyrocephalines, but its deduced phylogenetic position may not be accurate enough, as the species used were limited. For more extensive examination of the

phylogenetic position of *P. daicoci* among marine ancyrocephalines, 28S rDNA data were used for the ML and MP analyses, following Wu et al. [11]. The phylogenetic tree was depicted as ((*P. daicoci*, *Haliotrema epinepheli* + *H. cromileptis*), *Bravohollisia* spp. + *Caballeria intermedius*) (Fig. 11). These *Haliotrema* spp. and *Bravohollisia* spp./*C. intermedius* are parasites of Serranidae and Haemulidae (both Perciformes), respectively, while *P. daicoci* is a parasite of Dactylopteridae (Scorpaeniformes), quite distant from the former two families. Morphologically, these *Haliotrema*, *Bravohollisia* and *Caballeria* are different from *Parancyrocephaloides* in having a dorsal bar, but similar in having a penis without an accessory piece. *Bravohollisia* and *Caballeria* may be closer to *Parancyrocephaloides* in that they have rather thick and massive hamuli containing canals [28], though the node of the clade ((*P. daicoci*, *Haliotrema epinepheli* + *H. cromileptis*), *Bravohollisia* spp. + *Caballeria intermedius*) was not supported with a statistically significant BS value. In this study, the subfamily status of *Parancyrocephaloides* remains unsettled until these morphological features and polyphyletic nature of Ancyrocephalinae [8,18] are properly evaluated.

In the present ML and MP analyses, together with Šimková et al. [8,18], it is reasonable to conclude the ancyrocephaline clades consisting of *Ancyrocephalus percae*, *Urocleidus similis* and *Cleidodisucus pricei* from freshwater fishes as Ancyrocephalinae sensu stricto. On the other hand, subfamily status of marine ancyrocephalines including *P. daicoci* has not yet been settled. Further study will be needed to organize the phylogeny of marine ancyrocephalines, using a wider range of taxa and more DNA markers as suggested by Wu et al. [11].

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Legends for Tables 1 & 2 and Figures 1–11.

Table 1. List of DNA sequences 18S rRNA used for the molecular analyses with parasite species and GenBank accession numbers.

Table 2. List of 28S rRNA sequences used for the molecular analyses with parasite species and GenBank accession numbers.

Fig. 1. *Parancyrocephaloides daicoci* Yamaguti, 1938, ventral view. A: Entire body, B: Anterior body. Scale bars: 0.2 mm.

Fig. 2. Haptoral sclerites of *Parancyrocephaloides daicoci* Yamaguti, 1938. A: Specimen in ammonium picrate-glycerin medium (not mounted in Canada balsam), B: Ventral hamulus (a), dorsal hamulus (b), ventral bar (c), a pair of dorsal bars (d), marginal hook (e) after mounted in Canada balsam. Scale bars: 50 μ m.

Fig. 3. Male copulatory organs of *Parancyrocephaloides daicoci* Yamaguti, 1938 (ammonium picrate-glycerin specimens). Left two are drawings of permanent specimens and the photograph represents the right permanent specimen before dehydration. Scale bar: 50 μ m.

Fig. 4. Dorsal hamuli and two bars of *Parancyrocephaloides dactylopteri* Yamaguti, 1968 holotype (USNM 1359362). Scale bar: 20 μ m.

Fig. 5. Whole worm of *Gobioecetes biwaensis* n. g., n. sp., holotype, dorsal view. Scale bar: 0.1 mm.

Fig. 6. Haptoral sclerites of *Gobioecetes biwaensis* paratype from *Rhinogobius* sp. at Sento Imperial Palace, Kyoto. A: Specimen in ammonium picrate-glycerin medium (not mounted in Canada balsam), differential interference contrast microscopy, B: Drawings of A (a, ventral Hamulus; b; dorsal Hamulus; c, marginal hook, d; ventral bar) after mounted in Canada balsam. Scale bars: 30 μ m.

Fig. 7. Morphological variations in ventral (upper row) and dorsal (lower row) hamuli of *Gobioecetes biwaensis* paratypes. Specimens of far left in two rows are same as in Fig. 5B, and all others were from *Rhinogobius* sp. BW in Lake Biwa. Scale bars: 30 μ m.

Fig. 8. Male copulatory organs of *Gobioecetes biwaensis* paratypes. Left two are from *Rhinogobius* sp. at Sento Imperial Palace, Kyoto and right two are from *Rhinogobius* sp. BW in Lake Biwa. Scale bars: 20 μ m.

Fig. 9. Maximum likelihood tree inferred from partial 18S rRNA gene analyses with Gyrodactylidea and Capsalidea used as outgroups. The log likelihood of the tree was

-3864.8693, and the parameter of Gamma distribution was 0.2708 (five categories). Numbers on internal nodes indicate bootstrap values.

Fig. 10. Maximum parsimony tree inferred from partial 18S rRNA gene analyses with Gyrodactylidea used as the outgroup. The most parsimonious tree with length = 751 is shown. Numbers on internal nodes indicate bootstrap values.

Fig. 11. Maximum likelihood tree inferred from partial 28S rRNA gene analyses with Ancylo-discoidinae used as the outgroup. The log likelihood of the tree was -10410.2675, and the parameter of Gamma distribution was 0.4688 (five categories). Numbers on internal nodes indicate bootstrap values by Maximum-Likelihood (left) and Maximum Parsimony (right) analyses. Clades 1-14, proposed by Wu et al. (2007) [11], were reproduced in the present analysis.

Parasite species	GenBank Accession No
Capsalidea	
<i>Benedenia</i> sp.	AJ228774
<i>Capsala martinieri</i>	AJ276423
<i>Encotyllabe chironemi</i>	AJ228780
Gyrodactylidea	
<i>Gyrodactylus salaris</i>	Z26942
<i>Udonella caligorum</i>	FJ946831
Monocotylidea	
<i>Calicotyle affinis</i>	AJ228777
<i>Dictyocotyle coeliaca</i>	AJ228778
<i>Leptocotyle minor</i>	AJ228784
<i>Troglocephalus rhinobatidis</i>	AJ228795
Dactylogyridea	
Tetraonchinae	
<i>Anoplodiscus cirruspiralis</i>	AJ287475
<i>Sundanonchus micropeltis</i>	AJ287579
<i>Tetraonchus monenteron</i>	AJ490159
Dactylogyrynea	
Dactylogyridae	
Ancylodiscoidinae	
<i>Thaparocleidus siluri</i>	AJ490164
<i>Thaparocleidus vistulensis</i>	AJ490165
Ancyrocephalinae	
<i>Ancyrocephalus percae</i>	AJ490166
<i>Cleidodiscus pricei</i>	AJ490168
<i>Haliotrema angelopterum</i>	AY820609
<i>Haliotrema cromileptis</i>	EU523144
<i>Haliotrema ctenochaeti</i>	EU836220
<i>Haliotrema epinepheli</i>	EU836222
<i>Haliotrema fleti</i>	EU836224
<i>Haliotrema pratasensis</i>	EU836230
<i>Pseudohaliotrema sphincteroporus</i>	AJ287568
<i>Thylacicleidus</i> sp.	AJ490169
<i>Urocleidus similis</i>	AJ490167
Dactylogyrynae	
<i>Dactylogyrus anchoratus</i>	AJ490161
<i>Dactylogyrus difformis</i>	AJ490160
Pseudodactylogyrynae	
<i>Pseudodactylogyrus anguillae</i>	AJ490162
<i>Pseudodactylogyrus bini</i>	AJ490163
<i>Pseudodactylogyroides apogonis</i>	AB065115
<i>Pseudodactylogyrus haze</i>	AB065114
Diplectanidae	
<i>Diplectanum aequans</i>	AJ276439
<i>Furnestinia echeneis</i>	AF294953
<i>Lamellodiscus elegans</i>	AF294956
<i>Lamellodiscus erythrini</i>	AJ276440
Pseudomurraytrematidae	
<i>Pseudomurraytrema ardens</i>	AJ228793

Parasite species	GenBank Accession No
Ancyrocephalinae	
<i>Aliatrema cribbi</i>	AY820612
<i>Bravohollisia gussevi</i>	DQ157665
<i>Bravohollisia maculatus</i>	DQ537363
<i>Bravohollisia parvianchoratus</i>	DQ537362
<i>Bravohollisia</i> sp.	DQ537365
<i>Bravohollisia rosetta</i>	DQ537364
<i>Caballeria intermedius</i>	DQ537366
<i>Cichlidogyrus sclerosus</i>	DQ157660
<i>Cichlidogyrus</i> sp. 1	DQ537367
<i>Cichlidogyrus</i> sp. 2	DQ537368
<i>Euhaliotrema chrysotaeniae</i>	AF026115
<i>Euryhaliotrema</i> sp. HBDD	DQ537374
<i>Euryhaliotrematoides annulocirrus</i>	AY820613
<i>Euryhaliotrematoides aspistis</i>	AY820614
<i>Euryhaliotrematoides berenguelae</i>	AY820615
<i>Euryhaliotrematoides grandis</i>	AY820616
<i>Euryhaliotrematoides microphallus</i>	AY820617
<i>Euryhaliotrematoides pirulum</i>	AY820618
<i>Euryhaliotrematoides triangulovagina</i>	AY820619
<i>Euryhaliotrematoides</i> sp. HQDD	DQ537369
<i>Haliotrema angelopterum</i>	AY820620
<i>Haliotrema anguiformis</i>	DQ537375
<i>Haliotrema aurigae</i>	EU836198
<i>Haliotrema bihamulatum</i>	DQ537378
<i>Haliotrema chenhsintaoi</i>	DQ537371
<i>Haliotrema cromileptis</i>	EU523146
<i>Haliotrema digyroides</i>	EU836200
<i>Haliotrema epinepheli</i>	EU836201
<i>Haliotrema fleti</i>	EU836203
<i>Haliotrema geminatohamula</i>	DQ157649
<i>Haliotrema johnstoni</i>	DQ157664
<i>Haliotrema kurodai</i>	EU836205
<i>Haliotrema nanaoensis</i>	DQ537373
<i>Haliotrema platycephali</i>	FJ767866
<i>Haliotrema scyphovagina</i>	AY820622
<i>Haliotrema shenzhenensis</i>	DQ537372
<i>Haliotrema subancistroides</i>	EU836210
<i>Haliotrema spirotribiforum</i>	DQ157656
<i>Haliotrema</i> sp. HBDQY	DQ537370
<i>Ligophorus leporinus</i>	DQ537380
<i>Ligophorus vanbenedenii</i>	JN996802
<i>Metahaliotrema mizellei</i>	DQ157647
<i>Metahaliotrema geminatohamula</i>	DQ157646
<i>Protyrodactylus alienus</i>	DQ157650
<i>Protyrodactylus hainanensis</i>	DQ157653
<i>Protyrodactylus</i> sp. 1	DQ157651
<i>Protyrodactylus</i> sp. 2	DQ157652
<i>Scutogyrus longicornis</i>	HQ010035
Ancylo-discoidinae	
<i>Thaparocleidus asoti</i>	DQ157669
<i>Thaparocleidus varicus</i>	DQ157668
<i>Thaparocleidus</i> sp. NY1	DQ157670
<i>Thaparocleidus</i> sp. NY2	DQ157671

A

B

A

a

b

c

B

0.1