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Trichodina hokkaidoensis (Ciliophora: Peritrichia) isolated from olive flounder (*Paralichthys olivaceus*) in Korea

Han-Seul Cho[*], U-Hwa Nam[**] and Jeong-Ho Kim[*†]

[*]*Department of Aquatic Life Medicine, Gangneung-Wonju National University, Gangneung, 25457, Korea*
[**]*Department of Marine Bioscience, Gangneung-Wonju National University, Gangneung, 25457, Korea*

We described *Trichodina hokkaidoensis* Mizuno, Matsuda, Nishikawa and Ito, 2022 from olive flounder *Paralichthys olivaceus* by morphological observation and molecular analysis. Morphological parameters of our specimen from 4 different sampling sites (Gangneung, Jeju, Wando, Taean) mostly coincided with those of *T. hokkaidoensis*. Some morphometric parameters of this trichodinid showed some inconsistency, depending on the sampling locations, but all of their partial small subunit ribosomal DNA sequences (1,182 bp) showed 100% homology with *T. hokkaidoensis*, originally described from artificially reared juvenile barfin flounder *Verasper moseri* from Japan. *T. hokkaidoensis* is known to cause epidermal damages to the host fish. However, there was no considerable pathological lesions in the olive flounder harboring *T. hokkaidoensis* in this study. The pathogenicity of *T. hokkaidoensis* against olive flounder needs to be investigated.

Key words: *Trichodina hokkaidoensis*, *Trichodina*, olive flounder, *Paralichthys olivaceus*, parasite

INTRODUCTION

Trichodinid ciliates are known as ectoparasitic or symbiotic to various aquatic animals. When the hosts are kept under less than optimal conditions, they can invade their hosts and sometimes cause serious damages on the skin or gills (Lom, 1958). Many trichodinids have been reported from farmed fish species, known to cause mild damage to serious mortalities depending on the hosts' immune status and environmental conditions (Mizuno et al., 2016; Nilsen, 1995; Xu, 2007). Most of these trichodinids, however, have been described solely based on morphological parameters, without molecular information. Given the low host specificity, high intraspecific variability and interspecific similarity of them, molecular data of trichodinids are indispensable for clear identification and further establishment of control methods in aquaculture farms.

Olive flounder (*Paralichthys olivaceus*) is one of the commercially important aquaculture fish species and exclusively propagated in Korea, occupying 46.2% (37,240 metric ton) of total aquaculture production in 2018 (KOSTAT, 2018). Mortalities due to infectious diseases are thought to be one of the big obstacle against stable olive flounder production; scuticociliates infection is considered as the biggest cause of mortalities occurring in olive flounder aquaculture farms, followed by emaciation disease (myxosporean infection) and viral hemorrhagic septicemia (Shim et al., 2019). Trichodinids are also frequently observed in olive flounder farms and considered as a potential pathogen (Cho et al., 2008; Jung et al., 2012). However, they have never been clearly identified at the species level in Korea. In the present study, we

[†]Corresponding author: Jeong-Ho Kim
Tel: +82-33-640-2851; Fax: +82-33-640-2955
E-mail: jhkim70@gwnu.ac.kr

collected several trichodinid species from olive flounder and identified one of them as *Trichodina hokkaidoensis* using morphological criteria and molecular approach.

Materials and Methods

Sample collection and morphological observation

Olive flounder samples were collected from aquaculture farms located in Jeju (n=18, mean total length 33.3 cm) and Wando (n=5, mean total length 38.8 cm). Additional fish samples were also purchased from local fisheries markets in Gangneung (n=20, mean total length 27.7 cm) and Taean (n=4, mean total length 25.7 cm), respectively. All of these fish samples were collected in 2022. Fresh skin and gill smears were made with several randomly selected fish samples from each sampling site and air-dried. The smears were impregnated with the dry silver nitrate technique of Foissner (1991), to reveal the detailed structure of adhesive disc. The impregnated specimens were observed and measured under a light microscope equipped with a camera, at 1,000X magnification. All the measurements were expressed in micrometers with the range (mean ± S.D.). The scheme and terminology for describing the structure of the adhesive disc followed the system proposed by Lom (1958) and Van As and Basson (1989) (Fig. 1).

Molecular analysis

For molecular analyses, a single cell was carefully collected from the mixture of fresh scraped olive flounder mucus and sterile seawater by using a micropipette, and each cell was carefully washed with sterile seawater several times. Genomic DNA was extracted with QIAamp DNA mini kit (Qiagen, Germany), according to the manufacturer's instruction. The small subunit (SSU) rRNA gene was amplified with the primer set (Tricho18S-01F and Tricho18S-

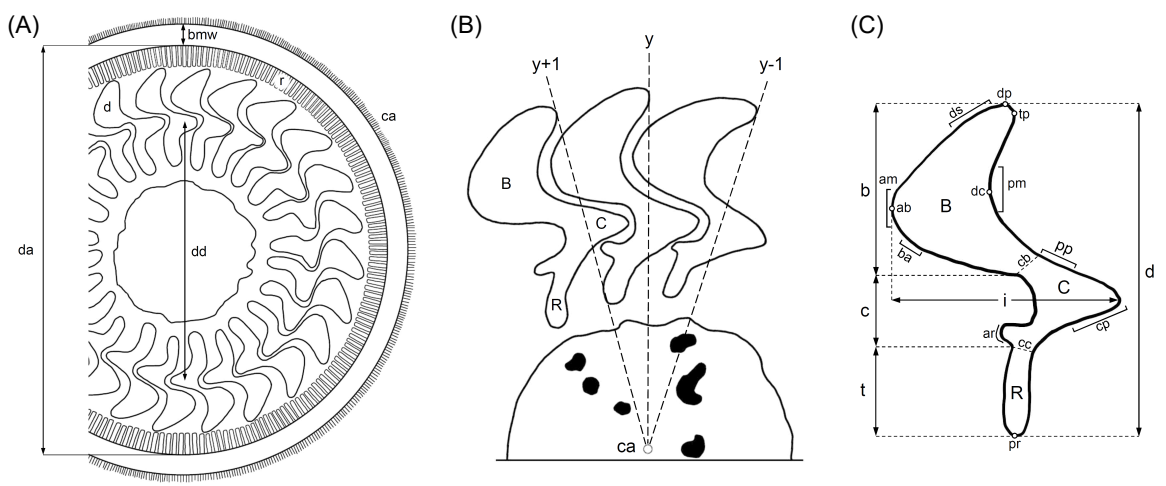

Fig. 1. Diagrams to illustrate description of skeletal part of adhesive disc (A) and denticles (B, C) based on Lom (1958) and Van As and Basson (1989). Abbreviations: (A): bmw, border membrane width; ca, cilia of adoral zone; da, diameter of adhesive disc; dd, diameter of denticulate ring; r, radial pins. (B): B, blade; C, central part; ca, center of adhesive disc; R, ray. (C): ab, apex of blade; am, anterior margin of blade; ar, apophysis of ray; ba, apophysis of blade; cb, section connecting blade and central part; cc, section connecting central part and ray; cp, central conical part; dc, deepest point of curve; ds, distal surface of blade; dp, distal point of blade; pm, posterior margin of blade; pp, posterior projection; pr, point of ray; tp, tangent point; b, length of blade; c, width of central part; d, length of denticle; i, denticle span; t, length of ray.

Table 1. Oligonucleotide primer set used for PCR identification of trichodinids.

Primer	Sequence(5′-3′)	Condition	Reference
Tricho18S-01F	CCAACCCTCGGGTTGCGTGGAC	95°C(30sec)-58.5°C(30sec)-	This study
Tricho18S-01R	GGAATTCCTCGTTCACGACCC	72°C(1min), 35 cycles	

01R) developed in this study (Table 1), using 2 µl extracted DNA as template, 2 µl for the primer set in total volume of 20 µl *AccuPower*® PCR Premix (Bioneer, Korea) containing 1U Tag DNA polymerase, 250 µM dNTPs (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 9.0), 30 mM KCl and 1.5 mM $MgCl_2$. The PCR conditions for the SSU rRNA gene were as follow: 30 seconds for initial denaturation at 95°C, annealing for 30 seconds at 58.5°C, extension for 1 min at 72°C, 35 cycles The PCR products were purified using *AccuPrep*® Gel Purification Kit (Bioneer, Korea) according to the manufacturer's instruction. 1-2 ng/µl of purified PCR products were directly sequenced by ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA).

The obtained sequences and 18 sequences of other trichodinids retrieved from the NCBI GenBank database (Accession numbers are given in Fig. 3) were aligned and the phylogenetic trees were constructed based on the neighbor-joining method using MEGA 7 (Kumar et al., 2016).

Results and Discussion

As almost all fishes examined had at least a few ciliates on the skin or gills, and as many fish were infected concurrently by several trichodinid species, no attempt was made to determine prevalence or intensity of infection for each parasite species. Moreover, the number of individual trichodinid species was insufficient for conducting morphological description and molecular analysis together. Therefore, we selected the most dominant species in 4 sampling batch for further analyses and described below.

Our specimen is characterized by its small size with disc-shaped body (Fig. 2). The body diameter of our specimens measured 40.7 to 53.8 µm (Gangneung), 36.7 to 53.5 µm (Jeju), 27.2 to 42.4 µm (Wando), 30.5 to 43.9 µm (Taean). The diameter of the adhesive disc was 34.0 to 48.2 µm (Gangneung), 31.8 to 45.1 µm (Jeju), 24.2 to 36.0 µm (Wando), 27.0 to 35.5 µm (Taean), and the diameter of the denticle ring was 20.3 to 26.7 µm (Gangneung), 15.9 to 26.4 µm (Jeju),

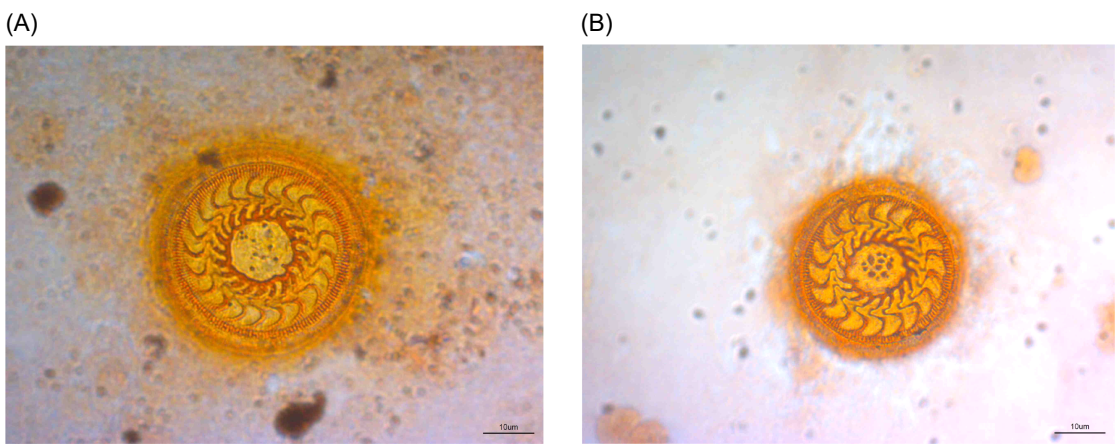

Fig. 2. Photomicrographs of *T. hokkaidoensis* from olive flounder. Silver nitrate staining. (A) Gangneung sample, (B) Jeju sample.

12.6 to 21.1 μm (Wando), 14.8 to 21.6 μm (Taean), respectively. The number of denticles ranged from 18 to 22 (Gangneung), 18 to 23 (Jeju), 19 to 24 (Wando), 17 to 24 (Taean), and the number of radial pins per denticle was 8 to 10 (Gangneung), 7 to 9 (Jeju), 5 to 9 (Wando), 6 to 9 (Taean). Border membrane width was 2.8 to 5.5 μm (Gangneung), 1.7 to 5.8 μm (Jeju), 1.6 to 5.0 μm (Wando), 1.3 to 4.1 μm (Taean). The blades were relatively broad, robust, sickle-shaped and located between y and y+1 axes. The tangent point was below distal point of blade and not sharp. A blade of apex was present, often extending beyond the y+1 axis. Anterior margin of blade was also often located beyond the y+1 axis. Blade apophysis and connection to central part was unclear. Posterior projection was unrecognizable. The length of central part was 2.3 to 3.8 μm (Gangneung), 1.9 to 3.6 μm (Jeju), 1.3 to 2.8 μm (Wando), and 1.2 to 2.3 μm (Taean). Ray short and slightly round. Anterior apophysis of ray was often elongated to the y+1 axis of the central part. The length of ray was 2.2 to 3.9 μm (Gangneung), 2.2 to 3.4 μm (Jeju), 1.2 to 3.7 μm (Wando), and 1.6 to 4.3 μm (Taean), respectively. Connection between ray and central part was well developed. Length ratio of ray to blade was 0.4 to 0.7, depending on the sampling locations. All of these morphometric data were presented in Fig. 1, 2 and Table 2. The slide specimens are deposited in the Department of Aquatic Life Medicine, Gangneung-Wonju National University (specimen number GWFPC-2201~2204).

At present, 3 trichodiniid species (*Trichodina jadranica*, *T. circinantis* and *Paratrichodina obliqua* have been previously reported from olive flounder (Xu et al., 2001; 2002). We compared our morphometric data and denticle morphology with them; Our specimen has well-developed robust, sickle-shaped blade, rod-like rays of various length and adoral spirals with more than 360°, all of which are the characteristics of the genus *Trichodina* (Basson and Van As, 1989). When compared with other 2 *Trichodina* species from *P. olivaceus*, our specimen has smaller denticle span with elongated central conical part of denticle, slightly curved ray with elongated anterior ray hypophysis and indistinct posterior projection. Moreover, length ratio of ray to blade in our specimen is less than 1.0. All of these morphological characteristics most closely coincided with those of recently described *T. hokkaidoensis* from barfin flounder (*Verasper moseri*) (Mizuno et al., 2022). Intra-species morphometric variations of trichodinid ciliates are often observed. This is probably due to different host species or different environmental conditions (e.g., sampling location, water temperature) and molecular data are suggested to solve to solve these problems. DNA sequence analysis of all the specimen from different sampling sites showed that their partial SSU RNA sequences (n=7; GenBank accession number: OP445699.1, OP445700.1, OP445702.1, OP445706.1, OP445707.1, OP811526.1, OP811528.1) robustly formed a single clade with *T. hokkaidoensis* (GenBank accession number: LC598228.1), with the 100% sequence homology among all the samples included in the *T. hokkaidoensis* clade (Fig. 3). Therefore, we identified all of our trichodinid ciliates from 4 different sampling locations as *T. hokkaidoensis*.

Trichodinids are common ectoparasites or commensals of aquatic animals and they can induce chronic dermatitis of the fins and body surface with varying degrees when under the stressful conditions of aquaculture. More than 300 *Trichodina* species have been described up to date, but many of them are still necessary to be confirmed or re-described, because many *Trichodina* species are morphologically very similar and sometimes concomitant infection of more than one *Trichodina* species are found in the same host (Tang et al., 2017). Moreover, intraspecific morphological variations are also reported (Xu, 2007). Molecular approach for clear identification and phylogenetic analysis of trichodinids are urgently needed, and recent publications adapt both of morphological and molecular approach for description.

This is the first detailed morphological and molec-

Table 2. Morphometric comparison of trichodinids in this study with T. hokkaidoensis from barfin flounder and other congeners reported from olive flounder.

Species	This study 1	This study 2	This study 3	This study 4	*Trichodina hokkaidoensis*	*Trichodina jadranica*	*Trichodina circinantis*	*Paratrichodina obliqua*
Host	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Verasper moseri*	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Paralichthys olivaceus*
Site	Skin	Skin	Skin	Skin	Skin	Skin	Gills	Gills
Locality	Gangneung, Korea	Jeju, Korea	Wando, Korea	Taean, Korea	Hokkaido, Japan	Qingdao, China	Wando, Korea	Qingdao, China
Source	This study	This study	This study	This study	Mizuno et al., 2021	Xu et al., 2001	Xu et al., 2002	Xu et al., 2001
Body diameter (da+2Xbmw)	40.7-53.8 (48.1±3.5)	36.7-53.5 (43.7±4.2)	27.2-42.4 (36.6±3.1)	30.5-43.9 (36.6±2.7)	29.1-45.4 (37.2±3.4)	30.0-40.0 (35.2±2.8)	30.0-40.0 (35.0±3.0)	20-22
Diameter of adhesive disc (da)	34.0-48.2 (40.2±3.8)	31.8-45.1 (37.0±3.4)	24.2-36.0 (30.7±2.6)	27.0-35.5 (31.0±2.1)	26.1-40.2 (33.6±2.9)	24.0-34.0 (28.5±2.7)	25.0-36.0 (30.4±2.9)	17-19
Border membrane width (bmw)	2.8-5.5 (4.5±0.6)	1.7-5.8 (3.5±0.8)	1.6-5.0 (3.2±0.8)	1.3-4.1 (2.8±0.8)	1.3-4.5 (2.3±0.6)	3.0-4.0 (3.3±0.4)	2.0-3.0 (2.5±0.4)	1.5
Diameter of denticular ring (dd)	20.3-26.7 (23.4±1.7)	15.9-26.4 (21.0±2.4)	12.6-21.1 (18.0±1.8)	14.8-21.6 (17.7±1.8)	16.2-23.5 (19.8±1.6)	13.0-20.0 (16.2±1.7)	16.0-22.0 (18.7±2.0)	9-10
Number of denticle	18-22 (20.5±0.8)	18.0-23.0 (20.6±1.3)	19-24 (21.2±1.1)	17-24 (20.4±1.3)	19-23 (21.1±0.9)	18-23 (20.1±1.2)	22-26 (24.2±1.2)	19-21
Radial pins per denticle (number)	8-10 (N=27)	7-9 (N=24)	5-9 (N=43)	6-9 (N=40)	7-8	7-8	6	5
Denticle span (i)	4.0-6.7 (4.9±06)	3.6-6.0 (4.7±0.6)	4.8-7.5 (6.0±0.5)	4.3-6.9 (5.7±0.5)	3.9-6.7 (5.1±0.6)	7.5-9.0 (7.9±0.4)	6.0-9.0 (7.4±0.7)	5-5.5
Denticle length (b+c+t)	9.2-13.1 (11.0±0.9)	8.3-10.8 (9.7±0.6)	6.3-9.5 (8.3±0.7)	5.7-9.9 (8.3±0.9)	3.7-6.9 (5.6±0.6)	5.0-6.5 (6.0±0.4)	4.0-5.0 (4.3±0.4)	2.5
Blade length (b)	3.7-6.5 (5.4±0.7)	4.0-5.4 (4.7±0.4)	3.3-4.7 (4.0±0.4)	3.1-5.1 (4.1±0.5)	2.5-4.6 (3.4±0.5)	3.5-4.5 (3.9±0.3)	2.5-3.5 (2.7±0.3)	-
Width of central part (c)	2.3-3.8 (3.0±0.4)	1.9-3.6 (2.5±0.4)	1.3-2.8 (2.0±0.4)	1.2-2.3 (1.6±0.3)	1.3-2.5 (1.7±0.3)	1.5-2.5 (2.1±0.2)	1.0-1.5 (1.2±0.3)	0.5-1.0
Ray length (t)	2.2-3.9 (3.2±0.4)	2.2-3.4 (2.6±0.3)	1.2-3.7 (2.6±0.5)	1.6-4.3 (2.9±0.5)	1.8-4.3 (2.7±0.4)	1.5-2.0 (1.9±0.2)	2.0-4.5 (3.5±0.5)	1-1.5
Number of specimens measured	30	27	45	40	54	20	25	-
span : length	4.9 : 11.0	4.7 : 9.7	6.0 : 8.3	5.7 : 8.3	5.1 : 5.6	7.9 : 6.0	7.4 : 4.3	-
span / length	0.4	0.5	0.7	0.7	0.9	1.3	1.7	-

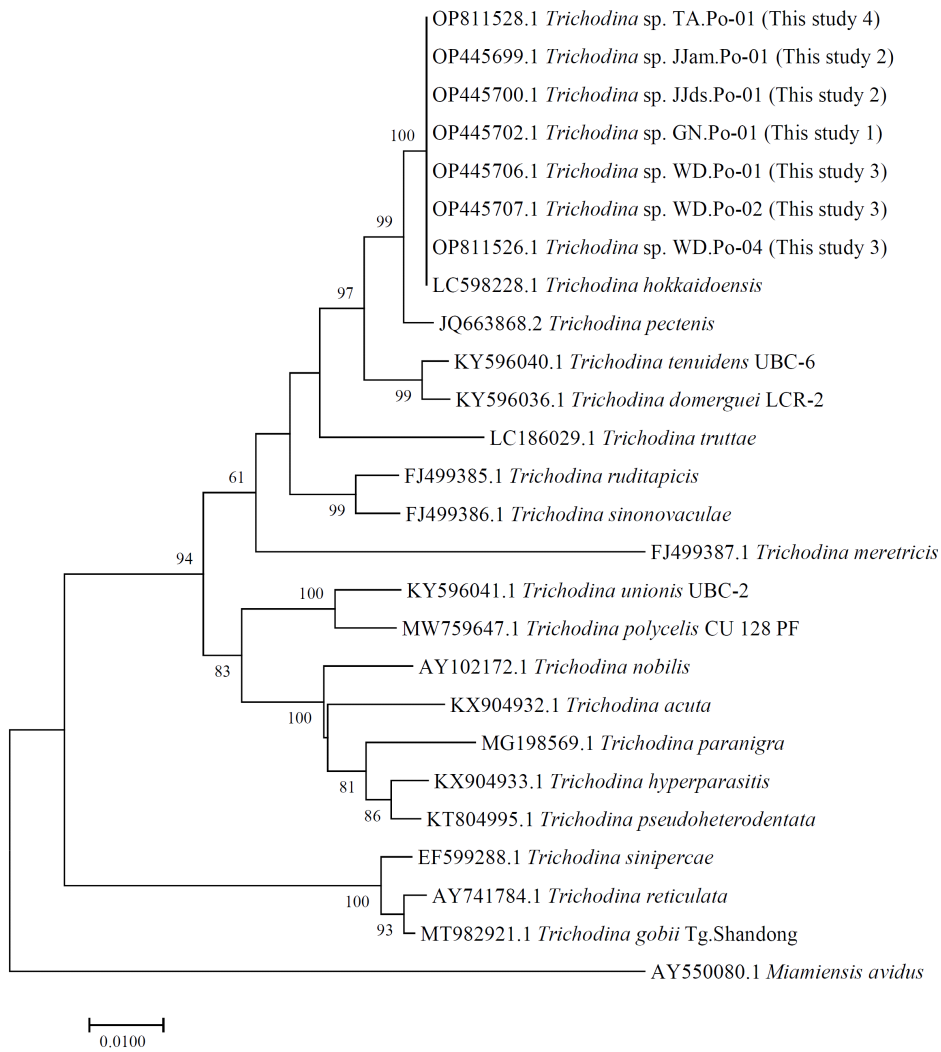

Fig. 3. Molecular phylogenetic trees showing the genetic relationships among trichodinid ciliates based on the partial 18S rDNA sequences (1,132bp).

ular description of trichodinids in Korea. Other trichodinids morphologically different from *T. hokkaidoensis* undoubtedly existed in our study, but unfortunately, we could not obtain sufficient number of samples for further analyses. There have been 3 trichodinids described from olive flounder up to date (Xu et al., 2001; 2002), but neither of their genetic information are available and await further study.

Trichodina hokkaidoensis is known to cause excessive mucus production, skin erosion and necrosis, resulting retarded growth in juvenile barfin flounder in Japan (Mizuno et al., 2022). However, we could not find distinct evidence that *T. hokkaidoensis* cause pathological lesions in olive flounder in this study; some fish harboring *T. hokkaidoensis* had mild erosion and excessive mucus on their body surface, but other trichodinids were also found from them. Moreover, apparently healthy-looking fish also harbored *T. hokkaidoensis*. The pathogenicity of this trichodinid species against olive flounder needs further inves-

tigation.

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