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Morphological and molecular characterization of *Trichodina* (Ciliophora: Peritrichia) species from cultured starry flounder (*Platichthys stellatus*)

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Trichodinid ciliates were found from the skin scrapings of culture starry flounder (*Platichthys stellatus*) obtained from a local fisheries market in Gangneung. Skin scrapings were prepared and stained by either Giemsa solution or silver nitrate impregnation, for morphological observation. PCR analysis was also conducted to make molecular identification and phylogenetic analysis. Morphological parameters obtained from our specimens were the most similar with *Trichodina hokkaidoensis* of barfin flounder from Japan and also olive flounder from Korea. The partial 18S rDNA sequences of our specimen (n=7) also showed 100.0% similarity with those of barfin flounder and olive flounder. Several trichodinids are known to have a broad host specificity and this is thought to be the case for *T. hokkaidoensis*. Starry flounder is a strong candidate for land-based aquaculture production, as a target species for prospective aquaculture diversification and therefore pathogenic potentials of this ciliate should be considered.

Key words: *Trichodina hokkaidoensis*, *Trichodina*, starry flounder, *Platichthys stellatus*, parasite

Introduction

Starry flounder (*Platichthys stellatus*) occurs around the margin of the North Pacific, as far as Korea and Japan on the western side of the Pacific and as far as California, the USA on the eastern side of the Pacific (Hart, 1973). They are considered as valuable game and food fish across their distribution range, and artificial propagation of this species have been conducted in Korea, being considered as an alternative aquaculture species for olive flounder (NIFS, 2019).

Several infectious diseases have been reported in cultured starry flounder since their artificial propagation started; some bacterial diseases are known to cause considerable mortalities and a commercial vaccine for preventing streptococcosis is available in Korea (Cho et al., 2008, 2013; Kim et al., 2022). Moreover, several fish-pathogenic viruses are also reported (Park et al., 2009; Won et al., 2013). On the other hand, the information on parasitic diseases of starry flounder is rather scarce and there is only one report of a pathogenic enteric myxosporean parasite *Enteromyxum leei* found in cultured starry founder (Shin and Lee, 2023). In this study, we found trichodinid ciliates on the skin of cultured starry flounder and identified them by morphological observation and molecular analysis.

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Materials and Methods

Starry flounder (n=12, mean body length=26.0 cm,

mean body weight=261.6 g) were obtained from a local fisheries market in Gangneung. The supplier stated that the fish were originated from Jeju island. All fish were examined for trichodinids by using a dissecting microscope, and then the gills and skin smear specimens were prepared and impregnated by the dry silver method (Foissner, 1991) or stained by conventional Giemsa solution. Observation was conducted under a light microscope equipped with a camera. All measurements of morphological characteristics were expressed in micrometers following the system proposed by Lom (1958), and the detailed description of denticles followed the system recommended by Van As and Basson (1989) (Fig. 1A, B).

The isolated trichodinids were washed with sterile sea water several times and transferred individually to a 1.5 ml PCR tube. The total genomic DNA of each trichodinid was extracted by QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. PCR amplification of the 18S rRNA gene was conducted using the primer set developed in our previous study (Cho et al., 2022). In addition, PCR amplification of the 28S rRNA gene was performed with the primer set developed in this study. All the detailed information on the PCR primers and conditions are described in Table 1. The amplicons were purified using AccuPrep gel purification kit (Bioneer, Korea) according to the manufacturer's instruction and sequenced by ABI prism 3730 XL DNA analyzer (PE Applied Biosystems, USA). Phylogenetic tree was constructed with the obtained sequences and other trichodinid sequences retrieved from the Gen Bank, based on the neighbor-joining method using MEGA 7 (Kumar et al., 2016).

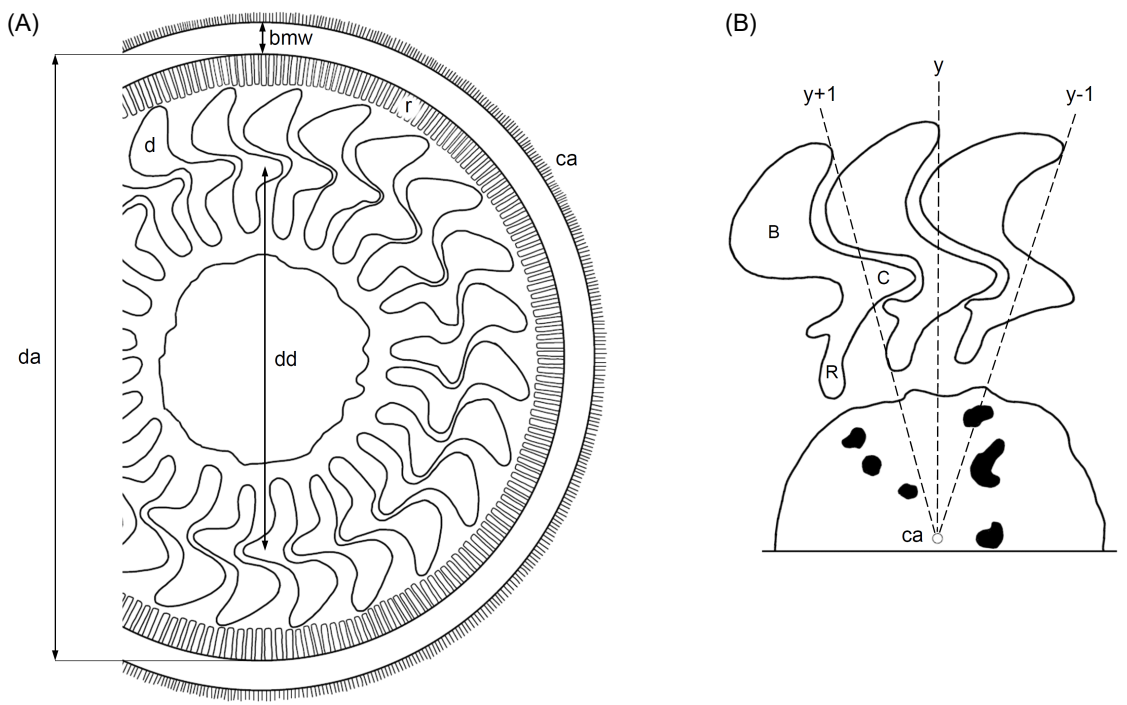

Fig. 1. Diagrams to illustrate description of skeletal part of adhesive disc (A) and denticles (B) based on Lom (1958) and Van As and Basson (1989). Abbreviations: (A): bmw, border membrane width; ca, cilia of adoral zone; da, diameter of adhesive disc; dd, diameter of denticulate ring; r, radial pins. (B): B, blade; C, central part; ca, center of adhesive disc; R, ray.

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

Primer	Sequence(5′-3′)	Condition	Reference
Tricho18S-01F	CCAACCCTCGGGTTGCGTGGAC	95°C(30sec)-58.5°C(30sec)-72°C(1min), 35 cycles	Cho et al., 2022
Tricho18S-01R	GGAATTCCTCGTTCACGACCC		
Tricho28S-01F	AAGCATATCAGTAAGCGGAGG	95°C(30sec)-51.6°C(30sec)-72°C(1min), 35 cycles	This study
Tricho28S-03R	GCTAGTTCATTCGGCAGGT		
Tricho28S-02F	GAGTCGKGTTGTTTGGGATTGC	95°C(30sec)-49.6°C(30sec)-72°C(1min), 35 cycles	This study
Tricho28S-04R	CCTTGGAGACCTGATGCGGTTA		

Results and Discussion

We found trichodinid ciliates from the skin scrapings of all the individual fish examined (n=12), regardless of external symptoms; a few fish had mild erosions over the dorsal body surface but most of them had no visible external symptoms. There were no trichodinid ciliates from the gill specimens prepared. The slide specimens are deposited in the Department of Aquatic Life Medicine, Gangneung-Wonju National University (specimen number: GWFPC-2205~2219).

All measurements were conducted with 40 specimens. Small-sized trichodinid with flat-shaped body, 34.0-44.2 μm in diameter (Fig. 2A, B). Adhesive disc 28.3-39.2 μm in diameter surrounded by a finely striated border membrane of 1.1-4.1 μm in width. Denticle ring 15.9-24.3 μm in diameter. The number of denticle 16-24. Denticle span 5.0-7.8 μm; blade length 3.5-5.5 μm ; central part width 1.3-2.4 μm; ray length 1.1-3.5 μm (Table 2). Blades broad, well-developed, almost sickle-shaped and nearly filling the gap between y and y+1 axes. Anterior margin of blade smooth, slightly curving toward prominent blade apex. Posterior margin of blade forming deep curve, deepest point almost at the same level as apex of anterior margin. Tangent point generally not sharp and below distal blade surface. Sections connecting blade and central part well developed. Central part well developed and conical. Posterior projection absent. Ray connection strongly developed and thick. Ray robust and long. Point of ray rounded. Apophysis of ray well-developed. All of these morphometric data were presented in Fig. 1, 2 and Table 2.

The 18s rRNA gene sequence of our specimens had 1,067 bp and were deposited in GenBank (GenBank

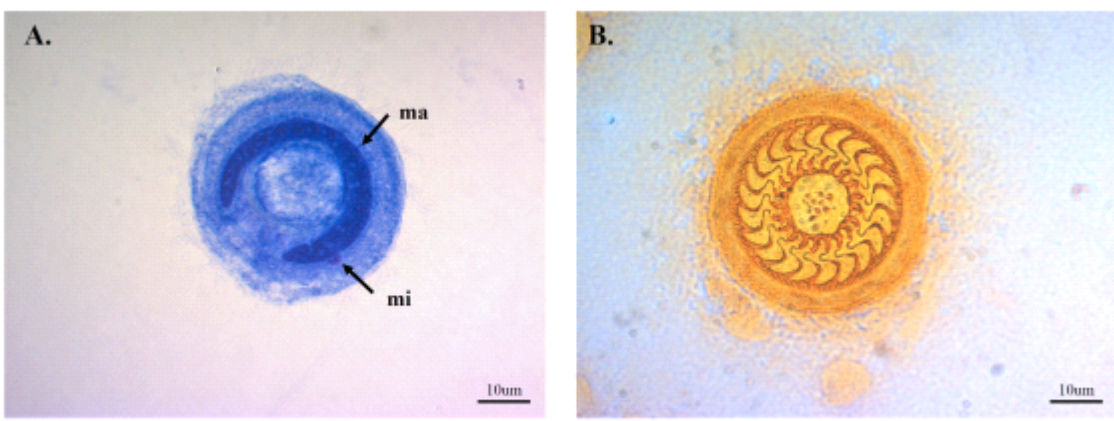

Fig. 2. Photomicrographs of *T. hokkaidoensis* from starry flounder. (A) Giemsa staining, (B) silver nitrate staining. Abbreviations: ma, macronucleus; mi, micronucleus

Table 2. Morphometric data comparison of trichodinid ciliates in this study and *T. hokkaidoensis* from barfin flounder and olive flounder.

Host	*Platichthys stellatus*	*Verasper moseri*	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Paralichthys olivaceus*	*Paralichthys olivaceus*
Site	Skin	Skin	Skin	Skin	Skin	Skin
Locality	Gangneung (Jeju)	Hokkaido, Japan	Gangneung, Korea	Jeju, Korea	Wando, Korea	Taean, Korea
Species	This study	*Trichodina hokkaidoensis*	*Trichodina hokkaidoensis*	*Trichodina hokkaidoensis*	*Trichodina hokkaidoensis*	*Trichodina hokkaidoensis*
Source	This study	Mizuno et al. (2022)	Cho et al. (2022)	Cho et al. (2022)	Cho et al. (2022)	Cho et al. (2022)
Number of speciemen measured	40	54	30	27	45	40
Body diameter (da+2*bmw)	34.0-44.2 38.1±2.8	29.1-45.4 37.2±3.4	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
Diameter of adhesive disc (da)	28.3-39.2 32.5±2.5	26.1-40.2 36.6±2.7	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
Border membrane width (bmw)	1.1-4.1 2.8±0.9	1.3-4.5 2.3±0.6	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
Diameter of denticular ring (dd)	15.9-24.3 19.5±1.9	16.2-235. 19.8±1.6	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
Number of denticle	16.0-24.0 21.74±1.7	19-23 21.1±0.9	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
Radial pins per denticle (nu)	6.0-10.0	7.0-8.0	8.0-10.0	7.0-9.0	5.0-9.0	6.0-6.0
Denticle span (i)	5.0-7.8 6.4±0.6	3.9-6.7 5.1±0.6	4.0-6.7 4.9±0.6	3.6-6.0 4.7±0.6	4.8-7.5 6.0±0.5	4.3-6.9 5.7±0.5
Denticle length (b+c+t)	7.0-10.4 8.4±0.7	3.7-6.9 5.6±0.6	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
Blade length (b)	3.5-5.5 4.6±0.5	2.5-4.6 3.4±0.6	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
Width of central part (c)	1.3-2.4 1.9±0.3	1.3-2.5 1.7±0.3	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
Ray length (t, thorn)	1.1-3.5 1.9±0.4	1.8-4.3 2.7±0.4	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

accession number: OQ940391.1, OQ940392.1, OQ 940393.1, OQ940394.1, OQ940395.1, OQ940396.1, OQ940397.1). The most similar species by BLAST search in GenBank were *T. hokkaidoensis* isolated from barfin flounder (GenBank accession number: LC598228.1, 100.0%) and olive flounder (GenBank accession number: OP445699.1, OP445700.1, OP44 5701.1, OP445702.1, OP445703.1, OP445706.1, OP 445707.1, OP445708.1, OP811526.1, OP811527.1, OP811528.1, OP811529.1, 100.0%). The second most similar species was *T. pectinis* (GenBank accession number: JQ663868.2, 99.6%) (Table 3). The phylogenetic tree also revealed that our specimens robustly clustered in a single clade with *T. hokkaidoensis* mentioned above (Fig. 3). Unfortunately, it was not possible to construct a phylogenetic tree based on the 28S RNA sequences because these sequences of any trichodinid ciliates were unavailable in GenBank when retrieved. We compared the 28S rRNA gene sequences (1,649-1,665 bp in length) of our specimens (GenBank accession number: OQ943976.1, OQ943 977.1, OQ943979.1, OQ943981.1, OQ943982.1, OQ 943983.1) and those of *T. hokkaidoensis* from olive flounder obtained by the authors (GenBank accession number: OQ943985.1, OQ943986.1, OQ 943987.1, OQ943990.1, OQ943994.1). The sequence similarity among them was 99.8-100.0% (data not shown). Therefore, we considered our specimens from starry flounder as *T. hokkaidoensis*.

Taxonomy of trichodinid ciliates has been traditionally conducted based on the shape and dimension of the denticles in the adhesive disc. Measurement of each parameter can be obtained from the analysis of micrographs of silver-impregnated specimens. However, high degree of variation in denticle morphology within a population of some species or even within the same individual is problematic when conducting identification (Islas-Ortega et al., 2022). Recently, molecular identification has become a new trend to distinguish morphologically similar trichodinid species and reveal the phylogenetic relationships

Table 3. Genetic distance among trichodinid ciliates estimated by 18s rRNA sequences (partial, 1,067 bp)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	OQ940391.1 *Trichodina* sp. JJ.Ps-01 (This study)	-														
2	OQ940392.1 *Trichodina* sp. JJ.Ps-02 (This study)	0.00%	–													
3	OP445699.1 *Trichodina hokkaidoensis* JJam.Po-01	0.00%	0.00%	–												
4	OP445700.1 *Trichodina hokkaidoensis* JJds.Po-01	0.00%	0.00%	0.00%	–											
5	OP445702.1 *Trichodina hokkaidoensis* GN.Po-01	0.00%	0.00%	0.00%	0.00%	–										
6	OP445706.1 *Trichodina hokkaidoensis* WD.Po-01	0.00%	0.00%	0.00%	0.00%	0.00%	–									
7	OP811528.1 *Trichodina hokkaidoensis* TA.Po-01	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	–								
8	LC598228.1 *Trichodina hokkaidoensis* 1-SM-2019	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	–							
9	JQ663868.2 *Trichodina pectenis*	0.38%	0.38%	0.38%	0.38%	0.38%	0.38%	0.38%	0.38%	–						
10	KY596039.1 *Trichodina domerguei* HL-2	1.23%	1.23%	1.23%	1.23%	1.23%	1.23%	1.23%	1.23%	1.23%	–					
11	KY596040.1 *Trichodina tenuidens* UBC-6	1.81%	1.81%	1.81%	1.81%	1.81%	1.81%	1.81%	1.81%	2.00%	1.23%	–				
12	OP445704.1 *Trichodina* sp. WD.Po.GI-01	1.52%	1.52%	1.52%	1.52%	1.52%	1.52%	1.52%	1.52%	1.61%	1.33%	1.81%	–			
13	LC186029.1 *Trichodina truttae*	2.97%	2.97%	2.97%	2.97%	2.97%	2.97%	2.97%	2.97%	3.07%	3.27%	3.96%	2.88%	–		
14	FJ499387.1 *Trichodina meretricis*	6.89%	6.89%	6.89%	6.89%	6.89%	6.89%	6.89%	6.89%	7.20%	7.42%	7.52%	6.90%	7.73%	–	
15	AY550080.1 *Miamiensis avidus*	12.35%	12.35%	12.35%	12.35%	12.35%	12.35%	12.35%	12.35%	12.24%	12.01%	12.00%	12.01%	12.79%	12.56%	–

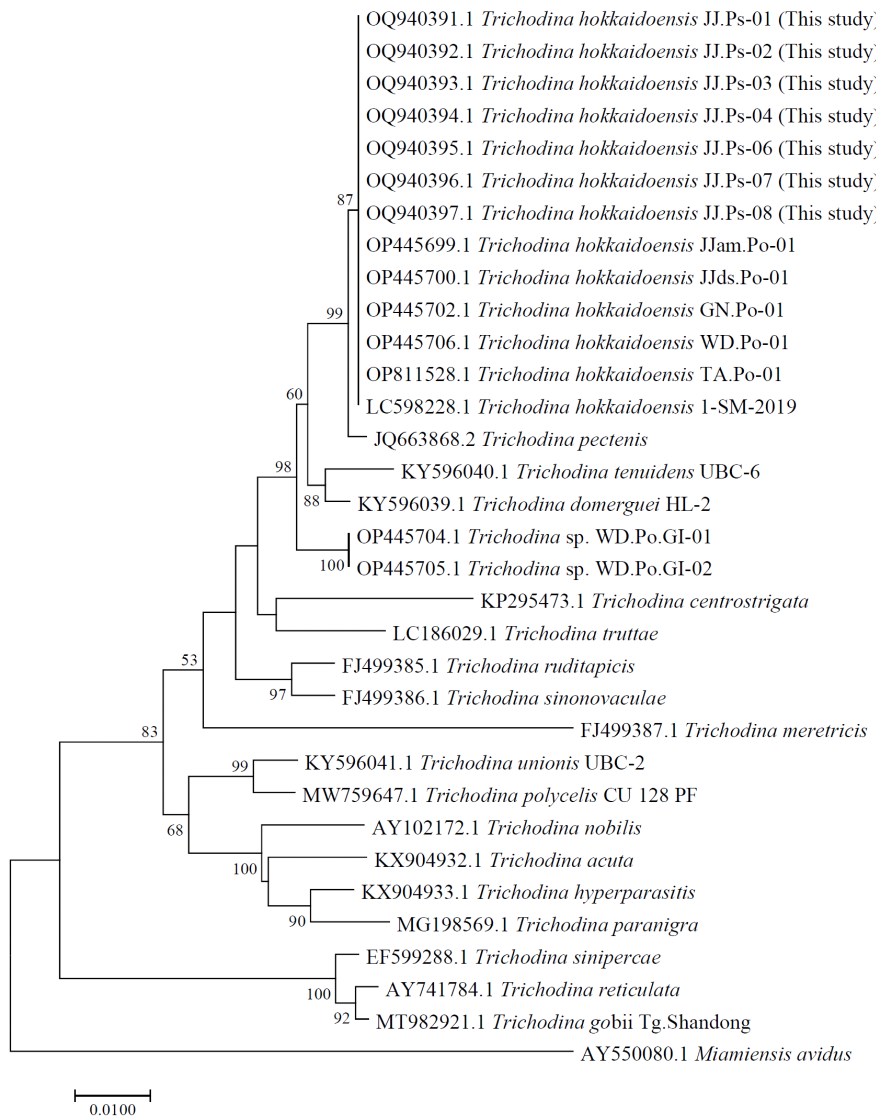

Fig. 3. Phylogenetic tree constructed based on 18s rRNA gene sequences (partial, 1,067 bp) of *Trichodina* species.

among the generic level of trichodinids as well. But still it is difficult to make a clear identification because sometimes multiple infection occurs in the same individual host and in this case, it is challenging to isolate each individual trichodinid from the same host for further analyses. Moreover, genetic information on trichodinid ciliates are limited, and the number of 18s rRNA sequences are available in the GenBank is 36. More sequence data are urgently needed to make a clear identification and analyze phylogenetic relationships among trichodinids.

Trichodina hokkaidoensis was firstly described from barfin flounder, *Verasper moseri* in Japan (Mizuno et al., 2022), and then recently isolated from olive flounder, *Paralichthys olivaceus* in Korea (Cho et al., 2022). We also found *T. hokkaidoensis* from starry flounder in this study. Several *Trichodina* species (e.g., *T. acuta, T. heterodentata, T. jadranica,*

T. nigra) are known to occur worldwide with a broad host range (de Jager and Basson, 2019; Islas-Ortega et al., 2020; Wang et al., 2022). Therefore, it is not surprising to find *T. hokkaidoensis* from starry flounder. In Korea, starry flounder has been artificially propagated mainly in Jeju island and east coast of Korea, to diversify aquaculture species biased to olive flounder and rockfish (*Sebastes schlegeli*). They are frequently reared in the facilities where olive flounder was previously propagated or in the same aquaculture facilities, with olive flounder. In either case, it is possible that *T. hokkaidoensis* might have transferred from olive flounder to starry flounder. It would be interesting to investigate trichodinids fauna of natural population of pleuronectid fishes, to clarify the host range of *T. hokkaidoensis*.

Trichodinid ciliates may be attracted to host mucus, sloughed epithelial cells or bacteria feeding on mucus of the host, and consequently colonize the skin, fins or gills of the host with their adhesive discs and cilia, causing pathological reactions and even death of the host. This phenomenon occurs particularly when the fish hosts are kept under less than optimal environmental conditions (Valladao et al., 2013; Wang et al., 2022). Hence they can be considered as opportunistic pathogens or commensals. *T. hokkaidoensis* is known to be pathogenic against juvenile barfin flounder (Mizuno et al., 2022). But, the pathogenicity of *T. hokkaidoensis* against starry flounder is currently unclear; Given that the larval stage of fish hosts is considered the most susceptible to trichodinids, the pathogenic potentials of *T. hokkaidoensis* should be investigated against starry flounder, particularly for fry and juveniles.

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