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First report of *Paranophrys marina* (Protozoa, Ciliophora, Scuticociliatia) isolated from olive flounder *Paralichthys olivaceus* in Korea: morphological and phylogenetic analysis

Hyun-Sil Kang[1†], Ilson Whang[2†] and Jae-Kwon Cho[1]

[1]Southeast Sea Fisheries Research Institute, National Institute of Fisheries Science (NIFS) of Korea, Tongyeong 53085, Republic of Korea
[2]National Marine Biodiversity Institute of Korea (MABIK), 75, Jangsan-ro 101beon-gil, Janghang-eup, Seochun-gun, Chungchungnam-do 33662, Republic of Korea

Scuticociliates are one of the serious parasitic threats faced by the marine aquaculturists worldwide. To date, *Uronema nigricans*, *Philasterides dicentrarchi, Miamiensis avidus*, *Uronema marinum*, and *Pseudocohnilembus persalinus* have been reported as the important culprit species causing scuticociliatosis in fish. The present paper reports the finding of an additional scuticociliate isolate from the gill of diseased olive flounder *Paralichthys olivaceus* in Korea. Based on the morphological characteristics, a scuticociliate in this study was identified as *Paranophrys marina*. Phylogenetic analysis placed *P. marina* as a sister lineage to three species of *Pseudocohnilembus* and *Mesanophrys carcini* within the order Philasterida.

Key words: *Paranophrys marina,* scuticociliatosis, olive flounder, morphology, SSU rRNA, phylogeny

Introduction

Scuticociliates are one of the serious parasitic threats in aquaculture industry worldwide. They provoke severe systemic infection through rapid invasion of internal organs and finally induce mass mortality in host populations (Cheung *et al.* 1980; Bassleer 1983). To date, several scuticociliates have been reported as the culprits of scuticociliatosis in marine fish including *Uronema nigricans* in southern bluefin tuna (Munday *et al.* 1997; Crosbie & Munday 1999), *Philasterides dicentrarchi* in sea bass, turbot and olive flounder (Dragesco *et al.* 1995; Iglesias *et al.* 2001; Parama *et al.* 2003; Kim *et al.* 2004a), *Uronema marinum* in olive flounder (Jee *et al.* 2001), *Pseudocohnilembus persalinus* in olive flounder (Kim *et al.* 2004b) and *Miamiensis avidus* in olive flounder (Jung *et al.* 2005; Jung *et al.* 2007; Song *et al.* 2009). In addition, an unidentified scuticociliate was also reported as the cause of mortality in olive flounder (Yoshinaga & Nakazoe 1993).

The farming of olive flounder *Paralichthys olivaceus*, which is one of major marine fish species raised by fish farmers, is widespread in Korea. Chun (2000) recorded that serious outbreaks of scuticociliatosis from cultured olive flounder in Korea were first noticed in the early 1990s. Until now, *Uronema marinum* (Jee *et al.* 2001), *Philasterides dicentrarchi* (Kim *et al.* 2004a), *Pseudocohnilembus persalinus* (Kim *et al.* 2004b), or *Miamiensis avidus* (Jung *et al.* 2005; Jung *et al.* 2007; Song *et al.* 2009) have been shown to be the causative agents of scuticociliatosis in olive flounder in Korea.

†Corresponding author: Hyun-Sil Kang
Tel: +82-55-640-4731; Fax: +82-55-641-2036
E-mail: kanghs97@korea.kr

The present study reports the finding of another scuticociliate species screened during an outbreak of scuticociliatosis from farmed olive flounder in Korea. Based on morphological characteristics, we identified this scuticociliate as *Paranophrys marina*. Moreover, the complete small subunit ribosomal RNA (SSU rRNA) gene of *P. marina* was sequenced for classification and identification of its phylogenetic position.

Materials and Methods

Ciliate isolation and *in vitro* culture

Scuticociliates were isolated from the gill tissue of diseased olive flounder *Paralichtys olivaceus* obtained from a local fish farm in Jeju, Korea in 2004. When live ciliates were observed under the light microscope in wet preparation of gill tissue, the gill tissue had nearly been destroyed by the ciliates' feeding action. Live ciliates were cultured in sterilized seawater containing 1% yeast extract and 1% proteose peptone as primary culture medium at 15°C for 5 days. After 5 days in culture, ciliates were cloned several times using a series of dilutions from the original culture until one ciliate remained in each well of a 96-well tissue-culture plate containing primary culture medium. The cloned ciliate was sub-cultured and maintained in the same medium on a petri-dish at 15 °C.

Staining and microscopic characteristics

Cultured ciliates were wet-mounted and observed under a differential-interference-contrast (DIC) microscope. For the study of the somatic and oral infraciliature, the ciliates were stained by the wet Chatton-Lwoff silver nitrate impregnation method described by Foissner (1991), and the silver carbonate impregnation method described by Ma *et al.* (2003). Stained ciliates were examined by light microscopy and measured using an ocular micrometer and image-analyzing software (Image-Pro Plus 3.0, USA).

Nuclear DNA extraction, PCR amplification of SSU rRNA and sequence analysis

Cultured ciliates were collected by centrifugation at 1000 × g for 10 min and washed with sterilized artificial seawater. Genomic DNA was extracted using the QIAmp DNA Mini kit (Qiagen, Germany), and the concentration of total genomic DNA was measured on a SmartSpec™ Plus Spectrophotometer (Bio-Rad, USA). Based on SSU rRNA sequences of scuticociliates in GenBank (Accession No. Z22881, AY103190, U83128), primer SSUF 5'-AACCTGGTT GATCCTGCCAG-3' and primer SSUR 5'-GATCYW TCTGCAGGTTCACCTAC-3' were designed to amplify the SSU rRNA sequences of the ciliate. PCR reactions were performed in a 50 µl PCR reaction mixture containing 20 pmol of each primer, 2.5 U of Ex Taq polymerase (Takara, Japan), and 50 ng of genomic DNA. The reaction was processed for 30 cycles using a Takara PCR Thermal cycler (Takara, Japan) at 95°C for 30 s, 55°C for 35 s, and 72°C for 2 min with pre-denaturation at 95°C for 5 min. The amplified product was ligated into pBluescript II SK(-) and used to transform *Escherichia coli* DH10b (Stratagene, USA). Recombinant plasmid was prepared by the alkaline lysis method using the Accuprep™ Plasmid Extraction kit (Bioneer, Korea). Sequencing reactions were carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 DNA sequencer (Applied Biosystems, USA) according to the manufacturer's instructions. To determine the complete sequences of SSU rRNA, we used the SSU-IF 5'-CGGTAATTCCAGCTCCAAT AG-3' with the universal primers SK and T7.

Phylogenetic analyses

The sequences were aligned with other SSU rRNA gene sequences using ClustalW 1.80 (Thompson *et al.* 1994). The sequence similarity and evolutionary distances were calculated between pairs of nucleotide sequences using the Kimura two-parameter model. A distance matrix tree was then constructed using the

neighbor-joining (NJ) methods (Saitou & Nei 1987) with the MEGA 4.0 program. The confidence estimate was obtained based on bootstrap generation of 10,000 replicates. The nucleotide sequences used in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Apofrontonia dohrni*, AM072621; *Anophryoides haemophila*, U51554; *Cardiostomatella vermiforme*, AY 881632; *Cohnilembus verminus*, Z22878; *Cyclidium plouneouri*, U27816; *Cyclidium porcatum*, Z29517; *Dexitrichides pangi*, AY212805; *Entodiscus borealis*, AY541687; *Entorhipidium tenue*, AY541688; *Entorhipidium triangularis*, AY541690; *Frontonia leucas*, AM072622; *Ichthyophthirius multifiliis*, U17354; *Lembadion bullinum*, AF255358; *Mesanophrys carcini*, AY103189; *Metanophrys similis*, AY314803; *Miamiensis avidus*, AY550080; *Ophryoglena catenula*, U17355; *Paranophrys magna*, AY103191; *Paramecium caudatum*, AF217655; *Paramecium multimicronucleatum*, AF255361; *Paramecium putrinum*, AF255360; *Paramecium woodruffi*, AF255362; *Parauronema longum*, AY212807; *Parauronema virginianum*, AY392128; *Philasterides dicentrachi*, AY 642280; *Plagiopyliella pacifica*, AY541685; *Pleuronema coronatum*, AY103188; *Pseudocohnilembus hargisi*, AY833087; *Pseudocohnilemus marinus*, Z22880; *Pseudocohnilemus persalinus*, AY835669; *Tetrahymena corlissi*, U17356; *Thyrophylax vorax*, AY541686; *Uronema elegans*, AY103190; *Uronema marinum*, AY551905.

Results and Discussion

Morphological characteristics of *Paranophrys marina*

Morphological characteristics of the scuticociliate under study are shown in Fig. 1 and Table 1. The body shape was generally slim and slender type with a sharply pointed anterior and narrowly rounded posterior end. Cell size was approximately 40 (range,

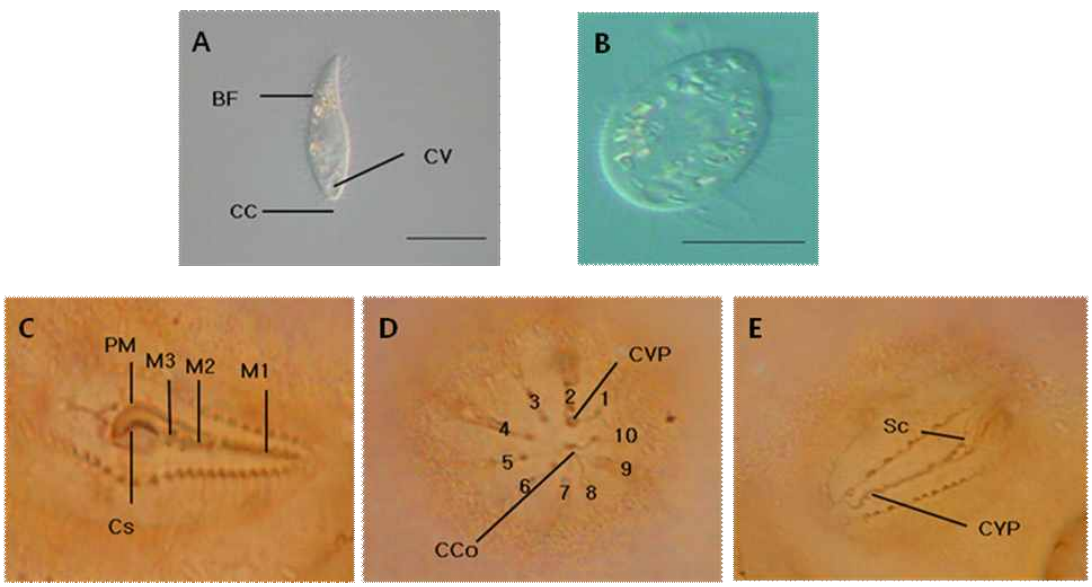

Fig. 1. Live observation and silver impregnated specimens of *Paranophrys marina*. (A) live *P. marina* ciliate *in vivo*. (B) live ciliate *in vivo* in fast growth phase. (C) buccal field of infraciliature. (D) caudal view of silverline system. (E) ventral view of silverline system. BF: buccal field; CC: caudal cilium; CCo: caudal cilium complex; Cs: cytostome; CV: contractile vacuole; CVP: contractile vacuole pore; CYP: cytopyge; M1, 2, 3: membranelles 1, 2, 3; PM: paroral membrane; Sc: scutica. Scale bar = 20 μm.

Table 1. Morphometric characterization of *Paranophrys marina* isolated from flounder.

Character	Min	Max	Mean	SD	n
Body length (μm)	36.1	48.7	41.5	3.13	40
Body width (μm)	17.1	24.6	20.4	2.04	40
Number of somatic kineties	9	10	10	0.51	38
Length of buccal field* (μm)	15	21.3	19.2	1.42	32
Length of membranelle 1 (μm)	5.1	9.3	7.9	0.97	36
Length of membranelle 2 (μm)	2.3	3.8	3.2	0.26	34
Length of membranelle 3 (μm)	0.6	1.5	1.0	0.21	33
Length of paroral membrane (μm)	7.9	10.4	9.0	0.62	25
Number of macronucleus	1	1	1	0	40
Number of micronucleus	1	1	1	0	40

* - distance from apex to posterior end of paroral membrane
Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples; SD-standard deviation.

33-46) × 11 (range, 8-15) μm *in vivo*. Cytoplasm looked transparent, which was filled with many transparent food vacuoles in the growth phase (Fig. 1B). A number of small light reflecting granules and several bar- shaped crystals were also observed (Fig. 1B). A contractile vacuole (CV) was located in posterior end and contained one caudal cilium (CC) about 11.5 (9-14) μm in length (Fig. 1A). The morphometric characteristics of scuticociliates stained using silver impregnation are given in Table 1. The ciliate was variable in size, ranging from 39.1 to 48.7 μm in length, and from 17.1 to 24.6 μm in width (n = 40). The Buccal field (BF) consisted of tripartite membranes on the left side and paroral membrane (PM) on the right side (Fig. 1C and Table 1). Membranelle 1 (M1) was slightly apart from anterior pole and located in the anterior portion of buccal cavity. It was well-developed with 2 long rows of kinetids and 7.9 μm in length. Membranelle 2 (M2) was just posteriorly located on M1, and was 3.2 μm in length. Membranelle 3 (M3) was close to M2 with 3 short rows and 1.0 μm in length. The paroral membrane began near the anterior end of M2, and terminated near the posterior end of the buccal cavity. The anterior portion of PM was straight and the posterior portion was curved around the cytostome (Cs) (Fig. 1C & Table 1). Ten somatic kineties were longitudinally arranged on both ends of the body. Continuing on to the caudal cilium complex (CCo), silverline of kinety 10 crossed over the caudal area and had a linkage between kinety 5 and 6 (Fig. 1D). The contractile vacuole pore (CVP) was positioned on the posterior end of kinety 2. A cytopyge (CYP), located between kinety 1 and 10, showed a wavy line at the posterior end in the ventral area (Fig. 1E). One macronucleus appeared in the middle of the body with one anteriorly-positioned micronucleus.

Based on the body shape of ciliate, organization of oral apparatus, the number of somatic kineties and position of contractile vacuole pore, we identified the scuticociliate screened in this study as *Paranophrys marina.* It was originally described by Thompson and Berger (1965) from hydroids (*Plumularia sp.*) and was re-evaluated by Song *et al.* (2002) after isolating from farmed scallop (*Argopecten irradians*). The only dissimilarity between the two descriptions was the body shape of this ciliate: Song *et al.* (2002) described the body shape as slim, spindle-shaped with a pointed anterior end, while Thompson and Berger (1965) depicted their form with a rounded anterior end. In the present study, a pointed shape was observed in the anterior end with a sharp apical pole in live and fixed specimens, which is consistent with Song *et al.* (Table 2).

Table 2. Morphological comparison of *Paranophrys marina* in literatures

Character	*P. marina** (Thomson & Berger, 1965)	Present study*	*P. marina*** (Song et al. 2002)
Body length × width (μm) *in vivo*	-	33-46 × 8-15	30-45 × 10-15
Body length × width (μm) *fixed*	39.3 × 19.2	41.5 × 20.4	-
No. of somatic kineties	10	10	10
Length of buccal field (μm)	19.6	19.2	about 2/5
Length of membranelle 1 (μm)	8.4	7.9	very long
Length of membranelle 2 (μm)	2.8	3.2	short
Body shape	Rounded both anterior and posterior	Slim, slender with pointed anterior	Slim, spindle shaped with pointed anterior
Position of contractile vacuole pore	end of SK2	end of SK2	end of SK2
Host	Hydroid	Flounder	Scallop
Sample location	Washington, USA	Jeju, Korea	Qingdao, China

*- Data from silver nitrate impregnation
**- Data from protargol impregnation

Phylogenetic analysis

The *P. marina* SSU rRNA sequence showed 96.3% identity with the *Parauronema longum* SSU rRNA gene sequence (GenBank accession no. AY212807). The SSU rRNA sequence of *P. marina* was aligned with SSU rRNA sequences of 34 other ciliates from three subclasses (Scuticociliatia, Hymenostomatia and Peniculia) in the GenBank database. After the removal of ambiguous sequences in the alignment site, a total of 1620 nucleotides remained for phylogenetic analysis. Phylogenetic analysis demonstrated that the subclass Scuticociliatia is supported as a monophyly in which it forms a polyphyletic clade (Order Philasterida and Pleuronematida). Within the Philasterida, *P. marina* and *Metanophrys similis* form as a monophyletic clade which is a sister lineage to three species of *Pseudocohnilembus* and *Mesanophrys carcini* (Fig. 2).

To date, molecular information has not been elucidated for *Paranophrys marina*. According to the analysis of SSU rRNA sequence data, *P. marina* SSU rRNA sequence was 96.3% homologous to that of *Parauronema longum*, indicating that these two species are closely related. Phylogenetic analysis displayed that *P. marina* is included in the order philaterida, within the subclass Scuticociliatia, the phylum Ciliophora, as a monophyletic clade which is a sister lineage to three species of *Pseudocohnilembus* and *Mesanophrys carcini*.

P. marina is reported as an ectocommensal within the mantle cavity of farmed scallop *Argopecten irradians* in China (Song *et al.* 2002). It is also known that *Paranophrys* sp. are opportunistic secondary parasites of cultured prawn *Penaeus chinensis*, in which they colonize pre-existing wounds, and subsequently invade the hemolymph and damage various organs including the gills (available at: http://www.pac.dfo-mpo.gc.ca/sci/shelldis/pages/cildsp_e.htm).

Although the present study reports that *Paranophrys marina* is another scuticociliate species screened during an outbreak of scuticociliatosis from farmed olive flounder in Korea, it cannot be ruled out that scuticociliatosis may have occurred in conjunction with other scuticociliate species. Further investigations are needed to prove the pathogenicity of *P. marina*.

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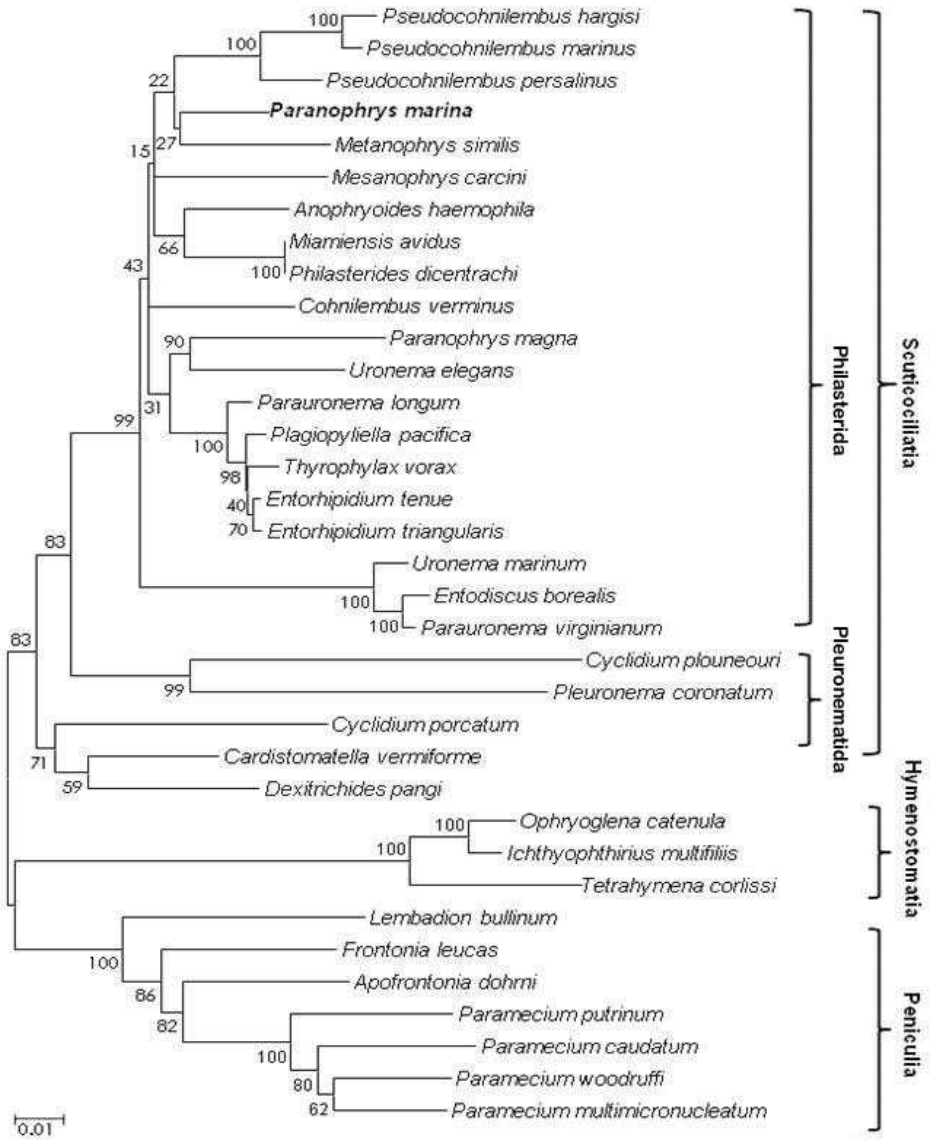

Fig. 2. A phylogenetic tree of SSU rDNA sequences of *P. marina* and scuticociliates constructed by the NJ method. Numbers at the nodes are bootstrap values representing their robustness (10,000 replicates). The new sequence is shown in boldface.

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