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Occurrence of scuticociliatosis in olive flounder *Paralichthys olivaceus* by *Phiasterides dicentrarchi* (Ciliophora: Scuticociliatida)

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ABSTRACT: In the course of identifying scuticociliates recently obtained from systemically infected olive flounder *Paralichthys olivaceus* in Korea, we found a scuticociliate species whose small subunit ribosomal RNA (SS rRNA) gene was not amplified by species-specific primers previously designed for *Uronema marinum* and *Pseudocohnilembus persalinus*. By studying morphological characteristics of wet-mounted and stained specimens, we identified the species as *Philasterides dicentrarchi*, which has been reported to cause systemic infection in the European sea bass *Dicentrarchus labrax* and turbot *Scophthalmus maximus*. In this study, we compared morphological characteristics of our specimens with previously reported *Philasterides* species, including *P. dicentrarchi*, and sequenced the SS rRNA gene in order to design *P. dicentrarchi* specific primers. This is the first report on scuticociliatosis caused by *P. dicentrarchi* from marine fish in Asia.

KEY WORDS: *Philasterides dicentrarchi* · Scuticociliatosis · Olive flounder · Morphology · SS rRNA gene · PCR-based

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INTRODUCTION

Free-living scuticociliates belonging to the genera *Uronema*, *Miamiensis* and *Philasterides* are the causative agents of scuticociliatosis in farmed marine fish (Thompson & Moewus 1964, Cheung et al. 1980, Dragesco et al. 1995, Munday et al. 1997, Iglesias et al. 2001, Jee et al. 2001). These opportunistic histophagous parasites are characterized by their high potential for systemically invading the fish and leading to high mortalities of the host.

Recently we reported that *Uronema marinum* and *Pseudocohnilembus persalinus* were the species causing scuticociliatosis in olive flounder *Paralichthys olivaceus* farmed in Korea. We rapidly differentiated these 2 species with multiplex PCR using 2 species-specific small subunit ribosomal RNA (SS rRNA) gene primers (Kim et al. 2004, this issue). However, in the course of identifying scuticociliates recently obtained from systemically infected olive flounder in Korea, we found another scuticociliate species whose SS rRNA gene was not amplified by the above species-specific primers. Using the comparison of morphological characteristics of wet-mounted and stained specimens, we identified the species as *Philasterides dicentrarchi* which has been reported to cause systemic infection in the European sea bass *Dicentrarchus labrax* (Dragesco et al. 1995) and in turbot *Scophthalmus maximus* (Iglesias et al. 2001).

In this study, we compared the morphological characteristics of our specimens with previously reported *Philasterides* species including *P. dicentrarchi*, and sequenced SS rRNA genes in order to design *P. dicentrarchi* specific primers. This is the first report on scuticociliatosis caused by *P. dicentrarchi* from marine fish in Asia.

MATERIALS AND METHODS

Ciliates. Ciliates were isolated from brain, gill and ulcerated skin of olive flounders *Paralichthys olivaceus*

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collected from a local fish farm in Korea, and were cultured in minimum essential medium (MEM, Sigma) supplemented with 10% fetal calf serum or in filtered seawater supplemented with autoclaved yeast extracts at 20°C.

Staining and microscopic characteristics. Live cells were examined with differential interference contrast microscopy and smears stained with Giemsa were prepared to visualize cytoplasmic organelles. The Chatton-Lwoff silver nitrate (Foissner 1991) and silver carbonate impregnation methods (Ma et al. 2003) were used for revealing the infraciliature and silverline system. Measurements were carried out with a microscope equipped with an ocular micrometer and image analyzing software (Image Tools ver. 3.0, UTHSCSA).

Nuclear DNA extraction, PCR amplification of SS rRNA and sequence analysis. Approximately 1×10^7 cells of cultured ciliates were pelleted by centrifugation at 900 × *g* for 5 min at 4°C and washed 3 times with Hank's balanced salt solution (HBSS, pH 7.4, Sigma). Genomic DNA was extracted using Accuprep® Genomic DNA Extraction Kit (Bioneer). Fifty ng of genomic DNA was used in 20 µl of PCR reaction mixture containing 10 pmoles of each primer and 0.5 U of *Taq* DNA polymerase (Takara Bio). The reaction was carried out for 30 cycles using an automated thermal cycler (iCycler, BioRad) at 95°C for 30 s, 55°C for 30 s and 72°C for 2 min, with an initial denaturation at 95°C for 2 min. Two oligonucleotide universal SS rRNA primers, U 1F (5'-AACCTGGTTGATCCTGCCAG-3') and U 1R (5'-TGATCCATCTGCAGGTTCAC-3') were used to amplify the SS rRNA gene. The amplified PCR product was cloned into a PCR 2.1-TOPO plasmid using the TOPO TA Cloning® Kit (Invitrogen), and the recombinant plasmid was purified using an Accuprep® Plasmid Extraction Kit (Bioneer) for the sequencing experiment. The sequencing reaction was carried out using BigDye terminator (Applied Biosystems) according to the manufacturer's recommendations and the sequences were analyzed with an Automated DNA Sequencer (ABI Prism 377, Applied Biosystems).

Polymerase chain reaction (PCR)-based identification. The SS rRNA gene sequence of *Paralichthys dicentrarchi* obtained in this study was aligned with the previously reported SS rRNA genes of *Uronema marinum* (GenBank accession no. AY551905) and *Pseudocohnilembus persalinus* (GenBank accession no. AY551906). Based on the sequence comparisons, *P. dicentrarchi* specific PCR primers were designed and used to attempt to amplify the DNA extracted from *P. dicentrarchi*, *U. marinum* and *P. persalinus*. The sequence and location of each forward and reverse primer are shown in Fig. 2. Five µl of amplified PCR product were separated on a 1.0% agarose gel and visualized using ethidium bromide staining. The expected size of the PCR product was 326 bp.

Table 1. Morphometric comparisons of the present specimens with *Philasterides armatalis* and *P. dicentrarchi* described previously

	Present specimen	*P. armatalis*	*P. dicentrarchi*	*P. dicentrarchi*
Body length (BL)	48.7 ± 2.0 (46.0–52.1)	57.7 ± 6.71 (42–74)	35.14 ± 4.8 (23–43)	33.6 ± 4.2 (25–43)
Body width (BW)	33.8 ± 3.2 (26.8–35.8)	21.2 ± 3.39 (16–29)	18.53 ± 2.5 (12–25)	19.5 ± 3 (15–28)
Length of buccal field (BF)	32.0 ± 2.1 (26.2–34.6)	20.6 ± 1.71 (18–25)	–	15 ± 1.7 (11–18)
BF : BL	0.3–0.5	0.36 ± 0.04 (0.3–0.44)	1/3 of total body length	0.43 (0.35–0.48)
M1 length	5.6 ± 0.7 (4.8–6.8)	–	2.31 ± 0.32 (2–3)	2.6 ± 0.3 (2–3)
M1 width	1.9 ± 0.3 (1.6–2.4)	–	1.02 ± 0.22 (0.6–1.8)	1.0 ± 0.1 (1–1.3)
M2 length	6.1 ± 0.6 (5.5–7.1)	–	2.91 ± 0.42 (2–4)	3.0 ± 0.1 (3.5–2.7)
M2 width	4.5 ± 0.6 (3.9–5.8)	–	2.03 ± 0.42 (1.2–3)	2.0 ± 0.2 (1.5–2.5)
M3 length	1.2 ± 0.2 (0.9–1.6)	–	0.84 ± 0.24 (0.2–1.5)	0.9 ± 0.1 (0.7–1)
M3 width	3.5 ± 0.42 (2.8–4.1)	–	1.8 ± 0.28 (1.2–2.1)	1.7 ± 0.1 (1.5–2)
PM1 length	8.7 ± 1.1 (7.5–11.0)	–	4.14 ± 0.49 (3.5–5)	3.9 ± 0.2 (3.5–4.5)
PM2 length	10.3 ± 3.0 (6.6–14.9)	–	5.97 ± 0.99 (4.5–8)	5.0 ± 0.5 (4–6)
Distance apex–M1	5.2 ± 1.1 (3.7–6.9)	–	3.74 ± 0.66 (3–5)	3.4 ± 0.6 (2.5–5)
Distance M1–M2	2.4 ± 0.4 (2.0–3.1)	–	1.1 ± 0.33 (0.5–2)	1.3 ± 0.2 (1–1.7)
Distance M2–M3	1.2 ± 0.2 (0.8–1.6)	–	0.61 ± 0.2 (0.2–0.9)	0.4 ± 0.1 (0.3–0.7)
Macronucleus diameter	15.8 ± 2.0 (13.3–18.4)	13.4 ± 2.45 (9–18)	6.42 ± 1.11 (4–8)	7.0 ± 1 (5–9)
Number of somatic kineties	14.0 ± 1.1 (13–15)	16.8 ± 0.54 (16–18)	14.5 ± 0.4 (13–15)	13–14
Position of CVP	Posterior end of kinety 2	Entirely posterior end	Between kinety 1 & 2	Posterior end of kinety 2
Geographical location	South Korea	China	France	Spain
Host species	*Paralichthys olivaceus*	Water from mollusc farm	*Dicentrarchus labrax*	*Scophthalmus maximus*
Source	Present study	Song (2000)	Dragesco et al. (1995)	Iglesias et al. (2001)

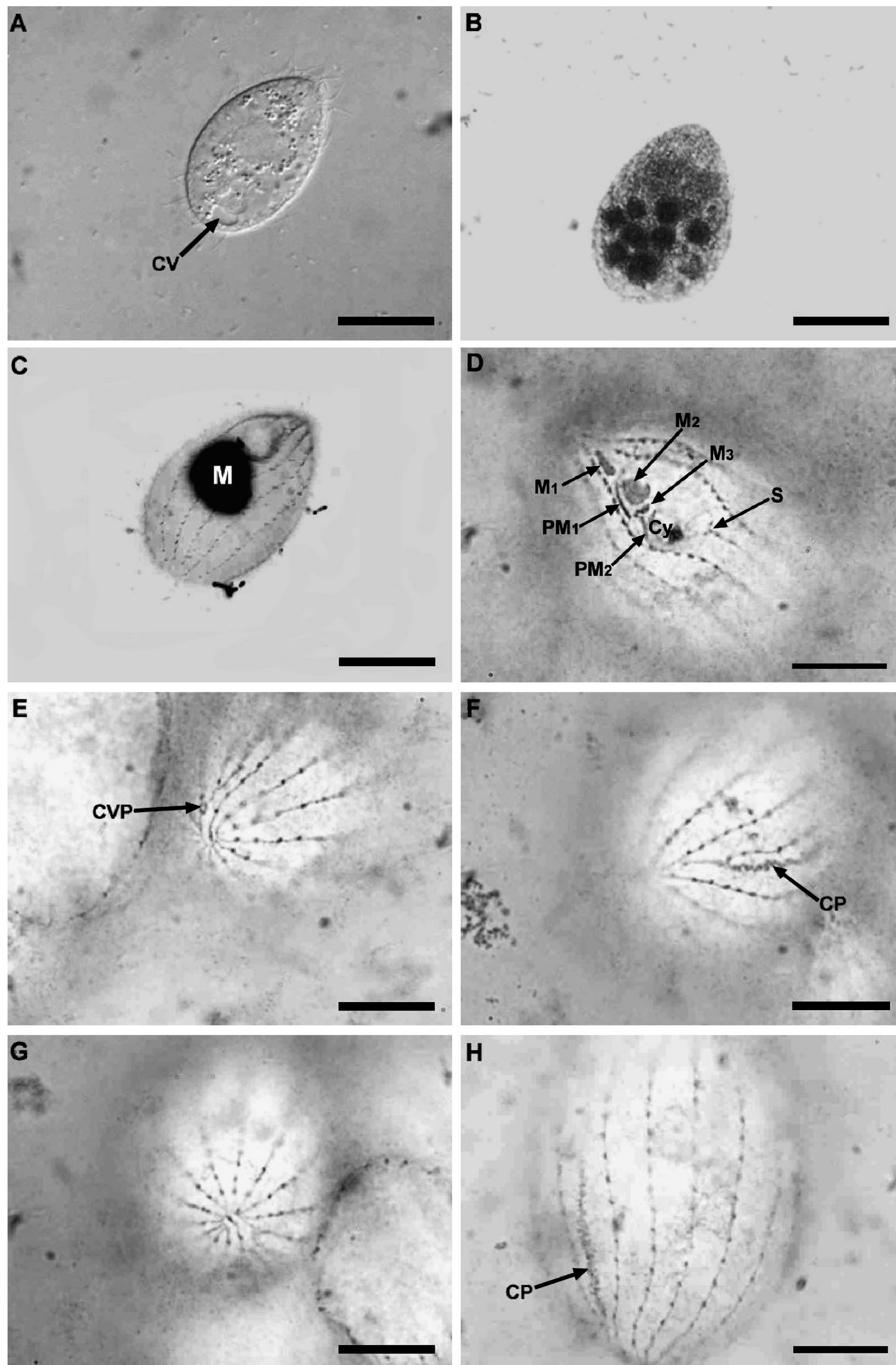

Fig. 1. *Philasterides dicentrarchi* isolated from the skin and brain of olive flounder *Paralichthys olivaceus*. (A) Live cell with a contractile vacuole (cv) at the posterior end. (B) Giemsa-stained ciliate containing numerous food vacuoles. (C) Silver carbonate impregnated ciliate. M: macronucleus. (D) Wet silver impregnated ciliate. The buccal apparatus was composed of a bi-partite paroral membrane (PM_1, PM_2), the 3 oral membranelles (M_1, M_2 and M_3), the cytostome (Cy) and the Scutico-vestige (S). (E,F,G, H) Posterior end of a silver impregnated ciliate. The cytoproct (CP) merged with the first kinety, and the contractile vacuole pore (CVP) opened at the end of second kinety. The somatic kineties except the last one are connected to each other by a circular polar ring. Scale bars are 20 µm in A–C and 10 µm in D–H

RESULTS

Morphological characteristics

All measurements were based upon 50 silver carbonate impregnated specimens (Table 1). The ciliate body was ovoid (Fig. 1A–C) with an oval or irregular-shaped macronucleus (Fig. 1C) and a posteriorly situated contractile vacuole (Fig. 1A). The posterior half of the body was filled with numerous food vacuoles (Fig. 1B). The ciliate body was covered with 13 to 15 somatic kineties (Fig. 1C) and a non-ciliated director meridian began just posterior to the cytostome and merged with the first kinety at the posterior end (Fig. 1E,F). The cytoproct was situated posteriorly on this meridian (Fig. 1F,H). At the posterior end, one caudal cilium present in the center and all kineties except the last one merged to an argentophilic line and formed a caudal cilium complex (Fig. 1E,G). The buccal apparatus consisted of 3 membranelles on the

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1     AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCT 60
61    AAGTATAAATAGTATACAGTGAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTATTT 120
121   GATAATGGAAAGCTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCTGTCAAAC 180
181   CCGACCTTTGGAAGGGTTGTATTTATTAGATATTAAGCCAATATTCCTTCGGGTCTATTG 240
241   TGGTGAATCATAGTAACTGATCGAATCTCTTCACGAGATAAATCATTCAAGTTTCTGCCC 300
301   TATCAGCTTTCGATGGTAGTGTATTGGACTACCATGGCAGTCACGGGTAACGGAGAATTA 360
361   GGGTTCGGTTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG 420
421   CGCGTAAATTACCCAATCCTGATTCAGGGAGGTAGTGACAAGAAATAACAACCTGGGGGC 480
481   CTCACGGCCTTACGGGATTGTAATGAGAACAATTTAAACGACTTAACGAGGAACAATTGG 540
541   AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTT 600
601   GTTGCAGTTAAAAAGCTCGTAGTTGAACTTCTGCATGTGCCCAGTTCTGGGCTTCGGTCA 660
661   GCTGTGGTGTATGCATCCGCTTGCAAAGCTAGACCGGTCTTCATTGATCGACTAGTGGAG 720
721   TAGGCTCTTTACCTTGAAAAAATTAGAGTGTTTCAGGCAGGCAATGGCTCGAATACATTA 780
781   GCATGGAATAATGGAATAGGACTTTTGTCCATTTGGTTGGTTATTGGACATAAGTAATGA 840
841   TTAAAAGGGACAGTTGGGGGCATTAGTATTTAATTGTCAGAGGTGAAATTCTTGGATTTA 900
901   TTAAAGACTAACTTATGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAA 960
961   GTTAGGGGATCAAAGACGATCAGATACCGTCCTAGTCTTAACTATAAACTATACCGACTC 1020
           PD1F ──►
1021  GGAATCGGACCGGCTTATAAAACTGGTTCGGCGCCGTATGAGAAATCAAAGTCTTTGGGT 1080
1081  TCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCA 1140
1141  GGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAAACATG 1200
1201  GGTGGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTT 1260
1261  CTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCT 1320
1321  AAATAGTACGTTGATGCACAATTGGCGTTACTTCTTAGAGGGACTATGCGCTTTGAAACG 1380
                        ◄── PD1R
1381  CATGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCCGCACGCG 1440
1441  CGCTACAATGACTCGCTCAGAAAGTACTTCCTGGTCCGGAAGGATTCGGGTAATCTTTTA 1500
1501  AATACGAGTCGTGTTAGGGATCGATCTTTGTAATTATGGATCTTGAACGAGGAATGCCTA 1560
1561  GTAAGTGCAAGTCATCAGCTTGTACTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1620
1621  CGCTCCTACCGATTTCGAGTGATCCGGTGAACCTTCTGGACTGAGCACGCTTGCGTGAAC 1680
1681  GGGAAGTTAAGTAAACCTAATCACTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAG 1740
1741  GTGAACCTGCAGATGGATCA 1760
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Fig. 2. Sequence of the SS rRNA gene of *Philasterides dicentrarchi* (GenBank AY642280). The 2 oligonucleotide primers (PDF1 and PDR1) specific for *P. dicentrarchi* are shown in black boxes

left side of the buccal cavity and a bipartite paroral membrane on the right side (Fig. 1D). Membranelle 1 (M_1) was triangular or spindle-shaped, positioned near the apical pole and was clearly separated from other membranelles. M_2 was trapezoidal-shaped and located close to M_3. M_3 was small and parallel to the base line of M_2. The paroral membrane had 2 distinct parts. The first part of the paroral membrane (PM_1) ran along the side of the M_2 and was slightly straight. It began at the anterior part of M_2 and ended at the posterior terminus of the M_3. The second part of paroral membrane (PM_2) was longer than the first one and slightly curved. It started from the posterior end of M_3.

SS rRNA gene sequence and PCR-based identification

The SS rRNA sequence of *Philaterides dicentrarchi* was 1760 bp (Fig. 2). The sequence was deposited in GenBank under the accession number AY642280. The present *P. dicentrarchi* SS rRNA sequence shared high homology with SS rRNA sequences from other scuticociliate species, and showed 95% identity with the *Anophryoides haemophila* SS rRNA gene sequence (GenBank accession no. U51554).

Using the oligonucleotide primers constructed based on the variable regions of the SS rRNA gene sequence, *P. dicentrarchi* was clearly distinguished from *Uronema marinum* and *Pseudocohnilembus persalinus* (Fig. 3).

Fig. 3. Ethidium bromide-stained 1.0% agarose gel showing PCR amplification by *Philasterides dicentrarchi* specific primers. Lane 1, *Philasterides dicentrarchi*; Lane 2, *Uronema marinum*, Lane 3, *Pseudocohnilembus persalinus*; Lane M, 1 kb molecular weight ladder (Bioneer)

DISCUSSION

In the genus *Philasterides*, 3 species: *P. armata*, *P. dicentrarchi* and *P. armatalis*, have been reported (Grolière 1980, Dragesco et al. 1995, Song 2000). *P. armata* clearly differs from our specimens by possessing a markedly higher number of somatic kineties (26 to 32) and is found in freshwater habitats. *P. armatalis* is distinguished from the present specimens by cell shape (a cylindrical form), higher somatic kinety number (16 to 18) and the position of the contractile vacuole pore (entirely terminal). Although the cell dimensions of the present specimens were larger than those of *P. dicentrarchi* described by Dragesco et al. (1995) and Iglesias et al. (2001), the cell shape, somatic kinety number, paroral membrane and membranelles morphology and the position of the contractile vacuole pore were identical with that species. Therefore, we identified the present specimens as *P. dicentrarchi* which has been reported from sea bass and turbot cultured in Europe. Since turbot was imported from Europe and has been cultured at several fish farms in Korea, it cannot be excluded that the occurrence of scuticociliatosis by the present *P. dicentrarchi* in olive flounder may have originated in imported turbot carrying the ciliates.

Since there is no known chemotherapeutic treatment against internal infection of scuticociliates, development of a vaccine for scuticociliatosis is one of the most promising and effective control measures. Although immobilization antigen (i-antigen) on the surface of pathogenic ciliates has been studied as a target for inducing protective antibodies (Clark et al. 1995, Iglesias et al. 2003), there is a marked variability of i-antigen in ciliates, even in the same species under different conditions (Lin et al. 2002, Wang et al. 2002). The occurrence of scuticociliatosis in olive flounder by various ciliate species demonstrated in this study suggests that common and more stable target antigens should be explored to develop an effective vaccine.

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