Vol. 62: 239–244, 2004 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published December 13

Pseudocohnilembus persalinus (Ciliophora: Scuticociitida) is an additional species causing scuticociliatosis in olive flounder *Paralichthys olivaceus*

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ABSTRACT: In the present study, *Pseudocohnilembus persalinus* was first reported as a species causing scuticociliatosis in olive flounder *Paralichthys olivaceus*. Based on the stained specimens, *P. persalinus* was clearly differentiated from *Uronema marinum*, which has been shown to be a cause of scuticociliatosis in farmed olive flounder in Korea from its characteristic oral infraciliature structure. The 1754 bp small subunit ribosomal RNA (SS rRNA) gene sequence of *P. persalinus* showed 95% homology with the partial sequence of *P. hargisi* SS rRNA. Moreover, multiplex PCR based on the species-specific amplification of the SS rRNA gene sequence enabled us to distinguish *P. persalinus* from *U. marinum* in a simple and rapid manner. *P. persalinus* was clearly differentiated from *U. marinum* even when the host was infected simultaneously with both species. These data suggest that the multiplex PCR procedure would make it possible to avoid the cumbersome and time-consuming procedures of morphological analysis for the definitive identification of ciliates.

KEY WORDS: *Pseudocohnilembus persalinus* · Scuticociliatosis · Olive flounder · Morphology · SS rRNA gene · Multiplex PCR

INTRODUCTION

Scuticociliatosis caused by histophagous opportunistic scuticociliates is recognized as one of the most important worldwide parasitological problems affecting cultured marine fish. The ciliates are characterized by their high potential for systemically invading and destroying tissues, leading to high mortalities of the host. Several scuticociliate species have been reported as causative agents of scuticociliatosis in farmed marine fish including *Philasterides dicentrarchi* in sea bass (Dragesco et al. 1995) and turbot (Iglesias et al. 2001), *Uronema nigricans* in bluefin tuna (Munday et al. 1997), *Uronema marinum* in olive flounder (Jee et al. 2001), and other unidentified species which affect olive flounder (Yoshinaga & Nakazoe 1993).

The present paper reports the finding of another scuticociliatosis causing species, *Pseudocohnilembus persalinus*, from cultured olive flounder in Korea. From the comparison of morphological characteristics of stained specimens and the small subunit ribosomal RNA (SS rRNA) gene sequences, *P. persalinus* was clearly distinguished from *Uronema marinum*, which has been shown to be a culprit of scuticociliatosis in farmed olive flounder in Korea (Jee et al. 2001). The definitive identification of ciliate species by morphological characteristics relies on time-consuming and laborious staining techniques. Therefore, in this study, we discriminated between these 2 species by

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multiplex PCR based on the specific primers of SS rRNA genes.

MATERIALS AND METHODS

Ciliates. Ciliates were isolated from brain, gill or ulcerated skin of olive flounder *Paralichthys olivaceus* collected from several local fish farms 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. 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 concentrated by centrifugation at 900 × *g* for 5 min, and then stained by the wet Chatton-Lwoff silver nitrate method described by Foissner (1991) or by the silver carbonate impregnation method described by Ma et al. (2003). To observe nuclei, ciliates were stained with Giemsa solution. The stained ciliates (n = 50) were measured using an ocular micrometer and image analysis software (UTHSCSA Image Tool ver. 3.0). Drawings of impregnated specimens were made with the aid of a camera lucida.

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 the Accuprep® Genomic DNA Extraction Kit (Bioneer). Fifty ng of genomic DNA was used in 20 µl of PCR reaction mixture containing 10 pmol of each primer and 0.5 U of *Taq* DNA polymerase (Takara). 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′-AACCTGGTTGATCCGCCA-G-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).

Multiplex PCR. The SS rRNA gene sequences of *Pseudocohnilembus persalinus* and *Uronema marinum* obtained from the present study were aligned with the previously known SS rRNA genes of *U. marinum* (GenBank accession no. Z22881 partial sequence) and *P. hargisi* (GenBank accession no. AY212806). Based on sequence comparison, 3 hyper-variable regions were selected to construct PCR primers for the species-specific amplification of SS rRNA gene segments using multiplex PCR. The sequence and location of each forward and reverse primer used are shown in Fig. 3. To perform the multiplex PCR, equal amounts of 4 oligonucleotides (10 pmol each) were included in a PCR reaction. The reaction mixture (20 µl) was carried out for 30 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. Five µl of amplified PCR product was separated using a 1% agarose gel and visualized using ethidium bromide staining. The expected sizes of the PCR products are 421 and 722 bp for *P. persalinus* and *U. marinum*, respectively.

RESULTS

Morphological characteristics of *Pseucohnilembus persalinus*

The ciliates were elongated, spindle-shaped (Fig. 1A) and measured 29 to 42 µm in length and 15 to 19 µm in width. A large globular macronucleus and a small micronucleus were located at the level of the buccal entrance (Fig. 2G). The posterior cytoplasm was filled with numerous food vacuoles (Fig. 2H). The somatic ciliatures were sparsely arranged, consisting of 8 to 11 bi-polar kineties made up of 15 to 18 kinetids.

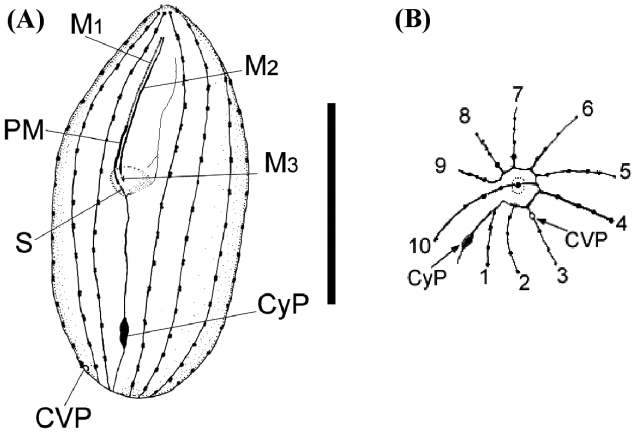

Fig. 1. *Pseudocohnilembus persalinus*. (A) Ventral view of silver impregnated specimen. CyP: cytopyge; CVP: pore of contractile vacuole; M_1–M_3: membranelles 1–3; PM: paroral membrane; S: scutico-vestige. Scale bar = 15 µm. (B) Caudal view of silver impregnated specimen

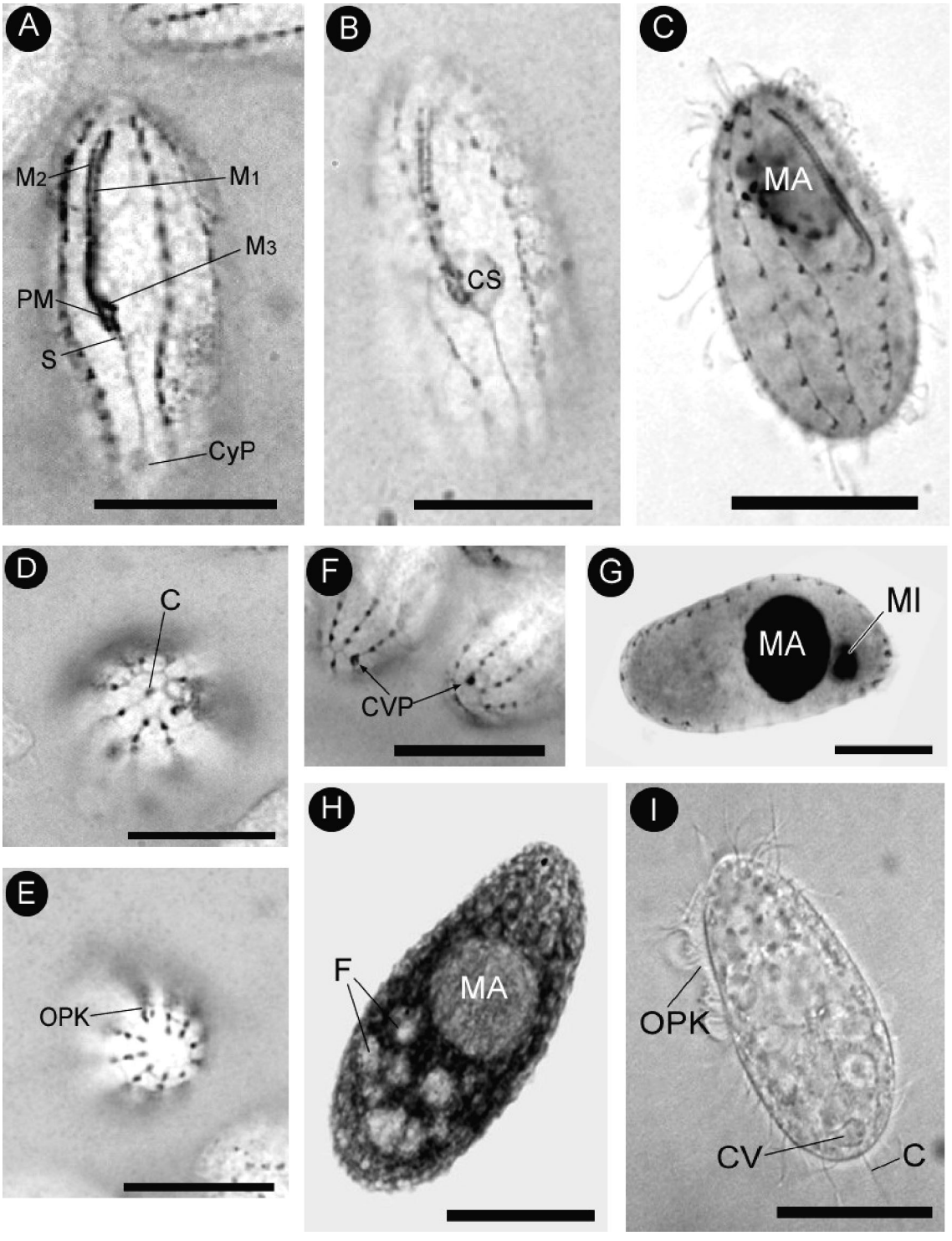

Fig. 2. *Pseudocohnilembus persalinus.* Silver impregnated ciliates. (A,B) Wet silver impregnated; (C) silver carbonate impregnated. Buccal apparatus composed of paroral membrane (PM), 3 oral membranelles (M_1–M_3), cytostome (CS), scutico-vestige (S), cytopyge (CyP), macronucleus (MA). (D). Posterior end of wet silver impregnated ciliate. C: caudal cilium. (E) Apical pole of wet silver impregnated ciliate. OPK: oral polykinetids. (F) Wet silver impregnated ciliate contractile vacuole pore (CVP) at posterior pole. (G) Silver carbonate impregnated ciliate with macro- (MA) and micronucleus (MI). (H) Giemsa-stained ciliate showing a macronucleus (MA) and numerous food vacuoles (F). (I) Live ciliate observed under the differential interference contrast microscope. CV: contractile vacuole. Scale bars = 10 µm

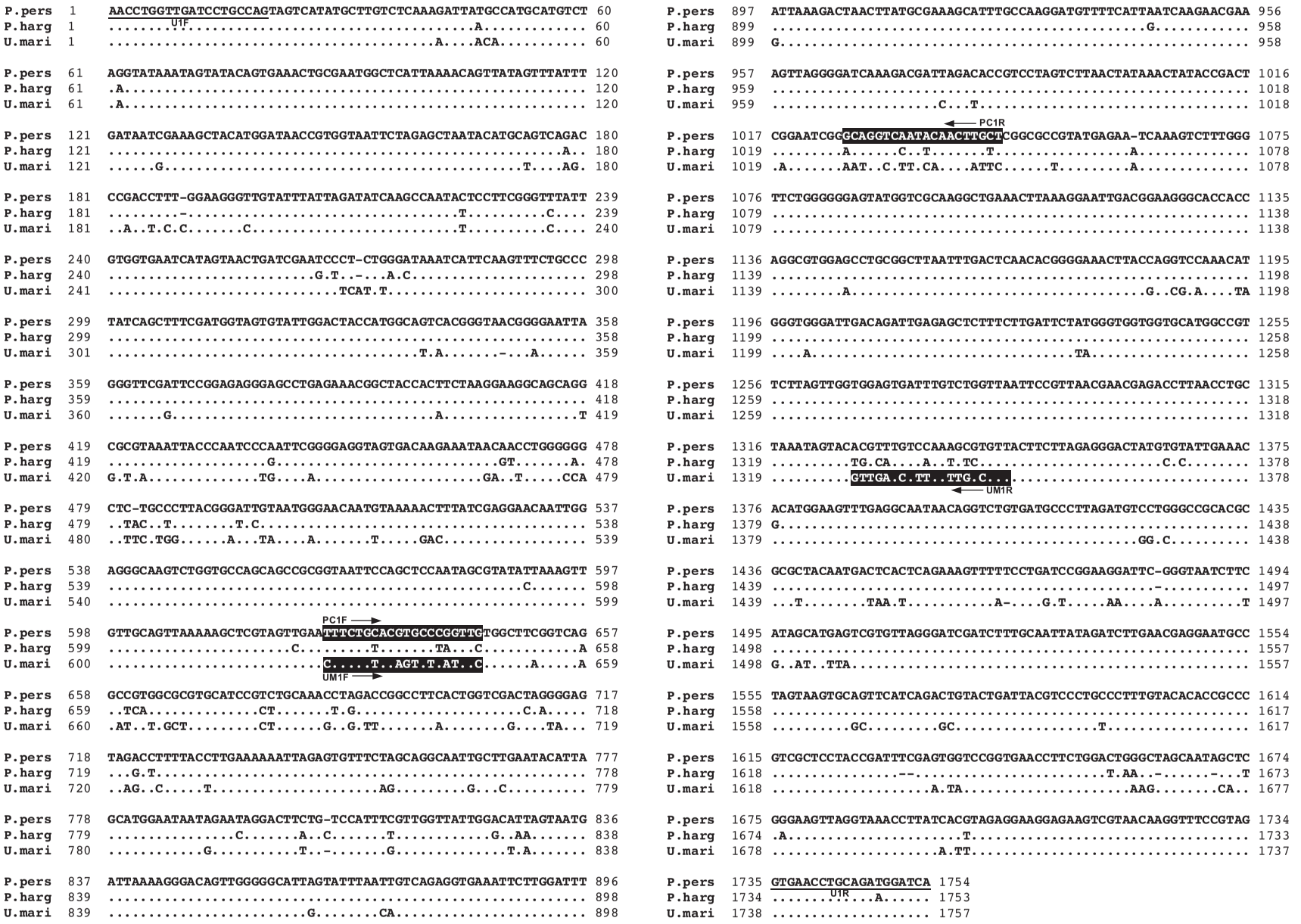

Fig. 3. Sequence alignment of SS rRNA genes from 3 scuticociliate species: *Pseudocohnilembus persalinus* (*P. Pers*; present study, GenBank AY551906), *P. hargisi* (*P. harg*; GenBank AY21280) and *Uronema marinum* (*U. mari*; present study, GenBank AY551905). Two universal primer sequences for SS rRNA gene (U 1F and 1R) are underlined. The 4 oligonucleotide primers (PC 1F, UM 1F, PC 1R and UM 1R) used for multiplex PCR are shown in black boxes

A non-ciliated director-meridian began just posterior to the scutica and merged with the first kinety at the posterior pole (Figs. 1B & 2D). The cytopyge, a thick argentophilic patch, was located subcaudally (Figs. 1 & 2A). A contractile vacuole pore (CVP) opened at the posterior end of the third somatic kinety (Figs. 1 & 2F,I).

The buccal field was 15 to 18 µm in length and occupied the anterior half of the body (Figs. 1A & 2A–C). The buccal apparatus consisted of 3 membranelles: M_1 was a single row and located just anterior to the paroral membrane (PM); M_2 was also a single row and was parallel to M1 and PM; M_3 was very small and located at the end of the PM (Figs. 1A & 2A–C,E,I). The paroral membrane was gently curved and positioned at the right side of the buccal cavity (Figs. 1A & 2A–C).

SS rRNA gene sequence and multiplex PCR

The SS rRNA sequences of *Uronema marinum* and *Pseudocohnilembus persalinus* determined from this study were 1757 and 1754 bp, respectively. These sequences were deposited in GenBank under the accession numbers AY551905 for *U. marium* and AY551906 for *P. persalinus*. The present *U. marinum* SS rRNA sequence exactly matched when aligned with the previously known 1680 bp of the partial *U. marinum* sequence (GenBank Z22881) except for 6 nucleotide bases, possibly indicating that there might be some sequence differences in SS rRNA genes among *U. marinum* strains. The 1754 bp of the SS rRNA gene sequence from *P. persalinus* shared high homology with those from other scuticociliate species, and showed 95% identity with the *P. hargisi* SS rRNA gene partial sequence (GenBank AY212806) (Fig. 3).

Using the 4 oligonucleotide primers which were designed based on the variable regions on the SS rRNA sequences, *Pseudocohnilembus persalinus* and *Uronema marinum* were clearly distinguished from each other by multiplex PCR. Most of the PCR reactions generated a PCR product which clearly matched the size of the SS rRNA gene segment amplified either from *P. persalinus* (421 bp) or *U. marinum* (722 bp). Multiplex PCR amplification of DNA of scuticociliate species isolated from fish sampled at several olive flounder farms located in Hansando, Pohang-A or Haeundae revealed a single band of 412 bp, indicating the fish should be infected only with *P. persalinus* (Fig. 4). On the other hand, only a 722 bp amplification product was obtained from olive flounder sampled from Jeju, suggesting that *U. marinum* was responsible for scuticociliatosis from this site. Interestingly, the multiplex PCR products with DNA of scuticociliates isolated from the fish sampled from Pohang-B displayed both sizes of PCR bands (421 and 722 bp), indicating the fish were infected with both *P. persalinus* and *U. marinum*.

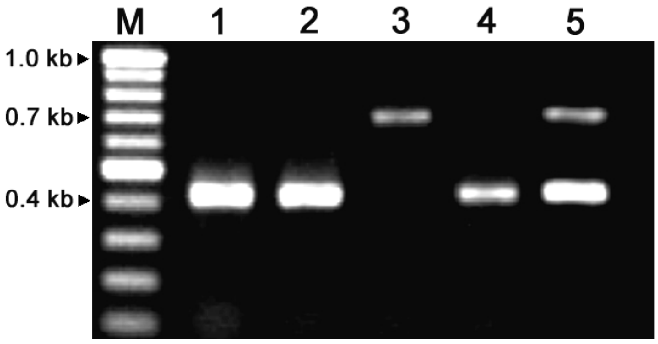

Fig. 4. Ethidium bromide-stained 1.0% agarose gel showing the multiplex PCR products amplified with DNA of scuticociliates isolated from diseased olive flounder collected at Hansando (Lane 1), Pohang-A (Lane 2), Jejudo (Lane 3), Haeundae (Lane 4) and Pohang-B (Lane 5). Lane M is a 1 kb molecular weight ladder (Bioneer)

DISCUSSION

In the present study, *Pseudocohnilembus persalinus* was first reported as a species causing scuticociliatosis in olive flounder *Paralichthys olivaceus*. *P. persalinus* was originally described by Evans & Thompson (1964) based on silver nitrate impregnated specimens and was recently described again by Song (2000). Although there are different opinions on the taxonomic identity of this species (Thompson 1965, 1966, Foissner & Wilbert 1981, Song 2000), morphological characteristics of the present specimens coincided well with the characteristics of *P. persalinus* (Table 1).

Table 1. *Pseudocohnilembus persalinus.* Morphometric comparisons between the present specimens and those of Song (2000). CVP: contractile vacuole pore

Characteristics	Present specimens	Song (2000)
Body length (µm)	29–42	25–43
Body width (µm)	15–19	15–23
Length of buccal field (µm)	15–18	12–21
Length of buccal field: body length ratio	0.41–0.60	0.46–0.59
Number of somatic kineties (SK)	8–11	9–11
Number of basal bodies in SK1	15–18	17–21
Position of CVP, located at the end of SK	SK3	SK3
Habitats	Marine	Marine
Geographical distribution	South Korea	China

Based on the stained specimens, *Pseudocohnilembus persalinus* was clearly differentiated from *Uronema marinum* by its characteristic oral infraciliature structure. Moreover, the multiplex PCR based on the species-specific amplification of SS rRNA gene sequence enabled us to distinguish *P. persalinus* from *U. marinum* in a simple and rapid manner. *P. persalinus* was clearly differentiated from *U. marinum* even in the host infected with both species. These data suggest that the multiplex PCR procedure would make it possible to avoid the cumbersome and time-consuming procedures of morphological analysis for the definitive identification of ciliates.

Although several attempts have been made to treat scuticociliatosis by various chemotherapeutics (Iglesias et al. 2002, Quintela et al. 2003), there is at present no effective *in vivo* chemotherapeutic treatment, especially for internal infections. Therefore, development of an effective vaccine may be the best way to control scuticociliatosis. Recently, Iglesias et al. (2003) reported that *Philasterides dicentrarchi* expressed immobilization antigens (i-antigens) on its surface which were recognized by the turbot immune system and which might be useful for vaccination. However, they pointed out the existence of different serotypes of the ciliate and that there were outbreaks of the disease in turbot by another scuticociliate, *U. marinum*, which probably expresses i-antigens of its own. Thus, identification and differentiation of ciliate species responsible for scuticociliatosis in a host species should be the first step for development of effective vaccines.

Although in the present study *Pseudocohnilembus persalinus* was reported as another species causing scuticociliatosis, it cannot be ruled out that other scuticociliate species may be involved in scuticociliatosis in olive flounder and more extensive studies are needed.

Acknowledgements. This study was supported by a grant from the Ministry of Maritime Affairs and Fisheries, Republic of Korea.

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Editorial responsibility: Wolfgang Körting, Hannover, Germany

Submitted: February 23, 2004; Accepted: July 16, 2004
Proofs received from author(s): November 3, 2004