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# Complete small subunit rRNA gene sequence of the scuticociliate *Miamiensis avidus* pathogenic to olive flounder *Paralichthys olivaceus*

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ABSTRACT: Eight isolates of *Miamiensis avidus* (scuticociliates) were collected from olive flounder *Paralichthys olivaceus* with symptoms of severe ulcers and haemorrhages at several culture farms in 1999 and 2003. Cloned strains were produced and the complete small subunit ribosomal RNA gene (SSU rRNA) of each strain was sequenced for classification and phylogenic study. The SSU rRNA is 1759 bp in length and the sequence was deposited in the GenBank under accession number AY550080. All 8 strains exhibited the same sequence, but this sequence did not match any previously deposited scuticociliate SSU rRNA sequence. Phylogenetic analysis placed *Miamiensis avidus* in a sister lineage to *Cohnilembus verminus*, *Pseudocohnilembus hargisi* and *P. marinus*.

KEY WORDS: Scuticociliatida · *Miamiensis avidus* · Small subunit rRNA · Olive flounder · *Paralichthys olivaceus* · Phylogeny



## INTRODUCTION

Scuticociliates are serious pathogens in worldwide marine aquaculture, causing mass mortalities in fish and crustaceans such as the olive flounder *Paralichthys olivaceus* (Yoshinaga & Nakazoe 1993, Jee et al. 2001, Kwon et al. 2003), turbot *Scophthalmus maximus* (Dyková & Figueras 1994, Sterud et al. 2000, Iglesias et al. 2001), sea bass *Dicentrarchus labrax* (Dragesco et al. 1995), southern bluefin tuna *Thunnus maccoyi* (Munday et al. 1997), American lobster *Homarus americanus* (Cawthorn et al. 1996, Cawthorn 1997), blue crab *Callinectes sapidus* (Messick & Small 1996) and seahorse *Hippocampus erectus* (Thompson & Moewus 1964). In Korea, commercially significant occurrences of scuticociliatosis in the olive flounder were first noted in 1990 (Chun 2000). The ciliate infects the gills, skin, muscles and visceral organs, including the intestine, heart, and brain. It is highly histophagous and destroys infected tissues. The causative agent of scuticociliatosis in the olive flounder in Korea was identified by virtue of its morphological characteristics as *Uronema marinum* (Jee et al. 2001). A very similar disease caused by a scuticociliate in the olive flounder was also reported in Japan (Yoshinaga & Nakazoe 1993). The ciliate infested the internal tissues and caused mass mortalities in fry and juveniles. However, detailed identification has not been conducted yet.

Although various silver impregnation methods have been used to identify Scuticociliatida (Corliss 1953, Foissner 1991), there is still some confusion in the identification of certain species. Song & Wilbert (2000) re-identified various marine scuticociliates and reported that 14 forms had either been misidentified or were junior synonyms. Small subunit rRNA (SSU rRNA, 18S rRNA) sequences have proven useful for identifying and comparing distantly related organisms (Hillis & Dixon 1991, Hirt et al. 1995) because they are reasonably large (about 1800 nucleotides in length), highly conserved, and because available data have increased rapidly within the last few years. The information from these partial and complete SSU rRNA sequences has corroborated some taxonomic relationships based on ultrastructural and other morphological characters (Elwood et al. 1985).

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In this study, we produced 8 cloned strains of scuticociliates, identified as *Miamiensis avidus*, from olive flounder exhibiting severe symptoms of ulcer and haemorrhage in skeletal muscle. The SSU rRNA gene of the ciliates was sequenced for classification and identification of their respective phylogenic positions.

## MATERIALS AND METHODS

Scuticociliates were collected from 8 different culture farms in the Wando, Jindo, and Yosu areas of Korea in 1999 and 2003; details are shown in Table 1. All olive flounder showed typical symptoms of ulcers and haemorrhages in skeletal muscle. Live ciliates were observed in wet preparations of gills, skeletal muscle and brain.

The brain was dissected and washed 3 times in Eagle's minimum essential medium (EMEM) containing high concentrations of antibiotics (500 IU $ml^{-1}$ penicillin and 500 µg $ml^{-1}$ streptomycin). Small pieces of brain were inoculated onto chinook salmon embryo (CHSE-214) cells in 25 $cm^2$ tissue-culture flasks. After 3 to 7 d, each strain was cloned 5 times using the limiting dilution method in 96 well tissue-culture plates of cultured CHSE-214 cells. The CHSE-214 cells were cultured in EMEM, supplemented with 10% foetal bovine serum (FBS), penicillin (50 IU $ml^{-1}$) and streptomycin (50 µg $ml^{-1}$) at 20°C. The ciliates were sub-cultured and maintained in CHSE-214 cells, and grew to a titre of $10^5$ to $10^6$ cells $ml^{-1}$ in 5 to 7 d.

Cultured ciliates (1 ml) were harvested by centrifugation at 2000 × *g* for 5 min. The cells were washed with EMEM without FBS, suspended with 170 µl of TE (Tris-EDTA) buffer, to which 20 µl of Proteinase K (20 mg $ml^{-1}$; TaKaRa) and 10 µl of 10% sodium dodecyl sulfate (SDS) was added. The mixture was incubated at 55°C for 2 h. Total nucleic acids were extracted using Trizol regent (Gibco BRL) and chloroform. Nucleic acids were precipitated with isopropanol, resuspended with distilled water, and stored at –20° C until use. Universal primers A (5′-ACCTGGTTGATCCTGCCAGT-3′) (primer 1) and B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (primer 6) as described by Sogin (1990) were used to amplify the SSU rRNA gene of the ciliates. PCR reactions were performed in a final reaction mixture volume of 20 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 100 pM of each primer, 0.2 mM each dNTP, 1 U of *Taq* DNA polymerase, and template DNA. PCR amplification was performed in a GeneAmp 2400 DNA thermal cycler (Perkin Elmer) with 40 amplification cycles (93.5°C for 30 s, 50°C for 30 s, and 72°C for 2 min). The purity and size of the amplified products were analysed by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and visualized under UV light.

Table 1. *Miamiensis avidus*. Scuticociliate isolates from Korean flounder *Paralichthys olivaceus* included in this study

| Strain | Sampling site | Sampling date (d/mo/yr) | Fish body weight (g) |
|---|---|---|---|
| SJF-03A | Wando | 21/10/2003 | 208 |
| SJF-03B | Wando | 21/10/2003 | 170 |
| SJF-03C | Wando | 9/11/2003 | 33 |
| SJF-03D | Jindo | 28/11/2003 | 88 |
| WS1 | Wando | 20/08/2003 | 190 |
| YK1 | Youngkwang | 26/11/2003 | 200 |
| YK2 | Youngkwang | 26/11/2003 | 182 |
| YS1 | Yosu | 22/05/1999 | 70 |

Amplified PCR products of the expected length were cut from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were ligated into the pCR 2.1 T-vector (Invitrogen) using standard protocols. The subcloned plasmid was transformed into *Escherichia coli* TOP10 (Invitrogen). This plasmid was extracted from cultured *E. coli* TOP10 and used for sequencing. Sequencing was performed by the dideoxynucleotide chain-termination method in an automated ABI PRISM 310 DNA sequencer (PE Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems) and the universal primer set of M13 and RV. DNA samples were sequenced in both directions and from several separate amplifications with terminal primers (primers 1 and 6) and internal primers. The internal primers used were primer 2 (5′-CTATCAGCTTTCGATGGT-3′), primer 3 (5′-GTAGGCTCTTTACCTTGA-3′), primer 4 (5′-CAAATCACTCCACCAACT-3′), and primer 5 (5′-ACGACTTCTCCTTCCTCT-3′).

The sequences were aligned with other SSU rRNA gene sequences using Genetyx Win Ver. 5.1 software. Sites containing gaps were excluded from phylogenetic analysis to reduce systematic errors. Alignments were analysed with the MEGA program (Kumar et al. 1994) to produce neighbour-joining (NJ) trees using the Kimura 2-parameter model. Confidence in the NJ trees was determined by analysing 1000 bootstrap replicates using the MEGA program. The nucleotide sequences used in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Anophryoides haemophila* U51554, *Cohnilembus verminus* Z22878, *Cyclidium glaucoma* Z22879, *Cyclidium plouneouri* U27816, *Dexitrichides pangi* AY212805, *Glauconema trihymene* AY169274,

*Mesanophrys carcini* AY103189, *Metanophrys similis* AY314803, *Paralembus digitiformis* AY297715, *Paranophrys magna* AY103191, *Parauronema longum* AY212807, *Pleuronema coronatum* AY103188, *Pseudocohnilembus hargisi* AY212806, *Pseudocohnilembus marinus* Z22880, *Schizocaryum dogieli* AF527756, *Uronema acuminata* U83128, *Uronema belkae* AF182821, *Uronema elegans* AY103190, and *Uronema marinum* Z22881.

## RESULTS AND DISCUSSION

The ciliate in this study, was morphologically identified as *Miamiensis avidus* Thompson & Moewus (1964) when stained with silver nitrate and silver carbonate. It had a pointed anterior and rounded posterior end and measures 26 to 40 × 16 to 28 µm. A paroral membrane consisting of 2 parts separated by a narrow gap and 3 oral polykinetids, forms the buccal apparatus. The number of ciliary rows was 13 to 14 and the last kinety was terminated midway along membranelle 1. The contractile vacuole was in the second end of the somatic kinety. *M. avidus* is opportunistic ectoparasite which was originally isolated from seahorses (Thomson & Moewus 1964). The ciliate also detected from olive flounder in China, and its morphological characteristics were well described by Song & Wilbert (2000). However, no molecular information has so far been presented on *M. avidus*. The SSU rRNA of the *M. avidus*, in this study was 1759 bp in length, including the regions corresponding to the forward (20 nucleotides [nt]) and reverse (24 nt) amplification primers. The sequences of all 8 strains obtained from olive flounder farms in 4 different regions, were 100% homologous. The sequences were deposited in GenBank under accession number AY550080. The sequences did not show 100% identity to any other deposited sequence; the sequence was 95.62% homologous to that of *Anophryoides haemophila*, 94.78% to that of *Parauronema longum*, 94.21% to that of *Schizocaryum dogieli* and 94.13% to that of *Cohnilembus verminus*. The guanine + cytosine (GC) content was 44.61%, which is in the same range as that of most other ciliates. Phylogenetic analysis showed that the ciliates are included in the phylum Ciliophora within the subclass Scuticociliatida, as a sister lineage to *Cohnilembus verminus*, *Pseudocohnilembus hargisi*, and *P. marinus* (Fig. 1).

A similar scuticociliate infection in the olive flounder was reported with the same symptoms in Korea (Jee et al. 2001), where the arrangement and shape of the buccal structure led researchers to identify the ciliate as *Uronema marinum*, using the silver staining method. However, all 8 strains isolated from 8 different locations exhibited morphological characteristics of *Miamiensis avidus*. We conducted experimental infections of *M. avidus* with the YS1 and SJF-03D strains, and confirmed severe ciliate infection in muscle, brain, and gills, with the same symptoms as seen in naturally infected fish, including ulcers around the mouth and in the skeletal muscles (data not shown). From these results, we hypothesize that *M. avidus* is a major species of scuticociliate pathogenic to olive flounder in the southern and western coastal areas of Korea. Further studies need to be conducted on the possible existence of other species of scuticociliate in culture farms and their pathogenicity to olive flounder.

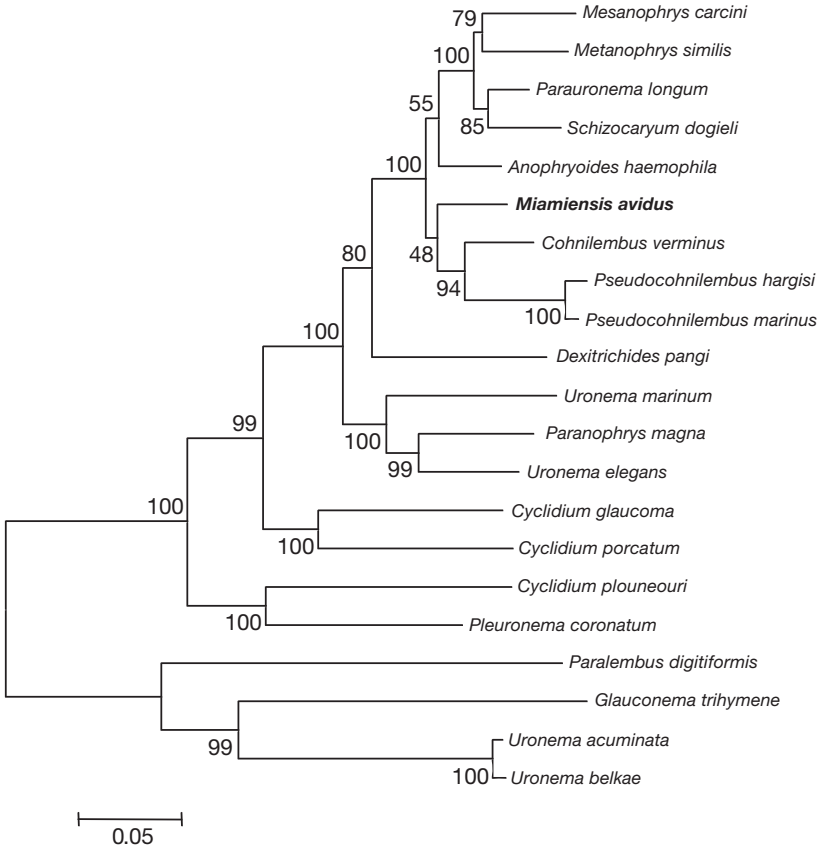


Fig. 1. A small subunit rRNA tree derived from evolutionary distances showing the phylogenic position of *Miamiensis avidus*. The numbers at the nodes represent the bootstrap percentages of 1000 replicates, in the neighbour-joining (NJ) method. The new sequence is shown in boldface

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