A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MOLECULAR GENETIC, MORPHOLOGICAL CHARACTERIZATION OF SCUTICOCILIATES ISOLATED FROM MARINE FISH AND DEVELOPMENT OF RECOMBINANT VACCINE AGAINST *Miamiensis avidus*

Kang, Hyun-Sil

Department of Marine Biotechnology
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

2006. 12

Molecular genetic, morphological characterization of scuticociliates isolated from marine fish and development of recombinant vaccine against *Miamiensis avidus*

Hyun-Sil Kang
(Supervised by professor Jehee Lee)

A thesis submitted in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY

2006. 12.

This thesis has been examined and approved by

Thesis director,
Sung-Ju Jung, Professor of Aqualife Medicine

Choon-Bok Song, Professor of Marine Biotechnology

Young-Don Lee,
Professor of Marine and Environmental research institute

Moon-Soo Heo, Professor of Marine Biotechnology

Jehee Lee, Professor of Marine Biotechnology

Date

DEPARTMENT OF MARINE BIOTECHNOLOGY
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

CONTENTS

요 약 문

전 세계적으로 넙치, 돌돔, 자주복 등의 양식어종들로부터 다양한 종의 스쿠티카충이 감염되는 것으로 보고되어져 있다. 본 연구에서는 제주도의 해산어 양식장들로부터 수집된 스쿠티카 종들로부터 은 염색 방법을 이용하여 형태학적 특징을 분석하였으며, 그 결과 6종의 스쿠티카충을 형태, 섬모하부구조, silverline system 등에 기초하여 동정하였다.

각 종들의 형태를 살펴보면 *Paranophrys marina*는 뾰족한 선단과 둥근 후부를 갖는 가늘고 긴 체형으로 뚜렷하게 긴 membranelle 1(M1)이 몸체의 선단부에 위치하고, paroral membrane (PM)은 M2의 앞까지 신장되어 있다. 10개의 섬모열과 1개의 수축포공이 제2번 섬모열 후단부에 위치하며 한 개의 대핵과 소핵이 몸체의 가운데에 존재하고 있다. *Paralembus digitiformis*는 선단부에 snout를 갖고 있는 가늘고 폭이 좁은 형 또는 계란형의 체형을 갖고 있다. 뚜렷하게 발달된 membranelle 2(M2)를 포함하는 3개의 membranelle과 M2의 중간까지 뻗어있는 PM이 구강부에 넓게 위치하고 있으며, 21-23개의 섬모열과 한 개의 수축포공이 8번과 9번 섬모열 사이의 후단부에 위치하고 있다. 그리고 한 개의 대핵과 소핵이 몸체의 중간에 위치하고 있다. *Miamiensis avidus*는 뾰족한 선단과 둥근 후부를 갖고 있는 두터운 난형으로 3개의 membranelle과 두 개로 나뉘어진 PM이 구강부에 위치하고 있었다. 또한, 이 종은 평균 13개의 섬모열을 갖고 있으며, 한 개의 수축포공이 제2 섬모열 후단부에 위치하고 있다. *Parauronema virginianum*는 뭉툭한 선단과 둥근 후부를 갖는 방추형의 체형으로, 구부기관은 거의 비슷한 길이의 M1과 M2를 포함한 3개의 소막과 M2의 중간까지 신장되어 있는 PM이 몸체의 중간부에 위치하고 있다. 이 종은 대개 12-13개의 섬모열과 제2 섬모열의 후단부에 한 개의 수축포공을 갖고 있다. 점차적으로 작아지는 선단과 둥근 후부를 갖고 있는 방추형의 *Pseudocohnilembus persalinus*는 특이적인 3개의 membranelle과 PM이 구강부에 위치하고 있다. M1과 M2는 서로 평행하며, M3는 PM의 중간에 위치하고 있다. 평균 10개의 섬모열과 한 개의 수축포공이 제2 섬모열 후

단부에 존재한다. 길고 가는 선단과 둥근 후부를 갖는 *Pseudocohnilembus longisetus*는 서로 평행한 두 개의 membranelle과 지그재그형의 PM이 구강부에 위치하고 있다. 평균 9개의 섬모열, 3번과 4번 섬모열에 두 개의 수축포공을 갖고 있으며, 한 개의 대핵과 소핵이 세포의 중앙에 위치하고 있다.

또한, 형태분류된 6종의 스쿠티카충에 대한 유전학적 분석을 수행하기 위해 genomic DNA를 분리하여 SSU rDNA를 PCR 증폭하여 그 염기서열을 결정하였다. 각각의 스쿠티카종으로부터 증폭된 SSU rDNA는 1754 ~ 1759 뉴클레오타이드의 염기서열을 갖고 있다. 이들의 염기서열을 이용하여 BLAST program을 이용하여 분석한 결과, 3종의 스쿠티카충은 (*M. avidus, P. virginianum, P. persalinus*) 이미 알려진 종에 유의하게 일치한 반면, 나머지 3종 (*P. marina*, *P. digitiformis*, *P. longisetus*)은 스쿠티카충에 감염된 병어로부터 새롭게 동정하였다. 이들 서열을 이용하여 계통발생학적 위치를 분석한 결과, 6종의 SSU rDNA는 Philasterida내 단계통군을 형성하였다.

*Miamiensis avidus*는 해산어에 스쿠티카증을 유발하는 주요 기생충성 병원체이다. 본 연구에서는 *M. avidus*로 부터 38 kDa 항원 단백질에 대한 cDNA와 이 유전자를 이용한 재조합 단백질 생산 및 효능 검증에 관한 연구를 수행하였다. 38 kDa의 항원 유전자는 1096 bp로 294개의 아미노산을 암호화하는 882 bp의 ORF를 갖고 있으며, 이 유전자로부터 예측된 분자량은 29679 Da이다. 아미노산 서열에 기초하여 분석된 단백질의 이차구조는 N과 C 말단에 소수성 영역인 signal peptide와 GPI-anchor addition site를 갖고 있다. 또한 60~267번째 아미노산은 cysteine-rich domain으로 이 영역내 시스테인을 포함한 알라닌, 세린과 리신이 다량으로 존재하고 있다.

*M. avidus*의 38kDa 항원 유전자 단편을 합성 올리고뉴클레오타이드를 이용하여 조합한 후, pGEX-4T-1 발현 벡터에 클로닝하여 GST 융합 단백질로 발현시켰다. 융합 단백질을 분리하여 38 kDa 항원 단백질의 단클론 항체와 반응시켜 항원성을 확인하였으며, 그 효능을 naive flounder에 면역화하여 스쿠티카충체의 감염시켰다. 그 결과, 감염 24일째 면역화된 그룹의 사망률은 대조구의 60%에 비교하여 10-15%로 낮게 관찰되었다. 또한, 면역화된 그룹의 항

체 역가도 대조구보다 높은 OD 수치가 관찰되었다. 이들 결과로부터 GST-ScuAg 융합 단백질은 *M. avidus* 감염에 대하여 유용한 백신으로써의 사용가능성을 시사하고 있다.

INTRODUCTION

Scuticociliates belonging to the subclass Scuticociliatia are ciliated protozoa, which are frequently occurred in eutrophic coastal or saprobic maricultural waters. They sometimes act as an opportunistic histophagous pathogen and cause an economic loss of mariculture in Asia and Europe. This subclass is divided into three orders namely Philasterida, Pleuronematida and Thigmotrichida (Lynn and Small, 1997). Taxonomical and systematic studies on scuticociliates have been widely carried out on the basis of their morphological and morphogenetic characteristics over the past two decades.

Recently, scuticociliatosis is a series of parasite diseases in fresh and mariculture fish, which increases the attention regarding as an important threat element in aquaculture industry. This parasitic disease provokes a severe systemic infection through rapid internal organ infiltration by binary fission and finally induces a mass mortality. To date, several species of scuticociliates have been reported as the causative agents in the disease outbreaks of scuticociliates. In marine fish, *Uronema nigricans, Philasterides dicentrachi, Uronema marinum, Pseudocohnilembus persalinus* and *Miamiensis avidus* are well known scuticociliates (Dyvoka and Figueras, 1994; Dragesco et al., 1995; Munday et al., 1997; Iglesias et al., 2001; Jee et al., 2001; Kim et al., 2004a and b; Jung et al., 2005). In addition, *Anophryoides haemophila* and *Mesanophrys chesapeakensis* are described in Crustacea (Cawthorn et al., 1996; Messick and Small. 1996). Although several species of scuticociliates have caused serious problems, studies on parasitic disease causative factors are delayed due to the confusion of an imprecise taxonomic system.

Since the 1960s with the application of various silver impregnation

methods, most of the taxonomic studies on ciliate have progressively changed. Morphological research on the order Scuticociliatia has revealed biological diversity in the ciliates such as species variation as well as physiological and ecological phenotypes (Song and Wilbert, 2002). Silver impregnations help to identify ciliates however, drawbacks such as staining difficulty, misidentification due to species variation, or insufficient data for previously reported strains exist. Molecular methods based on DNA analysis provide additional information to identify phylogenetic relationships and resolve uncharacterized ciliates within the subclass or genus for classical taxonomy. Many genes including small subunit rDNA (SSU rDNA), large subunit rDNA, tubulin, histone, heat shock protein 70 and DNA polymerase α are mostly used to reconstruct phylogenetic trees (Miao et al., 2001). Because the SSU rDNA sequence can be widely used to identify the species, the partial or complete sequences of several protists have been determined. However, the molecular data on scuticociliates are comparatively small and incomplete compared to other ciliates. Therefore, analysis of SSU rDNA and morphological characteristics will discern the taxonomic relationship of unidentified or identified genera.

Many researchers have developed effective treatments or protective methods against scuticociliatosis. The previous scuticocilatosis studies focused on an *in vitro* cultivation (Yoshinaga and Nakazoe, 1993; Iglesias et al., 2003a), infection routes (Parama et al., 2003; Jin et al., 2003), and antigenicity (Iglesias et al., 2003b) in pathologic aspects, drug resistance, and chemotherapeutic material screening (Iglesias et al., 2002; Leiro, et al., 2004; Parama et al., 2004; Parama et al., 2005) in therapeutic research. Although pathologic study provides valuable information to investigate the disease, it does not provide the solution for effective treatment. Vaccination against the parasite is a possible alternative to chemical treatments

because fish acquiring immunity against the infective parasite could survive when infected by the parasite (Xu et al., 2006). Research on the vaccination against a few parasitic diseases such as *Cryptobia salmositica*, *Ichthyophthirius multifiliis* and *Amyloofinium ocellatum* has been studied. (Smith et al. 1992; Woo, 1997; He et al., 1997). Recently, the presence of an immobilizaton antigen in *P. dicentrachi* and its protective effect against challenge infection were demonstrated (Iglesias et al., 2003). Immunization with formalin-killed cells of *M. avidus* in olive flounder also induced protective immune response against parasites (Jung et al., 2006). These observations provide useful information for recombinant vaccine development.

In the present study, we isolated 6 strains of scuticociliates from mariculture farms in Jeju Island. These SSU rDNA sequences and morphological characteristics of ciliates were used to identify and classify their respective phylogeny and to identify major pathogen against scuticociliatosis. *M. avidus* has been reported as a major histophagous pathogen of scuticociliatosis in olive flounder in Korea. We have previously identified a major antigen protein of 38 kDa of *M. avidus* by immunoblot assay (unpublished data). Therefore, as the next step in this study, we attemped to develop the vaccine against *M. avidus*. Here, we cloned and characterized the cDNA of 38 kDa scuticociliate antigen (ScuAg) protein. For gene expression, a fragment of 38 kDa ScuAg gene was synthesized using synthetic oligonucleotides and it induced the protein in a prokaryotic expression system. The fusion protein was purified and its effectiveness on immunized flounder against *M. avidus* was investigated.

PART I. MORPHOLOGY AND GENETICAL STUDIES ON MARINE SCUTICOCILIATES ISOLATED FROM JEJU ISLAND

1. ABSTRACT

Several species of scuticociliates have been implicated in systemic infections of mariculture fishes. In the present study, eight isolates of scuticociliates were collected from several fish farms in Jeju. Its morphological features using silver impregnations investigated isolated strains. As a result, a total of 6 marine scuticociliates were identified based on morphology, infraciliature, and silverline system. *Paranophrys marina* was slender shape with pointed anterior and rounded posterior ends. A distinctly long M1 was positioned near the anterior part of body and PM extended to the anterior part of M2. Ten somatic kineties, one contractile vacuole pore located on the posterior end of the second kinety. One macro- and micronucleus were existed in the middle part of the body. *Paralembus digitiformis* was elongated with slender or thick oval shape. Three membranelles with highly developed M2 and PM that extended to the middle part of M2 were widely distributed in the buccal field. Twenty one-twenty three somatic kineties and one contractile vacuole pore posteriorly positioned between somatic kineties 8 and 9. One macro- and micronuclues were centrally located in the body. *Miamiensis avidus* consisted of a pointed anterior end and rounded posterior end. Three membranelles and bipartite paroral membranes were occupied the buccal field. Most of the thirteen somatic kineties and one contractile vacuole pore located in the posterior end of the second kinety. *Parauronema virginianum* contained blunt anterior and narrowly rounded posterior ends. Three membranelles with almost same length M1 and M2 and PM extended to

the middle part of M2 were composed the buccal cavity in the middle part of the body. Twelve to thirteen somatickineties and one contractile vacuole pore positioned at the posterior end of somatic kinety 2. *Pseudocohnilembus persalinus* was spindle-shaped with a tapered anterior end and rounded posterior end. Three specialized membranelles and PM were located in the buccal field. M1 and M2 were parallel to each other and M3 was located at the middle part of PM. Ten somatic kineties and one contractile vacuole pore located at the posterior end of somatic kinety 2. An elongated shape with a tapered anterior end and rounded posterior end was observed in *Pseudocohnilembus longisetus*. Two parallel membranelles (M1 and M2) and PM with a zig-zag pattern occupied the buccal field. Nine somatic kineties, two contractile vacuole pore were located at the posterior end of somatic kineties 3 and 4. One macro- and micronucleus were centrally located at the body.

Moreover, genomic DNA was isolated from these strains and SSU rDNA sequences were by polymerase chain reaction (PCR). The amplified PCR products were cloned into a vector, and subjected to nucleotide sequencing. All the amplified SSU rDNA of 6 scuticociliates were ranged from 1754 to 1759 nucleotides in length. The phylogenetic positions of those species within the order Scuticociliatia were deduced as a monophyletic group in the order Philasterida.

MATERIALS AND METHODS

1. Isolation and maintenance of scuticociliates

Scuticociliates were isolated from diseased fishes in several mariculture farms located in coastal area of Jeju Island. Ciliates were observed under the light microscope in wet preparations of gill, muscle, skin, fin and brain from the diseased fish. The infected tissues were washed 3 times with sterilized seawater (containing 1% yeast extract and 1% proteose peptone) and cultivated at 15℃ for 5 days. After 5 days, each strains were cloned using serial dilution of primary culture medium in a 96 well tissue culture plate.

The cloned ciliates were maintained in two types of media, which Millport S (NaCl 1.5g, $MgCl_2 \cdot 6H_2O$ 0.25g, KCl 0.04g, $CaSO_4$ 0.012g per 100 mL distilled water) and sterilized seawater with 2% brain heart infusion broth (final concentration, 0.5%) with *vibrio sp.* depending on the growth rate. Axenic culture with Chinook salmon embryo-214 (CHSE-214) cell lines which were cultivated at modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL ampicillin and 100 mg/mL streptomycin) was also performed. Table 1 displays the strains of ciliate isolated from Jeju Island. These strains were used for morphological and genetic studies.

2. Morphological analysis

Silver impregnations (silver carbonate and silver nitrate) were used for revealing the infraciliature and silverline system. Measurements were performed under 1000-× magnification using image-analyzing software (Image -Pro Plus 3.0, USA).

Table 1. The strains of scuticociliate isolated from marine fishes in Jeju Island

Species	Group	Origin	Sampling date
Paranophrys marina	GLS	Flounder	2004.6
Paralembus digitiformis	LS	Flounder	2003.7
Miamiensis avidus	MS	Turbot	2003.4
		Pufferfish	2002.11
			2003.10
Parauronema virginianum	SS1	Red seabream	2003.1
		Rock bream	2002.12
Pseudocohnilembus persalinus	SS3	Flounder	2004.12
Pseudocohnilmebus longisetus	SS3	Schlegel's black rock fish	2005.8

2.1. Silver carbonate impregnation

The ciliates were concentrated by centrifugation at 2000 rpm for 5 min. After removal of the supernatant, the pellets were resuspended with remaining 1 mL supernatant. One mL of 10% formalin was added into resuspended solution and mixed for 2 min. Distilled water was added up to 10 mL and centrifuged as above. Washing steps were repeated 3 times with distilled water. After final centrifugation, 10% formalin was added into resuspended solution and mixed by circular motion for 2 min. An appropriate volume of fixed ciliates were transferred onto a concave slide and mixed with the same volume of Fernandez- Galiano's solution for 1 min at room temperature. The slide was placed on 60℃ hot plate and the solution was mixed by circular motion until it turned a golden or brown color. The impregnated specimen was observed under the light microscope and the reaction was terminated by adding 5% sodium thiosulfate ($Na_2S_2O_3$).

To prepare permanent slides, the specimens were washed by distilled water at least 3 times to remove $Na_2S_2O_3$. In the final washing step, the supernatant was removed after centrifugation and the pellets were resuspended with a small volume of distilled water. The same volume of well-impregnated ciliates and albumen-glycerol (1:1) were added onto a clean slide, mixed thoroughly and then spread mixture to become a thin layer. After that, the slides were dried in the oven at 60 ℃ for 30 min. Then, the slides were subjected to alcohol dehydration (70%-80%-90%-100%-100%: ethanol) and embedded to xylene. Finally, the slides were mounted with mounting medium.

2.2. Silver nitrate impregnation

The ciliates were concentrated by centrifugation at 2000 rpm for 5 min.

To the concentrated ciliates 2 times volume of Champy's fixative was added and fixed for 15 min. The supernatant was removed by centrifugation, and post-fixed with Da Fano's fluid for 5 min. This step was repeated until solution has became the same color as the Da Fano's fluid. After that, a small piece of concentrated ciliate and molten gelatin was placed onto a warmed slide and the mixture was spread by mounting needle to form a thin layer of gelatin. The slides were immediately transferred into a cold chamber and incubated until the gelatin hardened. Then, the slides were washed with distilled water for a few seconds and kept on 1% silver nitrate solution for 30 min at 4℃. The slides were washed again with cold distilled water and irradiated with a ultraviolet light (<254 nm) for 10-15 min until slides turned a golden or brown color . The intensity of the impregnated specimens was observed under light microscope and then terminated the staining reaction. To prepare the permanent specimen, the slides were transferred immediately into prechilled 70% alcohol and underwent alcohol dehydration (100% alcohol, 2 times) for 10 min. Afterward, slides were embedded in xylene for 10 min at 2 times and mounted with mounting medium.

3. Genetic analysis

3.1. Small subunit ribosomal DNA amplification by PCR

The cultured ciliates were pelleted by centrifugation at 2000 rpm for 10 min. Pellets were washed with sterilized Millport S and genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Germany). The concentration of isolated nucleic acids was measured at 260 nm using SmartSpec^{TM} Plus Spectrophotometer (Bio-rad, USA).

To amplify the small subunit ribosomal DNA (SSU rDNA), we designed the forward (SSUF 5′-AACCTGGTTGATCCTGCCAG-3′) and

reverse primers (SSUR 5′-GATCYWTCTGCAGGTTCACCTAC-3′) based on SSU rDNA sequences of scuticociliate in GenBank. PCR reactions were performed in a final volume of 50 uL containing 10× Ex Taq buffer, 50 pmol of each primer, 2.5 mM dNTPs, 2.5 U of Ex Taq DNA polymerase (Takara, Japan) and 50 ng of genomic DNA. The PCR reaction was processed for 30 cycles using a Takara PCR Thermal cycler (Takara, Japan) at 95℃ for 30 s, 55℃ for 35 s and 72℃ for 2 min with pre-denaturation at 95℃, 2 min. The amplified products were analyzed using electrophoresis on 1% agarose gel.

PCR products were cut from the gel and extracted using the Accuprep™ Gel purification kit (Bioneer, Korea). Purified DNAs were ligated into pBluescript II SK(-) and transformed *Escherichia coli* DH10b (Stratagene, USA). Recombinant plasmid was prepared by alkaline lysis method using Accuprep™ Plasmid Extraction kit (Bioneer, Korea). Sequencing reaction was carried out with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI 377 DNA sequencer (Applied Biosystems, USA). To determine complete sequences of SSU rDNA, we used the internal primer SSU1F (5′-CGGTAATTCCAGCTCCAATAG-3′) with the universal primers SK and T7.

3.2. Data analysis

BLAST N searching and pairwise alignmemt program calculated the similarity of the determined nucleotide sequences of scuticociliates. The sequences of scuticociliates were aligned using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic tree and Kimura two-parameter distance were determined by the MEGA 3.1 and neighbor-joining method, respectively. Confidence estimate was obtained on the basis of bootstrap generation of 10,000 replicates.

RESULTS

1. Ciliate isolation and culture

The morphology of isolated ciliates from several mariculture farms was observed under both the inverted and differential light microscopes and it showed variable sizes. Based on the size and buccal appearance under the microscope, we categorized the list of ciliates and investigated the optimal medium for the further study (Table 1).

First, all types of scuticociliate were cultured in an axenic culture using CHSE-214 fish cell lines. Scuticociliates of GLS and LS group were not grown in the CHSE-214 cell line. The GLS and LS groups died one day after inoculation. On the other hand, scuticociliates of MS group grew well in the CHSE-214 cell lines. They attached to the bottom of the culture plate and destroyed the fish cell lines. After 5 or 7 days, they completely digested the cells and reached the highest scuticociliate density in culture plate. In the case of SS groups they also attached to the bottom of plate and started the cell lysis on 1 day after inoculation. However, they did not grow well as compared to the MS group and did not completely digest the cells, therefore leaving cell debris. A few days later, bacteria were grown in culture plate and used as a food for the SS groups.

Secondly, we investigated the optimal culture medium for scuticociliates not growing in the axenic CHSE-214 cell line. Both the GLS and LS groups grew well in sterilized sea water containing 0.5% BHI broth and *vibrio sp*. MS and SS groups grew well in Millport S containing 0.5% BHI broth and *vibrio sp* as well.

2. Morphological characteristics

Silver impregnations of scuticociliates isolated from several mariculture farms located in coastal area of Jeju Island were performed to observe the infraciliature and silverline system. As a result, ciliates of each group showed distinct morphological characters. In the GLS group, one species of scuticociliate was identified as *Paranophrys marina* based on infraciliature of the buccal apparature. This ciliate was isolated from diseased flounder, *Paralichthys olivaceus* located at Jeju on June 2004. It was observed that ectoparasite found on gill tissue, had destroy the gill tissue by a feeding action.

Morphological characteristics of scuticociliate are shown in Table 2 and Figure. 1. The body shape is generally slim and slender type with a sharply pointed anterior and narrowly rounded posterior ends. Cell size was about 40 (range, 33-46) × 11 (range, 8-15) ㎛ *in vivo*, sometimes appeared a giant body shape in the growth phase. The cytoplasm appeared colorless and often filled with many small light reflecting granules and bar-shaped crystals in culture (Fig. 1A). A contractile vacuole pore was posteriorly located and had one caudal cilium about 11.5 (9-14) ㎛ length. Movement was active or very quiet when they were fed bacteria in the Petridish bottom.

The length and width of silver impregnated specimens were about 41.5 × 20.4 ㎛. The buccal cavity is consisted of tripartite membranes on the left side and a paroral membrane on the right side. Membranelle 1 (M1) was slightly away from the anterior pole and located in the anterior portion of the buccal cavity. It was well-developed with 2 long rows of kineties and 7.88 ㎛ in length. Membranelle 2 (M2) is posteriorly located on M1 and 3.22 ㎛ in length. Membranelle 3 (M3) was close to M2 with 3 short rows and 0.99 ㎛ in length. The paroral membrane (PM) started near

the anterior end of M2 and terminated near the posterior end of the buccal cavity (Fig. 1B). The anterior portion of PM was straight while the posterior portion had zig-zag row that curved around the cytostome.

Ten somatic kineties were longitudinally arranged on both ends of body, except for kinety n. Kinety n terminated in front of M1. Silverline of kinety n crossed over the caudal area, continuing onto the caudal cilium complex and linked between kineties 5 and 6 (Fig. 1C). One contractile vacuole pore located in the posterior end of kinety 2. A cytoproct was located between kineties 1 and 10 and showed a wavy line at the posterior end in the ventral area (Fig. 1D). One macronucleus appeared in the middle of the body with one anteriorly positioned micronucleus. Based on these observations, we identified our ciliate as *Paranophrys marina*.

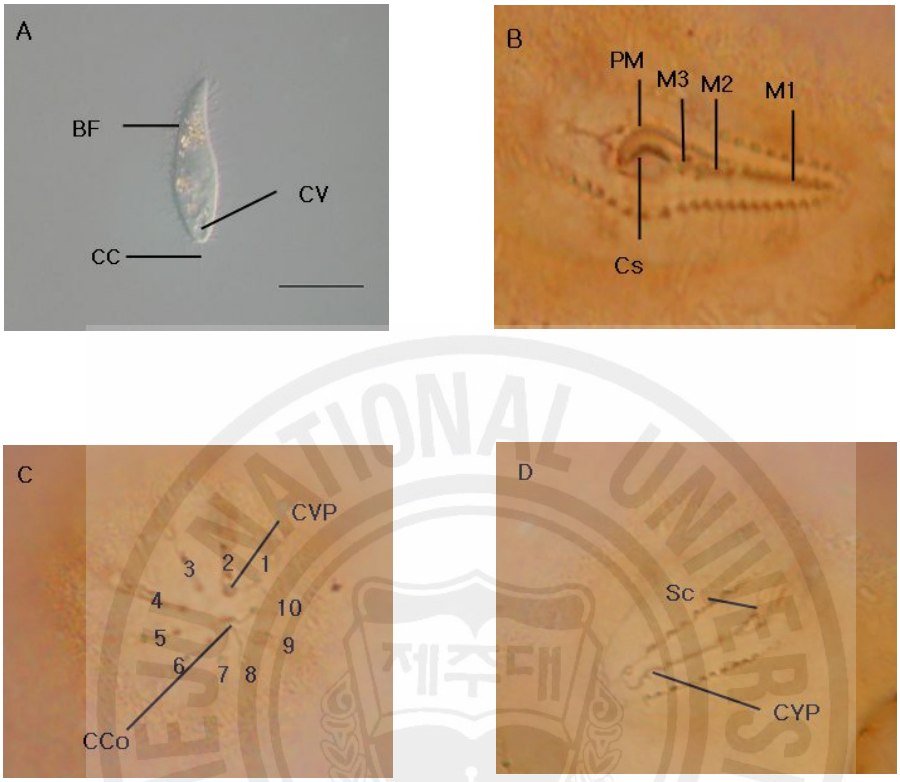

Fig. 1. Silver impregnated specimens and live observation of *Paranophrys marina*. A: live ciliate *in vivo*, B: buccal field of infraciliature, C: caudal view of silverline system, D: ventral view of silverline, BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, Cs: cytostome, CV: contractile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

Table 2. Morphometric characterization of *Paranophrys marina*. All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples; SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	36.1	48.7	41.5	3.13	40
Body width	17.1	24.6	20.4	2.04	40
Number of somatic kineties	9	10	10	0.51	38
Length of buccal field*	15	21.3	19.2	1.42	32
Length of membranelle 1	5.1	9.3	7.9	0.97	36
Length of membranelle 2	2.3	3.8	3.2	0.26	34
Length of membranelle 3	0.6	1.5	1.00	0.21	33
Length of paroral membrane	7.9	10.4	9.00	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

Table 3. Morphological comparison of *Paranophrys marina* Thompson, 1965 and Song, 2002

Character	*P. marina** (Thomson & Berger, 1965)	Present study**	*P. marina**** (Song et al. 2002)
Body length × width (㎛) *in vivo*	-	33-46 × 8-15	30-45 ×10-15
Body length × width (㎛) fixed	39.3 ×19.2	41.5 × 20.4	-
No. of somatic kineties	10	10	10
Length of buccal field	19.6	19.2	about 2/5
Length of membranelle 1	8.4	7.9	- very long
Length of membranelle 2	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 silver nitrate impregnation

***- Data from protargol impregnation

Scuticociliate of the LS group was isolated from diseased flounder of mariculture farm at Jeju on June 2003. It seemed to be an endoparasite that was observed in ulcer tissue of fishes with feeding behavior on cells.

The body shape was usually elongated and longer form with a pointed anterior and rounded posterior ends. (Fig. 2A). Also, it became thick oval or rounded shape with a distinct snout in the anterior area when they completely consumed the food in culture (Fig. 2B). Cell size was about 39 × 13 ㎛ *in vivo*. The cytoplasm was lighter often filled with many small granules and reflecting bar-shaped crystals. The contractile vacuole pore was small and caudally located near the right side on ventral area with one single caudal cilium about 7-14 ㎛ long. Locomotion was very slow or inactive when they were feeding on bacteria in the Petrfidish bottom. When swimming, it had a continuous spiraling motion along it's body's axis.

The length and width of silver nitrate impregnated specimens were 40.6 × 21.2 ㎛. The buccal cavity was widely distributed in the body, consisting of 3 membranelles on the right side (Fig. 2C). M1 was slightly away from the apical pole and had a triangular shape with 3 rows in about 3.1 ㎛ long. M2 was located near the posterior end of M1 and was highly developed. It was approximately 16.4 ㎛ long with 3 or 4 longitudinal rows. M3 was very small and located posterior end of M2 with 2 or 3 short rows. PM was positioned on the right side of the buccal cavity and extended to the middle part of M2 from the posterior buccal cavity.

Somatic kineties of this ciliate were mostly 21-23. Kineties of located right of the buccal field in the anterior pole were densely arranged and extended to the pointed snout (Fig. 2D). In addition, kinety first to third were distinctly shorter when compared with other kineties in the posterior end. Silverline of the last kinety connected to the caudal cilium complex

and crossed over to the polar fiber near kinety 10 (Fig. 2E). One contractile vacuole pore was located at the posterior end between kineties 8 and 9. A cytoproct was short with a wavy line at the posterior end (Fig. 2F). One macronucleus with anr anteriorly positioned micronucleus positioned in the middle of the body. According to these observations, we identified this ciliate as *Paralembus digitiformis*.

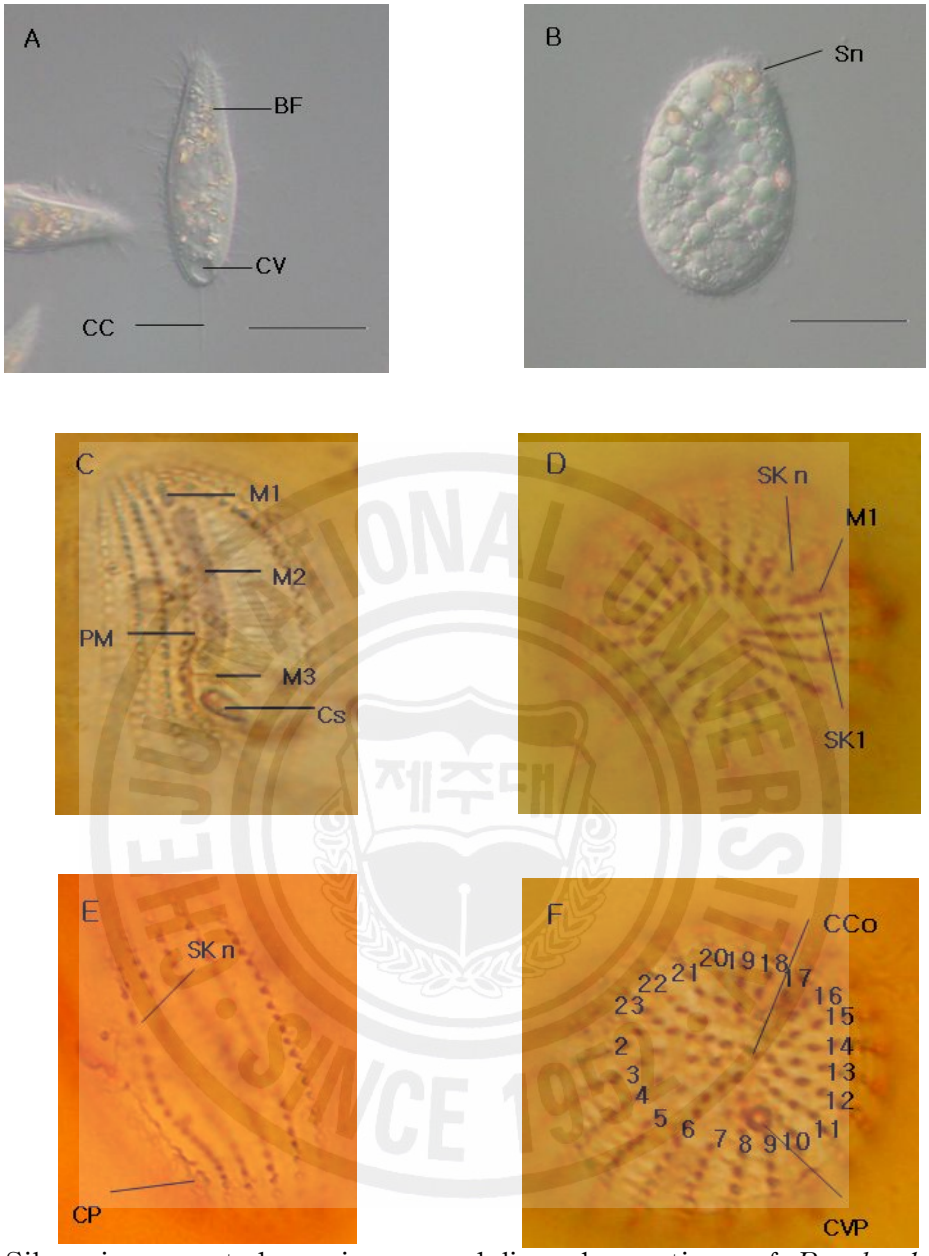

Fig. 2. Silver impregnated specimens and live observations of *Paralembus digitiformis*. A,B: *in vivo*. C: infraciliature of buccal field, D: apical pole area. E: ventral view of silverline, F: caudal view of silverline, BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, Cs: cytostome, CV: contractile vacuole, CVP: contractile vacuole pore, CP: cytopyge, M1, 2, 3: membranelles 1, 2, 3, PM: paroral membrane, Sn: snout, Scale bar= 20 ㎛.

Table 4. Morphometric characterization of *Paralembus digitiformis*. All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples, SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	31.7	47	40.6	4.34	40
Body width	17.3	25.6	21.2	1.89	40
Number of somatic kineties	21	23	22	0.75	21
Length of buccal field*	24.7	29.6	28.0	1.43	15
Length of membranelle 1	2.2	4.6	3.1	0.52	39
Length of membranelle 2	13.8	18.8	16.5	1.26	40
Length of membranelle 3	0.5	0.9	0.7	0.13	26
Length of paroral membrane	11.4	15.9	14	1.11	27
Number of macronucleus	1	1	1	0	0
Width of macronucleus	6.5	13.3	9.9	1.40	40
Number of micronucleus	1	1	1	0	0

*- distance from apex to posterior end of paroral membrane

Table 5. Morphological comparison of *Paralembus digitiformis* in present and literatures

Character	*P. digitiformis** (from 2 strains)	Present study**	*P. rostrata* ***
Body length × width (㎛) *in vivo*	-	33-55×8-35	-
Body length × width (㎛) fixed	25-37 × 18-29 39-62 × 15-26	40.6 × 21.2	24-45 × 15-26
No. of somatic kineties	25 22-23	21-23	ca. 32
Length of buccal field	14-19 22-31	24.7- 29.6	20-25
No. of rows of basal bodies in membranelle 1	3 3	3	5
No. of rows of basal bodies in membranelle 2	3-4 4	3-4	5-6
Body shape	thick oval slender	elongated, thick oval	oval
Position of contractile vacuole pore	end of SK8 and 9	end of SK8 and 9	-
Host	molluscs	flounder	-
Sample location	Qingdao, China	Jeju, Korea	Roscoff, France
Data source	Song et al. 2000	Original	Groliere, 1974

*- Data from protargol impregnation

**- Data from silver nitrate impregnation

***- Data from protargol impregnation

Scuticociliates of MS group were isolated from diseased turbot and pufferfish of two mariculture fish farms at Jeju from November 2002 to October 2003. The isolated scuticociliates resembled a histophagous parasite, which widely infected several organs of fish, including the brain destroyed organs or tissue by feeding on cells or tissue components.

These strains were drop-like or ovoid with a pointed anterior end and widely rounded posterior end. *In vivo* they approximated 27 (range, 22-34) × 14 (range, 10-21) ㎛. Many food vacuoles and crystals of bar-shape are filled the cytoplasm. Contractile vacuole pore laid on the posterior end of body with one caudal cilium 9.22 um long (Fig. 3A). Movement was almost slow or inactive when it was attached to bacteria or foods in the culture. Swimming was continuous and showed forward movement in culture medium.

The buccal apparatus consisted of 3 membranelles on the left side of the buccal cavity and a bipartite paroral membrane on the right side (Fig. 3B). M1 was spindle-like or triangular shape with 2 rows, away from the anterior pole. M2 was trapezoid-shaped and longer than M1, along with 4-5 rows. M3 was small and positioned close to M2 with 2 to 3 rows. PM is composed of two distinct parts (PM1 and PM2), which are generally joined together near by the M3. Moreover, the anterior part (PM1) of PM extended to the anterior end of M1. The posterior part (PM2) consisted of zig-zag row and curved around the cytostome. Somatic kineties averged 13 to 14 and they were longitudinally arranged at both ends of body except for the buccal cavity. Specifically, last kinety is terminated along anterior end or middle of M1. Silverline of last kinety continued on the caudal cilium complex and crossed over between kineties 7 and 8. A single contractile vacuole pore was located near the posterior end of the second kinety (Fig. 3D). Cytoproct was positioned in between kineties 1 and n

near the posterior end with an irregular shape (Fig. 3C). One macronucleus with spherical or ellipsoid-shape located at the anterior part of the body. Also, a single micronucleus was positioned close to macronucleus or embedded in the macronucleus. According to these observations, we identified this ciliate as *Miamiensis avidus*.

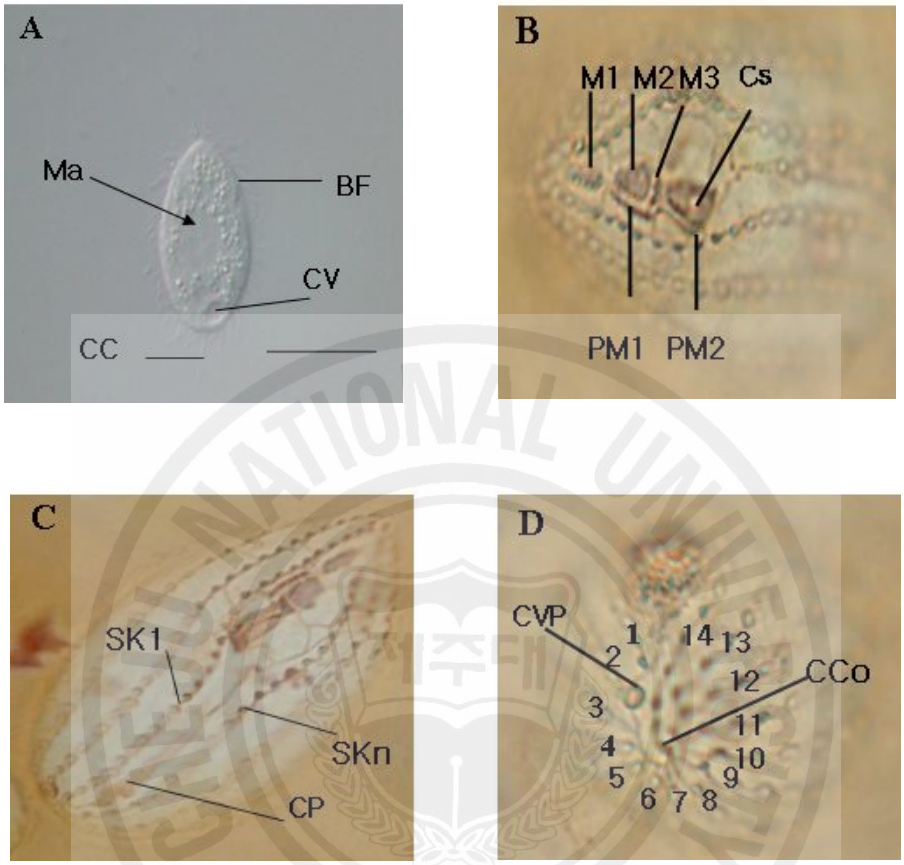

Fig 3. Silver impregnated specimens and live observation of *Miamiensis avidus*. A: *in vivo*, B: infraciliature of buccal field, C: ventral view of silverline system, D: caudal view of silverline system. BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, Cs: cytostome, CP: cytopyge, CV: contractile vacuole, CVP: contractile vacuole pore, Ma: macronucleus, M1, 2, 3: membranelles 1, 2, 3, PM1, 2: paroral membrane 1, 2, SK 1, n: somatic kinety 1, n, scale bar= 20 ㎛.

Table 6. Morphometric characterization of two populations of *Miamiensis avidus*, population 1 (1st line), population 2 (2nd line). All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples, SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	34.4	44.5	37.9	2.38	37
	32.4	44.5	38.6	2.98	40
Body width	18.6	26.1	21.9	1.98	37
	16.1	26.6	21	2.59	40
Number of somatic kineties	12	14	13	0.63	26
	12	14	13	0.53	26
Length of buccal field*	15.5	19.3	17.6	1.03	25
	15.7	20.6	17.9	1.22	32
Length of membranelle 1	2.3	3.4	3.1	0.24	29
	2.5	3.7	3.2	0.25	34
Length of membranelle 2	2.6	3.4	2.9	0.21	30
	2.8	3.9	3.3	0.31	34
Length of membranelle 3	0.5	0.9	0.7	0.11	30
	2.8	3.9	0.7	0.11	30
Length of paroral membrane 1	3.9	5.2	4.5	0.43	20
	3.8	5.3	4.7	0.42	28
Length of paroral membrane 2	3.9	7.4	5.7	0.79	20
	5.5	8.6	6.8	0.74	29
Length of macronucleus	5.3	9.4	7.1	1.19	40
	5.5	8	6.9	0.68	40
Width of macronucleus	5.2	8.9	6.9	1.04	40
	5.7	8.6	7.2	0.75	25
Width of micronucleus	1.3	2.3	1.8	0.33	28
	1.4	2.4	1.9	0.26	25

*- distance from apex to posterior end of paroral membrane

Table. 7. Morphological comparison of *Miamiensis avidus* from the present study and literatures

Character	*M. avidus* (from 2 strains)	*M. avidus*	Present study (from 2 strains)	*Philasterides dicentrachi*
Body length × width (㎛) *in vivo*	-	30-45 × 20-35	22-34 × 10-21	30-42 × 10-22
Body length × width (㎛) fixed	31.9 × 16.1 39.9 × 20.1	28-41 × 23-32	37.9 × 21.9 38.6 × 21	23-45× 11-20 26-40×12-25
No. of somatic kineties	10-12 10-13	13-14	12-14	13-15
Length of buccal field/ body length	about 1/2	2/5-1/2	about 1/2 about 1/2	about 1/3-2/5
Length of M1* (㎛)	2.6 3.0	ca. 3-4	3.05 3.20	ca. 2-3
Length of M2** (㎛)	2.8 3.6	ca. 3-4	2.94 3.32	ca. 2-4
Life style	ectoparasite, histophagous	ectoparasite, histophagous	ectoparasite, histophagous	ectoparasite, histophagous
Position of CVP***	end of SK2	end of SK2	end of SK2	end of SK2
Host	sea horse	flatfish	turbot, pufferfish	sea bass
Sample location	Miami, USA	Qingdao, China	Jeju, Korea	(?), France
Data source	Thomson and Moewus (1964)	Song et al. (2000)	Original	Dragesco et al.(1995)

* M1, membranelle 1

** M2, membranelle 2

*** CVP, contractille vacuole pore

In the SS groups, three species of scuticociliates were identified based on the infraciliature of buccal apparature. Scuticociliates of the SS1 group were isolated from the rock bream and pufferfish of two mariculture fish farms from December 2002 to October 2003. These parasites were observed in the gill or ulcer tissue of the fishe with other scuticociliates, a little bigger size when compared with other scuticociliates of the SS group.

The body was elongated or slender like with a bluntly pointed anterior end and rounded posterior end. Cell size measured 32-40 × 12-16 ㎛ *in vivo*, filled with several shining globules and barlike crystals in the cytoplasm (Fig. 4A). The contractile vacuole was small and located at the posterior end with one caudal cilium approximate 11-12 ㎛ long. It moved quickly and had a rotating motion along it's body axis while swimming.

The silver impregnated specimens were 34.3 × 13.2, and 30.3 × 14 ㎛ in populations 1 and 2, respectively. The buccal apparatus was positioned in the middle of the body with 3 membranelles and a paroral membrane. M1 was short and located away from the anterior end of the body. M2 was almost the same length as M1 with 2-3 rows of kineties. M3 was much smaller than M1 and M2. The anterior portion of PM was straight and terminated near the middle of M2, but posterior portion was curved along the right side of the cytostome. Twelve to thirteen somatic kineties were arranged longitudinally except for the buccal cavity. The silverline of the last kinety connected on the caudal cilium complex and linked near the kinety 8 or 9 (Fig. 4B). A single contractile vacuole pore was located near the posterior end of the second kinety. One macronucleus and micronucleus were located at the middle of the body. According to above observations, we identified this ciliate as *Parauronema virginianum*.

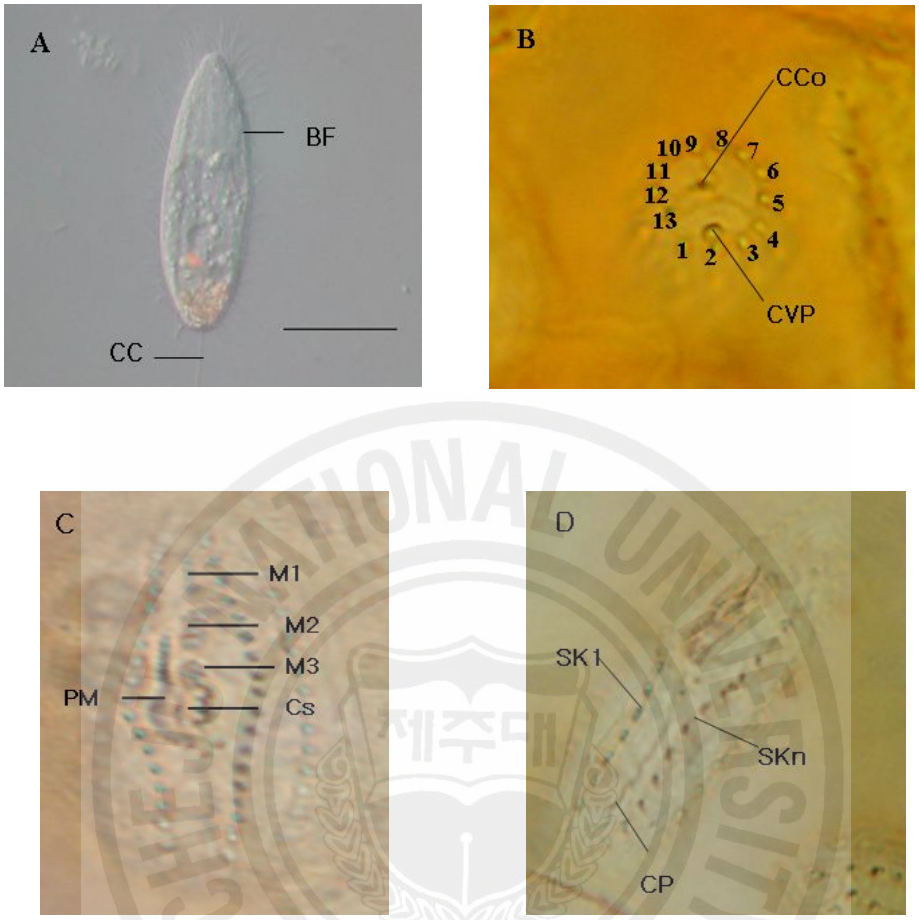

Fig. 4. Silver impregnated specimens and live observation of *Parauronema virginianum*. A: *in vivo*, B: caudal view of silverline, C: infraciliature of buccal field, D: ventral view of silverline, BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, CP: cytopyge, Cs: cytostome, CV: contractile vacuole, CVP: contractile vacuole pore, M1, 2, 3: membranelles 1, 2, 3, PM: paroral membrane 1, SK 1, n: somatic kinety 1, n, Scale bar= 20 ㎛.

Table 8. Morphometric characterization of two populations of *Pararuronema virginianum*, population 1 (1st line), population 2 (2nd line).. All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples, SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	28.3	37.8	34.3	2.40	20
	26.9	36.3	30.3	2.58	31
Body width	12.1	14.4	13.2	0.81	20
	11.3	20	14	1.61	31
Number of somatic kineties	12	14	13	0.64	20
	11	13	12	0.62	16
Length of buccal field*	17.7	20.5	19.2	0.75	20
	15.4	18.6	16.8	0.86	20
Length of membranelle 1	2.2	3.3	2.8	0.29	20
	2.0	3.2	2.5	0.35	20
Length of membranelle 2	2.1	3	2.6	0.23	20
	2.1	2.9	2.5	0.22	20
Length of membranelle 3	0.7	1.7	1.3	0.28	20
	0.9	1.9	1.4	0.28	20
Length of paroral membrane	6.7	8.0	7.4	0.34	20
	6.5	8.2	7.3	0.45	20
Number of macronucleus	1	1	1	0	20
	1	1	1	0	30
Number of micronucleus	1	1	1	0	20
	1	1	1	0	30

*- Distance from apex to posterior end of paroral membrane

Table 9. Morphological comparison of *Parauronema virginianum* from present study and literatures

Character	*P. virginianum* (from 2 strains)	Present study *P. virginianum*	
Body length × width (㎛) *in vivo*	-	×	-
Body length × width (㎛) fixed	23-29 × 12-16 29-36 × 12-16	28-38 × 12-14 27-36 × 11-20	ca. 28 × 12
No. of somatic kineties (SK)	11-12	12-13(11-14)	13
Length of buccal field: cell length	ca1/3- 1/2	ca 1/2	ca 1/3 -1/2
No. of contractile vacuole pores (CVP)	1	1	2 (1-3)
Position of CVP	SK2	SK2	SK2
Sample location	Virginia, USA	Jeju, Korea	Solar lake, USA
Data source	Thompson (1965)	original	Wilbert & Kahan(1981)

Two species of SS2 and SS3 groups had buccal apparatus with similar Infraciliature. Both strains formed a cyst when food was scarce in the culture medium (Fig. 5A). Scuticociliate of SS2 group was isolated from the gill of diseased young flounder in December 2004. Cell size measured 43 (range, 27-43) × 14 (range, 10-21) ㎛ *in vivo*. The body shape of this ciliate was usually spindle-shaped with a tapered anterior end and rounded posterior end (Fig. 5B). Cytoplasm was colorless and filled with many granules, which are more or less greenish in culture. The contractile vacuole was small and positioned near the posterior end of the body with one caudal cilium about 11 ㎛ long. It usually moved fast fast or it was quiet when feeding on bacteria or foods in the Petridish bottom.

The buccal apparatus is consisted of 3 specialized membranelles and a paroral membrane (Fig. 5C). M1 and PM were only distinguishable in well-impregnated specimens. M1 was parallel to M2 and positioned just anterior the PM with a single row. M2 was very long compared with other membranells and close to M1 and PM. M3 was very small and positioned around the half of the PM. PM was gently curved and located at the right side of the buccal cavity. Somatic kineties averaged 10 and they were sparsely arranged except for first and last kinety on the buccal area. Silverline of the last kinety continued to the caudal cilium complex and linked near the kinety 5 or between kineties 4 and 5 (Fig. 5E). A contractile vacuole pore (CVP) laid on the end of third somatic kinety. Rarely were two CVPs found at the end of kineties 2 and 3. A cytoproct was located subcaudally appearing as a thick argentophilic patch (Fig. 5D). One macronucleus was centrally located in the body. Based on the above observations, we identified our ciliate as *Pseudocohnilembus persalinus*.

Scuticociliate of SS3 group was isolated from Schlegel's black rockfish in August 2005. The body was elongated with a tapered anterior end and

rounded posterior end. The anterior end was slightly toward the right side of the body. The cell size was about 28 (range, 24-34) × 12 (range, 10-17) ㎛ *in vivo*. The Cytoplasm was colorless and filled with many globules in culture (Fig. 6A). The contractile vacuole pore was small and positioned near the posterior end of body with one caudal cilium measuring 11 ㎛ long.

The buccal apparatus occupied the anterior half of the body and 2 membranelles (Fig. 6B). M1 was parallel to M2 with a single row and located anterior of PM. M2 was very long and close to M1 and PM. PM is gently curved and located at the right side of the buccal cavity. Usually, 9 somatic kineties were found and they were evenly arranged except for first and last kineties. Silverline of the last kinety continued on the caudal cilium complex and joined with kineties 4, 5 and 6 (Fig. 6D). However, most often it was near the kineties 4 and 5. Two contractille vacuole pores were located the end of the 3 and 4 kineties. The cytoproct was located subcaudally as a thick argentophilic patch (Fig. 6C). Finally, we identified our ciliate as *Pseudocohnilembus longisetus*.

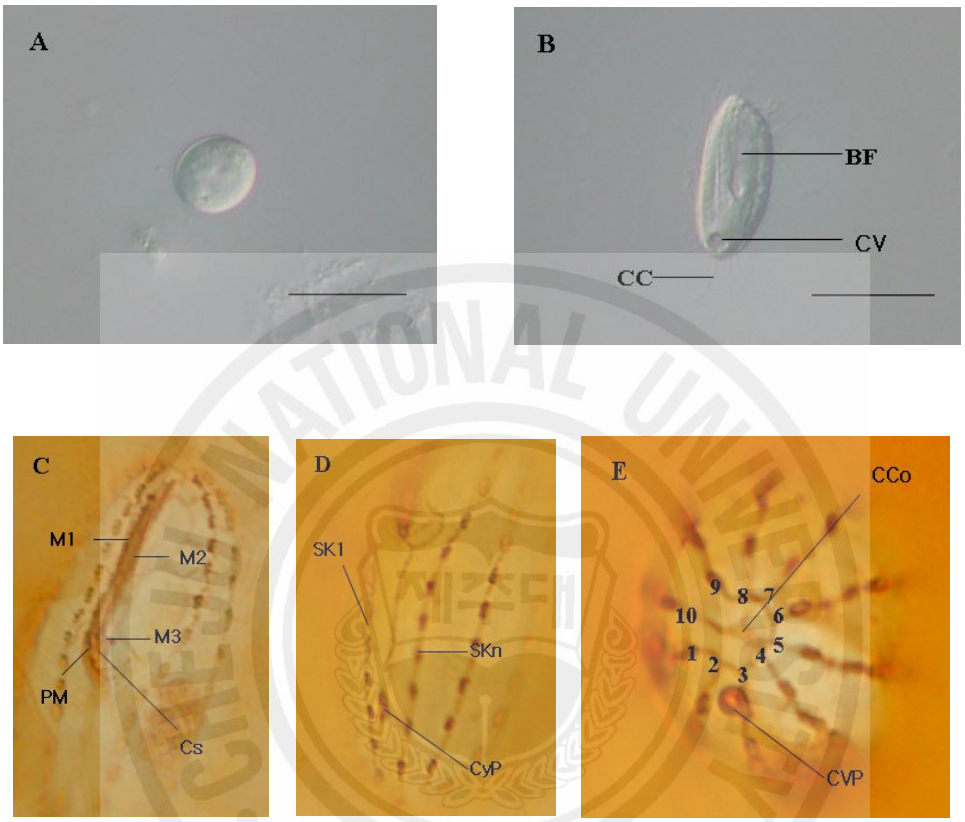

Fig. 5. Silver impregnated specimens and live observations of *Pseudocohnilembus persalinus*. A: Cyst form *in vivo*, B: live ciliate *in vivo*, C: infraciliature of buccal field, D: dorsal view of silverline, E: caudal view of silverline, BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, Cs: cytostome, CV: contractile vacuole, CVP: contractile vacuole pore, CyP: cytopyge, M1, 2: membranelles 1, 2, PM: paroral membrane, SK 1, n: somatic kinety 1, n, Scale bar= 20 ㎛.

Table 10. Morphometric characterization of *Pseudocohnilembus persalinus*. All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples, SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	21.8	32.4	26.6	2.40	32
Body width	11.2	16.1	13.8	1.34	32
Number of somatic kineties	9	10	10	0.18	30
Length of buccal field*	14.2	16.8	15.8	0.70	18
Length of membranelle 1	6.6	8.7	7.6	0.63	28
Length of membranelle 2	10.5	15.1	12.0	0.85	27
Length of membranelle 3	0.4	0.7	0.5	0.12	18
Length of paroral membrane	4.7	7.8	6.0	0.78	30
Number of macronucleus	1	1	1	0	0
Width of macronucleus	5.7	8.4	7.1	0.70	20
Number of micronucleus	1	1	1	0	0

*- Distance from apex to posterior end of paroral membrane

Table 11. Morphological comparison of *Pseudoconilembus persalinus* from present and literatures

Character	*P. persalinus*	Present study	*P. persalinus*
Body length × width (㎛) *in vivo*	-	27-43 × 10-21	-
Body length × width (㎛) fixed	25-40 × 15-25	22-32 × 11-16	20-38 × 10-18
No. of somatic kineties	9-11	10(9-10)	8-9
Length of buccal field: cell length	ca 1/2	ca 1/2	ca 1/2
No. of contractile vacuole pores (CVP)	1	1 (seldom 2)	2 (1-3)
Position of CVP	SK3	SK3 (When two, SK3 & 4)	SK3 (when two, near SK3 &4)
Sample location	China	Jeju, Korea	Great salt lake, USA
Data source	Song (2000)	Oiginal	Evans & Thompson (1964)

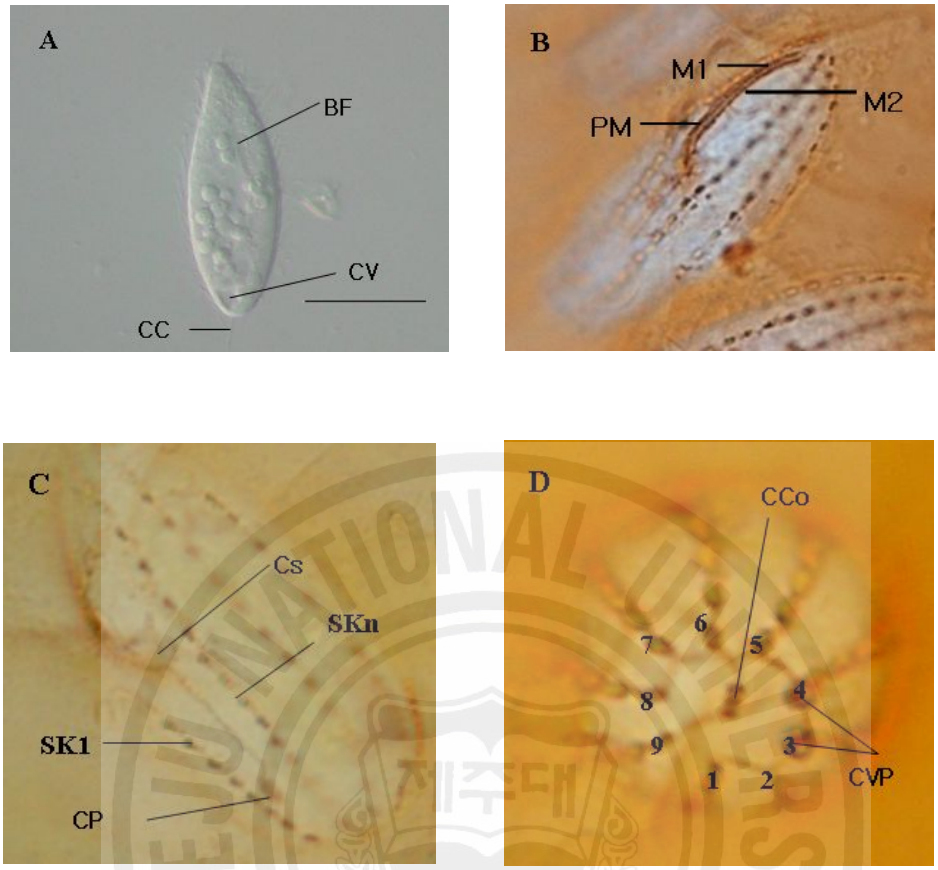

Fig. 6. Silver impregnated specimens and live observation of *Pseudocohnilembus longisetus*. A: *in vivo*, B: infraciliature of buccal field, C: ventral view of silverline system, D: caudal view of silverline system. BF: buccal field, CC: caudal cilium, CCo: caudal cilium complex, CP: cytopyge, Cs: cytostome, CV: contractile vacuole, CVP: contractile vacuole pore, M1, 2: membranelles 1, 2, PM: paroral membrane, SK 1, n: somatic kinety 1, n, Scale bar= 20 ㎛.

Table 12. Morphometric characterization of *Pseudocohnilembus longisetus*. All measurements in ㎛. Max-maximum; Mean-arithmetic mean; Min-minimum; n-number of samples, SD-standard deviation.

Character	Min	Max	Mean	SD	n
Body length	30.6	39.2	34.4	2.07	35
Body width	10.8	14.9	12.7	0.92	35
Number of somatic kineties	9	10	9	0.33	30
Length of buccal field*	15.6	19.9	17.6	1.16	22
Length of membranelle 1	6.8	9.2	8.2	0.58	31
Length of membranelle 2	11.7	16.1	13.9	1.19	31
Length of paroral membrane	6.2	9.5	7.6	0.77	25
Number of macronucleus	1	1	1	0	0
Number of micronucleus	1	1	1	0	0

*- distance from apex to posterior end of paroral membrane

Table 13. Morphological comparison of *Pseudocohnilembus longisetus* from present and literatures

Character	*P. longisetus*	Present study	*P. longisetus*
Body length × width (㎛) *in vivo*	-	ca. 28 × 12	-
Body length × width (㎛) fixed	ca. 27 × 12	ca. 34 × 13	ca. 29 × 14
No. of somatic kineties (SK)	11 (10-11)	9	10(9-11)
Length of buccal field: cell length	ca. 1/2	ca. 1/2	ca. 1/2
No. of contractile vacuole pores (CVP)	1	2	2(1-3)
Position of CVP	between SK2 and 3	SK3 & 4	mostly SK3 &4 (SK3-5)
Cyst formation	yes	yes	no observe
Sample location	Florida, USA	Jeju, Korea	Nelly Island, Antarctica
Data sources	Evans & Thompson (1964)	Orignal	Thompson (1965)

3. Genetic characteristics

SSU rDNAs of 6 different scuticociliates were PCR amplified using SSUF and SSUR primers (Fig. 7). To determine the SSU rDNA sequences, PCR products were cloned and sequenced. The complete SSU rDNA sequences of both *Paranophrys marina* and *Miamiensis avidus* were 1759 nucleotides. Their GC contents were 43% and 44%, respectively. SSU rDNA of *P. marina* showed 96% similarity with the *Parauronema longum* 16S SSU rRNA sequence (AY212807), while *M. avidus* was completely matched with the deposited *M. avidus* (AY550080) in GenBank. *Paralembus digitiformis* and *Parauronema virginianum* were the same length of 1757 nucleotides and their GC contents were 44% and 42%, respectively. They showed 95% and 99% similarity to *M. avidus* (AY550080) and *P. virginianum* (AY392128) SSU rRNAs in GenBank, respectively. SSU rDNA sequence of *Pseudocohnilembus persalinus* and *Pseudocohnilembus longisetus* were 1754 nucleotides and GC contents were 44 and 45%, respectively. *P. persalinus* showed 99% similarity to the *P. persalinus* (AY835669) 18S rRNA sequence, while *P. longisetus* is highly matched with the *P. hargisi* 18S rRNA sequence (AY833087, 98% similarity). Therefore, the 3 species of scuticociliates SSU rDNA were newly identified among the 6 scuticociliates according to genetic analysis.

Table 14 shows similarity and evolutionary distance values for the nucleotides that were calculated pairwise for 6 scuticociliates including two ciliates of Hymenostomatia. The evolutionary distance value for *P. digitiformis* and *M. avidus* is 0.038 indicating that these two species are closely related. The most closely related species to *P. longisetus* was *P. persalinus*. *P. digitiformis and M. avidus* comprise the same clade that was more closely related to *P. marina* than were two species of *Pseudocohnilembus* genus. On the other hand, *P. virginianum* showed a

long distance relationship compared with other scuticociliates. As shown in Fig. 14, 6 scuticociliates comprised a monophyletic group of the subclass Scuticociliatia. Moreover, *P. persalinus* and *P. longisetus* formed a monophyletic clade and branched to *P. marina* with a low bootstrap value (50%). *P. marina* was a sister group to a lineage that includes *P. digitiformis* and *M. avidus*. Also, the 6 species of scuticociliates within the order Philasterida formed a monophyly with bootstrap value and were separate from the two species (*Cyclidium* and *Pleuronema*), which belong to the order Pleuronematida as shown in Fig. 15.

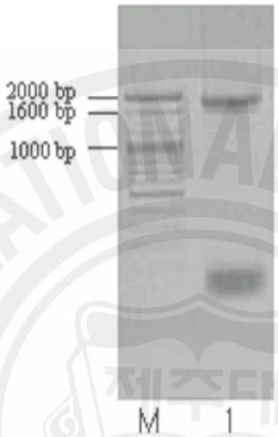

Fig. 7. Electrophoresis of scuticociliate SSU rDNA PCR product on 1% agarose ge. Lane M: 100 bp DNA Ladder (Bioneer), Lane 1: Amplified SSU rDNA

```
AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATGGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCTGTTAAAC CCGACTTTCG TGGAAGGGTT GTATTTATTA     210
GATATCAAGC CAATATTCCT TCGGGTCTAT TGTGGTGAAT CATAGTAACT GATCGAATCC TTTTTTAGGA     280
TAAATCATTC AAGTTTCTGC CCTATCAGCT TTCGATGGTA GTGTATTGGA CTACCATGGC AGTCACGGGT     350
AACGGAGAAT TAGGGTTCGA TTCCGGAGAG GGAGCCTGAG AAACGGCTAC CACATCTAAG GAAGGCAGCA     420
GGCGCGTAAA TTACCCAATC CCGATTCGGG GAGGTAGTGA CAAGAAATAA CAACCTGGGG GACCTAGTCC     490
TTACGGGATT GCAATGAGAA CAATGTAAAA GACTTATCGA GGAACAATTG GAGGGCAAGT CTGGTGCCAG     560
CAGCCGCGGT AATTCCAGCT CCAATAGCGT ATATTAAAGT TGTTGCAGTT AAAAAGCTCG TAGTTGAACT     630
TCTGCTCAGT TACAGTTCTG GCTTCTGTCA AGCTGTTAAT TGTGCATCCG CTTGCAAACT TAGCCCGGCC     700
TTCACTGGTC GACTAAGGGA GTAAGCCCTT TACTTTGAAA AAATTAGAGT GTTTCAAGCA GGCAATGGCT     770
CGAATACATT AGCATGGAAT AATGGAATAG GACTTTTGTC CATTTGGTTG GTTATTGGAC ATGAGTAATG     840
ATTAAAAGGG ACAGTTGGGG GCATTAGTAT TTAATTGTCA GAGGTGAAAT TCTTGGATTT ATTAAAGACT     910
AACTTATGCG AAAGCATTTG CCAAGGATGT TTTCATTAAT CAAGAACGAA AGTTAGGGGA TCAAAGACGA     980
TCAGATACCG TCCTAGTCTT AACTATAAAC TATACCGACT CGGAATCGGA CAGGCTTCTT AAACTTGTTC    1050
GGCGCCGTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCG CAAGGCTGAA ACTTAAAGGA    1120
ATTGACGGAA GGGCACCACC AGGCGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGGA AACTTACCAG    1190
GTCCAAACAT GGGTGGGATT GACAGATTGA GAGCTCTTTC TTGATTCTAT GGGTGGTGGT GCATGGCCGT    1260
TCTTAGTTGG TGGAGTGATT TGTCTGGTTA ATTCCGTTAA CGAACGAGGC CTTAACCTGC TAAATAGTAC    1330
TGCGATGTCC AATCGTAGTT ACTTCTTAGA GGGACTATGC GTATTGAAGC GCATGGAAGT TTGAGGCAAT    1400
AACAGGTCTG TGATGCCCTT AGATGTCCTG GGCCGCACGC GCGCTACAAT GACTTATTCA GAAAGTTTTG    1470
CCTGGTCCGG AAGGATTCAG GGTAATCTTT GTAATACAAG TCGTGTTAGG GATCGATCTT TGCAATTATA    1540
GATCTTGAAC GAGGAATGCC TAGTAAGTGC AGTTCATCAG ACTGTACTGA TTACGTCCCT GCCCTTTGTA    1610
CACACCGCCC GTCGCTCCTA CCGATTTCGA GTGGTCCGGT GAACCTTCTG GATTGGATAA GCAATTGTCC    1680
GAGAAGTTAA GTAAACCTAA TCACTTAGAG GAAGGAGAAG TCGTAACAAG GTTTCCGTAG GTGAACCTGC    1750
AGAAAGATC                                                                       1759
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Fig. 8. Complete sequences of small subunit rDNA gene from *Paranophrys marina*

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AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATGGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCTGTTAAAC CCGACTTCGC GGAAGGGTTG TATTTATTAG     210
ATATCAAGCC AATATTCCTT CGGGTCTATT GTGGTGAATC ATAGTAACTG ATCGAATCCC TATGGGATAA     280
ATCATTCAAG TTTCTGCCCT ATCAGCTTTC GATGGTAGTG TATTGGACTA CCATGGCAGT CACGGGTAAC     350
GGAGAATTAG GGTTCGGTTC CGGAGAGGGA GCCTGAGAAA CGGCTACCAC TTCTAAGGAA GGCAGCAGGC     420
GCGTAAATTA CCCAATCCTG ATTCAGGGAG GTAGTGACAA GAAATAACAA CCTGGGGGAC ATCTGTCCTT     490
ACGGGATTGC AATGAGAACA ATTTAAAAGA CTTATCGAGG AACAATTGGA GGGCAAGTCT GGTGCCAGCA     560
GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAAGTTG TTGCAGTTAA AAAGCTCGTA GTTGAACTTC     630
TGTGTGTGCC CAGTTCTGGC TTCGGTCAAG CTGTGGTGCT CACATCCGCT TGCAAACCTA GTCCGGCCTT     700
CATTGGTCGA CTAGGGGAGT AGGCTTTTTA CTTTGAAAAA ATTAGAGTGT TTCAAGCAGG CAATGGCTCG     770
AATACATTAG CATGGAATAA TGGAATAGGA CTTTTGTCCA TTTGGTTGGT TATTGGACAT GAGTAATGAT     840
TAACAGGGAC AGTTGGGGGC ATTAGTATTT AATTGTCAGA GGTGAAATTC TTGGATTTAT TAAAGACTAA     910
CTTATGCGAA AGCATTTGCC AAGGATGTTT TCATTAATCA AGAACGAAAG TTAGGGGATC AAAGACGATT     980
AGACACCGTC CTAGTCTTAA CTATAAACTA TACCGACTCG GAATCAGCCC GGCTTCTAAA ACTGGGTTGG    1050
CGCCGTATGA GAAATCAAAG TCTTTGGGTT CTGGGGGGAG TATGGTCGCA AGGCTGAAAC TTAAAGGAAT    1120
TGACGGAAGG GCACCACCAG GCGTGGAGCC TGCGGCTTAA TTTGACTCAA CACGGGGAAA CTTACCAGGT    1190
CCAAACATGG GTGGGATTGA CAGATTGAGA GCTCTTTCTT GATTCTATGG GTGGTGGTGC ATGGCCGTTC    1260
TTAGTTGGTG GAGTGATTTG TCTGGTTAAT TCCGTTAACG AACGAGACCT TAACCTGCTA AATAGTATGC    1330
TGATGCACAA TTGGTATTAC TTCTTAGAGG GACTATGCGT ATTGAAGCGC ATGGAGGTTT GAGGCAATAA    1400
CAGGTCAGTG ATGCCCTTAG ATGTCCTGGG CCGCACGCGC GCTACAATGA CTGACTCAGA GAGTACTTCC    1470
TGGTCCGGTA GGATTCAGGG TAATCTTTAT AATATCAGTC GTGTTAGGGA TCGATCTTTG CAATTATAGA    1540
TCTTGAACGA GGAATGCCTA GTAAGTGCAT GTCATCAGCA TGTACTGATT ACGTCCCTGC CCTTTGTACA    1610
CACCGCCCGT CGCTCCTACC GATTTCGAGT GATCCGGTGA ACCTTCTGGA CTGGACACAT TAGTGTTCGG    1680
GAAGTTAAGT AAACCTAATC ACTTAGAGGA AGGAGAAGTC GTAACAAGGT TTCCGTAGGT GAACCTGCAG    1750
AAGGATC                                                                        1757
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Fig. 9. Complete sequences of small subunit rDNA gene from *Paralembus digitiformis*

```
AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATGGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCTGTCAAAC CCGACCTTTG GAAGGGTTGT ATTTATTAGA     210
TATTAAGCCA ATATTCCTTC GGGTCTATTG TGGTGAATCA TAGTAACTGA TCGAATCTCT TCACGAGATA     280
AATCATTCAA GTTTCTGCCC TATCAGCTTT CGATGGTAGT GTATTGGACT ACCATGGCAG TCACGGGTAA     350
CGGAGAATTA GGGTTCGGTT CCGGAGAGGG AGCCTGAGAA ACGGCTACCA CATCTAAGGA AGGCAGCAGG     420
CGCGTAAATT ACCCAATCCT GATTCAGGGA GGTAGTGACA AGAAATAACA ACCTGGGGGC CTCACGGCCT     490
TACGGGATTG TAATGAGAAC AATTTAAACG ACTTAACGAG GAACAATTGG AGGGCAAGTC TGGTGCCAGC     560
AGCCGCGGTA ATTCCAGCTC CAATAGCGTA TATTAAAGTT GTTGCAGTTA AAAAGCTCGT AGTTGAACTT     630
CTGCATGTGC CCAGTTCTGG CTTCGGTCAA GCTGTGGTGT ATGCATCCGC TTGCAAAGCT AGACCGGTCT     700
TCATTGATCG ACTAGTGGAG TAGGCTCTTT ACCTTGAAAA AATTAGAGTG TTTCAGGCAG GCAATGGCTC     770
GAATACATTA GCATGGAATA ATGGAATAGG ACTTTTGTCC ATTTGGTTGG TTATTGGACA TAAGTAATGA     840
TTAAAAGGGA CAGTTGGGGG CATTAGTATT TAATTGTCAG AGGTGAAATT CTTGGATTTA TTAAAGACTA     910
ACTTATGCGA AAGCATTTGC CAAGGATGTT TTCATTAATC AAGAACGAAA GTTAGGGGAT CAAAGACGAT     980
CAGATACCGT CCTAGTCTTA ACTATAAACT ATACCGACTC GGAATCGGAC CGGCTTATAA AACTGGTTCG    1050
GCGCCGTATG AGAAATCAAA GTCTTTGGGT TCTGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA    1120
TTGACGGAAG GGCACCACCA GGCGTGGAGC CTGCGGCTTA ATTTGACTCA ACACGGGGAA ACTTACCAGG    1190
TCCAAACATG GGTGGGATTG ACAGATTGAG AGCTCTTTCT TGATTCTATG GGTGGTGGTG CATGGCCGTT    1260
CTTAGTTGGT GGAGTGATTT GTCTGGTTAA TTCCGTTAAC GAACGAGACC TTAACCTGCT AAATAGTACG    1330
TTGATGCACA ATTGGCGTTA CTTCTTAGAG GGACTATGCG CTTTGAAACG CATGGAAGTT TGAGGCAATA    1400
ACAGGTCTGT GATGCCCTTA GATGTCCTGG GCCGCACGCG CGCTACAATG ACTCGCTCAG AAAGTACTTC    1470
CTGGTCCGGA AGGATTCGGG TAATCTTTTA AATACGAGTC GTGTTAGGGA TCGATCTTTG TAATTATGGA    1540
TCTTGAACGA GGAATGCCTA GTAAGTGCAA GTCATCAGCT TGTACTGATT ACGTCCCTGC CCTTTGTACA    1610
CACCGCCCGT CGCTCCTACC GATTTCGAGT GATCCGGTGA ACCTTCTGGA CTGAGCACGC TTGCGTGAAC    1680
GGGAAGTTAA GTAAACCTAA TCACTTAGAG GAAGGAGAAG TCGTAACAAG GTTTCCGTAG GTGAACCTGC    1750
AGAAGGATC                                                                       1759
```

Fig. 10. Complete sequences of small subunit rDNA gene from *Miamiensis avidus*

```
AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AAATTAACAC ATGCATGTCT AAGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATGGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCTGTCAAGC CCGACTTTTT GGAAGGGTTG TATTTATTAG     210
ATATCAAGCC AATATTCCTT CGGGTCTATT GTGGTGAATC ATAGTAACTG ATCGAATCCC TTCTTGGGAT     280
AAATCATTCA AGTTTCTGCC CTATCAGCTT TCGATGGTAG TGTATTGGAC TACCATGGCT TTAACGGGTA     350
CGGAGAATTA GGGTTCGATT CCGGAGAGGG AGCCTGAGAA ACGGCTACCA CATCTAAGGA AGGCAGCAGT     420
GGTGAAAATT ACCCAATCCT GATTCAGGGA GGTAGTGACA AGAAATAGAA ATCTGGGCCA CTTTCGTGGT     490
TACGGAATTT AAATGAGAAC AATTTAAAAG ACTTATCGAG GAACAATTGG AGGGCAAGTC TGGTGCCAGC     560
AGCCGCGGTA ATTCCAGCTC CAATAGCGTA TACTAAAGTT GTTGCAGTTA AAAAGCTCGT AGTTGAACTT     630
CTGTACAGTC TCAGTTCTGG CTTAGGTCAA GCTGTTGGTT GTACATCCGC TTGCAAAGCT AGTTCGGCCT     700
TAACTGGTCG GCTGGTAGAG TAAGCCTTTT ACTTTGAAAA AATTAGAGTG TTTCAGGCAG GCAATGGCTC     770
GAATACATTA GCATGGAATA ATGGAATAGG ACTTTTGTCC ATTTGGTTGG TTATTGGACA TGAGTAATGA     840
TTAAAAGGGA CAGTTGGGGG CATTGGTATT TAACAGTCAG AGGTGAAATT CTTGGATTTG TTAAAGACTA     910
ACTTATGCGA AAGCATTTGC CAAGGATGTT TTCATTAATC AAGAACGAAA GTTAGGGGAT CAAAGACGAT     980
CAGATACCGT CCTAGTCTTA ACTATAAACT ATACCGACTC AGAATCAGAA TGGCCTTTCA AACTATTCTG    1050
GCGCTGTATG AGAAATCAAA GTCTTTGGGT TCTGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA    1120
TTGACGGAAG GGCACCACCA GGCGTGGAAC CTGCGGCTTA ATTTGACTCA ACACGGGGAA ACTTACGAGC    1190
GCAAAACTAG GGTAGGATTG ACAGATTGAG AGCTCTTTCT TGATTCTTAG GGTGGTGGTG CATGGCCGTT    1260
CTTAGTTGGT GGAGTGATTT GTCTGGTTAA TTCCGTTAAC GAACGAGACC TTAACCTACT AAATAGTATG    1330
TTGATCTTTA ATCGGCATTA CTTCTTAGAG GGACTATGTG TATTGAAACA CATGGAAGTT TGAGGCAATA    1400
ACAGGTCTGT GATGCCCTTA GATGTGGTCG GCCGCACGCG CGTTACAATG ATAAATTCAG AAAGTTTACC    1470
TGGTTCGGAA GAATTCAGGG TAATCTTTGT AATATTTATC GTGTTAGGGA TCGATCTTTG CAATTATAGA    1540
TCTTGAACGA GGAATGCCTA GTAAGTGCGC TTCATCAGAG CGTACTGATT ACGTCCCTGT CCTTTGTACA    1610
CACCGCCCGT CGCTCCTACC GATTTCGAAT TATCCGGTGA ATCATCTGGA CTGAAGCAGC AATGCATCGG    1680
GAAGTTTGAT AAACCTTATA ATTTAGAGGA AGGAGAAGTC GTAACAAGGT TTCCGTAGGT GAACCTGCAG    1750
AAGGATC                                                                         1757
```

Fig. 11. Complete sequences of small subunit rDNA gene from *Parauronema virginianum*

```
AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AGGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATCGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCAGTCAAAC CCGACCTTCG GAAGGGTTGT ATTTATTAGA     210
TATCAAGCCA ATACTCCTTC GGGTTTATTG TGGTGAATCA TAGTAACTGA TCGAATCCCT CAGGGATAAA     280
TCATTCAAGT TTCTGCCCTA TCAGCTTTCG ATGGTAGTGT ATTGGACTAC CATGGCAGTC ACGGGTAACG     350
GGGAATTAGG GTTCGATTCC GGAGAGGGAG CCTGAGAAAC GGCTACCACT TCTAAGGAAG GCAGCAGGCG     420
CGTAAATTAC CCAATCCCAA TTCGGGGAGG TAGTGACAAG AAATAACAAC CTGGGGGGCT CTGCCCTTAC     490
GGGATTGTAA TGGGAACAAT GTAAAAACTT TATCGAGGAA CAATTGGAGG GCAAGTCTGG TGCCAGCAGC     560
CGCGGTAATT CCAGCTCCAA TAGCGTATAT TAAAGTTGTT GCAGTTAAAA AGCTCGTAGT TGAATTTCTG     630
CACGTGCCCG GTTGTGGCTT CGGTCAGGCC GTGGCGCGTG CATCCGCCTG CAAACCTAGA CCGGCCTTCA     700
CTGGTCGACT AGGGGAGTAG GCCTTTTACC TTGAAAAAAT TAGAGTGTTT CTAGCAGGCA ATTGCTTGAA     770
TACATTAGCA TGGAATAATA GAATAGGACT TTTGTCCATT TCGTTGGTTA TTGGACATTA GTAATGATTA     840
AAAGGGACAG TTGGGGGCAT TAGTATTTAA TTGTCAGAGG TGAAATTCTT GGATTTATTA AAGACTAACT     910
TATGCGAAAG CATTTGCCAA GGATGTTTTC ATTAATCAAG AACGAAAGTT AGGGGATCAA AGACGATTAG     980
ACACCGTCCT AGTCTTAACT ATAAACTATA CCGACTCGGA ATCGGACAGG TCATTATAAC TTGTTCGGCG    1050
CCGTATGAGA AATCAAAGTC TTTGGGTTCT GGGGGGAGTA TGGTCGCAAG GCTGAAACTT AAAGGAATTG    1120
ACGGAAGGGC ACCACCAGGC GTGGAGCCTG CGGCTTAATT TGACTCAACA CGGGGAAACT TACCAGGTCC    1190
AAACATGGGT GGGATTGACA GATTGAGAGC TCTTTCTTGA TTCTATGGGT GGTGGTGCAT GGCCGTTCTT    1260
AGTTGGTGGA GTGATTTGTC TGGTTAATTC CGTTAACGAA CGAGACCTTA ACCTGCTAAA TAGTACGCGT    1330
TTGTTCAAAG CGTGTTACTT CTTAGAGGGA CTATGCGTAT TGAAGCGCAT GGAAGTTTGA GGCAATAACA    1400
GGTCTGTGAT GCCCTTAGAT GTCCTGGGCC GCACGCGCGC TACAATGACT CACTCAGAAA GTTTTTCCTG    1470
ATCCGGAAGG ATTCGGGTAA TCTTCATAGC ATGAGTCGTG TTAGGGATCG ATCTTTGCAA TTATAGATCT    1540
TGAACGAGGA ATGCCTAGTA AGTGCAGTTC ATCAGACTGT ACTGATTACG TCCCTGCCCT TTGTACACAC    1610
CGCCCGTCGC TCCTACCGAT TTCGAGTGGT CCGGTGAACC TTCTGGACTG GGCTAGCAAT AGCTCGGGAA    1680
GTTAGGTAAA CCTTATCACT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA CCTGCAGAAG    1750
GATC                                                                            1754
```

Fig. 12. Complete sequences of small subunit rDNA gene from *Pseudocohnilembus persalinus*

```
AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AGGTATAAAT      70
AGTATACAGT GAAACTGCGA ATGGCTCATT AAAACAGTTA TAGTTTATTT GATAATTGAA AGCTACATGG     140
ATAACCGTGG TAATTCTAGA GCTAATACAT GCAGTCAAAC CCGACCTTCG GAAGGGTTGT ATTTATTAGA     210
TATCAAGCCA ATACTCCTTC GGGTTTATTG TGGTGAATCA TAGTAACTGA TCGAAGCTCT CTGAGCTAAA     280
TCATTCAAGT TTCTGCCCTA TCAGCTTTCG ATGGTAGTGT ATTGGACTAC CATGGCAGTC ACGGGTAACG     350
GGGAATTAGG GTTCGATTCC GGAGAGGGAG CCTGAGAAAC GGCTACCACT TCTAAGGAAG GCAGCAGGCG     420
CGTAAATTAC CCAATCCCAA TTCGGGGAGG TAGTGACAAG AAATAACGTC CTGGGGGGCT TATGCTCTTA     490
CGGGTTCGTA ATGGGAACAA TGTAAAAACT TTATCGAGGA ACAATTGGAG GGCAAGTCTG GTGCCAGCAG     560
CCGCGGTAAT TCCAGCTCCA ATAGCGTATA TTAAAGTTGT TGCAGTTAAA AAGCTCGTAG TCGAATTTCT     630
GTACGTGCCT AGTTCTGGCT TCGGTCAAGC TTAGGCGCGT GCATCCGCTT GCAAACCTAG ACCGGCCTTC     700
ACTGGTTGAC TAGGGGAGTA GGCTTTTTAC CTTGAAAAAA TTAGAGTGTT TCTAGCAGGC AATTGCTTGA     770
ATACATTAGC ATGGAATAAT AGAATAGGAC TTCTGCTCCA TTTCGTTGGT TATTGGGCAA AAGTAATGAT     840
TAAAAGGGAC AGTTGGGGGC ATTAGTATTT AATTGTCAGA GGTGAAATTC TTGGATTTAT TAAAGACTAA     910
CTTATGCGAA AGCATTTGCC AAGGATGTTT TCATTGATCA AGAACGAAAG TTAGGGGATC AAAGACGATT     980
AGACACCGTC CTAGTCTTAA CTATAAACTA TACCGACTCG GAATCGGACA GGTCAATACA ACTTGTTCGG    1050
CGCCGTATGA GAAATCAAAG TCTTTGGGTT CTGGGGGGAG TATGGTCGCA AGGCTGAAAC TTAAAGGAAT    1120
TGACGGAAGG GCACCACCAG GCGTGGAGCC TGCGGCTTAA TTTGACTCAA CACGGGGAAA CTTACCAGGT    1190
CCAAACATGG GTGGGATTGA CAGATTGAGA GCTCTTTCTT GATTCTATGG GTGGTGGTGC ATGGCCGTTC    1260
TTAGTTGGTG GAGTGATTTG TCTGGTTAAT TCCGTTAACG AACGAGACCT TAACCTGCTA AATAGTACTG    1330
GCGTGTATAA CGTCTGTTAC TTCTTAGAGG GACTATGCGC ATTGAAACGC ATGGAAGTTT GAGGCAATAA    1400
CAGGTCTGTG ATGCCCTTAG ATGTCCTGGG CCGCACGCGC GCTACAATGA CTCACTCAGA AAGTTTTTCC    1470
TGATCCGGAA GGATTCGGGT AATCTTCATA GCATGAGTCG TGTTAGGGAT CGATCTTTGC AATTATAGAT    1540
CTTGAACGAG GAATGCCTAG TAAGTGCAGT TCATCAGACT GTACTGATTA CGTCCCTGCC CTTTGTACAC    1610
ACCGCCCGTC GCTCCTACCG ATTTCGAGTG GTCAGGTGAA TCTTCTGGAT CGAGCAGCAA TGTTTGAGAA    1680
GTTAGGTAAA CCTTATCACT TAGAGGAAGG AGAAGTCGTA ACAAGGTTTC CGTAGGTGAA CCTGCAGAAA    1750
GATC                                                                            1754
```

Fig. 13. Complete sequences of small subunit rDNA gene from *Pseudocohnilembus longisetus*

Table 14 . SSU rDNA nucleotide similarity (upper half, %) and evolutionary distance (lower half) for scuticociliates and ciliates from other class.

	P. dig	*M. avi*	*P. per*	*P. lon*	*P. mar*	*P. vir*	*I. mul*	*T. the*
P. dig	-	96.2	94.4	93.2	95.4	91.8	84.1	82.4
M. avi	0.038	-	94.2	93.6	95	91.4	84.1	82.6
P. per	0.056	0.058	-	97.2	94.8	90.4	83.5	81.7
P. lon	0.068	0.064	0.028	-	93.9	89.3	82.7	80.9
P. mar	0.046	0.050	0.052	0.061	-	92.2	84.3	82.8
P. vir	0.082	0.086	0.096	0.107	0.078	-	82.4	81.4
I. mul	0.159	0.159	0.165	0.173	0.157	0.176	-	94.3
T. the	0.176	0.174	0.183	0.191	0.172	0.186	0.057	-

Abbrevation: *P. dig* - *Paralembus digitiformis*; *M. avi* - *Miamiensis avidus*; *P. per* - *Pseudocohnilembus persalinus*; *P. lon* - *Pseudocohnilembus longisetus*; *P. mar* - *Paranophrys marina*; *P. vir* - *Parauronema virginianum*; *I. mul* - *Ichthyophthirius multifiliis*; *T. the* - *Tetrahymena thermophila*

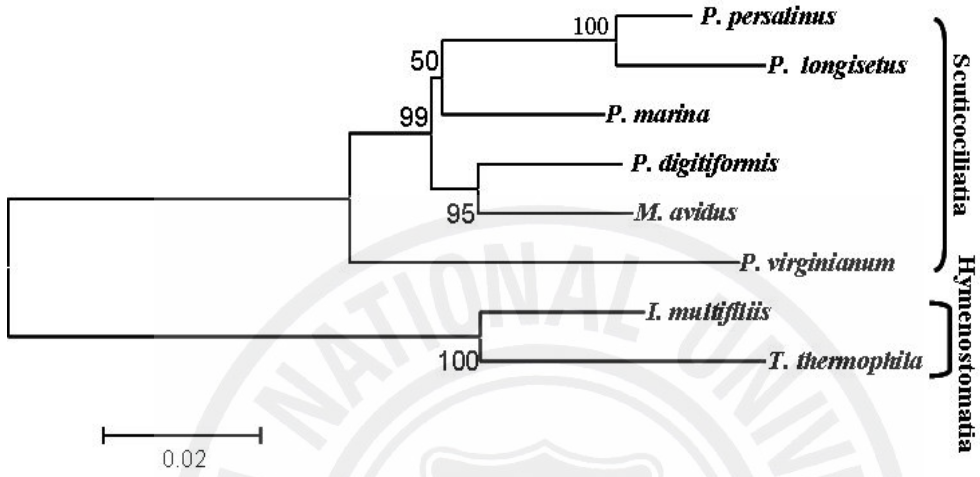


Fig. 14. A phylogenetic tree of scuticociliates SSU rDNA sequences constructed by the Neighbor-joining method with MEGA 3.1. Number at the nodes are boostrap values representing their robustness (10,000 replications). Two species considered in the tree construction were *Ichthyophthirus multifiliiss* (U17354), *Tetrahymena thermophila* (X56165).

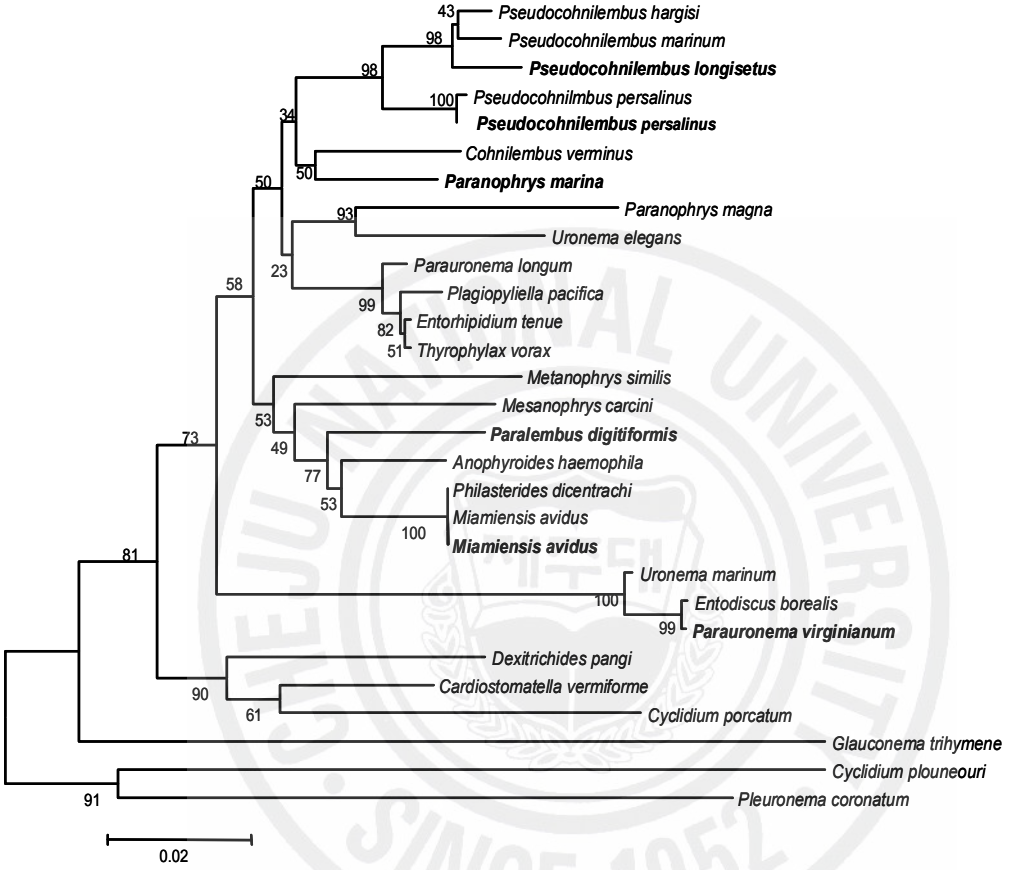


Fig. 15. A phylogenetic tree of scuticociliates SSU rDNA sequences constructed by the Neighbor-joining method with MEGA 3.1. Number at the nodes are boostrap values representing their robustness (10,000 replications). The new sequences are represented in boldface.

4. DISCUSSION

Scuticociliates is classified as order Scuticociliatida including suborders Pleuronematina, Pseudocohnilembina and Philasterina, the class Oligohymenophorea and the phylum Ciliophora by Small (1967). Interestingly, it was observed in the scutico-field during stomatogenesis in all ciliates including these orders. Also, origin of opisthe field, buccal organelles organization and buccal organelles migration reversely processed compared with the Hymenostomatida ciliate's stomatogenic characters.

Scuticociliatosis is a parasitic disease of cultured marine fish including flounder in the land-based culture systems. Inspection of the diseased fish revealed several types of scuticociliates. Presently, we isolated several species of scuticociliates from parasitic infected mariculture fishes. GLS and LS strains were found in the gill and skin tumor in flounder with skin darkening, respectively. On the other hand, the MS type were abundantly presented in systemically infected fishes with severe external symptoms such as skin, caudal fin and mouth necrosis, gill congestion, and head inflammation. SS groups were observed in the gill or other organs by themselves or along with the MS type. Therefore, even if scuticociliates infect the gill and skin, the fatal pathogenicity is regarded as the histophagous pathogen in a few strains.

To date, no avaliable information about outbreak patterns depending on species and the condition of damage for scuticociliatosis can be found. Recently, some researchers have investigated the causative agents of this disease by morphological and molecular biological analysis. To investigate the pathologic relation of the scuticociliate as a primary pathogen in marine fish, mass culture of isolate ciliates *in vitro* were required. Some scuticociliates of the subclass Scuticociliatida can grow in diverse media,

ranging from bacteria to axenic media. For instance, *U. nigricans* use bacteria as a food *in vitro*, while *P. dicentrachi* and *M. avidus* use FBS and lipids in axenic culture medium (Nerad and Daggett, 1992; Crosbie and Munday, 1999; Iglesias et al., 2003).

We could grow the isolated ciliates in the presence of bacteria or in fish cell lines in axenic medium. These culture methods were used to obtain optimal growth conditions for further experiments on morphology and molecular analyses. As a result, *M. avidus* strains could be cultivated in the axenic culture medium with CHSE-214 fish cell lines. Those ciliates used the fish cells as a food and rapidly increased cell density in 5 to 7 days. When completely fed on the cells, their morphological shapes changed and could survive in this medium up to 1 month until subculturing them. By contrast, different scuticociliates (*P. marina*, *P. digitiformis*, *P. virginianum*, *P. persalinus* and *P. longisetus*) of other groups could only grow well in the sterilized seawater (33‰) or Milliport S medium (about 15‰) containing *Vibrio* sp based on the difference of salinity concentration. In this manner, the ability of ciliate culture to proliferate *in vitro* was displayed across different stages of evolution from bacteriophages to histophages according to their food preferences (Fenchel, 1968; Plunket and Hidu, 1978). Plunket and Hidu (1978) described *Uronema marinum* in which isolated form sick oyster culture would grow only on bacteria when provided with several food sources such as bacteria, algal cells and oyster tissue. Therefore, we concluded that *M. avidus* was a histophagous ciliate as a primary parasitic pathogen in mariculture. On the other hand, other ciliates were bacteriophages and could not be major fish pathogens but have a commensalism relationship with histophagous pathogens.

Although, several silver impregnation techniques have been widely used

to evaluate taxonomy of ciliates including scuticociliates, there exists confusion among or within the species for identification. To complement this problem, morphological characteristics along with molecular analysis of several ciliates have been studied. In the present study, we identified a total of 6 scuticociliates that were isolated from several fishes in Jeju by silver impregnations and SSU rDNA analysis.

Thompson and Berger (1965) originally reported about *P. marina* and Song et al. (2002) described it again. According to the Thomson and Berger description, this ciliate had a rounded shape at both the anterior and posterior ends along with elongated buccal cavity. M1 was especially very long and occupied most of the buccal cavity. Ten somatic kineties and PM started at the posterior end of M2. All features of *P. marina* were very similar to our strain except for the body shape described in Table 2. Our strain had a pointed anterior and rounded posterior ends *in vivo*. These observations are completely matched with Song's result. Moreover, when we analyzed SSU rDNA sequence, it did not show any significant similarity to SSU rDNA sequences of scuticociliates (96% similarity to *Parauronema longum*) in GenBank. Finally, we identified this strains as *P. marina* by morphological characteristics and also determined its full-length of SSU rDNA sequence.

Kahl (1931) originally reported 2 species of genus *Paralembus*, and Groliere (1974) described *P. rostrata* again. However, *P. digitiformis* had never been reported until Song and Wilbert (2000) described it. *P. rostrata* was very large with thick oval shape, while *P. digitiformis* had two types of body shape with oval or slender shapes depending on the population by Song and Wilbert description (Table 4). M1 was small and slightly away from the apex, while M2 was long and well developed with 3 to 4 rows. Somatic kineties were observed 25 or 22-23 and PM was extended to the

middle part of M2. When our strain was compared with the China populations, infraciliature of the buccal field and the numbers of somatic kineties corresponded to the china population 2 described by Song and Wilbert (2002). Interestingly, two types of body shapes were observed in our strain during the culture period. A thick oval or plump shape was observed in the strain at primary period in culture. Many globules like food vacuole were also present in the cytoplasm (Fig. 2B). After one week culture, the body shape became the slender form. Variation of cell shape or size in culture could not considered as the taxonomic standard as Song and Wilbert description. On the basis of the morphological characteristics, we analyzed the SSU rDNA of *P. digitiformis.* Consequently we could not identified the strains due to low sequence similarity (95%). Finally, we identified our strain as *P. digitiformis* based on morphological characters.

M. avidus was first reported as a facultative parasite in sea horse by Thompson and Moewus (1964). Since then Song and Wilbert (2002) investigated the living morphology of *M. avidus.* Recently, complete SSU rDNA sequences and pathogenicity of this strain against scuticociliatosis of flounder in Korea were identified by Jung et al. (2005). Our strains were also present as parasites that seriously infected two maricultured fishes. When our strains processed the silver impregnations, their morphological features completely resembled the original descriptions, except for the cell and buccal area sizes(Table 7). At the same time, we performed their SSU rDNA sequence analysis using PCR and BLAST search. Our data perfectly matched with *M. avidus* (AY550080) reported by Jung et al. (2000). Another interesting result was that our data revealed a difference of three nucleotides against *P. dicentrachi* with 99% similarity. *P. dicentrachi* was well known as a histophagous parasite of sea bass, turbot, and flounder in Europe and Korea. Its morphological characteristics,

pathogenicity, infection route, and genetical analysis have been widely investigated by several researchers (Dragesco et al. 1995; Iglesias et al. 2001; Parama et al. 2003; Kim et al. 2005a; Parama et al. 2006). However, some authors suggested that *P. dicentrachi* was a junior synonym of *M. avidus* due to the similarity between morphology and genetic composition (Song and Wilbert, 2000, Parama et al., 2006). According to Song and Wilbert description (2000), they only difference between *M. avidus* and *P. dicentrachi* were body length and the buccal area. Therefore, our strains were identified as *M. avidus* by the first description including the findings Song and Wilbert (2000).

First, Thompson (1967) described *P. virginianum* as two species from the Virginia coast and then Wilbert and Kahan (1981) reported additional strains. Our strains have an elongated shape with a blunt anterior end and rounded posterior end with the buccal cavity positioned in the middle of the body. Our two populations differed in the number of somatic kineties and silverline of caudal cilium complex compared to the Virginia strains (12-13 vs 11-12, SK8 or 9: SK6 or 7), while those matched with the strains isolated from Solar lake (12-13 vs 13, SK8 or 9 vs SK 7 or 8) by Wilbert and Kahan (Table 9). To complete this observation, we subsequently analyzed the SSU rDNA analysis. As a result, our strains showed the 99% sequence similarity to *P. virginianium* (AY392128). Thus, , we identified our strains as a *P. virginianum*.

Evans and Thompson (1964) first descrived *Pseudocohnilembus* genus based on silver nitrate impregnations and Song (2000) recently supplemented the data with living morphological information of *P. persalinus.* We isolated two species of *Pseudocohnilembus* containing two long membranes positioned at the right side of buccal cavity as described originally. One strain had usually 10 somatic kineties and one contractile

vacuole pore at kinety 3. Particularly, the buccal apparatus of this strain had small size of M3, which positioned at the posterior end M1 and close to the middle part of PM. These observations were very similar to *P. persalinus*, which was isolated in China (Table 11). The other strain had a relatively larger size than did the former strain, 9 somatic kineties, and 2 contractile vacuole pores at kineties 3 and 4. When compared to the former and reference strains in *Pseudocohilembus* genus, M3 could not be identified in the buccal cavity. Based on these results, the latter strain was very close to *P. longisetus* as described by Thompson (1965). SSU rDNA sequence determinations supported this classification. The former strain had 99% sequence similarity with *P. persalinus* (AY835669), while the latter strain showed 98% similarity to *P. hargisi* SSU rRNA sequences. Therefore, our two strains were identified as *P. persalinus* and *P. longisetus*, respectively.

According to the SSU rDNA sequences obtained in present study, the monophyly of the subclass Scuticociliatia and the order Philasterida was confirmed with bootstrapping values in phylogenetic analysis (Figs. 14 and 15). The molecular phylogenetic analysis completely confirmed that *M. avidus* and *P. dicentrachi* were considered synonyms along with their morphological comparisons (Song and Wilbert, 2000). Thompson and Berger (1965) assigned *P. marina* to the family of Uronematidae. In the present study, *P. marina* was a sister group to morphospecies of the genus *Pseudocohnilembus* despite the difference of the buccal organelles arrangement. Scuticociliates and other ciliate group have been reported using phylogenetic trees based on molecular data. However, sometimes those data could not match the morphological characteristics. For instance, the genus *Anophryoides* suggested a synonym with the *Paranophrys* by Song and Wilber (2000). However, Shang et al. (2003) described that this

genus is phylogentically closed to the *Mesanophrys*. Additional molecular information of scuticociliates including large subunit rDNA, histone and Hsp80 could yield more data to resolve the phylogenetic relationship of this order.

We isolated a total of 6 scuticociliates from diseased marine fishes using morphological and genetic analyses. As a result, we identified 4 additional species of scuticociliate when compared to other reports diseased fishes. These data support that several species of scuticociliates may be involved in the outbreak of scuticociliatosis. Therefore, these results may be useful in molecular investigations and even for unskilled researcher to identify scuticocliates by traditional methods.

PART II. VACCINE DEVELOPMENT TO PROTECT MARINE FISH FROM SCUTICOCILIATOSIS

ABSTRACT

Miamiensis avidus is primary parasitic pathogen of scuticociliatosis in mariculture fish. This work reports about cDNA encoding a 38 kDa scuticociliate antigen protein and recombinant protein production from *M. avidus*. The 38 kDa scuticociliate antigen (ScuAg) gene fragment was cloned into *E. coli* expression vector and the recombinant protein was produced as a fusion protein. With the purified fusion protein, we investigated the efficacy of recombinant vaccine to protect fish against this scuticociliate. The full length of cDNA was 1096 bp with an open reading frame of 882 bp encoding 294 aa. The predicted molecular mass encoded by the 38 kDa gene was 29679 Da. Based on its deduced amino acid sequence, the secondary structure of the protein was predicted and the protein contained hydrophobic amino acid domains at its N- and C-termini that were characteristic of a signal peptide and GPI-anchor addition sites, respectively. IA cysteine-rich domain was observed from aa 60 to 267 and cysteine, alanine, serine and lysine residues were highly distributed within that domain.

A gene fragment of 38 kDa ScuAg of *M. avidus* was assembled from synthetic oligonucleotides and cloned into pGEX-4T-1 expression vector. The recombinant protein was successfully expressed as GST-fusion (GST-ScuAg) protein. The purified fusion protein strongly reacted with monoclonal antibody of 38 kDa protein. Naive flounders were immunized with purified GST-ScuAg fusion protein and then challenged. The survival rate of the immunized groups was 10 to 15% compared to the control group, which was 60% at 24 days after challenging. The antibody titer of

immunized fish groups also had a higher OD value than did the control group. All these observations suggest that the recombinant GST-ScuAg fusion protein can be a useful vaccine against *M. avidus* infection.

MATERIALS AND METHODS

1. Ciliate isolation and culture

We collected different types of scuticociliates with scuticociliatosis from several mariculture farms. From them, ciliate isolated from flounder brain tissue was used for in this study.

The brain was dissected and washed 3 times with Eagle's minimum essential medium (EMEM) containing a high concentration of antibiotics. A small piece of brain was inoculated with Chinook salmon embryo (CHSE-214) in a tissue culture flask and cultivated at 20℃ for 5 or 7 days. Then, the cultivated ciliates were cloned using serial dilution in a 96 well plate containing CHSE-214. Isolated ciliate was identified as *M. avidus* based on the morphologyand genetic characteristics, which were maintained under axenic condition.

2. Western blotting

Cultured ciliates were collected by centrifugation at 2000 ×g for 5 min. The pellets were resuspended with phosphate-buffered saline (PBS) and lysed in a SONOPLUS ultrasonic homogenizer. The sonicated lysate seperated according to the different sizes of protein by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% separation gel at 70V for 45 min. Separated proteins were electrophoretically transferred to Polyvinylidene Difluoride (PVDF) membrane and blocked with 10% skim milk for 2 h. The membrane was washed 3 times with PBS and incubated 1:1000 of anti-scuticociliate rabbit antibody, followed by 1:1000 of anti-rabbit IgG goat antibody conjugated alkaline phosphatase. Nitrobluetertazorium (NBT) and 5- bromo-4-chloro-3-indoylphosphate (BCIP) were used to detect the alkaline phosphatase.

3. Ciliary Antigen preparation and N-terminal sequencing

Miamiensis avidus were deciliated using a modified method described by Dickerson et al. (1989). Scuticociliates from axenic culture were harvested by centrifugation at 3000 ×g for 10 min. Pellets were washed with EMEM without EBS, suspended with ice-cold deciliation buffer containing 12.5 mM sodium acetate, pH 7.5, 6% ethanol and 3 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was rapidly passed 10 times through a 3 mL syringe with 18 gauge needle. Deciliated cells were observed under a light microscope. The suspension was centrifuged at 1000 ×g for 5 min. The supernatant was transferred in a new e-tube and centrifuged to collect cilia at 16,000 × g for 15 min at 4℃. The resulting cilia were stored at -70℃.

To isolate membrane proteins of cilia Triton X-114 solution was used as described by Bordier (1981). Collected cilia were suspended in 10 mM Tris-HCl, pH 7.5, and then an ice-cold extraction buffer (300 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2% (v/v)Triton X-114) was added. The resulting suspension was centrifuged to remove cytoskeleton components at 16,000 ×g for 10 min at 4℃. The supernatant was then transferred to a new e-tube, incubated at 30℃ for 5 min and applied on a sucrose cushion (6% (w/v) sucrose, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.06% (v/v) Triton X-114). After centrifugation at 1000 ×g for 5 min, two layers were observed in the e-tube namely, a detergent and aqueous phase. The supernatant was discarded and 9 volumes of cold acetone were added into a detergent phase, then resuspended by vortexing and incubated on ice for 30 min. After incubation, the tube was centrifuged at 16,000 ×g for 15 min at 4 ℃ and removed the acetone. Finally, the extracts of membrane protein were resuspended in 10 mM Tris-HCl, pH 7.5 and stored at -70℃.

Isolated membrane protein of cilia was resuspended in 2×SDS sample

buffer and electrophoresed on 12% polyacrylamide gel. Proteins were transferred onto a PVDF membrane in buffer containing 10 mM CAPS, 5 mM dithiothreitol (DTT), 10% (v/v) methanol at 300 mA for 3 h. Membrane was stained Coommassie brilliant blue R-250 for 5 min and destained in buffer containing 50% (v/v) methanol and 10% (v/v) acetic acid. The membrane was air-dried and band was excised to determine the N-terminal sequences of protein. Sequencing was performed according to manufacturer's protocol.

4. mRNA isolation and synthesis of the first strand cDNA

Cells of scuticociliate were harvested with centrifugation at 3000 ×g for 5 min. Messenger RNA from scuticociliate was extracted using Micro FastTrack mRNA isolation system (Invitrogen, USA).

In brief, pellet were resuspended with 1mL lysis buffer and incubate at 45 ℃ for 20 min. DNA was sheared from the lysate by passing it through of a sterile syringe after adding NaCl, and then it was mixed with oligo(dT) cellulose. The oligo(dT) cellulose was washed to remove DNA, protein, cell debris and non-polyadenylated RNA by using binding buffer and low salt wash buffer. Polyadenylated RNA was eluted with 100 uL of elution buffer and precipitated with glycogen, sodium acetate and 100% ethanol. Concentration of purified RNA was measured and stored at -70℃ until use.

Next, 50 ng of mRNA was used to synthesize cDNA using the Cloned AMV first strand cDNA synthesis kit (Invitrogen, USA). Briefly, RNA was incubated with 300 ng of oligo(dT) primer and 2 uL of 10 mM dNTPs for 65 ℃ for 5 min. After incubation, 4 uL of 5× cDNA synthesis buffer, 1 ul 0.1M DTT, 1 uL RNaseOUT and 1 uL Cloned AMV RT (15U/uL) were added to the annealed mixture and incubated at 50℃ for 1 h. Heating at

85℃ for 5 min terminated the reaction and the resulting cDNA was stored at -20℃.

5. Cloning of the 3′ end of 38 kDa ScuAg cDNA

A primer Scu-1F (5′-GCNGCNGAYTTYAARTGYCCNGGNACNGARGCNGCNG-3′) corresponding to N-terminal sequences of 38kDa Scuticociliate antigen protein (ScuAg), analyzed by N-terminal sequencing of protein, was used with oligo (dT_{20}) primer to amplify the 3′ end of 38kDa ScuAg cDNA. The reaction mixture containing 10× Ex Taq buffer, 2.5 mM dNTPs, 100 ng first strand cDNA, 50 pM of primers and 1U of Ex Taq polymerase (Takara, Japan) was performed 30 cycles of amplification at 94℃ for 30 sec, annealing at 45℃ for 30 sec, extension at 72℃ for 1 min with initial denaturation at 95℃ for 1 min and final extension at 72℃ for 5 min.

The resulting PCR product was electrophoresed on 1.5 % agarose gel and DNA was purified using QIAEX Gel extraction kit (Qiagen, Germany). Purified DNA was ligated into the digested pBluescript II SK(-) (Stratagene, USA) and transformed into *E. coli* DH10b. Recombinant clone was extracted by Accuprep^{TM} plasmid extraction kit (Bioneer, Korea) for sequencing. Sequencing reactions was carried out with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI 377 DNA sequencer (Applied Biosystems, USA).

6. Cloning of 5′ end of 38 kDa ScuAg cDNA

First strand cDNA was terminally transferred by poly (G) tail in the 5′ end of cDNA using terminal deoxynucleotidyltransferase to amplify the 5′ end of 38 kDa ScuAg cDNA. First, synthesized first strand cDNA was purified using QIAquick PCR purification kit (Qiagen, Germany) and incubated the mixture at 37℃ for 1 hr containing 5× terminal transferase

buffer, 10 mM GTP and 6.5U TdT (Amersham Pharmacia, UK). The reaction was stop by heating at 80℃ for 5 min.

This cDNA was used as a template for PCR to amplify the 3′ end of cDNA and PCR was performed under the same conditions except for primer extension at 72℃ for 30 sec with Scu1R (5′-GTAGTA GCATCACAAGCATCACCATC-3′) and oligo$(dC)_{18}$. Cloning and sequencing was processed as above.

7. Synthesize of synthetic ScuAg DNA and *in vitro* mutagensis

Oligonucleotides ranging from 80 to 90 nucleotides were synthesized by commercial oligo providers. First, synthesized oligonucleotide were diluted in 50 pmole and then processed kinase reaction to add the phosphate into 5′ end of the oligonucleotides. After that, the oligonucleotides were performed in 5 separate tubes ligation reaction for assembling of a synthetic ScuAg DNA. After that, the assembled oligonucleotide was seperated on 12% polyacrylamide/urea sequencing gels and visualized by shadow of UV-light. Bands of acrylamide containing DNA of expected size were excised from the gel. Excised gels were crushed in an elution column by adding of 10 mM Tris-EDTA and incubated at 37℃ for overnight. The next day, the columns were fractionated by gravity. Fractionated solutions were concentrated by ethanol precipitation and dissolved in distilled water. After assembly of synthetic ScuAg was complete, 2 uL of assembled product was amplified in PCR reactions using Herculase® Hotstart DNA polymerase (Stratagene, USA) and its respective primers, ScuAgF (5′-GAATGATTAAATTAATTGTTATAGTAGC-3′) and ScuAgR (5′-GATCAAATGAAGAAAGCAGC-3′), according to complement sequences of both ends of ScuAg cDNA. Reaction conditions for amplification were 10 cycles at 95℃ for 30 sec, 52℃ for 30 sec, 72℃ for

55 sec in first round and subsequently 15 cycles at 95℃ for 30 sec, 52℃ for 30 sec, 72℃ for 1 min 5 sec and final extension at 72℃ for 2 min. Amplified product was purified by agarose gel purification and cloned into pBS SK(-). Recombinant clone was sequenced by universal primers.

During the synthetic process, mutations were introduced into 4 sites of synthetic ScuAg. To correct sequences of DNA, we designed 4 sets of primer based on the codon usage of *E. coli* and performed *in vitro* mutagenesis.: set 1; Mut1F (5′-GATTCTGTTCTAGTACCAATAATCAATGTGC-3′) and Mut1R (5′-CATTGATTATTGGTACTAGAACAGAATCCAGC-3′), set 2; Mut2F (5′-GTTATCTAACAACCAAACTTGTGCTGC-3′) and Mut2R (5′-GCACAAGTTTGGTTGTTAGATAACAAGC-3′), set 3; Mut3F (5′-GTGTTGCTTTGGCCGCCGCAGGAGCAGCATG-3′) and Mut3R (5′-GCTGCTCCTGCGGCGGCCAAAGCAACACATC-3′), set 4; Mut4F (5′-GATGCTGATTGTACATCCGCAAAATGTG-3′), and Mut4R (5′-CATTTTGCGGATGTACAATCAGCATCTTTAGTG-3′). Synthetic ScuAg was used as a template for PCR reaction with Herculase polymerase and first set primers. Process for amplification were 10 cycles at 95℃ for 30 sec, 50℃ for 1 min, 50℃ for 30 sec, 72℃ for 3 min 50 sec in first step, and 15 cycles at 95℃ for 30 sec, 50℃ for 30 sec, 50℃ for 30 sec and extended at 72℃ for 4 min as a second step. Amplified PCR product was purified by agarose gel purification and incubated with 2U *Dpn*I (New England Biolabs, USA) for 2 h at 37℃. After that, the reaction was terminated by heating at 80℃ for 20 min and transformed into *E. coli* DH10b. Transformed clone was extracted by Accuprep^{TM} plasmid extraction kit and then performed second mutagenesis. This procedure was repeated 4 times. Finally, the resulting construct was cloned into pBSII SK(-) and sequenced again by universal primers.

8. Expression of recombinant protein using *E. coli*

Synthetic ScuAg was reamplified from mutagenesised ScuAg by PCR to remove the signal peptide and hydrophobic domain of C-terminal using SE-F1 (5′-GAGAGAGGATCCGCTGATTTTAAATGTCCTGGTACTGAA-3′) and SE-R1 (5′-GAGAGACTCGAGTTATCATTCACCGTTAGAAGAGGAAGCCT-3′), SE-F2 (5′-GAGAGACATATGGCTGATTTTAAATGTCCTGGTACTGAA-3′) and SE-R2 (5′-GAGAGACTCGAGTTATCATTCACCGTTAGAAGAGGAAGCCT-3′) containing *Bam*HI and *Xho*I, *Nde*I and *Bam*HI, respectively. These fragments were double digested with their respective enzymes and cloned into pGEX-4T-1 and pET-11a at their respective enzyme sites. The resulting constructs called pGST-ScuAg and pHis-ScuAg, respectively were sequenced again by the same primers above.

To induce protein expression, pHis-ScuAg and pGST-ScuAg were transformed into BL21(DE3). Transformed clones were inoculated in 3 mL LB-amplicillin (100 ㎍/mL) broth and cultured at 37℃ for overnight. The next day, overnight culture was diluted 1:10 in fresh LB-ampicillin broth and incubated at 30℃ for 1hr 30 min. After that, 250 μL culture was harvested by centrifugation at 3000 ×g for 5 min and we used uninduced control for SDS-PAGE. The remaining culture was added IPTG to a final concentration of 1 mM. Cells were harvested 3h after the addition of IPTG by centrifugation resuspended in distilled water, and boiled in 2×SDS sample buffer at 95℃ for 5 min. The lysates were electrophoresed on 10% polyacrylamide gel and stained with Coommassie brilliant blue.

9. Protein purification

ScuAg fusion protein was purified by affinity chromatography on glutathione resin as follows. Transformed single colony was inoculated in 50 mL LB-ampicillin (100 ㎍/mL) broth and vigorously incubated for

overnight at 37℃. The next day, overnight culture was reinoculated in a fresh 1L LB-ampicillin broth and the bacteria were cultivated until the OD_{600} reached about 0.8. IPTG was added to a final concentration of 0.5 mM and protein expressed at 30℃ for 3 h. By centrifugation, bacteria were harvested and resuspended with 5 mL/g PBS the bacteria. To protect the protein from lysis, a protease inhibitor tablet added and then the mixture was subsequently sonicated by ultrasonicater. The lysate was centrifuged at 8000 ×g for 30 min at 4℃ and resuspended the supernatant with 2 volumes of PBS buffer. The resulting solution was loaded into charged glutathione sepharose 4B resin column. The column was then washed with 10 volumes of PBS and eluted with an elution buffer containing 10 mM reduced glutathione, 50 mM Tris-HCl, pH 9.5. The optical density (OD) of the eluted protein fractions was measured using a spectrophotometer and the fractions over the 0.1 OD were collected. These fractions were dialyzed with PBS for overnight at 4℃. Finally, dialyzed protein was eletrophoresed to observe the fusion protein on 10% separation gel and we also conducted an immunoblot assay.

10. Immunization of fish and challenge test

Young flounder, *Paralichthys olivaceus* were used for a challenge test which had a mean weight and body size of 10 g and 10 to 12 cm, respectively. All fish were neither infected by scuticociliates nor any other ectoparasite. The four groups of 40 fishes were maintained in a 20 L aquarium at 20℃ for 10 days before the experiment. Each fish was immunized by intraperitoneal injection of 0 ㎍ (PBS only), 10 ㎍ and 50 ㎍ protein in 50 uL PBS and boosted with the same dose of protein after 2 weeks. The control group was maintained without vaccine treatment for the experimental period. Four days after the boost injection, blood was

collected from caudal fin of 20 fishes from each group to prepare serum using a 1 mL syringe and then centrifuged at 2000 rpm for 5 min at 4℃. The resulting sera were transferred into a new e-tube and stored at -70℃ until enzyme linked immunosorbent assay (ELISA).

For the challenge test, scuticociliates were prepared for centrifugation at 1500 rpm for 5 min and then the cell were counted using a hematocytometer. The seawater of all experimental tanks was drained only 5 L remained in aquarium and the fishes were immersed with 3.96×10^4 ciliates/mL for one day. The next two days seawater was supplied (5 L/day) to the tanks. At the 3 days postchallenge, the fish were supplied commercial diet and 5 L seawater was changed daily. During the experimental period, dead and moribund fish were removed from the tanks and observed cumulative mortality.

11. ELISA

Miamiensis avidus cultivated in CHSE-214 was sonicated by ultrasonicator and used as an antigen for ELISA. The antigen was diluted in 1:1000 coating buffer and added with 100 uL antigen to each well in a 96 well plate. The plates was then incubated for overnight at 4℃. The next day, the plates were rinsed 3 times with PBS-tween (PBS-T). To block non-specific binding, the plate was incubated in 10% skim milk at room temperature for 1 h. After that, plate was washed again with PBS-T and incubated in flounder serum by serial two times dilution at room temperature for 2 h, followed by anti-flounder IgM mouse monoclonal antibody and 1:3000 anti-mouse polyvalent immunoglobulins peroxidase conjugate for 1 h, individually. The plate was then incubated in developing solution for 5 min and the reaction was terminated by diluted sulfuric acid. Finally, the antibody titers were determined using absorbance at 492 nm.

RESULTS

1. Identification of antigen protein from *M. adivus*

Lysate of *M. avidus* was injected into a rabbit to identify the antigen protein. Four weeks after injection, sera from the immunized rabbit were collected and immunoblotting using the total ciliate protein was performed. As a result, four major bands of protein were detected with molecular size of 72, 52, 48, and 38 kDa. Among these bands, the 38 kDa band showed the strongest intensity. Also, this pattern was observed in immunoblotting using monoclonal antibody for *M. avidus,* which was used to identify the cilia protein of scuticociliate by indirect immunofluorescent staining (data not shown). Therefore, we selected the antigenic protein of 38 kDa for vaccine development against scuticociliatosis.

2. Analysis of N-terminal amino acid sequence and cDNA sequence of 38 kDa ScuAg

To design the primers to characterize the 38 kDa scuticociliate antigen (ScuAg) cDNA, we isolated the membrane proteins from scuticociliates by Dickerson's method. Isolated proteins were separated by SDS-PAGE and blotted into PVDF membrane. The band of the dominant 38 kDa protein was excised from the membrane and subjected to the N-terminal sequencing by Edman degradation (Fig. 16). Consequently, an amino acid sequences of 20 residues was identified as AADFKCPGTEAAVNCDSAIQ. The degenerated Scu1F primer was designed based on N-terminal amino acid sequences of 38kDa protein and approximately 1000 bp of 3′ partial fragment was amplified. The flanking region of the 5′ end of cDNA was obtained by 5′ RACE with Scu1R primer based on the sequences of 3′ flanking region. A partial 5′ and 3′ end of cDNA was cloned and the

complete sequence was determined

The nucleotide and putative amino acid sequence of 38kD ScuAg cDNA are shown in Fig. 17. The cDNA sequence is 1096 bp including an open reading frame of 882 bp encoding a putative 294 amino acids (aa) with 69 bp of 5′ untranslated region (UTR) and 142 bp of 3′ UTR. Two TAA triplets and 1 TAG triplet encode the glutamine within the coding region, along with TGA stop codon at position of 952-954 nt. The presence of TAA and TAG encoding glutamine in cDNA indicates that scuticociliate also utilizes a non-standard genetic code in which TAA and TAG specifically encode glutamine or glutamic acid rather than stop codons in hymenostomatid ciliates (Tondravi et al., 1990; Clark et al., 1992 and 1999).

Deduced amino acid sequence of the 38 kDa ScuAg was analyzed by universal network algorithms to find out specific functional characteristics of the protein. The signal peptide was predicted from methionine to alanine in N-terminal region and a cleavage site of the signal peptide was between alanine and alanine (17 and 18 aa, respectively). This result supported the determined N-terminal amino acid sequence of 38 kDa protein. The cysteine rich region was positioned after the signal peptide and periodically distributed specific cysteine segments were observed from 67 to 267 aa region. Two small amino acids and 17 hydrophobic amino acids present at C-terminal predicted a potential cleavage and signal site for glycosylphosphatidylinositol(GPI)-anchor protein by Big-GPI program. Moreover, three potential N-glycosylation sites and five N-myristoylation sites were identified by PHD program. O-glycosylation site also was observed at the residue of 60 aa. Based on this observation, predicted molecular weight and isoelectric point of 38 kDa ScuAg was 29679 Da and 7.53, respectively.

Secondary structure prediction using the PHD program showed that the

protein has a membrane helix structure at both N- and C-terminal ends and randomly coiled loop inside. The predominant amino acid residues were alanine (18.4%), cysteine (12.2%), threonine (11.9%) and lysine (9.5%).

3. Construction of synthetic scutica antigen gene and its *E. coli* expression

To express 38 kDa ScuAg gene in *E. coli* we constructed a synthetic ScuAg gene to exchange the genetic code for glutamine and other preferred proteins based on a *pichia* expression system. Twenty oligonucleotides were synthesized that spanned the coding region of the 38 kDa ScuAg gene and the ligation of the oligonucleotides were performed in 5 separate tubes. The product was combined in two reactions and separated on a denaturing polyacrylamide gel. As a result, the assembled fragment showed two bands, which on a gel had a small difference in size and then they were purified (data not shown). The bigger size band was used as a template and full length of ScuAg gene was amplified using high-fidelity polymerase and then it was cloned. Cloned synthetic ScuAg have 4 point mutations at positions 307, 500, 658 and 694 nt. To correct these sequences, we designed 4 sets of mutagenic primer and processed *in vitro* site-directed mutagenesis. Finally, 885 bp of synthetic ScuAg gene were completed (Fig. 18).

To clone into the *E. coli* expression vector, the truncated gene of 780 bp was amplified by PCR except for the 5′ and 3′ hydrophobic signal sequences. The amplified fragment was cloned into two different bacterial expression vectors and the recombinant proteins were induced. In the case of pHIs-ScuAg, the protein of expected size was not detected in SDS-PAGE of bacterial lysate after IPTG induction. On the other hand, cloned ScuAg in a pGEX 4T-1 vector produced soluble fusion protein

around 52 kDa, which was the predicted molecular weight of GST (26 kDa) and synthetic ScuAg (26 kDa) (Fig. 19A). Fusion protein was purified by glutathione sepharose 4B resin and then overdialyzed. The yield and purity of GST-ScuAg fusion protein purified from 1L bacterial culture was 15 mg and over 90%, respectively. Antigenicity of the purified fusion protein was tested with monoclonal antibody against 38 kDa ScuAg. The monoclonal antibody recognized the fusion protein (Fig. 20).

4. Vaccination and challenge test

To test the effectives of recombinant GST-ScuAg as a vaccine, young flounder were immunized with 10 and 50 μg of fusion protein without adjuvant and then challenged with 3.96×10^4 ciliates/mL of *M. avidus*. Mortality was observed 8 days postchallenge and continued until 24 days postchallenge (Fig. 21). In the control group injected with PBS, cumulative mortality was 60% at 24 days postchallenge. To contrast, cumulative mortality in the vaccinated group receiving 10 ug and 50 ug was 10% and 15%, respectively. These results demonstrated the protective effect of recombinant vaccine against *M. avidus* infection.

5. Antibody against recombinant 38 kDa ScuAg protein

Sera of 20 fish in each group were prepared to test antibody reactivity against recombinant protein at 4 days after the second immunization. Figure 22 shows the result of antibody reactivity showed the OD value against sera of fish immunized with recombinant vaccine and PBS. Antibody reactivity of fish sera for 10 and 50 ug vaccinated groups showed higher OD values than did the group immunized with PBS. Of the immunized 20 fish with 10 ug and 50 ug, 35% (7/20) showed a high antibody titer, but 25% (5/20) of the control group showed also had a high titer.

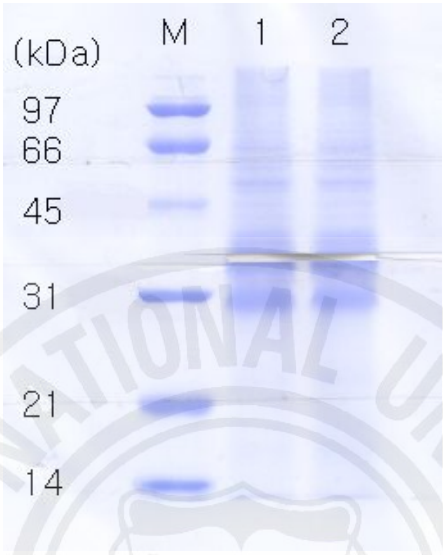


Fig. 16. PVDF membrane blotting of membrane proteins extracted from *M. avidus*. Lane M: Molecular Weight Standards, Low Range (BIO-RAD), Lane 1 and 2: Purified membrane proteins

Fig. 17. Nucleotide and deduced amino acid sequence of the 38 kD ScuAg cDNA. Signal peptide and N-terminal analysis results indicated by arrow head and underlined, respectively. N- and O- glocosylation sites showed by box and italic letter, respectively.

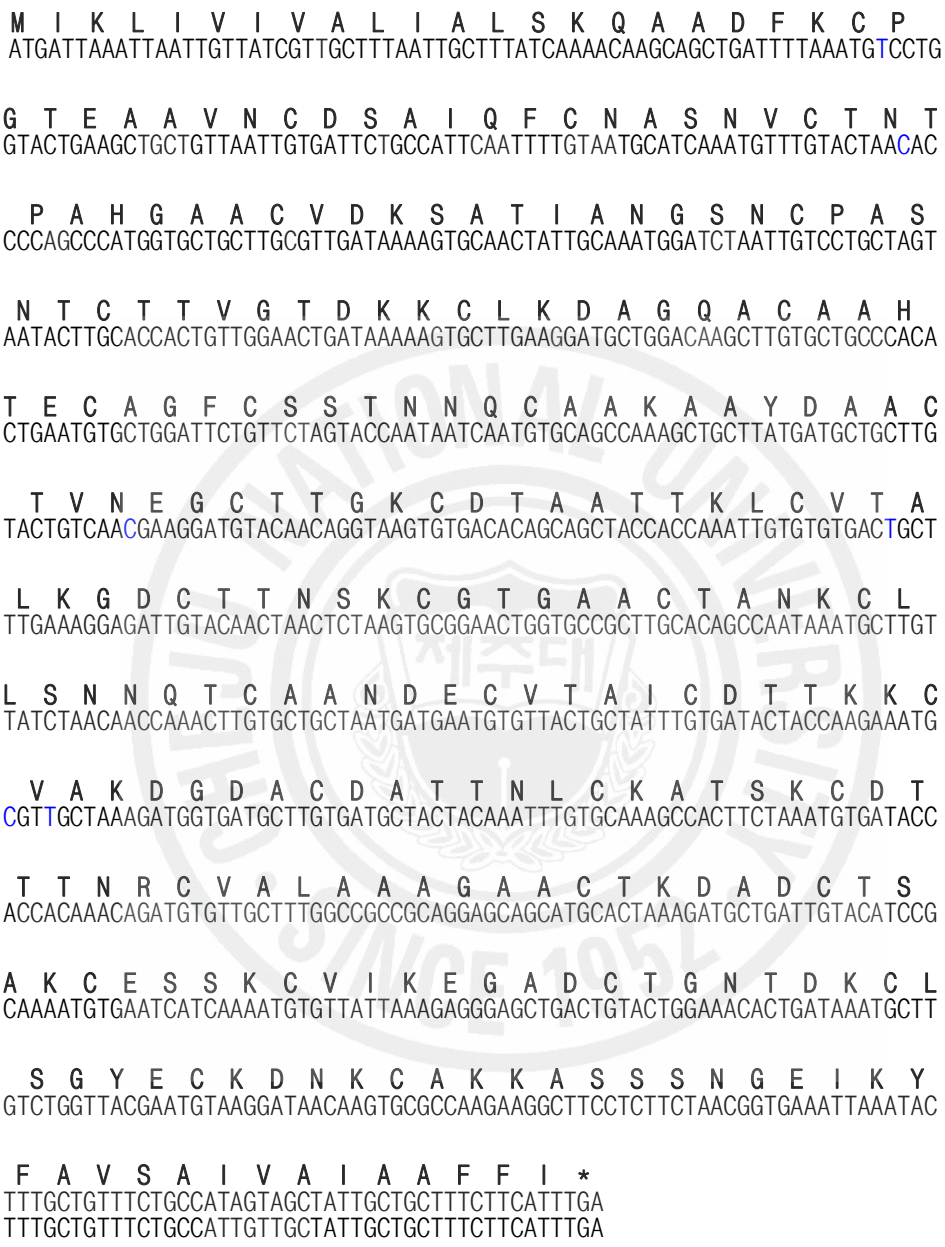


Fig. 18. Complete nucleotide sequences of synthetic 38 kDa ScuAg gene.

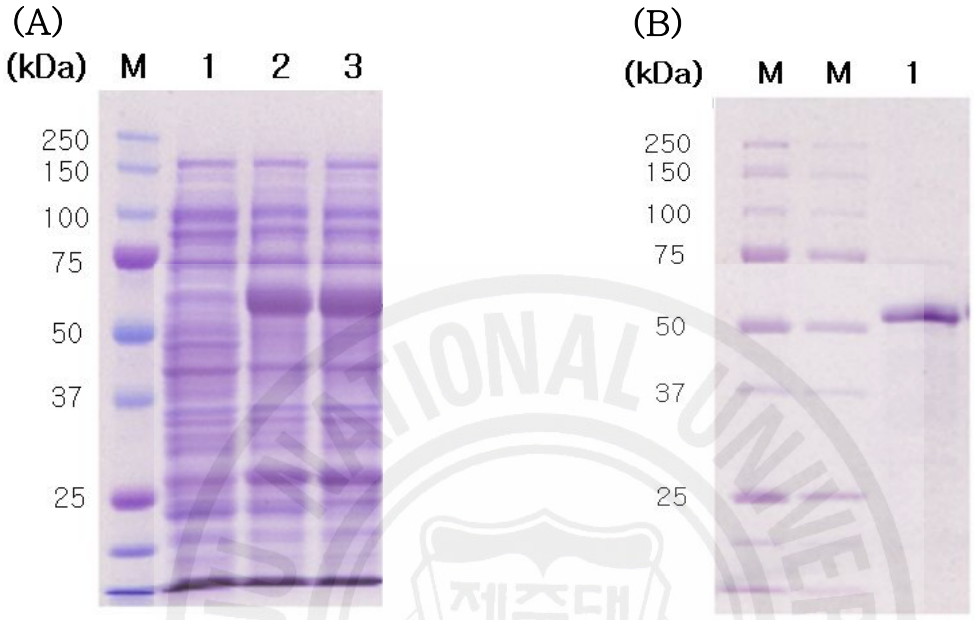


Fig. 19. SDS-PAGE analysis of synthetic ScuAg gene expressed in BL21(DE3) (A) and purified recombinant ScuAg protein using glutahione sepharose 4B (B). A: Bacterial expression of synthetic ScuAg in pGEX-4T-1. Lane M: dual color size marker (Bio-rad), Lane 1: cell lysates without IPTG induction , Lane 2 and 3: cell lysates with IPTG indction. B: Purified recombinant ScuAg protein using glutathione sepharose 4B resion. Lane M: Precision plus protein standard (Bio-rad), Lane 1: purified Scu-Ag fusion protein.

Fig. 20. Western blot analysis of purified GST-ScuAg protein using glutahione sepharose 4B resin. Lane M: Broad range of prestained molecular standard (Bio-rad), Lane 1 and 2: Purified GST- ScuAg fusion protein was load 10 ul and 15 ul, respectively.

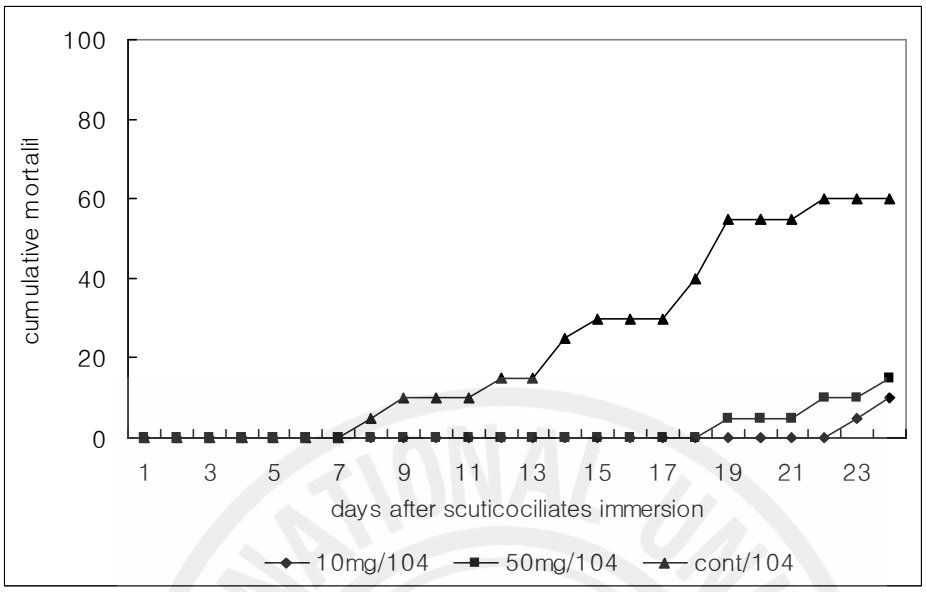
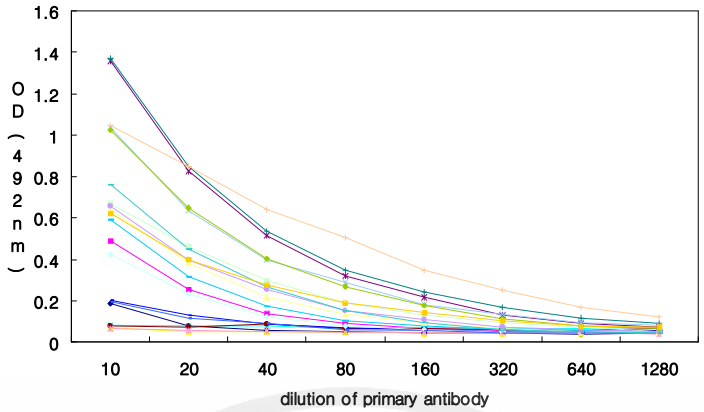
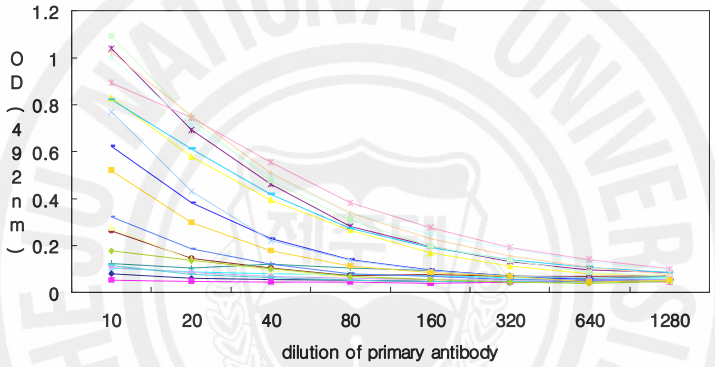


Fig. 21. Cumulative mortality of vaccinated juvenile flounder *Paralichthys olivaceus* challenge with *M. avidus*. Each group infected with 3.96×10^{4} ciliates/mL of *M. avidus* at 4 days post-second injection.

(A)



(B)



(C)

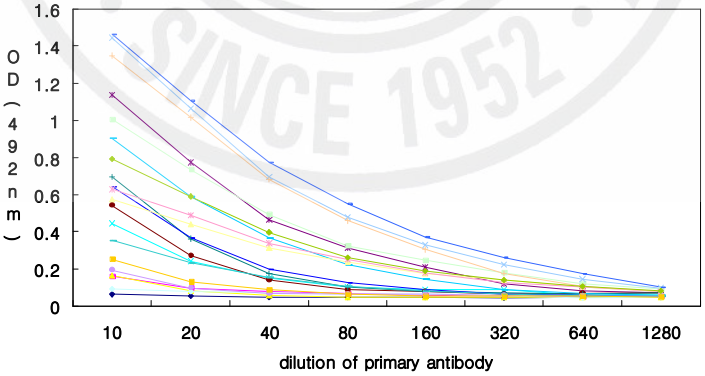


Fig. 22. Antibody detection against 38 kDa ScuAg in immunized fish serum (A) fish sera immunized with PBS, (B) fish sera immunized with 10 ug recombinant vaccine, (C) fish sera immunized with 50 ug recombinant vaccine

DISCUSSION

Scuticociliatosis is a parasitic disease that frequently outbreaks in mariculture fish and induces high infectivity and mass mortality in juvenile or young flatfish in Asia and Europe. This type of disease is caused by scuticociliates such as *M. avidus* and *U. marinum* and they act as an ectoparasitic protozoan. They enter into the body of fish, feed on cells or tissue components, rapidly divide by binary fission, and finally cause death of the host. Chemotherapeutic methods to control this pathogen by using several compounds either natural or synthesized were employed. (Iglesias et al., 2002; Parama et al. 2004 and 2005; Leiro et al. 2004). However, chemotherapeutic control has several drawbacks. One is that it only acts on ectoparasites and causes increasing environmental contamination. To resolve these problems, many researchers focus on immunophylaxis to stimulate fish immunity by a self immune system. For instance, parasitic disease of freshwater fish known as 'white spot disease' was tested by immunization with live or killed tomites of *I.multifiliis* and showed an effective protection against this pathogenic parasite (Sin et al. 1996). Similarly, purified 55 kDa i-antigen of *I. multifiliis* was used a vaccination experiment and reported as an immobilization antigen, which showed high survival rate for the ciliate challenge (Wang and Dickerson, 2002). However, these methods highlights the high costs to produce an antigen protein and establishment of the pure *in vitro* culture system. For this reason, the production of recombinant antigen using recombinant technology could be a good solution given the economic aspects and practical application.

To date, identity of the precise antigen of scuticociliates is unknown. We have previously studied immune responses and antigenicity against *M.*

avidus and, consequently identified a 38 kDa membrane antigen protein composed of ciliary membrane proteins of scuticociliate. Antisera against this ciliate protein induced immobilization, agglutination and lysis of ciliates *in vitro* and it strongly recognized the membrane protein of 38 kDa by indirect immunofluorescent and Western blot assay (data unpublished). These observations suggest that *M. avidus* has a membrane antigen that like other ciliates express immobilization antigen (i-antigen) on their surface. The scuticociliate pathogen *P. dicentrachi* of turbot also demonstrated the existence of i-antigen (Iglesias et al., 2003).

At the first step of vaccine development, we cloned cDNA encoding a 38 kDa antigen protein from *M. avidus* and characterized it. cDNA described here was 1.1 kb encoding around 30 kDa and its N-terminal sequences matched with N-terminal amino acid sequence analysis using the mature 38 kDa protein. However, a discrepancy between the predicted size of cDNA encoding antigen protein (29679 Da) and SDS-PAGE analysis of mature protein (38kDa) was found. This may be due to the electrophoretic mobility of mature protein due to secondary structure modification and structural anomalies such as glycosylation and periodic repetitive sequence segment, respectively (Ozaki et al., 1983; Clark et al. 1992). The other interesting functional features of the deduced amino acids were a hydrophobic region at C-terminal and repetitive cysteine segments in a cysteine rich region. It has been identified in several i-antigen genes of free-living ciliates (Capdeville et al., 1987; Clark et al. 1999; Lin et al. 2002). Furthermore, it has been reported that GPI anchor proteins link to plasma membrane by signaling of hydrophobic C-terminal. A GPI anchor addition occurs 3 amino acids upstream of a hydrophobic C-terminal that provides a GPI anchor addition and cleavage site (Udenfriend and Kodukula, 1995). Analysis of deduced amino acid sequence of the 38 kDa

antigen predicted the two amino acids at positions 271-272 (alanine and serine, residues) and hydrophobic region at positions 277-294 aa, respectively. Therefore, 38kDa antigen protein of *M. avidus* could be link to the plasma membrane by GPI anchor addition. Investigation of the actual cleavage and GPI anchor addition site require further study.

A cysteine motif in the 38 kDa antigen gene of *M. avidus* presented a high order repetitive, $C-X_{4,5}-C$ motif within the cysteine rich region without tandem repeat amino acid domain. It has been found that tandem repeat amino acid domains exist within the i-antigen gene, which showed high similarity between these domains. Several I-antigens of *I. multifiliiss* have the $C-X_{1,2}-C$ or $C-X_{2,3}-C$ repeats with repeated domains (Clark et al., 1999; Lin et al., 2002). The variant specific proteins of *Giardia lamblia* also has the $C-X_{2,3}-C$ motif that showed the zinc binding activity *in vitro* (Zhang et al., 1993). The association of cysteine residues with zinc finger proteins has been reported to show protein-protein and protein-DNA interactions through structural conformation with zinc or metal ions (Berg and Shi, 1996). A similar arrangement of cysteine residues and relatively rich alanine, serine, and lysine in the primary amino acid sequence indicate that the 38 kDa antigen protein also could bind zinc or metal ions *in vitro* and *in vivo*, and it is very important for the structure and function of this protein.

Due to the presence of TAA and TAG codons encoding glutamine in cDNA of the 38 kDa antigen, the antigen cDNA cannot be used to express recombinant protein in conventional systems. We constructed the synthetic antigen gene using long oligonucleotides covering the coding sequence and high fidelity DNA polymerase. It allowed us to substitute the preferred codon usage of *P. pastrois* for the sequences of 38 kDa antigen gene sequences, finally leading to expression of recombinant protein in *E. coli*.

Most of i-antigens contain the tandem repetitive amino acid domains sharing high homology that create a problem when designing synthetic genes (Tondravi et al., 1990; Clark et al., 1999). Even if antigen gene of *M. avidus* showed the cysteine rich region, no tandemly repetitive domains were observed. Therefore, we easily assembled the oligonucleotides in separate reaction tubes by ligation process and subsequently product was purified.

Synthetic ScuAg gene including *Pichia* standard codon usage was successfully expressed in prokaryotic system. When it was cloned in two different expression vectors, pHis-ScuAg did not give any identifiable induced bands as expressed with six-histidine residues in N-terminal. It may be due to the unstable properties of the antigen expressed in *E. coli.* On the other hand, pGST-ScuAg showed a highly expressed protein as a fusion protein and it was easily purified after binding to glutathione-sepharose resin. The purified fusion protein strongly reacted to a monoclonal antibody of 38 kDa, proving its availability as a vaccine in fish.

To test the efficacy of the GST-ScuAg as a vaccine, naive flounder were immunized with two different doses of the antigen (10 and 50 ug). Both vaccinated groups were protected against the ciliate challenge, whereas the control group (PBS injection) had a high mortality rate (Fig. 20). Particularly, the fish group vaccinated with 10 ug GST-ScuAg showed a lower mortality as compared the receiving 50 ug vaccinated group. To prove that the low mortality of the immunized fish was linked to the high amount of antibody produced, we analyzed antibody titers of fishes using ELISA before the challenge test. As a result, both immunized groups with vaccine produced high levles of antibody. Similarly, fish immunized with 10 ug of 55 kDa i-antigen purified from *I. multifiliis* showed a high survival

rate after the challenge test. The antibody titer was also maintained at a high level at 9 months after postchallenge (Wang and Dickerson, 2002). It strongly suggests that synthetic protein of the 38 kDa antigen plays a role as a protective immunogen against *M. avidus*, which triggers the humoral immune response of fish.

In conclusion, the results of the present study demonstrate that the histophagous scuticociliate *M. avidus* expresses the 38 kDa immobilization antigen on its surface and could be used as candidate gene for vaccine development against scuticociliatosis. Finally, we induced a protective effect against this parasite. However, several researchers reported that the outbreaks of scuticociliatosis are caused by several species of scuticociliates such as *P. dicentrachi*, *U. marinum*, *P. persalinus*, and *U. nigricans*. Therefore, further studies will be performed to identify whether the common antigen (38 kDa antigen) or another antigen is expressed in other scuticociliates.

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감사의 글

이 논문을 완성하기까지 부족함이 많았던 저에게 항상 용기와 학문에 정진할 수 있도록 조언과 격려를 아끼지 않으셨던 이제희 교수님께 깊은 감사를 드립니다. 바쁘신 중에도 저의 논문을 심사해주신 정성주 교수님, 송춘복 교수님, 이영돈 교수님, 허문수 교수님께 감사를 드립니다. 또한 학위 과정동안 많음 가르침을 주신 전유진 교수님과 여인규 교수님, 짧은 시간동안 많은 조언을 아끼시지 않으셨던 박근태 선생님께도 깊은 감사를 드립니다.

학위 과정동안 많은 실험 준비 및 도움을 준 분자 유전학 실험실의 오철홍, Mahanma, Chamilani, Anoja, Wang ning, Wan qiang, 이영득에게 고마움을 전합니다. 그리고, 이 논문의 결과가 나오기까지 여러 실험에 도움과 조언을 주신 해양연구소의 송영보 박사님, 임봉수 박사님과 이치훈 선배님, 실험 과정동안 많은 도움을 준 허성표, 강형철 후배를 비롯한 발생학 실험실원들과 멀리 호주에서 열심히 일하고 있는 Prashani 에게도 감사의 마음을 전합니다.

제가 대학원에 들어와 졸업하기까지 함께 길을 걸우주신 진창남 선생님께 깊은 감사와 함께 해양생물공학과 선후배들에게도 고마움을 표합니다. 마지막으로 이 길을 걸어올 수 있도록 믿어주시고 기다려주신 부모님과 남동생에게 감사의 말을 전하고 싶습니다.