THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Detection and characterization of the causative pathogen of Scuticociliatosis in olive flounder (*Paralichthys olivaceus*) in Jeju Island

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Detection and charaterization of the causative pathogen of Scuticociliatosis in olive flounder (*Paralichthys olivaceus*) in Jeju Island

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Abstract

The disease known as "Scuticociliatosis" is one of the major problems in farmed olive flounder (*Paralichthys olivaceus*) and is considered as a parasitic disease with significant economic implications in aquaculture due to its high mortality rate and inadequate treatment options. In particular, when the juvenile olive flounder is infected with Scuticociliates during their early stages, the parasites, including *Miamiensis avidus* and *Uronema marinum*, penetrate various organs, such as the gills, skin, eyes, brain, and internal organs, causing substantial challenges in fish farming. Therefore, the prevention of infections and multifaceted research and approaches are crucial to minimize losses.

In this study, we conducted a six-month disease monitoring from July to December 2022, targeting 21 aquaculture farms in Jeju, South Korea. The focus was on two species of Scuticociliates, namely *M. avidus* and *U. marinum*, which are commonly associated with infections in domestic farmed olive flounder. We measured the weight and length of all olive flounder used

in the analysis and observed external and internal symptoms suspected of Scuticociliatosis to analyze disease occurrence patterns. For the diagnosis of infections caused by these two species, we conducted polymerase chain reaction (PCR) on DNA extracted from the muscle tissues of the fish.

Out of the total 3,352 farmed olive flounders used in the experiment, *M. avidus* was detected in 234 individuals, while *U. marinum* showed negative results in all 3,352 fish. The 234 *M. avidus*-positive individuals were confirmed to have 100% sequence identity with 10 reference *M. avidus* 18S ribosomal RNA gene sequences. Additionally, we attempted classification using the cytochrome *c* oxidase 1 (*cox* 1) gene and found a correlation between *cox* 1 gene types and serotypes through related research.

This study investigated the trends in Scuticociliatosis outbreaks in the region of Jeju, which is geographically isolated and accounts for half of South Korea's farmed olive flounder production. The analysis of disease characteristics presented in this research can serve as fundamental data for the prevention of Scuticociliate infections in the future.

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1. Introduction

In 2022, the production of olive flounder *(Paralichthys olivaceus)* in the Jeju region reached approximately 10,800 tons, accounting for approximately 49.8% of the total domestic olive flounder production (KOSIS, 2022). Jeju Island have an optimal conditions for olive flounder aquaculture, with the highest number of land-based tank aquaculture facilities for olive flounder production in South Korea, total 244 facilities (KOSIS, 2021). However, as the number of olive flounder aquaculture facilities in Jeju continues to increase, along with long-term farming practices, various diseases affecting cultured fish and issues like mass mortality of olive flounder have emerged as significant challenges in the region. According to Shim (2019), a survey conducted in 2015 on olive flounder farms in Jeollanam-do and Jeju revealed a mortality rate of 59.94% due to scuticociliatosis, with particularly high mortality rates observed in Jeju (66.83%) compared to Jeollanam-do (39.19%).

Scuticociliatosis is one of the prominent parasitic diseases affecting cultured olive flounder (Jung *et al.*, 2006). When infecting juvenile olive flounder, this disease is characterized by symptoms affecting not only the gills and body surface but also various internal organs, including brain (Jee *et al.*, 2001; Jin *et al.*, 2003; Jin *et al.*, 2006). Fish suffering from scuticociliatosis usually have macroscopic skin and fin lesions, such as ulcers and dark patches, exophthalmia and distension of the abdominal cavity (Piazzon *et al.*, 2014). In South Korea, four species have been identified as causative agents of scuticociliatosis in olive flounder: *Miamiensis avidus* (Jung *et al.*, 2005), *Uronema marinum* (Jee *et al.*, 2001), *Pseudocohnilembus persalinus* (Kim *et al.*, 2004b), and *Philasterides dicentrarchi* (Kim *et al.*, 2004a). Among these, Jung *et al.* (2007) reported that *P. dicentrarchi* and *M. avidus* are considered to be the same species (Jung *et al.*, 2005; Jung *et al.*, 2006). *M. avidus* is a

strong pathogen that can cause primary infection. The ciliates rapidly invade and proliferate in the skin and gills, as evidenced by the large numbers found at these locations in infected fish. They then consume both host cells and body fluids, and spread to the internal organs in the absence of any additional pathogens such as secondary bacterial invaders (Jung *et al.*, 2007). Also, the pathology of scuticociliate-infected flounder is very similar to that reported by Cheung *et al.* (1980) in marine fish infected *U. marinum* and that reported by Dykova & Figueras (1994) in turbot infected with a histophagous ciliate. The present ciliate is highly invasive and destructive to host tissue, ingesting host cells or tissue debris. It is noted that the present ciliate may be a histophagous species, acting on a serious pathogen in cultured flounder (Jee *et al.*, 2001). In previous studies, the observed sizes of scuticociliates were documented as follows: for *M. avidus*, the length was 31.35 ± 3.87 μm and the width was 18.5 ± 3.04 μm (Jung *et al.*, 2007), while for *U. marinum*, the length measured 34.4 μm and the width was 16.4 μm (Jee *et al.*, 2001). According to Chueng *et al.* (1980), *U. marinum* infecting marine angelfish in aquariums was reported to occur within a salinity range of 21 to 33 ppt and at temperatures ranging from 8 to 28°C. *M. avidus* proliferation takes place within a pH range of 6 to 9, and it can survive and proliferate within a temperature range of 10 to 30°C, with an optimal range between 10 to 25°C, as reported by Bae *et al.* (2009) and Jin *et al.* (2007a). In this study, we aimed to identify the causative species of scuticociliatosis through disease monitoring. As a result, the causative species of scuticociliatosis was confirmed to be *M. avidus*. Therefore, further characterization analyses were intended to be conducted on the obtained *M. avidus*-positive specimens.

In this study, we investigated antigenic differences between isolates of *M. avidus* in Jeju region as an initial step in the development of an effective vaccine against scuticociliatosis. To develop an effective vaccine, it is important to clarify any antigenetic differences between isolates since

antigenetic variation in fish pathogens has been widely reported (Song *et al.*, 2009). Immunobilization assays and immunoblot analysis using serotype-specific antisera have been used for differentiation of the serotypes (Song *et al.*, 2009). In addition, we used three serotype-specific PCR primer sets based on determined ORFs for fast detection and identification of *M. avidus* serotypes.

On the other hand, the SSU rDNA sequence analysis approach is a universally applicable tool; however, it has limitations in its ability to distinguish closely related species (Jung *et al.*, 2011). To overcome the diagnostic limitations, a rapid, precise assay needs to be developed for identifying the pathogenetic scuticociliate species (Whang *et al.*, 2013). The cytochrome *c* oxidase subunit 1 gene has recently been proposed as a marker for animal species identification (Herbert *et al.*, 2003) due to the faster evolutionary rate of the mitochondrial genome compared to the nuclear genome, potentially enhancing its utility in distinguishing closely related species (Jung *et al.*, 2011). Based on the study by Jung *et al.* (2011), which demonstrated a causal relationship between serotype and genotype. This study aimed to determine the matching of *cox* 1 types and serotypes using the results obtained. Thus, this study sought to delineate the disease characteristics and trends of scuticociliatosis occurring in Jeju Island based on the serotype and *cox* 1 type results of *M. avidus*.

To effectively establish mitigation strategies for diseases occurring in cultured organisms, accurate monitoring of disease occurrence on a per-farm and monthly basis is crucial. In this study, we aimed to assess the prevalence of the causative agent of scuticociliatosis in olive flounder from July to December 2022. We employed the polymerase chain reaction (PCR) method to investigate the monthly incidence rates of scuticociliatosis and conducted genetic analysis of the scuticociliatosis causative agent to determine its phylogenetic classification. As part of further experiments to elucidate

specific patterns between serotypes and *cox* 1 types, a detailed characterization of the *M. avidus* strain obtained in this study was conducted.

2. Materials and methods

2.1. Experimental fish

The olive flounders used in this experiment were transported from 21 aquaculture facilities located within Jeju Island to the laboratory to investigate the presence of scuticociliatosis infection. This experiment was conducted from July to December 2022, spanning a period of six months, and a total of 3,352 individuals were analyzed during the investigation period. All olive flounders used in the analysis we measured their weight and length, and external and internal symptoms were observed and recorded for further analysis. Additionally, during the investigation period, microscopic examination was performed to confirm the presence of scuticociliatosis infection in the samples used for the experiment. Microscopic examination involved collecting tissue samples from the gill and affected areas of the body surface of the olive flounders and observed at magnifications ranging from 100 to 400 times using an optical microscope. In cases where external symptoms due to scuticociliatosis were distinctly visible in specific areas, those areas were additionally sampled for analysis.

2.2. DNA extraction

The muscle tissue was isolated from the olive flounder used in the experiment, and DNA was isolated by using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Each tissues were quantified to 50 mg and proceeded to the experiment. Quantities of 50 mg of each tissue were mixed with 180 μL of ATL buffer and 20 μL of proteinase K, and the reaction was carried out at 56 ℃ until the tissue dissolved. After the reaction, 200 μL of AL buffer was added and incubated for 10 minutes. Following this, 200 μL of 100% ethanol was added, and the mixture was transferred to a spin column and centrifuged at 6,000 × g for 1 minute. The column was transferred to a new tube and 500 μL of AW1 buffer was added, followed by centrifugation at 6,000 × g for 1 minute. Subsequently, 500 μL of AW2 buffer was added, and centrifugation was carried out at 20,000 × g for 3 minutes to complete the washing process. The column was then transferred to a microtube, and 50 μL of AE buffer was used to finally elute the DNA. The isolated DNA was stored at -80 ℃ for use in the experiment.

2.3. Polymerase chain reaction (PCR)

2.3.1. Conventional PCR

PCR was conducted for the diagnosis of infections caused by two causative agents of scuticociliatosis using DNA extracted from olive flounder muscle tissue. PCR was carried out using specific primer sets for the detection of *M. avidus* (323 bp) and *U. marinum* (316 bp), as detailed in Table 1. In microtubes, 1 μM of each primer, 2.5 mM of each dNTP, 10 × IP-Taq buffer, 2.5 U IP-Taq DNA polymerase (LaboPass^{TM} IP-Taq 500 units 2.5 U/μL, COSMO GENETECH, Korea), and the DNA isolated from the tissue were added, and distilled water was added to made a final volume of 20 μL for the PCR mixture. The PCR conditions included an initial pre-denaturation step at 94 ℃ for 5 minutes, followed by 30 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 53 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds. A final extension step was carried out at 72 ℃ for 5 minutes. The amplified products obtained after PCR were subjected to electrophoresis on a 1% agarose gel using 1 × TAE buffer as the running buffer and SYBR® Safe gel stain 10,000X in DMSO (Invitrogen, USA). The PCR products were visualized using a UV detector.

Table 1. Primer sequences used in the PCR for the Scuticociliatida in *Paralichthys olivaceus*

Target	Purpose	Primer	Oligonucleotide Sequences	Product size (bp)	Reference
Miamiensis avidus	Detection	Tma-F	TACTTGAATAGAGCTAGATGGGACT	323	K.R. patent No. 10-1000530 (2010)
		Tma-R	GGGAGAAGTGGATTTAAAGTCTACT		
Uronema marinum	Detection	Tum-F	GCTTTGGAGGGTGAAAAAGTTTAG	316	
		Tum-R	GAATTTAGTGAGAGAAAGTTGCCTTT		
Miamiensis avidus	Sequencing	Ma-F	ACTGATCGAATCTCTTCACG	1,216	This study
		Ma-R	GGAAGTACTTTCTGAGCGAG		

2.3.2. Serotype multipex PCR

Serotype PCR was conducted on all DNA samples that exhibited *M. avidus* positive in the previous conventional PCR. In order to determine the serotype of *M. avidus*-positive DNA samples, a multiplex PCR assay was employed. This multiplex PCR included the use of serotype I primer set (443 bp), serotype II primer set (604 bp), and serotype III primer set (350 bp) simultaneously. In microtubes, 1 μM of each primer, 2.5 mM of each dNTP, 10 × IP-Taq buffer, 2.5 U IP-Taq DNA polymerase (LaboPass^{TM} IP-Taq 500 units 2.5 U/μL, COSMO GENETECH, Korea), and the DNA isolated from the tissue were added, followed by the addition of distilled water to made a final volume of 20 μL for the PCR mixture. The PCR conditions are detailed in Table 3. After PCR is done, the amplified products were subjected to electrophoresis on a 1% agarose gel using the same method as described earlier. The PCR products were visualized using a UV detector.

2.3.3. Cytochrome *c* oxidase subunit 1 gene type PCR

To determine the cytochrome *c* oxidase subunit 1 (*cox* 1) gene type of *M. avidus*, PCR was carried out using the *cox* 1 type I primer set (379 bp) and *cox* 1 type II primer set (379 bp) with the same composition as in the conventional PCR method (Table 2). The PCR conditions are provided in Table 3. Following PCR, the amplified products were analyzed in a manner consistent with the previous experimental procedure to interpret the results.

Table 2. Primer sequences used in the PCR for the *M. avidus* (Serotype, cytochrome *c* oxidase subunit 1 gene)

Type	Target	Primer	Oligonucleotide Sequences	Product size (bp)	Reference
serotype	serotype Ⅰ	Forward	CAGCAGCTACTGTTGCTAATCC	443	Motokawa *et al.*, 2018
		Reverse	AGCAGTACATGCGGTAGCAC		
	serotype Ⅱ	Forward	AAATGCCCTGGTACTGAAGC	604	
		Reverse	CTGCAGCAGCTAAAGCTACAC		
	serotype Ⅲ	Forward	CGCCTTATTAGCTCTCTTCTTAGC	350	
		Reverse	AGCAGTACAAGCATCGGAAG		
cytochrome *c* oxidase subunit 1 gene	*cox* 1 Type Ⅰ	Type Ⅰ &Ⅱ - orf 143 F	GAATGTTATTAAGGTATAAGGATA	379	K.R. patent No. 10-2254461 (2021)
		Type Ⅰ - orf 143 R	TCAAACGTATAAATATGTATACTTC		
	cox 1 Type Ⅱ	Type Ⅰ &Ⅱ - orf 143 F	GAATGTTATTAAGGTATAAGGATA		
		Type Ⅱ - orf 143 R	AACGTATAAATACAGATGCTTT		

Table 3. PCR conditions for the detection of Scuticociliatida in *Paralichthys olivaceus*

Target (purpose)	Pre-denaturation	PCR condition (temp./Time)	Cycle no.	Elongnation
Miamiensis avidus (Detection) *Uronema marinum* (Detection)	94 ℃/ 5 min	94 ℃(30″) - 53 ℃(30″) - 72 ℃(30″)	30	72 ℃/ 7 min
Miamiensis avidus (Sequencing)	94 ℃/ 5 min	94 ℃(30″) - 56 ℃(30″) - 72 ℃(40″)	35	72 ℃/ 5 min
Miamiensis avidus Serotype (Ⅰ,Ⅱ,Ⅲ)	94 ℃/ 4 min	94 ℃(30″) - 60 ℃(30″) - 72 ℃(1′)	35	72 ℃/ 7 min
Miamiensis avidus *cox* 1 Type (Ⅰ,Ⅱ)	94 ℃/ 4 min	94 ℃(30″) - 54.2 ℃(30″) - 72 ℃(30″)	35	72 ℃/ 7 min

2.4. Phylogenetic analysis

In order to investigate variations in the nucleotide sequence of *M. avidus* through DNA sequencing, custom-designed primers were used in the experiment. To determine the nucleotide sequence of *M. avidus*, PCR was performed using the Ma-F / Ma-R primer set (1,216 bp) with the same composition as in the previous experiment (Table 1). The PCR conditions involved an initial pre-denaturation step at 94 ℃ for 5 minutes, followed by 30 seconds of denaturation at 94 ℃, 30 seconds of annealing at 60 ℃, and 30 seconds of extension at 72 ℃ for a single cycle. This cycle was repeated 35 times, followed by a final post-extension step at 72 ℃ for 5 minutes. The PCR amplification products were subjected to gel electrophoresis and purified using the Gel purification kit (Bioneer, Korea) before being sent for DNA sequencing (Solgent, Korea). For nucleotide sequence phylogenetic analysis, alignment was performed using the MEGA 11 program version 11.0.13 (Tamura *et al.* 2021). Four *M. avidus* nucleotide sequences (JU.1, JU.2, JU.3, JU.4) isolated from four different aquaculture facilities were aligned along with ten 18S ribosomal RNA gene nucleotide sequences of *M. avidus* registered in GenBank. Additionally, 17 nucleotide sequences belonging to Philasterida within the Scuticociliatia group were included. In the case of the seventeen nucleotide sequences from Philasterida within Scuticociliatia, the SSU rRNA sequences for phylogenetic analysis were selected following the guidelines provided by Kang *et al.* (2021). The genetic distances between nucleotide sequences were determined by performing a maximum-likelihood phylogenetic tree analysis using the Mega 11 program version 11.0.13 (Tamura *et al.* 2021) to understand the evolutionary relationships among the sequences.

3. Results

3.1. Confirmation of external symptoms caused by Scuticocilliatosis infection

All olive flounders used in the experiment were thoroughly examined for both external and internal symptoms. Distinctive external symptoms included body erosion, body pigmentation changes, body ulcers, fin loss, and erosion, while internal symptoms included liver hemorrhaging, kidney hypertrophy, and spleen hypertrophy. Fish infected with scuticociliatosis exhibited characteristic external symptoms, distinguishing them from other bacterial and viral diseases (Fig. 1). Fig. 1a to 1f depict fin loss and erosion, fin ulceration, gill hemorrhage, body posterior erosion and ulceration, eye hemorrhage and ulceration, and tail fin erosion, respectively.

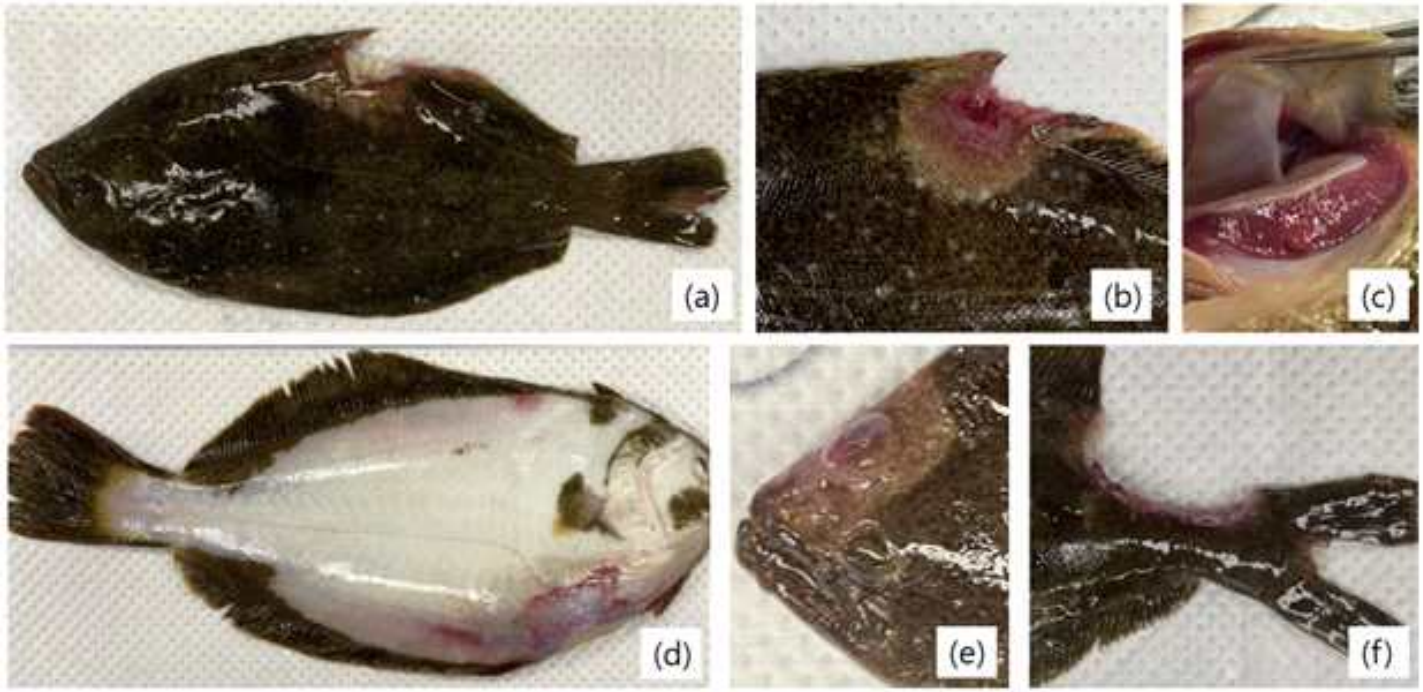


Fig. 1. Olive flounder infected with *M. avidus* (a-f). (a) fin erosion; (b) skin ulceration; (c) lesions in the gills; (d) haemorrhages on large area of the skin; (e) severe haemorrhages and ulcers on eyes; (f) severe corrosion of fin and tail.

3.2. Microscopic analysis

The size of scuticociliate observed in the gills and body lesions of the fish specimens used in the experiment had an average length of 31.33 μm (± 3.34 μm) and a width of 16.67 μm (± 2.25 μm). For the sake of experimental accuracy, only individuals displaying a clear external morphology of scuticociliates were selected for observation. The observed scuticociliates are depicted in Fig. 2, illustrating the appearance of scuticociliates observed in the gills (Fig. 2 A~D) and in the muscle tissue (Fig. 2 E~F).

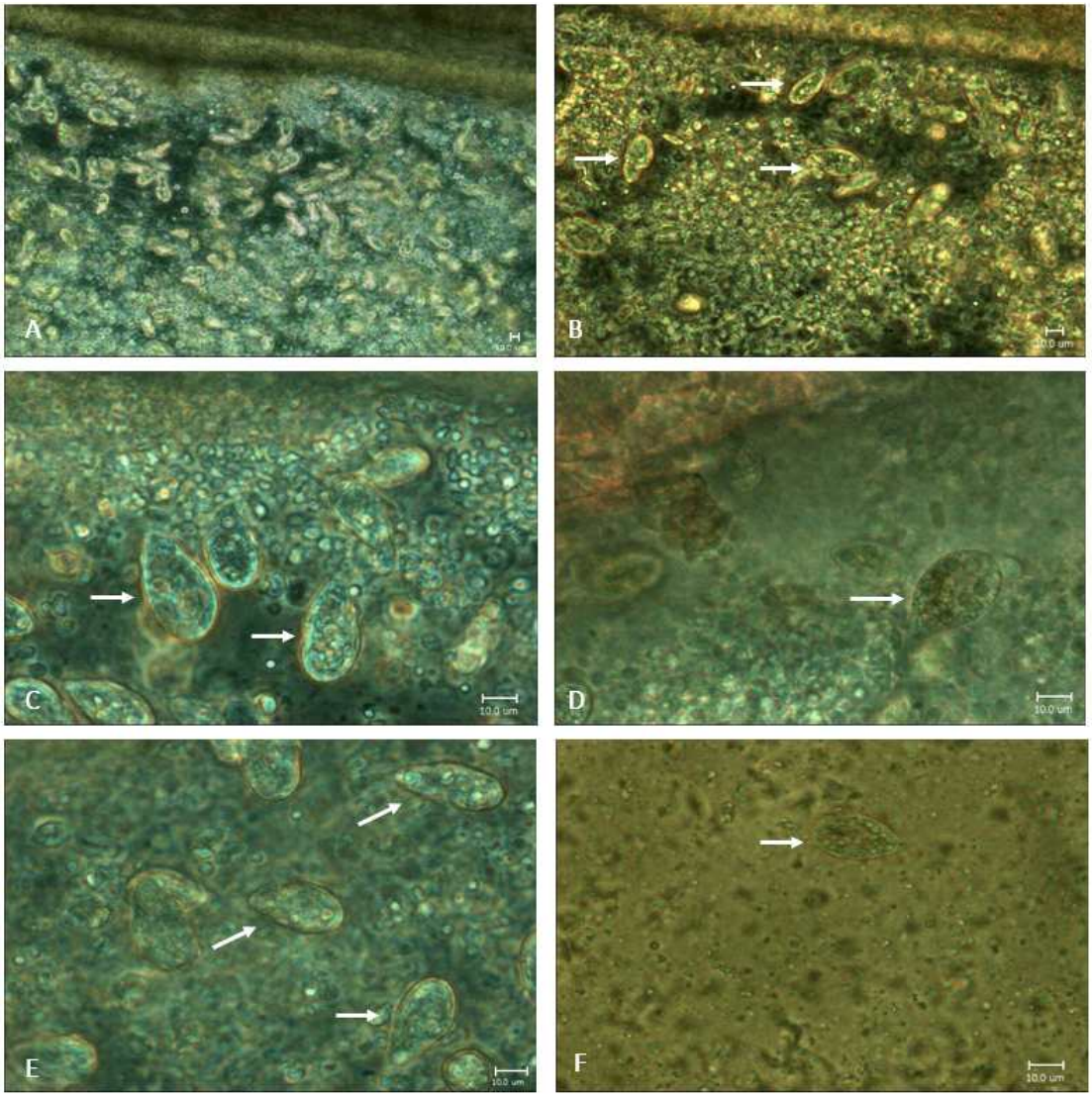


Fig. 2. Light micrograph of the gills (A-D) and muscle (E-F) in the infected olive flounder. Scuticociliates are shown as arrows. (A) x100; (B) x200; (C-F) x400. Scale bar = 10 μm.

3.3. Conventional PCR results

The monthly diagnosis of scuticociliatosis and the detection rate of the causative agent, *M. avidus*, in olive flounder used during the research period from July to December 2022 are presented in Table 4. Out of a total of 3,352 farmed olive flounder specimens used in the experiment, *M. avidus*, one of the causative agents of scuticociliatosis, was detected in 234 of them. In contrast, *U. marinum*, which was not indicated in Table 4, showed negative results in all 3,352 specimens used in the experiment. Among the months of the infection survey period, the detection rate of *M. avidus* in August was the highest at 15.1% (92 out of 609 specimens). When comparing the four aquaculture facilities from July to December, Farm A had the highest positivity rate at 16.3%, with 196 out of 1,204 specimens during the investigation periods. It was observed that the detection rate of *M. avidus* increased from 9.9% to 15.1% in July and August, which corresponded to a period of rising water temperatures. On the other hand, from September to November, during a period of decreasing water temperatures, the detection rate of *M. avidus* decreased. However, in December, the detection rate was 11.1%, showed a different trend.

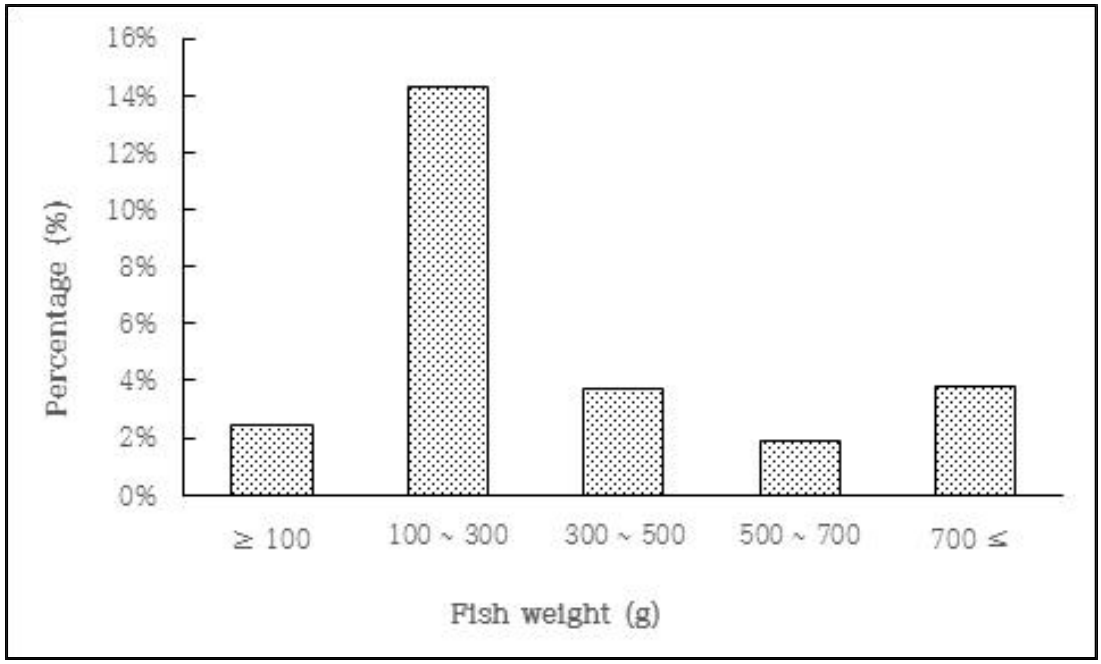
Table 4. Total detection rate of *M. avidus* infections in cultured olive flounder in Jeju from July to December in 2022

Month	No. of olive flounder infected by *M. avidus* / Total No. of olive flounder examined (Percentage of detection rates)					
	Aquafarm					
	Farm A	Farm B	Farm C	Farm D	Other Farms	Total
Jul	69/288 (24.0)	2/176 (1.1)	0/158 (0.0)	0/98 (0.0)	-	71/720 (9.9)
Aug	74/212 (35.0)	14/153 (9.2)	1/152 (0.7)	3/64 (4.7)	0/28 (0.0)	92/609 (15.1)
Sep	25/348 (7.2)	2/197 (1.0)	0/128 (0.0)	0/153 (0.0)	0/20 (0.0)	27/846 (3.2)
Oct	21/266 (7.9)	0/239 (0.0)	0/185 (0.0)	0/116 (0.0)	0/22 (0.0)	21/828 (2.5)
Nov	7/50 (14.0)	0/97 (0.0)	0/38 (0.0)	0/20 (0.0)	-	7/205 (3.4)
Dec	0/40 (0.0)	11/44 (25.0)	3/30 (10.0)	2/30 (6.7)	-	16/144 (11.1)
Total	196/1,204 (16.3%)	29/906 (3.2%)	4/691 (0.6%)	5/481 (1.0%)	0/70 (0.0%)	234/3,352 (7.0%)

3.4. Distribution patterns by fish size

3.4.1. Weight-based distribution

The olive flounders obtained during the research period were categorized into weight-based groups, and the *M. avidus* positivity rates are illustrated in Fig. 3. Out of the 3,352 samples, the specimens were divided into five groups based on weight; ≤100 g, 100~300 g, 300~500 g, 500~700 g, and 700 g ≤, and the positivity rates were calculated for each group. The group with the highest positivity rate among the weight-based categories was the 100~300 g group, showing a positivity rate of 14.3%. The 300~500 g and 700 g ≤ groups exhibited positivity rates of 3.8%. The group with the lowest positivity rate was the 500~700 g group, with a positivity rate of 1.9%.

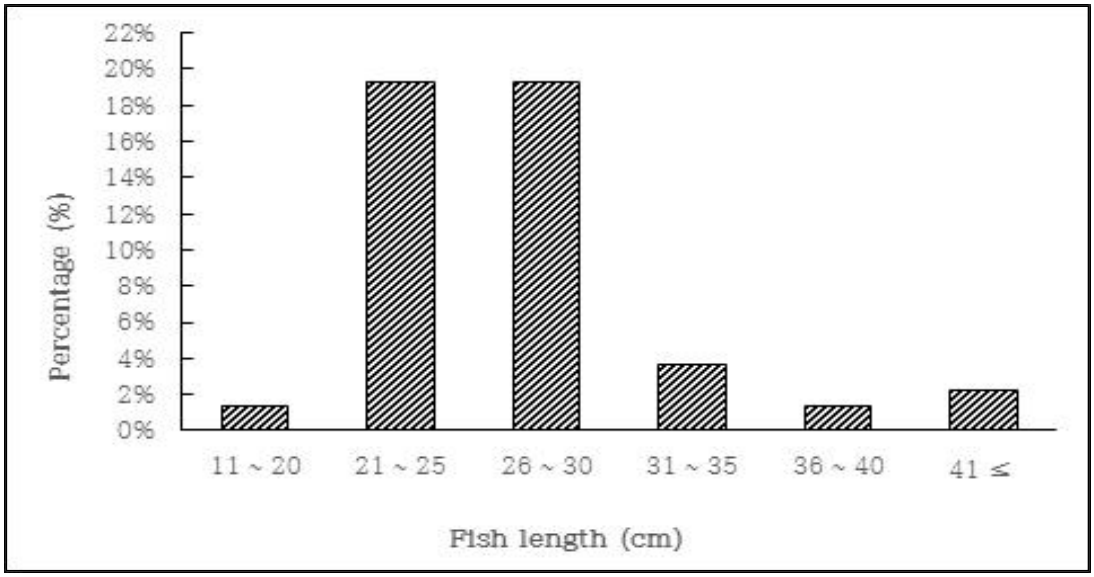


Month	Division of fish weight (g)					Total.
	≥ 100	100 ~ 300	300 ~ 500	500 ~ 700	700 ≤	
Jul	0/13	69/409	1/216	1/53	0/29	71/720
Aug	0/1	73/288	8/166	4/86	7/68	92/609
Sep	0/2	10/265	17/398	0/148	0/33	27/846
Oct	0/123	3/112	16/425	2/116	0/52	21/828
Nov	1/81	0/20	6/88	0/14	0/2	7/205
Dec	5/26	7/39	3/68	1/11	0/0	16/144
Total.	6/246 (2.4%)	162/1,133 (14.3%)	51/1,361 (3.8%)	8/428 (1.9%)	7/184 (3.8%)	234/3,352

Fig. 3. Weight-based distribution of *M. avidus* within individual olive flounder.

3.4.2. Length-based distribution

The olive flounder specimens obtained during the research period were categorized into length-based groups, and the *M. avidus* positivity rates are presented in Fig. 4. The groups were defined based on the length of the fish, ranging from the smallest individuals at the beginning of the study period to the following categories: 11~20 cm, 21~25 cm, 26~30 cm, 31~35 cm, 36~40 cm, and 41 cm ≤. There were a total of six groups. The highest positivity rate among the six groups was the 21~25 cm size group, representing juvenile fish, in which 41 out of 212 specimens. The 26~30 cm size group exhibited a similar positivity rate, with 129 out of 667 specimens testing positive, resulting in a 19.3% positivity rate for both groups. The 31~35 cm size group, which had the largest sample size (1,276 olive flounders), showed 46 positive, accounting for a positivity rate of 3.6%. Among the six groups, the lowest positivity rate was observed in the 36~40cm size group, consisting of 756 olive flounder specimens, of which 10 specimens tested positive, indicating the lowest rate at 1.3%.



Month	Division of fish length (cm)						Total.
	11 ~ 20	21 ~ 25	26 ~ 30	31 ~ 35	35 ~ 40	41 ≤	
Jul	0/8	25/142	45/217	1/234	0/94	0/25	71/720
Aug	0/0	8/19	66/205	7/186	6/133	5/66	92/609
Sep	0/1	0/14	12/167	14/370	1/241	0/53	27/846
Oct	0/123	0/0	3/40	15/354	3/238	0/73	21/828
Nov	0/79	0/4	2/12	5/69	0/39	0/2	7/205
Dec	3/11	8/33	1/26	4/63	0/11	0/0	16/144
Total.	3/222 (1.4%)	41/212 (19.3%)	129/667 (19.3%)	46/1,276 (3.6%)	10/756 (1.3%)	5/219 (2.3%)	234/3,352

Fig. 4. Length-based distribution of *M. avidus* within individual olive flounder.

3.5. Phylogenetic analysis results

The phylogenetic analysis results of the *M. avidus* nucleotide sequences from four aquaculture facilities, along with the 10 nucleotide sequences of the *M. avidus* 18S ribosomal RNA gene, and 17 sequences belonging to Philasterida within the Scuticociliatia, are presented in Fig. 4. In this study, the isolated *M. avidus* nucleotide sequences were confirmed to exhibit 100% identity with the 10 reference *M. avidus* 18S ribosomal RNA gene sequences, indicating genetic similarity. Furthermore, the *M. avidus* sequences from the four aquaculture facilities showed significant differences compared to the 17 nucleotide sequences belonging to Philasterida within the Scuticociliatia. Specifically, they exhibited 92.87% identity with *U. marinum* (GenBank accession no. AY551905), the highest identity of 99.82% with *Philasterides dicentrachi* (GenBank accession no. AY642280) and the lowest identity of 91.92% with *Entodiscus borealis* (GenBank accession no. AY541687). The other nucleotide sequences belonging to Philasterida within the Scuticociliatia was 96% with *Anophyroides haemophila*, 95.73% with *Entorhipidium triangularis*, 95.72% with *Parauronema longum*, 95.72% with *Plagiopyliella pacifica*, 95.63% with *Entorhipidium tenue*, 95.01% with *Thyrophylax vorax*, 94.92% with *Cohnilembus verminus*, 94.57% with *Pseudocohnilemus persalinus*, 94.30% with *Metanophrys similis*, 93.86% with *Pseudocohnilemus marinus*, 93.77% with *Pseudocohnilembus hargisi*, 93.39% with *Mesanophrys carcini*, 93.09% with *Uronema elegans*, 92.72% with *Paranophrys magna*, 92.04% with *Parauronema virginianum.*

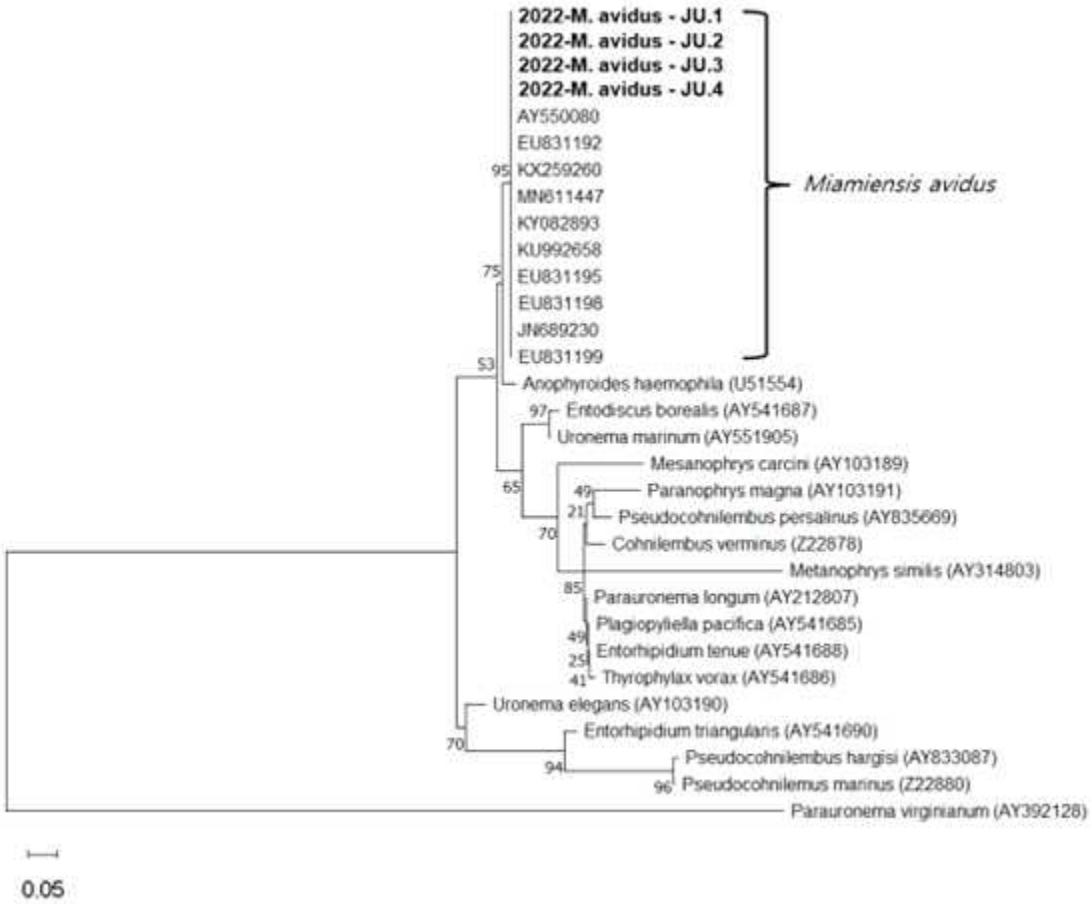


Fig. 5. A phylogenetic tree of SSU rRNA sequences of *M. avidus* and Philasterida of Scuticociliatia constructed by Mega 11 program. The numbers at the nodes represent the bootstrap percentages of 1,000 replicates, in the maximum-likehood (ML) method. The four different aquaculture farm sequence is shown in boldface.

3.6. PCR results of *M. avidus* characteristics

3.6.1. Serotype PCR results

During the analysis period, serotype analysis was conducted on all 234 individuals that tested positive for *M. avidus*. Among the three serotypes, serotype II was detected in 190 samples, while serotype I was detected in only four samples. In contrast, not a single instance of serotype III was detected among the 234 samples. In cases where no bands were observed in the results of the three serotype PCRs, it was denoted as "ND" (Non-detective). Based on the aquaculture farm, the serotype trends were investigated, and the results showed the dominance of serotype II in all four aquaculture facilities. The serotype PCR analysis results are presented in Table 5.

Table 5. Serotype analysis results using positive *M. avidus* samples

Serotype PCR results					
Aquafarm	Serotype				Total*
	Serotype Ⅰ	Serotype Ⅱ	Serotype Ⅲ	ND**	
Farm A	3	164	0	28	195/196
Farm B	0	19	0	9	28/29
Farm C	1	3	0	0	4/4
Farm D	0	4	0	1	5/5
Total	4	190	0	38	232/234

(* Serotype results / Total *M. avidus* positive sample, ** Non detective)

3.6.2. Cytochrome *c* oxidase subunit 1 gene type PCR results

During the investigation, PCR was conducted for the cytochrome *c* oxidase subunit 1 gene types I and II on the obtained 234 *M. avidus* - positive samples. The genetic trends specific to each aquaculture facility were investigated. In cases where no bands were observed in the PCR results for the two types of cytochrome *c* oxidase subunit 1 genes, it was denoted as "ND" (Non-detective). Notably, *cox* 1 type II was the most dominant genotype detected. The analysis results are presented in Table 6.

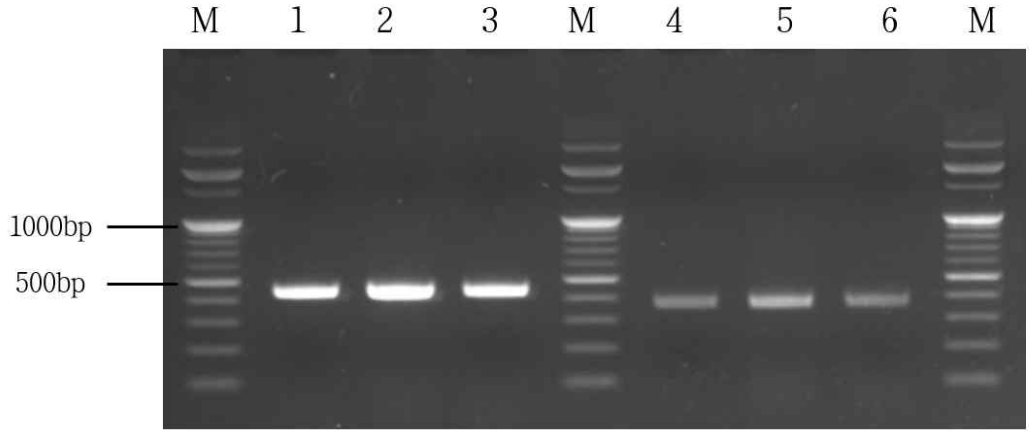
Table 6. Cytochrome *c* oxidase subunit 1 gene type analysis results using positive *M. avidus* samples

cytochrome *c* oxidase subunit 1 type gene PCR results				
Aquafarm	cytochrome *c* oxidase subunit 1 gene type			Total*
	cox 1 type I	*cox* 1 type II	ND**	
Farm A	2	102	91	195/196
Farm B	0	7	21	28/29
Farm C	1	2	1	4/4
Farm D	0	3	2	5/5
Total	3	114	115	232/234

(* *cox* 1 type results / Total *M. avidus* positive sample, ** Non detective)

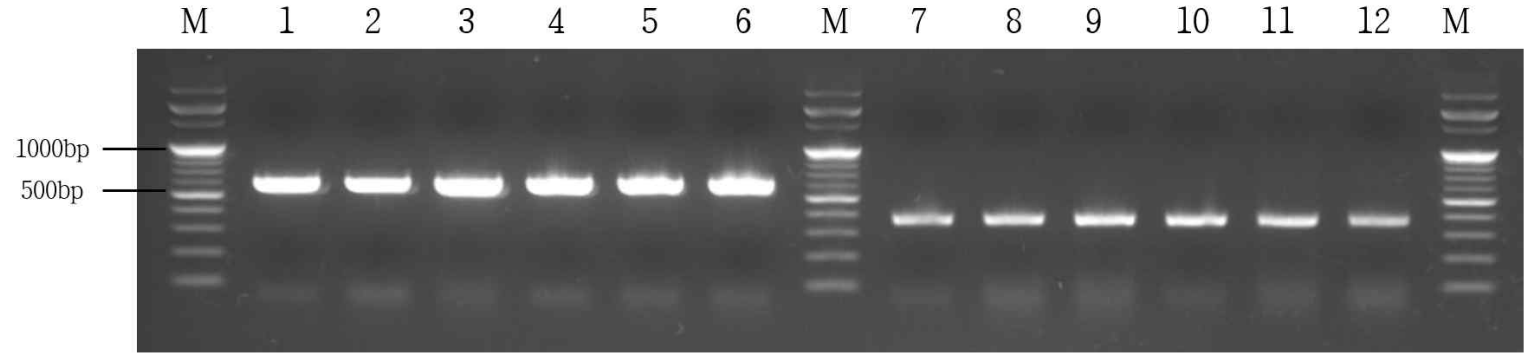
3.6.3. Serotype and *cox* 1 gene type PCR results

A specific pattern was observed between serotypes and cytochrome *c* oxidase subunit 1 gene types in the PCR results. In cases where serotype I was identified in a sample, *cox* 1 type I was simultaneously detected, and a similar pattern was observed when serotype II was detected, with *cox* 1 type II being concurrently identified (Table 7). Among the 234 *M. avidus*-positive samples, serotype I and *cox* 1 type I were detected together in three samples. The results of the gel electrophoresis for these cases are presented in Fig. 6. Furthermore, when serotype II and *cox* 1 type II were detected together, it was observed in 113 samples, with results obtained from samples collected in July and August presented in Fig. 7, and those from September to December shown in Fig. 8. To understand the distribution patterns of serotypes and gene types, the results were tabulated according to the four aquaculture facilities in Table 8. Instances of serotype I and *cox* 1 type I co-occurrence were found in Farms A and C, while in all four aquaculture facilities, the most common finding was the co-occurrence of serotype II and *cox* 1 type II. To further understand the distribution patterns between serotypes and gene types, monthly results were presented in Table 9. Excluding the results from October, November, and December, it was noted that from July to September, the most frequent occurrence was the co-detection of serotype II and *cox* 1 type II.



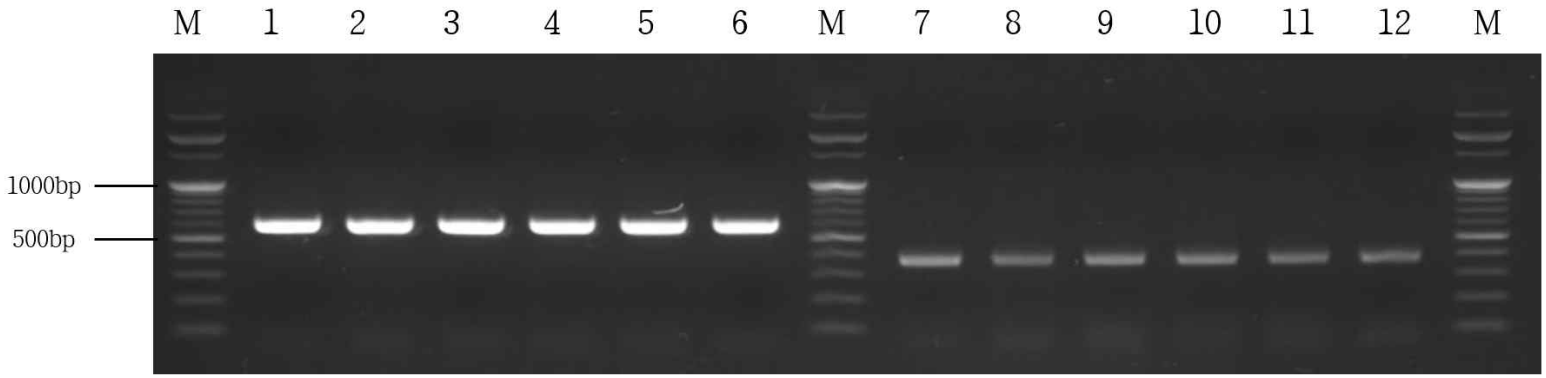
	M. avidus - serotype Ⅰ			*M. avidus* – *Cox* 1 type Ⅰ		
Product size	443bp			379bp		
Lane No.	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
PCR results	+	+	+	+	+	+
DNA sample	22.08.29 – no.30	22.09.29 – no.36	22.11.23 – no.10	22.08.29 – no.30	22.09.29 – no.36	22.11.23 – no.10
Farm	Farm C	Farm A	Farm A	Farm C	Farm A	Farm A

Fig. 6. Detection of the *M. avidus* serotype Ⅰ and cytochrome *c* oxidase subunit 1 gene type Ⅰ by conventional PCR using the serotype Ⅰ (lane 1-3) or *cox* 1 Type Ⅰ (lane 4-6) primer set.



	M. avidus - serotype Ⅱ						*M. avidus* – *Cox* 1 type Ⅱ					
Product size	604bp						379bp					
Lane No.	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12
PCR results	+	+	+	+	+	+	+	+	+	+	+	+
DNA sample	22.07.19 – no.4	22.07.22 – no.14	22.08.01 – no.18	22.08.02 – no.11	22.08.03 – no.14	22.08.10 – no.46	22.07.19 – no.4	22.07.22 – no.14	22.08.01 – no.18	22.08.02 – no.11	22.08.03 – no.14	22.08.10 – no.46
Farm	Farm A	Farm A	Farm A	Farm A	Farm A	Farm B	Farm A	Farm A	Farm A	Farm A	Farm A	Farm B

Fig. 7. Detection of the *M. avidus* serotype Ⅱ and cytochrome *c* oxidase subunit 1 gene type Ⅱ by conventional PCR using the serotype Ⅱ (lane 1-6) or *cox* 1 Type Ⅱ(lane 7-12) primer set.



	M. avidus - serotype Ⅱ						*M. avidus* – *Cox* 1 type Ⅱ					
Product size	604bp						379bp					
Lane No.	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Lane 11	Lane 12
PCR results	+	+	+	+	+	+	+	+	+	+	+	+
DNA sample	22.09.07 – no.10	22.09.14 – no.29	22.09.27 – no.8	22.10.04 – no.20	22.11.23 – no.17	22.12.22 – no.1	22.09.07 – no.10	22.09.14 – no.29	22.09.27 – no.8	22.10.04 – no.20	22.11.23 – no.17	22.12.22 – no.1
Farm	Farm A	Farm B	Farm A	Farm A	Farm A	Farm D	Farm A	Farm B	Farm A	Farm A	Farm A	Farm D

Fig. 8. Detection of the *M. avidus* serotype Ⅱ and cytochrome *c* oxidase subunit 1 gene type Ⅱ by conventional PCR using the serotype Ⅱ (lane 1-6) or *cox* 1 Type Ⅱ(lane 7-12) primer set.

Table 7. Total results of the *M. avidus* serotype and cytochrome *c* oxidase subunit 1 gene type by conventional PCR

Total results (Sample no. 232)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	3	-	-
	II	-	113	1
	ND	1	77	37

Table 8. Positive counts divided by the aquaculture farms of the *M. avidus* serotype and cytochrome *c* oxidase subunit 1 gene type by conventional PCR

Farm A (Total. 195)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	2	-	-
	II	-	101	1
	ND	1	63	27

Farm B (Total. 28)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	-	-	-
	II	-	7	-
	ND	-	12	9

Farm C (Total. 4)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	1	-	-
	II	-	2	-
	ND	-	1	-

Farm D (Total. 5)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	-	-	-
	II	-	3	-
	ND	-	1	1

Table 9. Positive counts divided by the months of the *M. avidus* serotype and cytochrome *c* oxidase subunit 1 gene type by conventional PCR

July (Total. 71)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	-	-	-
	II	-	38	-
	ND	-	23	10

August (Total. 92)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	1	-	-
	II	-	47	-
	ND	-	27	17

September (Total. 25)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	1	-	-
	II	-	16	-
	ND	-	6	2

October (Total. 21)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	-	-	-
	II	-	7	1
	ND	-	6	7

November (Total. 7)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	1	-	-
	II	-	2	-
	ND	1	3	-

December (Total. 16)		Serotype		
		I	II	ND
cytochrome *c* oxidase subunit 1 gene type	I	-	-	-
	II	-	3	-
	ND	-	12	1

4. Discussion

This study aimed to monitor and analyze the genetic correlation of the causative agent responsible for scuticociliatosis in olive flounder (*Paralichthys olivaceus*) collected from aquaculture farms in Jeju Island. As a result, characteristic external symptoms were observed in olive flounder infected with scuticociliatosis, including erosion and ulceration of the body surface, as well as dark discoloration from the head to the caudal fin, confirming results consistent with previous research (Jin *et al.*, 2006; Jung *et al.*, 2007; Kang *et al.*, 2015). In a study conducted by Song *et al.* (2009), comparing and analyzing the pathogenicity of four species of scuticociliates, namely *M. avidus*, *U. marinum*, *P. persalinus*, and *P. hargisi*, through direct intraperitoneal injection into olive flounder, *M. avidus* was reported as a major species exhibiting strong pathogenicity in olive flounder. In this study, the average size of detected *M. avidus* was found to be consistent with the findings reported by Jung *et al.* (2007), with a length of 31.33 μm (± 3.34 μm) and a width of 16.67 μm (± 2.25 μm).

Scuticociliatosis-induced mortalities occur throughout the year, but they are particularly exacerbated during periods of high water temperatures, primarily due to the enhanced proliferation of scuticociliates (Bae *et al.*, 2009). In this study, we also observed an increase in detection rates during the high temperature months of July (9.86%) and August (15.83%), aligning with the period of elevated water temperatures. Conversely, from September to November, when water temperatures decrease, we recorded lower average detection rates at 3.09%, consistent with previous reports (Jin *et al.*, 2007b). Aquaculture facilities in the Jeju region utilize a combination of groundwater and natural seawater for rearing, which results in relatively minor seasonal environmental fluctuations compared to other regions (Jin *et al.*, 2007b). It is

believed that the optimal temperature for the proliferation of the causative agent of scuticociliatosis in cultured olive flounder corresponds with the early summer rearing water temperatures in Jeju, contributing to higher incidence rates during the summer months. Additionally, it is worth noting that the sedimentation of feed and organic matter in the sub-bottom of the rearing tanks may create a favorable environment for scuticociliate proliferation, contributing to year-round outbreaks in olive flounder aquaculture facilities (Iglesias *et al.*, 2001; Jin *et al.*, 2007a).

Regarding the relationship between fish weight and disease incidence, scuticociliatosis has been reported to predominantly affect juvenile olive flounder weighing less than 300 grams (Kim *et al.*, 2012). A study by Kim *et al.* (2020) reported that among the groups infected and uninfected with scuticociliatosis from 2015 to 2017, individuals weighing 335 grams or less comprised 75% of the scuticociliatosis-infected group. In this study, we also observed a high proportion (14.3%) of individuals infected with *M. avidus* falling within the 100~300 grams weight range, consistent with previous findings (Kim *et al.*, 2012; Kim *et al.*, 2020). Additionally, dividing the population based on size distribution, the groups of 21 to 25 cm and 26 to 30 cm showed a significantly high positivity rate of 19.3% for *M. avidus*. The analysis of body length and weight would be helpful in clinically estimating future scuticociliatosis infections or predicting periods with a higher likelihood of increased occurrences of scuticociliatosis.

Due to the similar size and morphology of the causative agents responsible for scuticociliatosis, accurate species identification has been challenging, leading to research efforts focusing on genetic and morphological characterization (Kim *et al.*, 2004a; Kim *et al.*, 2004b). In this study, we conducted phylogenetic analysis to determine the genetic classification for precise identification. The nucleotide sequences of the pathogens detected in four aquaculture facilities showed 100% similarity with GenBank registered

M. avidus nucleotide sequences, further confirming their genetic identity.

Research on the serotypes of pathogens is a prerequisite in vaccine development, as generating immunity necessitates immunization against the same serotype when various serotypes exist. Therefore, investigating whether *M. avidus*, isolated from all aqua-farming regions in South Korea, represents a single serotype or if multiple serotypes exist is crucial information for vaccine development. According to Song *et al* (2009), they proposed three serotypes of *M. avidus* based on western blotting profiles and immobilization assays. But, these methods require pathogen isolation from diseased fish and hence they take time and effort (Motokawa *et al*., 2018). Therefore, we used three serotype-specific PCR primer sets based on determined ORFs for determination and fast detection of *M. avidus* serotypes. Until now, most *M. avidus* have been found to belong to serotypes Ⅰ and Ⅱ, and these serotypes are widely distributed throughout Japan and Korea (Motokawa *et al*., 2018). In this study, as in previous research, the results also demonstrated a predominance of serotype II over serotype I, while serotype III was not detected. In recent times, the vaccines being introduced in South Korea incorporate antigens of both *M. avidus* serotype I and II. The currently approved vaccine for scuticociliatosis includes both *M. avidus* serotypes I and II. Therefore, based on the serotype analysis of *M. avidus* strains found in Jeju Island in this study, it is expected that the subsequent vaccination will have a remarkable preventive effect against scuticociliates, leading to an improvement in olive flounder aquaculture productivity.

In the case of scuticociliatosis, species identification is typically carried out through the silver impregnation method or PCR-based classification. However, these classification methods encounter challenges when dealing with species that share similar morphological characteristics or exhibit varying forms based on their life stages. This can result in misclassifications or the reclassification of previously identified species as new ones. To address these issues and

achieve more accurate classification, contemporary research efforts incorporate DNA-based identification alongside traditional methods. Small subunit ribosomal DNA (SSU rDNA) has proven to be a valuable tool for unambiguous identification and comparison of various organisms (Hirt *et al.*, 1995; Dunthorn *et al.*, 2008). Nevertheless, SSU rDNA-based identification sometimes fails to align with morphological classification, and the criteria for determining whether a certain level of homology indicates conspecificity are not always clear. In response to these challenges, Hebert *et al.* (2003) proposed the use of the mitochondrial cytochrome-c oxidase 1 (*cox* 1) gene, commonly referred to as a 'DNA barcode' for biological identification of animal species. The mitochondrial genome evolves more rapidly than the nuclear genome, and specific regions of mitochondrial genomic sequences have been reported to be useful for distinguishing closely related species or for taxonomic purposes (Hebert *et al.*, 2003). Also according to Whang *et al* (2013), they indicated that the *cox* 1 sequence divergence (23.5%) between the four ciliate species such as *Pseudocohnilembus longisetus*, *P. persalinus*, *U. marinum* and *M. avidus* was about three times higher than those of the SSU rDNA sequence (8.3%). Thus, this result indicates that the hyper-variable sequences of the *cox* 1 gene may represent a useful diagnostic DNA barcoding region for differential identification of scuticociliate species (Whang *et al.*, 2013). Based on the study by Jung *et al.* (2011), which demonstrated a causal relationship between serotype and genotype, this study aimed to determine the matching of *cox* 1 types and serotypes using the results obtained. Therefore, in this study *M. avidus* isolated from four aquaculture facilities in the Jeju region exhibited a dominance of *cox* 1 type II over *cox* 1 type I. In the serotype results, the detection rate of serotype II was higher than that of serotype I. In our study, out of the 232 *M. avidus* samples, results revealed a matching between *cox* 1 type II and serotype II in 113 samples, while *cox* 1 type I and serotype I matched in 3 samples. The results

of this study also exhibited a high level of matching, and this trend was found to be similar to the findings of Jung *et al* (2011). This results to propose a possibility of the *cox* 1 genes and the serological grouping of the organism.

Efforts to develop treatments and evaluate their effectiveness are ongoing to combat scuticociliatosis, a disease causing high mortality rates in olive flounder and significant economic losses in marine fish farming (Harikrishnan *et al.*, 2010; Lee *et al.*, 2017). However, to date, there is no effective alternative for controlling the causative agent of scuticociliatosis in cultured olive flounder (Lee *et al.*, 2017). Therefore, in this study, we conducted scuticociliatosis monitoring in Jeju Island's olive flounder aquaculture facilities and performed phylogenetic analysis of the isolated strains. The study conclusively identified *M. avidus* as the primary species causing scuticociliatosis in the Jeju region, with no instances of *U. marinum* found among 3,352 samples, enabling an assessment of the disease's incidence trend. Therefore, this study aimed to depict the disease characteristics of scuticocilaites in Jeju Island through the results of *M. avidus* serotypes and *cox* 1 types. This study could be useful to further assess the biological and antigenetic diversities in relation to intraspecific variations of the *cox* 1 gene, and also serve as fundamental data for disease prevention strategies.

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제주지역 양식 넙치(*Paralichthys olivaceus*)를 대상으로 한 스쿠티카증(Scuticociliatosis) 원인병원체의 검출 및 특성 분석

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요약

스쿠티카증은 넙치에 발생하는 질병 중 큰 문제를 일으키는 질병 중 하나이며 치료 대책이 미비하고 폐사율이 높아 넙치 양식 현장에서의 경제적 손실을 불러일으키는 기생충성 질병이다. 특히 치어기의 넙치에 스쿠티카충이 감염될 경우 아가미와 체표를 비롯한 안구, 뇌, 내부 장기 등에 침투하는 특징을 지니고 있어 양식 현장에서 어려움을 겪는 질병이다. 따라서, 감염예방이 중요하며, 피해를 줄이기 위해서는 다각적인 연구와 접근이 필요할 것으로 보인다.

본 연구에서는 제주지역에 위치한 21개소 양식장을 대상으로 2022년 7월부터 12월까지 6개월간 국내 양식 넙치에 주로 감염되는 것으로 보고된 2종의 스쿠티카충 (*Miamiensis avidus, Uronema marinum*) 질병 모니터링을 실시하였다. 또한, 분석에 사용한 모든 넙치의 체중과 체장을 측정하고 스쿠티카증으로 의심되는 외부증상과 내부증상을 관찰하여 질병 발생 패턴을 분석하고자 하였다. 스쿠티카증을 일으키는 원인체 2종에 대한 감염 진단 검사를 위해 넙치의 근육 조직에서 추출한 DNA를 대상으로 polymerase chain reaction (PCR)법을 사용하였다. 실험에 사용된 총 3,352마리의 양식 넙치 중 234마리의 넙치에서 스쿠티카증 원인체 중 하나인 *M. avidus*가 검출되었다. 반면, *M. avidus*의 결과와는 달리 *U. marinum*의 경우, 본 실험에 사용된 3,352마리에서 모두 음성 결과를 보였다. PCR 검사를 통해 얻어진 234개의 *M. avidus* 양성 개체의 경우 reference로 이

용된 10개의 *M. avidus* 18S ribosomal RNA gene 염기서열과 100%의 상동성을 지니는 것으로 확인되어 *M. avidus* 18S ribosomal RNA gene의 10개의 염기서열과 유전적으로 유사한 것으로 확인되었다. 추가적으로 cytochrome *c* oxidase 1 (*cox* 1) gene을 이용하여 분류하고자 하였으며, 또한 함께 이루어진 혈청형에 관한 연구를 통해 *cox* 1 gene type이 혈청형과 서로 관련이 있음을 확인하였다.

이로써, 본 연구는 양식 넙치에 많은 피해를 입히고 있는 스쿠티카증에 대하여 지리적으로 격리되어 있으며 우리나라 양식 넙치 생산량의 절반을 차지하고 있는 제주도 지역의 스쿠티카증 발병 동향을 조사하고 질병 특성 분석 결과를 제시하였다. 이는 향후 스쿠티카 감염증을 예방하기 위한 기초자료로 활용될 수 있을 것이다.

감사의 글

2번의 사계절이 지나, 졸업이라는 결실을 맺게 되었습니다. 오늘이 오기까지 많이 웃고 가끔은 울기도 하며 교수님과 박사님의 제자로 수많은 교훈과 연구자의 자세를 배웠습니다. 실험실의 일원으로 석사과정을 보내며 부족하지만 이런 저의 옆에서 따스한 지도와 조언, 끊임없이 힘을 주신 분들에게 이 글을 통해 감사하는 마음을 담아보려 합니다.

먼저, 저의 지도교수님이신 정준범 교수님께 감사드립니다. 석사과정 동안 교수님은 저에게 많은 조언과 응원을 아낌없이 해주시며 항상 온화한 미소로 저를 응원해주셨고, 불편한 점이나 필요한 도움이 없는지 보살펴 주셨기에 힘들고 어려운 순간마다 큰 힘이 되어주셨습니다. 그리고, 전려진 박사님께 감사드립니다. 박사님께서는 제 옆에서 섬세하고 꼼꼼한 지도와 조언으로 제가 더 성장할 수 있도록 해주셨고, 따듯한 말씀들로 큰 힘을 주셨습니다. 석사과정 동안 교수님과 박사님께 듣고 배웠던 많은 교훈을 바탕으로 앞으로 사회에 나가서도 교수님과 박사님께 부끄럽지 않은, 자랑스러운 제자가 되도록 항상 노력하겠습니다. 또한 바쁘신 와중에도 제 논문을 심사해주시고 지도해주신 김준환 교수님께도 감사의 인사를 전합니다.

다음으로, 제가 2년이라는 시간 동안 즐겁고 행복한 석사과정을 보낼 수 있었던 큰 원동력, “어병진단학 실험실”의 기둥인 실험실 방장 예지 언니, 든든한 선배이자 기생충 파트 파트너 영준 오빠, 언제나 친절하고 다정다감한 지은 언니, 내 반쪽이자 쌍둥이 영은이, 실험실 all-라운더 응준이에게 여러분이 있기에 제가 더 성장할 수 있었고, 즐거운 실험실 생활을 할 수 있었습니다. 그리고, 6년간의 긴 제주도에서의 학교생활 동안 제 옆에서 큰 힘이 되어주었던 친구들 모두에게 너무 고맙다는 말을 전하고 싶습니다.

마지막으로, 제 옆에서 언제나 힘을 주고 응원해주는 아빠, 엄마, 동생 영준이에게 너무 감사합니다. 대학 생활, 석사과정 동안 아낌없는 지원과 응원을 해주신 아빠와 엄마 두 분 덕분에 무사히 석사 졸업을 하게 되었고, 부모님 덕분에 저는 부족함 없이 세상에서 가장 행복하고 밝은 25살 딸로 성장했습니다! 이제는 사회로 나아가 아빠와 엄마의 자랑스러운 딸이 되겠습니다. 아빠, 엄마, 영준이, 그리고 도비 너무너무 사랑하고 감사합니다.