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Removal of two pathogenic scuticociliates *Miamiensis avidus* and *Miamiensis* sp. using cells or culture filtrates of the dinoflagellate *Alexandrium andersonii*

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ABSTRACT

Scuticociliatosis, which is caused by parasitic protistan pathogens known as scuticociliates, is one of the most serious diseases in marine aquaculture worldwide. Thus, elimination of these ciliates is a primary concern for scientists and managers in the aquaculture industry. To date, formalin and other toxic chemicals have been used as anti-scuticociliate agents, but issues regarding their secondary effects often arise. Consequently, development of safer methods is necessary. To find out a safe method of controlling scuticociliate populations in aqua-tanks or small-scale natural environments, cultures of 14 phototrophic dinoflagellates were tested to determine whether they were able to control populations of the common scuticociliates *Miamiensis avidus* and *Miamiensis* sp. isolated from Korean waters. Among the dinoflagellates tested, both cells and culture filtrates of *Alexandrium andersonii* effectively killed *M. avidus* and *Miamiensis* sp. The minimal concentration of cells and equivalent culture filtrates of *A. andersonii* to kill all *M. avidus* cells within 48 h of incubation was ca. 2500 and 4500 cells ml^{-1}, respectively; whereas those needed to kill all *Miamiensis* sp. cells were ca. 1000 and 4500 cells ml^{-1}, respectively. It was estimated that 1 m^3 of the stock culture containing 20,000 *A. andersonii* cells ml^{-1} could eliminate all *M. avidus* cells in 7 m^3 of waters within the aqua-tanks on land and all *Miamiensis* sp. cells in 19 m^3 of waters within 48 h. None of the brine shrimp *Artemia salina* nauplii incubated with concentrations of 50–4500 *A. andersonii* cells ml^{-1} for 24 h was dead. Furthermore, none of the flounder *Paralichthys olivaceus* juveniles incubated with a mean concentration of ca. 2280 *A. andersonii* cells ml^{-1} for 96 h was dead. Therefore, *A. andersonii* cultures may be used as a safe biological method for controlling populations of scuticociliates and can replace toxic formalin. The results of this study provided the basis for developing the method to control scuticociliate populations and understanding interactions between scuticociliates and phototrophic dinoflagellates in marine ecosystems.

1. Introduction

Scuticociliates are parasite protistan pathogens that often cause massive mortality of fishes, crustaceans, and molluscs (Iglesias et al., 2001; Kim et al., 2004a; Harikrishnan et al., 2010). Scuticociliatosis, which is caused by scuticociliates, occurs in areas around the world, including Australia, China, Denmark, Israel, Korea, Spain, and the USA (Harikrishnan et al., 2010). More than 20 species belong to the subclass Scuticociliatia, such as *Miamiensis avidus* (syn. *Philasterides dicentrarchi*), *Uronema marinum,* and *Anophryoides haemophila*, have been revealed to be facultative parasites that destroy host tissues (Thompson and Moewus, 1964; Cheung et al., 1980; Cawthorn et al., 1996; Harikrishnan et al., 2010). For many years, they have been regarded as one of the most important and dangerous pathogens because they cause large-scale mortality of fish in the aquaculture industry (Thompson and Moewus, 1964; Jee et al., 2014). Scuticociliatosis

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was revealed to cause most (35.9%) of the mortality in the flounder farms in South Korea from May to November 2011 (Jee et al., 2014). To minimize loss caused by scuticociliatosis, several methods of eliminating the causative scuticociliates have been used or suggested. Although there are treatments such as formalin treatment, chemotherapy, and immune stimulants (e.g., Iglesias et al., 2002; Harikrishnan et al., 2010; Kang and Kim, 2015), adverse secondary effects owing to the use of these materials have often arisen. Thus, development of safer methods is necessary. Several biological methods such as extracts of land plants and macroalgae and copepod predation have been developed because they are safer than toxic chemicals (Berk et al., 1977; Rieper, 1985; Besiktepe and Dam, 2002; Dam and Lopes, 2003; Kang, 2006; Kang et al., 2014; Mallo et al., 2016). Microalgae, including dinoflagellates, capable of effectively removing scuticociliates by feeding, physical contact, or culture filtrates, also, need to be explored. Therefore, it is worthwhile to attempt to discover such dinoflagellates.

There are ca. 2500 species and 400 genera of dinoflagellates (Taylor et al., 2008). Some dinoflagellates were reported to lyse and kill other protists by producing chemicals (Tillmann and John, 2002; Deeds and Place, 2006; Kim et al., 2016; Lee et al., 2016). In particular, several dinoflagellates belonging to the genus *Alexandrium* lyse the bodies of a diversity of protozoans, including dinoflagellates, microflagellates, and free-living ciliates (Fulco, 2007; Tillmann et al., 2008; Kim et al., 2016); *Alexandrium pohangense* lyses the ciliate *Tiarina fusus*; *Alexandrium tamarense* lyses the ciliates *Favella taraikaensis, Eutintinnus* sp., and *Rimostrombidium caudatum*. Parasitic scuticociliates, however, may respond differently to these dinoflagellates than free-living ciliates. Therefore, diverse dinoflagellates, including *A. tamarense* and *A. pohangense,* should be tested to determine whether dinoflagellates are able to kill scuticociliates.

In this study, two scuticociliates were isolated each from the brain of the olive flounder *Paralichthys olivaceus* and from an infected larva of the brackish-water snail *Clithon retropictus* in Korea. These two ciliates were identified as *Miamiensis avidus* and *Miamiensis* sp. To determine which dinoflagellates could kill these ciliates effectively, 14 phototrophic dinoflagellate species belonging to the genera *Alexandrium, Coolia, Gymnodinium, Karenia,* and *Prorocentrum* were tested. Furthermore, the survival of *Miamiensis avidus* and *Miamiensis* sp. as a function of the concentrations of cells and equivalent culture filtrate of *Alexandrium andersonii* were measured because this dinoflagellate was determined to kill these ciliates most effectively in a screening test. Also, bioassay for toxicity of *A. andersonii* using *Artemia salina* nauplii and *P. olivaceus* juveniles was analyzed to check whether *A. andersonii* could be used a safe materials to mitigate scuticociliates. Results of this study provided the basis for developing an effective method of controlling populations of scuticociliates in aqua-tanks and understanding interactions between scuticociliates and dinoflagellates in marine ecosystems.

2. Materials and methods

2.1. Collection and culture of scuticociliates and dinoflagellates

The scuticociliate *Miamiensis avidus* J1 was isolated from the brain of *Paralichthys olivaceus* (body weight = 530 g) which was collected from an aqua-tank in Jeju island in February 2016 and then anesthetized with MS-222 (Tricaine Methanesulfonate) in the laboratory at Kunsan National University. The water temperature was 17.3 °C in the aqua-tank. The scuticociliate *Miamiensis* sp. B1 was isolated from an infected larva of *Clithon retropictus* in waters off Bieung Island, western Korea in May 2016 when water temperature and salinity were 20.8 °C and 31.3, respectively (Table 1).

Cultures of *Miamiensis avidus* were maintained in seawater with proteose-peptone (1 mg 100 ml^{-1} seawater) and yeast, whereas cultures of *Miamiensis* sp. were maintained in seawater with autotrophic bacteria at 20 °C under an illumination of 20 μE $m^{-2}s^{-1}$ provided by cool-white fluorescent lights in a 14:10 h light-dark (LD) cycle.

Clonal cultures of the phototrophic dinoflagellates, including *Alexandrium andersonii*, *Alexandrium catenella*, *Alexandrium fraterculus*, *Alexandrium hiranoi*, *Alexandrium insuetum*, *Alexandrium leei, Alexandrium minutum*, *Alexandrium pacificum*, *Alexandrium pohangense, Alexandrium tamarense*, *Coolia malayensis*, *Gymnodinium catenatum*, *Karenia mikimotoi*, and *Prorocentrum rhathymum*, were either established by two single-cell isolations by our team or obtained from culture centers (Table 1). These dinoflagellate cultures were maintained photosynthetically in enriched f/2 seawater media (Guillard and Ryther, 1962) at 20 °C under 20 μE $m^{-2}s^{-1}$ in a 14:10 h LD cycle. Cultures in the exponential phase were used in these experiments.

2.2. PCR amplification, DNA sequencing, and phylogenetic analysis

In order to amplify the DNA of scuticociliates, the single-cell PCR method was used as in Ki et al. (2005); five cells from each of *Miamiensis avidus* and *Miamiensis* sp. were transferred to a 0.2-ml

Table 1
Isolation and maintenance conditions for the scuticociliates and phototrophic dinoflagellates used in the present study.

Organism	Strain number	Location	Date	T	S
Miamiensis avidus	J1	Jeju, Korea	2016 02	17.4	NA
Miamiensis sp.	B1	Bieung Isalnd, Korea	2016 05	20.8	31.3
Alexandrium andersonii	AAJH201505	Jinhae, Korea	2015 05	22.2	32.2
Alexandrium catenella	WEB-ALEX-03	Busan, Korea	2013 08	24.7	26.4
Alexandrium fraterculus	WEB-ALEX-04	Yeosu, Korea	2013 09	23.4	32.8
Alexandrium hiranoi	NIES-612	Japan	1984 08	NA	NA
Alexandrium insuetum	CCMP2082	Uchiumi Bay, Kagawa, Japan	1985 06	NA	NA
Alexandrium leei	CCMP2955	Singapore Strait	NA	NA	NA
Alexandrium minutum	CCMP113	Ria de vigo, spain	1987 09	11–16	NA
Alexandrium pacificum	CCMP3434	Port Phillip Bay, Australia	1988 03	NA	NA
Alexandrium pohangense	PHAlex1409	Pohang, Korea	2014 09	23	31
Alexandrium tamarense	CCMP1493	Hong Kong	1992 07	NA	NA
Coolia malayensis	CMJJ20910	Jeju, Korea	2009 10	21.1	28.6
Gymnodinium catenatum	GC111-3	NA	NA	NA	NA
Karenia mikimotoi	KMKS1408	Doopori, Korea	2014 08	26.2	33.0
Prorocentrum rhathymum	PRJJ0907	Jeju, Korea	2012 06	11.4	13.3

Sampling location and date; water temperature (T, °C); salinity (S) for isolation; NA, not available.