Available online at: www.mbai.org.in doi:10.6024/jmbai.2021.63.2.2192-10

Rare occurrence and culture of heterotrichous ciliate, *Folliculinopsis producta*

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Received: 20 May 2020 Accepted: 17 Sep 2021 Published: 20 Oct 2021

Original Article

Abstract

The occurrence of the marine heterotrichous ciliate in Indian waters was reported after a gap of four decades. These ciliates were recorded during a routine survey from the Chennai coast, and an attempt was also made further to rear them in captive conditions. The specimens were identified as *Folliculinopsis producta* after silver impregnation staining and *in vivo* observations. These organisms have solitary dark green colored contractile cells, which are housed into green colored semi-translucent sessile tube-like lorica of 300-419 μm length. The total length of the cells ranged from 520 to 950 μm at a fully extended active state and 180 to 361 μm in retracted resting state. Two peristomal lobes in different sizes (104-152 μm and 172-205 μm) with intense ciliary activity in the anterior part of the cell were observed. The water quality parameters such as salinity of 30$\pm$1 psu, temperature of 28$\pm$2 °C, pH of 8.1$\pm$0.2, and dissolved oxygen of 6.0$\pm$1.5 mg/L were identified as optimum for the growth of *F. producta* in captive conditions. They were fed with live feed consisting of bacteria, microalgae *Isochrysis galbana* and diatom *Thalassiosira subtilis*. The swarming of planktonic juveniles was observed in 3 days after the addition of the combined diet. The free swimming juveniles were settled in the substratum after six h to 48 h. Subsequently, offspring populations ranging from 2000 to 5000 individuals/ml in a school were observed for ten days. The swarm of juveniles was appeared on the water surface and are characterized by their phototactic movement. The offspring's length ranged from 98-170 μm during free-swimming stage, 244-290 μm during the settlement stage. Later they developed lorica. Thus the present study provides baseline scientific insights as this will go a long way in furthering research on their biology and its role in the marine environment.

Keywords: *Marine ciliate, bottle animalcule, culture, optimization, feeding*

Introduction

The heterotrichous folliculinid ciliates are widely distributed in coastal and marine environments around the world (Das, 1953; Hu *et al*., 2019; Montano *et al*., 2020). They are microscopic animals commonly called 'Bottle Animalcule' with highly specialized cells inside the flask-like lorica (tube). Generally, folliculinids are found attached to the submerged substrates such as algae, tubes of annelids, shells of barnacles

and molluscs. The free-swimming planktonic juvenile form is also a part of their life cycle. They inhabit littoral zones and deep warmer hydrothermal vents (Kouris *et al.*, 2007). Around 25 species of folliculinids belonging to six genera were recorded from different parts of the world (Andrews, 1923; Das, 1953; Scheltema, 1973; Hu *et al.*, 2019). The occurrence of these folliculinids in Indian coastal waters dates back to 1979 at Cochin and 1953 in Chennai (Das, 1953; Mohan, 1979). The ecological significance of these organisms is not well known. Nevertheless, the marine ciliated protozoans play a significant role in the biogeochemical cycles and energy flow in the coastal environments (Stout, 1980; Caron, 1991). A few studies have also revealed their animal association and putative disease causative agents in corals (Montano *et al.*, 2020). True to this, the growth of folliculinids is reported to cause different coral diseases in Indo-Pacific, Caribbean Sea and Micronesia regions (Croquer *et al.*, 2006a, 2006b; Sweet *et al.*, 2019; Montano *et al.*, 2020). The folliculinid ciliates are endosymbiotic with chemolithoautotrophic bacteria and their exocellular association with microbes (Kouris *et al.*, 2007). Further, a few recent studies also reported the occurrence, re-description of species and genomic phylogeny of folliculinids (Song *et al.*, 2003; Ji *et al.*, 2004; Guo *et al.*, 2008; Miao *et al.*, 2009). Previous attempts on culture maintenance mainly focused on *in vivo* examination of folliculinids under a microscope for identification within two weeks (Das, 1947; Song *et al.*, 2003). The continuous culture of folliculinids has not been described in any previous reports with laboratory-controlled conditions, feeding, substratum and duration of development. As the information on its ecological significance, nutritional value and bioactive compounds are less, culturing these organisms could facilitate further studies to fulfil the lacunae that exist in this line. Accordingly, in the present study, a description of the rare occurrence of *F. producta* is made from the Chennai coastal waters, and an attempt was also made to culture the same in the lab conditions.

Material and methods

Collection of samples

The assemblages of folliculinids were observed in the glass aquarium tanks on the shells of barnacles, submerged objects and walls of culture tanks. The culture tanks were originally maintained for barnacle culture for the toxicological studies on their larvae. The seawater was collected from the Chennai coast (12.80 N, 80.24 E) for culturing the above species. They were fed with mixed species of diatom, *Thalassiosira subtilis* and *Isochrysis galbana* with a partial seawater exchange at weekly intervals. A few batches of folliculinid assemblages were removed from the tanks using a surgical blade and mounted on the glass slide using the Pasteur pipette for *in vivo* morphological observation.

Identification and morphological examination

The specimens were observed under the microscope in-vivo at 200 to 1000 times of magnifications (Nikon, H600L). The species were identified by morphological and morphometric characteristics as re-described by Ji *et al.* (2004). The characteristics of the cell and lorica were examined separately for identification. The lorica shape, size, colour and number of rings were examined and noted. Similarly, the number of somatic kineties, shape and size of cells, peristomal lobes during the extended active stage and retracted resting stages were also studied in in-vivo photomicrographs. The characteristics of planktonic free-swimming life stages were also examined in-vivo up to settling on the substratum. Adequate specimens were preserved in Lugol's fixative solution, and the microscopic structures were analysed in preserved specimens. The specimens were stained with silver impregnation by the Protargol method for enhanced better visibility of cilia and nucleus (Dieckmann, 1995; Ji and Wang, 2018).

Culture maintenance

The laboratory culture of folliculinids was maintained for three months under controlled conditions. The temperature, salinity and pH were maintained at ambient environmental conditions during the culture period. The development of free-swimming life stages, their settlement on the substrate and population growth in the culture were considered as parameters to be studied. Feeding was optimized with the following strategies such as live-feed composition, density and frequency of feeding them with different compositions of live feeds. The boiled starch solution was added as a carbon source to enrich the bacterial population as a food. The heterokont microalgae *Isochrysis galbala* and marine diatom *Thalassiosira subtilis* were also fed along with bacteria. For safe handling and utilization of live assemblages of folliculinids for various applications like bioassay, the nylon fibers were used as an artificial substrate. Pieces of the nylon fibers of 0.5 cm length and 1mm diameter were kept floating on the seawater as the substrate. The folliculinids were characteristically seen attaching among the existing assemblages. Therefore, ideally, green colored nylon fiber was used to simulate the color of assemblages.

Results

The morphological characteristics of *Folliculinids* are presented in Table 1 and Fig. 1. The present specimen was identified as *Folliculinopsis producta* based on the morphometric characteristics.

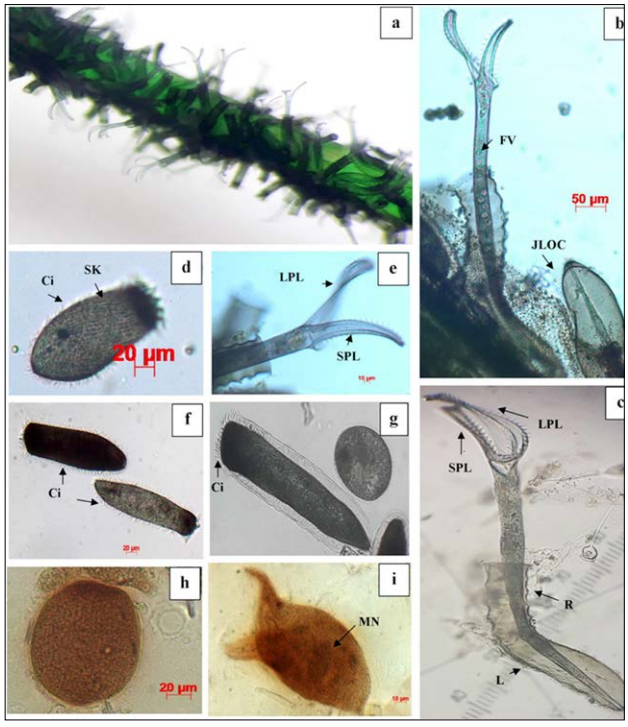

Fig. 1. Microscopical images of *F. producta* in-vivo and after staining. (a) Colonies of *F. producta* on the nylon fiber (b & c) a fully extended cell from the lorica (d,f & g) different stages of free-swimming juveniles (e) differential peristomal lobes (h&i) stained of free-swimming and sedentary cell. (LPL: Long peristomal lobe; SPL: Short peristomal lobe; R: Ridges of lorica; L: Lorica; FV: Food vacuole; MN: Macronucleus; Ci: Cilia; SK: Somatic kineties; JLOC: Jelly-like outer cover

Table 1. Morphometric characteristics of *Folliculinopsis producta* (in vivo). Values in the parenthesis indicate the sample size (n), measurements are in micrometers

	Total length (μm)	Diameter (μm)
Cell at retracted stage	180-361 (12)	48-69 (10)
Cell at fully extended stage	520-950 (10)	23.5-39.4 (8)
Peristomal Lobes	104- 152 (Short) (8) 172-205 (Long) (9)	16- 22 (Short) (5) 28-36 (Long) (5)
Lorica	300-419 (12)	69-82 (neck) (10) 86-92 (base) (5)
Juvenile (Free-swimming stage)	98-170 (8)	64-85 (8)
Juvenile (Settled stage)	244-290 (10)	48-54 (10)
Number of somatic kineties	80-100 (6)	
Color	Green to dark green	

Taxonomy

Class : Heterotrichea Stein, 1859
Order : Heterotrichida Stein, 1859
Family : Folliculinidae Dons, 1914
Genus : Folliculinopsis Fraure-Fremiet, 1936.
Species : *Folliculinopsis producta*; Synonym: *Folliculina producta*, Wright, 1859.

Description

The cell (is referred to as 'body') are highly contractile, semi-translucent, the cytoplasm is green to dark green in color with 10-20 micronucleus, the body is covered with several longitudinal lines of cilia, peristomal lobes are unequal in size with marginal ciliary activity (Fig.1a,b,c&i). The posterior part of the body is slender and attached to the lorica. The body length ranged from 520 to 950 μm, width from 23 to 39 μm in the extended stage while in retracted stage, the body length varied from 180 to 361 μm and width from 48 to 69 μm (Table 1).

Lorica is vase-shaped, green to dark green in color, translucent, mostly covered with a thick jelly-like layer. The lorica has 7-10 circular ridges/thickenings in the neck region (Fig.1a, b,c & e). The length and outer diameter of the lorica ranged from 300 to 419 μm and 69 to 92 μm, respectively. The juveniles were planktonic, their colour is green to dark green in colour, and ciliary activity was observed along the body that helps their locomotion and feeding. Early planktonic juveniles (98-110 μ L $\times$ 64-85 μ W) were almost circular in shape. They were elongated during the growth until settling on the substratum (244-290 μ L $\times$ 48-54 μ W). The ciliary action in juveniles creates a circular process at the anterior end of the body (Fig.1d, f, g & h). The anteroposterior longitudinal pigmented lines with cilia are termed as the somatic kineties. Enumeration of somatic kineties was found to be 80 to 100 lines in the sedentary animal.

Culture conditions

The culture was maintained in natural seawater collected from the Chennai coast. The population of *F. producta* was raised at water temperature of 26-30 ºC, salinity of 29-31 psu, pH of 7.9–8.3 and dissolved oxygen level of 4.5-7.5 mg/L. The light intensity was maintained at 2000 to 2500 lux with 12 h light and dark cycle. Combined live feeds such as microalgae *Isochrysis galbana* (10^4 cells/ml), *Thalassiosira subtilis* (10^3 cells/ml) and bacteria (starch solution added for development of microbial population) was given as daily nourishment. Water exchange was done at a weekly interval. Asexual mode of reproduction such as cell division/binary fission was observed (Das, 1953).

The planktonic juveniles were developed after three days with a combined diet, and six h to 48 h later, they started settling onto substratum. Subsequent offsprings were observed after 7 to 10 days. The juveniles were characterized for their photo-tactic movement at the surface water in a group. The population density of juveniles ranged from 2000 to 5000 individuals/ml in a group.

Discussion

Folliculinids inhabit fresh and marine waters. A few of them are known to form the extensive blue mats in deep sea hydrothermal vents from their pigments (Kouris *et al.*, 2007). Folliculinids have a symbiotic association with bacteria, and some of them have also been reported as potential threats to the Caribbean corals (Croquer *et al.*, 2006a; Sweet *et al.*, 2019; Montano *et al.*, 2020). Generally, reports on their occurrences are relatively less globally and rare in India which might be owing to the lack of appropriate sampling method, preservation and poor knowledge of their biology. Information and records will increase if these issues are fixed. The present study reports the occurrence of *Folliculinopsis producta* from Indian coastal waters after a gap of four decades against the previous report in 1979. Das (1953) reported *Parafolliculina indica* and *Folliculina andrewsi syn. Folliculinopsis producta* from Chennai coastal water. The characteristics of *Folliculinopsis producta* were originally described by Wright (1859) and was re-described based on the specific numbers of somatic kineties. The number of somatic kineties was reported as 100 for *F. andrewsi* and 60-70 for *F. producta* (Das, 1953; Ji *et al.*, 2004). The *Folliculinopsis producta* and *Folliculinopsis andrewsi* resemble morphologically despite the differences in their numbers of somatic kineties. The size of *Folliculinopsis producta* reported in coastal waters of India (length-520 to 950 μm; width-23 to 39 μm) is comparable with the ones recorded in Qingdao (length-800 to 1000 μm; width-30 to 40 μm) (Wright, 1859). The size difference among the populations reported elsewhere is perhaps due to the different life stages after the settlement on the substratum and prevailing environmental conditions (Ji *et al.*, 2004). Additionally, a recent study reported the genomic rRNA phylogeny of *F. producta* that they are strongly related to *Eufolliculina* and clustered with stentor *Mari-stentor* (Miao *et al.*, 2009, 2005; Schmidt *et al.*, 2007). The present study optimized the ambient environmental parameters for the long-term (3 months) culture maintenance of *Folliculinopsis producta* for the first time. Accordingly, the water temperature of 26-30 ºC, salinity of 29-31 psu, pH of 7.9 to 8.3 and dissolved oxygen of 4.5-7.5 mg/L were found optimum ranges for the population growth of *F. producta* equally, proper feeding also played a crucial role in the culture.

The study by Kouris *et al.* (2007) reported that the folliculinid is well known for its feeding on microbes and symbiotic association with bacteria. Therefore, the culture was initially maintained with bacterial feed alone, as reported previously by other researchers (Song *et al.*, 2003; Ji *et al.*, 2004), but this did not yield more offspring and faster growth. Hence the combination of feeds such as microalgae *Isochrysis galbana* (10^4 cells/ml), *Thalassiosira subtilis* (10^3 cells/ml) and bacteria was fed. The ingestion of microalgae into the buccal cavity through the ciliary activity of peristomal lobes was observed *in-vivo*. The microalgal di*et alo*ne provides nourishment for free swimming planktonic juveniles. However, the growth and settlement of free swimming planktonic juveniles have been promoted by the algal diet supplemented with a microbial population. The microbial populations may be provided as cultured yeast cells or by adding rice grain or boiled starch solution as a carbon source. Ciliated protozoa like *Vorticella* spp., *Paramecium* spp. fed with bacteria were reported to crash cultures within a few days due to the intense bacterial activity and unknown reasons (Bick, 1972). However, advantageously, it was observed that the combined diet consisting of microalgae, diatoms and microbes with periodical water exchange enhanced the laboratory culture of folliculinids. Interestingly, after a week, the folliculinids were grown on the nylon fiber with a density of 50 to 230 individuals/0.5 cm fiber substrate. These populations on the fibers can be handled with ease and used for the *in-vivo* bioassays.

In situ culture of folliculinids under ambient conditions would be an advantage for further understanding of (1) their biological functions such as feeding, growth rate, reproduction and physiological aspects and (2) for exploring bioactive compounds, biochemical values, the chemistry of the pigments etc. These organisms could also be a potential source of research material for studying environmental health due to their faster growth rate, the large size of the single cell, sedentary nature and rapid activities like extension, contraction and ciliary action.

Acknowledgements

Authors are thankful to the Ministry of Earth Sciences, Govt. of India for implementing the Marine Ecotoxicology programme at National Centre for Coastal Research (NCCR), Chennai [MoES/EFC/28/2018-PC-II dated 12/11/2018]. The authors thank the Director, NCCR for providing the necessary facilities and encouragement for the present work. This article has the NCCR contribution Number NCCR/MS384.

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