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Visualization of Thecal Plates of Lightly Armored Dinoflagellates *Cryptoperidiniopsis brodyi* and *Pfiesteria piscicida* (Dinophyceae)

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Abstract

Early studies claimed that heterotrophic dinoflagellates *Pfiesteria piscicida* and related genera may produce a putative water-soluble toxin that causes death of fish and other marine animals. Several methods were tested to visualize plate morphology of *Cryptoperidiniopsis brodyi* and *Pfiesteria piscicida*. Cellulose plates of cells were exposed and visualized by a membrane stripping method using Triton X-100. While calcofluor M2R white stain could readily bind to the thecal plates, details of the plate tabulation were difficult to observe. Fixation with osmium tetroxide (OsO_4) produced well preserved cells with little morphological distortion, but thecal plates could not be visualized. Scanning electron microscopy (SEM) observation using the membrane stripping method showed distinctive plate tabulations between *C. brodyi* and *P. piscicida* suggesting that this method is a useful tool for morphological identification of lightly armored dinoflagellates.

Key Words : *Cryptoperidiniopsis brodyi*, Harmful algal blooms, Membrane stripping method, Plate tabulations, *Pfiesteria piscicida*, Red tide

1. Introduction

Armored dinoflagellates possess thecal plates that are specific in number and arrangement. The number and orientation of these plates are important characters to identify armored heterotrophic dinoflagellates[1]. Several techniques have been developed to visualize dinoflagellates plates. One of the most rapid and simple methods uses the fluorescent stain calcofluor white (fluorescent brightness 28). This fluorescent stain specifically binds cellulose and other β-linked glucans[2] and readily binds to dinoflagellate thecae, of which cellulose is a main component[3]. The stain has been used to distinguish small morphological details of thecate dinoflagellates such as *Alexandrium* species using light microscopy.

Definitive identification of armored dinoflagellates mainly relies on plate tabulation observed by scanning electron microscopy (SEM). Currently, there are several methods to visualize plate morphology. Fixation of specimens with formaldehyde or glutaraldehyde (GTA) is commonly used. However, thecal plates of armored species vary in thickness and cellulose plates of thinly armored dinoflagellates are hidden beneath the outer amphiesmal membranes in well-preserved cells. Suture-swelling techniques and cell-stripping with ethanol have been developed to expose the underlying cellulose plates[4,5]. Both methods showed inconsistency in stripping the outer membranes of swollen cells, especially in the critical sulcal area.

Membrane stripping method using the detergent Triton X-100 has been suggested as an effective approach to removing the outer membranes[6]. This method

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has been shown to allow visualization of patterns of thecal pores and sulcal plates that are difficult to observe using conventional methods. Alternatively, fixation with osmium tetroxide (OsO_4) could be used to observe whole cells[7]. This method is ineffective in removing the membranes but permits to visualization of intact cells that are not osmotically distorted and flagella are retained intact. In this study, several methods were tested to clearly visualize thecal plates of lightly armored dinoflagellates *Cryptoperidiniopsis brodyi* Steidinger et Litaker and *Pfiesteria piscicida* Steidinger et Burkholder.

2. Materials and Methods

2.1. Cultures

Four strains of *C. brodyi* (CBDE14, CBWA11, CBHU1, and CBDE2 from Australia) and two strains of *P. piscicida* (CCMP1975 and CCMP1974 from USA) were prepared for morphological study. Cultures were maintained in 15 psu f/2 medium at 20°C in the dark by adding *Rhodomonas salina* prey every 2-3 days. Before harvesting cultures for SEM, *R. salina* was added every day to increase cell densities of the dinoflagellates.

2.2. SEM fixation

Five ml of cultures and 5 ml of 4% osmium tetroxide were combined in a 1 : 1 ratio, mixed gently, and incubated for 30 min. To reduce cell damage due to osmotic change, cells were incubated with cell culture medium diluted in water to a concentration of 40%. Cells were rinsed with distilled water and stored at 4°C until dehydration was carried out. The samples were dehydrated in a graded methanol series for 15 min per step: 30, 50, 70, 90, 100, followed by 100% dry acetone. The mounted specimens were then critical-point-dried from CO_2 after a 30 min liquid CO_2 rinse, sputtercoated with gold-palladium, and examined under a JEOL 35C scanning electron microscope (JEOL, Tokyo, Japan).

2.3. Membrane stripping SEM protocol

Specimens for SEM observations were prepared using a membrane stripping method[6] to remove the outer membranes of lightly armored dinoflagellates and to allow visualization of plate tabulations. Samples were combined 1 : 1 with 15% Triton X-100 diluted in double distilled water, and incubated in ultrasonic water bath with gentle sonication at 20-29°C for 1 h. After centrifugation of cultures at 1500 x g, the cell pellets were incubated in 40% culture medium for 30 min at room temperature. The cells were fixed with 4% glutaraldehyde. Samples were dehydrated in a graded methanol series as described in the SEM protocol using osmium tetroxide for fixation.

2.4. Fluorescent stain with calcofluor white M2R

A working stock solution of 10 mg·mL^{-1} of calcofluor white M2R (Sigma Chemical Co.) was prepared with distilled water and stored at room temperature until use. Living dinoflagellates were fixed in 2% glutaraldehyde. A few drops of 1% calcofluor white solution were added to fixed samples on coverslips. Photographs were taken on an Axioskop 2 Plus microscope (Zeiss, Gottingen, Germany) with UV filter set connected to AxioCam HR digital camera (Zeiss, Gottingen, Germany). The UV filter arrangement was for 330-380 nm excitation and 420 nm emission wavelength.

3. Results

3.1. Fluorescent stain by calcofluor white M2R

Zoospores fixed in glutaraldehyde immediately fluoresced upon addition of calcofluor. Fig. 1 shows *P. piscicida* and *C. brodyi*cells stained with calcofluor. The fluorescence light micrograph (Fig. 1a) shows a triangular 1a (intercalary plate) that is one of the diagnostic characteristics of the species. Thecal plates staining by calcofluor has also been shown in other *Pfiesteria*-like species[8,9]. However, this method did not provide clear and complete plate patterns of *C. brodyi* and *P. piscicida* due to their possession of very thin thecal plates.

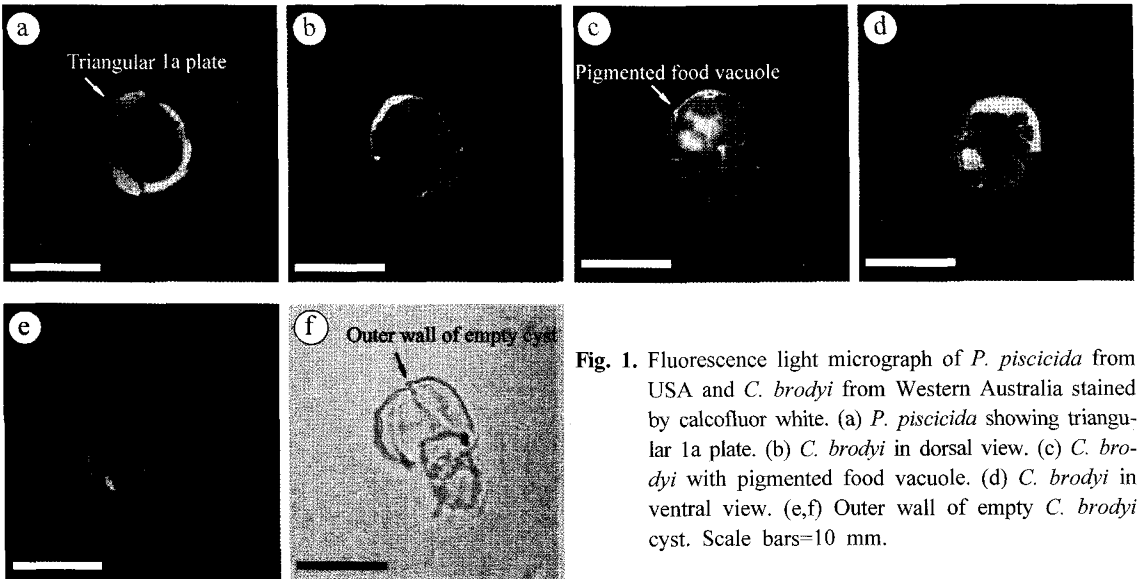

Fig. 1. Fluorescence light micrograph of *P. piscicida* from USA and *C. brodyi* from Western Australia stained by calcofluor white. (a) *P. piscicida* showing triangular 1a plate. (b) *C. brodyi* in dorsal view. (c) *C. brodyi* with pigmented food vacuole. (d) *C. brodyi* in ventral view. (e,f) Outer wall of empty *C. brodyi* cyst. Scale bars=10 mm.

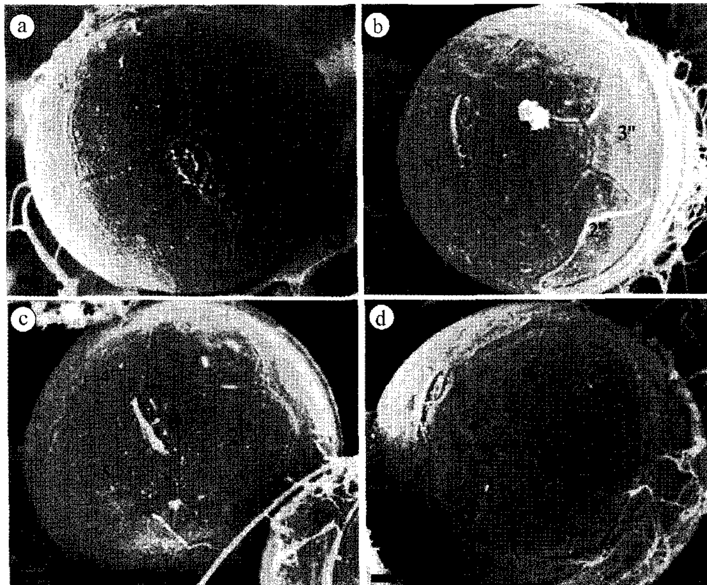

Fig. 2. Scanning electron micrographs of *C. brodyi* in apical views, treated by membrane stripping method. (a) CBDE14 from Derwent River, Tasmania. (b) CBWA11 from Brunswick River, Western Australia. (c) CBHU1 from Huon River, Tasmania. (d) CBDE2 from Sullivans Cove, Derwent River, Tasmania. Scale bars=1 mm.

3.2. Membrane stripping protocol for SEM

Good results were obtained when cells were stripped with 15% Triton X-100 for 1 h and treated by a post-stripping cell-swelling step. The apical pore complex and sulcal plates were clearly visualized by this protocol. Concentrations between 10 and 20% of Triton X-100 showed similar results in stripping the outer membranes of zoospores. Fig. 2a-d shows epithecae of

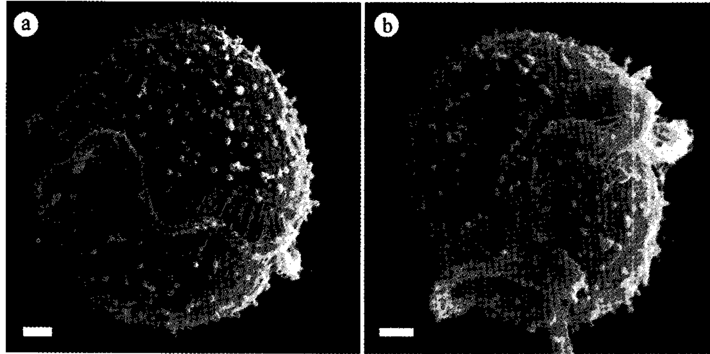

Fig. 3. Scanning electron micrographs of *C. brodyi*zoospores (CBWA12) fixed by 4% osmium tetroxide. (a) Dorsal view of *C. brodyi*. (b) Ventral view of *C. brodyi*. Scale bars=1 mm.

Australian *C. brodyi* consisting of plate Po, cp, X, 5' and 6". Larger cells were more readily stripped of their outer membranes than smaller cells.

3.3. SEM protocol using fixation with osmium tetroxide

This procedure preserved the outer membranes and flagella of zoospores. *Cryptoperidiniopsis brodyi* zoospores (CBWA11) were well preserved with little morphological distortion, and the transverse and longitudinal flagella were retained (Fig. 3). This protocol could minimize cell shrinkage or swelling, and permitted intact cells. However, the outer membranes of *C. brodyi* were not stripped, and plate structures were not visualized.

4. Discussion

4.1. Comparison between the osmium tetroxide fixative protocol and membrane stripping protocol

The number and arrangement of thecal plates are important features to identify armored dinoflagellates. Unarmored dinoflagellates are generally identified by morphological characters such as the apical groove, cingulum-sulcus juncture, and flagella[10]. Selection of a proper preparation protocol for SEM is critical for the morphological study of the dinoflagellates. Osmium tetroxide protocols for SEM have been successfully used for morphological study of many unarmored dinoflagellates. When this method was applied to *P. piscicida*, the cells retained their outer membranes and did not reveal their underlying cellulose plates. This protocol thus was of little value in taxonomy of *Pfiesteria* and closely related dinoflagellates.

To overcome the difficulty of removing the outer membranes of thinly armored species, several SEM preparation protocols were developed. Stripping off the membranes with ethanol or swelling of the sutures between cellulose plates have been suggested by Steidinger et al.[4] and Truby[11]. However, the two methods were inadequate to resolve the complete tabulation because the protocols resulted in incomplete stripping and excessive damage to cells[5,6]. The membrane stripping protocol using Triton X-100 was developed for lightly armored dinoflagellates by Mason et al.[6]. Triton X-100 can solubilize the outer membranes because the compound, a gentle non-ionic detergent, removes membrane-bound proteins[12]. In the present study, Triton X-100 could effectively remove outer membranes of *C. brodyi* and other related dinoflagellates. Although small and newly excysted zoospores were not stripped off cleanly, complete plate patterns of most large zoospores could be visualized. Zoospores can be enlarged in size in the presence of prey in their food vacuole, thus additions of prey cells prior to harvesting zoospores may facilitate the stripping of outer membranes of the cells.

4.2. Cells stained with calcofluor white

Calcofluor white could stain *P. piscicida* cells in a simple procedure including glutaraldehyde fixation. In a previous study by Fritz and Triemer[3], post-fixation in buffered 1% osmium tetroxide was added to the procedure for some species, but *P. pisicicda* could be readily stained without the post-fixation step. Partial thecal plate patterns of *P. pisicicda* could be visualized by calcofluor white, but this method was inadequate to reveal the complete plates.

5. Conclusions

Several methods were tested to visualize plate patterns of *C. brodyi* and *P. piscicida*. The membrane stripping protocol with Triton X-100 provided good quality SEM photographs for taxonomy of these armored dinoflagellates.

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