Visualisation of the Symbiosome Membrane Surrounding Cnidarian Algal Cells

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Abstract

Virtually nothing is known of the role of the cnidarian symbiosome, primarily due to the difficulty in visualising its membrane. We used the fluorescent dye MDY-64 to stain symbiosome membranes surrounding algae of the anthozoan *Zoanthus robustus*. MDY-64 did not stain cultured symbiotic dinoflagellates, confirming that this dye binds to a membrane of host cell origin. Another fluorescent dye, amino-chloromethylcoumarin (CMAC) stained the cytoplasm of both endoderm cells and algal cells from the zoanthid, a coral and an anemone. By drawing a suspension of endoderm cells from *Z. robustus* back and forth (5–7 times) through a hypodermic needle, we obtained approximately 73% of the algae in intact symbiosomes, with only 6% of the algae remaining in intact endoderm cells, and 21% free of both endoderm cell and symbiosome. About 15 additional passages of the cells through the needle removed the symbiosome membranes, leaving approximately 85% of the algae free of all host cell material. Use of detergents to remove the endoderm cell plasma membrane damaged both the symbiosome and algal membranes. Transmission electron microscopy showed variable numbers of membranes surrounding the algae. The ability to isolate dinoflagellate cells with and without symbiosome membranes will allow studies of the role of this membrane.

Keywords: Symbiosome, Cnidaria, fluorescent dyes, symbiosis, zoanthid

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1. Introduction

Many corals and other marine invertebrates depend upon their mutualistic association with symbiotic dinoflagellates. Photosynthetic products of the algae are transferred to the animal partner, and may satisfy a large part of the animal's nutritional requirements (Trench, 1971; Muscatine et al., 1983). In return, the algae may receive organic and inorganic nitrogen and phosphorus compounds, and sulphur amino acids from their hosts (Cates and McLaughlin, 1979; Deane and O'Brien, 1981a and b; Cook, 1983; Rahav et al., 1989).

In most marine coelenterates, the symbiotic algae are found within the host's endoderm cells (Muscatine et al., 1975), where each algal cell is surrounded by a host-derived membrane known as the perisymbiotic or symbiosome membrane (Rands et al., 1993). The symbiosome has been defined as "a membrane bound compartment containing one or more symbionts and certain metabolic components and located in the cytoplasm of eukaryotic cells." (Roth, 1988). The symbiosome membrane physically separates the alga from the endoderm cell, and all substances which enter and leave the alga must cross both host membranes.

Symbiosome formation is common in intracellular symbioses. The best studied of these are the symbioses between leguminous plants and nitrogen fixing bacteria (Price et al., 1987; Rosendahl and Jochimsen, 1995; Hernández et al., 1996) in which the transport properties of the symbiosome membrane play a crucial regulatory role. Organic carbon, primarily in the form of dicarboxylate, is actively transported from the host plant cells, across the symbiosome membrane and into the symbiotic bacteroid. In return, the bacteroids supply their host with fixed nitrogen (Ou Yang et al., 1990; Udvardi et al., 1990). If the symbiosome membrane lacks the dicarboxylate transport molecule, nitrogen fixation does not occur (Ronson et al., 1981; Finan et al., 1983). The symbiosome membrane is therefore critical to the functioning of these symbiotic associations, and is the site where the rate limiting step of carbon flux to the endosymbiont is found. In contrast, virtually nothing is known of the role of the symbiosome in cnidarian-dinoflagellate symbioses, although there is some evidence to suggest that the cnidarian symbiosome membrane controls the flux of inorganic phosphate from the host to the algal cell in the anemone *Anemonia viridis* (Rands et al., 1993). This lack of knowledge is primarily due to the difficulty in detecting the presence of the membrane, as it is closely appressed to the membranes of the symbiotic alga. As the symbiosome membrane defines the immediate environment of the endosymbiont, it may also play a vital role in the regulation of photosynthesis, algal respiration and the translocation of photosynthate to the host in cnidarian symbioses.

Unlike the tough cells which contain the bacteroids in the roots of leguminous plants, anthozoan cells are often fragile and may secrete copious

amounts of mucus, which has made the collection of host cells from these organisms difficult (Gates and Muscatine, 1992), and has hindered the study of these symbioses at the cellular level. While several reports have identified the endoderm cell membrane using light microscopy (Gates and Muscatine, 1992; Muscatine et al., 1994), until now the symbiosome membrane has only been viewed through the use of electron microscopy (Gates and Muscatine, 1992; Rands et al., 1993; Wakefield and Kempf, 2001).

We have developed a simple method to visualise these symbiosome membranes within living cnidarian endoderm cells, using fluorescent dyes, and to extract algae still in symbiosomes.   The ability to isolate symbiotic dinoflagellate cells with and without the symbiosome membrane, will allow studies of the role of this membrane in controlling fluxes of nutrients and other compounds between the symbionts.

2. Materials and Methods

Isolation of algae (dinoflagellates)

The zoanthid, *Zoanthus robustus* Carlgren, was used as the model organism for this study. Zoanthids were collected from two sites in the Sydney region (Latitude 33°50'S, 151°15'E); from Port Jackson at a depth of 9 m, and from Botany Bay at a depth of 3 m. Following collection, the zoanthids were maintained in aquaria at the University of Sydney, with a constant inflow of recirculated seawater, under a 12 h light : 12 h dark regime, with a light intensity of 35 μmol photons.$m^{-2}.s^{-1}$ from fluorescent lamps.

Endoderm cells were collected from zoanthids by removing the animal from the water and making a small incision in the side of the column with a sharp razor blade. Squeezing the zoanthid released the endoderm cells, which were collected using a pipette, and viewed using differential interference contrast (DIC) microscopy. This method allows the collection of large numbers of viable endoderm cells (about 40% of the endoderm cells were motile at the time of collection).   Following harvesting of the endoderm cells, zoanthids were returned to the aquarium; they reinflated within about 30 min and regained their original density of endoderm cells and symbiotic algae, within 2 to 4 weeks.

For comparison, intact endoderm cells containing algae were also collected from the coral *Plesiastrea versipora* (collected from Port Jackson) after they were expelled from the coral host following heat shock (Gates et al., 1992; Muscatine et al., 1991), and from the anemone *Aiptasia pulchella* (donated by Sydney Aquarium) by cutting the anemone in half and gently squeezing the body using a glass homogeniser.

In some instances, algae were also removed from these animal hosts using mechanical techniques. For the coral, the algae were collected by brushing the surface of the coral with a toothbrush (Ritchie et al., 1995) and centrifuging the slurry to pellet the algae. Algae were collected from the anemone by vigorously homogenising the whole animal in a glass homogeniser (Grant et al., 2001).

Staining of the symbiosome membrane with MDY-64

To view the symbiosome membrane, suspensions of endoderm cells (500 µl, $>5 \times 10^5$ cells/ml), were stained for 20 min with the steryl fluorescent dye MDY-64 ($C_{24}H_{24}N_4O$ from Molecular Probes) at a concentration of 2 µM in seawater. Following staining the cells were rinsed twice with 1.2 ml seawater, with centrifugation at 350 g for 1 min. The cells were then placed on a microscope slide and viewed under differential interference contrast (DIC) illumination and fluorescence (excitation 395–440 nm, beam splitter 460 nm, barrier filter 470 nm) using a Zeiss Axiophot fluorescence microscope. MDY-64 is a yeast vacuole membrane marker which fluoresces only when incorporated into a membrane. It binds to phospholipids but its specificity has not been further defined (Dr. Tony Russell, Molecular Probes, pers. comm.).

Staining of the cell cytoplasm with CMAC

The cytoplasm of endoderm cells was stained with the fluorescent dye CMAC (7-amino-4-chloromethylcoumarin; CMAC, Molecular Probes) at a concentration of 10 µM in seawater for about 45 min before being rinsed twice with seawater, and viewed with a fluorescence microscope (excitation 365 nm, beam splitter 395 nm, barrier filter 420 nm). CMAC passes freely through the cell membrane before undergoing what is believed to be a glutathione S-transferase-mediated reaction, producing a cell-impermeant reaction product (Zhang et al., 1992). After about 25 min incubation in CMAC, MDY-64 could be added to the cell suspension so that the cells could be stained with both dyes simultaneously.

Staining of cultured algae and algae free of host membranes

Cultures of symbiotic dinoflagellates, originally isolated from the anemone *Aiptasia tagetes*, the coral *Montipora verrucosa*, the jellyfish *Cassiopea xamachana* and the clams *Tridacna maxima* and *T. crocea*, were obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) culture collection (Hobart, Australia). Cultured algae were grown in f/2

medium (Guillard and Ryther, 1962) at 24°C, under a 16 h:8 h light:dark cycle, at an irradiance of 25 μmol photons.m^{-2}.s^{-1}. The f/2 medium was replaced monthly and the cells were subcultured every 3 months. Samples from each culture were stained with MDY-64, and a lack of staining confirmed that this dye did not bind to algal membranes. Suspensions of free algal cells which had been freshly isolated from macerated tissue of the coral *P. versipora* and the anemone *A. pulchella* were also stained with MDY-64. Freshly collected endoderm cells from *Z. robustus* were used as positive controls.

Isolation of algae in symbiosomes and algae free of host cell membranes

Zoanthid algae, still within intact symbiosomes, were isolated from the endoderm cells by mechanical disruption. A 500 μl suspension of endoderm cells ($>5 \times 10^5$ cells/ml) was drawn gently back and forth through a 23 gauge metal hypodermic needle fitted to a 5 ml syringe. Five to 7 plunges of the syringe were required to remove the majority of the endoderm cells without damaging the symbiosome membranes. A further 15 plunges of the syringe were required to free the algae from the symbiosomes. Following isolation, algae were collected in microfuge tubes and washed twice with 1.2 ml seawater by centrifugation at 350 g for 1 min. The fluorescent dyes MDY-64 and CMAC were then used to determine the extent to which endoderm cells and symbiosomes had been removed.

Isolation of algae using detergents

Detergents were also used to rupture the endoderm cell membrane in an attempt to release algae in intact symbiosomes. Triton X-100 (Calbiochem), Brij 45 (Technicon), decyl sucrose (Calbiochem), digitonin (Calbiochem) and sodium dodecyl sulphate (Calbiochem) were used individually within the range of 0.001% to 0.05% in filtered seawater, for between 2 and 15 min. After incubation with a detergent, the cells were washed 5 times by centrifugation with filtered seawater, and then drawn back and forth through the needle twice to rupture the endoderm cells before staining with MDY-64 and CMAC.

Fluorescence microscopy

Following staining, the proportion of algal cells surrounded by symbiosome membranes only and those remaining in symbiosomes within intact endoderm cells, was estimated by counting at least 100 cells per experiment. To ensure that the counted cells were selected randomly, the cells were initially viewed through a filter (excitation 546 nm, beam splitter 580 nm, barrier filter 590 nm),

which allowed only chlorophyll fluorescence to be seen. After an area had been selected, other filters were used to view the MDY-64 and CMAC staining as defined above

Transmission electron microscopy

Transmission electron microscopy (TEM) was also used to visualise animal and algal cell membranes. Preparations of intact endoderm cells, algae within symbiosomes and clean algal cells free of all host membranes, were examined using conventional fixation or freeze substitution techniques for transmission electron microscopy. Conventional preparation for TEM included immersion of samples in 2.5% glutaraldehyde in phosphate buffer, post-fixing in 1% osmium tetroxide and dehydration through an ethanol series (30–100%) and embedding samples in Spurr's low viscosity resin. For freeze substitution, cells from each preparation were pelleted by settlement, drawn into cellulose microcapillary tubes and quickly frozen in liquid nitrogen using a Leica MM80 E slam freezing machine. Freeze substitution was carried out using the method of Hohenberg et al. (1994). The resin blocks containing the sample tubes were cut with a diamond knife using a Leica Ultracut S ultramicrotome. The cut sections were stained with osmium vapour for 6 h to improve contrast, and viewed using a Philips EM400 transmission electron microscope (TEM).

Using conventional fixation techniques, the cell preparations were centrifuged at 350 g for 1 min, and fixed in 3% glutaraldehyde / 0.1 M phosphate buffer with 0.6 M sucrose to prevent cell lysis. Cut sections were stained with uranyl acetate and Reynolds lead citrate (Reynolds, 1963).

3. Results

When viewed with a fluorescence microscope, endoderm cells from *Z. robustus* that had been stained with the dye MDY-64 showed a bright green band surrounding each symbiotic alga (Fig. 1). When there was more than one alga in an endoderm cell (about 25% of the endoderm cells collected from *Z. robustus*), each alga was surrounded by its own green fluorescent band (Fig. 1). If an alga was in the early stages of division, a single green band surrounded both daughter cells. At a later stage of division, a green band was visible between the dividing cells. MDY-64 also bound to a host cell membrane surrounding the symbiotic algae in endoderm cells from the coral *Plesiastrea versipora* and the anemone *Aiptasia pulchella* (results not shown).

MDY-64 generally did not penetrate the alga. When MDY-64 penetration did occur, the algal cells appeared to be bleached under DIC illumination, and showed a lower intensity of chlorophyll autofluorescence (Fig. 2).

MDY-64 did not stain cultured algae from the anemone *Aiptasia tagetes*, the coral *Montipora verrucosa*, the jellyfish *Cassiopea xamachana* or the clams *Tridacna maxima* or *T. crocea* (Fig. 2). These algae were free of all animal cell material as they had been cultured autotrophically for many generations. Similarly, MDY-64 did not stain algal cells which had been freshly isolated from macerated *Z. robustus*, the coral *Plesiastrea versipora*, or the anemone *Aiptasia pulchella* providing further confirmation that this dye binds to a membrane of host cell origin. All algal cells showed only bright red chlorophyll autofluorescence unless they were damaged or degenerate (bleached cells with low chlorophyll autofluorescence) when MDY-64 penetrated the alga (Fig. 2) indicating that MDY-64 does not enter intact algae.

When zoanthid endoderm cells stained with CMAC were viewed under the fluorescence microscope, the cytoplasm of both the endoderm cell and the symbiotic algae fluoresced blue (Fig. 3). Cultured algae stained with CMAC showed results identical to those found in the zoanthid algal cells contained within the endoderm cells, with the dye visible between the chloroplasts (results not shown).

When endoderm cells were drawn back and forth through a needle (5–7 times) 72.5%±13.9% (mean ± S.D.) of algae within intact symbiosomes (n=10 experiments) were obtained, with only 6.4%±3.8% of the algal cells remaining in intact endoderm cells. The remainder of the algal cells (21.1%±11.3%) were free of host cell material, including the symbiosome membrane. A further 15 passages of the cells through the needle allowed us to obtain 85%±11.3% (n=6) free algae which did not stain with MDY-64 (Fig. 4). The remaining algae retained a symbiosome membrane, but none retained the endoderm cell cytoplasm.

When algae were isolated by the use of detergents, the largest number of algae isolated still within symbiosomes (65.1%±14.7%, n=14) was obtained with decyl sucrose (0.02% for 10 min). However, all detergents, including Triton X-100 (Calbiochem), Brij 45 (Technicon), decyl sucrose (Calbiochem) and digitonin (Calbiochem), at a range of concentrations between 0.001% to 0.05% caused damage to the symbiotic algae, resulting in penetration of MDY-64 into about 60% of the isolated algal cells.

Results from both types of TEM preparations proved inconclusive in identifying symbiosome membranes. Intact endoderm cells, isolated algae still within symbiosomes and algal preparations free of all host material, all showed variable numbers of membranes surrounding the algae, ranging from 4 to 14 (results not shown). While it was easier to distinguish the membranes on the samples which had been prepared using freeze substitution techniques, their numbers were just as variable.
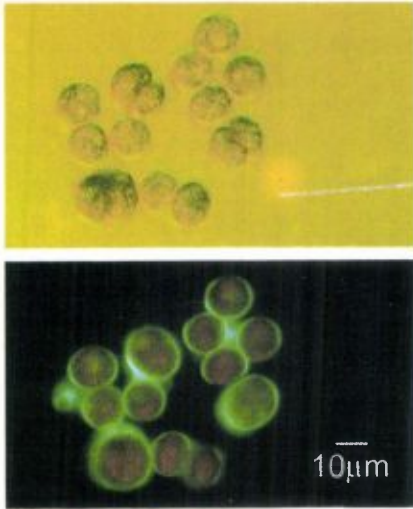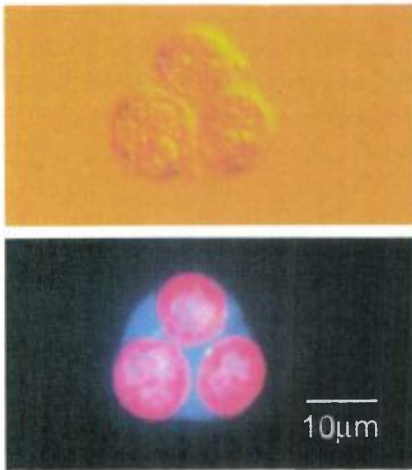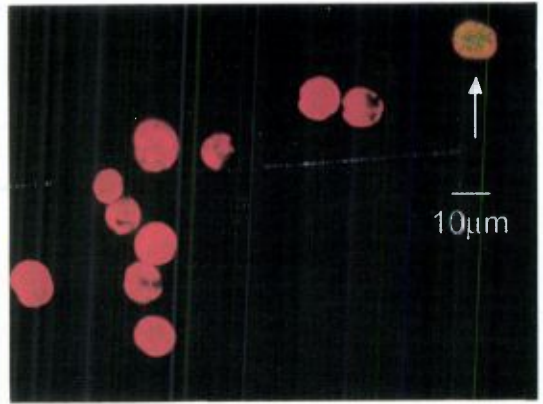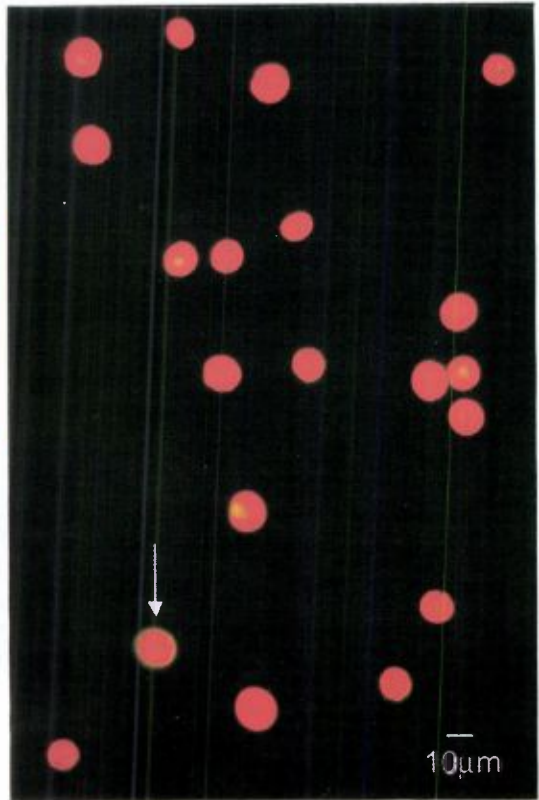
Figure 1a+b.

Figure 2.

Figure 3a+b.

Figure 4.

Figs. 1–4. See legends on opposite page.

4. Discussion

The method of endoderm cell isolation described here allows the collection of large numbers of endoderm cells with active flagella. Previous methods used enzymes (Gates and Muscatine, 1992), Ca^{2+} free seawater (Muscatine et al., 1998) or cold/heat shock (Gates et al., 1992; Muscatine et al., 1991), which resulted in the collection of relatively few endoderm cells containing symbiotic algae. These methods may also disrupt cellular metabolism. *Z. robustus* is therefore an ideal model for the study of cnidarian endoderm cells and symbiosomes, due to the ease of collection of algae in intact host cells.

MDY-64 did not stain cultured algae from the anemone *Aiptasia tagetes*, the coral *Montipora verrucosa*, the jellyfish *Cassiopea xamachana* or the clams *Tridacna maxima* or *T. crocea*. The fact that this dye did not stain these cultured algae, nor those which had been freshly isolated using mechanical techniques from locally collected cnidarians (*P. versipora* and *A. pulchella*) shows that this dye binds to a membrane of host cell origin. Given the location of the symbiosome membrane, and the staining patterns of MDY-64 on dividing algal cells, we believe that this membrane is the endoderm cell symbiosome.

Drawing the endoderm cells back and forth through a needle was shown to be an effective method of rupturing the endoderm cell plasma membrane to release algae still surrounded by intact symbiosome membranes. Additional drawing of the cells through the needle also effectively resulted in the removal of the symbiosome membranes. However, the force used to draw the cell suspension

Figs. 1–4. See opposite page.

Figure 1. Endoderm cells collected from the zoanthid *Zoanthid robustus*. a. Endoderm cells viewed under differential interference contrast microscopy. b. MDY-64 staining of the symbiosome band surrounding each algal cell.

Figure 2. MDY-64 staining of cultured symbiotic algae derived from the clam *Tridacna maxima*. Damaged cells showed penetration of MDY-64 into the algal cells (arrow), but intact cells did not stain with MDY-64 at all. Algae cultured from other symbiotic invertebrates showed similar results.

Figure 3. A single endoderm cell from the zoanthid *Z. robustus* containing 3 algal cells. a. Endoderm cell viewed under differential interference contrast microscopy. b. CMAC staining of the endoderm cell cytoplasm.

Figure 4. MDY-64 staining of symbiotic algae which had been isolated from the zoanthid *Z. robustus* following removal of the endoderm cell and the majority of the symbiosome membranes, by dragging the cells back and forth through a needle and syringe 20 times. Algal cells which were free of host cell material did not show any staining with MDY-64 whereas cells which retained the symbiosome membrane showed a green band around the algal cell (arrow).

back and forth through the syringe needed to be carefully controlled. In some of the early attempts many of the algal cells were ruptured using this technique. Both larger and smaller sized needles were also found to be less effective, resulting in fewer endoderm cells being removed and more damage to the algal cells respectively.

This method of removing host cell material was shown to remove the symbiosome membrane from more cells and be less damaging to the algal cells than the use of detergents. All detergents even at a concentration of 0.001%, resulted in MDY-64 penetration of about 60% of algal cells indicating damage.

A large number of membranes of algal origin surround symbiotic dinoflagellates in many marine cnidarian hosts, with the host-derived symbiosome membrane lying immediately outside them (Taylor, 1968; Rands et al., 1993). As the innermost of the animal cell membranes is the symbiosome membrane, and the animal and algal cell membranes are indistinguishable using light microscopy, it has been difficult to pinpoint the symbiosome membrane in previous ultrastructural studies of cnidarian endoderm cells (Smith, 1979). Results of our freeze substitution TEM studies were inconclusive in the identification of the symbiosome membrane, as the number of membranes surrounding each algal cell varied between free algal cells, between cells which retained the symbiosome membrane, and between intact endoderm cells. A possible explanation for this variation in the number of membranes surrounding the alga was given by Wakefield et al. (1998; 2000). Cultured symbiotic dinoflagellates are known occasionally to shed their thecae, when changing from the motile to the vegetative phase of their life cycle. These authors suggest that this process also occurs, although irregularly and at a slower rate, when the dinoflagellates live symbiotically inside invertebrate host cells. Unlike the cultured symbionts, symbiotic algae would accumulate these shed thecae, inside the symbiosome membrane, resulting in variable numbers of membranes surrounding the alga. Using immunogold labelling of fixed material isolated from the anemone *Aiptasia pallida*, Wakefield and Kempf (2001) recently concluded "that the definitive symbiosome membrane is a single host-derived membrane, whereas the remainder of the underlying apparent membranes surrounding the algal cell are symbiont-derived".

Our report is the first one describing a method for viewing the symbiosome membrane without damaging the endoderm cell or the symbiotic dinoflagellates. The fact that MDY-64 labels only the symbiosome membrane suggests that the symbiosome membrane differs in its composition from other animal or algal membranes in *Z. robustus*. The ability to identify the presence of the symbiosome membrane in intact endoderm cells is vital to further the understanding of the structural relationships between marine invertebrates and their symbiotic dinoflagellates. Specific staining will allow us to work with freshly isolated algae both with and without symbiosome membranes, thus

allowing us to identify control points in the nutritional interactions between host and symbiont which regulate these symbioses. Using this method, we have recently shown that in algae isolated from a coral and a zoanthid, symbiosomes are not required for host regulation of algal photosynthesis and that removal of symbiosome membranes by our method does not disrupt the physiological function of the algae (Grant et al., submitted). In addition, being able to identify the proportion of algal cells which are still surrounded by host cells and/or symbiosome membranes, may also help in understanding the variability which is inherent both within and between laboratories in experiments involving isolated symbiotic dinoflagellates.

Acknowledgments

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