Ultrastructural observation of a symbiotic dinoflagellate
Zooxanthella nutricula from radiolarians collected off
the northwestern coast of Okinawa Island, Japan

Tomoko Yuasa*1, Takeo Horiguchi2, and Osamu Takahashi3

* Corresponding author: T. Yuasa  E-mail: tyuasa@u-gakugei.ac.jp

1 Department of Biology, Tokyo Gakugei University, Koganei, Tokyo 184–8501, Japan
2 Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060–0810, Japan
3 Department of Astronomy and Earth Sciences, Tokyo Gakugei University, Koganei, Tokyo 184–8501, Japan

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Abstract  The ultrastructure of a symbiotic dinoflagellate Zooxanthella nutricula within the radiolarian Didymocyrhis tetrathalamus collected off the northwestern coast of Okinawa Island, Japan was examined in the symbiotic state and the cultured motile stage. In hospite, Z. nutricula had a coccoid morphology, and the typical dinoflagellate structures, i.e., cingulum, sulcus, and flagella were lost. The coccoid non-motile cells were surrounded by a perialgal envelope of the host radiolarian cytoplasm, and they possessed a dinokaryon with condensed chromosomes and mitochondria with tubular cristae. The chloroplast was located at the periphery of the cell and had one or two pyrenoids. In culture, however, the motile cells exhibited a typical cell shape of peridinioid dinoflagellates. The cell covering consisted of a plasma membrane and amphiousmal vesicles containing the thecal plates whose arrangement was congruent to the order Peridinales. The chloroplast was enclosed by three membranes and the pyrenoid was the double-stalked type. Pusules constructed by tubules with invaginations were present, but eyespot and trichocysts were lacked. Symbiotic dinoflagellates, such as Zooxanthella, Symbiodinium, and Amphidinium, have historically been referred to using the general term ‘zooxanthella(e)’ because of their similar appearances as yellow-brown spherical minute cells under light microscopy. However, the motile cells of Z. nutricula exhibited a typical peridinioid morphology and thecal plate arrangement that obviously differed from those of the other Symbiodiniaceae genera and Amphidinium. Considering the ultrastructural features and the currently available sequence database, it was suggested that Z. nutricula is a symbiotic dinoflagellate that lives specifically in holoplanktonic protist radiolarians.

Keywords  Dinoflagellate, Radiolaria, Symbiodinium, Symbiont, Zooxanthella nutricula

Introduction

Radiolaria (Acantharea, Taxopodida, and Polycystinea: Adl et al. 2019) are holoplanktonic protists that are widely distributed in tropical, subtropical, and even polar marine environments, occurring throughout the water column from the surface to several thousand meters depths (e.g., Boltovskoy 2017). Most of the radiolarians possess intra-cellular algal symbionts within their cytoplasmic bodies, and the holobionts have been shown to have exceptionally high rates of photosynthesis and to contribute to the primary production of the oceans (e.g., Decelle et al. 2015). Michaels (1988) estimated that it could occasionally account for ≥20% of the total primary production in the upper euphotic zone of oligotrophic oceans.

The algal symbionts in radiolarians generally appear as yellow-brown minute spheres. Huxley (1851) noted the presence of the algal symbiont in colonial radiolarians as “yellow cells” which was thought to be a part of the radiolarians. Brandt (1881) then first revealed that “yellow cells” of the colonial radiolarian Collozoum inerme (Müller 1856) were symbiotic dinoflagellates, and he
named them *Zooxanthella nutricula* Brandt 1881 as well as a monotypic genus *Zooxanthella* Brandt 1881. Since Brandt (1881)'s first description of *Zooxanthella nutricula*, several taxonomic revisions of *Z. nutricula* have been made, and its genus and/or species names were occasionally changed (Geddes 1882; Pascher 1911; Taylor 1974, 1984; Holland and Carré 1975; Blank and Trench 1986; Banaszak et al. 1993; Gast and Caron 1996, 2001; see also Yuasa et al. 2016).

On the other hand, the genus name *Zooxanthella* has been inappropriately used as a general term, ‘zooxanthella(e),’ which indicates any yellow-brown symbionts living within various hosts such as corals, sponges, sea anemones, jellyfish, nudibranchs, clams, and foraminifers in the marine environment (e.g., Blank and Trench 1986; Trench 1993; Rowan 1998). This inappropriate term and lack of taxonomic details of *Zooxanthella* have led to further confusion; for example, an attempt was made to transfer all species belonging to the genus *Symbiodinium* to the genus *Zooxanthella* as proposed by Loeblich and Sherley (1979), based on the recognition that *Zooxanthella* is an available name as well as its priority (Guiry and Andersen 2018). To address such ambiguity of the taxonomic affiliations due to the limited morphological observations done by light microscopy for the cells within a host cell, molecular techniques and ultrastructural observations of cultured motile cells of dinoflagellates isolated from the host cell have been applied. Probert et al. (2014) examined the morphology and molecular phylogenetic position of the free-living stage of dinoflagellates isolated from several different polycystine radiolarian hosts, and they identified a peridiniod dinoflagellate within the same radiolarian host from the same locality that Brandt (1881) had found and described as *Z. nutricula*. Since then, the validity of the genus name *Zooxanthella* for the peridiniod dinoflagellate symbionts of radiolarians has been widely accepted (e.g., Gottschling and McLean 2013; Yuasa et al. 2016; Guiry and Andersen 2018; LaJeunesse et al. 2018). This further emphasizes inappropriateness of the term ‘zooxanthellae(e)’ which often used to indicate common symbiotic dinoflagellates of corals, Symbiodiniaceae, which belong to the order Suessiales, not Peridiniales.

Although the original description by Brandt (1881) was of symbiotic cells in radiolarian hosts under light microscopy, no report of the ultrastructure of the symbiotic cells of *Z. nutricula* in hospite has been published. Here, we examined a symbiotic dinoflagellate *Z. nutricula* from radiolarians collected off the northwestern coast of Okinawa Island, Japan, and we describe the ultrastructural features of *Z. nutricula* in the symbiotic and cultured motile stages.

**Materials and methods**

**Sampling and culture conditions**

Materials were collected in July 2006 and March 2009 from the East China Sea, approximately 5 km off the northwest coast of Okinawa Island, Japan (26°37′N, 127°47′E), using a plankton net (60-cm circle opening with 37-µm mesh net). After collection, radiolarian specimens were carefully isolated and placed into six-well tissue culture dishes containing filtered seawater (0.2 µm pore size) for identification and incubation. To obtain cultured cells of *Zooxanthella nutricula*, the single cell of the host radiolarian *Didymocytis tetrathalamus* (Haeckel 1887) was micro-dissected on a slide glass under an inverted light microscope with a sterile razor blade. The cells were transferred from the slide glasses with a Pasteur pipette and were subsequently rinsed three times in sterile seawater. Approximately half of cells was directly used for polymerase chain reaction (PCR) amplifications, and the other half was used for clonal culture. The clonal culture was maintained in Daigo’s IMK medium for Marine Microalgae (Nihon Pharmaceutical, Tokyo, Japan) and incubated at 26°C with a 14:10 hour light:dark cycle.

**Light and electron microscopy**

Light microscopic observation of the cultured motile cells of *Z. nutricula* were examined using an OLYMPUS BX53 light microscope (OLYMPUS, Tokyo, Japan) equipped with a COOLPIX 950 digital camera (Nikon, Tokyo, Japan). For scanning electron microscopy (SEM), cultured cells of *Z. nutricula* were fixed in 4% OsO₄ on a 0.1% poly-L-lysine-coated glass plate for 10 min and subsequently used for dehydration. The dehydration protocol is described in Yuasa et al. (2016). The dried cells were coated with platinum-palladium in a JFC-1100
ion-sputter (JEOL, Tokyo, Japan) and examined with an S-4500 field emission scanning electron microscope (Hitachi, Tokyo, Japan). For transmission electron microscopic observation (TEM) of Z. nutricula under a symbiotic state, one cell of each two individuals of the host radiolarian D. tetrathalamus collected in 2006 and 2009 was embedded in 1.5% low-temperature-gelling agarose (Merck, Darmstadt, Germany) made up with seawater. A piece of the agarose gel with an embedded cell of D. tetrathalamus was initially fixed in 2.0% glutaraldehyde made up with 0.1 M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose. In a separate preparation for observation of the motile stages, the cultured cells of Z. nutricula were transferred to 1.5-ml Eppendorf tubes and centrifuged at 2000×g for 5 min, and then they also were fixed in 2.0% glutaraldehyde made up with 0.1 M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose. After that, each piece of the agarose gel of embedded cells of D. tetrathalamus and pellet of cultured cells was rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose before postfixation in 1.0% OsO₄ at room temperature for 2 h. Because the above fixation protocol did not preserve the detail of amphialeral structure, we employed following another method to fix cultured cells of Z. nutricula. Fifty microliters of 4% OsO₄ was added to 150 µL of culture medium containing large number of motile cells and the cells were fixed for 3 h at room temperature. After the postfixation with OsO₄, the subsequent method was same as described by Yuasa et al. (2016). The host radiolarian cell was observed using sections with thicknesses of 60 nm and the whole-cell observations of the cultured cells of Z. nutricula based on serial sections were made using sections with thicknesses of 90 nm. These sections were observed using a JEM-100S transmission electron microscope (JEOL).

**PCR amplification, cloning, and sequencing**

Nonmotile cells of Z. nutricula were obtained directly from the cytoplasm of a radiolarian D. tetrathalamus under an inverted light microscope by the above-mentioned method. The cultured motile cells were transferred to a 0.2-ml Eppendorf tube and centrifuged at 3000×g for 3 min, and the pellet was rinsed twice in distilled water. They were used as templates for amplification of small subunit ribosomal DNA (SSU rDNA) coding region. PCR primers, PCR and sequencing protocols are provided by Yuasa et al. (2016). Only the PCR products of Z. nutricula directly obtained from the cytoplasm of D. tetrathalamus were cloned in the pGEM-T Easy Vector System (Promega) using E. coli JM109 Competent Cells (Promega).

**Alignment and phylogenetic analysis**

Our determined sequences were aligned with the other dinoflagellate sequences obtained from GenBank. We generated alignments of SSU rDNA sequences using ClustalW ver. 1.81 (Thompson et al. 1994). Total of 44 taxa (1,521 bp) was used in the phylogenetic analyses for the data sets. Phylogenetic trees were constructed using maximum likelihood (ML) (Felsenstein 1981) and Bayesian analysis. To determine the best-fit model of DNA evolution, the alignment was subjected to hierarchical likelihood ratio tests in Modeltest v3.06 (Posada and Crandall 1998), indicated that GTR+I+G was the best-fit substitution model. ML analysis was performed with PAUP* version 4.0b10 (Swofford 2002). The ML tree was then analyzed using a heuristic search method with a TBR branch-swapping option and random taxon addition. The relative levels of support for nodes were assessed by calculating full heuristic bootstrap proportion values (BV) (Felsenstein 1985) based on 100 replicates. Bayesian analyses were carried out with MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). Trees were generated from two runs with one heated and three cold chains in the Markov chain Monte Carlo (MCMC). A total of 3,500,000 generations were calculated with trees sampled every 100 generations each other and with a prior burn-in of 875,000 generations (8,750 sampled trees were discarded). The remaining trees were used to construct both the majority-rule consensus tree and the posterior probabilities (PP) of the nodes.

**Results**

**Symbiotic state of Zooxanthella nutricula in a radiolarian Didymocyrtis tetrathalamus**

A light microscopic image of the radiolarian D. tetrathalamus showing brownish-orange to red-colored cytoplasm and numerous radiating axopodia that extend
Fig. 1  Light and transmission electron micrographs of Didymocyrtis tetrathalamus with Zooxanthella nutricula. (A) Living D. tetrathalamus with Z. nutricula as yellow-brown coccoid cells (up to 8 μm), distributed along the central capsule. Arrowhead indicates a coccoid cell of Z. nutricula. Scale bar=50 μm, (B) The perialgal envelope (arrowhead) encloses the coccoid cell of Z. nutricula. The lamellae consisted of three thylakoids are stacked and bent in the chloroplast. Scale bar=1 μm, (C) A section of a coccoid cell of Z. nutricula associated with D. tetrathalamus showing the nucleus with condensed chromosomes, mitochondria, peripheral chloroplast, and starch granules in the cytoplasm. Scale bar=2 μm, (D) A section of D. tetrathalamus showing the coccoid cells of Z. nutricula within the cytoplasm. Arrows indicate the central capsular wall of D. tetrathalamus and the arrowheads indicate the perialgal envelope. Scale bar=5 μm. Chl: chloroplast M: mitochondria, N: nucleus, Py: pyrenoid, S: starch granule, Si: siliceous shell of D. tetrathalamus.
toward the surrounding environment (Fig. 1A). In the symbiotic state, non-motile cells of *Z. nutricula* were almost coccoid, approximately 8 µm in diameter, and found in the host cytoplasm inside the siliceous cortical shell of *D. tetrathalamus* (Fig. 1A). Under TEM observations, the cells of *Z. nutricula* located in the extracytoplasm of *D. tetrathalamus* between the central capsular wall and siliceous cortical shell (Fig. 1D). Each cell contained a typical dinoflagellate nucleus and was enclosed by the perialgal envelope (Figs 1B, D). The cytoplasm was densely occupied by short and thick chloroplast profiles with one or two pyrenoids (Fig. 1C). The chloroplast profiles were situated around the periphery of the cell; the lamellae consisted of three thylakoids that were stacked and bent, and they were not always parallel to each other (Figs 1B, C, D). The pyrenoid was penetrated by several thylakoids (Fig. 1C). We did not detect any features of amphiesma and/or flagella in the cells (Figs 1C, D).

**Cultured motile cells of *Z. nutricula***

Cultured motile cells of *Z. nutricula* were examined by light and scanning electron microscopy (SEM), and the morphological features that we observed were almost the same as those described by Probert et al. (2014). The cultured cells were 11.4–12.9 µm long (mean 12.2 µm, n=10) and 9.4–10.6 µm wide (mean 10.4 µm, n=10). They had a slightly rounded conical epitheca and a hemispherical hypotheca (Fig. 2A). A single yellow-brown chloroplast, with one or two pyrenoids, was located at the periphery of the cell (Fig. 2A). No eyespot was observed. The cingulum was situated in the middle of the cell (Fig. 2B). Under culture conditions, the cells were essentially at a continuous motile stage and swam in rapid rotational movement. However, before the cell division that occurred once every few days, the motile cells attached to the bottom of the plastic culture dish and transformed into non-motile cells. During the non-motile phase, two daughter cells were produced within the cell covering (Fig. 2C) and were released from the parental cell covering. The remaining cell covering could be observed at the bottom of the culture dish (data not shown). Scanning electron microscopy revealed that the cells had a plate pattern of Po, x, 4′, 3a, 7″, 5c, 5s, 5″, and 1″″ (Fig. 3). The epitheca was somewhat larger than the hypotheca and conical with a raised apex (Figs 3A-D). The apical pore represented a complete circle and was encircled by a raised margin about 300 nm high (Fig. 3A). It was sealed by a thin cover plate (Fig. 3E).

The sections of the cultured cells showed typical dinoflagellate organelles, such as a dinokaryon with condensed chromosomes and mitochondria with tubular cristae (Figs 4A, B). A typically ovoid-shaped dinokaryon located in the middle or offset to one side of the cell extended from the epitheca to the hypotheca. The mitochondria were observed in both the anterior and posterior parts of the cytoplasm (Fig. 4A). A chloroplast was located peripherally and enclosed by three membranes (Figs 4A, 5A). Each lamella consisted of three thylakoids (Fig. 5A). The pyrenoid was circular and surrounded by a starch sheath (Figs 4A, B, C). The pyrenoid was the double-stalked type; that is, the pyrenoid matrix was connected with chloroplast at two channels and was pene-
Fig. 3  Scanning electron micrographs of the cultured motile cells of *Zooxanthella nutricula*. (A) Ventral view showing the five cingular plates, (B) Dorsal view, (C) Left lateral view. Small simple pores are scattered on the thecal plates (arrows), (D) Right lateral view, (E) Apical view. The anterior intercalary plates are asymmetrically arranged, (F) Antapical view showing the five postcingular plates and only one antapical plate. Scale bars=2 μm.
trated by several thylakoids (Fig. 4C). The pusule was constructed tubules with invaginations, and 2 to 10 tubular pusular vesicles were observed at the flagellar base (Figs 5D, E). The tubules consisting of double membranes were irregular in shape (Fig. 5D). Vacuoles with a single membrane containing electron-dense materials were present at the periphery of the cell (Fig. 5B, arrows). Some vacuoles opened to the outer cytoplasmic layer by way of a constricted opening (Fig. 5B, arrowhead).

Although we have made whole cell serial sections for 10 cells, we could not confirm the presence of an eyespot and trichocysts.

The cell covering was typical of thecate dinoflagellates, i.e., consisting of a plasma membrane and amphiesmal vesicles that contain the thecal plate (Fig. 6). A thecal plate was observed in each amphiesmal vesicle. Most of the thecal plates were thin and ~30 nm in thickness (Fig. 6C). In the apical region, we observed an apical fibrous complex that consisted of a group of fibers and a continuous fibrous layer underlying the apical pore complex (Fig. 6B). The fibrous structures with a striated pattern in the apical pore complex were observed just inside of the raised margin of the apical pore plates and the cover plate (Fig. 6B). In the apical pore region, several striated fibers extended from the apex straight downward (Fig. 6B). There was a gap between the sealing cover plate and the raised margin of the apical pore plate, where it formed the rim of the apical pore complex (Fig. 6B). The rim consisted of only an amphiesmal vesicle, which did not contain the thecal plate (Fig. 6B).

SSU rDNA sequences of Z. nutricula

We obtained full-length SSU rDNA sequences (1,795 bp) from the cells directly retrieved from the cytoplasm of D. tetrathalamus and the cells from the cultured strain. These two sequences were identical. The sequence was deposited in GenBank under accession number AB698452. Our obtained sequence completely corresponded with the sequences of Z. nutricula which were deposited in GenBank under accession numbers U52352-U52356 and U52911 by Gast and Caron (1996) and KF557491-KF557525 by Probert et al. (2014).

Our phylogenetic tree (Fig. 7) demonstrates that Z. nutricula from D. tetrathalamus formed a monophyletic
clade with the symbiont from the radiolarian *Collozoum caudatum* (Swanberg and Anderson 1981) and ‘*Scrippsiella velellae*’ Banaszak, Iglesias-Prieto and Trench 1993 (the sequence of this species has been deposited as *Scrippsiella nutricula* (Brandt 1881) under accession no. U52357) from the cnidarian *Velella velella* Linnaeus 1758 collected in the Sargasso Sea (BV: 100%; PP: 1.00) as reported by Probert et al. (2014). In addition, although the statistical support was not high, this clade was shown to be a sister to the clade of the so-called ‘dinotoms’ of *Unruhdinium nieu* (Liu et al. 2008) and *Durinskia dybowskii* (Woloszynska 1916) (Imanian et al. 2010) (BV: <50%; PP: 0.73).

**Discussion**

We observed the morphological features of *Zooxanthella nutricula* in hospite under light and electron microscopy and found that the cultured motile cells showed typical peridiniioid morphologies such as thecal plate arrangement as shown by Probert et al. (2014). The nucleus, chloroplast, mitochondria, and pusule also exhibited typical characteristics common in dinoflagellate organelles; however, a trichocyst was not observed despite the use of whole-cell serial sections, whereas
some minute scattered pores on the thecal plates were confirmed under SEM. The lack of a trichocyst is a specific characteristic also found in the Symbiodiniaceae and Suessiaceae.

The electron-dense materials contained in the vacuole with a single membrane (Fig. 5B, arrows) resemble those in the polyvesicle bodies observed in *Pileidinium ciceropse* Tamura and Horiguchi 2005 (Tamura and Horiguchi 2005), the virus particles in autolysosomes observed in *Plagiodinium belizeanum* Faust and Balech 1993 (Wakeman et al. 2018), and the virus-like particles within vacuoles observed in *Plagiodinium ballux* Yamada, Dawut, Terada and Horiguchi 2018 (Yamada et al. 2019). In the present study as well, we confirmed that the membrane surrounded the electron-dense materials and that it was open to the outside of the cell, but we could not confirm whether these dense materials were digested or infected virus, or why the membrane was open to the outside of the cell.

As shown in our molecular phylogenetic tree (Fig. 7), although the BV was low, the so-called ‘dinotom,’ characterized by the possession of a type D eyespot sensu Moestrup and Daugbjerg (2007) that was thought to be the remnant of a peridinin-type chloroplast (e.g., Horiguchi and Pienaar 1994), showed a monophyletic relationship with *Z. nutricula*. However, we found that *Z. nutricula* had no type D eyespot in the cytoplasm nor other type of eyespot, and the BV of the monophyletic clade was low. To reveal the relationship between ‘dinotoms’ and *Z. nutricula*, more detailed molecular phylogenetic studies using multiple genes are needed.

We observed some morphological differences between the ultrastructure of *Z. nutricula* and those of the coral symbiotic dinoflagellate Symbiodiniaceae, which had been called ‘zooxanthella(e).’ In both *Z. nutricula* and Symbiodiniaceae, the pyrenoids are surrounded by a starch sheath. However, some *Z. nutricula* possesses two pyrenoids, and the pyrenoid matrix are penetrated by sev-
Fig. 7  SSU rDNA phylogenetic tree based on the maximum-likelihood method (44 taxa, 1,521 nucleotide sites) for our obtained and other dinoflagellate sequences already in the database. Bootstrap values (left number) above 50% and posterior probabilities (right number) over 0.50 are given at the respective nodes. The sequence of ‘Scrippsiella velellae’ from the cnidarian Velella velella is now deposited as Scrippsiella nutricula (accession number U52357) in GenBank.
eral thylakoids, while Symbiodiniaceae possess a single pyrenoid in which thylakoids do not penetrate into the matrix (e.g., Hansen and Daugbjerg 2009; Yamashita et al. 2009; LaJeunesse et al. 2018). Furthermore, Symbiodiniaceae have a type E eyespot sensu Moestrup and Daugbjerg (2007) (e.g., Hansen and Daugbjerg 2009; Yamashita et al. 2009). It was suggested that Symbiodiniaceae could be attracted by the luminescence from reef-building corals and that their symbiotic relationships might be established (Aihara et al. 2019), whereas Z. nutricula lacks the eyespot that is commonly present in Symbiodiniaceae. In addition, under culture conditions, Z. nutricula swim in rotational movement continuously day and night, and before the cell division the motile phase transforms into the non-motile phase. On the other hand, as well known the Symbiodiniaceae species exhibit diel morphological alternations between a motile phase during the day and a coccolid phase at night (e.g., Yamashita and Koike 2015).

There are forty SSU rDNA sequences in GenBank that are identical to the sequences of Z. nutricula (accession no. KF557498) deposited by Probert et al. (2014), and all of these sequences were obtained from symbiotic algae living within radiolarians. Six of these sequences were from the symbiotic algae within the radiolarians Collozoum caudatum, Thalassicolla nucleate Haeckel 1887, Spongostaurus sp., and three unidentified specimens in the Sargasso Sea described by Gast and Caron (1996); the other thirty-four sequences are from symbiotic algae within radiolarians of twenty-five collodarian, one nassellarian, and eight spumellarian specimens in the North and South Pacific Oceans, the East China Sea, and the Mediterranean Sea reported by Probert et al. (2014).

The SSU rDNA sequences obtained from symbiotic algae within the radiolarian Didymocystis tetrathalamus in the present study were also identical to those of these radiolarian symbionts from various oceans. Brandt (1881) first defined Z. nutricula as a symbiotic dinoflagellate within a radiolarian Collozoum inerme, and since then the general term ‘zooxanthella(e)’ has been used as a general term for the symbiotic dinoflagellates within various metazoan and protistan hosts. However, considering the ultrastructural features and the currently available sequence database, we suggest that Z. nutricula is a symbiotic dinoflagellate that lives specifically in holoplanktonic protist radiolarians.

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