News of Osaka Micropaleontologists (NOM), Special Volume, no. 14, p. 1 – 4, Feb., 2009
ISSN 0287– 0436

A simple technique for extracting small subunit ribosomal DNAs from both the host and symbiont of a single radiolarian specimen

YUASA Tomoko[1)], TAKAHASHI Osamu[1)] and MAYAMA Shigeki[2)]

1) Department of Astronomy and Earth Sciences, Tokyo Gakugei University, Koganei, Tokyo 184-8501, Japan, and
2) Department of Biology, Tokyo Gakugei University, Koganei, Tokyo 184-8501, Japan
Corresponding author: T. Yuasa (tyuasa@u-gakugei.ac.jp)

(Recieved January 22, 2007; 1st review finished January 23, 2007; Accepted April 17, 2007)

Abstract

Here, we describe a simple and quick method for obtaining small subunit ribosomal DNA (18S rDNA) sequences from a single radiolarian cell. With this proposed technique, the 18S rDNAs of both the radiolarian host and its symbiotic algae can be simultaneously obtained from one radiolarian specimen without cross contamination. This new technique will be useful for obtaining 18S rDNA sequences from species that cannot be cultured and will be also available in small numbers from samples. Moreover, using this method, 18S rDNA can be obtained from both the host and the symbionts simultaneously. Therefore, this method can confirm whether the host–symbiont association of Radiolaria, which have still been unclear, are randomly distributed or highly specific.

Key words: Radiolaria, small subunit ribosomal DNA (18S rDNA), symbiont, single cell, DNA extraction

Introduction

Polycystine radiolaria (here we use "Radiolaria" *sensu lato* to include the classes Acantharea, Polycystinea, and Phaeodarea) are holoplanktonic protists, occurring exclusively in neritic and open ocean environments. They have roughly spherical cells that are approximately a few hundred micrometers in diameter and thread-like pseudopodia extending radially over the endoskeleton that facilitate a floating existence (e.g., Anderson, 1983). Since the first report of polycystine radiolaria (Meyen, 1834), many taxonomic and phylogenetic studies on living and fossil radiolarians have been conducted during approximately the past seventeen decades (e.g., Haeckel, 1887; Riedel, 1967). For example, the studies on cellular structures have focused on taxonomic classification at a higher level based on the cytological similarity of axopodial microtublines (e.g., Cachon et al., 1973). However, very little has been published on the physiology and ecology of these organisms. Our understanding of the radiolarians based only on skeletal and cytoplasmic evidence has caused the limitation to the construction of a natural taxonomic system of radiolaria. Classical morphological approaches combined with modern molecular genetic analyses hold promise for clarifying unresolved issues in the present radiolarian taxonomy. These approaches may be quite helpful in constructing a more natural system based on the genetic relationship between species.

Recently, molecular methods have increasingly been used in the study of the phylogenetic framework of eukaryotic organisms (e.g., Cavalier-Smith, 2004). In the study of radiolarians also, molecular methods have been employed during the last decade (e.g., Amaral Zettler et al., 1997; Polet et al., 2004; Yuasa et al., 2005). However, the application of molecular methods in the study of radiolarians has been delayed, because these organisms are difficult to reproduce in laboratory cultures. A severe constraint in the efforts for clarifying the phylogenetic framework of eukaryotes, such as radiolarians, has been the difficulty in obtaining DNA sequences from the species that cannot be cultured and that are only obtained in small numbers from samples.

Furthermore, within the polycystine radiolarians, various types of algae—dinoflagellates, prasinophytes, and prymnesiophytes—occur as intracellular symbionts (e.g., Anderson, 1976). In most polycystine radiolarians, algal symbionts are generally found in the rhizopodial network of the ectocytoplasm (Anderson, 1976). In order to infer the phylogenetic position of polycystine radiolarians and also to examine the diversity of their symbioses, we have developed a simple and quick method to simultaneously obtain the small subunit ribosomal DNA (18S rDNA) sequences of both radiolarians and their symbionts.

Yuasa et al. (2004) designed primers specifically for the spumellarians, which are solitary and have spongy or latticed

siliceous shells. The primers can amplify only host radiolarian 18S rDNA without amplifying the 18S rDNA of symbiotic algae. Here, we describe a method obtaining 18S rDNA from both the host and the symbionts simultaneously.

This method can confirm whether the host–symbiont associations of Radiolaria, which have still been unclear, are randomly distributed or highly specific.

Methodology

Recent methodological studies for extraction of DNA from single dinoflagellate cells have been carried out by using physicochemical and enzymatic lysis procedures (Marín et al., 2001), by direct PCR performed using cells subjected to freeze-thaw lysis (Ruiz Sebastián and O'Ryan, 2001; Edvardsen et al., 2003), and by using Chelex® (Bio-Rad) (Richlen and Barber, 2005). Similar to these methods, our technique is simpler than classical ones and it does not need cultivation. Moreover, 18S rDNAs from both the radiolarian host and the algal symbionts can be individually isolated from single radiolarian specimens.

Radiolarian samples were collected from surface seawater, the upper 3-m, by using a plankton net (50-cm net opening with a 37-µm mesh size) at one site (26°37′18″N, 127°47′35″E), located approximately 5 km northwest of Okinawa Island, Japan. The concentrated plankton samples were diluted in

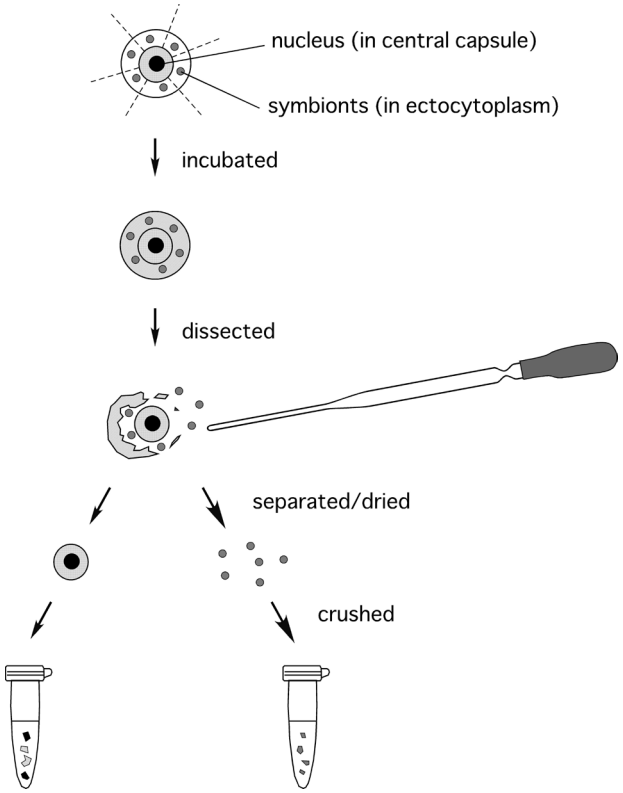

Fig. 1. Flowchart of the 18S rDNA extraction from a single radiolarian cell used in this study.

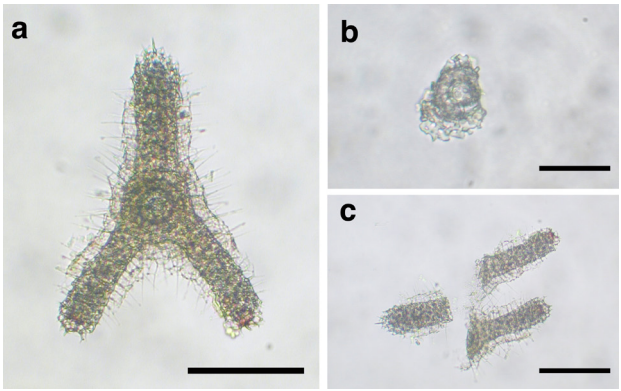

Fig. 2. Light micrographs (LM) of microdissections. a: LM of a specimen of *Euchitonia elegans* (Ehrenberg). b: LM of the central part (including central capsule) of *E. elegans* after microdissection. c: LM of the ectocytoplasm (including symbiotic algae) of *E. elegans* after microdissection.

polycarbonate jars with seawater collected from the same site and stored in cool box. The individual radiolarian specimens were isolated by using an inverted microscope or a binocular stereo-microscope and transferred to culture dishes containing paper-filtered seawater. The samples were then transferred into 0.5-ml microtubes containing 0.22-µm Millipore-filtered seawater and incubated at 75°C for 10 min and stored at –20°C until the 18S rDNA region amplifications were processed. Following incubation, each radiolarian cell was microdissected in sterile distilled water on a sterile glass slide with a sterile razor blade and glass needles to separate the central capsule that contained the nucleus from the ectocytoplasm and the symbiotic algae (Figs 1 and 2). The central capsule and the symbiotic algae from a single radiolarian specimen were placed on separate sterile glass slides and rinsed twice in sterile distilled water.

After this pretreatment procedure, each of the central capsule and the symbiotic algae were dried at 60°C for 30 min, and then crushed with a small fragment of a sterile cover glass (ca. 1-mm square) to tear open the organic membrane. The cover glass fragment and the remnants of the crushed central capsule, or the cover glass fragment and the remnants of the crushed symbiotic algae on each glass slide were scraped up with 4 µl of 0.2 µg/µl Proteinase K solution, 4 µl of phosphate-buffered saline (PBS), and 6 µl of sterile distilled water and inserted into a 0.5-ml microtube by using a Pasteur pipette. The samples were vortexed for approximately 5 s and briefly spun in a microcentrifuge at 10000 x g for 5-10 s and then incubated for 30 min at 37°C followed by 15 min at 80°C. Each solution (14 µl) was used for PCR amplification.

PCR amplification was carried out using the eukaryotic specific forward primer 90F (Hendriks et al., 1989)—5′-GAAACTGCGAATGGCTCATT-3′—and the reverse primer B (Medlin et al., 1988)—5′-CCTTCTGCAGGTTCACCTAC-3′—in a total volume of 50 µl containing 15.5 µl of sterile distilled water; 5 µl of 10′ Ex Taq™ Buffer (Mg^{2+}-free)

composed of 20mM Tris-HCl (pH8.0), 100mM KCl, 0.1mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT), 0.55% Tween 20, 0.5% Nonidet P-40, and 50% glycerol; 5 µl of $MgCl_2$ (25mM); 8 µl of dNTP mixture (2.5 mM each); 1 µl of each primer (100 µM); and 0.5 µl TaKaRa Ex Taq™ (TaKaRa). The thermal cycle was run on a MiniCycler (MJ Research) with the following cycle condition: an initial 3 min denaturation at 95°C followed by 35 amplification cycles of 1 min at 95°C, 2 min at 55°C, and 3 min at 72°C. The amplification products were checked for correct length on a 1.2% agarose gel and purified from the gel by using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and then cloned in the pGEM-T Easy Vector System (Promega) by using *Escherichia coli* JM109 Competent cells (Promega). Five clones of each of the amplified fragments from the inserts were sequenced using fluorescein isothiocyanate (FITC)-labeled primers and reagents from the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences) along with the sequencing protocol developed for the DSQ2000L DNA sequencer (Shimadzu). Forward and reverse sequences were aligned with Clustal W ver. 1.81 (Thompson et al., 1994).

Results and Discussion

The results of the experiments performed using polycystine radiolarian single cells are shown in Fig 3. In this paper, we examined three polycystine radiolarian species: *Dictyocoryne truncatum, Euchitonia elegans*, and *Dictyocoryne profunda* (Order Spumellarida, family Spongodiscidae). Since *D. profunda* does not harbor symbiotic algae (Takahashi et al., 2003), the PCR products from ectocytoplasm of *D. profunda* were not obtained (Fig. 3, lane 6). As indicated previously (Yuasa et al., 2005), the polycystine radiolarian sequences that we obtained were shown as a monophyletic group along with the colonial polycystine species of Amaral Zettler et al. (1999). On the other hand, the sequences of the PCR products from the ectocytoplasm of *D. truncatum* (Fig. 3, lane 2) branched within a clade of Class Haptophyta, and those from *E. elegans* (Fig. 3, lanes 4) branched in a clade of Class Dinophyta (data not shown).

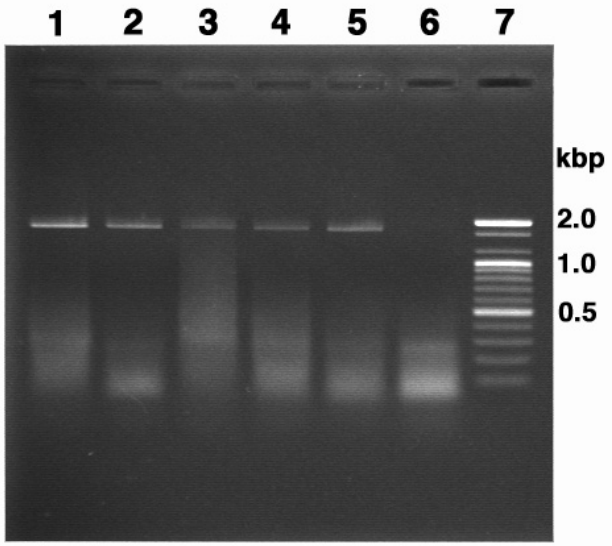

Fig. 3. Results of PCR amplification from three radiolarian specimens and their symbiotic algae. Lanes 1, 3, and 5 are 18S rDNA fragments from the nucleus in the central capsule of *Dictyocoryne truncatum*, *Euchitonia elegans*, and *Dictyocoryne profunda*, respectively. Lanes 2, 4, and 6 are 18S rDNA fragments from the symbiotic algae in the ectocytoplasm of *D. truncatum*, *E. elegans*, and *D. profunda*, respectively. Note *D. profunda* possesses no algal symbiont within the ectocytoplasm. Molecular weight standard: 100-bp ladder marker in Lane 7.

Our technique was successful in extracting 18S rDNAs of both the radiolarian host and the algal symbiont from only one radiolarian specimen. The results suggest that this technique will be useful for extracting 18S rDNAs from eukaryotes, such as radiolarians, that are difficult to culture. Furthermore, by using cloning in conjunction with micro-dissection, the 18S rDNAs from both the host and the symbiont can be obtained individually without any cross contamination. Anderson and Matsuoka (1992) showed that *D. truncatum* possesses algal and bacteroid endobionts within the central capsule. In this study, however, we could get only the radiolarian 18S rDNA sequence from the central capsule of *D. truncatum*. If algal endobionts/symbionts exist in the central capsule of some radiolarian species, we may need to modify this method.

Concluding remarks

This technique is inexpensive and time efficient, it comprises simple processes that involve no hazardous chemicals; moreover, it will also facilitate to document the morphology prior to obtaining sequence data from specimens used in taxonomic studies.

Despite knowledge of the existence of these symbioses among polycystine radiolarians (e.g., Anderson, 1983), identification of the symbionts has been hindered by the loss of diagnostic morphological features when the alga is in the symbiotic state. This method may be useful in revealing the history of radiolarian–algal symbiosis that may have triggered the diversification of polycystines in geologic time.

Acknowledgements

We are grateful to Noritoshi Suzuki of Tohoku University for his critical reviewing and helpful comments on the

manuscript. We would like to acknowledge Akihiro Takemura of the Tropical Biosphere Research Center of the University of the Ryukyus and Kjell R. Bjørklund of the Paleontological Museum of University of Oslo for their critical reading of early draft of the manuscript. We would like to thank Yoshikatsu Nakano and Shigeo Nakamura of the Tropical Biosphere Research Center of the University of the Ryukyus for their support and help. We wish to thank Hiroshi Nyunoya and Yasuhiko Matsushita of the Gene Research Center of Tokyo University of Agriculture and Technology for their valuable technical advice. This study was financially supported by the JSPS Research Fellowships for Young Scientists (No. 17-3353) granted to T. Yuasa; and Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture, Japan (Grant Nos 12640452 and 15540449) granted to O. Takahashi.

References

Amaral Zettler, L., Sogin, M.L. and Caron, D.A., 1997, Phylogenetic relationships between the Acantharea and the Polycystinea: a molecular perspective on Haeckel's Radiolaria. *Proceedings of the National Academy of Sciences*, **94**, 11411-11416.

Amaral Zettler, L., Anderson, O.R. and Caron, D.A., 1999, Towards a molecular phylogeny of colonial spumellarian radiolaria. *Marine Micropaleontology*, **36**, 67-79.

Anderson, O. R., 1976, Fine structure of a collodarian radiolarian (*Sphaerozoum punctatum* Müller 1858) and cytoplasmic changes during reproduction. *Marine Micropaleontology,* **2,** 251-264.

Anderson, O.R., 1983, *Radiolaria*, Springer-Verlag, New York.

Anderson, O.R. and Matsuoka, A., 1992, Endocytoplasmic microalgae and bacteroids within the central capsule of the radiolarian *Dictyocoryne truncatum*. *Symbiosis*, **12**, 237-247.

Cachon, J., Cachon, M., Febvre-Chevalier, C. and Febvre, J., 1973, Déterminisme de l'édification des systèmes microtubulaires stéréoplasmiques d'Actinopodes. *Archiv für Protistenkunde*, **115**, 137-153.

Cavalier-Smith, T., 2004, Only six kingdoms of life. *Proceedings of the Royal Society B*, **271**, 1251-1262.

Edvardsen, B., Shalchian-Tabrizi, K., Jakobsen, K. S. Medlin, L. K., Dahl, E., Brubak, S. and Paasche, E., 2003, Genetic variability and molecular phylogeny of Dinophysis species (Dinophyceae) from Norwegian waters inferred from single cell analyses of rDNA. *Journal of Phycology*, **39**, 395-408.

Haeckel, E., 1887, Report on the Radiolaria collected by H.M.S. Challenger during the years 1873-1876. *In* Thompson, C. W. and Murray, J. eds. *Report on the Scientific Results of the Voyage of the H.M.S. Challenger, Zoology, 18, 2 parts.* Her Majesty's Stationery Office, London. 1-1803.

Hendriks, L., Goris, A., Neefs, J., Van de Peer, Y., Hennebert, G. and De Wachter, R., 1989, The nucleotide sequence of the small ribosomal subunit RNA of the yeast *Candida albicans* and the evolutionary position of the fungi among the eukaryotes. *Systematic and Applied Microbiology*, **12**, 223-229.

Marín, I., Aguilera, A., Reguera, B. and Abad, J.P., 2001, Preparation of DNA suitable for PCR amplification from fresh or fixed single dinoflagellate cells. *BioTechniques*, **30**, 88-93.

Meyen, F. J. F., 1834, Beiträge zur Zoologie: gesammelt auf einer Reise um die Erde. Fünfte Abhandlung von F. J. F. Meyen. Über das Leuchten des Meeres und Beschreibung einiger Polypen und anderer niederer Thiere. *Nova Acta Physico-Medica. Academiae Caesariae Leopoldino-Carolinae. Naturae Curiosorum (Verhandlungen der Kaiserlichen Leopoldinisch-Carolinischen Akademie der Naturforscher)*, **16**, 125-216.

Medlin, L., Hille, J. E., Shawn, S. and Sogin, M. L., 1988, The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene,* **71**, 491-499.

Polet, S., Berney, C., Fahrni, J. and Pawlowski, J., 2004, Small-subunit ribosomal RNA gene sequences of Phaeodarea challenge the monophyly of Haeckel's Radiolaria. *Protist*, **155**, 53-63.

Riedel, W.R., 1967, Subclass Radiolaria. *In* Harland, W. B. et al. eds. *The Fossil Record*. Geological Society of London, Northern Ireland, 291-299.

Richlen, M.L. and Barber, H., 2005, A technique for the rapid extraction of microalgal DNA from single live and preserved cells. *Molecular Ecology Notes*, **5**, 688-691.

Ruiz Sebastián, C. and O'Ryan, C., 2001, Single–cell sequencing of dinoflagellate (Dinophyceae) nuclear ribosomal genes. *Molecular Ecology Notes*, **1**,329-331.

Takahashi, O., Mayama, S. and Matsuoka, A., 2003, Host-symbiont associations of polycystine Radiolaria: epifluorescence microscopic observation of living Radiolaria. *Marine Micropaleontology*, **49**, 187-194.

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

Yuasa, T., Takahashi, O. and Mayama, S., 2004, PCR primers for the amplification of the nuclear small subunit ribosomal DNA sequences from polycystine radiolarians. *The Japanese Journal of Protozoology*. **37**, 133-137.

Yuasa, T., Takahashi, O., Honda, D. and Mayama, S., 2005, Phylogenetic analyses of the polycystine Radiolaria based on the 18S rDNA sequences of the Spumellarida and the Nassellarida. *European Journal of Protistology*. **41**, 287-298.