

# Isolation and identification of endosymbiotic diatoms from planktonic and benthic species of foraminifera

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浮遊性および底生有孔虫から単離培養した細胞内共生珪藻

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## Abstract

Planktonic foraminifera, *Globigerina* sp. and benthic foraminifera, *Sorites orbiculus* were collected, respectively, at coastal areas and near beaches of Sesoko Island, Okinawa, Japan. From these foraminifera, spheroid endosymbionts without frustules were isolated and cultivated. Since these isolates formed frustules, they were identified as diatoms. The symbiotic diatom from planktonic foraminifera was *Amphora leeana* sp. nov. From *S. orbiculus* two diatom species, *Nitzschia* sp. and *Nanofrustulum shiloi* were recognized.

## Key index words

*Amphora leeana* sp. nov., benthic foraminifera, endosymbiotic diatoms, *Globigerina*, *Nanofrustulum shiloi*, planktonic foraminifera

## Introduction

Diatoms living as endosymbionts of foraminifera had been suggested by some researchers (e.g., Dietz-Elbrächter 1971, Shmalijohann & Röttger 1976, Leuternegger 1977, Berthold 1978), however, the identification of the symbionts was impossible *in situ*. Lee *et al.* (1979) succeeded in making symbionts form frustules by means of isolation from host and cultivation of isolates. Lee and his co-workers aggressively researched the taxonomy of the symbiotic diatoms and the relationship between symbiont and host foraminifera (e.g., Lee & McEnery 1980, Lee *et al.* 1980, Lee *et al.* 1982, Lee & McEnery 1983, Lee 1983, Lee *et al.* 1989, Lee *et al.* 1992), although other authors rarely studied the symbiotic diatoms (e.g., Rho *et al.* 1996). The hosts, which they examined in the course of their research, were larger benthic foraminifera, i.e., *Amphistegia lessonii*, *Amphistegia lobifera*, *Heterostegina depressa*, *Borelis schlumbergi*, *Operculina ammonoides*, *Calcarina calcar*, *C. gaudichaudi*, *C. defrancei*, *C. spengleri*, *Baculogypsina*

*sphaerulata*, *Neorotalia calcar*; they have found 20 taxa of symbiont diatoms.

Studies of planktonic foraminifera revealed endosymbioses of dinoflagellates (e.g. Be *et al.* 1977) and Chrysophycophyta (Gastrich 1987), but there was no finding of diatoms as intracellular symbiotic algae. In this study we isolated small spheroid algae (ca. 5  $\mu$ m) from planktonic *Globigerina* sp. collected from the East China Sea, and cultured it. Consequently, the alga formed a frustule and could be identified as a diatom taxon. We also isolated symbiotic algae from benthic *Sorites orbiculus* and could identify them as diatoms, because the frustules were formed during culture.

## Materials and methods

The planktonic foraminiferan species, *Globigerina* sp., was collected on October 8, 1998, from 3 m under the water surface in the East China Sea at 26° 36' 76"N, 127° 48' 98"E (a bottom depth of 211 m), which was located about 5 km WSW of Sesoko Island, Okinawa Pref. Japan. The benthic larger species, *Sorites orbiculus*, was collected from shallow reef habitat on Sesoko Island on June 2, 1999. They were

washed with filtered, sterilized sea water three times and cut by micro-knife under an inverted microscope. Cytoplasm, including yellow-brown spheroid algae, was raked up with "Denter-Systema<sup>®</sup>" (Lion Corp., Tokyo) toothbrush fiber (Mayama & Mayama 1995), and the endosymbionts were isolated with a capillary pipette. After washing by sterilized sea water and confirmation of being free of contamination using  $\times 40$  objective, the isolates were transferred into glass tubes multiplate holes, and pre-cultured under the condition of 20°C and L:D 12h:12h. Each tube or hole had five to eight cells at the beginning. One to three weeks later, the symbiotic algae, in which multiplication was recognized with  $\times 20$  objective, were transferred to petri dishes containing f/2 medium (Guillard & Ryther 1962) to obtain enough cells for the cleaning procedure.

Aliquot cells were harvested from a strain which had grown sufficiently, suspended in hydrogen peroxide, and cleaned by ultraviolet radiation (Swift 1967); then they were washed in distilled water. The samples were coated with platinum-palladium and observed by a scanning electron microscope (SEM), HITACHI S-5000.

### Results and discussion

#### Endosymbiotic diatom from planktonic foraminifera

*Amphora leana* Mayama et Nagumo sp. nov. Figs 1-9.

Valva minutissima semielliptica, margine dorsali convexa, margine ventrali directa, apicibus leviter rostratis, 4.6-7.0  $\mu\text{m}$  longae, 2.3-2.6  $\mu\text{m}$  latae. Raphe quasi directa, apicibus vincis approximates. Striae transapicales latera dorsales leniter radiatae 23-26 in 10  $\mu\text{m}$  latera ventrales brevissimae subtilissimaeque, ca. 40 in 10  $\mu\text{m}$ .

**Holotype**: TNS-AL-47999. National Science Museum Tokyo (TNS). (from clone A-40; Host: *Globigerina* sp.)

**Type locality**: East China Sea at 26° 36' 76" N, 127° 48' 98" E, about 5 km WSW of Sesoko Island, Okinawa Pref. Japan

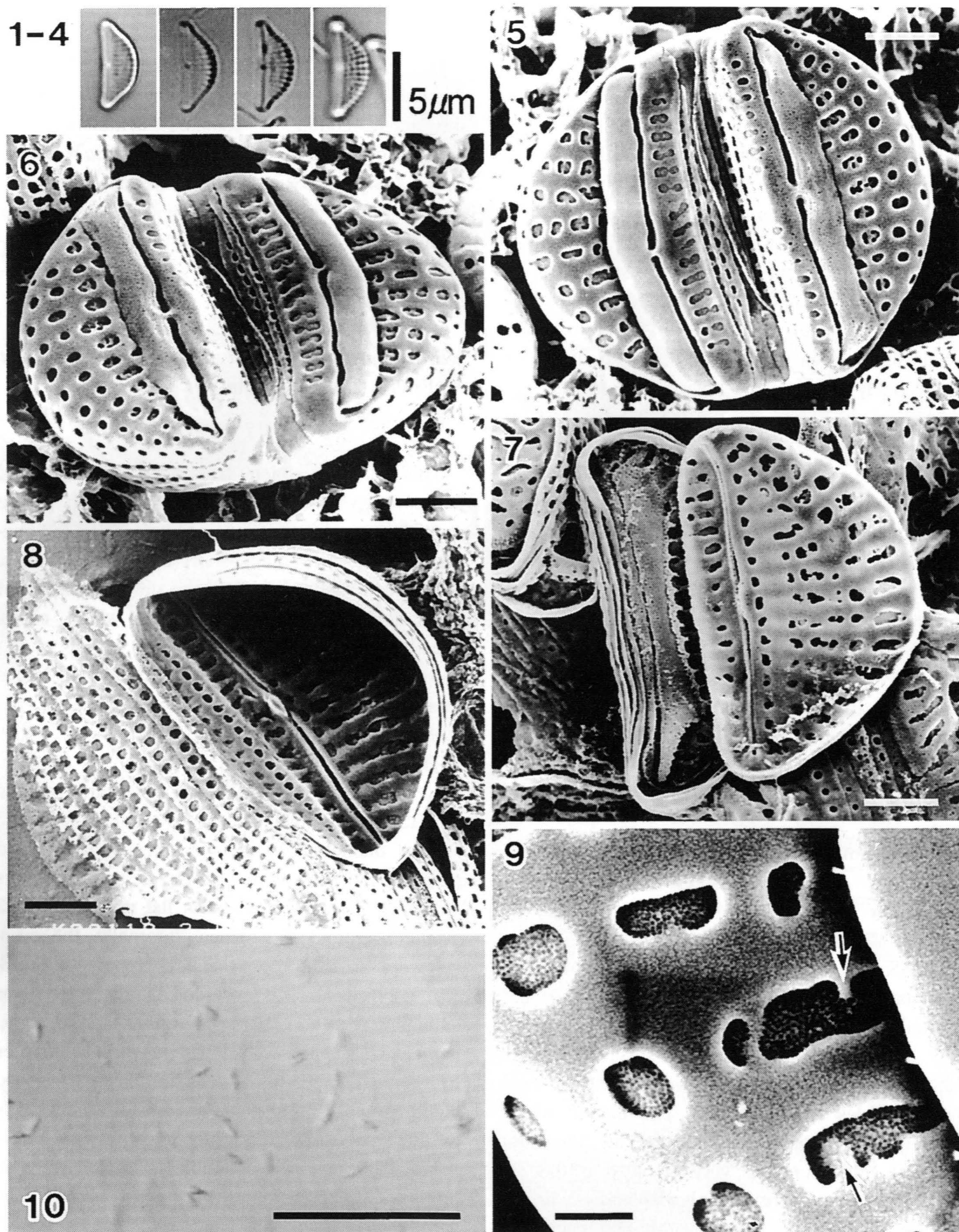
**Etymology**: The specific epithet *leana* is given

in honor of Professor John J. Lee of the City College, City University of New York, who first succeeded in isolating and cultivating diatom symbionts from larger foraminifera.

Valve small, semi-elliptical with convex dorsal and straight ventral margins (Figs 1-4). Valve ends faintly rostrate. Length 4.6-7.0  $\mu\text{m}$ . Width 2.3-2.6  $\mu\text{m}$ . Raphe almost straight, proximal ends close. Dorsal striae weakly radiate, ventral striae very short and not resolved. Striae 23-26 in 10  $\mu\text{m}$  dorsally, ca. 40 in 10  $\mu\text{m}$  ventrally.

SEM observations revealed the detailed structure of the endosymbiotic diatom from the planktonic foraminifera. The specimens showed a well developed raphe ledge and striae consisting of single rows of areolae that continue from valve face to mantle without any interruption along the valve face and mantle juncture (Figs 5,6). The shape of areola opening was variable (Figs 5,6,9), and each areola had hymenate pore occlusion on the inner aperture (Figs 7-9). The perforations of the hymen were a regular scatter type (Mann 1981) (Fig.9). Some areolae had extensions of siliceous rod-like flaps from the external edge of the areola opening; these flaps fused with the hymens. As the ultraviolet radiation we used is one of the mildest cleaning methods, we could easily observe a complete frustule (Fig.6) without any damaged bands. Each band was split and areolate (Figs 5,6,8). This and the valve features classified the taxon into *Amphora* Section Halamphora, as seen in modern taxonomic reference (Nagumo & Kobayasi 1990).

This species resembles *Amphora roettgeri* J.J. Lee et Reimer, which was originally described as an endosymbiotic diatom from larger foraminifera (Lee & Reimer 1984), though it is different in valve shape and striae density. *A. roettgeri* shows the valve with straight to slightly convex ventral margin, obtusely rounded ends, and finer dorsal striation, 30-32 in 10  $\mu\text{m}$ . Moreover, the difference is evident in SEM observation. The specimen from the same clone as the type of *A. roettgeri* has striae, each of which consists of a double row of poroids in the valve face but a single row in the dorsal mantle, and has hyaline costa along the valve face and mantle juncture (see Lee & Reimer 1984, fig.2). In addition, the figure shows the raphe ledge is insufficiently de-



**Figs 1-9.** *Amphora leana*, an endosymbiont isolated from *Globigerina* sp. Scale bars=1  $\mu\text{m}$  (Figs 5-8) or 0.2  $\mu\text{m}$  (Fig.9). **Figs 1-4.** Cultured specimens (Fig.2. Holotype). **Fig.5.** Front view of a whole frustule showing two valves with almost straight raphe branches. **Fig.6.** The whole frustule showing prominent dorsal raphe ledges, oblique view. **Fig.7.** The valve interior. Areolar occlusions, hymens, are partly broken. **Fig.8.** The valve interior and copulae with serial pores, oblique view. **Fig.9.** Detail of areolae showing hymenate pore occlusions with perforations of regular scatter type. Note siliceous rod-like flaps fused with the hymens (arrows). **Fig.10.** *Nitzschia* sp. isolated from *Sorites orbiculus*. Cells in culture plate were photographed using an inverted microscope with  $\times 20$  objective. Scale bar=100  $\mu\text{m}$ .

veloped.

As cultured symbiotic diatoms are usually dwarfs, abnormal morphology is likely to develop. In fact, the length of the raphe branches is different even in a pair of branches in a single valve (Fig.5). However, *Amphora* sp., presented by Lee & Reimer (1984, figs 1,3) and upon which they commented, "Possibly clonal variants of *A. roettgeri*", and the specimen identified as *A. roettgeri* (Lee *et al.* 1989, fig.10) seem to share the fine structures with our species, so that they are probably of the same taxon as *A. leeana*.

*Amphora* is known to be benthic at least in the middle or larger size species. If *A. leeana* is also benthic, the intracellular occurrence of *A. leeana* in the planktonic foraminifera may be somewhat mysterious. However, *Amphora tenerima* Aleem *et* Hust., which is a small diatom and frequently occurs as a larger foraminiferan endosymbiont in different hosts from different habitats (Lee *et al.* 1989, 1992), was also reported as plankton in Xiamen Bay of the East China Sea (Cheng *et al.* 1989). This occurrence implies that our endosymbiotic species also can grow as a free-living diatom, which is then caught by planktonic foraminifera.

#### Endosymbiotic diatoms from benthic foraminifera

##### *Nitzschia* sp.

Fig.10.

The *Sorites orbiculus* we collected was a common larger foraminifera around Sesoko Island (Hohenegger *et al.* 1999) and had many spheroid endosymbionts. Among several isolates transferred into microplate, two diatoms formed their frustules and multiplied. The diatom which showed the fastest multiplication in the first week of cultivation was very small (Fig.10). Because the valves were linear-lanceolate, ca. 10  $\mu\text{m}$  in length and ca. 3  $\mu\text{m}$  in width and each cell had two plastids arranged in series along the apical axis of the cell, this diatom was identified as *Nitzschia* sp. They were transferred to petri dishes containing f/2 medium for further multiplication; however, unfortunately they did not grow any more in the medium, so that identification of species level was impossible, as we could not obtain a sufficient amount of cells for the cleaning procedure.

Lee *et al.* (1989, 1992) described *Nitzschia frustulum* var. *symbiotica* J.J. Lee *et* Reimer as the most commonly isolated symbiont from larger foraminifera. Though *Nitzschia pandriiformis* var. *continua* and *N. laevis* were frequently recorded as other symbiotic *Nitzschia* (Lee *et al.* 1989), the valve shape of the cultured diatom most resembles that of *N. frustulum* var. *symbiotica*.

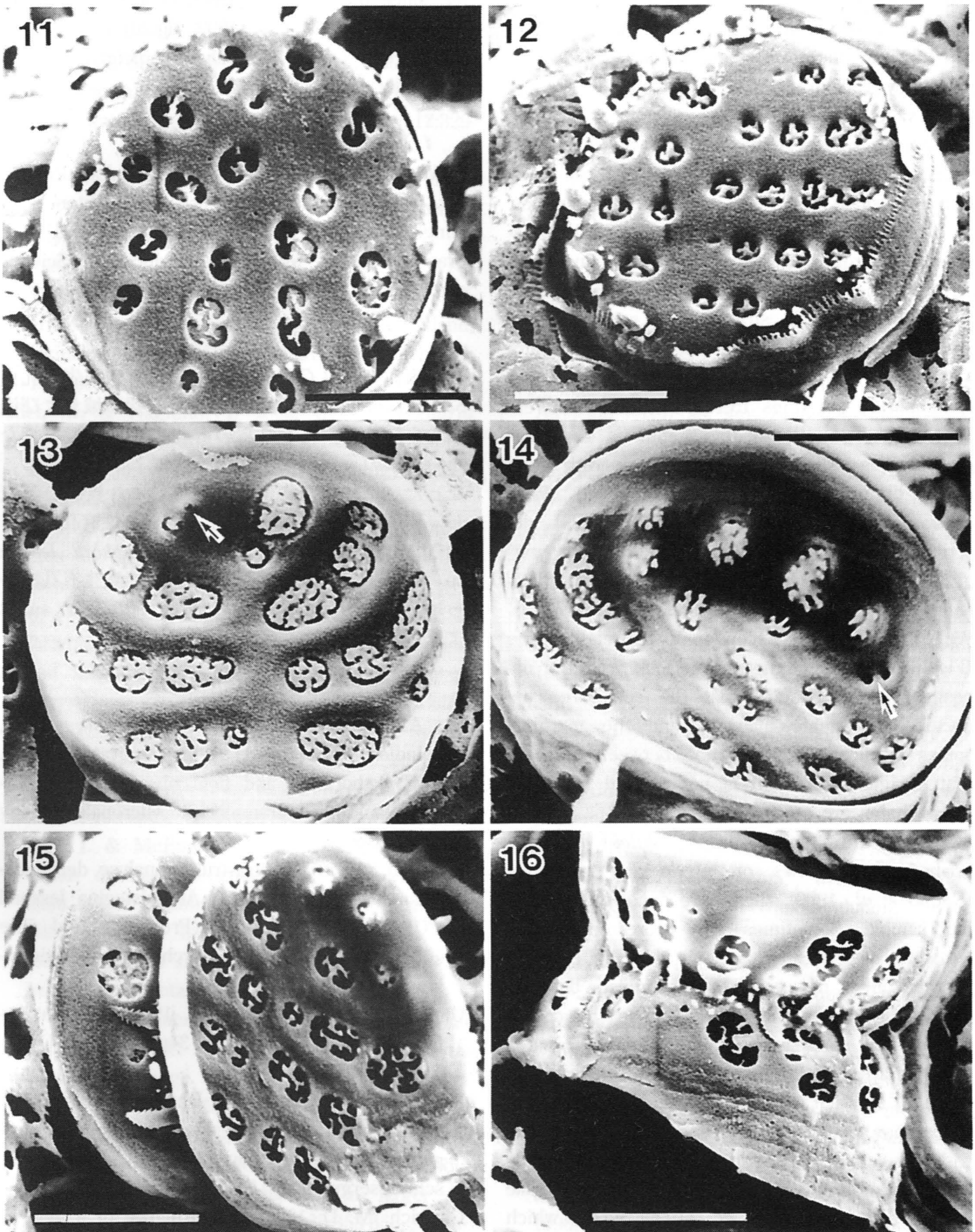
#### *Nanofrustulum shiloi* (J.J.Lee, Reimer *et* McEnery) Round, Hallst. *et* Paasche

Figs 11-16.

This diatom, which had been smaller than *Nitzschia* sp. and achieved second growth in the multiplate, proliferated after transfer into f/2 medium and formed many short chain-like colonies. SEM observation revealed the fine structure of fragilarioid species (Figs 11-16), which corresponds to that of *Fragilaria shiloi* J.J.Lee *et al.*, described originally as an endosymbiont in larger foraminifera, *Amphistegina lessonii* (Lee *et al.* 1980).

The valves observed were circular, as are centric diatoms, but with the sternum central (Fig.13) or slightly eccentric (Figs 11,12), 2.0-2.5  $\mu\text{m}$  in diameter. Marginal spines were located peripherally across the rows of areolae (Figs 11,12,16). The valve face was flat and with small granules around the spines. Internally, the areolae were arranged in a shallow trough (Figs 13-15). Each areola was occluded by dissected flaps extending from short marginal projections, namely vola, which developed to various degrees. In the specimens in which the flaps developed vigorously, the areola looks occluded internally as the vola extended slightly inward (Figs 13,14). A single pore, rarely a double pore, is located at one end of the sternum (Figs 13,14, arrow). The valvocopula was a split band (Fig.14).

Hallegraeff & Burford (1996) transferred *F. shiloi* to the genus *Pseudostaurosira* according to Williams & Round (1987), who divided traditionally defined *Fragilaria* into several genera. The areolae of *Pseudostaurosira brevistriata*, the generic type species shown by the latter authors, had only remnants of a closing plate, but the same species photographed by Idei & Nagumo (1995) showed distinctly dissected plates, volae.



**Figs 11-16.** *Nanofrustulum shiloi*, an endosymbiont isolated from *Sorites orbiculus*. Scale bars=1  $\mu$ m. **Fig.11.** The external front view of the valve face with marginal spines. **Fig.12.** Oblique view of the valve showing eccentric sternum and fringed copula. **Fig.13.** The valve interior showing well developed volae. There is a single pore at one end of the sternum (arrow). **Fig.14.** The valve interior showing double pores at one end of the sternum (arrow). **Fig.15.** Sibling valves and linking spines with hairs. **Fig.16.** Connection of sibling valves stabilized by spines. Note thin and delicate copulae.

The appearance of the specimen suggests the close relationship of *F. shiloi* to *Pseudostaurosira* with areolae; therefore, the nomenclatural combination by Hallegraeff & Burford (1996) seemed to be validated.

However, very recently Round *et al.* (1999) established a new genus, *Nanofrustulum*, and transferred *F. shiloi* to it. As generic characteristics, they pointed out the marginal spines with a basal, downwardly projecting spinula and fine hairs on the spines. They are surely unique characteristics, but not all strains and samples they showed bear them. In our specimens, the hairs were observed but the downwardly projecting spinula were not (Figs 15,16). The most differentiating characteristic of this genus is the marginal spines, which do not interlock with each other differently from *Fragilaria* and *Pseudostaurosira*, though Round *et al.* (1999) described *N. shiloi* as "linked by interlocking marginal spines." Each marginal spine merely attaches to the neighboring valve mantle (Fig.15). We observed an exceptional bifurcate spine (Fig.16) but even it never interlock with the opposite spine. It seems that the cells are connected by organic material, and the marginal spines stabilize the connection between cells, so that a rigid connection is not formed and only short chains are always observed.

Though the copulae of *N. shiloi* were described as a series of interlocking segments (Round *et al.* 1999), they were not segmented in our sample. Sometimes they were severed in certain places because of their delicacy (Fig.16). In fact, it is not easy to distinguish between genuine segments and artifacts when the copula is very thin and narrow. Copulae of many nanodiatoms are usually quite thin and delicate. In addition, surface tension caused by drying in preparation may make a cylindrical cingulum flat with distortion. Therefore, argument would be necessary for the segmented scale-like copulae explained by Round *et al.* (1999, fig.8), which resemble "quasifraction" in *Extubocellulus spinifera* and *Minutocellus pseudopolymorphus* (Hasle *et al.* 1983). The *Minutocellus polymorphus* we observed had obscurely segmented copulae in usual drying preparation, but not in critical point drying preparation (unpublished data). Nevertheless, segmented copulae are acceptable in *N. shi-*

*loi*, since the copulae termed concatemerous girdle bands, which Lee (1992, fig.20) observed in dwarf cells (<2  $\mu\text{m}$  in valve diameter), are undoubtedly segmented.

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We would like to thank Mr. S. Nakamura and Mr. Y. Nakano for assistance in plankton sampling. This study just barely was supported by a grant from the Ministry of Education, Science and Culture of Japan (No. 10836008).

#### 摘 要

浮遊性有孔虫 *Globigerina* sp. および底生有孔虫 *Sorites orbiculus* を沖縄県瀬底島周辺海域および浅瀬から採集した。これらの有孔虫から被殻を持たない球形の黄褐色の共生藻を単離し、培養をおこなった。これらの共生藻は培養により被殻を形成し、珪藻であることが判明した。浮遊性有孔虫から得られた共生珪藻は新種であり、*Amphora leeana* sp. nov. としてここに記載した。また底生有孔虫からは1個体中に2種の共生珪藻、*Nitzschia* sp. および *Nanofrustulum shiloi* が認められた。

#### References

- Be, A. W. H., Hemleben, C., Anderson, O. R., Spindler, M., Hacunda, J. & Tuntivate-Choy, S. 1977. Laboratory and field observations of living planktonic foraminifera. *Micropaleontology* **23**:155-179.
- Berthold, W. 1978. Ultrastrukturanalyse der endoplasmatischen Algen von *Amphistegina lessonii* d'Orbigny, Foraminifera (Protozoa) und ihre systematische Stellung. *Archiv für Protistenkunde* **120**:16-62.
- Cheng, Z., Gao, Y., & Liu, S. 1989. Nanodiatoms from Fujian Coast. 91pp. 34pls. China Ocean Press, Beijing.
- Dietz-Elbrächter, G. 1971. Untersuchungen über die Zooxanthellen der Foraminifere *Heterostegina depressa* d'Orbigny 1926. *Meteor Forschungsergebnisse*, **C6**:41-47.
- Gastrich, M. D. 1987. Ultrastructure of a new intracellular symbiotic alga found within planktonic foraminifera. *Journal of Phycology* **23**:623-632.
- Guillard, R. R. L. & Ryther, J. H. 1962. Studies of marine planktonic diatoms. I *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Canadian Journal of Microbiology* **8**:229-

- 239.
- Hallegraeff, G. M. & Burford, M. A. 1996. Some new or little known nanoplankton diatoms cultured from tropical and subtropical Australian shelf waters. *Nova Hedwigia, Beiheft* **112**:329-342.
- Hasle, G. R., Stosch, H. A. von, Syvertsen, E. E. 1983. Cymatosiraceae, a new diatom family. *Bacillaria* **6**:9-156.
- Hohenegger, J., Yordanova, E., Nakano, Y. & Tatzreiter, F. 1999. Habitats of larger foraminifera on the upper reef slope of Sesoko Island, Okinawa, Japan. *Marine Micropaleontology* **36**:109-168.
- Idei, M. & Nagumo, T. 1995. Genus *Fragilaria* (sensu stricto) and related genera in raphid diatoms. *Japanese Journal of Phycology* **43**:227-239. (in Japanese).
- Lee, J. J. 1983. Perspective on algal endosymbionts in larger foraminifera. *International Review of Cytology, Supplement* **14**:49-77.
- Lee, J. J. 1992. Taxonomy of algae symbiotic in foraminifera. 79-92. *In*:Reisser, W. (ed.) *Algae and symbioses: plants, animals, fungi, viruses, interactions explored*. Biopress Limited, Bristol.
- Lee, J. J., Faber Jr., W. W., Nathanson, B., Röttger, R., Nishihara, M. & Krüger, R. 1992. Endosymbiotic diatoms from larger foraminifera collected in Pacific habitats. *Symbiosis* **14**:265-281.
- Lee, J. J. & McEnery, M. E. 1980. The identification of diatoms isolated as endosymbionts from larger foraminifera from the Gulf of Eilat (Red Sea) and the description of 2 new species, *Fragilaria shiloi* sp. nov. and *Navicula reissii* sp. nov. *Botanica Marina* **23**:41-48.
- Lee, J. J. & McEnery, M. E. 1983. Symbiosis in foraminifera. 37-68. *In*:Goff, L. J. (ed.) *Algal symbiosis*. Cambridge University Press, Cambridge.
- Lee, J. J., McEnery, M. E., Kuile, B. T., Erez, J., Röttger, R., Rockwell, R. F., Faber Jr., W. W. & Lagziel, A. 1989. Identification and distribution of endosymbiotic diatoms in larger foraminifera. *Micropaleontology* **35**:353-366.
- Lee, J. J., McEnery, M. E., Röttger, R. & Reimer, C. W. 1980. The isolation, culture and identification of endosymbiotic diatoms from *Heterostegina depressa* D'Orbigny and *Amphistegina lessonii* D'Orbigny (larger foraminifera) from Hawaii. *Botanica Marina* **23**:297-302.
- Lee, J. J., McEnery, M. E., Shilo, M. & Reiss, Z. 1979. Isolation and cultivation of diatom symbionts from larger foraminifera (Protozoa). *Nature* **280** (5):57, 58.
- Lee, J. J. & Reimer, C. W. 1984. Isolation and identification of endosymbiotic diatoms. 327-343. *In*:Mann, D. G. (ed.) *Proceedings of the seventh International Diatom Symposium, Philadelphia, August 22-27, 1982*. Otto Koeltz, Koenigstein.
- Lee, J. J., Reimer, C. W. & McEnery, M. E. 1980. The identification of diatoms isolated as endosymbionts from larger foraminifera from the Gulf of Eilat (Red Sea) and the description of two new species, *Fragilaria shiloi* sp. nov. and *Navicula reissii* sp. nov. *Botanica Marina* **23**:41-48.
- Lee, M. J., Ellis, R. & Lee, J. J. 1982. A comparative study of photoadaptation in four diatoms isolated as endosymbionts from larger foraminifera. *Marine Biology* **68**:193-197.
- Leutenegger, S. 1977. Symbiosis between larger foraminifera and unicellular algae in the Gulf of Eilat. *Utrecht Micropaleontological Bulletins* **15**:225-239.
- Mann, D. G. 1981. Sieves and flaps: siliceous minutiae in pores of raphid diatoms. 279-300. *In*: Ross, R. (ed.) *Proceedings of the sixth symposium on recent and fossil diatoms*. Budapest, September 1-5, 1980. Otto Koeltz, Koenigstein.
- Mayama, N. & Mayama, S. 1995. The substitution of a "Denter-Systema®" tooth brush fiber for an eyelash in micromanipulation. *Diatom* **11**:88.
- Nagumo, T. & Kobayasi, H. 1990. Three *Amphora* species of the section *Halamphora* (Bacillariophyceae) found in Japanese brackish waters. 149-160. *In*:Simola, H. (ed.) *Proceedings of the tenth International Diatom Symposium, Joensuu, Finland, August 28-September 2, 1988*. Koeltz Scientific Books, Koenigstein.
- Rho, M. -C., Matsunaga, K., Park, Y. -H., Yasuda, K., Yamasu, T., Mayama, S. & Ohizumi, Y. 1996. A sulfonoglycolipid with Na<sup>+</sup>, K<sup>+</sup>-ATPase inhibitory activity, produced by a cultured unique diatom symbiot isolated from a larger foraminifera. *Planta Medica* **64**:552-554.
- Round, F. E., Hallsteinsen, H. & Paasche, E. 1999. On a previously controversial "Fragilarioid" diatom now placed in a new genus *Nanofrustulum*. *Diatom Research* **14**:343-356.

- Shmaljohann, R. & Röttger, R. 1976. The ultrastructure and taxonomic identity of the symbiotic algae of *Heterostegina depressa* (Foraminifera, Nummulitidae) Journal of Marine Biological Association U.K. 58:227-237.
- Swift, 5th, E. 1967. Cleaning diatom frustule with ultraviolet radiation and peroxide. Phycologia 6: 161-163.
- Williams, D. M. & Round, F. E. 1987. Revision of the genus *Fragilaria*. Diatom Research 2:267-288.

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