

## Shigeki Mayama and Ikuko Shihira-Ishikawa: Putative nucleoids scattered in chloroplast of *Pinnularia nobilis* (Bacillariophyceae)

Chloroplasts of the diatom identified as *Pinnularia nobilis* (Ehr.) Ehr. were stained with 4',6-diamidino-2-phenylindole (DAPI) and studied with both epifluorescence microscope and confocal laser scanning microscope (LSM). Numerous DAPI-fluorescent dots were observed throughout the chloroplast. DNase treatment confirmed that these dots contained DNA. LSM observation clarified that these DNA dots were located within chloroplasts. It was suggested the chloroplast nucleoids were scattered in this species.

*Key Index Words:* chloroplast nucleoids—confocal laser scanning microscopy—DAPI—*Pinnularia nobilis*—scattered DNA spots.

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Geitler (1937) described sieve-like perforations in chloroplasts of *Pinnularia nobilis* (Ehr.) Ehr. with light microscopy. Each of the perforations was about 0.5  $\mu\text{m}$  in diameter and they were scattered evenly throughout the plate-like chloroplasts. Further observations concerning this structure have not been published thereafter even in other diatoms. The perforations described by Geitler are identical to the scattered DNA spots in the chloroplasts of *P. nobilis* we first present in this paper.

*P. nobilis* was collected from a small mire in Hachigata, Saitama Pref. Some of the cells were isolated for culture and the others were cleaned with bleaching method for identification (Nagumo and Kobayasi 1990). For observations of the cleaned valves and live cells, a light microscope (Nikon SKE) and a microscope equipped with differential interference contrast (Nikon Optiphot) (DIC) were used respectively.

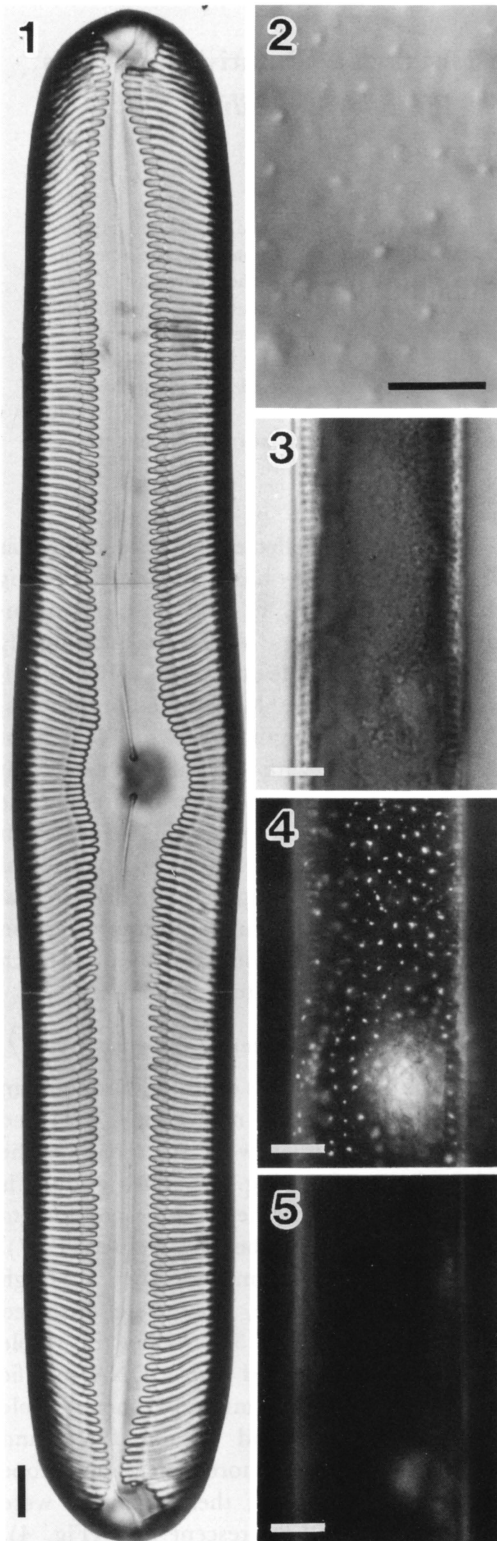
The isolated cells were cultured in Bold's Basal Medium (Bischoff and Bold 1963), to which was added 50 mg  $\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$  per liter (pH 6.8), and then diluted with distilled water to one-fifth. The cultures were maintained at 20°C under a cool white fluorescent light of 1500–2500 lux on a 12 : 12 (L : D) photoperiod.

The diatoms collected were identified as *P. nobilis* on the basis of the following characteristics (Fig. 1). Frustules rectangular in girdle view. Valves linear, slightly swollen in the middle portion, broadly rounded and sometimes slightly swollen in the ends, 246–309  $\mu\text{m}$  in length, 39–44  $\mu\text{m}$  in width. Raphe complex with the fold of raphe slit and the strongly sigmoid outer fissure. Striae radiate in the middle portion, convergent toward the ends, 5–6 in 10  $\mu\text{m}$ . Longitudinal band of striae, i.e. alignment of the internal aperture of alveoli in scanning electron microscopy, wide, about 2/3 width of each stria. Chloroplast plates, two per cell.

### Light and epifluorescence microscopy

We found many dots on the chloroplasts in *P. nobilis*. With DIC microscopy, it looked as if many granules were scattered on the chloroplast surface (Fig. 2). Each granule is about 0.5  $\mu\text{m}$  in diameter corresponding to the perforation described by Geitler (1937). Using bright field microscopy, although usually hardly visible, they were observed as scattered pits (Fig. 3). When the whole diatom cell was stained with a DNA specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI) (Kuroiwa and Suzuki 1980) and observed with an epifluorescence microscope (Olympus BH2-RFK), these granules were detected as DAPI-fluorescent dots (Fig. 4).

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These dots completely disappeared with DNase treatment (Fig. 5). For DNase treatment, 13 units·ml<sup>-1</sup> of DNase I (Type IV, Sigma) dissolved in 0.08 M sodium acetate (pH 5.0), 4 mM MgSO<sub>4</sub> and 0.025 M NaCl were applied to the specimens, followed by incubation at 30°C for 1.5 h. Before DNase application, frustules of the specimens had been broken partly near the apex with a micro-knife under a light microscope to improve penetration of the enzyme. In Fig. 5, the DAPI-fluorescence of the nucleus is scarcely visible, however, it entirely disappeared following 2 h in DNase. This result confirmed that the scattered DAPI-fluorescent dots contained DNA.

#### Confocal laser scanning microscopy (LSM)

The location of DAPI-fluorescent dots was examined in detail using LSM (Olympus LSM-GB200 UV-type) with "U-excitation". The levels of 10 serial optical sections observed in girdle view and 5 serial optical sections in valve view are diagrammed in Fig. 6. The real distances between two sections were 0.9 μm in girdle view and 1.4 μm in valve view. In Fig. 7, the photographs a-f were taken at the levels of optical sections a-f indicated in Fig. 6. The photograph of the uppermost section (Fig. 7a), the girdle bands and valves, was taken using twice the exposure time of the others, because an obvious fluorescence was not observed. This section shows the obscure images of red fluorescence being reflected from the autofluorescence of chloroplast to the girdle bands (Fig. 7a, middle part) and blue fluorescence which is possibly mitochondrial DNA located in alveolate striae (Fig. 7a, clear in right side and obscure in left) and non-specifically adherent DAPI to

Figs. 1-5. *Pinnularia nobilis*. Scale bars = 10 μm (Figs. 1, 3-5) or 5 μm (Fig. 2). Fig. 1. A whole cleaned valve. Fig. 2. Enlarged chloroplast showing scattered granules in girdle view. DIC. Figs. 3-5. The same cell stained with DAPI in girdle view. Fig. 3. Bright field illumination image. Fig. 4. Epifluorescence microgram showing scattered DAPI-fluorescent dots throughout chloroplast and fluorescence of nucleus (below). Before DNase treatment. Fig. 5. DNase treatment for 1.5 h. DAPI-fluorescent dots disappeared.

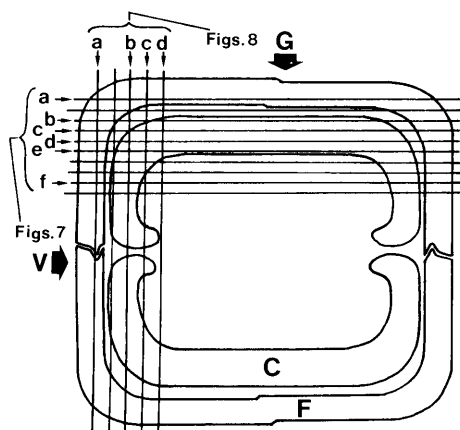


Fig. 6. Diagram showing a transapical section of the cell with lines indicating the levels of 10 serial optical sections observed in girdle view and 5 in valve view respectively. The optical sections at the levels indicated by arrows a-f and a-d are presented in Figs. 7a-f and Figs. 8a-d respectively. C=chloroplast, F=frustule, G=girdle view, V= valve view.

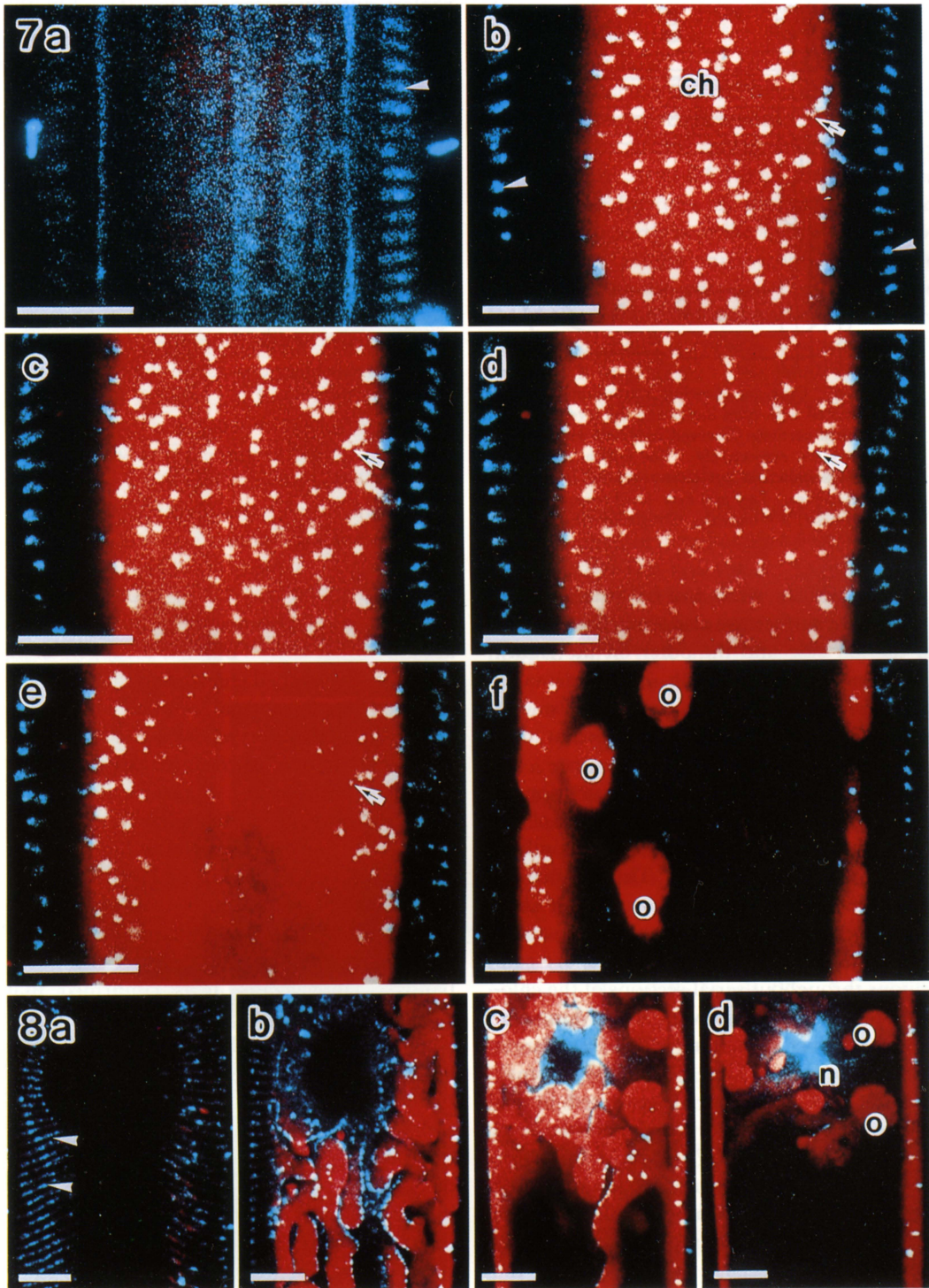
the valve surface, while two bacteria attached to the valve surface are also seen (Fig. 7a, one on the left side and the other on the right). The photographs b-e in Fig. 7 are the images of horizontal sections of a chloroplast. A number of scattered DAPI-fluorescent dots are observed in white. In this case, the DAPI-fluorescence is too strong to appear blue. Blue dots aligned on both sides of the chloroplast area are possibly mitochondrial DNA in the alveolate striae. The distance between sections b and e is  $2.7 \mu\text{m}$ , but the chloroplast is a little thicker than that, i.e. about  $3 \mu\text{m}$ . Most DAPI-fluorescent dots are continuous throughout serial sections of the chloroplast (Figs. 7b-e, arrows). The average diameters of dots in sections b and c are larger than in section d (Figs. 7b-d). Very small fluorescent dots are rarely scattered in the middle area of section e, i.e. the bottom level of the chloroplast (Fig. 7e). From this three dimensional observation, it is suggested DAPI-fluorescent dots are globular and are embedded in the chloroplast. The chloroplast-autofluorescence is not seen in the middle area of section f because of the chloroplast curvature, while three storage oil drops are visible due to the reflection of chlo-

roplast autofluorescence (Fig. 7f). On the right side of Fig. 7f, the band of chloroplast represents a longitudinal section of the lobed chloroplast extending immediately under the valve face. The discontinuity of the band shows the fimbriation of the chloroplast edge. The thickness of the chloroplast in this portion is about  $2.5 \mu\text{m}$  which is a little thinner than the distance estimated between sections b and e. DAPI-fluorescent dots are clearly located within the chloroplast itself, as shown in the chloroplast band in Fig. 7f. The left band of the chloroplast does not indicate the real thickness, as the chloroplast sinks slightly inward and the band is a tangential section of the chloroplast lobe.

Serial optical sections in valve view are shown in Figs. 8a-d. In the horizontal section of the valve, the alveolate striae with possible mitochondrial nucleoids are visible (Fig. 8a). The edge of the chloroplast is complicatedly lobed and DAPI-fluorescent dots are seen in the chloroplast (Fig. 8b). The dots are not only scattered but also arranged along the edge of the lobed chloroplast. In the next section (Fig. 8c), a part of the lobed chloroplast is still observed with DAPI-fluorescent dots in two patterns of arrangement. As DAPI-fluorescence of the nucleus is too strong, it sometimes causes flaring inside the cell. This flaring is visible as blue fluorescence in the upper middle of Figs. 8c and 8d and no part of the nucleus is included in these photographs. Fig. 8d shows a cross section of two chloroplasts extending under the girdle bands. Here again, it is confirmed that DAPI-fluorescent dots are located within the chloroplast. The thickness of the chloroplast is  $2.5\text{-}3.7 \mu\text{m}$  which includes the range of thickness estimated from the observation of serial sections shown in Figs. 7b-e.

LSM was useful, in this study, to analyze the three-dimensional structure of DNA spots in chloroplast in optical sections. It revealed that DNA spots were located in the chloroplast, suggesting these scattered DNA spots were chloroplast nucleoid (ct-nucleoid).

The scattered DNA spots were also observed in several species of *Pinnularia* (in



preparation) and the fine structures of DNA spots in their chloroplasts are under investigation. The TEM observations has already revealed that these DNA spots were not derived from invagination of mitochondria or endosymbiont into the chloroplast but corresponded to the discrete areas of stacking thylakoids. In addition to the results of DNase treatment and LSM observation, these TEM observations also suggest that the scattered DNA spots are ct-nucleoids. However, it has not been proven that these DNA spots comprise the chloroplast genome. Conclusive evidence of the scattered DNA spots being ct-nucleoid is under investigation.

Coleman (1979, 1985) and Kuroiwa et al. (1981) reported the ct-nucleoids in DAPI-stained diatoms. They have examined 12 species belonging to 12 genera and described that diatoms have ct-nucleoids arranged along the chloroplast edge, i.e. ring ct-nucleoids. The *P. nobilis* we observed presumably has the scattered ct-nucleoids in addition to the ring ct-nucleoids.

## References

- Bischoff, H. W. and Bold, H. C. 1963. Phycological studies, IV. Some algae from Enchanted Rock and related algal species. No. 6318. The Univ. of Texas Pub. 95 pp.
- Coleman, A. W. 1979. Use of fluorochrome 4'-diamidino-2-phenylindole in genetic and developmental studies of chloroplast DNA. *J. Cell Biol.* **82**: 299-305.
- Coleman, A. W. 1985. Diversity of plastid DNA configuration among classes of eukaryote algae. *J. Phycol.* **21**: 1-16.
- Geitler, L. 1937. Chromatophor, Chondriosomen, Plasmabewegung und Kernbau von *Pinnularia nobilis* und einigen anderen Diatomeen nach Lebendbeobachtungen. *Protoplasma* **27**: 534-543.
- Kuroiwa, T. and Suzuki, T. 1980. Circular nucleoids isolated from chloroplasts in a brown alga *Ectocarpus siliculosus*. *Exp. Cell Res.* **134**: 457-461.
- Kuroiwa, T., Suzuki, T., Ogawa, K. and Kawano, S. 1981. The chloroplast nucleus: distribution, number, size, and shape, and a model for the multiplication of the chloroplast genome during chloroplast development. *Plant Cell Physiol.* **22**: 381-396.
- Nagumo, T. and Kobayasi, H. 1990. The bleaching method for gently loosening and cleaning a single diatom frustule. *Diatom* **5**: 45-50.

### 真山茂樹・石川依久子： *Pinnularia nobilis* (Ehr.) Ehr. (珪藻綱) の葉緑体中に散在する核様体様の DNA 小粒

*Pinnularia nobilis* と同定される珪藻を DAPI 染色し、落射型蛍光顕微鏡および共焦点走査型レーザー顕微鏡で観察した。葉緑体には DAPI 蛍光を放つ点が多数散在しているが、その蛍光は DNase で処理することにより消失するため DNA を含有することが証明された。また、これらの DNA スポットは葉緑体中に存在することが共焦点走査型レーザー顕微鏡による光学的連続切片により確認された。珪藻はリング型の葉緑体核様体を持つとされていたが、*P. nobilis* はさらに分散型の葉緑体核様体を持つことが示唆された。(184 東京都小金井市貫井北町4-1, 東京学芸大学生物学教室)

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Figs. 7, 8. Serial LSM sections in *P. nobilis* stained with DAPI. Scale bars = 10  $\mu$ m. ch = chloroplast with DAPI-fluorescent dots. n = flare of nucleus fluorescence. o = storage oil. arrowhead = possible mitochondrial DNA. Figs. 7a-f. Serial optical sections from girdle inward. Arrows (b-e) indicate the serial sections of single DAPI-fluorescent dot. Figs. 8a-d. Serial optical sections from valve inward.