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Fine Structure of the Plastids Retained by the Foraminifer *Elphidium excavatum* (Terquem)

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Abstract

Previous studies showed that, when specimens of *Elphidium excavatum* harvested from field collections are fed diatoms, they retain more chloroplasts and survive longer than they do when fed diets of other types of algae. Does the host retain the chloroplasts other types of algae? To answer this question, foraminifera were fed diets of either a chlorophyte or a dinoflagellate and then studied by transmission electron microscopy (TEM). The algae used *Amphora coffeiformis* (diatom), *Amphidinium* sp. (dinoflagellate). and *Dunaliella salina* (green alga) have chloroplasts which are quite distinct from each other at the fine structural level. The experimental foraminifera were harvested directly from the field and thus already had a population of chloroplasts within their cytoplasm when the experiment started. Regardless of the type of alga used in the experimental diet, the ultrastructure of the retained plastids were typical of diatoms: 2–3 thylakoid stacks; a simple internal pyrenoid with a thylakoid crossing it; and a girdle lamella surrounding the outside of the chloroplast. Some of the plastids were undergoing digestion, because they had lost organization and we observed fusion of vesicles (presumed to be lysosomes) with the symbiosome vacuole. Cytoplasm within the inter-chamber canals of the foraminifera contained numerous vacuoles and vesicles, some of which contained algae in the process of digestion, others had isolated plastids. The plastids inside the chambers had protrusions on their edges

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corresponding to the positions of cannalicular pores connected to the intercameral canals. These pores were plugged by an electron dense material and no cytoplasmic connection was observed between the pores in the test and the intercameral cytoplasm.

Keywords: Foraminifera, *Elphidium excavatum*, kleptoplastids, chloroplast husbandry, chloroplast enslavement, fine structure of retained chloroplasts, algal plastids, symbiosis

1. Introduction

Recently we studied the phenomenon of chloroplast husbandry (chloroplast enslavement or kleptocloroplasty) by the salt marsh foraminifer *Elphidium excavatum* (Terquem). These studies were aimed at revealing whether the phenomenon was selective for particular types of algal chloroplasts and at determining their longevity within the foraminifera (Correia and Lee, 2000). The results suggested that only diatom chloroplasts were retained; chloroplasts originating from green algae or dinoflagellates seemed not to be kept by *E. excavatum* (Lee and Lee, 1989; Correia and Lee, 2000). The techniques used in those studies (epifluorescence and confocal scanning laser microscopy) however, limited us to counting the total number of plastids present, but did not give any information on their algal origin. The longevity study (Correia and Lee, 2002) showed that the sequestered chloroplasts remained fluorescent for at least 8 weeks.

After that time, the starved hosts seem to loose their viability and the fluorescence of the chloroplasts faded away. It was unclear whether the retained chloroplasts ran out of needed metabolites and underwent autolysis or whether they produced, or withdrew, molecular signals which renew host digestive activity by converting symbiosomes into phagolysosomes.

Previous studies of the fine structure of chloroplast-retaining foraminifera suggested that the plastids originated from partially digested diatoms or chrysophytes (Lopez, 1979; Leutenegger, 1984; Lee et al., 1988; Bernhard and Bowser, 1999). In one study (Lee et al., 1988) other algal organelles (mitochondria and nuclei) were found in a few symbiosomes of the foraminifer that could be useful in the diagnosis of plastid origin. The aim of this study was to examine the fine structure of the plastids/chloroplasts retained by individuals of *E. excavatum* and to compare them to the plastids of the different algae they were actually fed in their experimental diets.

2. Material and Methods

Sample collection

Samples were collected at Lake Tashmoo (Martha's Vineyard N 41°32' W 70°40') in the first week of August 2000. In this season it is easiest to collect large numbers of individuals of *Elphidium* spp. in this habitat. The procedures followed for the collection of specimens were described in Correia and Lee (2000). Individual specimens of *Elphidium excavatum* were picked from the sediment with sable paint brushes and divided into groups of 50 foraminifera. Each aliquot contained individuals of approximately the same size selected at random.

Experimental protocol

Each group of foraminifera was fed one of 3 axenic algal cultures and incubated at 25°C with a 12 hours light/12 hours dark cycle before a fluorescent light bank, or in a light tight box in complete darkness. The algae used were a diatom (*Amphora coffeiformis*), a chlorophyte (*Dunaliella salina*) and a dinoflagellate (*Amphidinium* sp.). The amount of algae added to each culture in a 250 ml tissue culture flask brought the final concentration of algae to 10^6 cells/ml. Half of the starved controls were incubated in the dark; the other half were incubated in a 12 hours light/12 hours dark cycle. For three consecutive weeks, 10 individuals were selected from each flask and prepared for TEM.

Preparation for TEM

Algae

In a fume hood, specimens were fixed in 4% glutaraldehyde in seawater for 1 hour at 4°C (ice bath). After several washes in sterile filtered seawater, the cells were post-fixed with 2% OSO_4 in sterile filtered seawater for 1 hour at 4°C. The specimens were then gently washed with distilled water and stained with a 2% solution of uranyl acetate in ethanol for 15 minutes. A progressive series of water-ethanol mixtures was used for dehydration, followed by two washes with propylene oxide and embedment in Embed 812® (Electron Microscopy Sciences). For embedment, the samples were infiltrated progressively with 1:2, 1: 1 and 2: 1 mixtures of resin and propylene oxide for at least 1 hour each. Before curing, the samples were further infiltrated with pure resin for 2.5 hours and overnight with the resin and the catalyst. The embedded specimens were then cured for 24–48 h until the resin achieved the desired

hardness. The chloroplasts of the algae fed to *Elphidium* were studied as controls, so that their fine structure could be compared to those of the plastids found in vacuoles within the host.

Foraminifera

Specimens were fixed in 4% glutaraldehyde in seawater for 1 hour at 4°C. After several washes in sterile filtered seawater, the cells were post-fixed with 2% OSO_4 in sterile filtered seawater for 30 minutes at 4°C. The specimens were then gently washed with distilled water and decalcified in a 1% solution of EDTA until the test appeared transparent when observed in a dissection microscope. At this point the decalcified specimens were quite fragile and needed to be processed further with great care. A progressive series of ethanol was used for dehydration, followed by two washes with propylene oxide and embedment in Embed 812®. During embedment. the samples were infiltrated with 1:2. 1:1 and 2:1 mixtures of resin and propylene oxide for at least 1 hour each. Before curing. the samples were further infiltrated with pure resin for 15 hours with the resin and the catalyst. The embedded specimens were then cured for 24–48 h at 60°C until the resin achieved the desired hardness.

The blocks obtained in the procedures described above were trimmed and sectioned using a Reichert-Jung Ultracut E microtome and a diamond knife (Dupont). The sections were collected on copper grids and some were stained with a 1% solution of uranyl acetate and 5% lead citrate. Observations of the sections were made using a Zeiss 902A TEM. Pictures were taken on Kodak ISO163 film and printed on Illford multigrade paper.

3. Results

Studies of the chloroplasts of the algae fed to *Elphidium* were made first to allow comparison to those of the plastids found in vacuoles (symbiosomes) within the host. The plastid structure *Amphora coffeiformis* was typical of a diatom (Round et al., 1990). It had two plastids, surrounded by four membranes. Inside the plastid were a series of more or less parallel lamellae, each composed of two and sometimes three stacked thylakoids which ran the length of the plastid (Figs. 1A–C). Surrounding was a girdle lamella which was continuous around the ends of the plastid (Fig. 1A). In ideal sections, we could recognize a membrane enclosing the chloroplasts and the nucleus (Fig. 1A). Each plastid contained a simple (not lobate) internal pyrenoid, lenticular in shape, and crossed by a single thylakoid (Figs. 1A–C). The pyrenoids were separated from the lumen of the plastid by a membrane (Fig. 1C). Pyrenoids varied in length from half the length of the plastid to approximately the full length. There were no large electron transparent vacuoles in log phase cells (Fig. 1A),

but they appeared as cells began stationary phase (Fig. 1B). The nucleus was central in the cell and varied from spherical to oval (Fig. 1A).

The structure of *Dunaliella salina* was typical of volvophyceans (Pickett-Heaps, 1975). In this alga, the chloroplast was cup-shaped and surrounded the central nucleus (Fig. 1D). The chloroplast had a typical invaginated pyrenoid surrounded by platelets of starch (Fig. 1D). Chloroplast lamellae enter the pyrenoid matrix (Fig. 1D). We did not see any suggestion of an eyespot (stigma) within the chloroplast membrane. Chloroplasts had thylakoids stacked in groups of 2–6 (Fig. 1E).

Amphidinium had a typical dinokaryon with condensed chromosomes, which have a fibrillar and banded appearance (Figs. 1F and G). The chloroplast of *Amphidinium* sp. had a stalked pyrenoid (Fig. 3F). The thylakoids in this type of chloroplast were stacked in groups of 3 but in most of the sections the thylakoids were compressed and it was difficult to count them (Fig. 1G).

Plastids were abundant in the cytoplasm of *E. excavatum* (Figs. 2A and B). Their distribution was random within the chambers. In the specimens harvested from the field and those fed experimental diets of either a green alga (*Dunaliella salina*) or a dinoflagellate (*Amphidinium* sp.), all fine structural evidence suggested that the plastids retained within the foraminiferal cytoplasm were only from diatoms.

Part of the cytoplasm of each chamber of the foraminifera was vacuolated (Figs. 2A and B). In some cases, remnants of the algal cytoplasm were present in the symbiosomes around the plastids (Fig. 2B). These algal remnants occasionally included the algal nucleus (Fig. 2B) and/or mitochondria, but most frequently only a dense granulation was observed (Fig. 3D). Diatom frustules were never observed in the internal cytoplasm of the foraminifera. The fine structure of every plastid/chloroplast retained within a vacuole in each section was examined with respect to thylakoid numbers, girdle lamellae, surrounding membranes and pyrenoid structure. The typical shape of the retained chloroplasts was oval to round and in some cases 2 lobes or plastids were evident in a single vacuole (Figs. 2 B–D). In the majority of the plastids, the pyrenoid was visible as a central structure separated from the lamellae of the chloroplasts by a membrane (Fig. 2C). In some sections, a single thylakoid could be seen crossing the pyrenoid longitudinally (Figs. 2C and D). The lamellae were formed in most specimens by 2 or 3 stacked thylakoids (Fig. 2D). The girdle lamella was usually thicker and had up to 4 stacked thylakoids (Fig. 2D). The lamellae were longitudinally oriented following the outline of the plastid, sometimes interrupting or curving to accommodate the pyrenoid (Figs. 2B–D). There was quite a range of structures found in vacuoles that did not contain robust plastids. Some contained residua from late stages in the digestion of food (Figs. 2G and H). Some of them were clearly algal cells in various stages

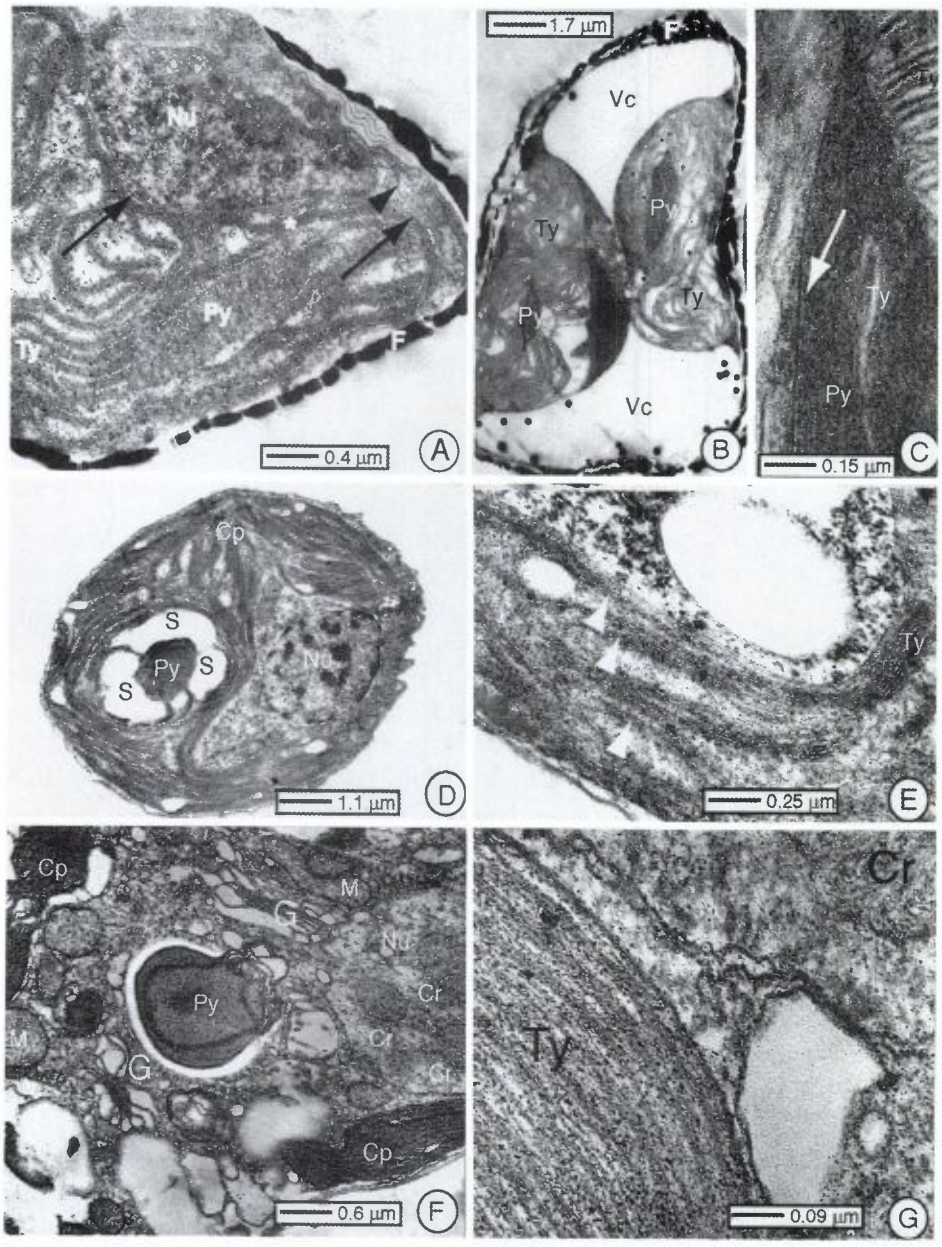

Figure 1. Algae used to feed *Elphidium excavatum* (Terquem). A–C: *Amphora coffeiformis*; D and E: *Dunaliella salina*; F and G: *Amphidinium* sp. Cp = chloroplast; Cr = chromosome; F = frustule; G = Golgi; M = mitochondria; Nu = nucleus; Py = pyrenoid; S = starch; Ty = thylakoids; Vc = vacuole. Black arrows = membrane surrounding chloroplast; white arrows = membrane surrounding pyrenoid;

of digestion (Fig. 2F) and others seemed to contain bacterial remains (Fig. 2G). The cytoplasm of foraminifera maintained in the laboratory for three weeks and those not fed *A. coffeiformis* was more highly vacuolated (Fig. 2H) than those which were fed this diatom species. Some of the retained plastids in the former group specimens were less organized and their thylakoid lamellae were less distinct (Figs. 2B and F). Although we searched specimens harvested from cultures fed chlorophytes or dinoflagellates very carefully we did not find any vacuoles containing intact chloroplasts which clearly came from those diets. Some of the partially digested remains in food vacuoles were most probably from the algae (chlorophytes or dinoflagellates) in their diets.

In general, plastids were separated from the rest of the cytoplasm by a symbiosome (vacuolar) membrane (Figs. 2C–E). Sometimes the symbiosome vacuoles were surrounded by small vesicles that were morphologically identical to lysosomes. In several instances we observed the "lysosomes" fusing with vacuoles containing the chloroplasts possibly converting them into phagolysosomal vacuoles (Figs. 2D–F).

We found pores in sections going through the edges of the chambers (Figs. 3A, C and E). A membrane separates the pore canal from the cytoplasm (Fig. 3D). The cytoplasm in the canal system between chambers was less dense than that found in the chambers (Figs. 3B, D and F), but it contained many fuzzy coated vesicles (Fig. 3D), vacuoles containing isolated chloroplasts (Fig. 3F), and vacuoles filled with the remains of partially digested food (Fig. 3D).

4. Discussion

The fine structure of the plastids found in the foraminifera fixed after collection, indicates that they are most probably all derived from diatoms. They had lamellae made of 2 or more stacked thylakoids. no starch accumulations inside the plastids, a girdle lamella and a simple internal pyrenoid bound by a membrane; characteristics that are common to diatom plastids (Dodge, 1973; Round et al., 1990). These results are in agreement with those from recent feeding and plastid longevity experiments (Correia and Lee, 2000; Correia and Lee, 2002), which showed that chloroplasts from green algae and dinoflagellates were not retained by *E. excavatum*. The differences in the

Figure 1. Continued.
black arrowhead = membrane enclosing nucleus and chloroplast; white arrowhead = stacks of different numbers of thylakoids; asterisks = girdle lamella. Scale bars as indicated in each figure.

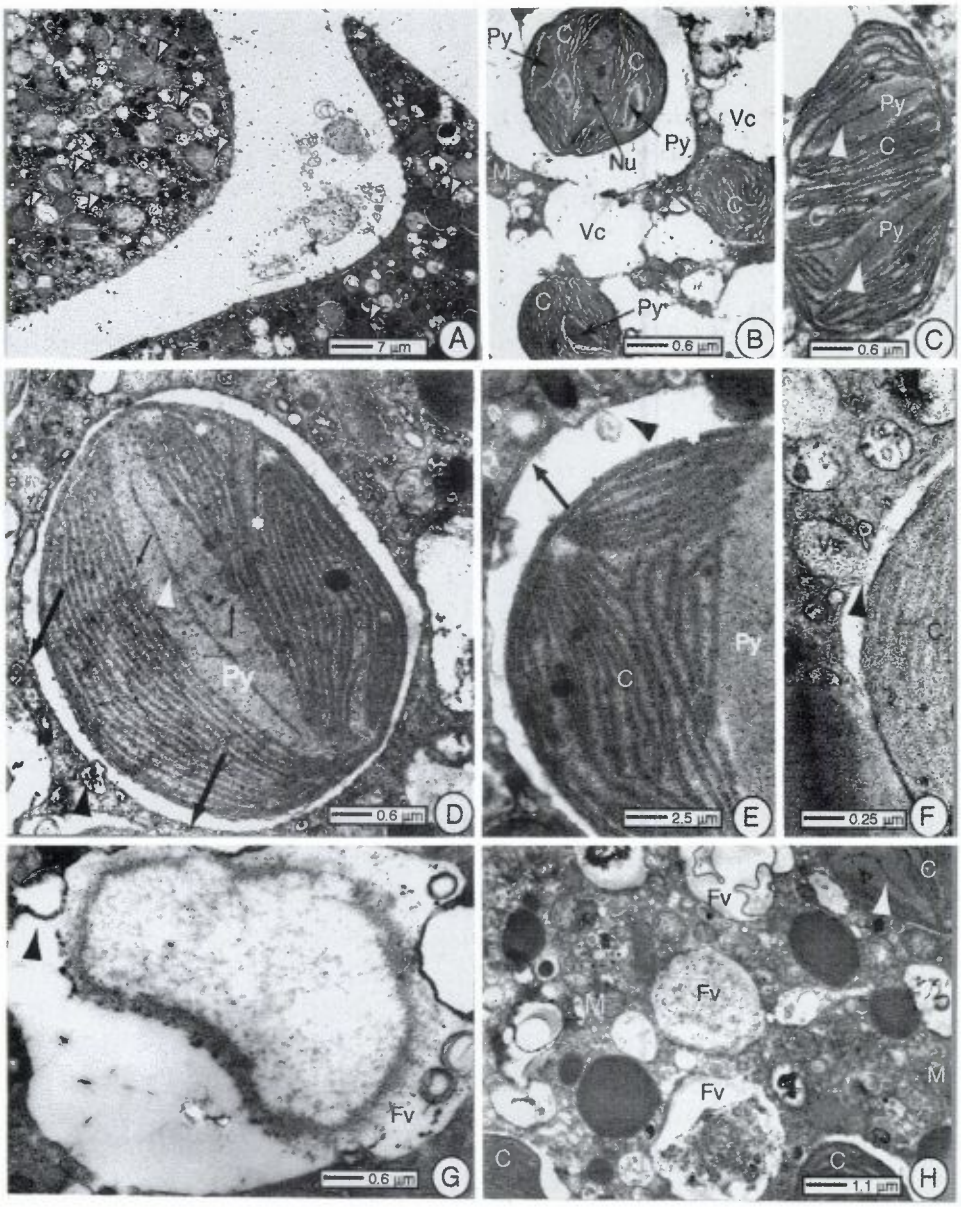

Figure 2. Cytoplasm of *Elphidium excavatum* (Terquem). A: Low magnification showing numerous chloroplasts (arrowheads); B–F: Algal plastids retained in the foraminifer's cytoplasm. Note thylakoid and pyrenoid structure; abundant vesicles around the plastids; some of the vesicles are fusing with the membrane(s) enclosing the plastid (black arrowheads); G and H: Food vacuoles with residues from digested material. C = chloroplast; F = food vacuoles; M = mitochondria;

numbers of plastids in the vacuoles of the foraminifera that were fed *A. coffeiformis* compared to those fed *D. salina, Amphidinium* sp., or starved, was good evidence that the former diatom plastids were being added to the cellular inventory of kleptochloroplasts while the latter were not. The results also agree with results published by Lopez (1979) and Bernhard and Bowser (1999). Cedhagen (1991) reported that the plastids in *Elphidium* originated from dinoflagellates. Based on the micrographs in that article, his interpretation is questionable because the structure of the plastids and pyrenoids in his figure seem to be from diatoms. Our present results seem in conflict with the results of feeding experiments by Lee and Lee (1989). They suggested that *Elphidium crispum* retained chloroplasts from green algae, even if in lower numbers than those of diatoms. Certainly *E. excavatum* did not retain the plastids of the *D. salina* it was fed. Perhaps there are differences between *Elphidium* species and between other chloroplast retaining genera even in the same habitat (e.g. *Haynesina germanica*). This remains to be tested in the future.

The results of the fine structure analysis are in agreement with pigment analyses of chloroplast husbanding foraminifera directly from a salt marsh (Knight and Mantoura, 1985). The pigments they extracted were from diatoms. They did not detect phaeopigments which shows that chlorophylls were not being degraded at the time the foraminifera were harvested. This suggests that the rate of turnover of the diatom plastids is low, because if the rate of diatom plastid digestion was higher, phaeopigments would have been detected by their methodology.

The septal canal cytoplasm we found in *E. excavatum* was similar to that described by Alexander and Banner (1984) in *Elphidium williamsoni*. This cytoplasm is comparable to that of pseudopods in ultrastructure. The fuzzy coated vesicles observed (Fig. 3D) are usually found in pseudopods and not in the internal cytoplasm (Travis and Bowser, 1991). Earlier, Hottinger (1978) suggested that "the living cytoplasm filling the interlocular spaces of nummulitids" has a "pseudopodial ultrastructure". It is interesting to note that the "pseudopodial cytoplasm" found in the septal canal contained isolated plastids, as well as remains from digestion (Figs. 3B, D and F) indicating that it is a passage way into and out of the cell chambers.

The pores in the test walls of *Elphidium excavatum* (Terquem) seem to be sealed by an organic layer (Figs. 3C and E). Although in some sections these

Figure 2. Continued.
Nu = nucleus; Py = pyrenoid; Vc = vacuole; Vs = vesicles. White arrowheads = thylakoids; large black arrows = membrane separating chloroplasts from host cytoplasm; small black arrows = pyrenoid membrane; asterisks = girdle lamella.

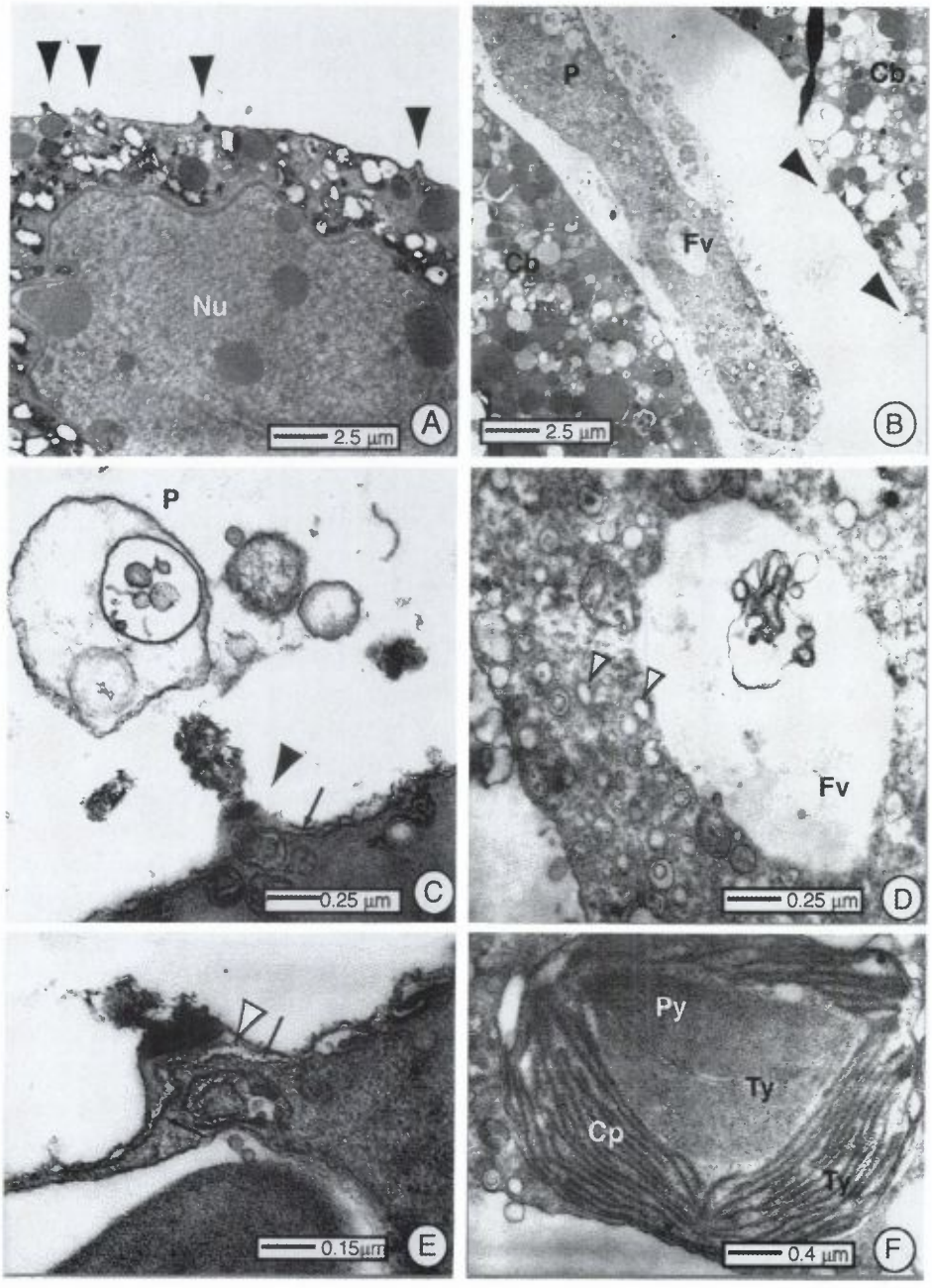

Figure 3. Test and pseudopods possibly running through the canal system. A, C and E: Micrographs showing the numerous pores found on the test of *Elphidium excavatum* (Terquem); B, D and F: Micrographs of pseudopods found inbetween chambers and though to run through the canal system.

pores were in the vicinity of the intercameral pseudopodial extensions (Fig. 3C), we found no evidence that there is a connection between the two. Alexander and Banner (1984) suggested that the organic "plug" covering the pores in a closely related form, *Haynesina germanica*, allow only the exchange of dissolved organic material, inorganic cations and dissolved gases. These pores lead to the inter-cameral canals (Banner et al., 1973).

Although we did not do the cytochemical procedures to test for phosphatase, definitive evidence of digestion, the micrographs we obtained strongly suggest that the symbiosomes containing retained chloroplasts eventually are converted to phagosomes, which allow lysosomal fusion. The molecular signaling events leading to the digestion of the retained plastids are an enticing prospect for the next stage in this research.

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Figure 3. Continued.
D: Vacuole with remains of digestion; F: Vacuole with isolated plastid. Black arrowheads = pores; white arrowheads = cell membrane; Cb = chamber; Cp = chloroplast; Fv = food vacuole; Fz = fuzzy coated vesicle; Nu = nucleus; P = pseudopod; Ty = thylakoids. Scale bars indicated in each figure.

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