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PLASTID MORPHOLOGY, ULTRASTRUCTURE, AND DEVELOPMENT IN *COLACIUM* AND THE LORICATE EUGLENOPHYTES (EUGLENOPHYCEAE)[1]

Patrick J. P. Brown[2]

Department of Cellular Biology, The University of Georgia, Athens, Georgia 30602, USA

Bożena Zakryś

Department of Plant Systematics and Geography, Warsaw University, Al. Ujazdowskie 4, PL-00-478 Warszawa, Poland

and

Mark A. Farmer

Department of Cellular Biology, The University of Georgia, Athens, Georgia 30602, USA

Chloroplast morphology was investigated in five species of euglenophytes: *Trachelomonas volvocinopsis* Swirenko, *Strombomonas verrucosa* (Daday) Deflandre, *Strombomonas costata* Deflandre, *Colacium mucronatum* Bourrelly et Chafaud, and *Colacium vesiculosum* Ehrenberg. All five species share a common plastid morphotype: disk-shaped plastids with a pyrenoid that protrudes asymmetrically toward the center of the cell and is capped by a single large grain of paramylon that conforms to the shape of the pyrenoid. Although plastids demonstrated some degree of diversity among the species studied, it was not consistent with current generic boundaries. The plastids of *S. verrucosa* show a developmental pattern similar to that of *Euglena gracilis*. The plastids divide during the early portion of the light phase after cell division, and pyrenoids are reduced or absent in dividing plastids. Developmental patterns of plastid replication also suggest that these five taxa share recent common ancestry with members of the genus *Euglena* subgenus *Calliglena*.

Key index words: **chloroplasts; *Euglena*; morphological evolution; *Strombomonas*; taxonomy; *Trachelomonas*; ultrastructure**

For decades plastid morphology has been the premier character for classifications within the genus *Euglena*. In her seminal monograph on the genus *Euglena*, Gojdics (1953) stated that "Chromatophores are the cytoplasmic features of *Euglena* that are the most conspicuous feature in the cell, and which show such constancy in a given species, that they have special value as taxonomic characters." Gojdics (1953) divided the genus *Euglena* into eight groups based primarily on plastid shape and size and to some extent on pyrenoid features. Pringsheim (1953) also divided *Euglena* into six groups relying primarily on plastid structure. Leedale (1967) formalized Pringsheim's groups into subgenera, suggesting that *Astasia* is not phylogenetically distinct from *Euglena*, but made no official taxonomic adjustment. The most recent taxonomy of *Euglena* by Zakryś (1986) reduces the number of subgenera to three (*Euglena*, *Calliglena*, and *Discoglena*), all defined by plastid architecture. Ultrastructural investigations from a variety of euglenophyte taxa (Dragos et al. 1979, Péterfi et al. 1979, Zakryś and Walne 1998, Zakryś et al. 2001) have served not only to bolster previous taxonomic schemes using plastid structure but have elucidated ultrastructural details that were absent from earlier analyses (Haller 1959, Mignot 1965, 1966, Leedale 1967, 1982, Buetow 1968). In this article "euglenid" refers to all members of the Euglenida (phagotrophs, phototrophs, and osmotrophs) and "euglenophyte" refers specifically to those euglenids that posses a plastid (including those that have secondarily lost them, e.g. *Astasia longa*).

Recent investigations suggest that the genus *Euglena* is not monophyletic (Linton et al. 1999, 2000, Milanowski et al. 2001, Müllner et al. 2001) and raise new questions about the utility of using *Euglena* subgeneric classifications in other euglenophyte taxa. Many of the plastid features used to delineate the three currently recognized subgenera of *Euglena* (Zakryś 1986) could be applied to other euglenophyte taxa. For example, the *Euglena* subgenus *Discoglena* is characterized by the presence of numerous lenticular chloroplasts without pyrenoids; the same types of plastids are found in many species of *Phacus* and *Lepocinclis*. Many of these *Phacus* and *Lepocinclis* species group with members of the genus *Euglena* in molecular phylogenies (Leander and Farmer 2001, Linton et al. 1999, 2000, Milanowski et al. 2001, Müllner et al. 2001).

Three of the five taxa in the current study belong to the only two loricate genera of euglenophytes, *Trachelomonas* Ehrenberg (1833) and *Strombomonas* Deflandre (1930). Most studies of loricate euglenophytes concentrate primarily on lorica morphology, often without mention of other cellular features (Conrad 1916, Deflandre 1926, Conforti et al. 1993, Conforti and Joo 1994, Conforti 1999, Shi et al. 1999). This emphasis on lorica morphology makes comparison with other eu-

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[2]Author for correspondence: e-mail pbrown@cb.uga.edu.

glenophytes difficult. *Colacium* Ehrenberg (1838) is the only colonial euglenophyte genus. During stationary phase growth, the individual cells of *Colacium* are connected by thick bifurcating mucilaginous stalks emanating from the anterior reservoir, resulting in a dendroid colony (Leedale 1967). This colonial habit has prompted some researchers to separate *Colacium* into its own family (Jahn 1951, Christen 1963, Popova and Safonova 1976, Compere 1989) or even into a separate order (Bourelly 1970, Tell and Conforti 1986) of equal rank to the other euglenids with a single emergent flagellum. The mucilaginous sheaths surrounding individual cells of *Colacium* are similar in appearance to those preceding the formation of the loricas in *Trachelomonas* and *Strombomonas*. This feature, along with similarities in plastid structure, led us to the hypothesis that *Colacium* might be closely related to the loricates.

Euglena gracilis has long been one of the model organisms for studies of the development and biochemistry of plastids. The genetic continuity of chloroplasts was proven using *E. gracilis* as a model system (Pringsheim and Pringsheim 1952, Schiff and Epstein 1965). Although the wealth of information available on *E. gracilis* is significant, there is danger if the studies on *E. gracilis* are extrapolated to include the rest of the euglenophytes. It is often assumed that what is true for *E. gracilis* is also true for the euglenophytes as a whole, but recent analyses using the nuclear 18S and chloroplast 16S rDNA sequence have shown that *E. gracilis* and its close relatives are potentially the most recently diverged of all euglenophytes (Linton et al. 1999, 2000, Leander and Farmer 2001, Milanowski et al. 2001, Müllner et al. 2001). This study not only reports on ultrastructure but also reexamines some of the fundamental plastid biology of the euglenophytes in an effort to refine some of the basic principles of euglenophyte taxonomy.

MATERIALS AND METHODS

Strains and culture conditions. The following strains were used: *Colacium mucronatum* Bourrelly et Chafaud (UTEX LB 2524), *Colacium vesiculosum* Ehrenberg (UW Łazienki), *Strombomonas costata* Deflandre (ACOI 2992), *Strombomonas verrucosa* (Daday) Deflandre (ACOI 2476 as *S. acuminata*), and *Trachelomonas volvocinopsis* Swirenko (SAG 1283-16). They were obtained from the following collections: UTEX, the Culture Center for Algae at the University of Texas; ACOI, Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen, Germany; and UW, Culture Collection of Algae at Department of Plant Systematics and Geography of Warsaw University, Poland. Cells were grown initially in biphasic soil–water medium and later transferred into ESSEX medium (ES-enriched Soil EXtract; see below). All cultures were maintained at 20 ± 1° C on a 12:12-h light:dark cycle.

ESSEX medium. ESSEX is a variation of Pringsheim's soil–water medium to which ES vitamins (Harrison et al. 1980) are added. The recipe is as follows: to 1 L dH_20 add 50 g garden soil, 0.2 g $NH_4MgPO_4{\cdot}6H_20$, 0.2 g $CaCO_3$, 0.2 g crushed barley, and 10 pieces of dry split peas. Heat to 70° C and maintain for 5 h, remove from heat, and cover with cheesecloth. Let stand 48 h at room temperature, decant the supernatant, filter through a 0.2-μm filter, and autoclave for 20 min. After the solution has cooled, add 1 mL of sterile ES vitamin solution (thiamine 0.1 $g{\cdot}L^{-1}$, cyanocobalamin 2 $mg{\cdot}L^{-1}$, biotin 1 $mg{\cdot}L^{-1}$) and dispense into sterile tubes in 10-mL aliquots.

TEM. Cells were pelleted by gentle centrifugation and fixed in 2% glutaraldehyde buffered in 0.1 M cacodylate for 1 h at 4° C. Cells were then post-fixed in 1% osmium tetroxide (OsO_4) for 1 h at 4° C and dehydrated in a graded ethanol series. The cells were infiltrated, embedded, and polymerized in Embed 812 epoxy resin (Polysciences, Warrington, PA, USA) and light gold/silver sections were cut. The sections were post-stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (model 100CX II, JEOL USA, Peabody, MA) operating at 80 KeV.

Confocal microscopy. Cells were collected by gentle centrifugation and lightly fixed with 1% glutaraldehyde and 0.5 M cacodylate for 30 min. After fixation the cells were rinsed twice in *Euglena* medium (Greenblatt and Schiff 1959) and mounted on glass microscope slides and sealed. The cells were then viewed on a confocal laser-scanning microscope (model MRC 600, Bio-Rad Life Sciences, Hercules, CA) at an excitation wavelength of 568 nm.

RESULTS

Pyrenoid structure. The five species under investigation share a distinct plastid morphology, unlike that of other euglenophytes. In cells undergoing normal growth, each species has 10 to 15 parietal disk-shaped plastids. Each plastid has a single large protruding pyrenoid on its cytoplasmic side (Figs. 1, A–D, and 2D) capped by a single large crystalline grain of the euglenid reserve polysaccharide paramylon (Fig. 1, E–H). Thylakoids are stacked in lamellae of three or five as is common for most euglenophytes but reduce to a stack of two upon entering the pyrenoid matrix (data not shown).

The pyrenoids of different species are stalked to various degrees. The pyrenoids of *C. vesiculosum* have almost no stalk and the pyrenoid is as wide at the proximal end as it is at the distal (Fig. 1, A and E). Other species exhibit a much greater degree of protrusion in which the pyrenoid appears to be "pinched" at the proximal end. This is quite evident in *C. mucronatum* in which the ratio of the width of the proximal end to the distal end is approximately 1:3 (Fig. 1, C and G). The pyrenoids of *T. volvocinopsis* are stalked but to a lesser degree (Fig. 1, B and F) than *C. mucronatum*. Both species of Strombomonas bear a stalked pyrenoid (Figs. 1D and 2), with that of *S. verrucosa* being the largest and most robust (Fig. 2D).

Plastid development. The plastids of *S. verrucosa* were studied in an effort to elucidate the progression of pyrenoid development in those plastids with an inwardly projecting pyrenoid. After cell division the pyrenoid appears as a small electron-dense region on the cytoplasmic side of the plastid and is almost immediately capped with a small grain of paramylon (Fig. 2A). Initially, the pyrenoid is a small homogeneous structure and does not possess thylakoids but has a robust paramylon cap (Fig. 2B). As material is added to the crystalline matrix of the pyrenoid, it begins to grow primarily along the axis perpendicular to the long axis of the plastid (Fig. 2C). This asymmetric growth "pulls" nearby thylakoids into the pyrenoid

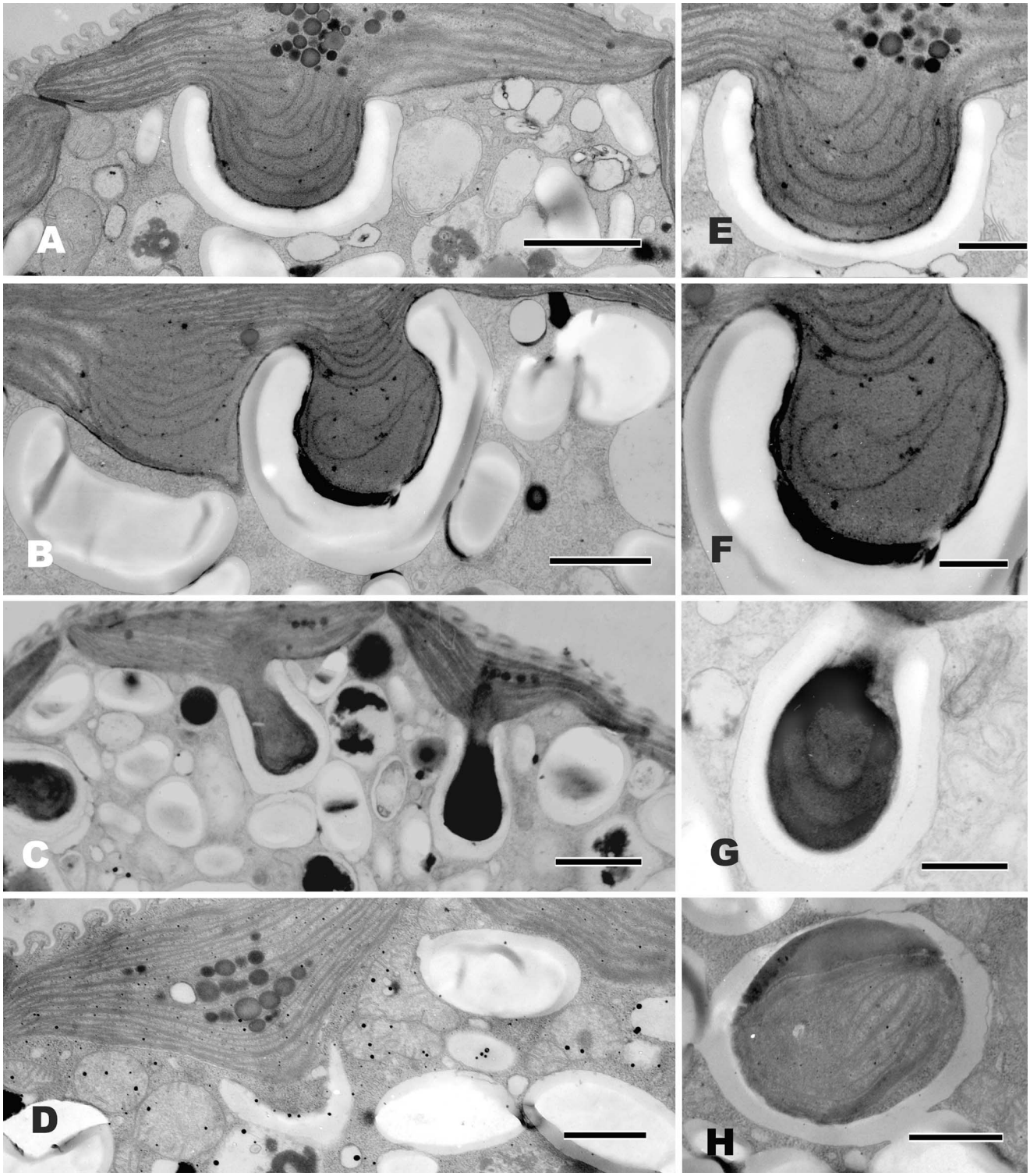

FIG. 1. Plastid and pyrenoid morphology of *Colacium vesiculosum*, *Trachelomonas volvocinopsis*, *Colacium mucronatum*, and *Strombomonas costata*. (A) Chloroplasts of *C. vesiculosum* are characterized by a pyrenoid that protrudes toward the center of the cell. The pyrenoid is not constricted at the proximal end. Bar, 1 μm. (B) The chloroplasts of *T. volvocinopsis* have similar pyrenoid morphology, but there is a slight constriction at the proximal end, resulting in the paramylon cap curving inward to fit the curve of the pyrenoid. Bar, 1 μm. (C) The chloroplasts of *C. vesiculosum* are highly constricted at the proximal end and are much more elongate than those of *C. vesiculosum*. Bar, 1 μm. (D) The chloroplasts of *S. costata* protrude inward but do not get as large as those of *S. verrucosa*. Bar, 1 μm. (E) High magnification of the pyrenoid of *C. vesiculosum* in which the thylakoid lamellae penetrate throughout the pyrenoid matrix and are contiguous through their entire length. Bar, 0.5 μm. (F) High magnification view of the pyrenoid of *T. volvocinopsis* with a unique "recurrent lamella" in the pyrenoid matrix. Bar, 0.5 μm. (G) Higher magnification of the pyrenoid of *C. mucronatum* in which the constriction at the proximal end is so pronounced the "neck" of the pyrenoid is absent from this oblique section. Bar, 0.5 μm. (H) Cross-section of the pyrenoid of *S. costata* in which the penetrating lamellae can be easily observed and are quite numerous. Bar, 0.5 μm.

matrix (Fig. 2C). At maturity the pyrenoid can be nearly as large as the plastid body and protrude inward to the nuclear region (Fig. 2D). This entire progression occurs over the course of 6 to 8 h, during the growth (light) phase of the cell cycle.

Using confocal laser-scanning microscopy, ultrastructural events in the development of *S. verrucosa* plastids can be correlated to gross morphological changes. Immediately after cell division the plastids are small and the pyrenoids are greatly reduced or even absent. During the early periods of the growth phase, the plastids increase in volume and become amorphous (Fig. 3A). Pyrenoids are few and difficult to distinguish with LM. By the peak of the growth phase (approximately 6–8 h after cell division) the plastids have nearly doubled in size and pyrenoids are easily visible. At this point the plastids begin to divide, resulting in numerous discoid plastids with prominent pyrenoids (Fig. 3B). Because the thylakoids penetrating the pyrenoid do not fluoresce, pyrenoids ap-

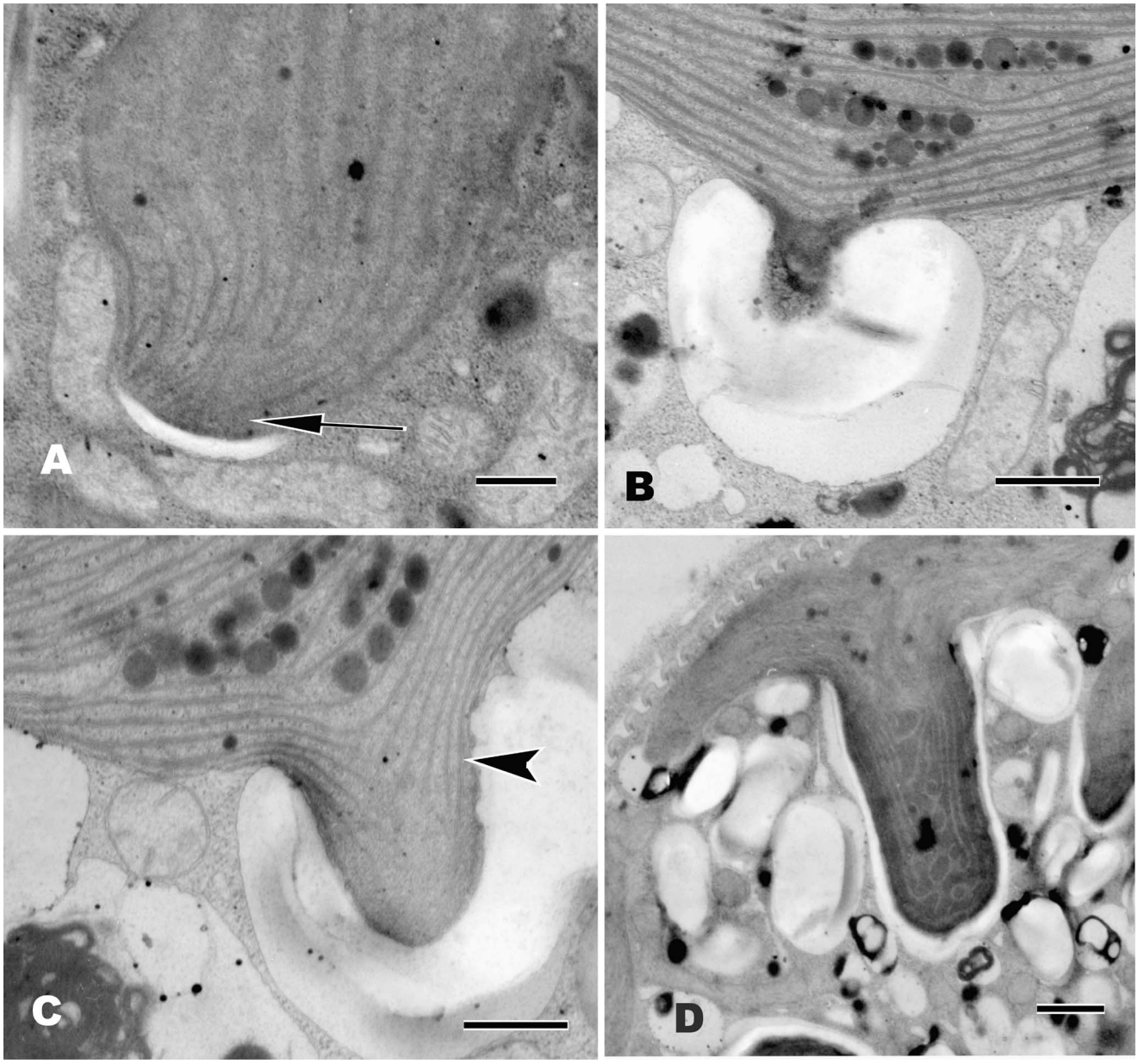

FIG. 2. Pyrenoid development follows an ordered series of events in *Strombomonas verrucosa.* (A) The earliest recognizable stage of what will become a pyrenoid is distinguishable by the electron-opaque region capped by a small thin grain of paramylon (arrow). Bar, 0.5 μm. (B) Later, a robust paramylon cap is added to the growing pyrenoid. Bar, 0.5 μm. (C) As more material is added to the pyrenoid matrix, thylakoid lamellae are "pulled" into the pyrenoid (arrowhead). Bar, 0.5 μm. (D) At maturation the pyrenoid is nearly as large as the plastid itself. Bar, 1 μm.

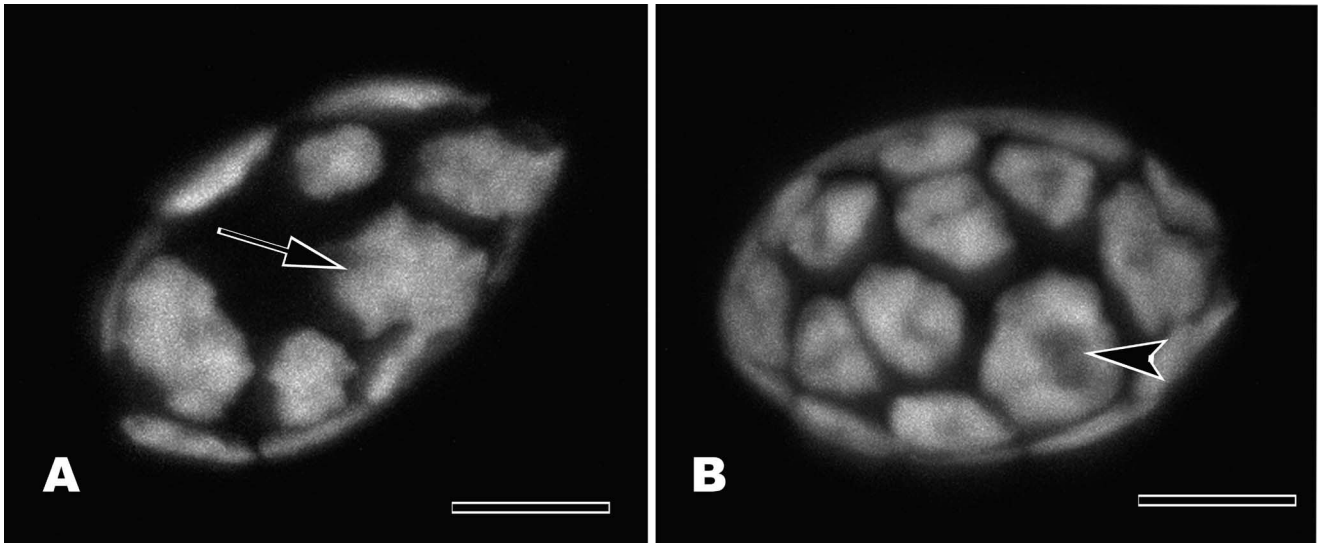

FIG. 3. (A) At the beginning of the light cycle the plastids of *Strombomonas verrucosa* are amorphous. A single large plastid (arrow) can be seen occupying a significant portion of the cell. Bar, 10 μm. (B) After 6 h in daylight the plastids have divided, and a central large pyrenoid can be seen as a fluorescence exclusion zone (arrowhead). Bar, 10 μm.

pear as dark spots, but only if the pyrenoid is large enough to constitute a significant portion of the entire plastid (Fig. 3B). After cell division daughter cells are then left with half of the plastids of the parent.

The plastids of the species under study also exhibited a form of plastid adhesion. In nearly every section of growing cells, plastids could be seen adhering to one another, often via an unidentified electron dense material (Fig. 4). In *T. volvocinopsis* adhesion was, more often than not, along the long-axis of the plastid. This results in an overlapping appearance (Fig. 4A). Most plastids in other species, however, were adherent end to end (Fig. 4, B and C). This was seen in members of all three genera studied, usually occurring along the long axis of the cell.

DISCUSSION

Taxonomic implications. The similarities of plastid and pyrenoid morphology in these five species, currently divided between three genera, lead us to conclude that the relationship between the loricates and *Colacium* may be closer than was previously believed. The observed variations in pyrenoid morphotypes do not delineate well-established taxonomic boundaries. The best example is stalk morphology of the pyrenoid. The pyrenoid of *C. vesiculosum* is very broad in face view, with no discernible constriction at the proximal end, whereas the mature pyrenoid of *C. mucronatum* is borne on a very slender stalk. These two morphotypes lie at opposite extremes of the entire diversity of pyrenoid stalk architecture, yet they occur within a single very well characterized genus. This leads to the conclusion that the finer details of stalk structure are potentially homoplasious; however, the mere presence of a protruding pyrenoid is, of itself, informative.

Although the species of *Trachelomonas* and both species of *Strombomonas* examined in this study possess stalked pyrenoids, there are species of *Trachelomonas* that apparently do not. Pringsheim (1953), while observing several species of *Trachelomonas* grown in culture, noted "the entire number of species of *Trachelomonas* observed can be divided into four groups: one with no pyrenoids, one with naked, one with inner, and one with double-sheathed pyrenoids. The majority of the species of *Trachelomonas* have, however, what I will call 'inner pyrenoids' protruding from the center of the concave surface of the chromatophores towards the middle of the cell." The inner pyrenoids and the naked pyrenoids (no associated paramylon) are barely visible in the light microscope and the reported absence of pyrenoids in many species of *Trachelomonas* may not be accurate. Pringsheim's description of inner pyrenoids accurately describes the pyrenoids we have observed in *Trachelomonas*, *Strombomonas*, and *Colacium*.

The most recent monographs of the euglenids (Huber-Pestalozzi 1955, Popova 1966, Popova and Safonova 1976) state that *T. volvocinopsis* does not have pyrenoids, whereas Pringsheim (1953) observed and reported them as inner pyrenoids. The culture of *T. volvocinopsis* used in this study is identical in every way to Huber-Pestalozzi's diagnosis adapted from Swirenko's own (Swirenko 1914). So although there are loricate taxa that probably have naked pyrenoids and those that definitely have diplopyrenoids (a centrally located pyrenoid capped on either side by a lens-shaped paramylon grain such as those seen in *Euglena agilis* Carter), a lack of pyrenoids altogether is possible but unlikely. Although Pringsheim (1953) states that some species of *Trachelomonas* (those with

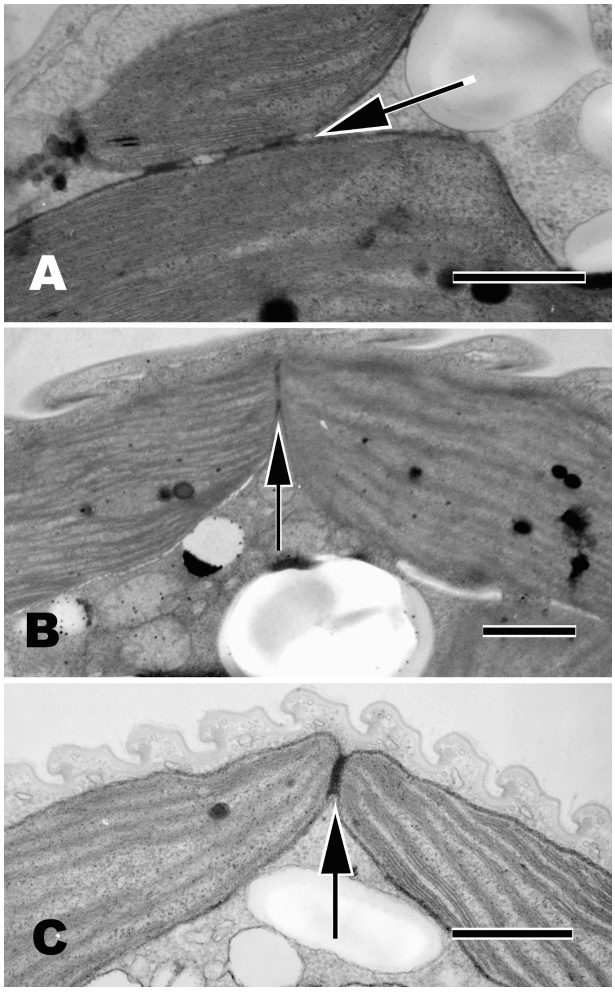

FIG. 4. Plastid adhesion in *Trachelomonas volvocinopsis*, *Strombomonas costata*, and *Colacium vesiculosum*. (A) The plastids of *T. volvocinopsis* are usually found apressed to one another along the overlapping surfaces of adjoining plastids; the adhesion is maintained by an electron-opaque material (arrows). Bar, 0.5 μm. (B) In *S. costata* the plastids adhere to one another along their margins and are pressed end to end. Bar, 0.5 μm. (C) In *C vesiculosum* the plastids adhere much as they do in *S. costata*. Bar, 0.5 μm.

numerous small chloroplasts like *T. abrupta*, *T. bulla*, and *T. varians*) have no pyrenoids, the absence of pyrenoids for these taxa should not be accepted as fact until this can be positively documented by EM.

In addition to the asymmetric pyrenoids, all five taxa in our study exhibited a phenomenon we term plastid adhesion in which plastids appear to be joined to one another, often by an electron-opaque material. This adhesion usually occurs along the lateral borders of the plastid, and when it extends down the length of the cell it forms a longitudinal ridge. This is a unique characteristic, not mentioned in previous reports of plastid ultrastructure, and offers another piece of evidence in support of a *Colacium*–loricate relationship. Unfortunately, the function of plastid adhesion in the cell has yet to be discerned.

The unusual plastid morphology seen in these five taxa is very distinctive and not found in any representative from the genera *Euglena*, *Phacus*, or *Lepocinclis*. Given the unusual nature of the pyrenoid's position within the plastid, as well as its asymmetric growth, it is reasonable to postulate some phylogenetic allegiance between the taxa in our study. In a recent study on euglenoid phylogeny, Linton et al. (2000) showed two major clades of euglenophytes. The first clade consisted of those species with rigid or semirigid pellicles and lenticular plastids devoid of pyrenoids at all stages of the cell cycle. This clade is comprised primarily of members of the genera *Phacus* and *Lepocinclis*, with some representatives of the genus *Euglena* subgenus *Discoglena*. The second clade comprises taxa whose plastids contain a single pyrenoid and have some association with granular paramylon. This includes members of the genus *Euglena* subgenera *Euglena* and *Calliglena*. Given that the plastids of the species in our study posses a single pyrenoid that is associated with granular paramylon, were they to be grouped solely on this character they would be included in this second clade. The results of the most recent molecular phylogeny that included two of these taxa (*Colacium vesiculosum* and *Strombomonas costata*) were consistent with this grouping (Milanowski et al. 2001). Milanowski et al.'s combined analysis showed a relationship between *C. vesiculosum*, *S. costata*, and another loricate, *Trachelomonas volvocina* Ehrenberg, and the genus *Euglena* subgenus *Calliglena*. Unfortunately, there was not sufficient resolution to work out the relationship between *Colacium*, the loricates, and *Euglena-Calliglena*. Molecular analyses of sequences from additional taxa will allow for the further testing of this hypothesis.

Plastid biology and development. Although numerous authors have dealt with plastid development and division in the euglenophytes, they have all used *E. gracilis* as their study organism (Cook et al. 1976, Pelligrini 1980, Ehara et al. 1990, García-Ferris et al. 1996). Ours is the first report of plastid division and pyrenoid development in euglenophyte taxa other than *E. gracilis*, but it appears that many of the phenomena reported for *E. gracilis* are maintained within euglenophytes with similar type plastids. In a very detailed examination, Pelligrini (1980) demonstrated that the plastids of *E. gracilis* do not divide in perfect synchrony with one another but maintain a division pattern compatible with the host cell compartment. This is very similar to what we observed in *S. verrucosa* and suggests that pyrenoid-containing discoid plastids, regardless of pyrenoid morphology, develop in a similar fashion. In the future, studies of plastid division in the genus *Euglena* subgenus *Euglena* should elucidate division and developmental mechanisms in the more complex stellate plastids. Also of interest are the aggregate plastids of *Eutreptiella*, *Eutreptia*, and *Tetreutreptia*, which possess many band-shaped plastids with a single pyrenoid, aggregated around a paramylon center (Walne et al. 1986, McLachlan et al. 1994).

These studies will lend themselves to the elucidation of the evolution of plastid development in the euglenophytes and give more clues as to the nature of the original symbiosis that defines the euglenophytes.

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