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NOVEL PELLICLE SURFACE PATTERNS ON *EUGLENA OBTUSA* (EUGLENOPHYTA) FROM THE MARINE BENTHIC ENVIRONMENT: IMPLICATIONS FOR PELLICLE DEVELOPMENT AND EVOLUTION[1]

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***Euglena obtusa* F. Schmitz possesses novel pellicle surface patterns, including the greatest number of strips (120) and the most posterior subwhorls of strip reduction in any euglenid described so far. Although the subwhorls form a mathematically linear pattern of strip reduction, the pattern observed here differs from the linear pattern described for *Euglena mutabilis* F. Schmitz in that it contains seven linear subwhorls, rather than three, and is developmentally equivalent to three whorls of exponential reduction, rather than two. These properties imply that the seven-subwhorled linear pattern observed in *E. obtusa* is evolutionarily derived from an ancestral bilinear pattern, rather than from a linear pattern, of strip reduction. Furthermore, analysis of the relative lateral positions of the strips forming the subwhorls in *E. obtusa* indicates that (1) the identity (relative length, lateral position, and maturity) of each strip in any mother cell specifies that strip's identity in one of the daughter cells following pellicle duplication and cell division, (2) the relative length of any given pellicle strip regulates the length of the nascent strip it will produce during pellicle duplication, and (3) pellicle pores develop within the heels of the most mature pellicle strips. These observations suggest that continued research on pellicle development could eventually establish an ideal system for understanding mechanisms associated with the morphogenesis and evolution of related eukaryotic cells.**

Key index words: **character evolution; electron microscopy; *Euglena*; Euglenida; morphogenesis; pellicle; ultrastructure**

Abbreviations: **DIC, differential interference contrast; *P*, the greatest number of pellicle strips that surround the circumference of a cell; *S*, the number of strips passing through each subwhorl between two successive terminating strips; *T*, the number of strips that reach the posterior tip of the cell; W_p, the number of whorls of exponential strip reduction**

A number of phylogenetic relationships within the Euglenophyta have been resolved in recent years due to the utilization of molecular and morphological data. For example, extensive taxon sampling and phylogenetic analyses using ribosomal DNA have resulted in the resurrection of the genus *Monomorphina* (Marin et al. 2003) and the designation of a novel genus, *Discoplastis* (Triemer et al. 2006). Moreover, morphological studies of the euglenid cytoskeleton, or pellicle, have confirmed the validity of separating the loricate genera *Trachelomonas* and *Strombomonas* (Brosnan et al. 2005) and have provided substantial evidence for a single, relatively late origin of chloroplasts in a phagotrophic euglenid ancestor (Leander 2004). Many relationships between and within well-supported genera are still poorly resolved (e.g., within *Euglena*; Triemer et al. 2006), and careful reexamination of morphological characters and their variability due to environmental factors is required to adequately define and delimit species, let alone uncover their evolutionary affinities (Kosmala et al. 2005, Nudelman et al. 2006).

Euglenid pellicle characters are numerous and variable enough to be used as tools in evolutionary inference (Leander and Farmer 2000a, 2001a,b, Leander et al. 2001), but relatively few taxa have been described with respect to the pellicle. Moreover, little is known about the development of, and relationships between, separate pellicle characters, information that is invaluable in studying character evolution and, in turn, making phylogenetic inferences based on character evolution (Mabee 2000).

In an effort to understand pellicle development and its role in pellicle character evolution, we proposed a model for the morphogenesis of pellicle strip reduction (Esson and Leander 2006), a character that was previously useful in phylogenetic and taxonomic studies (Leander and Farmer 2001a, Leander et al. 2001, Brosnan et al. 2005). Our

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research indicated that whorls of strip reduction, present in phototrophic euglenids, are the result of differences in developmental timing that affect strip elongation during pellicle replication prior to and during cell division. The strips forming each exponential whorl of reduction are the products of the same pellicle duplication event during cell division. In other words, pellicle reduction patterns are ''multigenerational,'' with successively younger (and shorter) strips forming successively anterior whorls of reduction (Esson and Leander 2006).

Anticipating a comprehensive description of the ultrastructure of the marine phototroph *Euglena obtusa* at a later date, this paper focuses on its pellicle surface patterns, which are the most complex found on any euglenid described so far. When interpreted in light of our morphogenetic model and previous work on pellicle morphogenesis (i.e., descriptions of dividing *Cyclidiopsis acus* cells; Mignot et al. 1987), our observations suggest that the relative maturity of pellicle strips influences the morphogenesis of pellicle surface patterns. The euglenid pellicle is an ideal system for studying developmental processes in eukaryotic cells because the dynamics of strip length and position can be easily viewed using SEM.

MATERIALS AND METHODS

Collection of E. obtusa. Sand substrate was collected with a spoon from Spanish Banks (English Bay, Vancouver, British Columbia; 49°17′N, 123°13′W) during low tide. The sand was placed in a vertical plastic cylinder with a 48 μm mesh filter (Sefar, Thal, Switzerland) attached to the bottom. Organisms were removed from the substrate by melting frozen, filtered seawater over the sand, causing the interstitial microorganisms to pass through the filter and into seawater within a petri dish below (Uhlig 1964).

LM and taxonomic identification. Cells were placed on a slide and either fixed with 2% glutaraldehyde in filtered seawater or left alive and viewed with a Zeiss Axioplan 2 Imaging microscope (Oberkochen, Germany). Differential interference contrast (DIC) images of 12 cells were taken using a Leica DC500 digital camera (Wetzlar, Germany). Cells were identified based on a key and description by Kim et al. (1998), comparison with drawings in Huber-Pestalozzi (1955), and comparison with the descriptions of Schmitz (1884) and Gojdics (1953).

SEM. Filtrate from the original sand samples was placed in a petri dish. A piece of filter paper mounted in the lid was saturated with 4% osmium tetroxide, and cells were fixed by placing the lid over the petri dish containing the filtrate (Leander and Farmer 2000a). Fixed cells were placed on Millipore filters (Billerica, MA, USA), dehydrated with an ethanol series, critical-point-dried with CO_2 in a Tousimis Samdri 795 critical point dryer (Rockville, MD, USA) and coated with a thin layer of gold and palladium using a Nanotech SEMprep II sputter coater. Samples were viewed on a Hitachi S4700 Scanning Electron Microscope (Pleasanton, CA, USA). Surface morphology data were collected from 10 cells.

TEM. Some 120–130 cells were individually isolated from the sand filtrate using a Pasteur pipette and fixed on ice for 1 h using 2% glutaraldehyde in filtered seawater. Cells were postfixed with 1% osmium tetroxide in filtered seawater for 1 h on ice. After rinsing twice with filtered seawater, cells were dehydrated with an ethanol series followed by acetone washes according to Leander and Farmer (2000a). The cells were then infiltrated with increasing ratios of resin to acetone and embedded in pure Epon 812 resin (resin and other chemicals manufactured by Canemco, Canton de Gore, Quebec, Canada); cells were finally centrifuged at high speed (5,900*g*) so that cells formed a pellet in the tip of an embedding capsule. Blocks were polymerized at 65°C. Ultrathin sections (70–80 nm) were cut on a Leica EM UC6 ultramicrotome (Vienna, Austria), placed on copper grids, poststained with uranyl acetate and lead citrate, and viewed using a Hitachi H7600 transmission electron microscope.

RESULTS

Cells were large and vermiform in shape (>100 μm when elongated) and underwent active metaboly. No flagella were observed ($n = 5$). The posterior end of the cell was consistently tapered, whether the cell was elongated (Fig. 1a and b) or compressed. A conspicuous red stigma (anterior to a large reservoir) and a large nucleus, located in the middle of the cell or toward the posterior end, were both visible with LM (Fig. 1b). Cytoplasmic paramylon grains were variable in abundance (Fig. 1b), but double paramylon caps were always

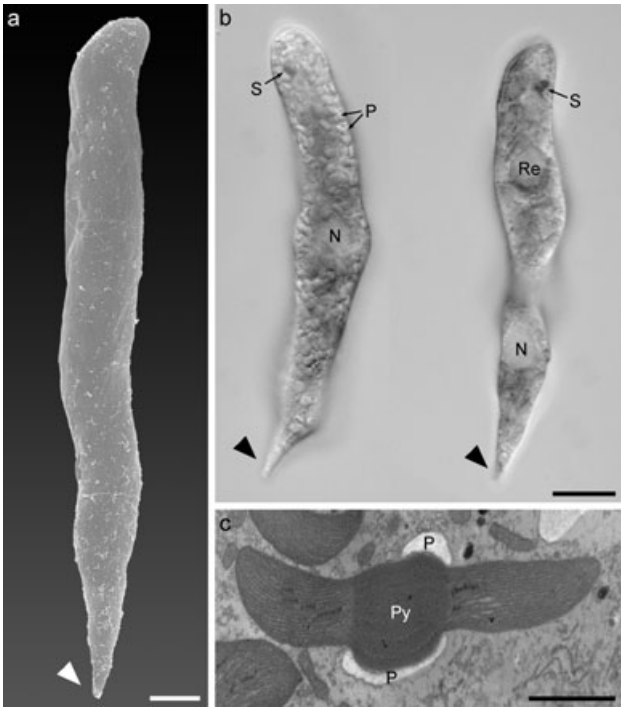

FIG. 1. General morphology of *Euglena obtusa*. (a) Scanning electron micrograph showing elongated cell with tapered posterior end (arrowhead). Scale bar, 10 μm. (b) Differential interference contrast (DIC) micrograph of two elongated cells with tapered posterior ends (arrowheads), nuclei (N), and stigmas (S). Numerous paramylon grains (P) are visible in the left cell. The right cell has a large inclusion inferred to be the reservoir (Re). Scale bar, 20 μm. (c) Transmission electron micrograph showing a transverse section of a plate-shaped plastid with a single pyrenoid (Py) surrounded by paramylon caps (P) on either side. Scale bar, 2 μm.

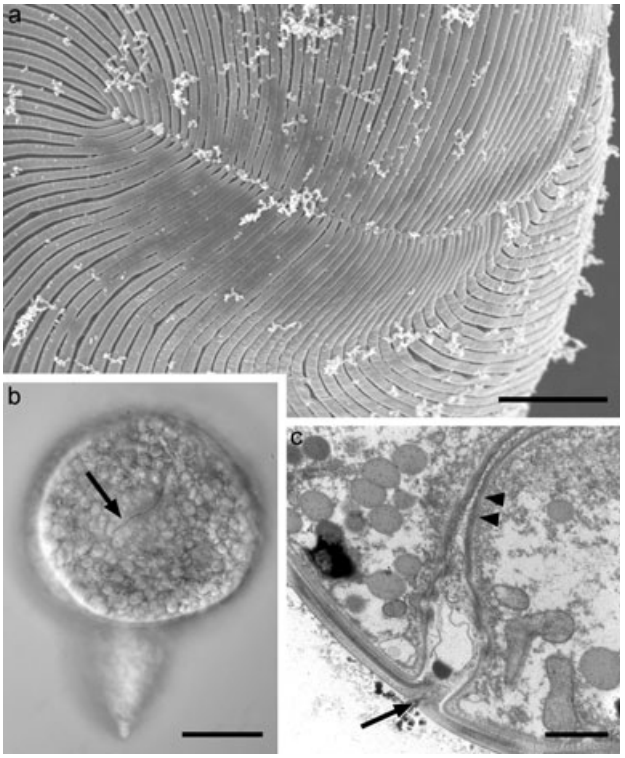

FIG. 2. The cryptic ''canal opening'' in *Euglena obtusa*. (a) Scanning electron micrograph showing 115 of 120 pellicle strips meeting along a line at the anterior end of the cell (five additional strips are outside the field of view in this image). The subterminal ''canal opening'' lies beneath this line. Scale bar, 2 μm. (b) Differential interference contrast (DIC) micrograph of a contracted cell fixed with glutaraldehyde. The anterior line where the pellicle strips meet above the ''canal opening'' is visible (arrow). Scale bar, 20 μm. (c) Transmission electron micrograph of a longitudinal section through the ''canal opening.'' An extremely small aperture (arrow) is visible between pellicle strips at the cell surface. The elongated canal narrows conspicuously (arrowheads) beneath the cell surface. Scale bar, 1 μm.

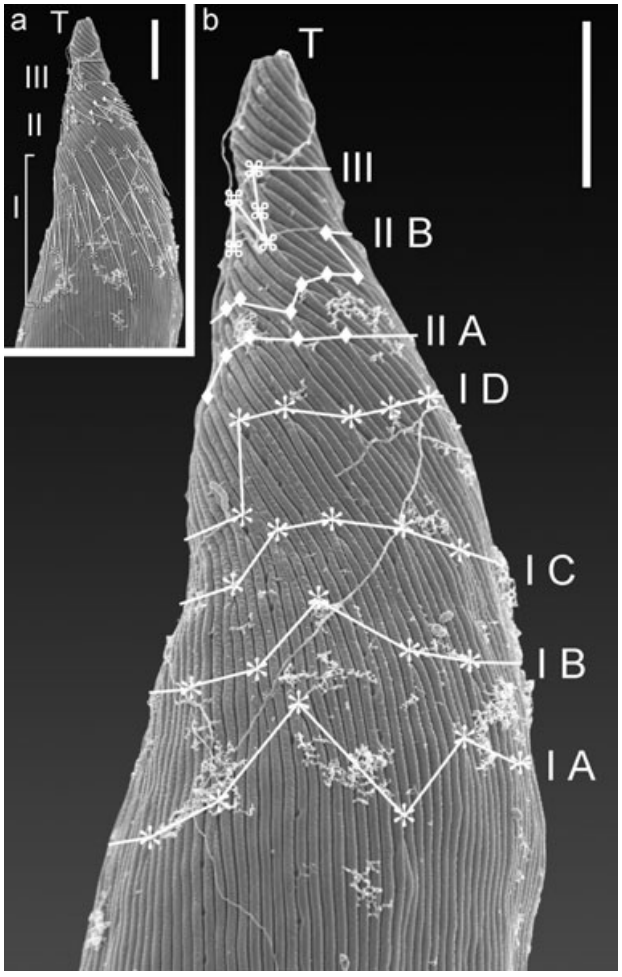

FIG. 3. Posterior strip reduction in *Euglena obtusa*. When every terminating strip is connected by a line (a), three whorls of exponential reduction become apparent: whorls I (*), II (◆), and III (⌘). Whorls I and II are staggered, and whorl I stretches over a relatively large portion of the cell length. Whorls I and II can be separated into four and two subwhorls, respectively (b): IA, IB, IC, and ID (*), and II A and IIB (◆). These subwhorls, with whorl III (⌘), form seven subwhorls of linear reduction, where seven pellicle strips pass between each pair of terminating strips in IA, six pass through IB, five pass through IC, four pass through ID, three pass through II A, two pass through IIB, and one passes through whorl III to meet at the posterior tip (*T*). The relative positions of the strips forming each subwhorl relative to the strips forming other subwhorls can also be observed. Scale bars, 5 μm.

associated with a single pyrenoid in the numerous plate-shaped chloroplasts (Fig. 1c).

When the anterior end was viewed using SEM, the pellicle strips (of which 115 were visible; about five additional strips were obscured due to the angle of the specimen; Fig. 2a) met along a compressed line before continuing into the canal, rather than descending into an open, circular canal opening as in other photosynthetic taxa. Anterior strip reduction surrounding the canal was not visible on the cell surface. This line was also observed in fixed, contracted cells under the light microscope (Fig. 2b). A longitudinal section viewed with TEM (Fig. 2c), however, revealed an aperture of <250 nm at the cell surface leading to a narrow, flattened canal beneath.

Posterior strip reduction. Cells possessed three whorls (from anterior to posterior: whorl I, whorl II, and whorl III) of exponential reduction ($W_p = 3$); at each whorl, every other pellicle strip terminated before reaching the posterior end of the cell. When the terminating strips of each whorl were connected, three staggered whorls (where the terminating strips forming a whorl vary in length) were observed (Fig. 3a). The strips forming whorl I were sorted according to their relative lengths, so that whorl I was separated into four ''subwhorls''; from anterior to posterior, these are designated subwhorls IA, IB, IC, and ID (Fig. 3b). Whorl II was separated into two subwhorls, IIA and IIB (Fig. 3b). Although in one cell whorl III seemed to form two subwhorls (not shown), this pattern was not conspicuous in other cells. The designation ''whorl III,'' therefore, is maintained in our consideration of posterior strip

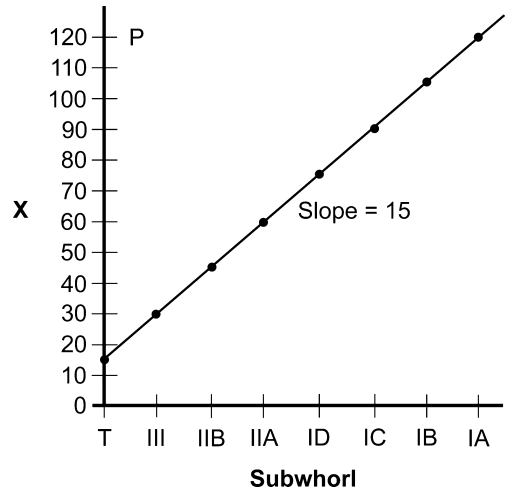

FIG. 4. Graph representing the linear pattern of posterior strip reduction in *Euglena obtusa*. *P* is the number of pellicle strips surrounding the cell periphery before strip reduction takes place. *X* is the number of strips surrounding the cell immediately before a whorl or subwhorl of strip reduction. *T* is the number of strips that reach the posterior tip of the cell.

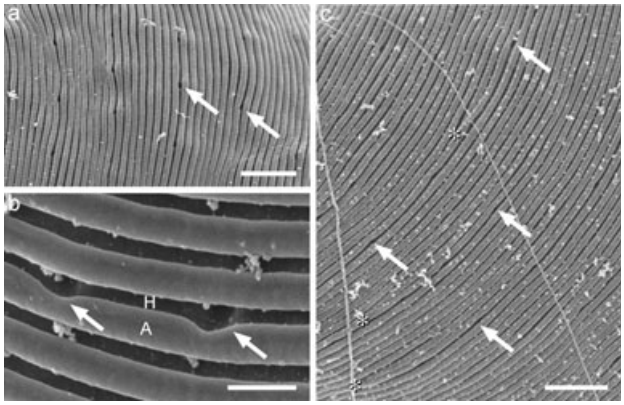

FIG. 5. The pattern of pellicle pores in *Euglena obtusa*. (a) Scanning electron micrograph showing pores (arrows), whose rows are separated by eight pellicle strips. Scale bar, 2 μm. (b) Scanning electron micrograph showing pores (arrows) located in the heel (H) region of a pellicle strip and the associated indentations in the arch (A) region of the same strip (abbreviations from Leander and Farmer 2001b). Scale bar, 500 nm. (c) Scanning electron micrograph of a cell (posterior is oriented to the bottom left of the image) showing pores (arrows) and associated indentations in the strips located immediately clockwise of the strips forming subwhorl IA (asterisks); these strips are inferred to be the strips that extend to the posterior tip of the cell. Scale bar, 2 μm.

reduction in *E. obtusa* (Fig. 3). Seven distinct subwhorls could be observed in all cells ($n = 10$). The length of the strips forming each subwhorl, however, was sometimes variable, and in three cells, the lines formed by connecting the ends of these strips crossed over one another at some points (not shown).

The number of strips passing through each subwhorl between two successive terminating strips (a value designated as *S*) decreased by one at each successive subwhorl, forming a linear pattern of strip reduction (Fig. 4). In subwhorl IA, there were seven strips between each pair of terminating strips ($S = 7$); in subwhorl IB, $S = 6$; in subwhorl IC, $S = 5$; in subwhorl ID, $S = 4$; in subwhorl IIA, $S = 3$; in subwhorl IIB, $S = 2$; and in whorl III, $S = 1$ (Fig. 3b). The number of pellicle strips converging at the posterior tip of one cell was 15.

Pellicle pores. Rows of pellicle pores between strips were observed on all 10 cells whose surface morphology was characterized. In cells where the number of strips between these rows could be determined ($n = 9$), eight strips separated rows of pellicle pores (Fig. 5a). In some cells, however, some rows of pores were separated by seven strips ($n = 3$), six strips ($n = 1$), or four strips ($n = 1$). Pores were located directly in the heel region of specific strips, creating an indentation in the arch region of the same strip (Fig. 5b). These indentations could be observed in nine of the 10 cells observed and were always in the same position. Rows of pellicle pores were located on the heel region of the strips that were located immediately to the left of the strips forming subwhorl IA (Fig. 5c). In other words, the strips bearing pores were the same 15 strips that ultimately converged at the posterior tip of the cell. Pores were rarely observed posterior to subwhorl IIB.

DISCUSSION

Pellicle morphogenesis and whorled strip reduction. The euglenid pellicle is a complex system incorporating the plasma membrane, proteinaceous strips, and underlying microtubules and endoplasmic reticulum (Murray 1984, Dubreuil and Bouck 1985). Prior to cell division, the protein strips forming the pellicle must be duplicated to ensure that each daughter cell has the same number of strips as the mother cell (Hofmann and Bouck 1976, Mignot et al. 1987, Bouck and Ngo 1996). Nascent pellicle strips are formed between mature strips, such that each mature strip alternates with a nascent strip (Hofmann and Bouck 1976). Mignot et al. (1987) demonstrated that each nascent strip is formed in a morphogenetic center associated with the ''heel'' (as defined in Leander and Farmer 2001b) of the strip to its right (see fig. 13 in Mignot et al. 1987). The strip heel and associated morphogenetic center are located on the left side of this mature strip (Fig. 6c). Thus, when the surface of the pellicle is observed, the morphogenetic origin (or parental strip) of a nascent strip can be inferred.

At least one microtubule underlying each nascent strip was previously located beneath the overhang of the mature strip to its left (Mignot et al. 1987). This, combined with the placement of the cleavage furrow during division in *E. gracilis*, seemed to imply a morphogenetic center associated with the strip

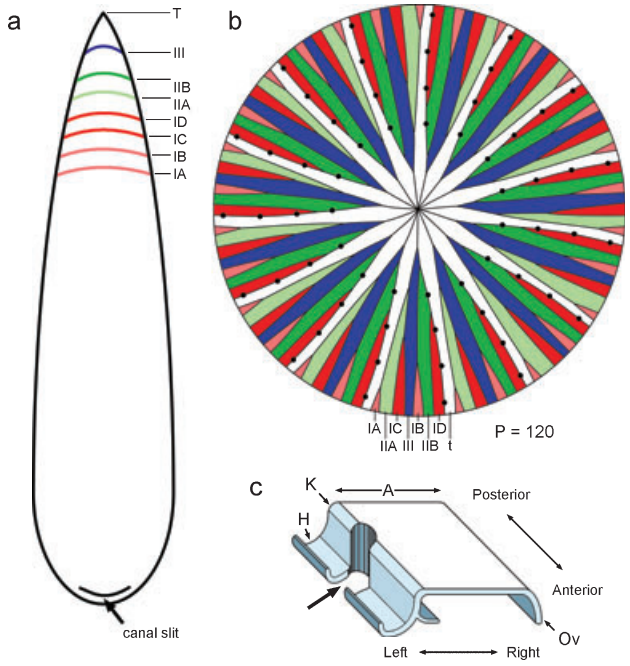

FIG. 6. A summary of pellicle strip reduction and pore placement in *Euglena obtusa*. (a) A drawing that depicts a cell in lateral view (posterior up) with the longitudinal placement and developmental origin of the seven subwhorls on the cell: whorl I, formed by the youngest pellicle strips, is divided into subwhorls IA (pink), IB (pink), IC (red), and ID (red). Whorl II, formed by the previous generation of strips, is divided into subwhorls IIA (light green) and IIB (green). Whorl III (blue) is formed by the oldest generation of terminating strips. (b) Illustration showing a cell viewed from the posterior end and using the same color scheme (with white used to denote strips that reach the posterior tip, *t*) to indicate the relative lengths and lateral (or transverse) positions of the strips forming each subwhorl. (c) A drawing of a strip section clarifying the orientation of pellicle strips and their ultrastructural components (terms are as defined by Leander and Farmer 2001b). If the cell posterior is oriented upward, the overhang (Ov) is located to the right, the keel (K) and heel (H) are located to the left, and the arch (A) is visible from the cell surface. Pores (arrow) are associated with the heel region of the strip. When viewing the cell in this way, the lateral (or transverse) order of strip identities from left to right (anticlockwise) is IA, IIA, IC, III, IB, IIB, ID, *t*. This pattern, if consistent around the circumference of the cell, necessitates that $P = 120$. Pores (black dots) are located in the heel region of *t* strips, giving the appearance of being located between ID and *t*.

overhang (Esson and Leander 2006). The work of Mignot et al. (1987), however, strongly implies its association with the heel of the adjacent mature strip. In the following discussion, therefore, we speculate that the ''parental strip'' of a given nascent strip is the mature strip to its right (that is, located immediately anticlockwise to the nascent strip). The orientation and relative positions of pellicle strips will be considered as they were described in the Results section: as if the cell were viewed laterally, with the posterior tip facing upward (Fig. 3). In this way, strip ultrastructure will be oriented as shown in Figure 6c.

The nascent strips originate in the anterior canal region and grow downward as cytokinesis takes place (Mignot et al. 1987). When this growth is terminated before the strips reach the posterior end of the cell, the shorter nascent strips alternate with longer mature strips and form an exponential whorl of reduction. Alterations in developmental timing and extent of strip growth throughout pellicle evolution have resulted in the diverse patterns of whorled reduction observed in phototrophic euglenids described so far (Leander and Farmer 2000a,b, Leander et al. 2001, Brosnan et al. 2005, Esson and Leander 2006).

Descriptive terminology. As novel patterns of posterior pellicle strip reduction are discovered, they should be described in a systematic way and integrated into a general framework of pellicle development and evolution. To do this, certain terms must be redefined and others must be invented. Leander and Farmer (2000a,b) designated the units of exponential, linear, and bilinear patterns of posterior strip reduction (where a unit constitutes all the strips terminating at the same time along the length of the cell) as ''whorls.'' It is only in exponential reduction, however, that every strip in a whorl or unit shares a common developmental origin: every strip in an exponential whorl was formed during the same round of cytokinesis (Esson and Leander 2006). Linear and bilinear ''whorls,'' on the other hand, are not necessarily developmentally unique from one another and are components of a more inclusive exponential whorl. For this reason, we contend that the term ''whorl'' should be restricted to those units containing all the strips on the cell produced during a single round of pellicle duplication and cytokinesis (i.e., exponential whorls). Nevertheless, the components of linear and bilinear patterns should be distinguished from one another, and we propose to use the term ''subwhorls'' in these contexts.

Furthermore, the notation used to number subwhorls—introduced by Leander and Farmer (2000a,b) and continued by Leander et al. (2001) and Esson and Leander (2006)—wherein the first subwhorl is indicated by a roman numeral and the second subwhorl is indicated by a roman numeral ''prime'' (e.g., I, I′, II, II′) is confusing because roman numerals are also used to indicate whorls of exponential reduction. In addition, this system is inadequate when faced with a pattern of reduction where one whorl of exponential reduction is divided into more than two subwhorls, such as in *E. obtusa*. For these reasons, we advocate the use of a roman numeral followed by a letter to indicate the order (longitudinal position) of subwhorls: the roman numeral indicates the exponential whorl of which the subwhorl is a component, while the letter indicates the relative position along the length of the cell occupied by the subwhorl. For example, the symbol ''IA'' indicates the most anterior subwhorl in the first (most anterior) whorl of exponential reduction. It should be noted at this point that

subwhorls with the same designation in different taxa are not necessarily homologous (see below).

The complex pattern of posterior strip reduction observed in *E. obtusa* and its implications for pellicle development and evolution require that we refer to individual strips throughout our discussion. For this reason, strips will be referred to using the same designation as the subwhorl to which they belong (e.g., ''IA strips''), and strips that reach the posterior tip [i.e., the oldest pellicle strips (Esson and Leander 2006)] will be designated ''*t* strips.''

Other terms used to discuss surface pellicle patterns will be retained from previous work (Leander and Farmer 2000a): the greatest number of pellicle strips that surround the circumference of a cell is designated *P*; the number of strips surrounding the cell periphery immediately anterior to a whorl or subwhorl of reduction is *X*; the number of strips that passes through a pair of terminating strips in a whorl or subwhorl is *S*; the number of exponential whorls of reduction is W_p; and the number of strips that reach the posterior tip is *T*.

Synthesis of pellicle surface patterns in E. obtusa. When studying euglenid pellicle characters with SEM, patterns of posterior reduction and strip number, *P*, can usually be observed directly on cells that lie either on their anterior or posterior end, so that the opposite end of the cell is completely visible. All cells examined in this study lay on their sides, making both direct anterior and posterior views difficult. Examination of the anterior end of one cell revealed 115 strips, and five more were obscured by the angle of this cell (as extrapolated by the space across the obscured region; Fig. 2a). Although *P* was not determined directly from this observation, other direct observations enabled us to confidently infer *P*. For instance, Leander and Farmer (2000a) showed that in a cell with three exponential whorls ($W_p = 3$), the number of strips surrounding the cell circumference immediately to the posterior of whorl I would be equal to half of *P*; after whorl II, this value would be 1/4 *P*; and after whorl III, it would be 1/8 *P*. As whorl III is the most posterior whorl in such a cell, all the strips remaining after passing through it would reach the posterior tip of the cell, so that $T = 1/8\ P$. Because $T = 15$, *P* for *E. obtusa* is inferred to be 120, which is also consistent with data shown in Figure 2a.

Moreover, the *P* of 120 congruently incorporates our observations of posterior strip reduction. The same repeating pattern, left to right, of strips was observed in nine cells: IA, IIA, IC, III, IB, IIB, ID, *t* (Fig. 6b). The consistency of this pattern indicates that strip reduction follows the same pattern around the circumference of the cell. Each repeating unit contained eight strips, so for a complete pattern of repetition, *P* must be divisible by eight: 120 strips divided by eight yields 15 strips, which is the total number of tip strips that converge at the posterior tip of the cell ($T = 15$). All of the observations of strip patterns near the anterior and posterior ends of *E. obtusa* are concordant with $P = 120$. It should also be noted that a cell with $P = 112$ and $T = 14$ would result in a complete pattern of repeating units consisting of eight strips each; however, this pattern was never observed.

A diagram of posterior strip reduction in *E. obtusa* is shown in Figure 6. The longitudinal positions of the subwhorls IA, IB, IC, ID, IIA, IIB, and III, and the relative lateral positions of their component strips are shown and coordinated with a *P* of 120. As inferred from the above calculations, there are 15 repeating units each comprised of eight strips, including one strip (*t*) per unit that reaches the posterior tip of the cell. Because each subwhorl comprises 15 strips, the number of strips surrounding the periphery of the cell reduces by 15 at each subwhorl, making the pattern of posterior strip reduction in *E. obtusa* mathematically ''linear'' (Fig. 4; Leander and Farmer 2000a).

Pellicle pores are located in the heel region of the strips that are located to the left of the terminating strips forming subwhorl IA; in other words, pores pierce the heels of the *t* strips (Fig. 6b). This finding is consistent with the observation that there are eight pellicle strips between rows of pores (Fig. 5a). According to our model of multigenerational whorl morphogenesis (Esson and Leander 2006), these strips are the oldest strips in the pellicle complex and, because $W_p = 3$, are at least four generations old. Semiconservative pellicle duplication and inheritance, however, requires that half of the *t* strips will be five or more generations old, because strips that have reached maturity will remain as *t* strips through subsequent cytokinetic events. It is conceivable that a single strip could be maintained throughout an infinite number of generations as long as it was always inherited by a daughter cell that survived to divide again. Although the number of rounds of cell division required for strip maturity can be inferred from posterior whorls of reduction, it is impossible to infer the absolute age of each *t* strip since they are all the same length irrespective of age.

In their description of pellicle pores, Leander and Farmer (2000a) state that pores are located in the articulation zone between strips, rather than within one strip per se. High magnification SEMs and the consistent presence of associated dents in the strip arch, however, suggest that, at least in *E. obtusa*, there is a strong association with one of the two strips bordering a row of pellicle pores, namely, the *t* strips (Fig. 5b).

Parallel evolution of linear posterior strip reduction. ''Linear'' strip reduction refers to the pattern of posterior strips formed when the lengths of strips comprising one or more whorls of exponential reduction are staggered so that the same numbers of strips terminate at several points along the length of the cell (the ''subwhorls''). Taxa previously

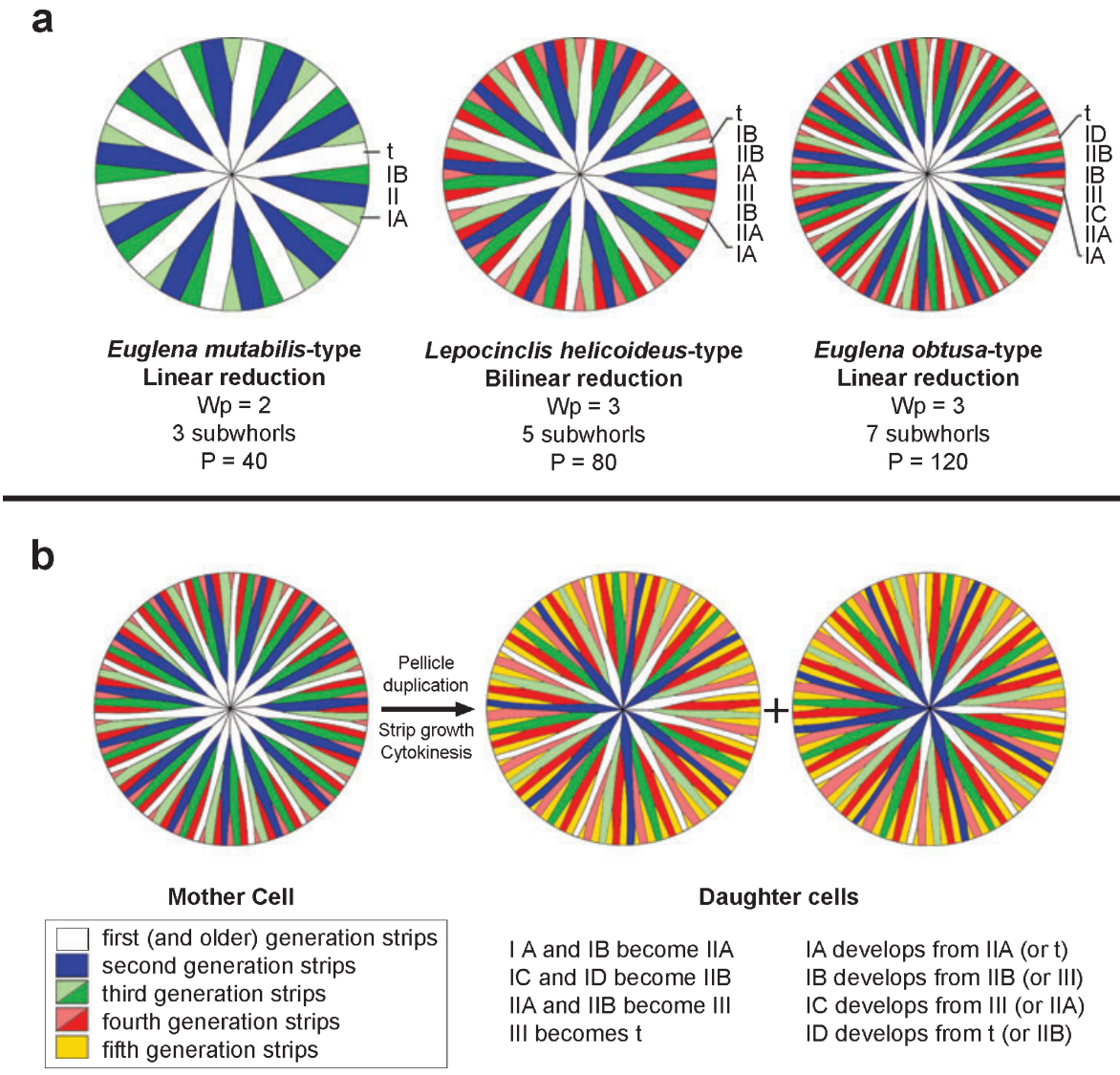

FIG. 7. Multigenerational linear and bilinear posterior strip reduction in phototrophic euglenids and a model for development of subwhorls in *Euglena obtusa*. (a) Developmental origins of subwhorls in euglenids with linear and bilinear strip reduction. In *Euglena mutabilis* ($P = 40$) (and potentially other taxa with similar patterns of reduction), there are three subwhorls of linear reduction that constitute two whorls of exponential reduction, formed by two respective generations of strips. Whorl I, formed by the youngest (third generation) strips, is divided into two subwhorls: IA (light green) and IB (green). Whorl II (blue) is formed by strips belonging to the previous (second) generation. Subwhorl IA is inferred to develop from whorl II, and subwhorl IB develops from the *t* strips. In *Lepocinclis helicoideus* ($P = 80$), three exponential whorls are differentiated into five subwhorls of bilinear reduction. Whorl I (fourth generation strips) is comprised of IA (pink) and IB (red), whorl II (third generation strips) is subdivided into IIA (light green) and IIB (green), and whorl III (second generation strips; blue) remains intact. Based on relative clockwise positions, IIA and IIB strips give rise to IA strips, while *t* and III strips give rise to IB strips. In *E. obtusa* ($P = 120$), the relative positions of strips are similar to those in *L. helicoideus*, but whorl I has further differentiated into four subwhorls, yielding seven subwhorls of linear reduction on the cell. Positions occupied by IA strips in *L. helicoideus* are occupied by IA and IC strips in *E. obtusa*, and those occupied by IB strips in *L. helicoideus* are occupied by IB and ID strips in *E. obtusa*. (b) An illustration of strip development and whorl inheritance in *E. obtusa*. Each pellicle strip in the mother cell produces a new strip (fifth generation strips) immediately clockwise to itself. These new strips grow to become whorl I in the daughter cells, while mature strips grow to form the next posterior whorl of exponential reduction. In *E. obtusa*, therefore, IA strips in the daughter cells develop from IA and IB strips (IIA strips in the daughter cells), IB strips develop from IC and ID strips (IIB strips in the daughter cells), IC strips develop from IIA and IIB strips (whorl III strips in the daughter cells), and ID strips develop from III and *t* strips (*t* strips in the daughter cells). Alternatively, a morphogenetic center located in the strip overhang would require the parent strips indicated in parentheses.

described as having linear (or ''pseudolinear'') reduction are *E. mutabilis* (with three subwhorls of linear reduction formed by two whorls of exponential reduction; Fig. 7) and *Eutreptia pertyi* (with two subwhorls of pseudolinear reduction formed by one whorl of exponential reduction) (Leander and Farmer 2000a, Leander et al. 2001). This pattern is similar to ''bilinear'' reduction, where there is an

equal number of terminating strips at each of several subwhorls, and then a second number of terminating strips at each of the remaining subwhorls (Leander and Farmer 2000b, Leander et al. 2001). Bilinear reduction has only been observed in one taxon, *Lepocinclis helicoideus* (=*Euglena helicoideus*), where 20 strips terminate at each of two subwhorls in one exponential whorl, and 10 strips terminate at each of two subwhorls of the second exponential whorl and at the intact third exponential whorl (Fig. 7; Leander and Farmer 2000b, Leander et al. 2001). The number of strips around the cell periphery reduces by 15 at each subwhorl in *E. obtusa* (Figs. 4 and 6b), resulting in a pattern of linear reduction over seven subwhorls (Figs. 3b, 4, 6b, and 7). The four subwhorls in whorl I of *E. obtusa* show a level of length differentiation within a single generation of pellicle strips that has not been observed until now. This level of length differentiation provides insight into the role of strip maturity in pellicle morphogenesis.

The seven-subwhorl linear pattern observed in *E. obtusa* is similar to the three-subwhorl linear pattern observed in *E. mutabilis* (Leander and Farmer 2000a) in that *P*, the number of strips surrounding the cell periphery, reduces by a constant number at each subwhorl. Considering the model of whorl morphogenesis and the evolutionary transformation previously proposed (Esson and Leander 2006), the pattern of strip reduction in *E. obtusa* is more likely derived from a five-subwhorl bilinear pattern like that observed in *L. helicoideus* (Leander and Farmer 2000b). Note, however, that we are not proposing that the bilinear pattern in *L. helicoideus* is specifically homologous to the pattern of strip reduction in *E. obtusa*.

As described above, the pattern of linear reduction observed in *E. mutabilis* is formed by two exponential whorls, or two generations of strips. The youngest generation is differentiated into two alternating sets of strips, forming subwhorls IA and IB (Fig. 7a). In light of the lateral position of these strips relative to those forming whorl II and the *t* strips (Leander and Farmer 2000a) and the association of a morphogenetic center with the heel of a mature strip (Mignot et al. 1987), we can infer that subwhorl IA developed from whorl II, and subwhorl IB developed from the *t* strips. The *L. helicoideus*–type of bilinear reduction is formed by three generations of strips. Two generations of strips are each differentiated into two subwhorls: IA and IB in the youngest generation, and IIA and IIB in the second youngest (Fig. 7a). The inferred developmental origin of subwhorl IA is shared between subwhorls IIA and IIB. The origin of subwhorl IB is divided between the *t* strips and whorl III. In linear strip reduction in *E. obtusa*, there are three generations of terminating strips as in *L. helicoideus*. However, in contrast to *L. helicoideus*, the youngest generation in *E. obtusa* is further differentiated into four subwhorls: IA, IB, IC, and ID. As such, the inferred developmental origins are more specifically discernable in *E. obtusa* than in *L. helicoideus*: subwhorl IA develops from subwhorl IIA, subwhorl IB develops from subwhorl IIB, subwhorl IC develops from whorl III, and subwhorl ID develops from the *t* strips. Alternatively, a morphogenetic center associated with the overhang would require that a nascent strip develop from the mature strip immediately to its left. Potential parent-nascent strip relationships are summarized in Figure 7b.

The inferred pattern of strip development and the fate of individual strips during subsequent strip duplications in *E. obtusa* are presented in Figure 7b. After nascent strips are produced, the strips forming the various subwhorls in the mother cell extend to assume new identities; that is, the strips become components of different posterior whorls and subwhorls (Esson and Leander 2006). These new identities will be the same for each set of strips regardless of the identity of their parental strips, as nascent strips belonging to each subwhorl are always located between the same two mature strips, either of which could be the parental strip. Strips forming subwhorls IA and IB become components of subwhorl IIA, subwhorls IC and ID become subwhorl IIB, subwhorls IIA and IIB converge to form whorl III, and the strips forming whorl III become *t* strips. Nascent strips will become mature strips (*t* strips) after three more rounds of cytokinesis.

It is significant to note that according to the proposed developmental scenario (i.e., the strip heel is the center of strip morphogenesis), nascent strip length could be a function of parent strip length as the relative length of nascent strips would be the same as the relative lengths of their inferred parental strips. This would provide a predictable framework for the relative lateral positions of each subwhorl's component strips. Subwhorl IA, comprising the shortest nascent strips, would develop from subwhorl IIA, the shortest mature strips; subwhorl IB, composed of slightly longer strips, would develop from subwhorl IIB, the mature strips with the corresponding relative length. Subwhorl IC, whose component strips are even longer, would develop from whorl III, the second-longest strips on the cell surface; and subwhorl ID, with the longest nascent strips in the pellicle, would develop from the longest pellicle strips, the *t* strips.

By contrast, if the morphogenetic center is localized in the overhang (rather than the heel), then nascent strip length would no longer be a function of parent strip length and might instead be influenced only by the relative maturity of parent strips. For example, IA strips, the shortest strips in whorl I, would develop from the oldest strips, the *t* strips. Nevertheless, both developmental scenarios implicate the influence of parental strips in determining the identity of nascent strips. Moreover, as pellicle strips mature after subsequent rounds of cell

TABLE 1. Relationship between patterns of pellicle pores and posterior exponential strip reduction in *Euglena* (based on data from Leander and Farmer 2000a and Leander et al. 2001). The number of whorls of exponential strip reduction (W_p) influences the number of strips reaching the posterior tip of the cell (T). In all taxa with a consistent number of strips between rows of pellicle pores, the number of strips with a row of pellicle pores is equal to the number of strips reaching the posterior tip of the cell, except in *E. myxocylindracea*, where only half of the tip strips have a row of pellicle pores. P refers to the total number of strips around the cell periphery.

Taxon	P	W_p	T	Number of strips between pores	Number of strips with pores
E. laciniata	40	2	10	4	10
E. myxocylindracea	40	2	10	8	5
E. terricola	40	2	10	4	10
E. stellata	40	2	10	4	10
E. cantabrica	48–56	1	24–28	2	24–28
E. obtusa	120	3	15	8	15

division, they converge in length at each subsequent posterior whorl of exponential reduction: strips of four lengths in whorl I converge to two lengths after one round of cell division; these in turn converge to one length over the next round of cell division (Figs. 6b and 7b).

Pellicle evolution and development: a potential model system? The position of pellicle pores in *E. obtusa* supports Leander and Farmer's (2000a) hypothesis that pellicle pores are associated with the most mature strips, and that strip morphology might change with subsequent cell divisions. By dividing P by the number of strips between rows of pores, one can infer how many pellicle strips bear pores (Table 1). In most of the taxa where the number of strips between rows of pellicle pores is relatively constant, the number of strips that reach the posterior tip of the cell is equal to the number of strips whose heel regions would be in contact with pellicle pores. The only known exception to this is *E. myxocylindracea*, in which only half of the 10 t strips bear pores. This finding is still consistent, however, with the hypothesis that pores are associated with mature strips, because the strips that reach the posterior tip of a cell, while being older than the other strips on that cell, were not all produced during the same pellicle duplication and cell division event. In a cell with $W_p = 2$, such as *E. myxocylindracea*, t strips must be at least three generations old, but half of the t strips will belong to one or more older generations (Esson and Leander 2006). The pattern of pellicle pores in *E. myxocylindracea* suggests, therefore, that strips must be at least four generations old before they form pellicle pores.

The constant relative positions and inferred morphogenetic origins of the strips forming the subwhorls in *E. obtusa* suggest that the developmental cues that help to direct the growth and final length of nascent strips are at least in part localized in the parental strip and the morphogenetic center near its heel (Mignot et al. 1987). Each strip comprises a complex of proteins intimately associated with the plasma membrane and underlying microtubules (Murray 1984, Dubreuil and Bouck 1988, Dubreuil et al. 1988), so the formation and elongation of nascent pellicle strips (and perhaps the formation of pellicle pores in mature strips) is dependent on underlying processes of protein deposition and microtubule formation and organization. These processes have not been thoroughly examined in the context of pellicle evolution and development in euglenid cells.

Leander and Farmer (2000a) have suggested that the formation of pellicle pores with strip maturity parallels the processes of flagellar maturation and identity change with each subsequent cell division in euglenids (Farmer and Triemer 1988, Brugerolle 1992) and other protists (Moestrup and Hori 1989, Nohynkova et al. 2006). There is merit to this argument because there is an integrated array of microtubules associated with basal bodies, flagellar roots, the feeding apparatus, and the pellicle in euglenids and related taxa (Willey and Wibel 1985, Surek and Melkonian 1986, Solomon et al. 1987, Simpson 2003). As such, the role of microtubule organization in the morphogenesis and character evolution of the euglenid pellicle should be examined closely using advanced microscopic and genetic approaches. The relative ease with which photosynthetic euglenids can be induced to divide and cytoskeletal development can be observed makes the euglenid pellicle an ideal system on which to perform more detailed analyses of morphogenesis in eukaryotic cells (Hofmann and Bouck 1976, Bouck and Ngo 1996, Esson and Leander 2006). In addition to helping us understand the cell biology and evolution of euglenids, further analyses of euglenid development have great potential for improving our understanding of fundamental processes associated with the diversification of eukaryotic cells.

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Bouck, G. B. & Ngo, H. 1996. Cortical structure and function in euglenoids with reference to trypanosomes, ciliates, and dinoflagellates. *Int. Rev. Cytol.* 169:267–318.

Brosnan, S., Brown, P. J. P., Farmer, M. A. & Triemer, R. E. 2005. Morphological separation of the euglenoid genera *Trachelomonas* and *Strombomonas* (Euglenophyta) based on lorica development and posterior strip reduction. *J. Phycol.* 41:590–605.

Brugerolle, G. 1992. Flagellar apparatus duplication and partition, flagellar transformation during division in *Entosiphon sulcatum*. *Biosystems* 28:203–9.

Dubreuil, R. R. & Bouck, G. B. 1985. The membrane skeleton of a unicellular organism consists of bridged, articulating strips. *J. Cell Biol.* 101:1884–96.

Dubreuil, R. R. & Bouck, G. B. 1988. Interrelationships among the plasma membrane, the membrane skeleton and surface form in a unicellular flagellate. *Protoplasma* 143:150–64.

Dubreuil, R. R., Rosiere, T. K., Rosner, M. C. & Bouck, G. B. 1988. Properties and topography of the major integral plasma membrane protein of a unicellular organism. *J. Cell Biol.* 107:191–200.

Esson, H. J. & Leander, B. S. 2006. A model for the morphogenesis of strip reduction patterns in phototrophic euglenids: evidence for heterochrony in pellicle evolution. *Evol. Dev.* 8:378–88.

Farmer, M. A. & Triemer, R. E. 1988. Flagellar systems in the euglenoid flagellates. *Biosystems* 21:283–91.

Gojdics, M. 1953. *The Genus* Euglena. University of Wisconsin Press, Wisconsin, Madison, 268 pp.

Hofmann, C. & Bouck, G. B. 1976. Immunological and structural evidence for patterned intussusceptive surface growth in a unicellular organism. *J. Cell Biol.* 69:693–715.

Huber-Pestalozzi, G. 1955. *Das Phytoplankton des Süsswasser, 4. Teil: Euglenophyceen.* E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany, 1,265 pp.

Kim, J. T., Boo, S. M. & Zakrys, B. 1998. Floristic and taxonomic accounts of the genus *Euglena* (Euglenophyceae) from Korean fresh waters. *Algae* 13:173–97.

Kosmala, S., Karnkowska, A., Milanowski, R., Kwiatowski, J. & Zakrys, B. 2005. Phylogenetic and taxonomic position of *Lepocinclis fusca* comb. nov. (=*Euglena fusca*) (Euglenaceae): morphological and molecular justification. *J. Phycol.* 41:1258–67.

Leander, B. S. 2004. Did trypanosomatid parasites have photosynthetic ancestors? *Trends Microbiol.* 12:251–8.

Leander, B. S. & Farmer, M. A. 2000a. Comparative morphology of the euglenid pellicle. I. Patterns of strips and pores. *J. Eukaryot. Microbiol.* 47:469–79.

Leander, B. S. & Farmer, M. A. 2000b. Epibiotic bacteria and a novel pattern of strip reduction on the pellicle of *Euglena helicoideus* (Bernard) Lemmermann. *Eur. J. Protistol.* 36:405–13.

Leander, B. S. & Farmer, M. A. 2001a. Evolution of *Phacus* (Euglenophyceae) as inferred from pellicle morphology and SSU rDNA. *J. Phycol.* 37:143–59.

Leander, B. S. & Farmer, M. A. 2001b. Comparative morphology of the euglenid pellicle. II. Diversity of strip substructure. *J. Eukaryot. Microbiol.* 48:202–17.

Leander, B. S., Witek, R. P. & Farmer, M. A. 2001. Trends in the evolution of the euglenid pellicle. *Evolution* 55:2215–35.

Mabee, P. M. 2000. The usefulness of ontogeny in interpreting morphological characters. *In* Wiens, J. J. [Ed.] *Phylogenetic Analysis of Morphological Data.* Smithsonian Inst. Press, Washington, DC, pp. 84–114.

Marin, B., Palm, A., Klingberg, M. & Melkonian, M. 2003. Phylogeny and taxonomic revision of plastid-containing euglenophytes based on SSU rDNA sequence comparisons and synapomorphic signatures in the SSU rRNA secondary structure. *Protist* 154:99–145.

Mignot, J. P., Brugerolle, G. & Bricheux, G. 1987. Intercalary strip development and dividing cell morphogenesis in the euglenid *Cyclidiopsis acus*. *Protoplasma* 139:51–65.

Moestrup, Ø. & Hori, T. 1989. Ultrastructure of the flagellar apparatus in *Pyramimonas octopus* (Prasinophyceae). II. Flagellar roots, connecting fibers, and numbering of individual flagella in green algae. *Protoplasma* 148:41–56.

Murray, J. M. 1984. Disassembly and reconstitution of a membrane-microtubule complex. *J. Cell Biol.* 98:1481–7.

Nohynkova, E., Tumova, P. & Kulda, J. 2006. Cell division of *Giardia intestinalis*: flagellar development cycle involves transformation and exchange of flagella between mastigonts of a diplomonad cell. *Eukaryot. Cell* 5:753–61.

Nudelman, M. A., Leonardi, P. I., Conforti, V., Farmer, M. A. & Triemer, R. E. 2006. Fine structure and taxonomy of *Monomorphina aenigmatica* comb. nov. (Euglenophyta). *J. Phycol.* 42:194–202.

Schmitz, F. 1884. Beitrage zur kenntnis der chromatophoren. *Jahrb. F. wiss. Bot.* 15:1–175.

Simpson, A. G. B. 2003. Cytoskeletal organization, phylogenetic affinities and systematics in the contentious taxon Excavata (Eukaryota). *Int. J. Syst. Evol. Microbiol.* 53:1759–77.

Solomon, J. A., Walne, P. L. & Kivic, P. A. 1987. *Entosiphon sulcatum* (Euglenophyceae): flagellar roots of the basal body complex and reservoir region. *J. Phycol.* 23:85–98.

Surek, B. & Melkonian, M. 1986. A cryptic cytostome is present in *Euglena*. *Protoplasma* 133:39–49.

Triemer, R. E., Linton, E., Shin, W., Nudelman, A., Monfils, A., Bennet, M. & Brosnan, S. 2006. Phylogeny of the Euglenales based upon combined SSU and LSU rDNA sequence comparisons and description of *Discoplastis* gen. nov. (Euglenophyta). *J. Phycol.* 42:731–40.

Uhlig, G. 1964. Eine einfache Methode zur extraction der vagilen, mesopsammalen Mikrofauna. *Helgol. Wiss. Meeresunters.* 11:178–85.

Willey, R. L. & Wibel, R. G. 1985. The reservoir cytoskeleton and a possible cytostomal homologue in *Colacium* (Euglenophyceae). *J. Phycol.* 21:570–7.