MORPHOLOGICAL EVOLUTION AND DEVELOPMENT OF THE EUGLENID CYTOSKELETON

by

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Abstract

In an effort to better understand character evolution in the cytoskeleton (pellicle) of euglenid protists, I used comparative and descriptive methods to investigate the morphological diversity and development of pellicle surface patterns formed by differences in strip length at the anterior and posterior ends of the cell (strip reduction). By observing dividing *Euglena gracilis* cells with scanning electron microscopy (SEM) and integrating these data with previous evolutionary and developmental research, I showed that these patterns result from the semiconservative duplication and subsequent intermittent growth of pellicle strips during cytoskeletal replication and cytokinesis. Furthermore, simple changes in the developmental timing of this process (heterochrony) resulted in the diversity of posterior strip reduction patterns observed in phototrophic euglenids. This model was then used to interpret the results of two studies describing pellicle surface patterns in other photosynthetic taxa. The first was a morphological description of the complex linear pattern of posterior reduction in the benthic marine phototroph, *Euglena obtusa*. The second was an investigation of the evolution of bilaterally symmetrical, "clustered" strip reduction patterns in the rigid genus *Phacus*, examined in the context of maximum likelihood (ML) and Bayesian phylogenetic analyses of combined nuclear small subunit and partial large subunit ribosomal genes (SSU rDNA and LSU rDNA, respectively). These studies, taken together, show that strip length and other pellicle characters (such as pore placement) are strongly influenced by age and perhaps other developmental factors (such as parental strip identity and cell polarity), but the underlying genetics and molecular biology of these factors are completely unknown. Finally, SEM was used for the first time to describe prearticular strip projections, a pellicle character that has been extensively studied using transmission electron microscopy (TEM). The novel character state revealed by this study shows that the diversity of this pellicle character is still poorly understood. The structural complexity of the euglenid pellicle and the developmental and evolutionary processes that resulted in its astonishing diversity could make it an ideal model system for studying cytoskeletal evolution and development once a robust genetic research framework is constructed.

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Co-authorship statement

All chapters were written in collaboration with Brian Leander, who performed several phylogenetic analyses and offered suggestions for improving taxon sampling, molecular alignments, phylogenetic analyses, and the clarity and organization of the manuscripts and figures. I was primarily responsible for selecting taxa to study, designing and performing experiments, analyzing data, writing all manuscripts, and creating all figures.

Chapter 1: INTRODUCTION

1.1 The study of character evolution

The study of biological evolution and systematics is a complicated and time-consuming undertaking. It is clear that the millions of species that are, or were, present on earth share a common ancestry; what is not quite clear is what the ancestors at different points in the biological hierarchy were like, or which of their numerous descendents are more closely related to one another. An excellent way of addressing these questions is to look for "family resemblance": any group of organisms that are closely related to one another *should* have more traits, or characters, in common than a group of distantly related organisms. Characters that are shared between organisms are also more likely to have been present in their last common ancestor. This is based on the (well supported) assumption that populations that are relatively distantly related to one another have had more time to be independently shaped by natural selection and other evolutionary processes and should therefore be more divergent from one another. The characters used to reconstruct phylogenies can include the range of shapes or quantities of a given structure, specific patterns of behavior, or the sequence of nucleotides in a particular segment of a particular gene (Brooks and McLennan 2002), giving the evolutionary biologist a vast array of clues which can be used to solve the puzzle of biodiversity and common descent.

Any pair of taxa will share some similarities and some differences – it is not always clear, however, which characters are better indications of a close evolutionary relationship. Characters are shaped by complex processes, and these processes are not always readily apparent. A character may change from one state to another and then revert to something resembling the original state. The state of a given character may be influenced by the state of another, apparently unrelated, character (interdependence). Different structures in unrelated organisms may also experience similar selective processes and evolve to assume similar appearance or biochemical attributes (convergence). Characters (or the taxa exhibiting them) may be excluded from an analysis because they seem unrelated to the problem at hand, or because the researcher is simply unaware of their existence (sampling problems). In order to successfully resolve

evolutionary relationships, the researcher must retrace the evolution of the characters themselves – and, given the difficulties outlined above, this requires understanding as much as possible about what influences the evolution of a given character. What is the function, if any, of the character? How does a change in character state affect this function? What other characters and processes influence this character and its function? What developmental processes are required to produce the character?

1.2 Eukaryotic cytoskeletal diversity and an introduction to the euglenid pellicle

The eukaryotic cytoskeleton integrates multiple complex components that function together to provide structural stability and facilitate fundamental cellular processes such as mitosis, feeding and locomotion. Unicellular eukaryotes (protists) display astounding cytoskeletal diversity according to their complex evolutionary history, incorporating lineage-specific configurations of widely conserved structural proteins (such as the tubulin in microtubules), or novel structural proteins (such as articulins). Environmental PCR surveys indicate that unicellular eukaryotic diversity is vast and poorly sampled. Most organisms represented by these sequences, furthermore, have not been described morphologically or taxonomically (e.g., Moreira and López-García 2002; Stoeck et al. 2006). Even where detailed morphological descriptions exist, there are little or no data pertaining to the biochemicals and developmental processes that yield a given cytoskeletal configuration. In well-studied protists that are readily cultured, such as the apicomplexan parasite *Toxoplasma gondii* (Mann and Beckers 2001, Gordon et al. 2008), the ciliate *Paramecium tetraurelia* (Pomel et al. 2006), and other members of the protistan group Alveolata (Gould et al. 2008), novel cytoskeletal proteins and their homologues continue to be discovered and characterized. The complexity described in these organisms, representing only a fraction of protistan diversity, serves to emphasize how little is known about cytoskeletal diversity and evolution in eukaryotes.

A compelling example of such complexity is the peripheral cytoskeleton, or pellicle, of the euglenids, which is formed by interactions between microtubules, the endomembrane system, the plasma membrane, and proteinaceous strips. While biologists have studied euglenid taxonomy

and ecology in some detail for over a century, it was the use of the electron microscope in the mid-twentieth century that revealed the remarkable complexity of the pellicle (e.g., Groupé 1947, Pitelka 1963, Kirk and Juniper 1964, Taylor 1999). Data have accumulated over the subsequent six decades, contributing to our knowledge of the pellicle's ultrastructure, biochemistry and development; these data in turn have been used to answer important questions regarding euglenid ecology, evolution and taxonomy. The research on pellicle development and diversity presented in this thesis has shed light on the complex interactions between the morphogenesis and evolution of this unique structural system. In order to understand these interactions, however, it is useful to first review euglenid diversity.

1.3 Overview of euglenid systematics

Euglenids (or euglenoids) are unicellular eukaryotic flagellates that occupy marine and freshwater sediments (Taylor 1967, Brown et al. 2002, Chapter 3), marine and freshwater planktonic communities, or more extreme habitats such as volcanic mud pools (Sittenfeld et al. 2002).

Euglenids include phagotrophic, osmotrophic, and phototrophic taxa. Phagotrophic euglenids consume either bacteria ("bacterivores") or microeukaryotes ("eukaryovores"), and glide on a substrate using a backward-trailing ventral flagellum and a dorsal flagellum held straight in front of the cell. Eukaryovores differ from bacterivores in the structure of the feeding apparatus and the pellicle (Fig. 1.1; Leander 2004, Leander et al. 2001a). Within the eukaryovores, phagotrophy was lost twice: once in a clade of cells that absorb nutrients from their surroundings ("primary osmotrophs") (Fig. 1.1; Leander et al. 2001a, Busse et al. 2003) and once in a diverse clade of photosynthetic euglenids. The latter group acquired photosynthesis via secondary endosymbiosis involving a eukaryovorous ancestor and a green algal prey cell (Fig. 1.1; Gibbs 1978; Montegut-Felkner and Triemer 1997, Linton et al. 1999, Busse et al. 2003, Leander 2004, Leander et al. 2001a, 2007). Gliding motility was also lost in both clades and replaced by swimming motility, where the anterior flagella pull the cells through the water column. Photosynthesis has been lost multiple times within the phototrophic clade, resulting in a

polyphyletic assemblage of colorless “secondary osmotrophs” (Linton et al. 2000, Müllner et al. 2001, Marin et al. 2003).

Multiple independent changes in motility, plastid number, cell shape, cytoskeletal configuration and other characters have accompanied the radiation of the phototrophic lineage. One flagellum was substantially reduced subsequent to the divergence of the Eutreptiales, a clade of marine phototrophs that possess two (or four, in the case of *Eutreptia pomquetensis*; McLachlan et al. 1994, Marin et al. 2003) emergent flagella – as a result, most phototrophic and secondarily osmotrophic euglenids have one emergent flagellum (Fig. 1.1a). While eukaryovores and early-diverging lineages of phototrophs can readily change the shape of their cells, at least two independent losses of cell plasticity have occurred in the phototrophic lineage (Fig. 1.1a, g-h). Members of the genera *Trachelomonas* and *Strombomonas* secrete mucilaginous shells, or loricae, that encase each cell (Fig. 1.1a, f). The number of flagella, plastids, and stored carbohydrate (paramylon) granules, combined with variations in cell or lorica shape and cell plasticity, have been used as characters to describe and delineate euglenid taxa, resulting in a large (and confusing) collection of genera, subgenera, species, and varieties (see, for example, Huber-Pestalozzi 1955). Over the past decade, molecular and morphological studies of cultures and cells individually isolated from the environment have significantly clarified euglenid systematics, especially within the phototrophic lineage (e.g., Linton et al. 1999, 2000, Marin 2003, Nudelman et al. 2003, Shin and Triemer 2004, Triemer et al. 2006) – taxonomy of phagotrophic euglenids, however, remains problematic due to poor knowledge of overall diversity and the difficulty in establishing cultures of these organisms (Busse et al. 2003, Breglia et al. 2007).

Phylogenetic analyses of small and/or large subunit ribosomal genes have been particularly informative. Several phototrophic genera, including *Euglena* Ehrenberg, *Phacus* Dujardin, and *Lepocinclis* Perty, were repeatedly found to be polyphyletic in these studies, resulting in several species being moved to different genera (Linton et al. 2000, Brosnan et al. 2003, Marin et al. 2003, Nudelman et al. 2003, Shin and Triemer 2004). Several species formerly placed within *Euglena* were moved to *Lepocinclis* (Marin et al. 2003, Kosmala et al. 2005), while a smaller group was removed to a new genus, *Discoplastis* (Fig. 1.1a; Triemer et al. 2006). The

genus *Monomorphina* Mereschowsky was resurrected by Marin et al. (2003) to incorporate species designated as *Lepocinclis* and *Phacus* that did not group with other members of these genera in phylogenetic analyses (Linton et al. 1999, 2000, Müllner et al. 2001). Additional species designated as *Phacus* were relocated to *Cryptoglena* Ehrenberg, the sister genus to *Monomorphina* (Fig. 1.1a; Marin et al. 2003, Nudelman et al. 2003).

While the widespread application of molecular phylogenetic methods has helped to ensure that photosynthetic genera are now monophyletic, several important relationships between and within these genera remain to be resolved. *Phacus* and *Lepocinclis*, for example, are accepted to be sister taxa, but it is unclear from molecular data alone when they diverged from other phototrophs; some molecular phylogenies and the possession of numerous disc-shaped plastids, suggest a sister relationship between *Discoplastis* and the rigid *Phacus*/*Lepocinclis* clade, whose members exhibit similar plastid morphology (Fig. 1.1a; Marin et al. 2003, Triemer et al. 2006, Chapter 4). The large number of described species and varieties in the taxonomic literature also needs to be revised. Many of the morphological characters upon which traditional euglenid taxonomy is based, such as the size and number of paramylon grains, the shape and number of plastids, and the shape of cell margins, have been shown to be variable within a given strain and therefore unreliable as taxonomic indicators (Conforti 1998, Nudelman et al. 2006, Kosmala et al. 2007a, b). No molecular data are available for the majority of these taxa. Reliable taxonomic indicators observed using light microscopy have been defined for select taxa, but only after a large number of strains and characters, morphological and molecular, were carefully observed and defined (Kosmala et al. 2005, 2007a, b).

Electron microscopy permits the observation of novel characters that can be incorporated into the ongoing study of euglenid systematics (Taylor 1976, 1999). Description and analysis of the pellicle, combined with the increasing number of available molecular phylogenies, have yielded taxonomically informative characters and revealed morphological markers of important transitions in euglenid evolution (Leander 2004, Leander and Farmer 2001b, Leander et al. 2001b, Brosnan et al. 2005).

1.4 Structure of the pellicle

Euglenids possess the anterior flagellar and microtubular roots typical of the Excavata, an emerging supergroup consisting of euglenozoans (i.e. euglenids, kinetoplastids and diplonemids; Simpson 1997) and several groups of anaerobic flagellates (e.g., diplomonads, retortomonads, heteroloboseans, oxymonads, and parabasalids)(Simpson 2003). These roots give rise to one set of microtubules that supports the feeding apparatus (or its vestige, in phototrophic euglenids) and two others that surround the anterior flagellar pocket or canal and continue around the periphery of the cell, finally reaching the posterior end (Guttman and Ziegler 1974, Willey and Wibel 1985, Surek and Melkonian 1986, Leander et al. 2007). This peripheral arrangement of microtubules is similar to the subpellicular corset of trypanosomes and is closely associated with the cytoplasmic surface of the proteinaceous strips that are arguably the most distinctive component of the pellicle (Fig. 1.2; Mikolajczyk 1975, Dubreuil and Bouck 1985, Mignot et al. 1987, Sherwin and Gull 1989, Bouck and Ngo 1996). The strips extend from the anterior to the posterior end of the cell beneath the plasma membrane and cover the entire cell surface (Leedale 1964, 1967, Sommer 1965, Schwelitz et al. 1970, Miller and Miller 1978, Murray 1984, Bouck and Ngo 1996, Leander et al. 2007). In addition to strips and microtubules, the pellicle incorporates a peripheral network of endoplasmic reticulum and in some cases is associated with muciferous bodies that empty through surface pores formed in strips (Fig. 1.2b-c; Arnott and Walne 1967, Leander and Farmer 2000a, Leander et al. 2001b, 2007).

While taxonomic variations in the number and arrangement of microtubules have been reported, the complex ultrastructure of the proteinaceous strips has been more successfully exploited as a source of phylogenetically informative characters (Leedale 1964, 1967, Leander and Farmer 2001a, b, Leander et al. 2001a, b, Kusel-Fetzmann and Weidinger 2008). Using transmission electron microscopy (TEM), researchers have found that strips vary in thickness and shape in transverse section (Dragos et al. 1997, Leander and Farmer 2001a). A strip's ultrastructure can vary in terms of the shape and width of its arch, the portion of the strip visible on the cell surface; its overhang, heel and hook, which together form the "articulation zone" between adjacent strips; and its keel, which is raised in some species (Fig. 1.2a; Leander and

Farmer 2001a, Leander et al. 2001a). Strips may also possess lateral projections, extensions of the pellicle strip that lie beneath the arch of the strip (postarticular projections) or the overhang of the adjacent strip (prearticular projections) (Figure 1.2c-g; Leedale 1964, Mikolajczyk 1975, Dragos et al. 1997, Leander 2004, Leander and Farmer 2001a, b, Leander et al. 2001b, 2007). They are absent in phagotrophs and primary osmotrophs (Fig. 1.2b), thread-like or comb-like in plastic phototrophs belonging to early-branching lineages (e.g., *Eutreptia* and *Euglena*; Fig. 1.2c-d), and tooth- or plate-like in rigid or semi-rigid phototrophs such as *Lepocinclis* and *Phacus* (Fig. 1.2e-g; Leander 2004, Leander et al. 2001a, b, 2007). Some authors postulate that the relative size and shape of strip projections is correlated with degree of cell plasticity (also referred to as euglenoid movement or "metaboly", a process that occurs via sliding between adjacent strips; Suzaki and Williamson 1985, 1986a, b), since their absence in eukaryovorous phagotrophs coincides with high pellicle plasticity while rigid phototrophs tend to have very robust projections (Dragos et al. 1997, Leander 2004, Leander and Farmer 2001b, Leander et al. 2001b). On the other hand, lateral strip projections are highly reduced in *Monomorphina*, a phototrophic genus with rigid cells, and robust in plastic taxa such as *Euglena ehrenbergii*; these data imply that cell rigidity is not solely influenced by the ultrastructure of lateral strip projections (Mikolajczyk 1975, Nudelman et al. 2006).

Examination of the cell surface using scanning electron microscopy (SEM) has also provided informative characters. The number of pellicle strips per cell is consistent within taxa but is highly variable between taxa, ranging from four to 120 strips (Leander et al. 2007, Chapter 3). Like the ultrastructure of strip projections, strip number (a variable designated as *P*; Leander and Farmer 2000a) seems to be correlated in part with metaboly. Bacterivores are more or less rigid and exhibit smaller *P* values (from four to twelve strips), while eukaryovores, which require a more flexible pellicle to ingest relatively large prey, have approximately 20 to 60 strips (Leander 2004; Leander et al. 2001a, b, 2007). The loss of phagotrophy, which probably reduced feeding behavior-based selective pressure on *P* values, resulted in multiple reductions of strip number: primary osmotrophs have 14-20 pellicle strips; the photosynthetic genera *Cryptoglena* and *Monomorphina* have 15 and 16 strips, respectively, and members of *Phacus* have 20 to 32 strips

(Leander and Farmer 2001b, 2000a, Leander et al. 2007, Chapter 4). Interestingly, the largest *P* values are also observed in phototrophic taxa, one of which (*Lepocinclis helicoideus*, *P* = 80) is only slightly plastic (Leander and Farmer 2001a, 2000b, Leander et al. 2007, Chapter 3).

Pellicle pores are visible on the cell surface in the eukaryovore *Peranema trichophorum* and in plastic phototrophs such as *Eutreptia* and *Euglena* (Leander and Farmer 2000a, Leander et al. 2001b). In many phototrophs, they occur in the heel region of specific strips and form discrete rows of pores on the cell surface (Arnott and Walne 1967, Leander and Farmer 2000a). The number of "unmarked" strips between pore-bearing strips varies between taxa: in *Euglena cantabrica*, for example, every other strip has pores, while every eighth strip possesses pores in *E. geniculata* (identified as *myxocylindracea*; Leander and Farmer 2000a, Zakrys et al. 2002). It has been proposed that the frequency of pore-bearing strips has increased over time (i.e. that eight strips between rows of pores is a relatively primitive state; Leander et al. 2001b) and that pellicle pores may indicate relative strip maturity (Leander and Farmer 2000a). How pores are related to strip maturity (and how this relationship will affect the interpretation of character state polarity regarding patterns of pellicle pores) remains to be described (Leander and Farmer 2000a, Chapter 3).

1.5 Patterns of pellicle strip reduction

A particularly intriguing pellicle surface character results when strips appear to abruptly end or join with their neighboring strips (Fig. 1.3). These discontinuities form patterns (a "whorl" or "vortex") at both the posterior and anterior ends of the cell and have been alternately explained as "bifurcations", "fusions", "terminations," or "undertucking" of adjacent strips (Groupé 1947, Guttman and Ziegler 1974, James 1963, Pitelka 1963, Kirk and Juniper 1964, Leedale 1964, Leander and Farmer 2000a).

These patterns were first visualized using light microscopy and silver staining methods (Jírovec 1929, Foissner 1977), but electron microscopy more readily allowed researchers to integrate accumulating descriptions of bifurcation or reduction patterns with other pellicle characters. Mathematical relationships between pellicle strip numbers at different points along the

length of the cell were identified - Bourrelly et al. (1976) observed that the number of pellicle strips around the cell periphery in *Euglena oxyuris* var. *minor* was twice that observed within the canal, after strip terminations at the anterior end. In their description of the doubling and subsequent halving (reduction) of strips at, respectively, the anterior and posterior ends of *Lepocinclis salina*, Conforti and Tell (1983) noted the similarity of these patterns to those observed in other euglenids, namely *E. oxyuris* var. *minor* (Bourrelly et al. 1976), *E. gracilis* (Rosowski 1977) and *Colacium mucronatum* (Rosowski and Willey 1977). They provided further evidence of strip fusion or reduction at the cell anterior and posterior in other species of *Euglena*, *Lepocinclis*, and *Phacus* (Conforti and Tell 1989). Dawson and Walne (1991) described strip reduction at the posterior end of *Eutreptia pertyi*, and Angeler (2000) showed that pairs of strips fused at both ends of the cell in the secondary osmotroph *Khawkinea pertyi*. Interestingly, Angeler et al. (1999) stated specifically that the primary osmotroph *Distigma* lacked any fused or terminating strips, maintaining a constant number of strips over the entire cell length.

High quality SEMs showed that strips did not fuse together (Guttman and Ziegler 1974, Leander and Farmer 2000a, b, 2001b, Leander et al. 2001b). Leander and colleagues placed patterns of strip reduction in a robust comparative and evolutionary framework (Leander and Farmer 2001b, 2000a, b, Leander et al. 2001a, b). Strip reduction is lacking or disorganized in phagotrophic and primary osmotrophic taxa (Leander and Farmer 2000a, Leander et al. 2001a, b). In phototrophic taxa, however, patterns are clearer and can be described mathematically (Leander and Farmer 2000a). Terminating strips were found to alternate with continuous strips to form radial patterns (termed “whorls” by Leander and Farmer, 2000a) of strip reduction at both the anterior end of the cell, around the canal opening, and at the posterior end of the cell, around the posterior tip (Fig 1.3b-c). In many taxa, continuous strips at the posterior end of the cell (and, infrequently, at the anterior end of the cell; Leander and Farmer 2000a, Brosnan et al. 2005) terminate to form another, more posterior whorl (or, in the case of anterior reduction, a more anterior whorl), where every other “continuous” strip terminates (Fig. 1.3d). The number of posterior whorls of reduction is consistent within taxa and can range from one (in *Eutreptia pertyi* and *Euglena cantabrica*; Leander et al. 2001b) to four (in *Euglena rustica*; Leander and Farmer

2000a, Brown et al. 2002). Because the number of pellicle strips surrounding the periphery of the cell reduces by half (i.e., exponentially) at each whorl, this form of reduction is described as "exponential reduction" (Leander and Farmer 2000a).

Posterior whorled reduction has been modified in some phototrophic lineages to form even more complex patterns. In members of the Eutreptiales (Fig. 1.3e-f), every other terminating strip within the single whorl of posterior reduction is longer than the adjacent terminating strip, effectively forming two whorls within a whorl. A comparable pattern occurs in *Euglena mutabilis*, where the first (that is, the most anterior) of two whorls is similarly divided. The number of terminating strips within each of these "whorls," and the second whorl, is equal, yielding a pattern of posterior reduction that can be described as linear (Leander and Farmer 2000a). *Lepocinclis helicoideus* has three whorls, the first and second of which are each divided into two whorls as in *E. mutabilis*. The number of terminating strips is constant over the first two "whorls" and then reduces by half and remains constant over the next two whorls, producing a "bilinear" pattern of posterior reduction (Leander and Farmer 2000b).

The three species of *Phacus* sensu stricto whose reduction patterns have been described exhibit further complexity (Leander and Farmer 2001b). *Phacus triqueter* has three misshapen whorls of exponential reduction, while *P. oscillans* has one whorl of exponential reduction and an additional terminating strip. *Phacus acuminatus* (*brachykentron*) has one elliptical whorl of posterior reduction in addition to two lateral clusters of terminating strips, with four strips in each cluster. Such a limited taxon sample makes it difficult to determine the evolutionary origin of these novel patterns.

Whorls of reduction have proven useful as a character in phylogenetic analyses and, in some cases, taxonomic delimitation (Leander et al. 2001b, Brosnan et al. 2005). Their adaptive significance, however, is unresolved. Leander et al. (2001b) suggested that posterior reduction might be correlated with posterior cell tip morphology: in a cell with a sharp tip, the tip should have fewer strips and therefore require more whorls of reduction than a cell with the same *P* value, but a blunter posterior tip (the "optimal packing hypothesis"). Examination of pellicle data,

however, indicated that there was no such correlation between tip morphology and the number of posterior whorls of reduction (Leander et al. 2001b).

It has been suggested (S. Bowser, Wadsworth Center, personal communication) that strip reduction patterns may play a role in euglenoid movement. The currently accepted model of metaboly requires sliding between adjacent pellicle strips, which allows strips to avoid deformation throughout most of the cell length – strips, however, are fixed at the cell anterior and posterior (Suzaki and Williamson 1985, 1986a). When paper models with twelve strips were created from a computer simulation of this model, some strips were required to bend, particularly at each end, to accommodate relative sliding (Suzaki and Williamson 1986b). These models were produced with strips of equal lengths, and no comparison between cells with different numbers of strips was made. It is possible that reducing the number of strips at the posterior end of the cell somehow reduces deformation or mechanical stress at strip ends, but no theoretical or experimental data have been presented to answer this question. It seems more likely at this point that reduction patterns are adaptively neutral, perhaps influenced by a related, but currently unknown, character or cellular process (Leander et al. 2007). Since developmental data have the potential to yield significant insight into character evolution (Mabee 1999), an understanding of the development of these patterns in the context of pellicle morphogenesis may provide clues as to their evolutionary significance (Chapter 2).

1.6 Pellicle duplication and cell division

No compelling evidence for sexual reproduction in euglenids has been presented. Cells reproduce asexually through mitosis and longitudinal cell division, facilitating the establishment of clonal cultures. Prior to cytokinesis, the pellicle is duplicated via semiconservative or intussusceptive growth – a new strip grows in the articulation zone between two mature strips, doubling the number of strips covering the cell. Subsequent to mitosis, flagellar replication and the division of the anterior canal, the cell divides. The cleavage furrow forms between two pairs of strips, located on opposite sides of the anterior canal, so that the number of strips is halved again; as one strip from each strip pair rearticulates with one strip from the opposite pair, each

daughter cell forms with the same number of strips as the mother cell (Hofmann and Bouck 1976, Bouck and Ngo 1996).

Semiconservative pellicle strip replication was inferred based on light microscopal observations (Pochmann 1953, Leedale 1967, Hofmann and Bouck 1976), but little was known about the process until electron microscopy was used to observe dividing cells. These studies (e.g., Sommer and Blum 1964,1965, Hofmann and Bouck 1976, Bré and Lefort-Tran 1978 Lefort-Tran et al. 1980, Mignot et al. 1987, Bouck and Ngo 1996) revealed important details of the complex process of pellicle duplication and its coordination with the cell cycle. Strip growth begins at the anterior of the cell near the canal opening where each nascent strip is first visible as a small protrusion between two mature strips (Sommer and Blum 1965, Hofmann and Bouck 1976, Gillott and Triemer 1978, Mignot et al. 1987, Dubreuil et al. 1992). New strips gradually grow in width and length until they assume a similar appearance to mature strips; at intermediate stages in growth (e.g., immediately after cytokinesis; Mignot et al. 1987), or subsequent to vitamin B_{12} deprivation (Bré and Lefort-Tran 1978, Lefort-Tran et al. 1980), however, nascent strips may be narrower than their mature neighbors (Leedale 1967).

An important evolutionary implication of this complex process was identified when it was proposed that the various strip numbers or *P* values observed in euglenids are the result of irregularities in strip replication and segregation (Leander 2004, Leander et al. 2001a,b, 2007). It was suggested that the evolution of eukaryovory was dependent on “strip doubling events” where a cell with a newly replicated pellicle failed to divide, resulting in a cell with twice as many strips as before (Leander et al. 2001b). If an increase in strip number is correlated with cell plasticity as described above, such an event would have resulted in a lineage of cells with the improved ability to engulf larger prey (Leander 2004; Leander et al. 2001a, 2007). The *P* values described for bacterivores, eukaryovores, and early-diverging phototrophs indicate that such an event took place at least twice: a bacterivore with 10 strips gave rise to a eukaryovore with 20 strips, which in turn gave rise to a cell with 40 strips or more, and this eukaryovore with 40 strips would have been capable of engulfing the green alga that gave rise to the secondary chloroplasts of euglenophytes (Fig. 1.4; Leander 2004, Leander et al. 2001a, 2007). It has also been

hypothesized that an ancient variation of the highly regulated process of pellicle duplication may have been instrumental in the initial evolution of multiple pellicle strips from a single, continuous layer of articulins or related proteins that reinforced the cytoskeleton of the ancestral euglenid (Leander et al. 2007). A related process resulting in “strip halving events” – where a cell divides without initially replicating its pellicle – is a possible mechanism for producing taxa with fewer strips than their ancestors. Smaller differences in strip number between certain related taxa, on the other hand, indicate that uneven segregation of strips through misplacement of the cleavage furrow, rather than a cell’s failure either to replicate the pellicle or to divide, has also been a common occurrence throughout pellicle evolution (Fig. 1.4; Leander et al. 2001b, 2007).

Mignot et al. (1987) suggested that a “morphogenetic centre”, visible in micrographs as an electron dense region surrounding two of the pellicle microtubules, was associated with the heel of each pellicle strip. These authors inferred that this centre was integral to the development of the adjacent nascent strip, perhaps through microtubule-associated transport. The semiconservative replication of the pellicle, accomplished through the precise placement of nascent strips, is obviously a highly coordinated and regulated process (Hofmann and Bouck 1976, Mignot et al. 1987). The mechanisms of this regulation, however, remain unclear, because our knowledge of pellicle development is restricted to relatively few descriptive microscopical studies. Nothing is known about the underlying genetics of pellicle morphogenesis, and descriptions of the cellular processes that produce the diversity of strip ultrastructure summarized above are extremely limited (Leedale 1967, Leander et al. 2007).

1.7 Thesis Goals and Scope

This thesis attempts to describe and synthesize comparative and developmental morphological data in order to better understand the complexities of pellicle evolution. Its focus is on the description of characters associated with pellicle surface patterns (particularly strip reduction), the taxonomic distribution of these characters, and the role that developmental processes have played in their evolutionary history. Chapter 2, “A model for the morphogenesis of strip reduction patterns in phototrophic euglenids: evidence for heterochrony in pellicle

evolution", synthesizes previous work on pellicle development and morphological evolution with novel SEM data of dividing *Euglena gracilis* cells to provide a developmental framework for the evolution of posterior whorls of strip reduction. Chapter 3, "Novel pellicle surface patterns on *Euglena obtusa* Schmitz (Euglenophyta), a euglenophyte from a benthic marine environment: Implications for pellicle development and evolution" uses this developmental framework to describe a novel and particularly complex pattern of whorled strip reduction in a phototroph with a large number (120) of pellicle strips. Chapter 4, "Evolution of distorted pellicle patterns in rigid photosynthetic euglenids (*Phacus* Dujardin)" discusses the comparative morphology and evolution of the novel patterns of strip reduction observed in the genus *Phacus* in the context of intrageneric relationships as inferred from a ribosomal DNA phylogeny. Chapter 5, "Visualizing the complex substructure of euglenid pellicle strips with SEM", departs from the focus on surface patterns and developmental processes. It describes a novel character state in prearticular strip projections, observed using scanning electron microscopy (SEM) rather than transmission electron microscopy (TEM) which has been used extensively and exclusively for describing this character in euglenids.

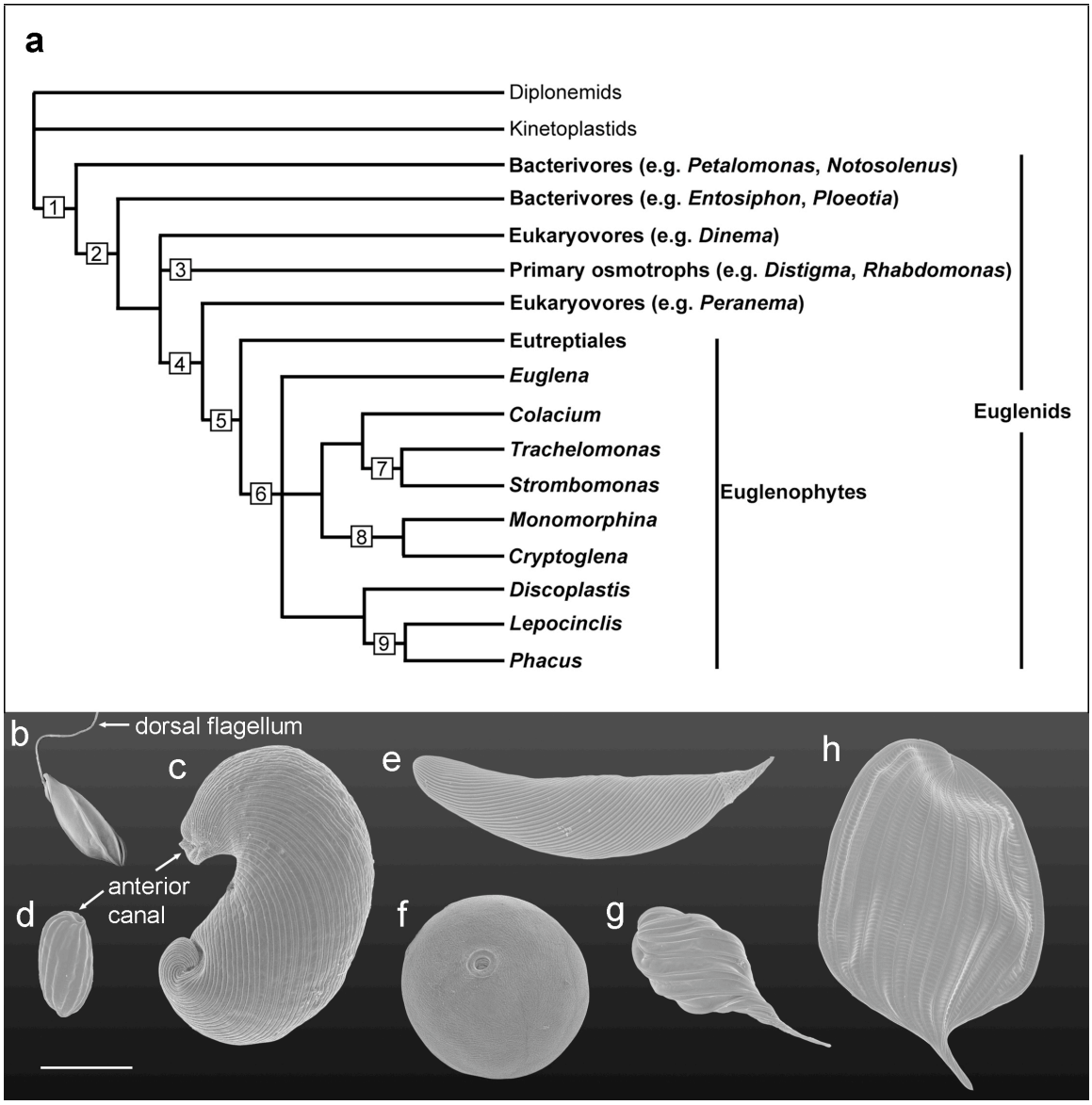

Figure 1.1. Euglenid diversity. (a) A summary of euglenid relationships and major evolutionary transitions based on previously published phylogenies (e.g., Leander et al. 2007). Euglenid taxa are shown in bold. 1. Acquisition of the pellicle or its precursor. 2. Acquisition of "rod-and-vane" feeding apparatus. 3. Reduction of feeding apparatus and loss of phagotrophy. 4. Strip doubling event from approximately 20 to 40 strips. 5. Secondary endosymbiosis of a green alga. 6. Reduction of one anterior flagellum. 7. Acquisition of mucilaginous lorica. 8-9. Independent secondary losses of cell plasticity. (b-h) Scanning electron micrographs (SEMs) of representative euglenids. (b) *Petalomonas cantuscygny*, a bacterivore. Previously published in Leander et al. 2007. (c) *Peranema* sp., a eukaryovore. (d) *Rhabdomonas* sp., a primary osmotroph. (e) *Euglena* sp., a plastic phototroph. (f) Lorica of *Trachelomonas* sp., a loricate phototroph. (g) *Monomorphina pyrum*, a rigid phototroph. (h) *Phacus* sp., a rigid phototroph. Scale bar, 10 μm.

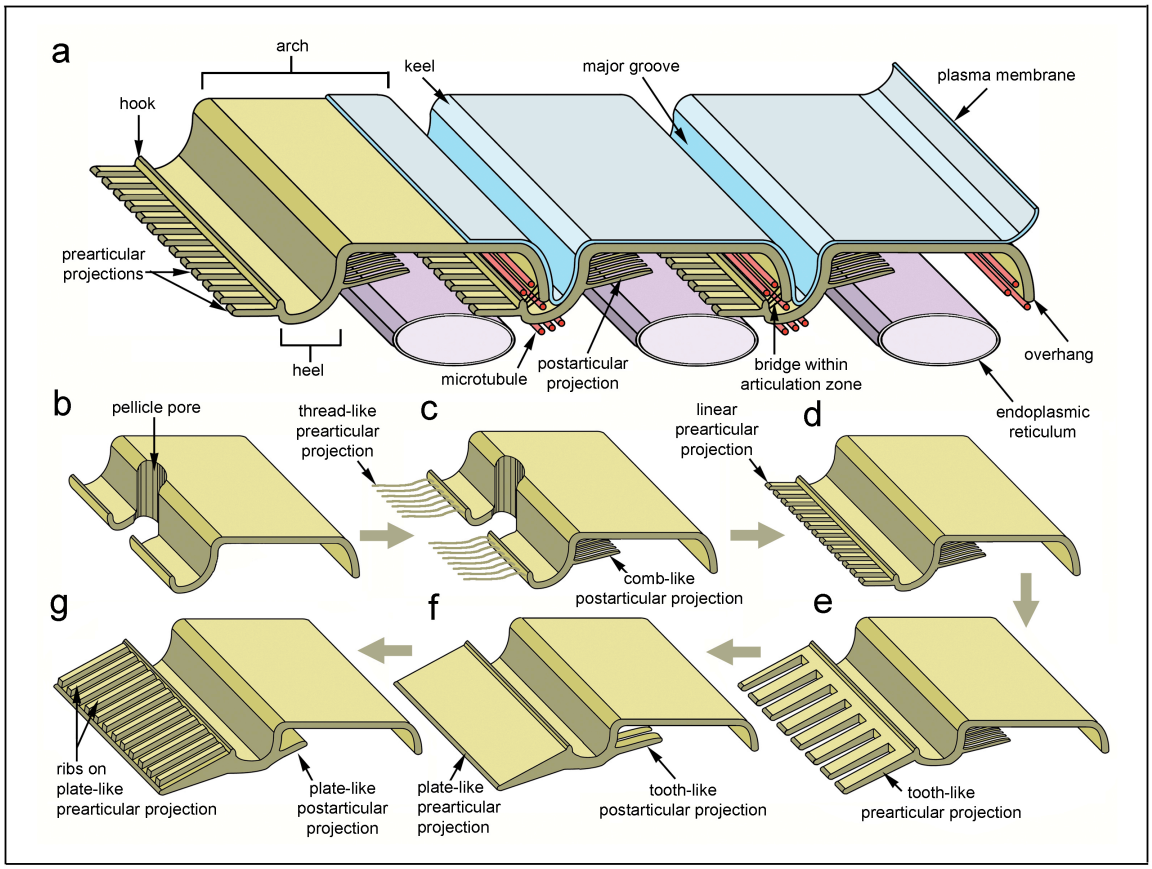

Figure 1.2. Pellicle ultrastructure. (a) A drawing of the complex ultrastructure of the pellicle, including proteinaceous strips, microtubules, endoplasmic reticulum and plasma membrane. Details of strip architecture include the strip arch, lateral strip projections, heel, hook, keel, overhang, and major groove, visible from the cell surface. (b-e) Transitions of character states in strip ultrastructure. (b) A strip typical of eukaryovorous euglenids, lacking lateral projections and possessing a pore through the heel region. (c-d) Strips similar to those found in many *Euglena* species, with pellicle pores and delicate strip projections. (e) *Phacus*- and *Lepocinclis*-like strips with more robust tooth-like prearticular projections. (f-g) Plate-like projections are found in several species of *Lepocinclis*. Plate-like prearticular projections may possess or lack ribs or ridges on their upper surface. Figure modified from Leander et al. 2007, © Wiley Periodicals, Inc.

Figure 1.3 (Next page). Patterns of strip reduction in phototrophic euglenids. (a) The posterior end of *Euglena obtusa*, showing multiple strip terminations (asterisks). Scale bar, 5 μm. (b) Strip reduction surrounding the anterior canal in *Euglena sanguinea*. There are approximately 25 reducing or terminating strips (asterisks) out of 51 strips surround the periphery of the cell. Scale bar, 2 μm. (c) The posterior end of *Euglena viridis* (*P* = 48), showing one whorl of posterior reduction formed by 24 alternately terminating strips, colored blue. Scale bar, 2 μm. (d) The posterior end of *Lepocinclis* sp. (*P* = 32) with two whorls of posterior reduction. Whorl I is formed by 16 alternately terminating strips, colored green. Whorl II is formed by the termination of every other strip passing through whorl I (8 of 16 strips), colored blue. Scale bar, 2 μm. (e-f) *Eutreptiella* sp. (*P* = 24) possesses one whorl of posterior reduction formed by twelve alternately terminating strips of two lengths (blue): every other terminating strip is longer than the terminating strip immediately adjacent to it. Scale bars, 4 μm. (e) When every consecutive terminating strip is connected, one misshapen whorl consisting of twelve terminating strips is produced. (f) When every other terminating strip is connected, two subwhorls (i.e. “whorls within a whorl”; see Chapter 3), each with six terminating strips, become apparent.

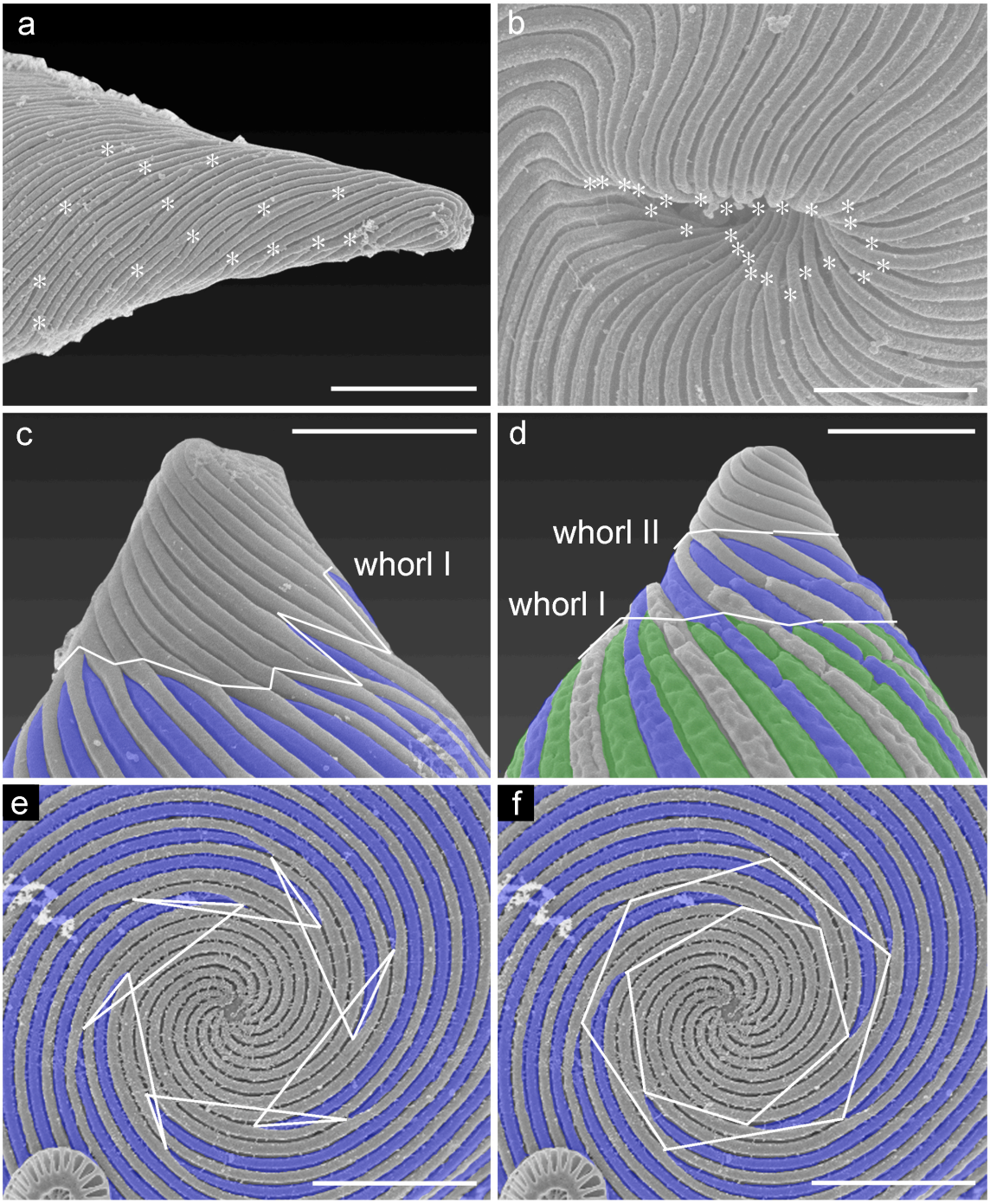

Figure 1.3

Figure 1.4 (Next page). Changes in *P*, the number of pellicle strips, due to modifications in strip duplication and cytokinesis. Subsequent to the acquisition of four pellicle strips in the bacterivores, pellicle duplication coupled with failure of the cell to divide ("strip doubling") led to a cell with eight strips. Cells with ten strips likely resulted from unequal distribution of duplicated strips during subsequent cytokinetic events. Strip doubling events occurred again at least twice, the first resulting in eukaryovorous cells with 20 strips, the second in plastic eukaryovores with 40 strips. A eukaryovorous cell similar to *Peranema* underwent a secondary endosymbiotic event with a green algal prey cell, giving rise to the first phototrophic euglenids, which had a similar number of strips. Unequal distribution of strips during cell division likely gave rise to the remaining diversity of strip numbers in euglenids. Bacterivores with ten strips gave rise to cells with 12 strips, while eukaryovores with 20 strips gave rise to the primary osmotrophs, whose strip numbers range from 14 to 20. Eukaryovores with 56 strips arose from cells with 40 strips. Different phototrophic lineages underwent multiple irregular pellicle duplications and divisions to produce cells with strip numbers ranging from 15 to 120. Figure modified from Leander et al. 2007, © Wiley Periodicals, Inc.

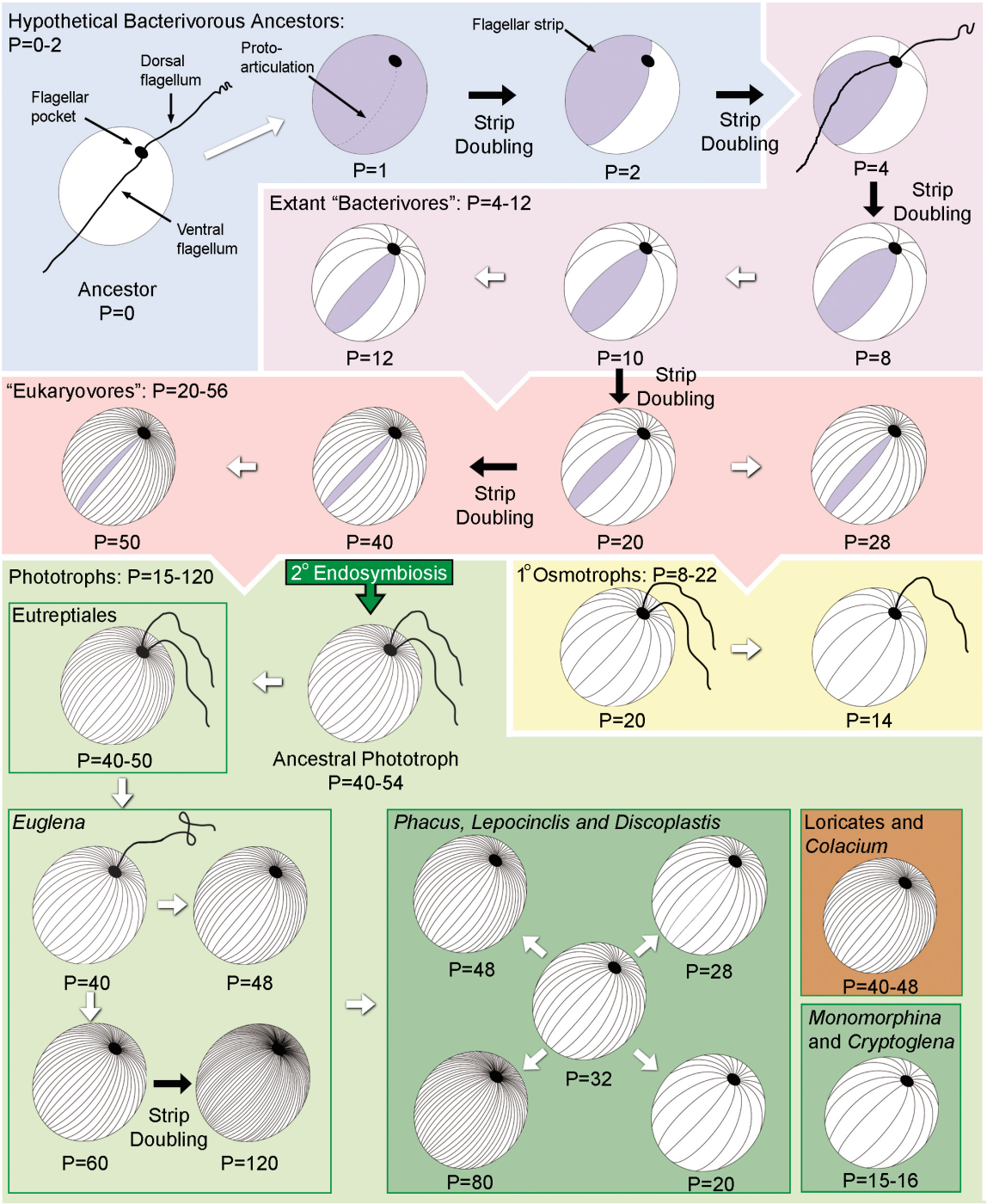
Hypothetical Bacterivorous Ancestors:
P=0-2
Dorsal flagellum
Proto-articulation
Flagellar pocket
Ventral flagellum
Ancestor
P=0
P=1
Strip Doubling
Flagellar strip
P=2
Strip Doubling
P=4
Strip Doubling
Extant "Bacterivores": P=4-12
P=12
P=10
P=8
Strip Doubling
"Eukaryovores": P=20-56
P=50
P=40
Strip Doubling
P=20
P=28
Phototrophs: P=15-120
2° Endosymbiosis
1° Osmotrophs: P=8-22
P=20
P=14
Eutreptiales
P=40-50
Ancestral Phototroph
P=40-54
Euglena
P=40
P=48
Strip Doubling
P=60
P=120
Phacus, Lepocinclis and Discoplastis
P=48
P=28
P=32
P=80
P=20
Loricates and Colacium
P=40-48
Monomorphina and Cryptoglena
P=15-16

Fig. 1.4

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Chapter 2: A MODEL FOR THE MORPHOGENESIS OF STRIP REDUCTION PATTERNS IN PHOTOTROPHIC EUGLENIDS: EVIDENCE FOR HETEROCHRONY IN PELLICLE EVOLUTION*

2.1 Introduction

Euglenids are predominantly free-living biflagellate eukaryotes that play important roles as consumers or producers in both marine and freshwater habitats: some feed on bacteria or other eukaryotes (phagotrophs), some absorb nutrients from the surrounding environment (osmotrophs), and others are capable of photosynthesis (phototrophs). Euglenids are closely related to kinetoplastids, together forming the two major subgroups within a larger clade of eukaryotes called the Euglenozoa. Kinetoplastids contain several lineages of small phagotrophs and important vertebrate parasites such as *Trypanosoma* spp., which can cause sleeping sickness and Chagas' disease. Photosynthesis within the Euglenozoa is limited to one subclade of euglenids that resulted from a secondary endosymbiotic event wherein a green alga was engulfed by an ancestral euglenid and its plastid retained by the predator (Gibbs 1978). Subsequently, the phototrophs underwent extensive diversification and now exhibit a wide variety of ultrastructural characters that appear to be the direct or indirect result of plastid acquisition (Leander 2004). In particular, phototrophs have acquired a light sensing complex comprised of a carotenoid-rich eyespot and a paraflagellar body, have undergone extensive reduction of the complex feeding apparatus that is present in phagotrophic relatives and have been subject to major changes in the structure of their cytoskeleton, or pellicle.

The pellicle is composed of the plasma membrane, protein strips, subtending microtubules, and endoplasmic reticulum. The laterally articulating pellicle strips run longitudinally or helically from the anterior canal region to the posterior end of the cell and create a striated pattern on the cell surface (Fig. 2.1) The proteinaceous strips are of primary interest as their structure, number, and orientation have profound effects on the size, locomotion, and feeding

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habits of euglenids: for example, species that feed on other eukaryotes tend to have more numerous, helically arranged strips. This condition is thought to facilitate sliding between strips (Suzaki and Williamson 1985) and associated rapid changes in cell shape (metaboly) that are advantageous for engulfing large prey (Leander 2004). More derived lineages of phototrophs, on the other hand, have fewer, longitudinally arranged strips with complex lateral projections underneath the cell surface (Leander 2004). These features are conserved within species and varied enough to provide useful characters for taxonomic identification and phylogenetic inference (e.g., Leander et al. 2001, Brosnan et al. 2005).

In some euglenid species, pellicle strips terminate before reaching the posterior end of the cell. In phototrophic euglenids and osmotrophic taxa whose ancestors were phototrophs (''secondary osmotrophs'' have lost photosynthesis), these terminations may form ''whorled'' patterns of reduction, so-called because the terminating strips form a circular pattern on the cell surface, and the total number of pellicle strips around the cell periphery is reduced at the point of strip termination. Whorls of reduction have not been observed in either phagotrophic euglenids or primary osmotrophs (osmotrophic taxa that diverged before the acquisition of secondary plastids; Angeler et al. 1999, Leander and Farmer 2000a). The most common form of whorled reduction is produced when every other strip at a particular point along the length of the cell terminates. This pattern is referred to as "exponential" reduction because at each whorl, the number of strips around the cell periphery is halved. Different species of phototrophs may possess one (W_p = 1), two (W_p = 2), three (W_p = 3), or four (W_p = 4) exponential whorls of reduction at the posterior end of the cell (Leander and Farmer 2000a).

In some phototrophs, an exponential whorl may be staggered; that is, every other terminating strip in the whorl is displaced from its neighbors. If this staggering is sufficiently pronounced, it effectively forms two separate whorls, followed by a third posterior exponential whorl (Leander and Farmer 2000a, Leander et al. 2001). After each whorl, the total number of strips is again reduced, but reduction follows a linear rather than an exponential function. When two exponential whorls are staggered in this manner and followed by a third exponential whorl, a

“bi-linear” pattern of five whorls is produced, and strip reduction follows one linear function over two whorls, then another linear function over the next three whorls (Leander and Farmer 2000b).

Sexual reproduction is unknown in euglenids. All reproduction is accomplished through asexual mitotic division. Before cell division, euglenids replicate the pellicle by doubling the number of strips (a value defined as “*P*”; Leander and Farmer 2000a; Fig. 2.1, a and b). Nascent strips form within the articulation zones between adjacent mother strips near the canal opening (the anterior end of the cell; Sommer and Blum 1965, Hofmann and Bouck 1976, Gillott and Triemer 1978, Dubreuil and Bouck 1985, Mignot et al. 1987, Bouck and Ngo 1996, Vismara et al. 2000). These nascent strips grow towards the posterior end as cell division progresses, and cytokinesis begins before nascent strips are fully grown. Two adjacent strips on each side of the canal are separated from one another and each is rejoined to one of the strips on the opposite side, forming a longitudinal cleavage furrow (Bouck and Ngo 1996; Fig. 2.1c-d). In this way, the number of strips can be evenly divided between the two developing daughter cells. When symmetrical cell division is complete, each daughter cell possesses the same number of strips as the mother cell. Minor variations in the number of strips in different individuals within a taxon indicate that asymmetrical division also occurs, whereby strips are unevenly distributed between daughter cells (Leander and Farmer 2000a, 2001, Leander et al. 2001). However, the processes by which posterior strip reduction develops and is inherited from generation to generation are unclear and have never been articulated (Leander et al. 2001).

In order to understand the origin, development and inheritance of strip reduction, we observed dividing cells belonging to several phototrophic euglenids using scanning electron microscopy (SEM), namely *Euglena gracilis*, *E. viridis*, *Phacus similis*, and *P. segretii*. Our work concentrated on dividing cells of *E. gracilis*, a taxon with three exponential whorls of posterior strip reduction. We were able to determine the fate of the exponential whorl formed by nascent strips, the sequence of whorl morphogenesis and the fates of the whorls already present in the mother cell. We have synthesized these data and propose a model of whorl morphogenesis that explains not only the inheritance of consistent strip reduction patterns, but also provides evidence that heterochrony has played a major role in the evolution of the euglenid cytoskeleton.

2.2 Materials and Methods

The following cultures were used in this investigation: *E. gracilis*, *E. viridis* (SAG 1223-17d), *P. segretii* (ACOI 1337), and *P. similis* (SAG 58.81). *E. viridis* and *P. similis* were purchased from Sammlung von Algenkulturen Göttingen (SAG); *P. segretii* was purchased from the Coimbra Collection of Algae (ACOI); *E. gracilis* was obtained from the Biology Program at the University of British Columbia. *E. viridis* and *P. similis* were grown in modified MES-volvox medium (Provasoli and Pintner 1959); *P. segretii* was grown in LM_7 medium (http://www.uc.pt/botanica/ACOI_M~1.htm). *E. gracilis* cells were grown in a standard *Chlamydomonas* medium (recipe available upon request) and exposed to a 12 h light/12 h dark cycle at 18˚ and 17˚C, respectively. *E. gracilis* cells were harvested for SEM 4.5 h after commencement of the dark cycle. All cells were fixed with osmium tetroxide according to Leander and Farmer (2000a), placed on Millipore filters and dehydrated with a series of increasing ethanol concentrations. Cells were critical point dried with CO_2, mounted on aluminum stubs and sputter coated with a mixture of gold and palladium. Samples were viewed using a Hitachi S4700 scanning electron microscope. One dividing cell each of *P. similis*, *P. segretii*, and *E. viridis*, and 42 cells of *E. gracilis* in various stages of cell division were observed in order to help substantiate our model.

2.3 Results and Discussion

2.3.1 Whorl I is formed by nascent strips

The developing, nascent strips in dividing *P. similis* alternate between mature strips (Fig. 2.2a), forming a whorl of exponential reduction anterior to the terminating strips present in the mother cell. During interphase in *P. segretii* (W_p = 2), strips that terminate at the anterior canal, forming an anterior whorl of reduction, also terminate before reaching the posterior cell tip and form the first posterior whorl of strip reduction (Fig. 2.2b). These strips are inferred to have been formed during the last round of cell division, which is consistent with reports that nascent strips

form alternating ‘‘minor’’ strips within the canal in the secondary osmotroph *Cyclidiopsis acus* (Mignot et al. 1987). In other words, the strips that terminate before entering the canal in phototrophic euglenids (and secondary osmotrophs) are inferred to be the developmental equivalents to the minor strips identified by Mignot et al. (1987) in *C. acus*, and thus are more recently formed than the neighboring strips that do enter the canal. During late cytokinesis in *E. viridis*, the anterior-most exponential whorl of reduction is visible near the point where the cells are joined at their posterior tips (Fig. 2.2c). The strips forming this whorl of reduction are narrower than several of their neighbors, indicating that they are younger and have been produced immediately before cell division. The widest strips, on the other hand, all extend to where the cells are joined at their posterior tips.

2.3.2 Formation of the cleavage furrow and the relative positions of furrow strips

Cell cleavage occurred along the articulation zone between pellicle strips on two opposite sides of the canal. On each side, two strips (one inferred from anterior termination to be nascent, and one mature), designated as ‘‘furrow strips,’’ separated along their lateral articulation zone beginning at the anterior end and continuing towards the posterior end. We designated the nascent strips as ‘‘n’’ and ‘‘n´,’’ and the mature strips as ‘‘m’’ and ‘‘m´,’’ such that m was adjacent to n and m´ was adjacent to n´ before the separation of furrow strips (Fig. 2.3, a, b, and d). As this separation progressed, each nascent furrow strip rearticulated with one of the mature furrow strips from the opposite side of the cell. As in the separation process, the rearticulation process necessarily progressed from the anterior end of the cell toward the posterior end. In specific terms, the nascent furrow strips, n and n´, articulated with the mature strips on the opposite side of the cell such that n articulated with m´ and n´ articulated with m (Fig. 2.3).

As the opposite pairs of strips separated and then rearticulated (Fig. 2.3), a cleavage furrow progressed from the anterior end to the posterior end of the cell, gradually forming two daughter cells. The rate of cleavage furrow formation actually exceeded that of nascent strip growth (Fig. 2.3c), which required the separation of two adjacent mature strips along their lateral

articulation zone near the posterior end of the cell. Upon the completion of cytokinesis, however, one daughter cell inherited strips n and m´ whereas the other daughter cell inherited strips n´ and m (Fig. 2.3d).This process of inheritance maintained the alternating pattern of nascent and mature strips.

In their study of cell division in the secondary osmotroph *C. acus*, Mignot et al. (1987) observed that nascent strips arose from a ‘‘morphogenetic center’’ in the articulation zone between the ‘‘overhang’’ of one strip and the ‘‘hook’’ of another (for definitions of ultrastructural terms, see Leander and Farmer 2001a). In this context, we can deduce that the nascent strips, such as n and n´, develop beneath the overhangs of adjacent mature strips, such as m and m´, respectively. It is also known that some of the microtubules underlying the nascent furrow strips are inherited from their respective mature furrow strips (Mignot et al. 1987). This developmental linkage and relative positioning of nascent furrow strips to mature furrow strips ensures that rearticulation occurs between a nascent and a mature strip in each daughter cell, which maintains consistent patterns of posterior strip reduction from one generation to the next.

Moreover, this consistency relies on the cleavage furrow developing between a mature strip and the nascent strip that developed from it; it is helpful to point out that the nascent strips are always on the right hand side of the mature strips from which they developed when viewed from the anterior end. The following hypothetical exercise reinforces the importance of the position of the mature furrow strips relative to that of the nascent ones in transferring a consistent pattern of strips from mother cell to daughter cells. If for example (1) the position of n´ was reversed and located to the left of m´ when viewed from the anterior end, (2) n remained to the right of m and (3) n´ and m´ were separated along their lateral articulation zones, then rearticulation would occur between n and n´ and m and m´. This would cause adjacent nascent strips n and n´ to terminate in whorl I in one daughter cell and adjacent mature strips m and m´ to continue past whorl I in the other daughter cell. This outcome would be inconsistent with the exponential and linear patterns of strip reduction observed in phototrophic euglenids so far.

The relative ages of the furrow strips may be important in determining the initial location of strip separation and the formation of the cleavage furrow. Unfortunately, with the SEM methods

described here, the relative ages of m and m´ are indeterminable without observing the posterior whorls of the cell and the fates of furrow strips simultaneously. In cells where the posterior whorls were visible, the cleavage furrow was not, which prevented us from identifying the furrow strips. Because strip age plays an important role in pellicle strip development, as evidenced by differences between canal strips (Mignot et al. 1987) and the morphogenesis of posterior patterns of reduction described here, determining the relative age of furrow strips might provide important further insight into pellicle morphogenetic processes and the evolutionary history of pellicle characters. An antibody labeling experiment using different sized latex beads, as an extension of the experiments conducted by Hofmann and Bouck (1976), wherein cells are allowed to duplicate their strips and divide after the labeling procedure, could prove useful for this purpose.

Interestingly, the location of the cleavage furrow did not always permit even distribution of strips between daughter cells. Two cells (Fig. 2.3a, b) in the early stages of cytokinesis had fewer strips than would be expected from cells whose interphase *P* value is 40. One (Fig. 2.3a) had *P* = 64 strips and the other (Fig. 2.3b) had *P* = 76 strips, indicating that the mother cells during interphase had *P* = 32 and *P* = 38, respectively. Moreover, strips were divided evenly between the daughter cells in the first instance, yielding two daughter cells with 32 strips each (Fig. 2.3a), whereas strips were unevenly divided in the second instance, yielding one daughter cell with 36 strips and one with 40 strips (Fig. 2.3b). Whether or not this is due to an inherent ‘‘deterministic’’ property present in the mature furrow strips m and m´ that is lacking in other pellicle strips, remains to be investigated.

2.3.3 Posterior strip reduction patterns in *E. gracilis*

In *E. gracilis*, the mode for *P* (the number of strips around the cell periphery) was 40 (range = 32–40, n = 17). All observed cells had an even number of strips. Cells exhibited an exponential pattern of posterior strip reduction with three whorls (Fig. 2.4a). When exponential reduction was prevented by insufficient strip number (in this case, a number that cannot be

exponentially reduced three times, such as 36 or 38), pseudoexponential reduction, where strips terminate asymmetrically in the most posterior whorl, was observed (Leander and Farmer 2000a).

Nascent strips formed at the anterior end of the cell and grew towards the posterior tip (Fig. 2.4b). These new strips were narrower than mature strips (Fig. 2.4c, d) and alternated between them. Cells commenced cytokinesis before nascent strips reached the posterior tip or the position of the first whorl of reduction (whorl I; Fig. 2.4c–f). Between the beginning and end of cytokinesis, cells had four (Fig. 2.4d) whorls of exponential reduction. At the end of cytokinesis, cells were joined just at their posterior tips and had three whorls of exponential reduction (Fig. 2.4f).

2.3.4 Multigenerational strips and posterior whorls of reduction: a model of inheritance

Although nascent strips have been known to be evenly distributed between daughter cells for some time (Hofmann and Bouck 1976), it was not clear how different generations of strips are incorporated into the defined patterns of posterior reduction that are conserved within phototrophic euglenid taxa. Here we propose a model of whorl morphogenesis that incorporates semiconservative pellicle strip inheritance and explains how a pattern of posterior strip reduction can be maintained through successive cell divisions in a taxon or cell lineage. It requires, however, that mature pellicle strips be capable of repeated elongation events after their initial synthesis. Mignot et al. (1987) found that this is exactly what occurs to half of the mature strips within the anterior canal of the secondary osmotroph *C. acus*. Before division in *C. acus*, the 16 minor strips that alternate between 16 major strips grow to the same size as the major strips. Subsequently, 32 nascent strips emerge between the 32 mature strips and grow to become the (16) minor strips in each of the daughter cells.

Likewise, our model proposes that the morphology (namely, the length) of strips at the posterior end of the cell also changes from generation to generation. In the example outlined in Fig. 2.5, a cell with $P = 32$ and $W_p = 1$ (i.e., one whorl of exponential reduction composed of 16 terminating strips) doubles its strips before cell division so that $P = 64$. Because nascent strips

alternate with mature strips, the cell immediately before cytokinesis has two exponential whorls, W_p = 2. The cell begins to divide before the nascent pellicle strips are fully grown. As nascent strips grow longer and the cleavage furrow progresses further toward the posterior of the cell, the strips forming whorl I in the mother cell grow longer as well. This causes whorl I to move toward the posterior tip of the cell and gradually disappear as the strips forming whorl I achieve the same length as the strips that reached the posterior tip in the mother cell. The nascent strips grow to reach the former position of whorl I, so that when division is complete each daughter cell has W_p = 1 like the mother cell. However, whorl I is now composed of a new generation of nascent strips, and the strips that formed whorl I in the previous generation now reach the posterior tips of the daughter cells and are intermixed with the strips formed in earlier generations.

This model is applicable to taxa with more than one posterior whorl, such as *E. gracilis*. During cell division, strips forming whorl III grow to the same length as the strips that reach the posterior tip; strips forming whorl II grow to the same length as the strips that formed whorl III; strips forming whorl I grow to the same length as the strips that formed whorl II; and nascent strips grow to the same width and length as the strips that formed whorl I (Fig. 2.6). Because each of these groups of strips contains twice as many strips as the set immediately posterior to it, this growth process results in a cell with twice as many strips and three exponential whorls of reduction, each containing twice as many terminating strips as the whorls in an interphase cell (although cell division begins before the growth process is complete). When cell division is complete, each daughter cell possesses the same number of strips and the same pattern of reduction as the mother cell. Thus, each whorl of reduction is composed of terminating strips belonging to different generations produced during previous cell divisions: the strips in whorl I were produced during the most recent round of cell division, the strips in whorl II are one generation older, and the strips in whorl III belong to a still older generation. The strips that reach the posterior tip of the cell are composed of at least two different generations (because during the last round of cell division younger strips from whorl III grew between and intermixed with the older strips already present at the tip). Strictly speaking, there can be as many as three or four generations represented by strips at the tip because whorl III strips are incorporated between tip

strips at each cell division. For the sake of simplicity, however, tip strips in interphase cells are color-coded as the same generation in all figures. Any interphase *E. gracilis* cell can therefore have strips representing up to six or seven generations: three generations represented by posterior whorls of reduction, and three or four generations represented by strips reaching the posterior tip of the cell (Fig. 2.6, inset).

2.3.5 Heterochrony and the diversity of posterior strip reduction patterns

Posterior patterns of pellicle strip reduction are indicators of phylogenetic relationships, and the ancestral state for euglenids is the absence of strip reduction altogether (Leander and Farmer 2000a, Leander et al. 2001; Fig. 2.7). According to our model, the origin of a whorled pattern of strip reduction involved the incomplete growth of nascent strips before division. That is, if the nascent strips failed to grow to the posterior tip, then each daughter cell would have one exponential whorl of strip reduction, assuming that all of the nascent strips terminated at the same point along the length of the cell. If the differential growth of nascent strips was repeated during subsequent rounds of cell division, then multiple whorls of exponential strip reduction could be produced (Fig. 2.7). This extension of the model is consistent with previous observations of different states for the number of posterior whorls of strip reduction: one whorl (e.g., *E. cantabrica*, Leander et al. 2001), two whorls (e.g., *E. laciniata*, Leander et al. 2001), three whorls (e.g., *E. longa*, Leander et al. 2001) and four whorls (e.g., *E. rustica*, Leander and Farmer 2000a, Brown et al. 2002). Moreover, differences in growth rate within one whorl of exponential reduction, such that every other terminating strip grew longer than its terminating neighbor, would result in a pseudolinear pattern of reduction, as observed in *Eutreptia pertyi* (Leander et al. 2001) (Fig. 2.7). Differential growth in whorl I of a cell with two exponential whorls of reduction would result in cells with a staggered exponential whorl, followed by a uniform exponential whorl. From this intermediate state, cells with three linear whorls of reduction, such as *E. mutabilis*, could be produced (Leander and Farmer 2000a). Likewise, an ancestral cell with three exponential whorls of reduction could give rise to cells with two staggered exponential whorls and one uniform

exponential whorl (e.g., *Lepocinclis oxyuris*, Leander et al. 2001). Cells exhibiting this state in turn could yield descendants with five bilinear whorls (e.g., *E. helicoideus*, Leander and Farmer 2000b) (Fig. 2.7).

Some phylogenetic analyses of morphological characters and nuclear ribosomal RNA gene sequences (SSU and LSU rDNA) have suggested that a pseudolinear pattern of posterior strip reduction evolved before one ‘‘clean’’ exponential whorl. *E. pertyi*, for instance, is among the earliest diverging phototrophic taxa and has a pseudolinear pattern of posterior strip reduction (Leander et al. 2001) (Fig. 2.7). It should be emphasized that a pseudolinear pattern is effectively a disorganized version of one exponential whorl of strip reduction (Fig. 2.7). However, the origin of one ‘‘clean’’ exponential whorl of strip reduction by a single generation of aberrant nascent strips is also a plausible hypothesis for the ancestral state in phototrophic euglenids. Taxa known to have one exponential whorl of reduction include *E. cantabrica* (Leander et al. 2001), *Phacus oscillans* (Leander et al. 2001, Leander and Farmer 2001b), *Lepocinclis salina* (Conforti and Tell 1983) and members of the loricate genus *Trachelomonas* (Brosnan et al. 2005). The current molecular phylogenetic framework for euglenids, however, suggests that all of these taxa diverged relatively recently (Brosnan et al. 2003, Marin et al. 2003). Nonetheless, it cannot be ruled out that we have not yet observed early-branching taxa with a single exponential whorl of strip reduction because of low taxon sampling or the extinction of taxa possessing the ancestral character states.

The contentious phylogenetic position of *E. mutabilis* is problematic because its unique linear pattern of strip reduction has been used as evidence for its affinity to the Eutreptiales when molecular data were inconclusive (Leander et al. 2001). Although *E. mutabilis* can branch relatively early (i.e., subsequent to the Eutreptiales) in molecular and morphological phylogenies (Leander et al. 2001, Marin et al. 2003; Nudelman et al. 2003), it does not always do so (Marin et al. 2003, Nudelman et al. 2003). The SSU and LSU rDNA sequences from *E. mutabilis* are highly divergent, which has led to long-branch attraction artifacts in previous analyses (Leander et al. 2001, Brosnan et al. 2003, Marin et al. 2003). The evolutionary pathway of whorl reduction proposed by Leander et al. (2001) inferred from mapping this character on to a tree that may be

affected by long-branch attraction and inadequate taxon sampling seems unsatisfactory in light of our developmental data. It should be noted, however, that according to our model the first two whorls in a species with linear reduction (i.e., whorls I and I´, produced by differential growth within whorl I) should be produced by one generation of nascent strips (Fig. 2.7). Furthermore, these whorls must coalesce during the next round of cell division to produce whorl II. If a cell had a pseudolinear pattern of posterior reduction, then the coalescence of whorls I and I´ (rather than their growth to the posterior end of the cell) to form whorl II and the production of whorls I and I´ by nascent strips during a single cell division event could result in daughter cells with three linear whorls of reduction. The multigenerational nature of the pellicle means that every other nascent strip is produced adjacently to a mature strip belonging to a different generation than its neighbors. Therefore, staggered and pseudolinear patterns of strip reduction are consistent with our model because it is possible that the relative ages of adjacent mature strips affect the growth rate of the nascent strips (Fig. 2.7). Morphological analyses of more phototrophic euglenid taxa, especially those belonging to the Eutreptiales, in the context of comprehensive molecular phylogenies (e.g., Marin et al. 2003) should help clarify these inferences.

2.3.6 Conclusions

Although the data presented in this paper provide a model for the maintenance of posterior whorls of strip reduction and compelling evidence for heterochrony in the evolution of this character, several key questions remain unanswered. The genetic basis for patterns of strip reduction is completely unknown. It remains to be discovered what triggered a change in morphogenetic processes and therefore posterior patterns of strip reduction. Knowledge of the genetic basis of whorl morphogenesis could help us to determine if the evolutionary pathways in Fig. 2.7 are reversible; this information could lead to a set of probabilities associated with the direction of character evolution that could be applied to phylogenetic analyses of morphological data using likelihood methods (Lewis 2001, Hibbett 2004). We are only beginning to understand developmental processes in single-celled eukaryotes in a framework that incorporates our

increasing understanding of the vast phylogenetic and architectural diversity exhibited by these organisms. Research focusing on these processes could provide significant insights into the foundations of cellular differentiation and organismal diversity.

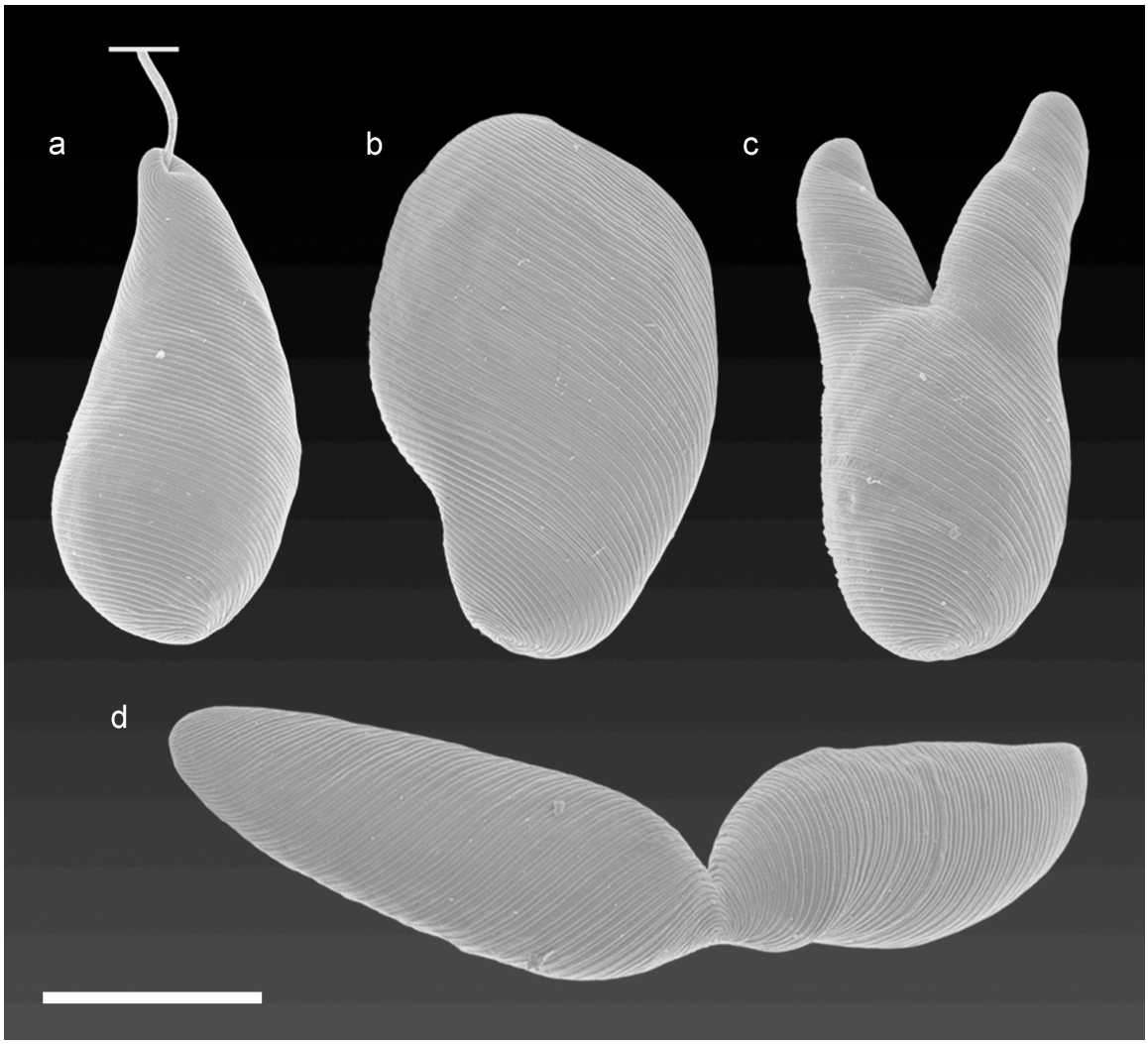


Figure 2.1. Longitudinal cell division in *Euglena gracilis*. Cells are shown (a) at interphase (*P* ~ 40), (b) after strip doubling (*P* ~ 80), (c) half way through cytokinesis, and (d) near the end of cytokinesis. Scale bar, 10 μm. *P*, the number of strips around the cell periphery.

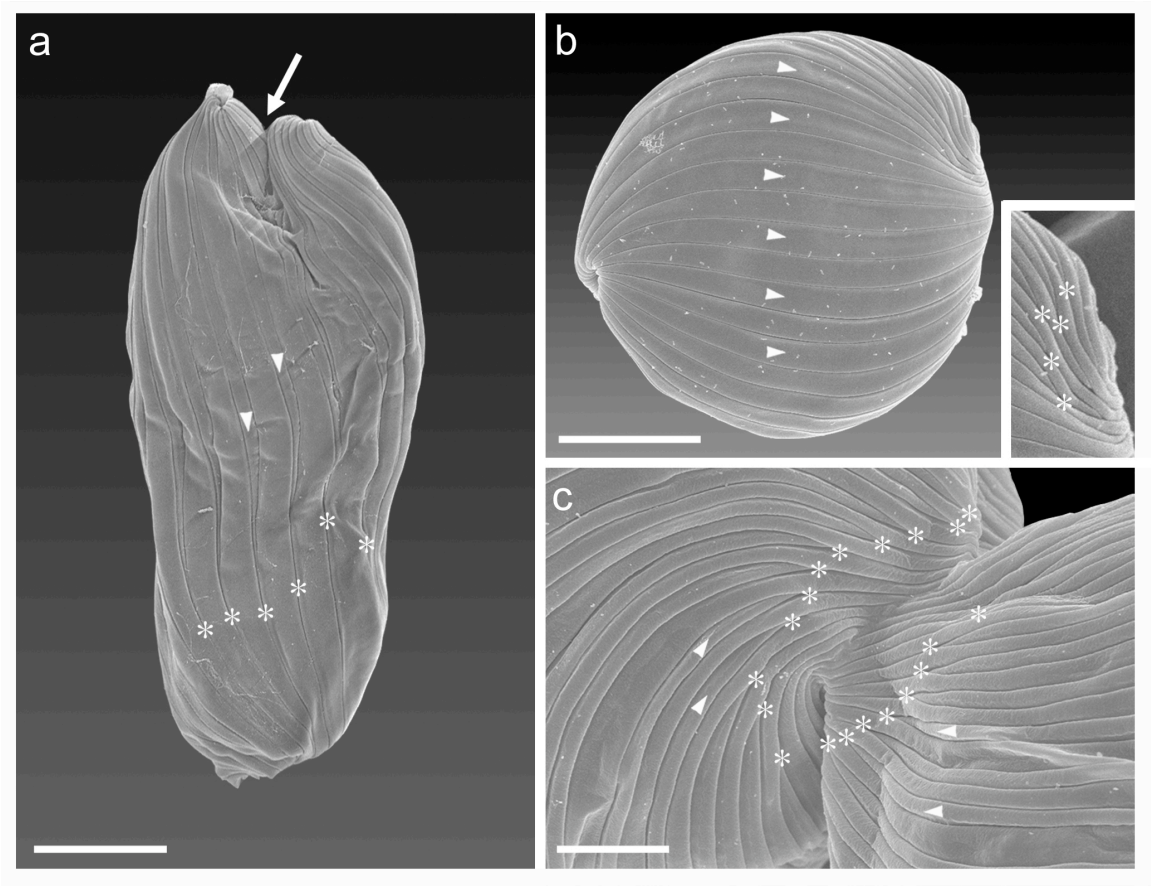


Figure 2.2. Evidence that whorl I in phototrophic euglenids is formed by nascent pellicle strips. (a) A *Phacus similis* cell beginning to divide. The nascent strips (arrowheads) are still growing towards the posterior end of the cell and appear to form an exponential whorl of reduction (asterisks); the cleavage furrow (arrow) has already begun to form. Scale bar, 5 μm. (b) *P. segretii* at interphase. Alternating strips (arrowheads) that terminate before entering the anterior canal (on the left) also terminate to form the first whorl of posterior reduction (inset, asterisks). Scale bar, 5 μm. (c) The cleavage furrow between two developing *Euglena viridis* cells near the end of cytokinesis. The two daughter cells are joined only at the posterior tip. Alternating strips (arrowheads), inferred from their relatively narrow width to be nascent, form an exponential whorl of reduction on each daughter cell (asterisks). Scale bar, 2 μm.

Figure 2.3 (Next page). Progression of the cleavage furrow and position of furrow strips during cell division in *Euglena gracilis*. Double arrows indicate separating furrow strips while arrows indicate rearticulating furrow strips; arrowheads indicate nascent strips and their direction of growth. Scale bars, 1 μm. (a) Anterior view showing the beginning of cytokinesis. The two pairs of furrow strips, m (mature) and n (nascent) on the left, and m´ and n´ on the right, have separated and rearticulated with strips from the opposite side of the cell: m´ with n, and m with n´. Each daughter cell has 32 strips surrounding the canal. (b) Strips m and m´ are further separated from n and n´, respectively, and rearticulated with n´ and n, respectively. The anterior ends of two daughter cells are now distinctly formed: the cell on the left has 40 strips; the cell on the right has 36 strips. (c) As cytokinesis progresses, the cleavage furrow appears to align with or surpass the extent of growth of the nascent furrow strips, n (located behind the cell) and n´. (d) A diagram summarizing the changing positions of furrow strips during cytokinesis. Furrow strips have been coded as follows: m´ is dark gray, m is mid-gray, n´ is light gray, and n is white-gray. After strip doubling, m is adjacent to n and m´ is adjacent to n´. The pairs of furrow strips are located opposite to one another, with the anterior canal between them. Cleavage furrow formation begins at the anterior of the cell, where m separates from n and m´ separates from n´. Where the strips have separated, m rearticulates with n´ and m´ rearticulates with n so that the anterior canal is divided into two. Separation of m and n, and m´ and n´ continues towards the cell's posterior end, while rearticulation between m and n´ and m´ and n continues in a corresponding fashion. As these processes take place, the cleavage furrow progresses and the two daughter canals are further separated from one another. Upon completion of cytokinesis, m´ is located adjacent to n on one daughter cell, and m is located adjacent to n´ on the other daughter cell.

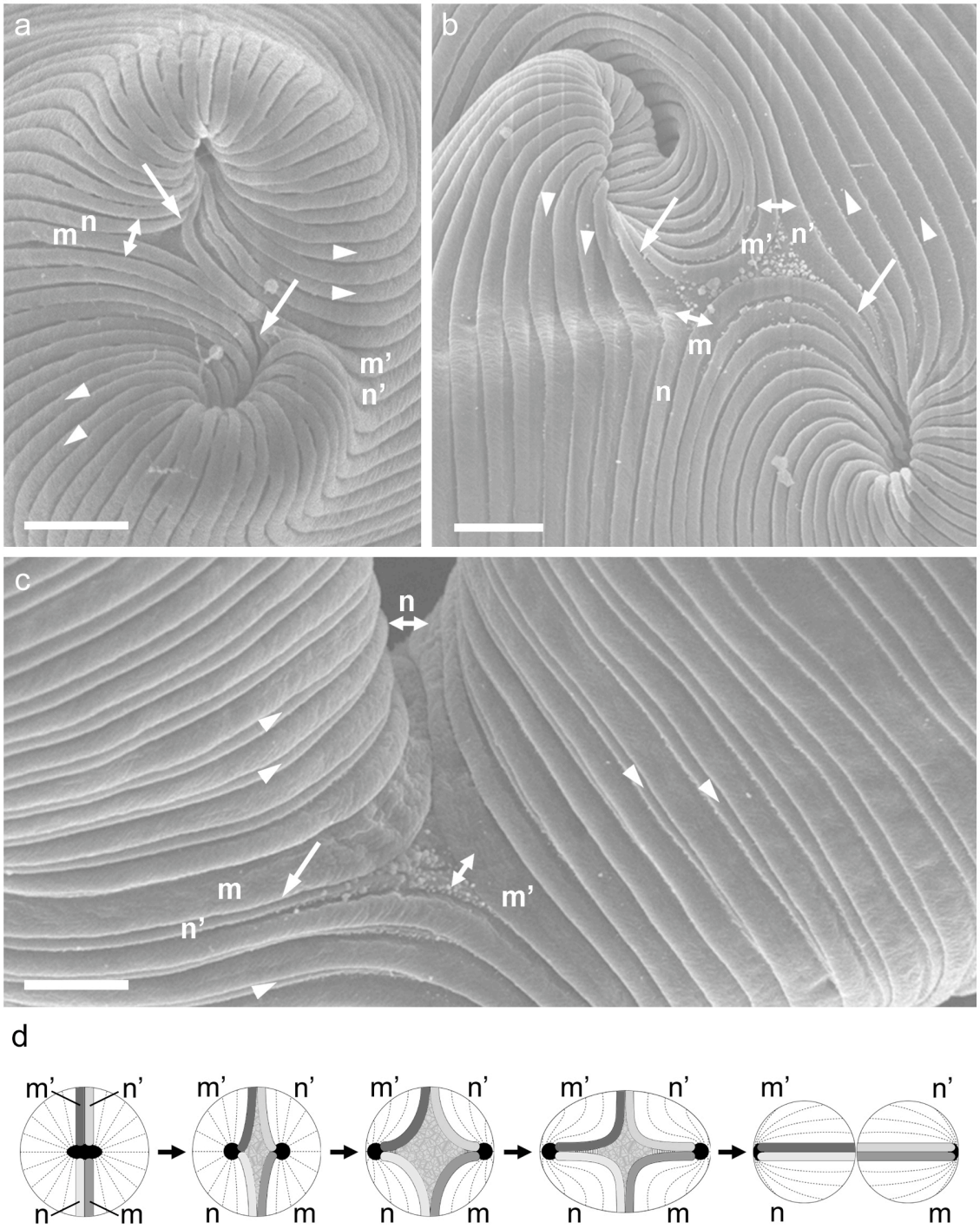


Figure 2.3

Figure 2.4 (Next page). Posterior whorls of reduction at different stages of cell division in *Euglena gracilis*. (a) An interphase cell (*P* = 40) with three whorls of exponential reduction. Scale bar, 1 μm. (b) A cell during strip doubling (*P* = 72). Posterior whorls remain unchanged from their position during interphase. Alternating nascent strips (visible strip reductions are indicated by squares) form a fourth whorl of exponential reduction. Scale bar, 5 μm. (c) The posterior end of a cell half way through cytokinesis. The whorl formed by nascent strips (squares) is closer to the posterior end; the three whorls present in the mother cell remain unchanged. Scale bar, 2 μm. (d) A cell near the end of cytokinesis. The nascent whorl has been disrupted by the cleavage furrow and now forms two whorls, one on each developing daughter cell. The three whorls present in the mother cell remain intact. Scale bar, 1 μm. (e) Another cell in late cytokinesis. The nascent whorl of reduction is again closer to the posterior end and apparently disrupted by the cleavage furrow in the upper portion of the image. Scale bar, 2 μm. (f) Newly formed daughter cells immediately before complete separation. Each has three exponential whorls of reduction: whorl I (squares) is formed by terminating nascent strips, whorl II by the strips that formed whorl I in the mother cell (asterisks), and whorl III by strips that formed whorl II in the mother cell. Scale bar, 2 μm.

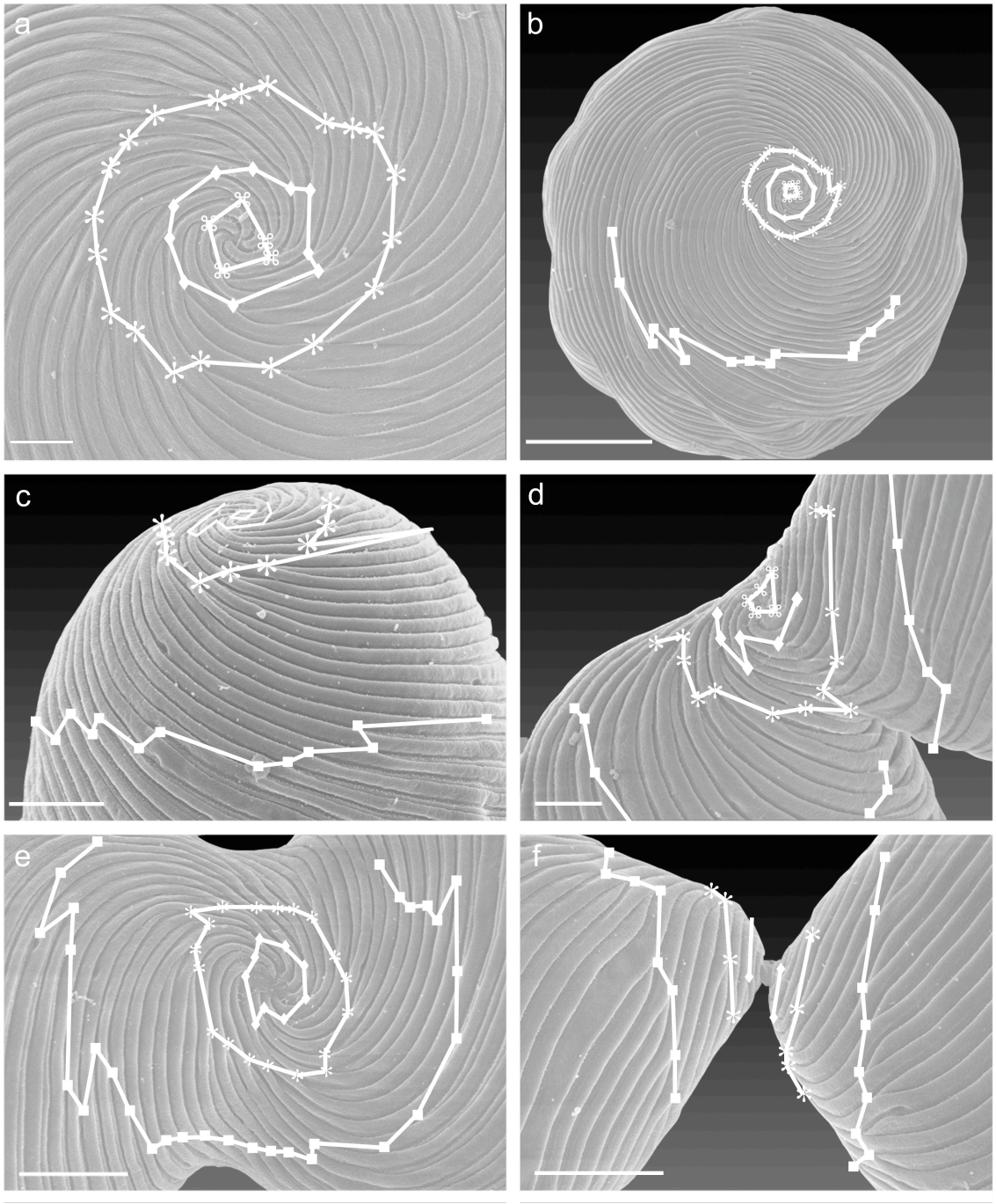
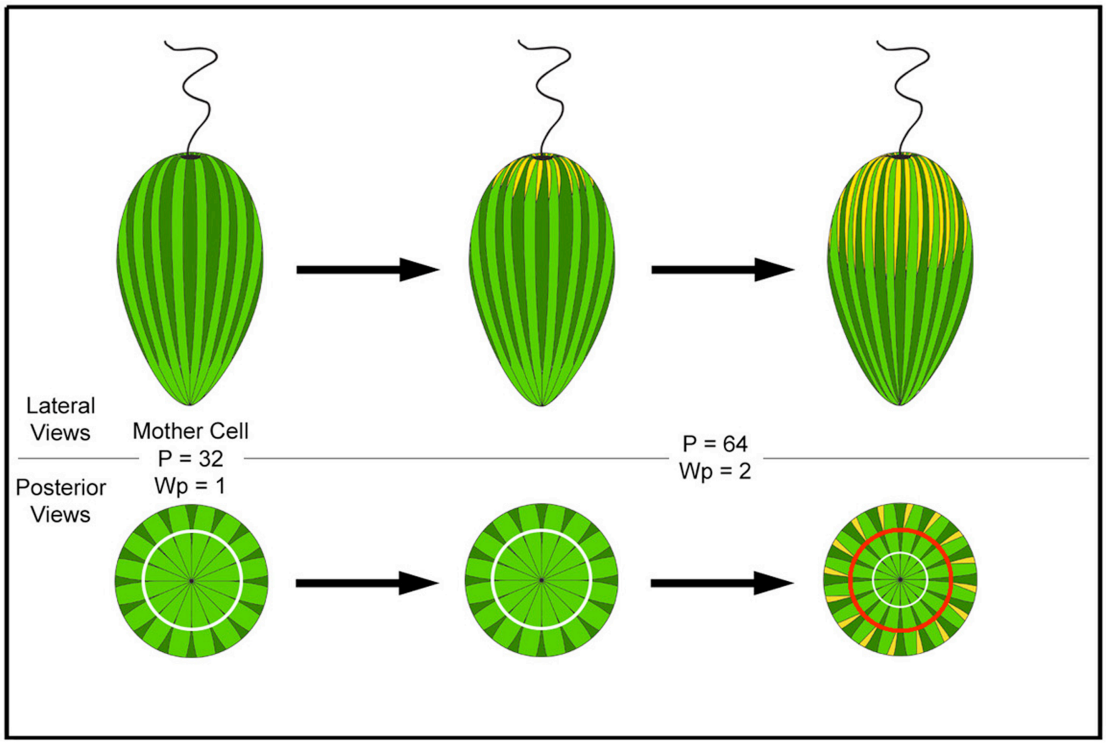


Figure 2.4

Figure 2.5 (Next page). A model for the maintenance of whorls of reduction on the posterior end of dividing euglenids. A hypothetical cell with a total strip number of 32 has one exponential whorl of reduction (white circle) composed of 16 pellicle strips (dark green) and 16 strips (light green) reaching the tip of the cell. During strip doubling prior to cell division, 32 nascent strips (yellow) extend from the canal towards the posterior end of the cell and form a second whorl of reduction (red circle). As the nascent strips continue to grow, the strips composing the original whorl of reduction begin to grow towards the posterior tip. The cell body begins to divide before the nascent strips are finished growing. After cell division pellicle strips are distributed evenly between two daughter cells: each cell has 16 mature strips (green) that were present in the mother cell, extending to the posterior tip, and 16 nascent, terminating strips (yellow) that form the new whorl of reduction. Eight of the strips at the posterior tip (dark green) belonged to the posterior whorl of reduction in the last generation. P, total strip number; W_p, number of posterior whorls of reduction.


Lateral
Views
Mother Cell
P = 32
Wp = 1
P = 64
Wp = 2
Posterior
Views

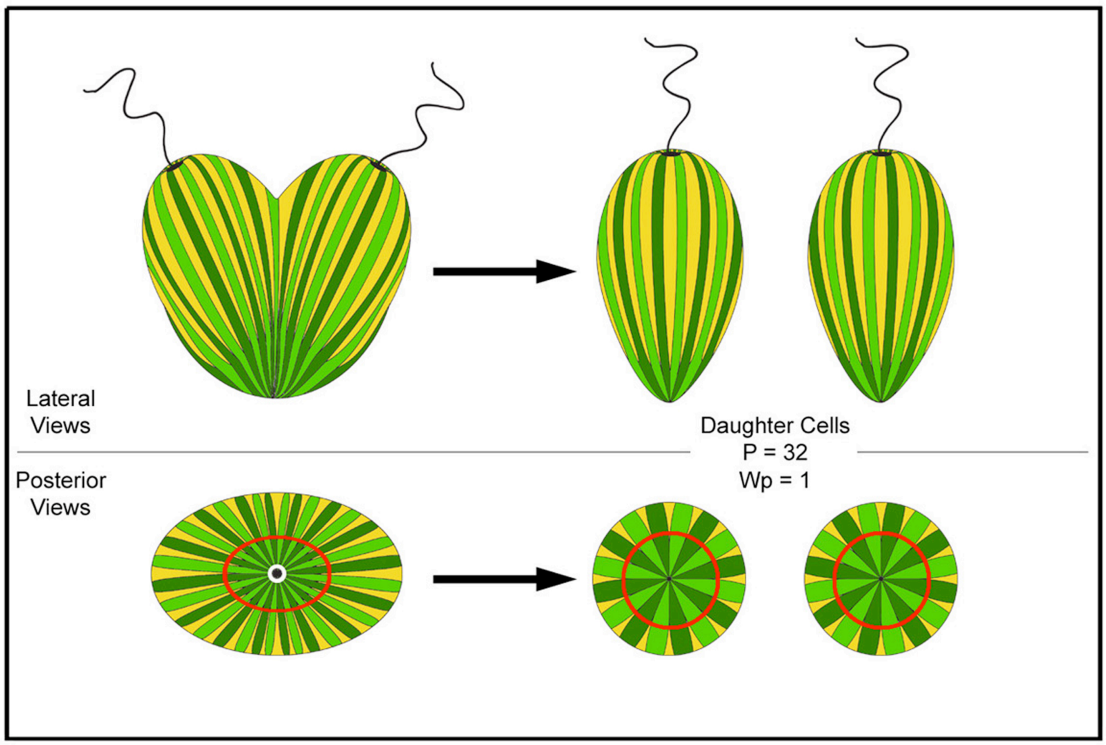

Lateral
Views
Daughter Cells
P = 32
Wp = 1
Posterior
Views

Figure 2.5

Figure 2.6 (Next page). A model of posterior whorl morphogenesis in *Euglena gracilis*. A mother cell has three posterior whorls of exponential reduction: whorl I (red), whorl II (green) and whorl III (blue). Before cell division, alternating nascent strips develop; as they lengthen towards the posterior end of the cell they form a fourth exponential whorl of reduction (yellow). Before the nascent strips are fully grown, the cleavage furrow and two daughter cells begin to form; the nascent whorl is now relatively close to the posterior of the cell. As the cleavage furrow progresses, the nascent whorl is disrupted and divided equally between the forming daughter cells. Whorl III is lost as the terminating strips forming it grow toward, and eventually reach, the posterior tip of the cell. The strips forming whorls I and II also grow slightly and are divided by the cleavage furrow, so that by the time cytokinesis is complete each daughter cell possesses three exponential whorls of reduction like the mother cell. In the daughter cells, whorl I is formed by nascent strips (yellow), whorl II is formed by strips that constituted whorl I in the mother cell (red), and whorl III is formed by strips from whorl II in the mother cell (green). Inset: *E. gracilis* with strips colored according to their relative age (uncolored strips are the oldest. Scale bar, 2 μm.

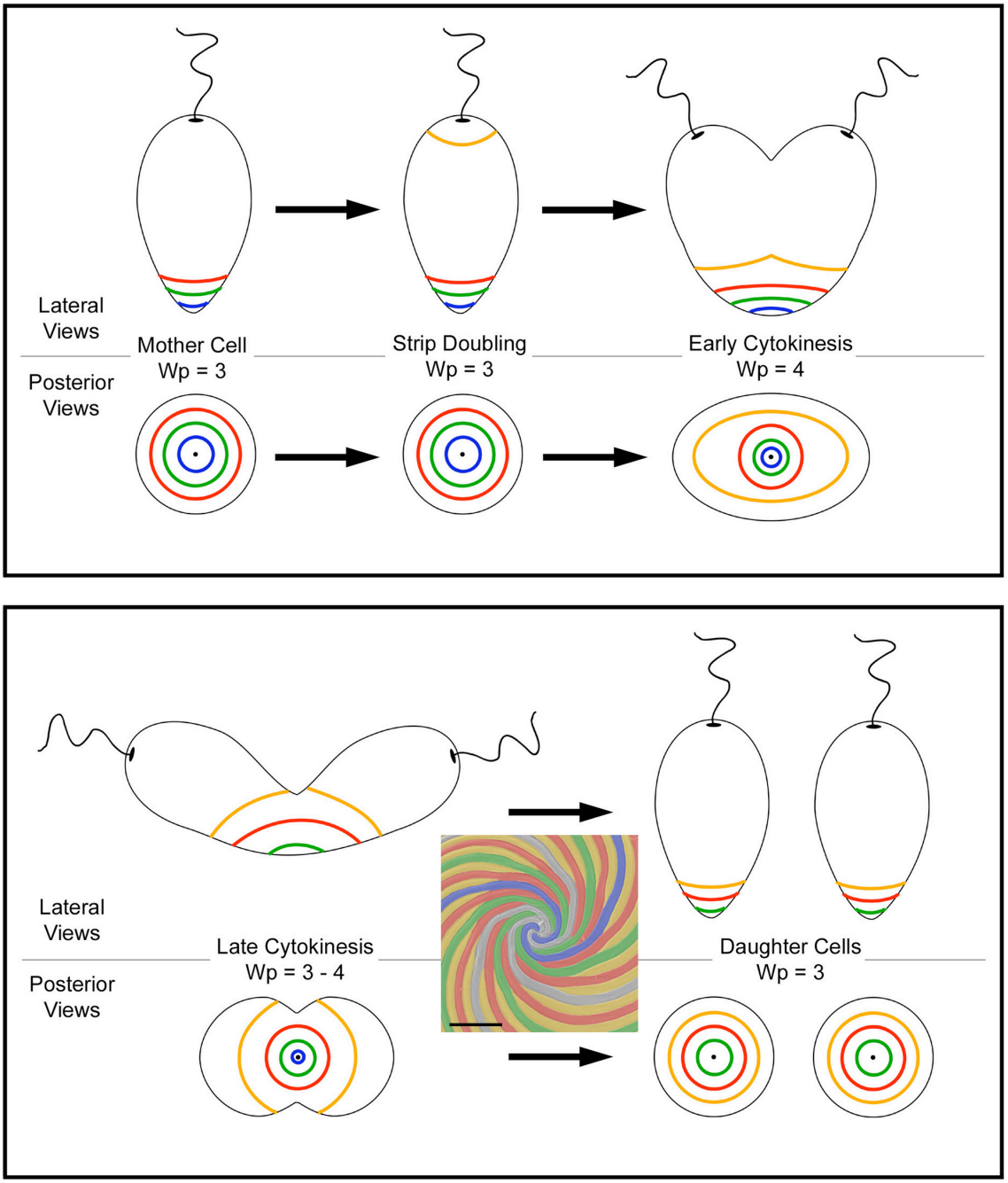

Lateral Views
Posterior Views
Mother Cell
Wp = 3
Strip Doubling
Wp = 3
Early Cytokinesis
Wp = 4
Lateral Views
Posterior Views
Late Cytokinesis
Wp = 3 - 4
Daughter Cells
Wp = 3

Figure 2.6

Figure 2.7 (Next page). Pathways for the heterochronic evolution of posterior patterns of strip reduction in phototrophic euglenids. An ancestral cell without posterior whorls of reduction could give rise to a cell with one exponential whorl of reduction if nascent strips did not completely extend to the posterior tip of the cell before cytokinesis. Through several repetitions of this event, cells with two, three, and four exponential whorls of reduction could be produced through time. Differences in growth rate between alternating strips within one exponential whorl of reduction, depending on their magnitude, could give rise to a staggered exponential whorl or to two linear whorls (which, though apparently separate, would both be formed by strips of the same generation). In this way an ancestral cell with one exponential whorl of reduction could give rise to a cell with a pseudolinear pattern of reduction. Alternatively, the ancestral state could give rise to pseudolinear reduction directly, which could, through homogenization of growth rate of nascent strips, give rise to one “clean” exponential whorl of reduction. An ancestral cell with two exponential whorls of reduction could give rise first to cells with two posterior whorls of reduction, one of which is staggered, then to cells with three whorls of linear reduction. A cell with three exponential whorls of reduction could likewise give rise to cells with two staggered whorls of reduction, which would in turn give rise to cells with five bilinear whorls of reduction. These character state changes are inferred to be reversible, as indicated by backward arrows.

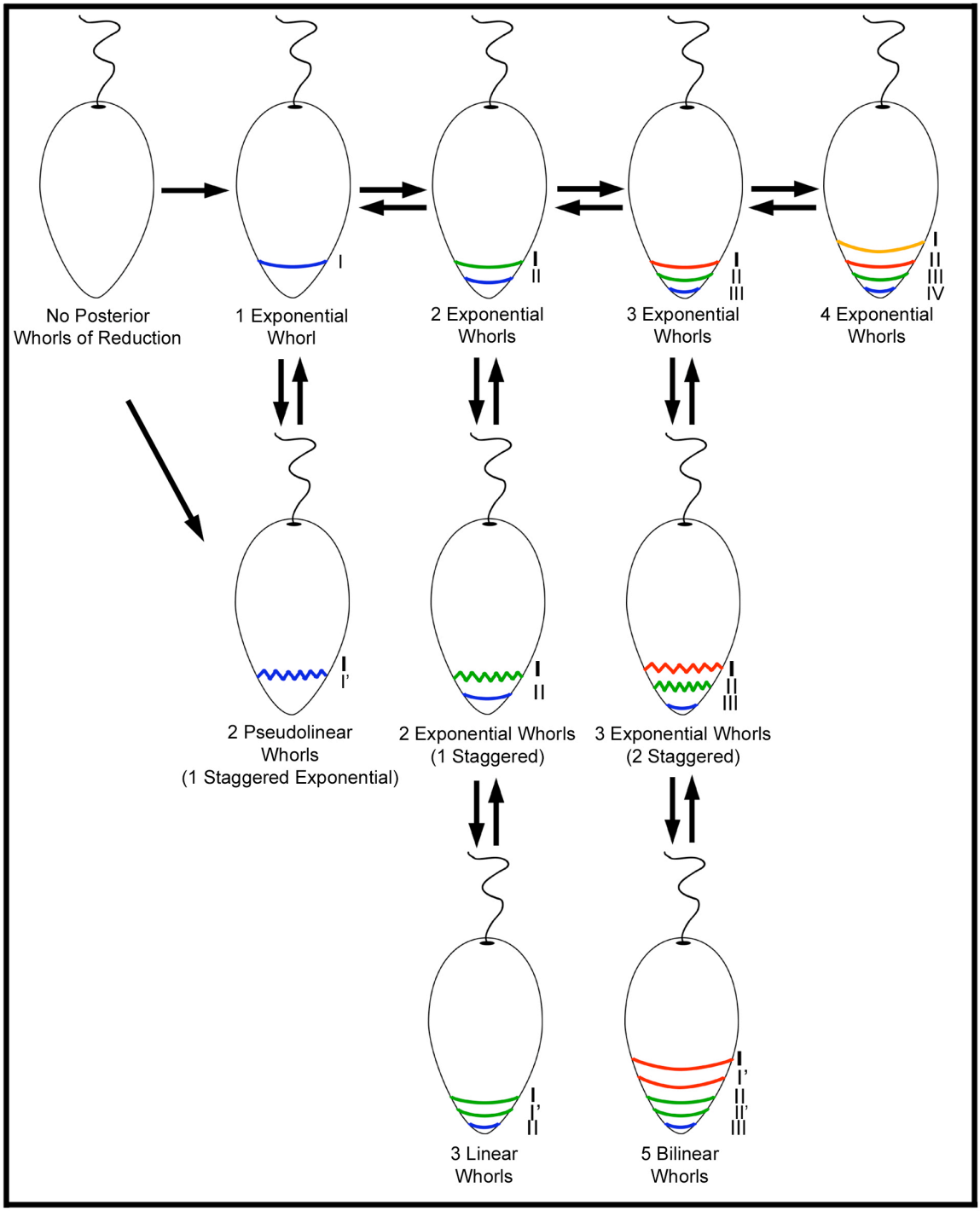

No Posterior
Whorls of Reduction
1 Exponential
Whorl
I
2 Exponential
Whorls
I
II
3 Exponential
Whorls
I
II
III
4 Exponential
Whorls
I
II
III
IV
2 Pseudolinear
Whorls
(1 Staggered Exponential)
I
I'
2 Exponential Whorls
(1 Staggered)
I
II
3 Exponential Whorls
(2 Staggered)
I
II
III
3 Linear
Whorls
I
I'
II
5 Bilinear
Whorls
I
I'
II
II'
III

Figure 2.7

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Chapter 3: NOVEL PELLICLE SURFACE PATTERNS ON *EUGLENA OBTUSA* SCHMITZ (EUGLENOPHYTA), A EUGLENOPHYTE FROM A BENTHIC MARINE ENVIRONMENT: IMPLICATIONS FOR PELLICLE DEVELOPMENT AND EVOLUTION*

3.1 Introduction

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),

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* A version of this chapter has been published: Esson, H. J. and Leander, B. S. 2008. Novel pellicle surface patterns on *Euglena obtusa* Schmitz (Euglenophyta), a euglenophyte from a benthic marine environment: Implications for pellicle development and evolution. Journal of Phycology 44:132-141.

a character that was previously useful in phylogenetic and taxonomic studies (Leander and Farmer 2001a, Leander et al. 2001, Brosnan et al. 2005). Our 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.

3.2 Materials and methods

3.2.1 Collection of *E. obtusa*

Sand substrate was collected with a spoon from Spanish Banks (English Bay, Vancouver, British Columbia; 49˚7´N, 123˚3´W) during low tide. The sand was placed in a vertical plastic cylinder with a 48 lm 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).

3.2.2 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).

3.2.3 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.

3.2.4 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 (5,900g) 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.

3.3. Results

3.3.1 General morphology

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. 3.1a 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. 3.1b). Cytoplasmic paramylon grains were variable in abundance (Fig. 3.1b), but double paramylon caps were always associated with a single pyrenoid in the numerous plate-shaped chloroplasts (Fig. 3.1c). 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. 3.2a) 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. 3.2b). A longitudinal section viewed with TEM (Fig. 3.2c), however, revealed an aperture of <250 nm at the cell surface leading to a narrow, flattened canal beneath.

3.3.2 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. 3.3a). 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. 3.3b). 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 reduction in *E. obtusa* (Fig. 3.3). 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. 3.4). 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. 3.3b). The number of pellicle strips converging at the posterior tip of one cell was 15.

3.3.3 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. 3.5a). 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. 3.5b). 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. 3.5c). 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.

3.4 Discussion

3.4.1 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. 3.6c). 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 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. 3.3). In this way, strip ultrastructure will be oriented as shown in Figure 3.6c.

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).

3.4.2 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*.

3.4.3 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. 3.2a). 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 3.2a.

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. 3.6b). 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 3.6. 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. 3.4; 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. 3.6b). This finding is consistent with the observation that there are eight pellicle strips between rows of pores (Fig. 3.5a). 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. 3.5b).

3.4.4 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 described as having linear (or ‘‘pseudolinear’’) reduction are *E. mutabilis* (with three subwhorls of linear reduction formed by two whorls of exponential reduction; Fig. 3.7) 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. 3.7; 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. 3.4 and 3.6b), resulting in a pattern of linear reduction over seven subwhorls (Figs. 3.3b, 3.4, 3.6b, and 3.7). 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. 3.7a). 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. 3.7a). 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 3.7b.

The inferred pattern of strip development and the fate of individual strips during subsequent strip duplications in *E. obtusa* are presented in Figure 3.7b. 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 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. 3.6b and 3.7b).

3.4.5 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 3.1). 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* (Leander and Farmer 2000a), 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.

Table 3.1. 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

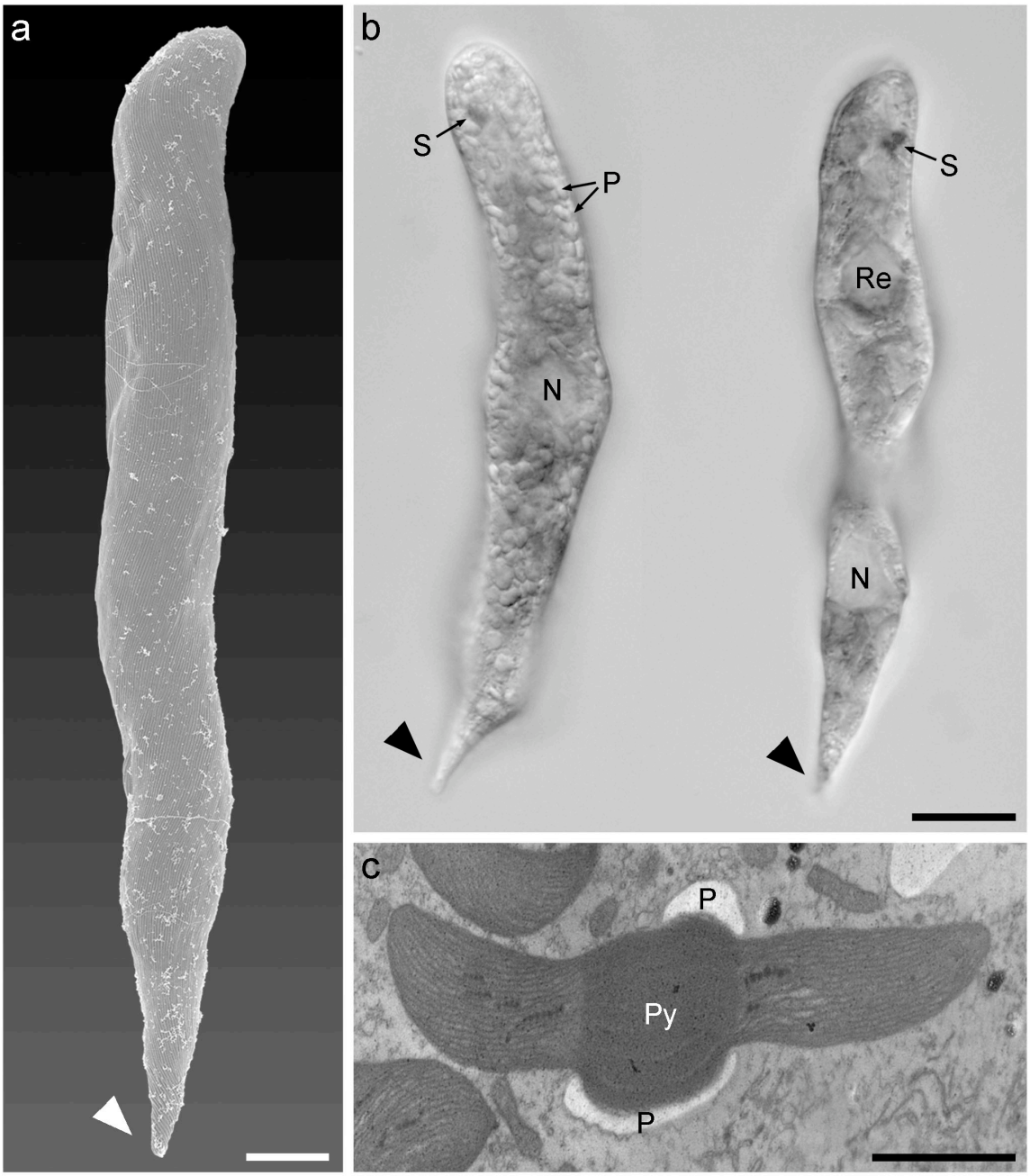

Figure 3.1. General morphology of *Euglena obtusa*. (a) Scanning electron micrograph showing elongated cell with tapered posterior end (arrowhead). Scale bar, 10 μm. (b) Differential interference contrast (DIC) micrograph of two elongated cells with tapered posterior ends (arrowheads), nuclei (N), and 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.

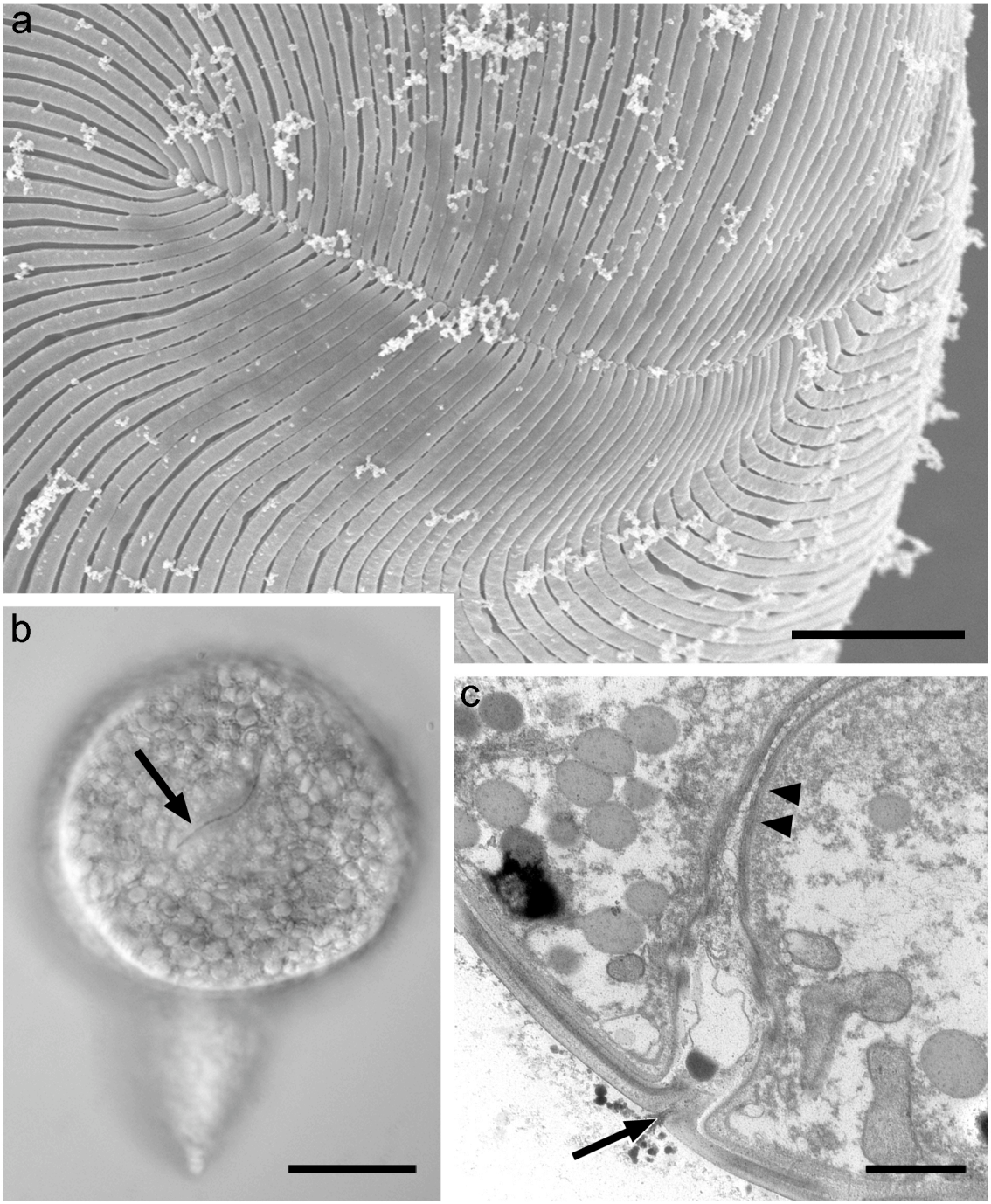

Figure 3.2. The cryptic "canal opening" in *Euglena obtusa*. (a) Scanning electron micrograph showing 115 of 120 pellicle strips meeting along a line at the anterior end of the cell (five additional strips are outside the field of view in this image). The subterminal "canal opening" lies beneath this 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.

Figure 3.3 (Next page). 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 IIA 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 though IB, five pass through IC, four pass through ID, three pass through IIA, 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.

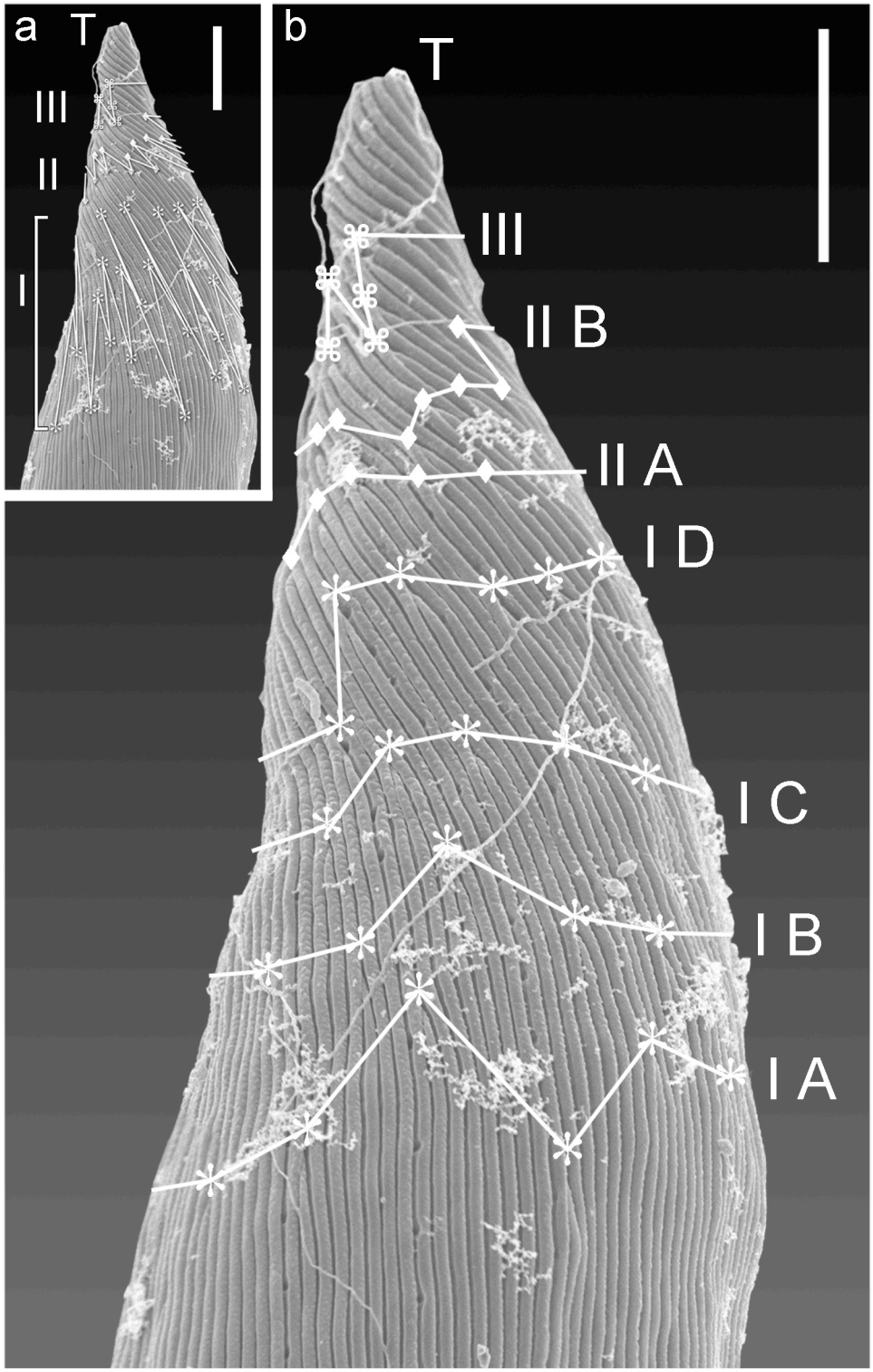

Figure 3.3

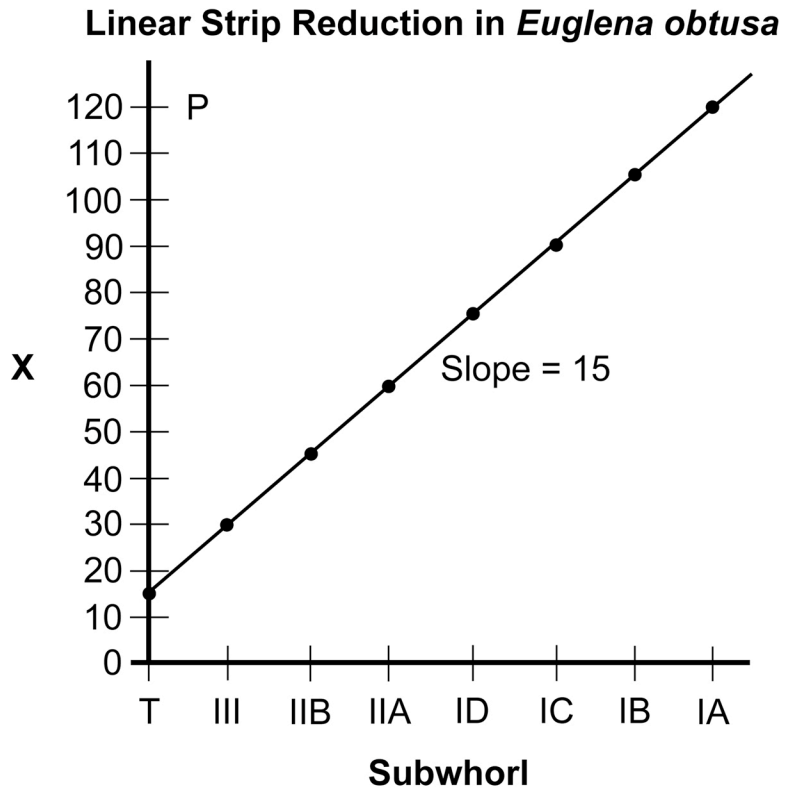

Figure 3.4. Graph representing the linear pattern of posterior strip reduction in *Euglena obtusa*. *P* is the number of pellicle strips surrounding the cell periphery before strip reduction takes place. *X* is the number of strips surrounding the cell immediately before a whorl or subwhorl of strip reduction. *T* is the number of strips that reach the posterior tip of the cell.

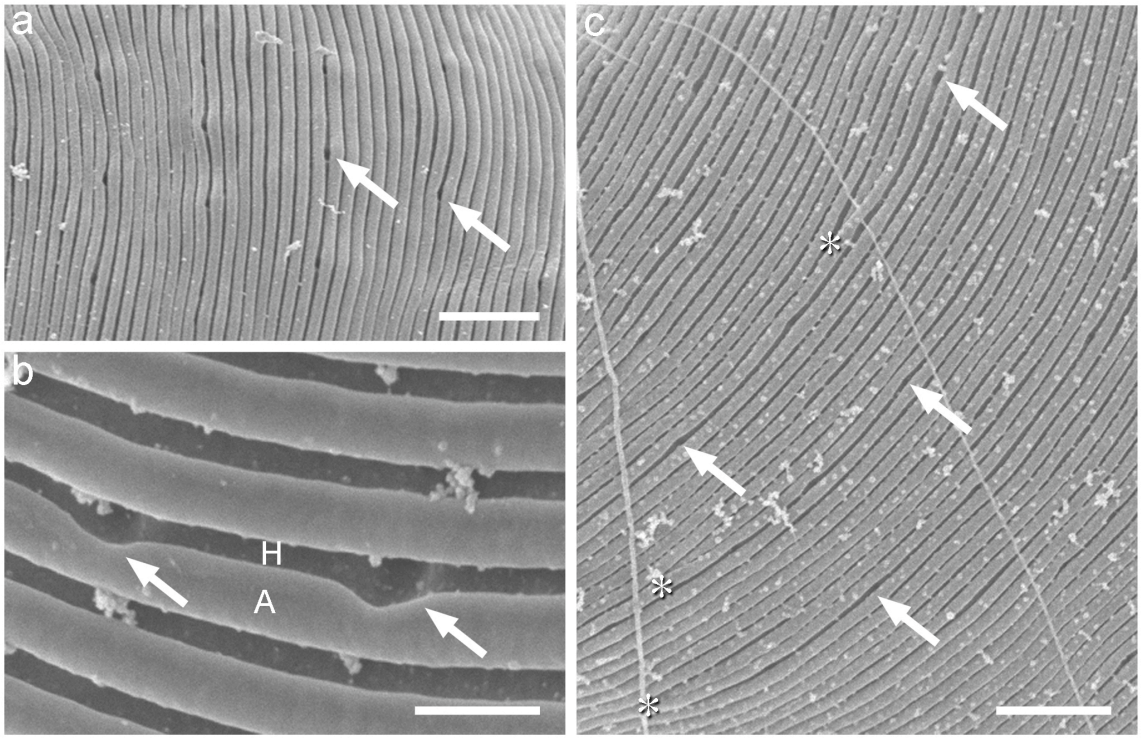

Figure 3.5. The pattern of pellicle pores in *Euglena obtusa*. (a) Scanning electron micrograph showing pores (arrows), whose rows are separated by eight pellicle strips. Scale bar, 2 μm. (b) Scanning electron micrograph showing pores (arrows) located in the heel (H) region of a pellicle strip and the 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.

Figure 3.6 (Next page). 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*.

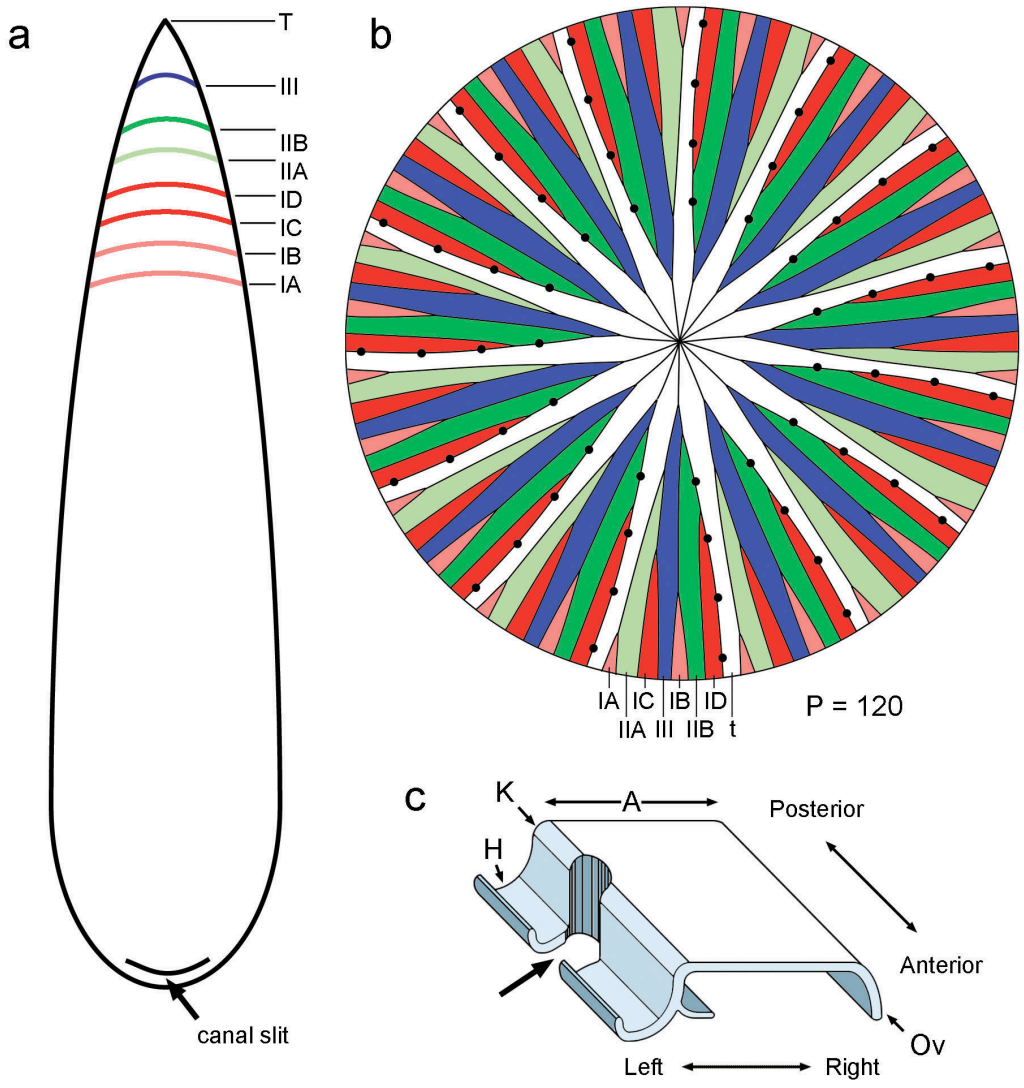
a
T
III
IIB
IIA
ID
IC
IB
IA
canal slit
b
IA
IC
IB
ID
IIA
III
IIB
t
P = 120
c
K
A
Posterior
H
Anterior
Ov
Left
Right

Figure 3.6.

Figure 3.7 (Next page). 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.

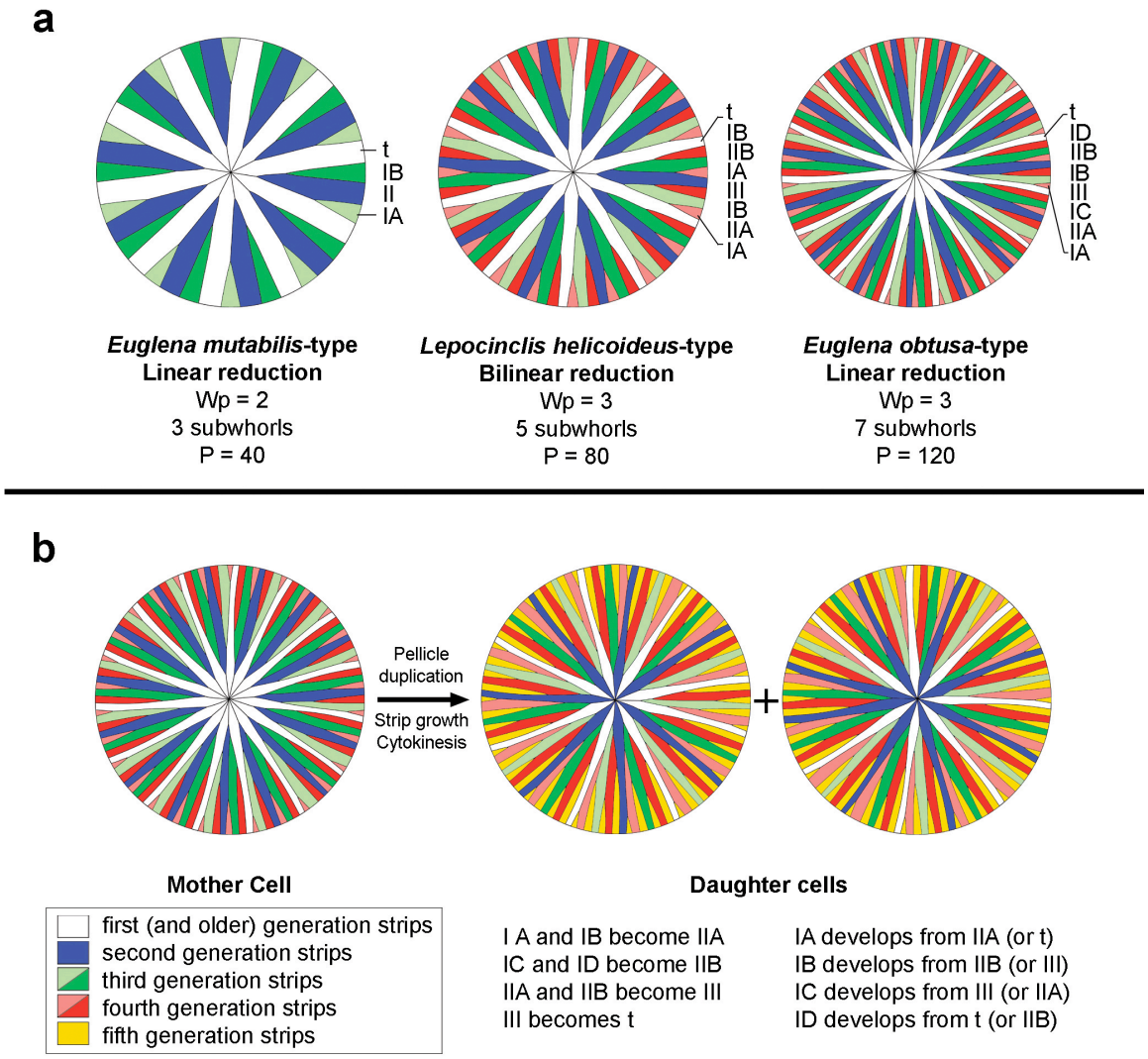
a
t
IB
II
IA
t
IB
IIB
IA
III
IB
IIA
IA
t
ID
IIB
IB
III
IC
IIA
IA
Euglena mutabilis-type
Linear reduction
Wp = 2
3 subwhorls
P = 40
Lepocinclis helicoideus-type
Bilinear reduction
Wp = 3
5 subwhorls
P = 80
Euglena obtusa-type
Linear reduction
Wp = 3
7 subwhorls
P = 120
b
Pellicle duplication
Strip growth
Cytokinesis
+
Mother Cell
first (and older) generation strips
second generation strips
third generation strips
fourth generation strips
fifth generation strips
Daughter cells
I A and IB become IIA
IC and ID become IIB
IIA and IIB become III
III becomes t
IA develops from IIA (or t)
IB develops from IIB (or III)
IC develops from III (or IIA)
ID develops from t (or IIB)

Figure 3.7

3.5 References

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Chapter 4: EVOLUTION OF DISTORTED PELLICLE PATTERNS IN RIGID PHOTOSYNTHETIC EUGLENIDS (*PHACUS* DUJARDIN)*

4.1. Introduction

4.1.1 Introduction to *Phacus* systematics

Phacus (Dujardin 1841) is a morphologically distinctive clade of photosynthetic euglenids that includes rigid cells that are dorsoventrally flattened. Most species have an elongated caudal process and longitudinally arranged pellicle strips (Fig. 4.1). Several species of *Phacus* consist of three lobes and are deltoid in transverse section, while other species have become twisted around their longitudinal axis in a corkscrew fashion (e.g., *P. inflexus* and *P. similis*, Fig. 4.1e; Huber-Pestalozzi 1955). Molecular phylogenetic analyses have demonstrated that the genus was polyphyletic, and several species formerly grouped within *Phacus* based on light microscopical observations have subsequently been moved to other rigid photosynthetic genera, namely *Monomorphina* and *Cryptoglena* (Marin et al. 2003). The molecular phylogenetic relationships within *Phacus* sensu stricto, however, remain poorly understood (Linton et al. 2000, Müllner et al. 2001, Brosnan et al. 2003, Marin et al. 2003, Nudelman et al. 2003, Triemer et al. 2006, Kosmala et al. 2007).

Comparative analyses of morphological data, particularly pellicle characters, are expected to help build a phylogenetic framework for understanding the overall diversity of *Phacus*. Kosmala et al. (2007) found that characters visible using light microscopy, such as the presence or absence of transverse struts, were good taxonomical characters in delimiting species, particularly *P. pleuronectes* and *P. orbicularis*. Leander and Farmer (2000a, b, 2001a, b, Leander et al. 2001) used scanning and transmission electron microscopy (SEM and TEM, respectively) to describe pellicle characters which, when incorporated into cladistic analyses and compared with molecular data, provided robust inferences about euglenid phylogeny. Their

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sampling of *Phacus*, however, turned out to include only three members of *Phacus* sensu stricto (other taxa belonged to *Lepocinclis* and *Monomorphina*; Leander and Farmer 2001b, Marin et al. 2003).

While the leaf-like morphology described by Dujardin (1841) is predominant in *Phacus* sensu stricto, a number of taxa described as *Phacus* (and not yet placed in other genera based on phylogenetic analyses) deviate from it in one or more characters. For example, *P. triqueter* and *P. warszewiczii* are conspicuously tri-lobed rather than being dorsoventrally flattened per se (Fig. 4.1h-j; Huber-Pestalozzi 1955, Leander and Farmer 2001b), and *P. warszewiczii* is illustrated with helically arranged pellicle strips (Huber-Pestalozzi 1955). Moreover, taxa such as *P. segretii* (Fig. 4.1g) and *P. stokesii* lack a caudal process and instead have rounded posterior ends (Huber-Pestalozzi 1955). Other taxa, such as *P. parvulus* and *P. pusillus*, are described as having extremely blunt caudal processes (Huber Pestalozzi 1955; Fig. 4.1k). To date, only one of these atypical taxa (*P. triqueter*) has been included in molecular or morphological phylogenetic analyses (Leander and Farmer 2001b, Marin et al. 2003).

One pellicle character that has been informative in previous studies of euglenid evolution and taxonomy is posterior strip reduction: patterns formed on the posterior cell surface by pellicle strips of different lengths (e.g., Leander and Farmer 2000a). The presence of uniquely modified patterns of posterior reduction in some species of *Phacus* indicate that it may be particularly useful in resolving relationships within the genus and forming inferences regarding pellicle character evolution (Leander and Farmer 2001b). Because of the complex evolutionary history and developmental processes underlying the formation of these patterns (Esson and Leander 2006, 2008), a brief review of their diversity and structure is included below.

4.1.2 Evolutionary significance of posterior strip reduction

The cytoskeleton of euglenids, in addition to a corset of microtubules and a network of endoplasmic reticulum, is reinforced by 4-120 proteinaceous strips that lie beneath the plasma membrane and extend longitudinally or helically from the anterior canal region to the posterior

end of the cell (Leander et al. 2007). The number of pellicle strips around the cell periphery is more or less consistent within species and is referred to using the variable "*P*" (Leander and Farmer 2000a). In photosynthetic euglenids, however, some strips are too short to reach the posterior end of the cell and instead terminate at a certain point along the length of the cell. The length of any particular strip depends on its relative age: pellicle strips are duplicated and inherited semi-conservatively, where existing strips resume and terminate growth with each subsequent round of cytokinesis. Just before cytokinesis, a new strip forms between every pair of existing strips, and these are the youngest strips on the pellicle of any cell (i.e. those strips formed during the most recent round of pellicle duplication and cytokinesis). The youngest strips are shorter than all other pellicle strips, while the oldest strips reach the posterior tip of the cell (Esson and Leander 2006).

Age-related length differentiation varies between species so that some species will have strips of two different lengths, while other species can have up to five different strip lengths (Leander and Farmer 2000a). Because pellicle duplication is semi-conservative, younger, shorter strips alternate with older, longer strips (Hofmann and Bouck 1976, Mignot et al. 1987, Bouck and Ngo 1996, Esson and Leander 2006); the younger strips terminate before reaching the posterior tip of the cell and form a radial pattern or "whorl" on the cell surface. The strips that lie between the strips forming a whorl are older strips that extend either to the posterior tip or to a more posterior whorl, depending on the relative age of the strips and the degree of length differentiation in the species (Fig. 4.2a; Leander and Farmer 2000a, Esson and Leander 2006, 2008).

The number of posterior whorls, denoted as "W_p", can therefore be described in terms of the degree of strip length differentiation in a given species or culture strain. Increased differentiation in strip lengths reflect higher W_p values and are thought to be the result of changes in developmental timing (i.e. heterochrony) associated with the termination and resumption of strip growth during pellicle duplication (Esson and Leander 2006). Some *Euglena* and *Lepocinclis* species exhibit length differentiation within a single whorl, indicating that factors other than age contribute to strip length (Leander and Farmer 2000a, b, Esson and Leander 2008).

The number of posterior whorls of reduction is consistent within a species and within some genera, such as *Trachelomonas* and *Strombomonas* (Brosnan et al. 2005). So far, posterior strip reduction patterns have been described for only three species of *Phacus*, and these patterns differ markedly from the radially symmetrical whorled reduction observed in all other photosynthetic taxa, including other rigid genera such as *Lepocinclis* (W_p = 1-3; Conforti and Tell 1983; Leander and Farmer 2000a, b; Leander et al. 2001) and *Monomorphina* (W_p = 2; Leander and Farmer 2001b; Leander et al. 2001; Nudelman et al. 2006). *Phacus triqueter* is described as possessing three whorls of strip reduction that are distorted by the three lobes of the deltoid-shaped cell (Leander and Farmer 2001b). *Phacus oscillans* has one whorl of strip reduction as well as an additional terminating strip, causing the number of strips around the cell periphery to reduce by half plus one (Leander and Farmer 2001b). *Phacus acuminatus* (identified as *Phacus brachykentron* in Leander and Farmer 2001b; Triemer et al. 2006) has one whorl of strip reduction that is distorted by the dorsoventral flattening of the cell; the terminating strips on the dorsal and ventral sides of the cell are closer to the posterior tip than the lateral terminating strips. Consequently, adjacent lateral strips terminate beneath whorl I, forming symmetrical clusters of four strips on either side of the caudal process (Leander and Farmer 2001b). This "clustered" posterior reduction has yet to be recorded in any other euglenid genus.

We studied the pellicle surface patterns of eight additional *Phacus* taxa – *P. acuminatus*, *P. longicauda* var. *tortus*, *P. pleuronectes*, *P. pusillus*, *P. orbicularis*, *P. segretii*, *P. similis*, and *P. warszewiczii* – and a close relative of the clade consisting of *Phacus* and *Lepocinclis*, namely *Discoplastis spathirhyncha*. By generating several new sequences, we ensured that SSU and partial LSU rDNA sequences were available from each of these species. This approach enabled us to (1) improve our understanding of *Phacus* diversity, (2) interpret pellicle characters in a molecular phylogenetic context, (3) examine the significance of pellicle diversity in reconstructing evolutionary trends along the *Phacus* lineage, and (4) establish a broader framework for understanding developmental processes associated with the diversification of the euglenid pellicle.

4.2 Materials and methods

4.2.1 Culture sources and conditions

Cultures were either purchased from culture collections or grown from single cells isolated from freshwater sources located in or near Vancouver, Canada; strain sources and numbers are listed in Table 4.1. Cultures were maintained in LM_7 (*P. segretii*, *P. longicauda* var. *tortus*, *P. acuminatus*, *P. inflexus*, *P. pleuronectes*, *P. warszewiczii*, *P. pusillus*; ACOI, http://www1.ci.uc.pt/botanica/ACOI_M~1.htm) or a modified soil water medium supplemented with either 1/8 of a pea (*P. orbicularis*) or vitamin B_{12} (*D. spathirhyncha*) (modified from Pringsheim 1946) at 17-18 ^{o}C with a 12 hour light:dark cycle.

4.2.2 Scanning electron microscopy and replicate observations

Cells in culture were placed in a petri dish whose lid was fitted with filter paper and fixed using osmium tetroxide vapor as previously described (Esson and Leander 2006). Cells were placed on filters and critical point dried with CO_2. Once the filters were mounted on stubs, cells were coated with either gold or a combination of gold and palladium. Stubs were viewed on a Hitachi S4700 scanning electron microscope.

While previous surface pattern descriptions (e.g., Leander and Farmer 2001b) were based on multiple cells with the same character clearly visible, the flattening and twisting of many *Phacus* species results in cells lying in positions where a given character, especially posterior strip reduction, cannot be clearly viewed in its entirety. Nevertheless, important information can be collected from a number of cells and synthesized to provide an accurate description of a given character. Between ten and fifty cells were observed for each taxon, and composite descriptions of relevant characters were created from these data.

4.2.3 DNA extraction, PCR amplification and cloning

Genomic DNA was extracted from *Phacus acuminatus*, *P. inflexus*, *P. longicauda* var. *tortus*, *P. orbicularis*, *P. pleuronectes*, *P. pusillus*, *P. segretii*, and *P. warszewiczii* using either a standard CTAB protocol (Breglia et al. 2007) or the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Nuclear SSU and LSU rDNA sequences were amplified on either a PTC-100 Peltier Thermal Cycler or a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA). Polymerase chain reaction (PCR) was performed using a total volume of 25 μl and the PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Small subunit (SSU) sequences were amplified as one or two fragments using combinations of the primers listed in Table 4.2; partial LSU sequences were amplified as one fragment using the primers in Table 4.2. Bands of the expected size were excised from agarose gel and cleaned using the UltraClean™ 15 DNA Purification kit (MO Bio, Carlsbad, California) according to instructions. Purified sequences were cloned using the TOPO TA kit (Invitrogen). Plasmids containing inserts were recovered using FastPlasmid Mini (Eppendorf) or GeneJet Plasmid Miniprep (Fermentas) kits and sequenced using BigDye 3.1, with forward and reverse vector primers and appropriate internal primers. Sequencing was performed using either a 3730S 48-capillary sequencer or a PRISM 377 automated sequencer (Applied Biosystems).

4.2.4. Molecular phylogenetic analyses

In addition to the sequences we obtained for this study, previously published nuclear SSU rDNA and partial LSU rDNA sequences for *Phacus* strains and other taxa were acquired from GenBank; strain information and accession numbers are listed in Table 4.1. Sequences from strains belonging to the same species as our own cultures (*P. orbicularis*, *P. pleuronectes* and *P. acuminatus*) were included to ensure that these cultures had been accurately identified using light microscopy. While an attempt was made to obtain the entire SSU gene for all taxa examined in this study (new SSU sequences ranged in length from 2110 to 2208 bases), only the second half

of the gene (977 bases) could be obtained from one taxon, namely *P. segretii*. New LSU sequences ranged in length from 1106 to 1654 bases.

Molecular phylogenetic analyses were performed on three alignments combining SSU and partial LSU sequences: (1) a 24-taxon alignment with nine outgroup sequences and 2026 sites (maximum likelihood analysis), (2) a 24-taxon alignment with nine outgroup sequences and 2024 sites (Bayesian analysis), and (3) a 23-taxon alignment (excluding *Phacus segretii*) with nine outgroup sequences and 2026 sites (maximum likelihood). Sequences were pairwise aligned in MacClade 4 (Maddison and Maddison 2000) using an alignment from a previously published study as our guide (Triemer et al. 2006). Sequences were further aligned by eye. Gaps and ambiguously aligned bases were excluded.

Phylogenetic analyses using maximum likelihood (ML) was performed on the first and third alignments using PhyML (Guindon and Gascuel 2003; Guindon et al. 2005), using a general time reversible (GTR) model of base substitutions (Rodríguez et al. 1990) and incorporating gamma distribution with four rate categories and invariable sites; 100 ML bootstrap replicates were performed using the same settings. The trees inferred from the first and third alignments were rooted in TreeView (Page 1996) using all nine outgroup taxa as the outgroup.

Bayesian analysis was performed on the second alignment (24 taxa and 2024 bases) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) under the GTR model with invariable sites, gamma distribution and four Monte-Carlo-Markov Chains (MCMC). A total of 2,000,000 generations were calculated with trees sampled every 50 generations and with a prior burn-in of 100,000 generations (i.e. 2000 sampled trees were discarded). A majority rule consensus tree was constructed from 38,000 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees. The Bayesian tree was rooted in TreeView using *Euglena viridis* as the outgroup.

4.3 Results

4.3.1 Description of clustered reduction

Clustered strip reduction in various forms appeared in all taxa whose surface morphology was examined, except for *D. spathirhyncha* and *P. warszewiczii* (Fig. 4.3a-b). In order to describe the differences in these patterns between taxa, it is helpful to identify the main components of clustered reduction and compare these patterns to radially symmetrical, whorled patterns (Fig. 4.2). The main features of two-whorled exponential reduction are summarized in Fig. 4.2a and d. Clustered reduction (Fig. 4.2b-c, e-f) is a distortion of whorled reduction that is often associated with dorsoventral cell flattening in *Phacus*. Length differentiation between different generations of strips still results in whorls of reduction, but the ventral and dorsal strips forming a whorl terminate closer to the posterior tip of the cell than the lateral strips do, resulting in whorls that are ovoid or otherwise misshapen rather than circular in outline. Furthermore, some mature strips that would reach the posterior tip in cells with regular whorled strip reduction terminate before reaching the posterior tip, forming clusters on either side of the cell (Fig. 4.2b).

The relationship between whorled and clustered strip reduction is best described in terms of the relative distance of strip terminations from the posterior tip of the cell, or real or apparent differential strip length (Fig. 4.2c). Clustered patterns are derived from developmental processes whereby strips in a given generation undergo unequal length differentiation, so that some strips terminate closer to the posterior end of the cell than their co-generational strips. For example, if dorsal and ventral strips in a whorl of reduction extend while the lateral strips belonging to the same whorl do not, that whorl acquires an ovoid shape. Similarly, if lateral strips that would normally reach the posterior tip of the cell shorten while ventral and dorsal tip strips retain their length, clusters are formed from the lateral strips (Fig. 4.2c, e-f).

4.3.2 Descriptions of pellicle surface patterns in *Discoplastis* and *Phacus*

Surface patterns and sample sizes for the taxa examined in this study are summarized in Table 4.3. *Discoplastis spathirhyncha* had P = 32 pellicle strips arranged in a clockwise helix (when viewed from the posterior end). Strips reduced over two whorls of exponential reduction (W_p = 2) of sixteen and eight terminating strips, respectively (Fig. 4.3a). Most cells observed had tips with four to seven strips instead of the predicted eight, but additional whorls were never detected – the few additional terminating strips observed near the posterior tip in some cells did not conform to any recognizable pattern. Cells gradually tapered over the posterior half to form a sharp caudal process.

Phacus warszewiczii had P = 32 pellicle strips that were arranged in an anti-clockwise helix when viewed from the posterior end (Fig. 4.3b). The helical pitch of the strips was reduced at the caudal process so that strips were arranged almost longitudinally (Fig. 4.1i, 4.3b). Strips reduced over three exponential whorls of reduction (W_p = 3), leaving four strips at the posterior tip. Struts were present on strips until they reached the caudal process (Fig. 4.3b).

Phacus segretii (P = 32) had more or less longitudinal strips that began to twist in an anti-clockwise direction near the posterior end of the cell, which completely lacked a caudal process (Fig. 4.1g). Two distorted whorls of exponential reduction (W_p = 2) were observed. The eight strips that comprised the second whorl usually formed a “figure eight” shape, when the posterior ends of terminating strips were connected by consecutive straight lines (Fig. 4.3c). In addition to the exponential whorls, there were two additional terminating strips that were located on opposite sides of the cell from one another; towards the anterior of the cell and prior to strip reduction, these two strips were separated from one another on both sides by 15 strips.

Phacus acuminatus (P = 32) had one flattened whorl of exponential reduction. Whorl I strips on the dorsal and ventral sides of the cell contributed to the short, blunt caudal process and terminated closer to the posterior tip than the lateral whorl I strips. Additional terminating strips formed a cluster on each side of the caudal process, leaving five to six strips at the posterior tip of

the cell (Fig. 4.4a-b). Pellicle strips maintained a longitudinal orientation over the entire cell surface. However, six cells showed a very slight clockwise twist at the posterior tip.

Phacus similis (*P* = 20) had longitudinally oriented strips and anti-clockwise twisted cells when viewed from the posterior end of the cell (Fig. 4.4c-d). Strips reduced over one whorl of exponential reduction that followed the deformation of the cell. Some whorl I strips extended down the caudal process near the posterior tip of the cell, and some terminated further up the cell on ridges formed by cell flattening and twisting (Fig. 4.4c-d). Additional terminating strips formed clusters of one to three strips on one side of the caudal process and two to four strips on the other side. The total number of cluster strips on a cell ranged from five to seven, leaving three to five strips at the posterior tip of the cell. As with *P. acuminatus*, some cluster strips terminated so close to the posterior tip of the cell that the exact number of strips in a cluster was difficult to determine (Fig. 4.4c).

Phacus pusillus cells had *P* values ranging from 20 to 26 strips (Table 4.3). Pellicle strips were longitudinal to slightly helical until they reached the posterior end of the cell, where the strips' helical pitch increased to produce a pronounced anti-clockwise pattern (Fig. 4.4e-f). Strips reduced over one whorl of reduction that was deformed along with cell flattening and twisting. In some cells, the terminating strips formed a figure eight pattern when connected by consecutive straight lines, similar to whorl II in *P. segretii* (Fig. 4.3c and 4.4f). In most cells, five or six strips reached the posterior tip, leaving four or five clustered strips in cells with *P* = 20 (20 strips less ten whorl I strips less five or six tip strips leaves four to five cluster strips) and seven or eight clustered strips in cells with *P* = 26 (Fig. 4.4e-f).

Phacus pleuronectes (*P* = 32) (Fig. 4.5a-b) and *P. orbicularis* (Fig. 4.5c-d) each had two flattened whorls of exponential reduction. Strips were longitudinally oriented along the cell body and twisted very slightly at the caudal process. Most of the caudal processes in *P. pleuronectes* were twisted anti-clockwise (19 of 21 cells); of these, 12 were twisted anti-clockwise along part of the process and then clockwise at the posterior tip. In *P. pleuronectes*, three to four strips reached the posterior tip of the cell, leaving two lateral clusters of two to three strips on one side and three strips on the other (Fig. 4.5a). In *P. orbicularis*, clusters were each composed of one or

two strips, leaving five to six strips at the posterior tip of the cell (Fig. 4.5c-d). Strips were oriented longitudinally down the entire length of the cell; however, the caudal process often displayed a slight clockwise twist (as viewed from the posterior end). In *P. orbicularis*, the strips exhibited robust transverse struts.

Phacus longicauda var. *tortus* had *P* = 32 strips and two flattened whorls of strip reduction (Fig. 4.5e-f), much like *P. pleuronectes* and *P. orbicularis*. Strips were oriented longitudinally along the cell and anti-clockwise at the cell posterior end; the cell body itself was twisted in a slight anti-clockwise helix. Whorls I and II were distorted due to twisting of the cell (Fig. 4.5f). Whorl I strips terminated before reaching the long, thin caudal process, while whorl II strips extended slightly past the base of the caudal process (Fig. 4.5e). Clusters of one to two strips on one side and two to three strips on the other side of the cell were present (Fig. 4.5f). Additional terminating strips along the extremely narrow caudal process resulted in only two to three strips reaching the sharp posterior tip of the cell (Fig. 4.5e). Transverse struts were present on pellicle strips over most of the cell but not the caudal process (Fig. 4.5f); the struts were less well defined than those in *P. warszewiczii* and *P. orbicularis*.

4.3.3 Phylogeny of *Phacus* as inferred from small and large subunit ribosomal DNA

While there were differences in topology between the maximum likelihood (ML) and Bayesian phylogenies, several important relationships were recovered in all analyses (results from 23-taxon analysis not shown). Maximum likelihood and Bayesian analyses recovered the monophyly of *Phacus* with high support (Fig. 4.6). *Phacus* and *Lepocinclis* formed well supported sister clades, and *Discoplastis* formed the basal sister lineage to these two clades with moderate statistical support. The early divergence of *P. warszewiczii* from the other *Phacus* species was strongly supported in the ML and Bayesian analyses (Fig. 4.6). A clade comprising *P. oscillans*, *P. similis*, *P. inflexus*, *P. parvulus* and both strains of *P. pusillus* – the so-called "*oscillans* clade" (after Marin et al. 2003) – was recovered with high support in all analyses. The *oscillans* clade consisted of two subclades: (1) *P. oscillans*, *P. similis* and *P. inflexus*, and (2) *P. parvulus* and

both *P. pusillus* strains. The position of the *oscillans*-clade within the genus, however, was unresolved. *Phacus longicauda* var. *tortus* and *P. triqueter* grouped together in the Bayesian analysis with high support but with weak support in the ML analysis of 24 taxa. When *Phacus segretii* was removed from the alignment prior to performing ML analyses, however, *P. longicauda* var. *tortus* and *P. triqueter* grouped together with high support (ML Bootstrap = 92; data not shown). In fact, the removal of *P. segretii* from the alignment increased support for several relationships. The separation of the clades containing (1) *P. pleuronectes*, *P. acuminatus* and the *oscillans* clade and (2) *P. longicauda* var. *tortus*, *P. triqueter* and *P. orbicularis*, was more highly supported (ML Bootstrap = 82; data not shown). The sister relationship between *Lepocinclis* and *Phacus* was also slightly better supported when *P. segretii* was excluded from analysis (ML bootstrap = 99; data not shown).

4.4 Discussion

4.4.1 A molecular phylogenetic framework for *Phacus*

Although some of the deeper branches within *Phacus* were not consistently recovered or highly supported in our molecular phylogenetic analyses, several conclusions can be drawn from these trees. *Phacus* as it is currently defined is monophyletic and all taxa used in this study that have been classified as *Phacus* should remain in the genus. *Phacus warszewiczii* is among the earliest diverging *Phacus* species, a conclusion reinforced by morphological data (see below). Since sequence data from *P. warszewiczii* was not incorporated in previous studies of euglenid molecular phylogenetics, this conclusion cannot be directly compared with any hypotheses regarding intrageneric relationships in *Phacus* resulting from those studies. Both Bayesian and ML analyses indicate that *P. longicauda* var. *tortus* and *P. triqueter* are closely related to one another in spite of their somewhat divergent morphology (Fig. 4.1c, h). However, the long branches associated with these taxa may be prone to the long-branch attraction artifact and we therefore interpret these results with caution. The precise phylogenetic position of *P. segretii*,

another morphologically derived taxon yielding long branches in our molecular analyses, proved elusive.

The so-called "*oscillans* clade", consisting of *P. oscillans*, *P. similis*, *P. inflexus*, both *P. pusillus* strains and *P. parvulus*, was recovered with high support in all analyses (Fig. 4.6). In previously published SSU and SSU/LSU phylogenies where any combination of these taxa is included, they invariably branch together with congruent topologies to the exclusion of other *Phacus* taxa (Brosnan et al. 2003, Marin et al. 2003, Triemer et al. 2006, Kosmala et al. 2007). The phylogenetic position of *Phacus parvulus* (ASW 08060) in our analyses suggests that this strain, or one or both of the *P. pusillus* strains, might be misidentified. Unfortunately, no other data are available at this time to confirm this speculation.

4.4.2 Evolution of clustered strip reduction patterns in *Phacus*

Clustered reduction was originally described in a strain of *Phacus acuminatus* (*brachykentron*; UTEX LB 1317) with one whorl of exponential reduction (W_p = 1) (Leander and Farmer 2001b). Prior to the study reported here, clustered reduction had not been observed in any other species of euglenid, even in the other two *Phacus* species previously examined: *P. oscillans* has a single additional terminating strip, while *P. triqueter* was reported as possessing three distorted whorls of exponential reduction (Leander and Farmer 2001b). Our SEM data show that clustered reduction is in fact widespread within *Phacus* (Fig. 4.7); its absence in *P. warszewiczii* indicates that clustered reduction was derived after the divergence of the genus from a photosynthetic ancestor with two or three whorls of strip reduction (W_p = 2 - 3) (Fig. 4.7-4.8). This character state is shared with *Discoplastis spathirhyncha* (4.3a) and all members of *Lepocinclis* for which posterior reduction has been described, with the exception of *L. salina* (W_p = 1; Conforti and Tell 1983; Leander and Farmer 2000a; Leander et al. 2001). We have shown that clustered reduction can be associated with two whorls of strip reduction (W_p = 2) as well as with one whorl of strip reduction (W_p = 1) (Fig. 4.8). The phylogenetic distribution of taxa with two-whorled clustered reduction suggests that this state evolved prior to taxa with a single whorl and

clustered reduction, which is limited to *P. acuminatus* and members of the *oscillans*-clade (Fig. 4.7-4.8).

The clusters of strips described by Leander and Farmer (2001b) were symmetrical – each cluster was comprised of four terminating strips positioned laterally on the cell. None of the species with clustered reduction described in this study had consistently symmetrical clusters, and most had consistently asymmetrical clusters. Members of the *oscillans* clade had particularly exaggerated asymmetry: the smaller cluster in *P. pusillus* was sometimes comprised of one strip; the additional terminating strip observed in *P. oscillans* (Leander and Farmer 2001b) appears to be the only remnant of the clusters that were present in its ancestors (Fig. 4.7-4.8).

Clustered reduction has also been minimized in *P. segretii* and *P. triqueter*. We interpret the two additional terminating strips in *P. segretii* as vestigial clusters (Fig. 4.8); they do not belong to either whorl I or whorl II and are therefore relatively mature strips. Furthermore, their location opposite one another is reminiscent of the location of lateral clusters of strip reduction observed in other taxa. *Phacus triqueter*, on the other hand, has distorted whorls but lacks strip clusters per se. Reexamination of electron micrographs used in a previous study (Leander and Farmer 2001b), combined with the distribution of character states inferred from the present study (Fig. 4.7) suggest that this taxon actually has two whorls of exponential reduction and several other terminating strips along the narrow caudal process, rather than three whorls of reduction. Although *P. triqueter* and *P. warzsewiczii* share a deltoid cell shape, the three whorls on *P. warszewiczii* are positioned on or near the caudal process and thereby avoid distortion by the pronounced deltoid shape of the cell. The whorls in *P. triqueter* are comprised of some strips that terminate on the caudal process and other strips that terminate further up the cell body, resulting in distorted whorls (see Fig. 4b in Leander and Farmer 2001b). This distortion seems to be a vestige of the clustered reduction present in *P. triqueter*'s inferred ancestor (Fig. 4.7-4.8).

The vestiges of clustered strip reduction in *P. oscillans*, *P. segretii* and *P. triqueter* raise some questions regarding the developmental origins of clustered reduction. While clusters and whorl distortion are related to dorsal-ventral flattening in *Phacus*, there appear to be other factors leading to the development and evolution of these patterns. *Phacus segretii* and *P. oscillans* have

rounded cells that do not pose any spatial restrictions on strips at their posterior ends that would necessitate clustered reduction (a modification of the “optimal packing hypothesis” as proposed, and somewhat refuted, by Leander et al. 2001). Similarly, the three-lobed cells of *P. triqueter* do not, in and of themselves, require distorted whorls, as *P. warszewiczii* clearly demonstrates (Fig. 4.8). *Phacus pusillus*, moreover, has clusters located on its ventral and dorsal surfaces (Fig. 4.4e-f), rather than its lateral margins, where space should be more restricted. It is possible that further taxon sampling will demonstrate *Phacus* species that have lost all traces of clustered reduction, indicating that as the degree of dorsoventral flattening is decreased, clusters of reduction are lost. On the other hand, flattened and twisted heterotrophic euglenids such as *Heteronema spirale*, a bacterivore that completely lacks posterior strip reduction (S. A. Breglia, University of British Columbia, personal communication), show that there must be other factors underlying the complex length differentiation patterns observed in *Phacus* and other photosynthetic euglenids.

The presence of clustered strip reduction in *Phacus*, but not in other photosynthetic taxa, suggests that there are developmental processes governing differential strip length that are peculiar to this genus of rigid cells. SEM studies similar to those previously undertaken (e.g., Esson and Leander 2006) should be integrated with previous observations of cell division in *Phacus* (Pochmann 1942) to better understand the interaction between bilateral symmetry and other developmental stages in pellicle duplication and cytokinesis, such as the placement of cleavage furrow strips (Esson and Leander 2006).

Two culture strains identified as the same species (*P. acuminatus*/*brachykentron* UTEX LB 1317 and *Phacus acuminatus* UBC) have similar, but not identical, patterns of strip reduction. Both strains have one whorl of exponential reduction supplemented by lateral clusters, but the clusters in the UTEX strain are unequivocally symmetrical (Leander and Farmer 2001b), while the clusters in the UBC strain appear to be asymmetrical in most cells. Furthermore, the strips are arranged in a conspicuous clockwise helix at the tip of the caudal process in the UTEX culture (see Fig. 5g in Leander and Farmer 2001b), while in the UBC culture a longitudinal orientation is maintained in most cells. These differences (indeed, all differences in posterior strip reduction described in this study, particularly cluster strip distribution) likely have taxonomic implications,

but they require scanning electron microscopy to observe. Strip reduction in both strains of *P. acuminatus* suggests that differences within closely related taxa such as species varieties are slight and, at this point, mainly qualitative, making posterior strip reduction an impractical taxonomic character to use in ecological or biogeographical investigations at this time.

4.4.3 Evolution of other pellicle surface characters in *Phacus*

The evolution of total strip number (*P*) in euglenids has previously been described in terms of behavioral ecology (i.e. a large *P* value facilitates metaboly via sliding between pellicle strips and, therefore, allows ingestion of large prey) and developmental processes (i.e. pellicle duplication combined with failure to divide has resulted in several “strip doubling” events throughout euglenid evolution; conversely, division combined with failure to duplicate the pellicle results in “strip halving” events) (Leander 2004, Leander et al. 2001, 2007). Photosynthesis originated in euglenids via a secondary endosymbiotic event involving eukaryovorous euglenids and green algal prey cells (Gibbs 1978, Leander 2004, Leander et al. 2007). In rigid photosynthetic euglenids like *Phacus*, behavioral and other locomotive requirements associated with predatory modes of feeding (e.g., gliding motility and metaboly) are no longer selected for. This could be one reason why many members of the *Phacus* and *Lepocinclis* clades have a relatively low number of strips ($P \leq 32$).

With a few exceptions [such as the larger, semi-rigid *L. helicoideus* (P=80) (Leander and Farmer 2000b) and the taxa with P = 20 strips described here], members of this clade possess P = 32 strips (Fig. 4.7-4.8; Leander et al. 2001). The taxa examined in this study, with the exception of the members of the *oscillans* clade, all have P = 32 strips – including *Discoplastis spathirhyncha*, which forms the sister lineage of the clade comprising *Lepocinclis* and *Phacus* (Marin et al. 2003) (Fig. 4.6). While *D. spathirhyncha* shares other morphological features with members of this clade (namely multiple disc-shaped plastids lacking pyrenoids; Triemer et al. 2006), further molecular and morphological work is required to more robustly resolve its relationship to these taxa and to determine whether these features are plesiomorphic or

synapomorphic (Triemer et al. 2006). Since *P* = 20 strips is shared by all members of the *oscillans* clade whose surface morphology has been described (that is, *P. oscillans*, *P. pusillus* and *P. similis*), it may be regarded as a synapomorphy for this clade. It is interesting to note, however, that the *P*-values recorded for *P. pusillus* in this study have a wider range than those recorded for other taxa (Table 3). It is unclear at this time what evolutionary and taxonomic implications this wide range may have.

Transverse struts were present on the pellicle strips in three of the taxa described in this study: *P. warszewiczii*, *P. longicauda* var. *tortus*, and *P. orbicularis*. Leander and Farmer (2001b) also observed struts in *P. triqueter* and to a much lesser degree *L. tripteris*. Because the relationships between these *Phacus* species are unresolved and *L. tripteris* is the only *Lepocinclis* species in which struts have been observed, it is impossible to make conclusive statements about the evolution of this character at this time. However, the presence of struts in both genera suggests that it was present in the most recent common ancestor of both *Phacus* and *Lepocinclis* (Fig. 4.7). Moreover, we observed fine, strut-like striations on the pellicle strips of *D. spathirhyncha* (Fig. 4.3a, inset), which suggests that this feature evolved before the most recent common ancestor of *Discoplastis*, *Phacus* and *Lepocinclis*.

With the exception of *P. warszewiczii*, most *Phacus* taxa have longitudinally arranged strips over most of the cell surface and a twisted caudal process. As previously observed by Leander and Farmer (2001b), however, the handedness of the posterior twist varies between taxa: some taxa, such as *P. acuminatus* (*brachykentron*) and *P. oscillans*, exhibit a clockwise helix when viewed from the posterior end; other taxa, such as *P. triqueter*, *P. longicauda*, and *P. pusillus*, have an anti-clockwise helix. Our observations affirm the observations by Leander and Farmer (2001b) that handedness of pellicle strip orientation is not phylogenetically informative. Members of the well-resolved *oscillans* clade have both clockwise and anticlockwise helices, and there is no certain relationship between the few taxa that exhibit a clockwise twist (*P. orbicularis*, *P. oscillans*, *P. acuminatus*, and *D. spathirhyncha*). Based on previous developmental research regarding the semi-conservative nature of pellicle duplication (e.g., Hofmann and Bouck 1976, Mignot et al. 1987, Bouck and Ngo 1996), Leander and Farmer (2001b) hypothesized that there

should be developmental constraints on the handedness of pellicle strip orientation. Similar studies using strains of *Phacus* (given the combination of pellicle rigidity and the usual change in strip orientation at the cell posterior in this genus) could be particularly informative regarding developmental mechanisms for the evolution of different strip orientations.

4.4.4 Conclusions

Based on the molecular and morphological data presented here, *Phacus* shares with its sister genus *Lepocinclis* the widespread possession of a semi-rigid or rigid pellicle and *P* = 32 pellicle strips. Furthermore, molecular phylogenetic support and the presence of 32 pellicle strips in the plastic taxon *D. spathirhyncha* suggest that *Discoplastis* is the sister taxon to the clade formed by *Phacus* and *Lepocinclis*. Patterns of clustered strip reduction are common in *Phacus*; however, they evolved after the divergence of *P. warszewiczii* and are not, therefore, considered a synapomorphy for the genus.

The posterior strip reduction patterns described in this study suggest that other, unknown factors contribute to strip length differentiation, which has previously been explained in terms of strip maturity (Esson and Leander 2006) and position relative to parental strips (Esson and Leander 2008). Strip clusters and strips that terminate outside of any particular exponential whorl are comprised of mature strips belonging to different generations that nevertheless terminate sooner than their co-generational strips on the ventral and dorsal cell surfaces. The presence of these clusters does not always appear to be directly correlated with cell flattening and twisting and often reflect modifications of ancestral states within the group. Therefore, distorted patterns of pellicle strips offer important insights into the development and evolutionary history of the cytoskeleton in *Phacus*, and have the potential to make contributions to our overall understanding of eukaryotic diversification.

Table 4.1. **Taxon names, strain identification and accession numbers of sequences used for molecular phylogenetic analyses in this study.**

Taxon	Strain Identification	GenBank Accession Numbers	
		SSU	LSU
Euglena viridis	SAG 1224-17c	AY523037	DQ140125
Discoplastis spathirhyncha[a]	SAG 1224-42	AJ532454	DQ140100
Colacium mucronatum[b]	UTEX 2524	AF326232	AY130224
Monomorphina pyrum[b]	UTEX 2354	AF112874	AY130238
Trachelomonas lefevrei	SAG 1283-10	DQ140136	AY359949
Lepocinclis ovum	SAG 1244-8	AF110419	AY130235
Lepocinclis steinii (*L. buetschlii* in Leander and Farmer 2000a)[b]	UTEX 523	AF096993	AY130815
Lepocinclis tripteris[b]	UTEX LB 1311	AF445459	AY130230
Phacus acuminatus[a]	UBC culture[c]	**GenBank**	**GenBank**
Phacus acuminatus (*P. brachykentron* in Leander and Farmer 2001b)[b]	UTEX LB 1317	AJ532481	AY130820
Phacus inflexus	ACOI 1336	**GenBank**	**GenBank**
Phacus longicauda var. *tortus*[a]	ACOI 1139	**GenBank**	**GenBank**
Phacus orbicularis	ASW 08054	AF283315	DQ140126
Phacus orbicularis[a]	UBC culture[d]	**GenBank**	**GenBank**
Phacus oscillans[b]	UTEX LB 1285	AF181968	AY1308238
Phacus cf. *parvulus*	ASW 08060	AF283314	DQ140127
Phacus pleuronectes	SAG 1261-3b	AJ532475	AY130824

Table 4.1 (continued)

Taxon	Strain Identification	GenBank Accession Numbers	
		SSU	LSU
Phacus pleuronectes[a]	UTEX LB54 via CCCM 7053	**GenBank**	**GenBank**
Phacus pusillus[a]	ACOI 1093	AJ532472	**GenBank**
Phacus pusillus	UTEX 1282	AF190815	AY130237
Phacus segretii[a]	ACOI 1337	**GenBank**	**GenBank**
Phacus similis[a]	SAG 58.81	AJ532467	AY130239
Phacus triqueter[b]	SAG 1261-8 (=UTEX LB1286)	AJ532485	**Triemer et al. (In press)**
Phacus warszewiczii[a]	ASW 08064	**GenBank**	**GenBank**

[a] Taxa for which pellicle surface morphology is described in this study.

[b] Taxa for which pellicle surface morphology was described by Leander and colleagues (Leander and Farmer 2001b; Leander et al. 2001; Leander and Farmer 2000a).

[c] Isolated from freshwater pond at the University of British Columbia.

[d] Isolated from freshwater pond near Boundary Bay, British Columbia.

Table 4.2. **Primers used in this study for amplification of ribosomal DNA.**

SSU Primer Name	Sequence
475 EUGF (forward)	5'-AAGTCTGGTGCCAGCAGCYGC3'
M917FD (forward)	5'-GGTGAAATTCTTAGAYCG-3'
PF1 (forward)[a]	5'-GCGCTACCTGGTTGATCCTGCC-3'
PHACF (forward)	5'-CTGTGAATGGCTCCTTACATCA-3'
EUGR (reverse)	5'-TCACCTACARCWACCTTGTTA-3'
FAD4 (reverse)[b]	5'-TGATCCTTCTGCAGGTTCACCTAC-3'
Inf 870R (reverse)	5'-CAAGAGGCTGCTTTGAGCACA-3'
PhR4 (reverse)	5'-CAGGTTCACCTACAACAACC-3'
R4 (reverse)	5'-GATCCTTCTGCAGGTTCACCTA-3'
LSU Primer Name	**Sequence**
1F (forward)[c]	5'-TTAAGCATATCACTCAGTGGAGG-3'
CIR (reverse)[c]	5'-GCTATCCTGAGGGAAACTTCG-3'

[a] Previously published by Keeling (2002).
[b] Previously published by Deane et al. (1998) and Keeling (2002).
[c] Previously published by Brosnan et al. (2003).

Table 4.3. **Summary of novel pellicle surface characters described in this study.** Alternate character states are indicated in brackets after the most frequently observed character state. Where character states could be observed directly, the number of cells displaying that state are shown as a fraction of the total sample size for that character, n. Where character states are composite reconstructions, the number of cells observed to arrive at that synthesis is given as n´.

Taxon	Pellicle surface characters					Number of cells observed (N)
	Number of strips around cell periphery (*P*)	Strip orientation	Number of posterior whorls (W_p)	Number of tip strips (*T*)	Number of strips in lateral clusters	
Discoplastis spathirhyncha	32 (31, 33) (8/n=10)	clockwise helical (18/n=18)	2 (12/n=12)	4-7 n´=9	-	18
Phacus acuminatus	32 (30) (8/n=10)	longitudinal (20/n=20)	1 (16/n=16)	5-6 n´=15	4-5 and 5-6 n´=14	20
Phacus longicauda var. *tortus*	32 (28,29) (3/n=5)	longitudinal followed by anti-clockwise posterior twist (18/n=18)	2 (6/n=6)	2-3 n´=9	1-2 and 2-3 n´=5	17
Phacus orbicularis	P=32 (3/n=3)	longitudinal followed by slight clockwise posterior twist (29/n=29)	2 (28/n=28)	5-6 n´=27	1 and 1-2. n´=12	30
Phacus pleuronectes	32 (30, 35, 36, 39, 40, 42) (3/n=9)	longitudinal followed by a slight anti-clockwise posterior twist (31/n=31)	2 (22/n=22)	3-4 n´=28	2-3 and 3 n´=23	31

Table 4.3 (continued)

Taxon	Pellicle surface characters					Number of cells observed (N)
	Number of strips around cell periphery (*P*)	Strip orientation	Number of posterior whorls (W_p)	Number of tip strips (*T*)	Number of strips in lateral clusters	
Phacus pusillus	20, 22, 23, 24, 26 (n=7)	longitudinal followed by anticlockwise posterior twist (39/n=39)	1 (29/n=29)	5-6 n´=28	1-4 and 2-5 n´=26	37
Phacus segretii	32 (29,30) (21/n=24)	longitudinal followed by posterior anticlockwise twist (31/n=31)	2 (27/n=27)	6 (5,7) (20/n=26)	1 and 1 (1 + 0, 1 + 1 +1) (22/n=27)	31
Phacus similis	20 (24) (15/n=19)	longitudinal with anticlockwise-twisted cell (30/n=30)	1 (23/n=23)	3-5 n´=30	1-3 and 2-4 n´=39	57
Phacus warszewiczii	32 (4/n=4)	anticlockwise followed by longitudinal posterior (31/n=31).	3 (28/n=28)	3-4 (2/n=2)	-	31

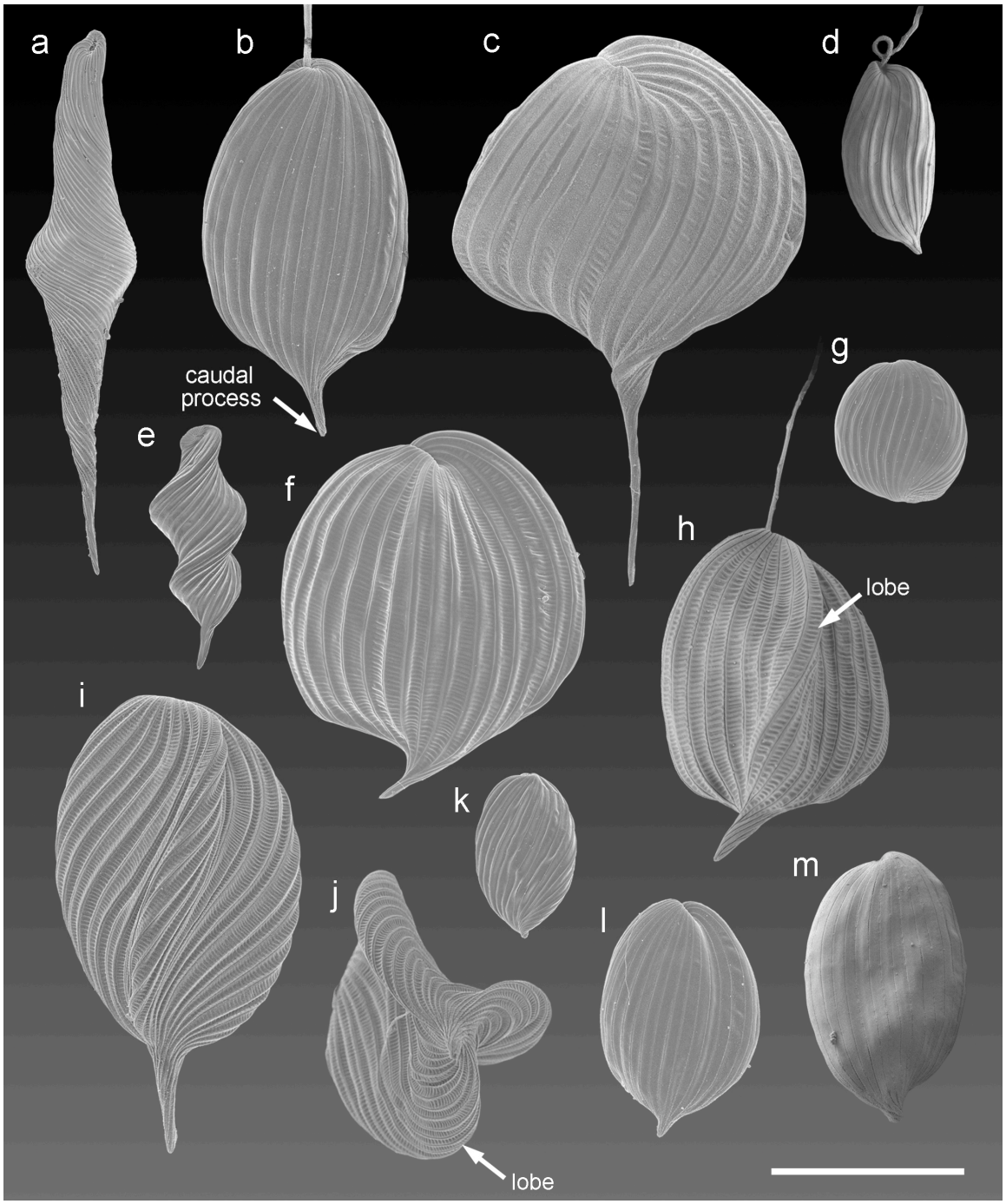

Fig. 4.1. Scanning electron micrographs (SEMs) showing the diversity of *Phacus*. (a) *Discoplastis spathirhyncha*, a closely related lineage to *Phacus* with 32 pellicle strips. (b) *Phacus pleuronectes*. (c) *Phacus longicauda* var. *tortus*. (d) *Phacus oscillans*. (e) *Phacus similis*. (f) *Phacus orbicularis*. (g) *Phacus segretii* showing the rounded posterior end of the cell. (h) *Phacus triqueter*. (i) *Phacus warszewiczii*. (j) Posterior view of *Phacus warszewiczii* showing three lobes of the deltoid shaped cell. (k) *Phacus pusillus*. (l) *Phacus acuminatus*, UBC isolate. (m) *Phacus acuminatus* (*brachykentron*), UTEX LB 1317. Scale bar, 20 μm).

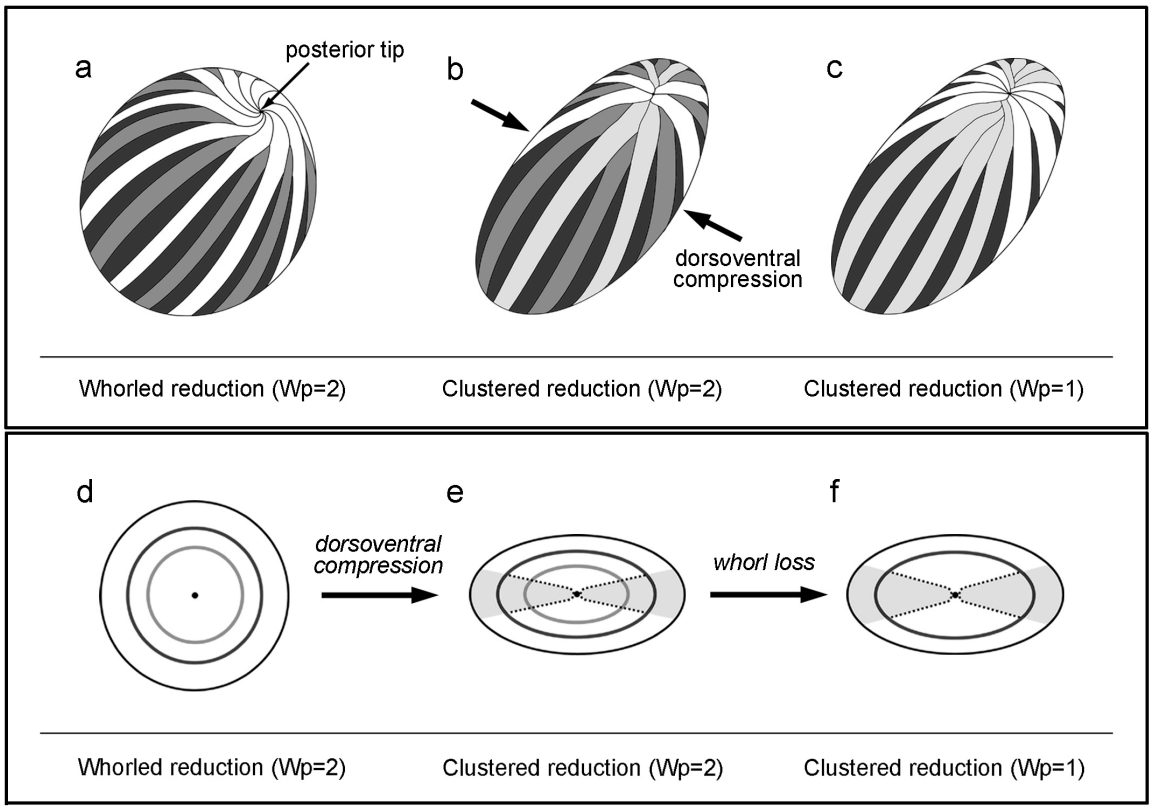

Fig. 4.2. Illustrations comparing whorled (ancestral state) and clustered (derived state) posterior strip reduction. (a) Whorled strip reduction is the result of length differentiation between pellicle strips of different generations: every alternate strip terminates before reaching the posterior of the cell, forming a radial pattern (i.e. a whorl) on the posterior cell surface. Because half of the strips terminate on each whorl, this pattern is also referred to as “exponential” strip reduction. A cell with two whorls of strip reduction, for example, has pellicle strips of three lengths: the younger, shortest strips (black) form the first, anteriormost whorl of posterior reduction. Slightly longer and older strips (dark grey) form the second whorl of reduction, and the longest and oldest strips (white) extend to the posterior of the cell. (b-c) Clustered strip reduction is a modification of whorled reduction (W_p = 2 or W_p = 1) that is associated with dorsoventral compression of cells. Dorsal and ventral strips belonging to whorl I (black) and whorl II (dark grey) now terminate closer to the posterior cell tip than co-generational lateral strips do. Furthermore, mature lateral strips (light grey) no longer extend to the posterior cell tip as they would in whorled reduction, but form clusters of adjacent terminating strips on either side of the posterior tip. The relationship between whorled reduction and different forms of clustered reduction is represented schematically in Fig. d-f. Dorsoventral compression of a cell with two whorls of exponential reduction (W_p = 2; d) results in a cell with two whorls of reduction supplemented by lateral clusters (W_p = 2; e). Loss of one of these whorls yields cells with one whorl of reduction with lateral clusters (W_p = 1; f). Dotted lines indicate the schematic outlines of clustered strips.

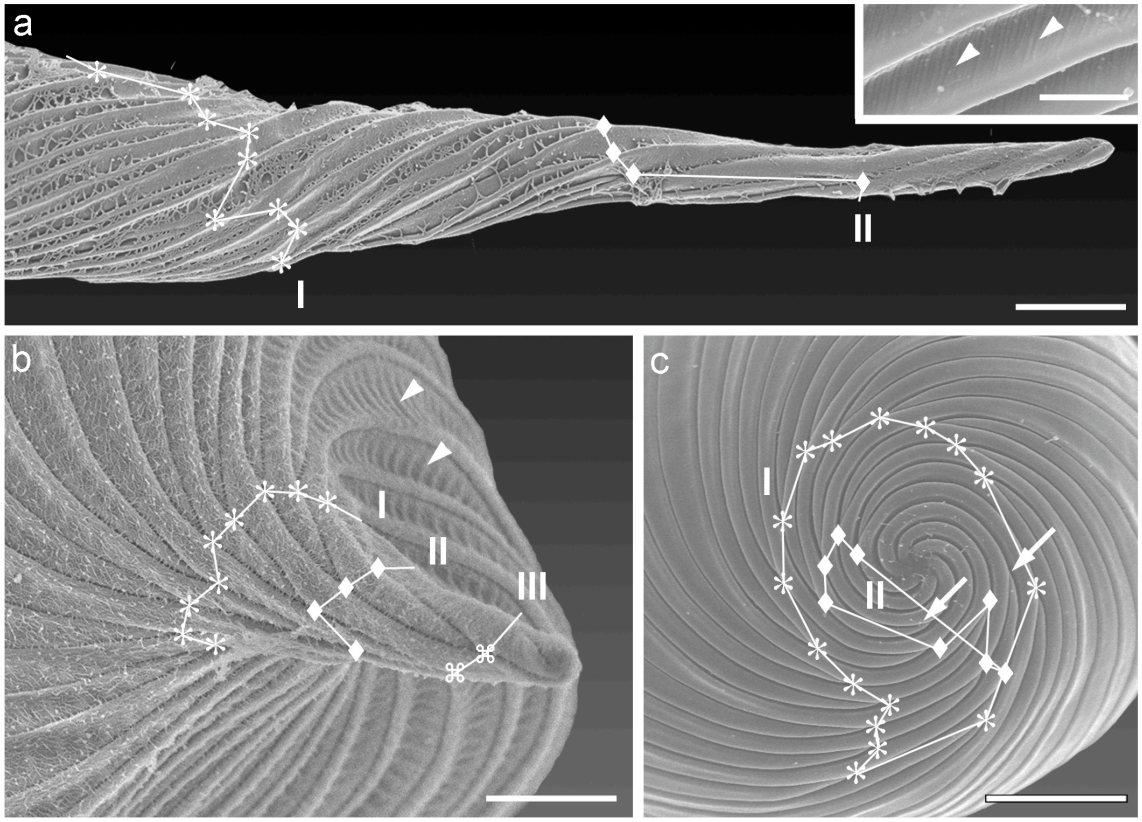

Fig. 4.3. Posterior strip reduction in *Discoplastis spathirhyncha*, *Phacus warszewiczii* and *P. segretii*. (a) *Discoplastis spathirhyncha* has two whorls of exponential strip reduction (W_p = 2) and 32 strips around the cell periphery (P = 32). Scale bar, 2 μm. Inset: High magnification SEM showing transverse strut-like striations on the pellicle strips. Scale bar, 0.5 μm. (b) *Phacus warszewiczii* has three whorls of exponential reduction on the caudal process (W_p = 3). Transverse struts (arrowheads) are present on the pellicle strips. Scale bar, 2 μm. (c) *Phacus segretii* has two whorls of exponential strip reduction (W_p =2); whorl I is slightly distorted, and whorl II forms an asymmetrical "figure eight" shape when adjacent terminating strips are connected by straight lines. Two additional strips (arrows) terminate on opposite sides of the cell. Scale bar, 2 μm.

Figure 4.4 (Next page). *Phacus* species with clustered strips associated with one whorl of exponential strip reduction (W_p = 1). (a-b) *Phacus acuminatus* (*P* = 32). (c-d) *Phacus similis* (*P* = 20). (e-f) *Phacus pusillus* (*P* = 20-26). (a) Posterior view of *P. acuminatus* showing a flattened whorl of strip reduction (asterisks) and symmetrical lateral clusters of four terminating strips (arrows). Scale bar, 5 μm. (b) Lateral view of *P. acuminatus*, showing whorl I strips (asterisks) extending up the dorsal/ventral surfaces of the caudal process and one lateral cluster of four terminating strips (arrows). Scale bar, 3 μm. (c) A lateral view of *P. similis* showing a distorted whorl of strip reduction (asterisks, white line) and a lateral cluster of three or four terminating strips (arrows). The posterior-most terminating strip (left arrow) is a tip strip effectively shortened by cell twisting. Scale bar, 4 μm. (d) A lateral view of *P. similis* showing a distorted whorl of strip reduction and two clustered strips (right arrows). Scale bar, 3 μm. (e) A view of *P. pusillus* showing a distorted whorl extending down the ventral or dorsal surface of the cell and a cluster of four terminating strips (lower arrows). The upper arrow indicates a terminating strip belonging to the cluster on the other side of the cell. Scale bar, 2 μm. (f) Posterior view of *P. pusillus* showing a figure eight-shaped whorl of reduction and two clusters (arrows) consisting of one and three terminating strips, respectively. Scale bar, 2 μm.

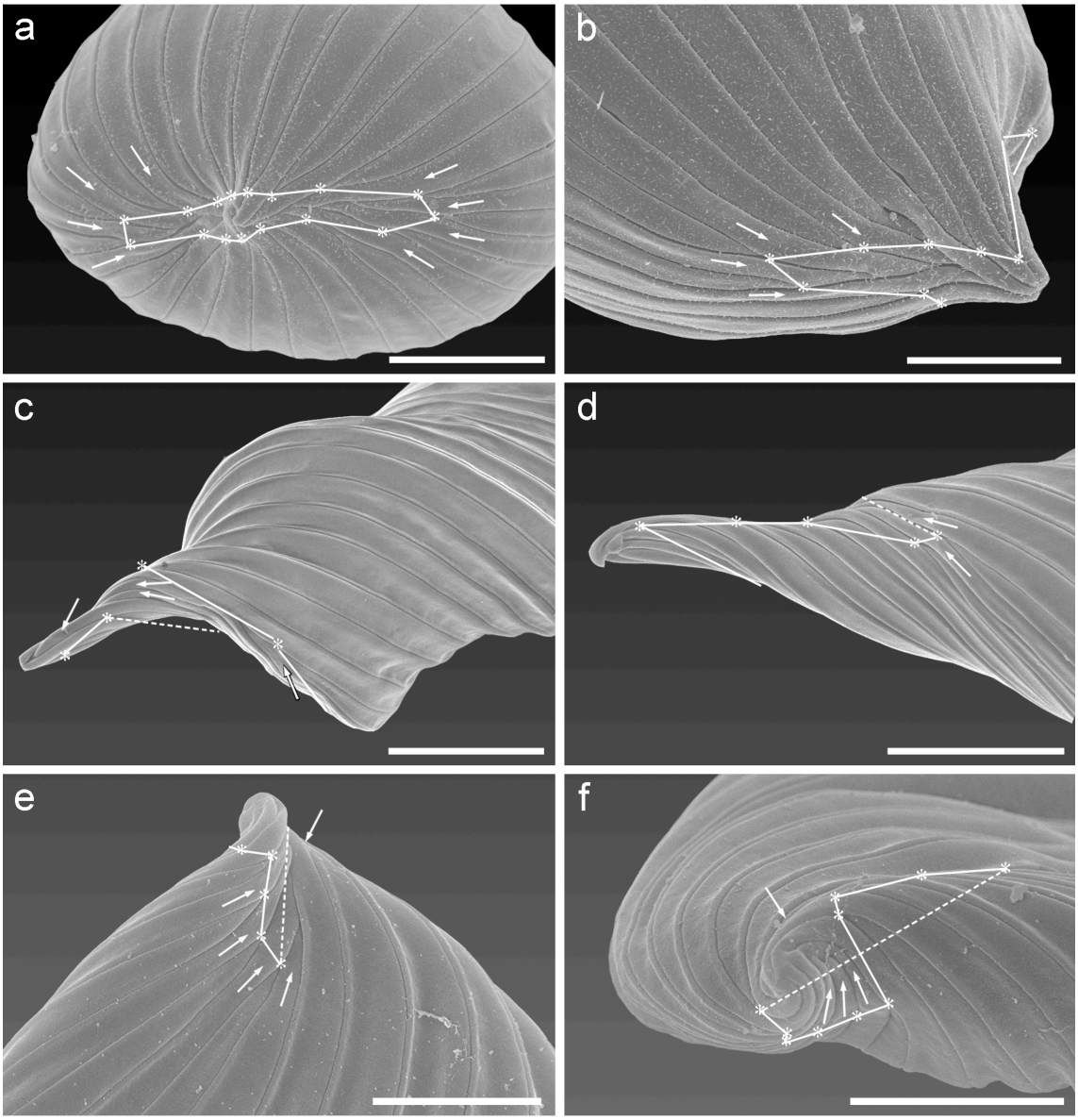

Figure 4.4

Figure 4.5 (Next page). *Phacus* species with clustered strips associated with two whorls of exponential strip reduction (W_p = 2). (a-b) *Phacus pleuronectes* (*P* = 32). (c-d) *Phacus orbicularis* (*P* = 32). (e-f) *Phacus longicauda* var. *tortus* (*P* = 32). (a) Posterior view of *P. pleuronectes* showing two dorsal-ventrally flattened whorls of strip reduction. Strips belonging to whorl I (outlined in white) do not extend down the caudal process, while strips belonging to whorl II (outlined in black) do extend down the caudal process. Lateral clusters are formed by three terminating strips on either side of the caudal process (arrows). Scale bar, 5 μm. (b) Lateral view of *P. pleuronectes* showing the positions and lengths of two clustered strips (arrows) relative to whorl I strips (asterisks) and whorl II strips (diamonds). Scale bar, 5 μm. (c) Lateral view of *P. orbicularis* showing two flattened whorls of strip reduction and one extra terminating strip (arrows) on both sides of the caudal process. Transverse struts (arrowheads) are present on pellicle strips. Scale bar, 10 μm. (d) View of the posterior end of *P. orbicularis* showing the relative positions and lengths of whorl I strips (asterisks), whorl II strips (diamond), and one extra terminating strip (arrow). Note that whorl II strips extend along the caudal process. Scale bar, 5 μm. (e) Lateral view of *P. longicauda* var. *tortus* showing a long, twisted caudal process. Whorl I strips (asterisks) terminate anterior to the caudal process, while whorl II strips (diamonds) occupy the base of the caudal process. Extra terminating strips (arrows) are also shown. Scale bar, 5 μm. (f) Posterior view of *P. longicauda* var. *tortus* showing an extremely flattened and twisted whorl I (white lines); whorl II (black lines) is distorted. Cluster strips (arrows) are arranged asymmetrically with two strips on one side of the caudal process and one strip on the other. Transverse struts (arrowheads) are visible on most strips but are absent on the caudal process and the most posterior region of the cell body. Scale bar, 5 μm.

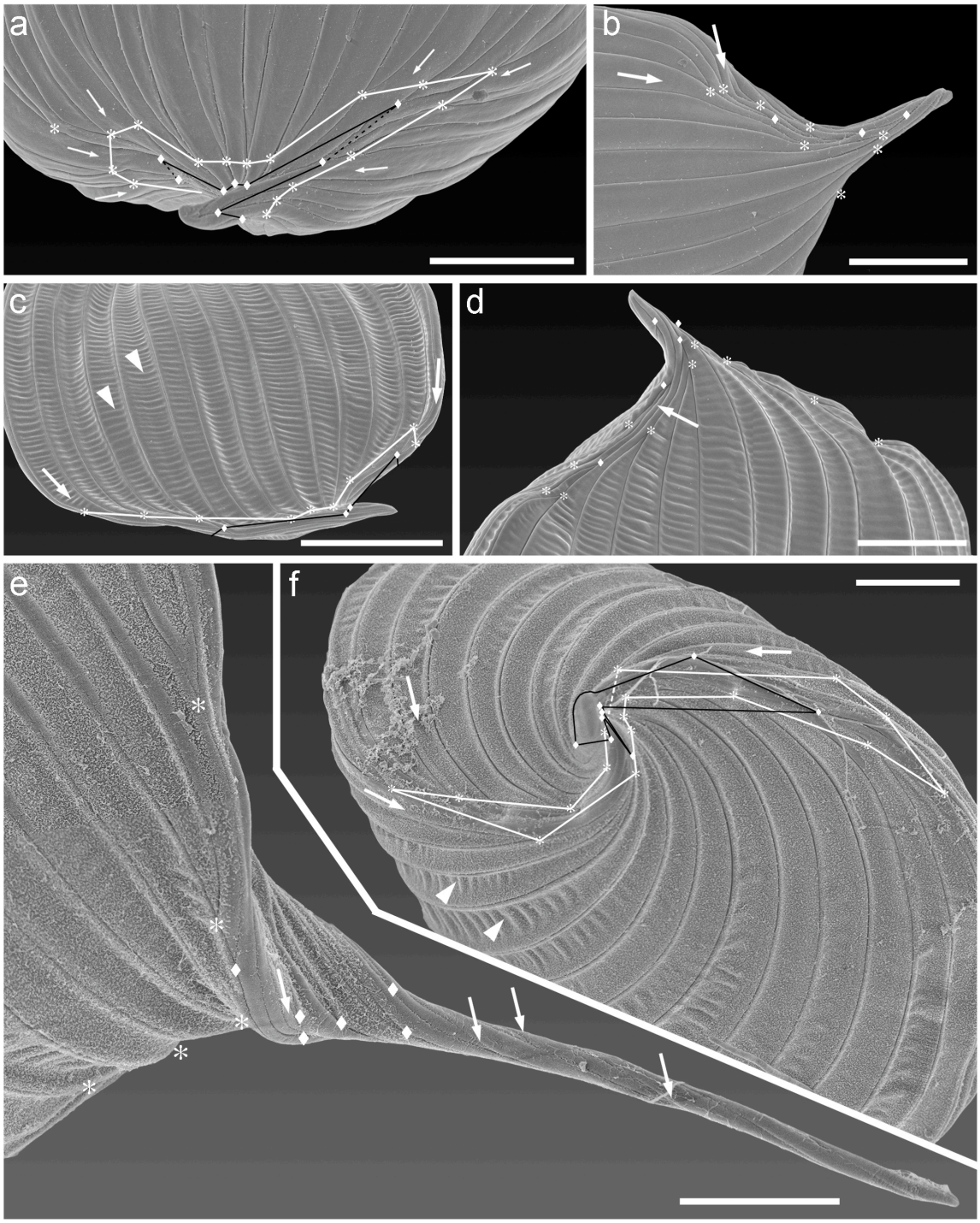

Figure 4.5

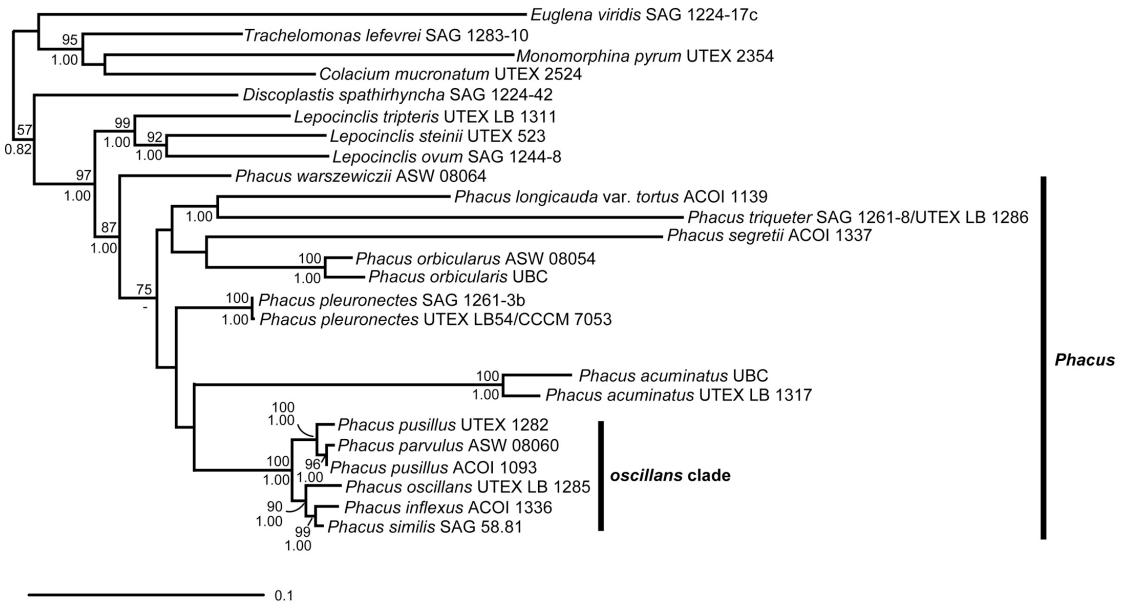

Figure 4.6. Rooted maximum likelihood tree of *Phacus* species and related photosynthetic euglenids inferred from combined SSU and partial LSU rDNA sequences. Maximum Likelihood (ML) bootstrap values above 55 are shown above the branches; Bayesian posterior probabilities (PP) above 0.80 are shown below the branches. Dashes indicate branches that were not recovered using Bayesian analysis.

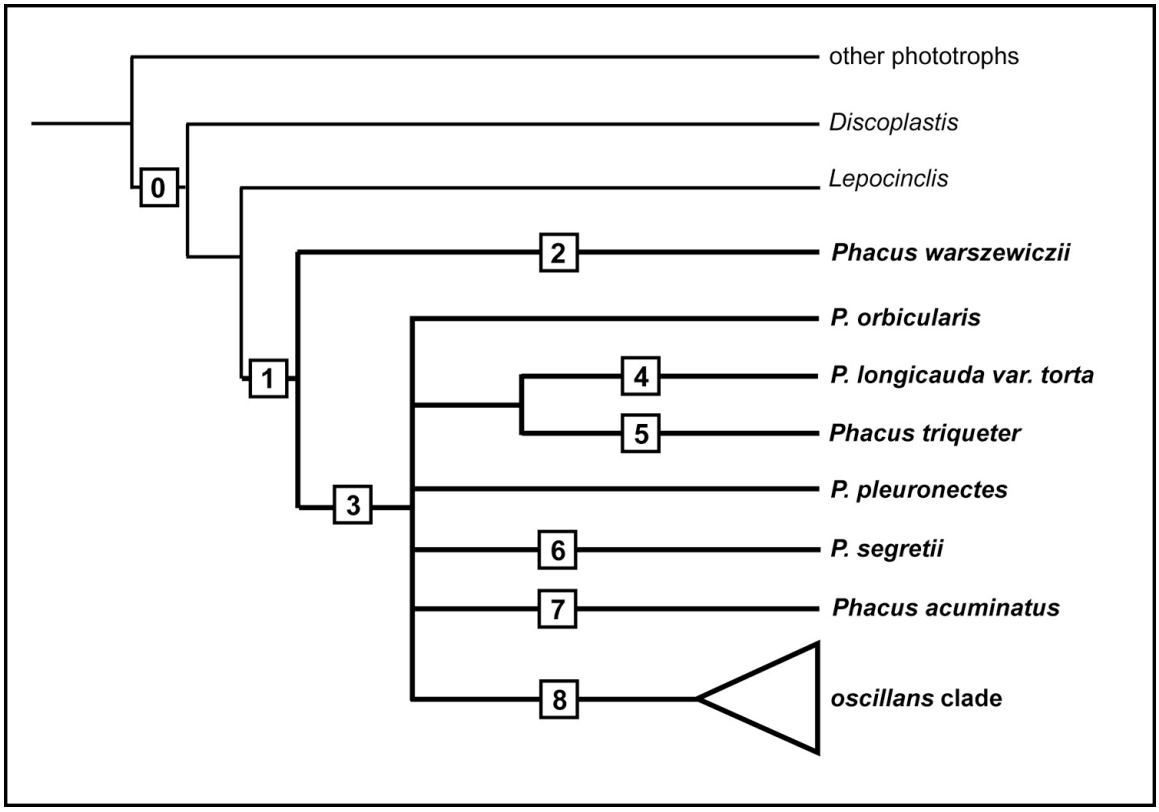

Figure 4.7. Hypotheses of character evolution in *Phacus* as inferred from the combined SSU/LSU phylogenetic analyses and comparative morphology. Position 0: The number of strips around the cell periphery stabilizes at 32 (P = 32); cells have two whorls of posterior reduction (W_p = 2) and are capable of metaboly. Position 1: Rigid cells with helically arranged strips, a caudal process and two or three undistorted, exponential whorls of strip reduction (W_p = 2 or W_p = 3) demarcate the origin of *Phacus*. Position 2: Potential acquisition of an additional whorl of posterior reduction (W_p =3) and deltoid cell shape. Position 3: The origin of longitudinal strip orientation, dorsoventral cell compression and clustered reduction associated with two whorls of exponential reduction. Position 4: Whorls of posterior strip reduction twisted. Position 5: Deltoid cell shape secondarily prominent and reduction of clusters. Position 6: Loss of the caudal process and reduction of clusters. Position 7: Secondary loss of one whorl of posterior reduction. Position 8: Secondary loss of one whorl of posterior strip reduction. Uneven strip reduction event from P = 32 strips, resulting in cells with P = 20 strips. Cells within this clade acquired stronger asymmetry in cluster distribution, and posterior reduction patterns became twisted.

Figure 4.8 (Next page). Illustration of the evolution of distorted posterior reduction patterns in *Phacus*. Taxon names with a given pattern are shown at the bottom of each box. The most recent ancestor of *Phacus* likely possessed a rigid pellicle and undistorted whorled reduction (W_p = 2 or W_p = 3) (position 1). These cells gave rise to both the deltoid cell shape and three whorls as seen in *P. warszewiczii* (W_p = 3; position 2) and flattened cells with two whorls and clustered strip reduction (W_p = 2) similar to those described here for *P. pleuronectes* and *P. orbicularis* (center) (position 3). Modification of this latter type of posterior strip reduction resulted in the remaining distortions described in this study. For instance, twisting of the cell and exaggerated elongation of the caudal process produced the misshapen whorls and extra terminating strips observed in *Phacus longicauda* var. *tortus* (top, center) (position 4). A secondary modification of the strip clusters associated with a prominent deltoid cell shape resulted in the pattern observed in *P. triqueter* (upper right) (position 5). Loss of the caudal process and reduction of strip clusters resulted in the pattern observed in *P. segretii* (right, second from top) (position 6). Loss of one whorl resulted in the pattern observed in *P. acuminatus* (right, second from top) (position 7). Comparative morphology indicates that this condition might be ancestral to the patterns of strip reduction observed in the *oscillans* clade (position 8). The absence of one whorl of strip reduction (like that in *P. acuminatus*), a reduction in the overall number of strips (P = 20, rather than 32) and increased asymmetry of the strip clusters produced the strip reduction patterns found in the *oscillans* clade (bottom) (position 8). Twisting of the flattened ancestral cell resulted in the distorted whorls observed here in *P. pusillus* and *P. similis* (bottom right and center).

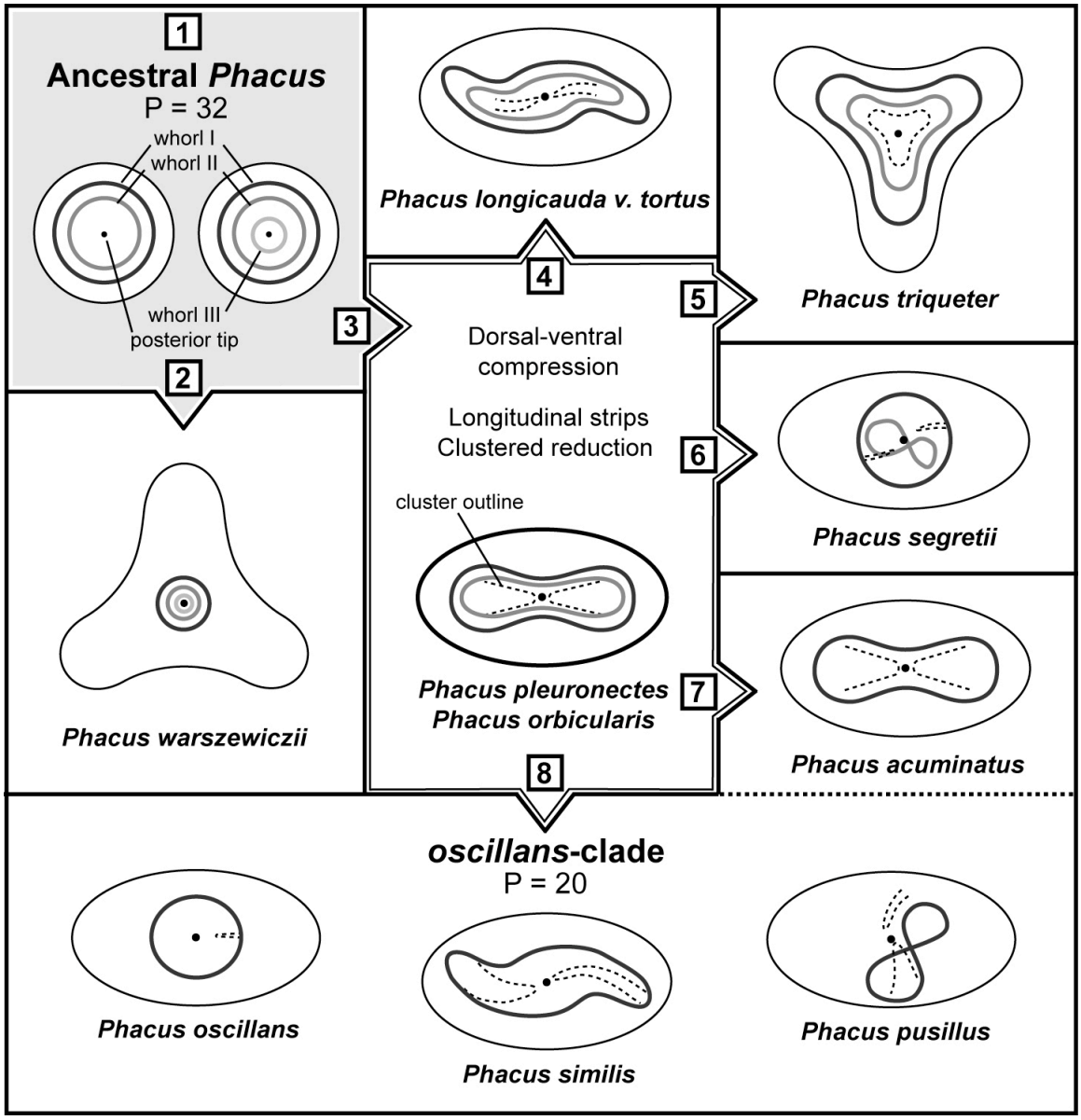
1
Ancestral Phacus
P = 32
whorl I
whorl II
whorl III
posterior tip
2
Phacus warszewiczii
3
Phacus longicauda v. tortus
4
Dorsal-ventral compression
Longitudinal strips
Clustered reduction
cluster outline
Phacus pleuronectes
Phacus orbicularis
5
Phacus triqueter
6
Phacus segretii
7
Phacus acuminatus
8
oscillans-clade
P = 20
Phacus oscillans
Phacus similis
Phacus pusillus

Figure 4.8

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Chapter 5: VISUALIZING THE COMPLEX SUBSTRUCTURE OF EUGLENID PELLICLE STRIPS WITH SEM*

5.1 Introduction

Euglenids comprise a diverse group of flagellates that includes lineages with different modes of nutrition: some feed on bacteria or microeukaryotes (phagotrophs), some absorb nutrients directly from the environment (osmotrophs), and some are photosynthetic (phototrophs). Euglenids share a novel cytoskeleton, referred to as the 'pellicle', consisting of the plasma membrane, a taxon-specific number of proteinaceous strips that extend from the anterior end of the cell to the posterior end, longitudinal microtubules that subtend the strips, and an underlying network of endoplasmic reticulum. The ultrastructure of the proteinaceous strips varies considerably between taxa, and detailed analyses of pellicle characters have significantly improved our understanding of euglenid behavior, development, and evolution (e.g., Leander 2004, Leander et al. 2001, 2007). While surface characters, such as relative strip length, can be observed rather straightforwardly with scanning electron microscopy (SEM; e.g., Brosnan et al. 2005, Esson & Leander 2006, 2008), other characters, such as the shape and thickness of pellicle strips in transverse section, must be viewed with TEM. This involves more time consuming fixation, staining, and sectioning protocols.

One of the characters previously recognized using TEM is the presence and morphology of lateral strip projections, defined by Leander & Farmer (2001a) as 'any proteinaceous extension branching from the heel [of the strip]'. These projections extend either below the arch (the portion of the strip visible on the cell surface) of the same strip (i.e. 'postarticular' projections) or beneath the overhang and arch of the adjacent strip (i.e. 'prearticular' projections); terms used here to describe strip ultrastructure are defined in Leander & Farmer (2001a). Strip projections are absent in phagotrophic euglenids, are delicately structured in 'plastic' photosynthetic euglenids (cells capable of euglenoid movement), and tend to be more robust in rigid photosynthetic

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euglenids (cells that are not capable of euglenoid movement) (Dragos et al. 1997, Leander 2004, Leander et al. 2001). However, some rigid photosynthetic euglenids, such as *Monomorphina aenigmatica*, apparently lack robust strip projections, indicating that there is not a complete correlation between strip projection morphology and the degree of euglenoid movement (Nudelman et al. 2006).

We were able to determine the structure of strip projections in disrupted cells of three rigid photosynthetic euglenids using SEM. This approach eliminated the need to perform three-dimensional reconstructions of strip substructure from thin sections viewed with the TEM. Here we describe the morphology of prearticular strip projections in *Lepocinclis fusiformis* (Carter) Lemmermann, *Phacus longicauda* (Ehrenberg) Dujardin var. *tortus* Lemmermann, and *Phacus segretii* Allorge & Lefevre for the first time, and compare these findings with previous descriptions of strip projections, derived from TEM, in other euglenid taxa.

5.2 Materials and methods

The following cultures were purchased from the Culture Collection at the University of Coimbra (ACOI): *Lepocinclis fusiformis* (strain number ACOI 1025), *Phacus longicauda* var. *tortus* (ACOI 1139), and *Phacus segretii* (ACOI 1337). Cells were prepared for SEM with osmium tetroxide vapor as previously described (Leander & Farmer 2000) with no additional steps taken to manually disrupt the cells. Fixed cells were transferred to millipore filters and critical point dried with CO_2. Filters were attached to stubs and sputter coated with gold or a mixture of gold and palladium. Samples were viewed using a Hitachi S4700 scanning electron microscope.

5.3 Results and discussion

Although most euglenid cells observed with SEM were intact (Fig. 5.1a, inset), a few had disrupted pellicles with two or more strips that were torn apart along their articulation zones (Fig. 5.1a). Prearticular projections could be observed where pellicle strips had disassociated (Figs 5.1b-d). Postarticular projections, which are relatively delicate as inferred from TEM (Leander et

al. 2001), were never observed, even when the underside of the strip arch was visible. Although postarticular strip projections might be absent in the three taxa described here, this is unlikely because postarticular strip projections are present in all previously examined lineages of *Phacus* and *Lepocinclis* (Leander & Farmer 2001a, b, Leander et al. 2001). It seems more probable that either (1) the postarticular projections were obscured by amorphous cytoplasmic components that remained attached to the underside of the pellicle strips, (2) delicate postarticular projections were firmly fixed to the underside of the arch making them invisible with SEM, or (3) the delicate structure of the postarticular projections was destroyed during the preparation of the cells for SEM.

Nonetheless, the prearticular strip projections were clearly visible in this study and consisted of a flat plate that extended from the strip hook and was covered with regularly spaced ridges oriented perpendicular to the longitudinal axis of the strip (Figs 5.1b-d, 5.2); this configuration was similar to that observed in some other *Lepocinclis* species (Leander & Farmer 2001a, b). However, in *L. fusiformis* and *P. longicauda* var. *tortus*, the ridges extended beyond the plate to form tooth-like structures or 'tooth-ridges' (Figs 5.1b, d, 5.2). The prearticular projections in *P. segretii* may also take the form of tooth-ridges, but evidence that the ridges extended beyond the underlying plate was uncertain because of lower preservation quality. Nevertheless, the tooth-ridge configuration represents a hybrid of two previously described morphologies for prearticular strip projections: ridged plates and tooth-like projections (Fig. 5.2). For instance, *L. helicoideus* and *L. oxyuris* have been shown to have prearticular projections in the form of ridged plates (Leedale 1964, Leander et al. 2001); whereas, *Euglena ehrenbergii* (Mikolajczyk 1975), *L. fusca* (Suzaki & Williamson 1985), *L. spirogyroides* (= *Euglena spirogyra*; Leedale 1964, Leander et al. 2001), *L. acus* (Dragos et al. 1997), *L. buetschlii*, *L. tripteris*, *Phacus acuminatus* (identified as *P. brachykentron*), and *P. oscillans* (Leander & Farmer 2001a, b, Leander et al. 2001) have been shown to have tooth-like prearticular projections without ridges (Fig. 5.2).

It is possible that these earlier reconstructions of prearticular projections, reporting the absence of ridges on top of the toothed prearticular plate in *Lepocinclis* and *Phacus*, reflect

incomplete or difficult to interpret observations derived from TEM studies. It is also possible that the tooth-ridge prearticular projections represent a novel state that has not been observed until now. The latter interpretation is consistent with previous observations that specific subcomponents in other microeukaryotes show little or no difference between the substructural details observed with either TEM or SEM (Sant' Anna et al. 2005). Nonetheless, because *Lepocinclis fusiformis*, on one hand, and *P. longicauda* var*. tortus* and *P. segretii*, on the other hand, are members of two different sister clades (Kosmala et al. 2005, Esson & Leander, unpublished observations), the tooth-ridge prearticular strip projections observed in these taxa (Figs 5.1b, d, 5.2) are probably widespread in both genera. However, we cannot currently infer whether tooth-ridge prearticular projections evolved convergently in several different lineages within the *Phacus-Lepocinclis* clade or were secondarily lost (modified) several times independently within this clade.

What we can confidently state is that the tooth-ridge projections described here with SEM represent a previously unrecognized substructure of euglenid strips that will serve as a guide for future reconstructions of prearticular strip projections in other species, whether by using SEM or TEM. Although SEM observations of pellicle strip projections should be consistent with TEM observations, SEM is much less time consuming and produces micrographs that are much easier to interpret. Continued experimentation with SEM protocols associated with cell disruption and fixation (e.g., by briefly applying pressure to cells prior to preparation for SEM; Leedale 1964) will hopefully help preserve the morphology of more delicate structures (e.g., postarticular projections) and facilitate an improved appreciation for the complexity of the euglenid cytoskeleton. This in turn will encourage more extensive taxon sampling within a molecular phylogenetic context, resulting in a better understanding of euglenid diversity and pellicle character evolution.

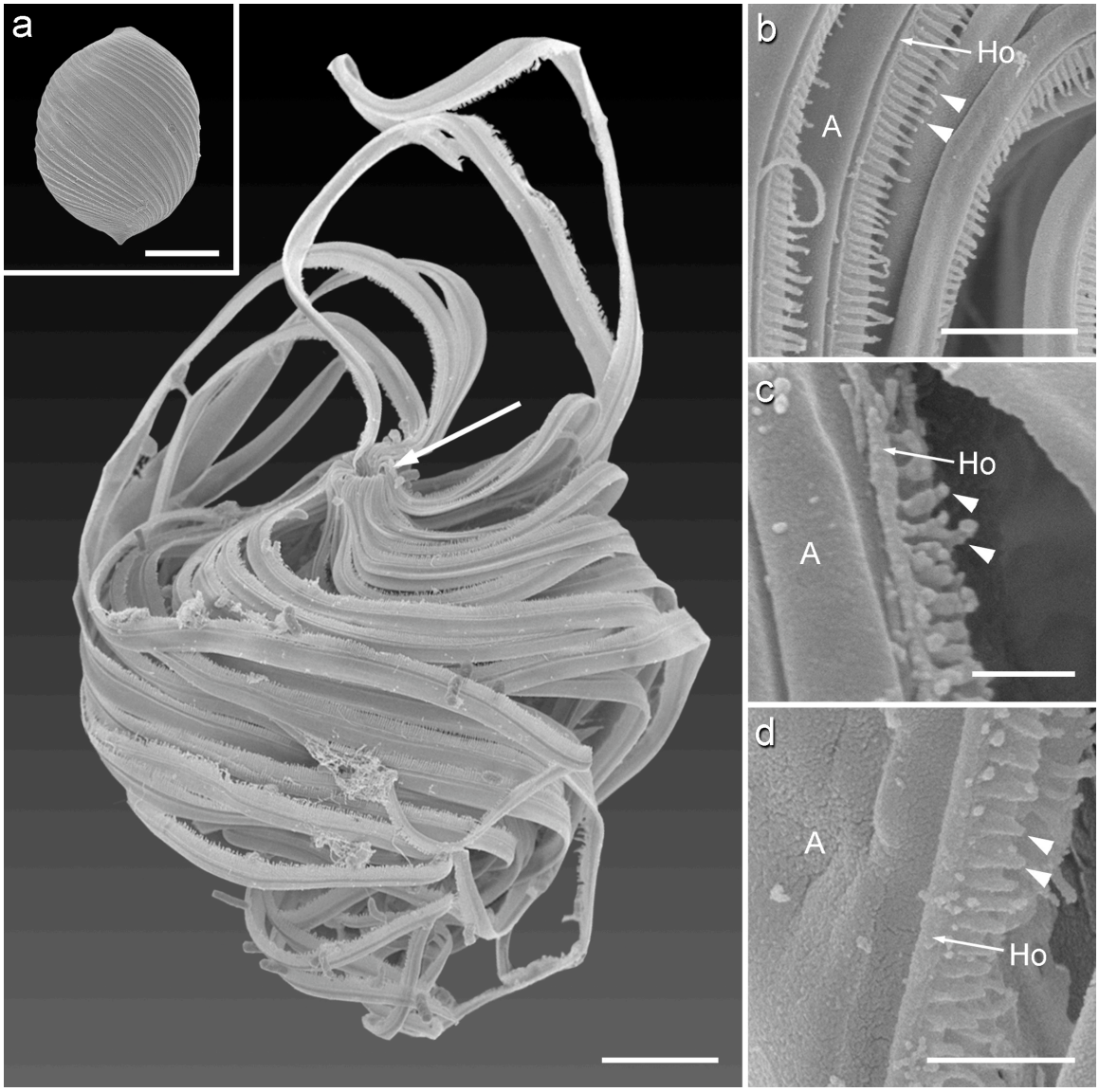

Figure 5.1. Scanning electron micrographs (SEMs) of rigid photosynthetic euglenids showing strip projections. (a) A disrupted cell of *Lepocinclis fusiformis* (ACOI 1025) showing separated pellicle strips that originate in the anterior canal region (arrow) and extend in a helical fashion toward the posterior end of the cell. Scale bar, 5 μm. Inset: An intact cell of *L. fusiformis*. Scale bar, 10 μm. (b) High magnification SEM of the *L. fusiformis* pellicle shown in (a); the anterior end of the cell is at the top of the micrograph. Prearticular projections consist of regularly spaced, tooth-like structures or 'ridges' (arrowheads) that are attached to the strip hook (Ho) and lie on top of a plate. The arch (A) of the strip lies to the left of the projections when the anterior end of the cell is oriented upwards. Scale bar, 1 μm. (c) High magnification SEM showing the prearticular strip projections in *Phacus segretii* (ACOI 1337). Ridges (arrowheads) extend from the strip hook (Ho) and over an underlying plate. A = arch. Scale bar, 0.25 μm. (d) High magnification SEM showing the prearticular strip projections in *P. longicauda* var. *tortus* (ACOI 1139). Ridges (arrowheads) extend beyond the edge of an underlying plate, similar to the projections in *L. fusiformis*. Ho = hook; A = arch. Scale bar, 0.50 μm.

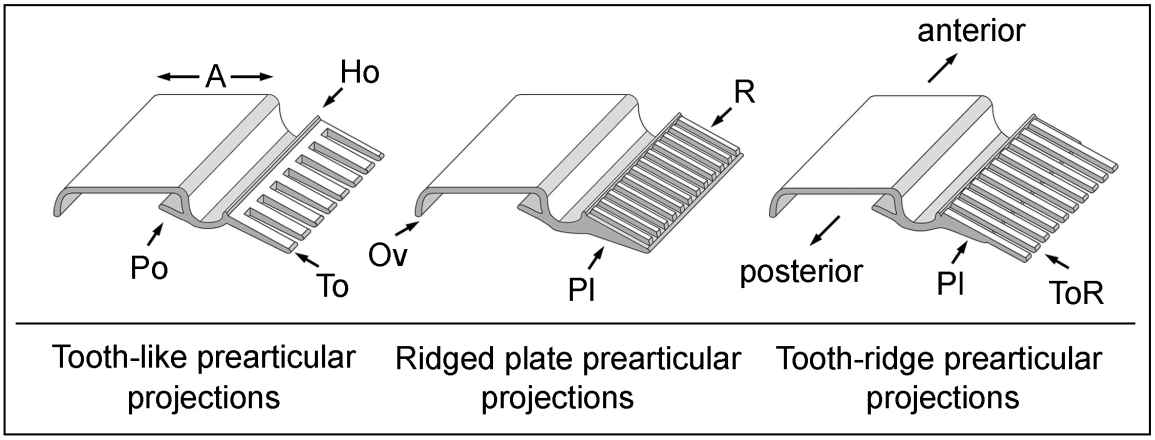

Fig. 5.2. Summary of three character states for prearticular strip projections described in *Lepocinclis* and *Phacus*. Strips are depicted so that their posterior end is oriented toward the lower left of the figure. The leftmost drawing illustrates tooth-like strip projections (To) previously described for members of the genus *Phacus*. The middle drawing shows plate-like projections (Pl) with regularly spaced ridges (R), such as those described for some *Lepocinclis* species and observed in *P. segretii*. The drawing on the right illustrates the plate-like projections (Pl) with overlying tooth-like ridges that extend beyond the plate (ToR), like those observed in *L. fusiformis* and *P. longicauda* var. *tortus*. A = arch; Ho = hook; Ov = overhang; Po = postarticular projection.

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Chapter 6: CONCLUDING REMARKS

6.1 Current understanding of the evolution and development of posterior whorls of strip reduction

The goal of this thesis was to further our understanding of the evolution and development of a complex system contained within a single cell; that is, the euglenid pellicle. Chapters 2-4 focused largely on one character associated with this system, patterns of posterior strip reduction. While researchers have known about these patterns for more than fifty years, systematic and mathematical descriptions of these patterns were only accomplished at the beginning of the present century (e.g., Leander and Farmer 2000a). The descriptive and comparative research presented here combines with previous work to improve our understanding of the evolutionary origin of these patterns. In addition to expanding our knowledge of the diversity of strip reduction patterns in euglenids (Chapters 3 and 4), this thesis lays the foundation for identifying and understanding the modifications in developmental timing that have given rise to these patterns and other pellicle surface characters, such as variations in strip number and pore spacing.

The morphogenetic model described in Chapter 2 may be considered a necessary first step in building this foundation. It synthesized previous studies on the morphogenesis and morphological diversity of the pellicle, particularly those that demonstrated (1) alternation of nascent and mature strips and semiconservative cytoskeletal replication (Hofmann and Bouck 1976, Mignot et al. 1987, Bouck and Ngo 1996), and (2) mathematical descriptions of exponential strip reduction (Leander and Farmer 2000a), I have shown that the newly formed, minor canal strips identified by Mignot et al. (1987) are identical to the strips forming the anterior whorl of reduction observed in many phototrophic euglenids (Leander and Farmer 2000a), and that these strips, in turn, form the anteriormost whorl of posterior strip reduction on the surface of the cell (Chapter 2). According to this model, strips belonging to subsequent (more posterior) whorls of reduction were produced during previous rounds of pellicle duplication and cell division. The relative positions of strips of varying age and length around the periphery of the cell produce exponential patterns of strip reduction (Leander and Farmer 2000a, Chapter 2).

The main drawback of this model is its reliance on inferences based on the synthesis of comparative morphological and developmental studies, rather than direct observations of strip growth during pellicle morphogenesis. Future work on whorl morphogenesis, relying, for example, on comparison between mutants with distinct surface pattern phenotypes, should allow researchers to more confidently describe the underlying cellular and genetic processes of pellicle development. It should be noted, however, that the consistency of the synthesized data and the usefulness of this model of whorl morphogenesis in explaining (1) the mathematical descriptions and diversity of known patterns of posterior reduction (Leander and Farmer 2000a, b, Leander et al. 2001, Chapter 2) and (2) the theoretical relationship between strip maturity and spacing of pellicle pores (Leander and Farmer 2000a; Chapter 3), strongly support its accuracy.

While the model described in Chapter 2 implicates relative maturity as a major determining factor of strip length, linear and bilinear patterns of strip reduction clearly indicate that it is not the only factor (Leander and Farmer 2000a, b, Chapter 3). The relative ages of adjacent strips are discernable in *Euglena obtusa*, making it possible to find a correlation between the age and identity of nascent (whorl I) strips and their respective parent strips (as inferred from comparison with the results of Mignot et al. 1987; Chapter 3). This correlation implies that parent strips somehow influence the development of their adjacent nascent strips, but the cellular mechanism of this influence is unknown. More research on the cell biology and biochemistry of pellicle morphogenesis is required to elucidate this mechanism (or mechanisms), but the "morphogenetic center" described by Mignot et al. (1987) is a good conceptual starting point for these investigations.

If relative strip maturity influences strip length in *Euglena gracilis* and *E. obtusa*, as hypothesized in Chapters 2 and 3, then the bilaterally symmetrical patterns of strip reduction described for *Phacus* in Chapter 4 indicate that, at least in some members of *Phacus*, the relative position of a strip around the circumference of the cell also influences its length. Laterally positioned strips usually terminate farther away from the posterior tip when compared to dorsoventrally positioned strips, forming the clusters of adjacent terminating strips easily recognized in *P. pleuronectes*, *P. orbicularis*, and *P. acuminatus* (Chapter 4). It is unclear how

strong the influence of circumferential position is on strip length: in the species mentioned above, clusters could be easily explained as a modification of the optimum packing hypothesis to accommodate dorsoventral cell flattening and a sharp caudal process (Leander et al. 2001b, Chapter 4). It is the modified strip reduction observed in taxa like *P. oscillans*, *P. pusillus* and *P. segretii* - where vestigial clusters consisting of as few as one strip do not lie precisely on the cell's lateral margins, and cell shape does not appear to limit strip number at the posterior tip - that implies the existence of factors other than optimum packing on the length of cluster strips or their vestigial counterparts. Perhaps these unknown factors are related to the morphogenetic centers in parental strips, as hypothesized in Chapter 3.

The model proposed in Chapter 2 does not explain the potential adaptive significance of strip reduction, which remains entirely unknown. The data presented in Chapter 4 and by Leander et al. (2001) show that posterior tip morphology has no direct correlation with patterns of posterior strip reduction. At this point in time, therefore, variations in strip reduction patterns are best interpreted as adaptively neutral modifications of complex developmental processes. These processes may or may not be linked to other pellicle characters that result from forces of natural selection (Leander et al. 2007).

Any relationship between posterior strip reduction and cell plasticity (see Chapter 1) has yet to be determined. The comparative work of Leander and colleagues (Leander and Farmer 2000a,b; 2001b; Leander et al. 2001) and the patterns described in Chapters 2 and 3 have not revealed an obvious correlation between the number of terminating strips (or the number of whorls of reduction) and the degree of euglenoid movement in a given species. The primary osmotroph *Distigma proteus* is highly plastic and completely lacks posterior reduction (Leander and Farmer 2000a, Leander et al. 2001), while plastic phototrophic euglenids may have one or more whorls of reduction – *Euglena mutabilis* has two whorls (yielding three subwhorls) of posterior reduction, and *E. obtusa* has three whorls separated into seven subwhorls (Leander and Farmer 2000a, Chapter 3). Rigid or semirigid phototrophs exhibit similar variations in posterior strip reduction between species (Leander and Farmer 2000a, 2001b, Chapter 4).

The model for euglenoid movement based on sliding between adjacent pellicle strips allows for no, or limited, movement between strips at the anterior and posterior ends of the cell and requires lateral deformation of strips at these locations (Suzaki and Williamson 1985, 1986). It is possible that the posterior end of a terminating strip, unlike the ends of tip strips, is not rigidly anchored to neighboring strips, which would allow the entire strip to slide freely between its longer neighbors. This would, in turn, leave fewer strips, and less surface area, subject to stress and deformation at the posterior end of the cell during euglenoid movement – a potential selective advantage. At the same time, the posterior tip of the terminating strip would change its relative distance from the posterior end of the cell throughout different stages of euglenoid movement. This hypothesis could be tested by using SEM to observe cells of a plastic phototroph, such as *Euglena mutabilis*, in different stages of metaboly (i.e. elongated, rounded, and bent cells) and measuring the relative distances of the free posterior tips of terminating strips in each stage. If subsequent analyses showed significant differences between these lengths during different stages of euglenoid movement, this would be evidence of an adaptive association between posterior strip reduction and metaboly.

6.2 Diversity of strip projections

Although not directly related to pellicle surface patterns, lateral strip projections are no doubt shaped, selectively and developmentally, by many of the same processes. For example, more delicate strip projections combined with a greater *P* value (i.e. the total number of strips on the cell surface) may be selected for in order to facilitate active metaboly (Dragos et al. 1997, Leander and Farmer 2001a, Leander 2004, Leander et al. 2001; see discussion in Chapter 1). Developmentally, the complex processes of protein deposition that occur during strip duplication must be coordinated, both to add sufficient length to specific strips in order to maintain surface patterns, and to build the complicated and diverse lateral projections on nascent strips. Although the Golgi apparatus and microtubules have been loosely implicated in these processes (Leedale 1967, Mignot et al. 1987), little more is known about the morphogenesis of strip ultrastructure.

The main question raised by the data presented in Chapter 5 is whether prearticular projections with tooth-ridge morphology represent a previously unobserved ultrastructural character state or one that was inaccurately described either as tooth-like or as a ridged plate in previous publications (e.g., Leander and Farmer 2001a). It seems that the best way of answering this question is to disrupt the pellicles of taxa whose prearticular projection ultrastructure has been described using TEM, and observe strip projections using SEM in order to detect any disparities between the data yielded by the two methods – it could be that SEM allows the visualization of greater morphological detail or provides a clearer context for understanding the ultrastructural organization of strip projections. If this is the case, disruption and SEM fixation methods should be improved in order to gather data pertaining to projection ultrastructure more accurately and efficiently (Chapter 5).

6.3 Future of the study of pellicle evolutionary development

The developmental and morphological complexity of the euglenid pellicle indicate that it may be an ideal model system for the study of cytoskeletal development and evolution. The myriad interactions between microtubules, pellicle strip proteins, and the associated endomembrane system and plasma membrane during development could be studied in far greater detail in an established model system than with the comparative methods described here. Such a system would subsequently shed light on the evolutionary processes that gave rise to the diversity of pellicle ultrastructure and the feeding and locomotory processes that rely on these variations (Leander 2004; Leander et al. 2007). While pellicle surface patterns are extremely complex, they are easily visualized with SEM and their component strips represent discrete units that can be readily quantified. Other features of strip ultrastructure, such as strip projection morphology, may also be readily observed using SEM following the refinement of fixation protocols (Chapter 5). The ease with which photosynthetic euglenids can be monoclonally cultured also favors the establishment of such a system.

The main obstacle to establishing euglenids as model organisms for cytoskeletal research is the lack of a robust genomic context for such work. Although expressed sequence tag

(EST) projects have begun for *Euglena gracilis* (Durnford and Gray 2006), no euglenid nuclear genome has been completely sequenced and cytoskeletal genes remain largely undescribed (exceptions include sequences for articulins, tubulins and actin; Marrs and Bouck 1992; Levasseur et al. 1994; Petersen-Mahrt et al. 1998). The sequencing and annotation of a euglenid genome would greatly facilitate the identification of potential cytoskeletal genes of interest based on sequence similarity, as is the case with other genes in other organisms (e.g., Dacks and Doolittle 2002, Dacks et al. 2008, Gould et al. 2008). Moreover, the extensive genomic and developmental data for other protists - including the closely related trypanosomes and more distantly related organisms such as *Giardia* and *Tetrahymena* – will provide some of the necessary building blocks for a preliminary comparative framework of eukaryotic cytoskeletal evolution and development. A euglenid genome would improve the taxonomic sample of this framework, yielding a more complete picture of the evolution of eukaryotic organisms and the developmental processes that shape them.

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