EVOLUTION AND COMPARATIVE MORPHOLOGY OF THE EUGLENOPHYTE PLASTID

by

PATRICK JERRY PAUL BROWN

(Under the Direction of Mark A. Farmer)

ABSTRACT

My doctoral research centered on understanding the evolution of the euglenophyte protists, with special attention paid to their plastids. The euglenophytes are a widely distributed group of euglenid protists that have acquired a chloroplast *via* secondary symbiogenesis. The goals of my research were to 1) test the efficacy of plastid morphological and ultrastructural characters in phylogenetic analysis; 2) understand the process of plastid development and partitioning in the euglenophytes; 3) to use a plastid-encoded protein gene to determine a euglenophyte phylogeny; and 4) to perform a multi-gene analysis to uncover clues about the origins of the euglenophyte plastid.

My work began with an alpha-taxonomic study that redefined the rare euglenophyte *Euglena rustica*. This work not only validly circumscribed the species, but also noted novel features of its habitat, cyclic migration habits, and cellular biology.

This was followed by a study of plastid morphology and development in a number of diverse euglenophytes. The results of this study showed that the plastids of euglenophytes undergo drastic changes in morphology and ultrastructure over the course of a single cell division cycle. I concluded that there are four main classes of plastid

development and partitioning in the euglenophytes, and that the class a given species will use is dependant on its interphase plastid morphology and the rigidity of the cell. The discovery of the class IV partitioning strategy in which cells with only one or very few plastids fragment their plastids prior to cell division was very significant. This partitioning strategy is unique to the euglenophytes and is correlated to the degree of 'euglenoid movement' in a given species.

An analysis of the PsaA gene from numerous euglenophytes had three main results: 1) the Eutreptiales are basal and paraphyletic; 2) there is some justification for a relationship between *Euglena anabaena* and *E. gracilis*; and 3) plastid genes are unsatisfactory for phylogentic analysis of the euglenophytes.

Finally I performed a multi-gene analysis of the PsaA, RbcL, and 16S rDNA genes from all major groups of eukaryotic algae. This showed that the euglenophyte plastid likely has its origin from within the green algal class Prasinophyceae.

INDEX WORDS: *Euglena, Eutreptia, Colacium, Trachelomonas, Strombomonas, Phacus,* Chloroplast, Plastid Evolution, Comparative Ultrastructure, Plastid Development, Plastid Partitioning, PsaA, Plastid Phylogeny, Introns.

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B.S., The University of Tennessee at Chattanooga, 1998

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

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May 2003

ACKNOWLEDGEMENTS

First I owe a huge debt of gratitude to Mark Farmer. I never imagined that a chance encounter in an East Tennessee toy store would result in the acquisition of a wonderful mentor, professional collaborator, and a fine friend. No one could ask for a major professor more dedicated not only to the academic success of his students, but also to their training as teachers, scientists, and professionals. I must also thank Mark for acquiring the NSF PEET (Partnerships for Enhancing Expertise in Taxonomy) grant (No. DEB 4-21348) that funded my research.

There have been many people who have helped me out along the path of my graduate career, beginning with my doctoral committee: Marcus Fechheimer, Marshall Darley, Russell Malmberg, John Logsdon, and David Porter. Very few students are fortunate enough to have a committee so accessible and willing to help. I also owe thanks to John Shields and Ruth Furukawa for their tireless technical help and expertise. Brian Leander, Rupal Thazath, Andrew Maselli, and Celeste Leander all gave me help, advice, and friendship and I thank them for it. I also would have never made it were it not for the help of Brett Rudolf and the entire cellular biology office staff.

I must thank my family, Jerry and Annette Brown, Melissa Brown, and Sean Brown who have been a constant fount of support and encouragement. Finally I have to thank my wife, Stacy Brown, whose love and support have made this accomplishment possible, I love you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Preface

"Out of the ground the Lord God formed every beast of the field and every bird of the sky, and brought them to the man to see what he would call them; and whatever the man called a living creature, that was its name."

Genesis 2:19

Since earliest recollection mankind has faced the daunting task of making some kind of sense of the amazing diversity of the world around him. Imagine if we were to look back in time to the earliest days of mankind as he struggled to make his living in the savannah. How would one hunter communicate the prey he had seen to another? How could a gatherer tell another where to find a plant that was good to eat? It is likely that as soon as humans developed fully formed language, the first words they used would have been to describe the world around them. The desire, or in the case of early humans, the absolute necessity to understand and describe biological diversity has been with us as long as language, but a systematic approach to understanding and communicating that diversity is a relatively recent phenomenon.

The most notable figure in biological diversity for nearly 2,000 years was Aristotle (384-322 B.C.). In fact, Mayr & Ashlock (1991) call Aristotle "the father of biological classification" and rightly so. Aristotle was the first to divide living creatures

into groups and some of the groups he named persist to this day (*e.g.* Diptera). It was not until Linnaeus (1707-1778) that a concerted effort was begun to organize and name the incredible diversity of life. Although Linnaeus rightly earns the title 'father of taxonomy', it was de Candolle (1813) who actually coined the word "taxonomy" from the Greek *taxis* ('arrangement') and *nomos* ('law'). The etymology of the word taxonomy brings me to the point of this exercise: what exactly is taxonomy, and what is its relation to systematics and classification? The latter part of the word taxonomy means literally 'law', and taxonomy is the creation of theories and rules by which we name biological groups. But how are those groups defined? This is the role of the systematist.

Systematics according to Mayr is simply put "...the science of the diversity of organisms" (Mayr & Ashlock 1991). This is a fitting definition and very helpful in understanding the often confusing relationship between systematics and taxonomy. Systematics gives us the means to understand the diversity of life while taxonomy gives us the means to communicate that diversity in a meaningful way, to others. So why do I spend this time relating these differences? I do it because this is the fundamental unifying theme throughout this body of work. The universality of systematics and taxonomy to all other branches of biological science is the common thread that runs throughout the following four chapters. My work ranges from pure alpha taxonomy (the circumscription and naming of species) to molecular systematics to comparative morphology. Only as a systematist and taxonomist can one person practice such wide and diverse panoply of science.

Introduction

Antony Van Leewunhoek was the first to glimpse a euglenid in 1674 (Van Leewunhoek 1674). Since that time these unique and enigmatic organisms have fascinated numerous scholars, scientists, and students. By far the most well known euglenid is the laboratory model organism *Euglena gracilis*, however *E. gracilis* is but one species among many that exhibit vast diversity in form, nutrition, and habitat. There are phagotrophic and osmotrophic euglenids from both freshwater and seawater, from shallow ponds to the deep ocean depths. But the most well known, and successful group of euglenids are those that have plastids; be they freshwater, brackish, or marine. Even though the plastid-containing euglenids are the most well-known and extensively studied (*E. gracilis* is one of these), very little is known about the relationships between these euglenids or about the basic biology of their plastids outside of what is known about *E. gracilis*. The work described herein is my contribution to our knowledge of the biology of the euglenid plastid and what that knowledge can elucidate in regards to euglenid systematics.

Nomenclature.

Euglenid taxonomy is currently in a state of flux; therefore there is no real standard taxonomic nomenclature with which to discuss the organisms that comprise this clade. What follows is a short note on usage of terms and their meanings. The largest well-defined clade within the Eukaryota that contains euglenids is the Euglenozoa (Cavalier-Smith 1981). This group is comprised of the Euglenids, Kinetoplastids, and the Diplonemids (Simpson 1997). The euglenids (Euglenida) are a rankless clade, subordinate to the Euglenozoa and are characterized by a specialized cortical

cytoskeleton, or pellicle (Simpson 1997). Within the Euglenida, there exists a monophyletic sub-assemblage consisting of organisms that have (or have had, and subsequently lost) a plastid. These organisms are referred to here as the euglenophytes. Historically photosynthetic euglenids have been referred to as the Division (phylum) Euglenophyta (VanDen Hoek *et. al.* 1995), Class Euglenophyceae (Fritsch 1961), or Order Euglenales (Stein 1878). These ranked names have appeared separately, together, and have many zoological counterparts (Leedale 1967). I prefer to use the term euglenophytes to refer to an unranked taxon within the Euglenida that consists of those organisms that have a plastid, or are descended from an organism that had plastids. The genera that make up the euglenophytes, including the well-known genus *Euglena*, are for the most part paraphyletic (Leander & Farmer 2001b, Linton *et. al.* 1999, 2001). For the time being, those taxa that are classically included in these genera will remain so, until such time as a large enough data set is available to make well-supported homology statements that can define new generic boundaries.

Euglenophyte origins.

The euglenophytes emerged *via* secondary symbiogenesis when a phagocytic eukaryotroph (Leander *et. al.* 2001) ingested a green alga (Lee 1977, Gibbs 1978,) sometime around 400 million years ago (Gray and Boucot 1989, Leander *et al.* 2001). Green algae, red algae, and the lesser-known glaucocystophytes all possess plastids originating from a primary endosymbiosis (Delwiche & Palmer 1997). The common ancestor of these organisms ingested a cyanobacterium and instead of digesting it, harbored it in their cytoplasm. The ingested cyanobacterium became a permanent endosymbiont and later a true organelle (Sagan 1967, Margulis 1981). Primary plastids

such as these are distinguished by the two membranes surrounding them; the inner membrane originated as the plasma membrane of the symbiont while the other is derived from the phagocytic vacuole of the host (Cavalier-Smith 1982). Like the heterokont algae, the haptophytes, the cryptophytes, the dinoflagellate algae, and the chlorarachniophytes, euglenophytes have a plastid derived from the ingestion of a eukaryotic alga, that has subsequently evolved into a dependent organelle (Lee 1977, Gibbs 1978, McFadden & Gilson 1995, Delwiche & Palmer 1997). These secondary plastids are characterized by the presence of three membranes as seen in the euglenophytes and most dinoflagellates, or four membranes typical of heterokonts, haptophytes, and chlorarachniophytes (Van Den Hoek 1995). The extra membranes are the remnant of the phagocytic vacuole of the host and when a fourth is present, the plasma membrane of the eukaryotic alga (Delwiche & Palmer 1997).

A Brief History of Euglenid Taxonomy

Taxonomy of the euglenids. The earliest taxonomy of the euglenids was by Stein (1878) who divided the euglenids into four families based on nutrition and flagellation. This caused some obviously closely related groups like *Phacus* and *Euglena* to be artificially separated into separate families equal in rank to other families of Infusoria. Klebs (1883) was the next to provide a classification for the euglenophytes and in many ways it was an improvement over Stein's. Klebs placed most of the euglenids into a single family. However, he still relied heavily on nutritive mode for separation of groups, resulting in the taxonomic separation of forms that are clearly very similar, but differ in nutritive mode (*e.g. Astasia* and *Euglena*). Senn (1900) was the first to place all of the euglenids into a single taxon, but again he relied heavily on nutrition to separate

taxa. Conrad (1916) was the first to tackle the already expansive loricate genus *Trachelomonas*. However, he ignored most cellular features and instead defined taxa almost exclusively on lorica morphology. Deflandre (1926, 1930) followed up on Conrad's work and separated those taxa with vase-shaped, aggregated, and unadorned loricas into a new genus, *Strombomonas*. The next major revision came when Jahn (1951) removed the only colonial genus of euglenid *Colacium* into its own order, equal in rank to all other euglenids with a single emergent flagellum. This was quickly amended by Hollande (1952) who placed *Colacium* alongside all the other euglenophytes with a single emergent flagellum. Hollande was also the first to rely less on nutritive mode, and instead based his taxonomy primarily on flagellation. The most widely used taxonomy today is that of Leedale (1967) who separated the euglenids into six orders under the class Euglenophyceae. Leedale's taxonomy was the first to use a variety of cellular features, taken together to try to create a taxonomy that could be explained by evolution. The last major revision to euglenid taxonomy was by Tell and Conforti (1986) who again tried to separate *Colacium* from the other euglenids, this time into its own order!

Taxonomy within the genus Euglena. Specialists have recognized for over 50 years that the genus *Euglena* is so diverse that it needs some sort of infra-generic classification. Every taxonomy of the genus *Euglena* has used plastid structure and morphology as either the sole character (Gojdics 1953, Zakrys 1986) or the major character (Pringsheim 1956, Leedale 1967). The first major attempt to sub-classify the genus was by Gojdics (1953) who divided *Euglena* into eight groups based solely and entirely on the size and shape of plastids. This was soon amended by Pringsheim who did take plastid morphology into account, but also used degree of rigidity, cell size, and

flagellation, as well as characters of the chloroplast that Gojdics did not, such as pyrenoid morphology and association with paramylon. Thus, Pringshiem divided the genus into six groups defined by circumscription of many cellular characters. It should be noted, however, that three of the six groups are defined primarily on plastid morphology, and the other three are consistent in their plastid morphology. Although Pringsheim was unclear as to whether or not he meant for his 6 'groups' to be taken as subgenera, Leedale cleared that ambiguity by clearly naming each of Pringshiem's groups as subgenera within *Euglena*. The most recent subgeneric taxonomy is that of Zakrys (1986) who again defines her subgenera based solely on plastid morphology. This time there are three subgenera: *Euglena; Calliglena;* and *Discoglena*. Subgenus *Euglena* is defined as having either one or two elongated axial chloroplasts or having "very numerous chloroplasts, without visible pyrenoids, aggregated into axial star-like groups". Subgenus *Calliglena* is characterized by having chloroplasts that are disc-shaped with a pyrenoid, and have "more or less incised margins". Subgenus *Discoglena* is defined as having numerous disc-like chloroplasts without pyrenoids (lenticular).

The Euglenophyte Plastid.

General plastid morphology. Numerous authors have expounded on the vast diversity of plastid morphology in the euglenophytes (Gojdics 1953, Leedale 1967) However, they usually disagree on exactly how diverse that morphology is. Gojdics (1953) described eight fundamental chromatophore (plastid) morphologies within the genus *Euglena* alone: 1) long and ribbon-like; 2) trough-shaped; 3) reticulate; 4) small discoid; 5) larger discoid; 6) bearing sheathed pyrenoids; 7) spindle shaped; and 8) different from the other seven types. Many of these distinctions are obviously artificial

(small discoid versus larger discoid), while others have more to do with the size and shape of the cell than with any specific and heritable trait of the plastid (*e.g.* trough shaped).

Pringsheim (1956) elaborated on Gojdics plastid designations, refining the definition of some and eliminating others. Pringsheim's principle criticism of Gojdics is her reliance on plastid size and shape, with no regard to other structures. For instance he points out "Gojdics neglects … the presence of naked pyrenoids in the chromatophores which she lists on their shapes and sizes, although these are relatively variable within groups of allied forms, and even in clones" (Pringsheim 1956 p. 41). Pringshiem also points out that Gojdics' group C (*Euglenae* with reticulate chromatophores) has, as he put it, "no right to exist" due to the lack of proof that such a form even exists. The result is that Pringsheim describes five basic plastid arrangements: 1) small lenticular chromatophores with no pyrenoids; 2) larger plate-like, often polygonal, lobed, or even star-shaped chromatophores with a diplopyrenoid; 3) star-like and ribbon-shaped with a paramylon center; 4) trough-like and with naked pyrenoids; and finally 5) plate-like with inner pyrenoids. Pringshiem goes into some detail about the etymology of plastids with a double-sheathed pyrenoid, but in the end concludes that the preferable term for this type of plastid/pyrenoid arrangement is 'diplopyrenoid' coined by Dangeard (1901).

Leedale (1967) followed with his description of five basic types of euglenophyte plastidome, based largely on Pringsheim's but codifying them into distinct groups and including the approximate size and number of the plastids. The plastidome is defined here as the total chloroplast complement of a cell, including accessory structures such as paramylon and pyrenoids. Leedale's groups were as follows: 1) numerous discoid

plastids from 2-5 μm in diameter with no pyrenoids; 2) discoid, elongated, or shield-shaped plastids, 5-10 μm in diameter with a 'naked' pyrenoid (a naked pyrenoid is a pyrenoid with no associated paramylon at any time in the life cycle); 3) large plate-like plastids 5-10 X 5-20 μm in size, each with a central pyrenoid that bears on either side a lens-shaped grain of paramylon and with margins that may be entire, lobed, dissected, or arranged as spiral arms around the cell cortex; 4) plate-like chloroplasts with 'inner' pyrenoids protruding from their inner surface that are capped by a cylindrical or spherical grain of paramylon; 5) chloroplast ribbons radiating from one, two, or three 'paramylon centers', each paramylon center being a pyrenoid surrounded by ovoid grains of paramylon.

The only correction that need be made to Leedale's categories is the separation of type 5 (those with a paramylon center) into two types. The first is the most common form of this type and is found in organisms such as *Euglena viridis* and *Euglena terricola* and I call these 'stellate' plastids. They are characterized by the fact that they are usually only one or two, very large plastids with a central pyrenoid and paramylon center with arms radiating outward from the center towards the cell periphery. The second type of plastid with a paramylon center is found in organisms such as *Tetreutreptia pomquitensis* and *Eutreptia eupharyngea*, and I call these 'aggregate' plastids. These are characterized by the presence of four to eight strap-shaped plastids with pyrenoids at one end. These individual plastids aggregate at the end with the pyrenoid, and the aggregation site is surrounded by paramylon into a paramylon center. It is only in recent years, with extensive electron microscopic sampling that this designation has been possible.

Plastid ultrastructure. Much of the early work on the ultrastructure of the euglenophyte plastid was done using *E. gracilis* as the model (Gibbs 1960, 1962). Ultrastructurally the euglenophyte plastid is quite unique. The euglenophyte plastid is surrounded by three membranes, a phenomenon seen only in the euglenophytes and some dinoflagellates. There is no evidence of a fourth membrane, or that there ever was one (Lee 1977), nor is there any association between the outermost plastid membrane and the endomembrane system of the host cell (Gibbs 1960, 1978, Leedale 1967, Lee 1977). It has been thought historically that thyllakoid lamellation is typically in stacks of three, but this is due primarily to the prevalence of *E. gracilis* (which does have three thyllakoids in a lamella) as the major euglenophyte of study. Ultrastructural research in a number of species other than *E. gracilis* has shown that lamellation can occur in stacks of 2 – 12 thyllakoids per stack (Dragos *et. al.* 1979, Zakrys and Walne 1998, Zakrys *et. al.* 2001, Brown *et. al.* 2002).

The storage product of euglenophytes (paramylon) is always deposited outside that plastid. Unlike the green algae, which deposit starch in the chloroplast stroma, the euglenophyte storage carbohydrate is always found in the cytoplasm. There are, however, close associations between the plastid and paramylon primarily at the pyrenoid as seen in inner pyrenoids, diplopyrenoids, and paramylon centers. Pyrenoids are composed primarily of the Calvin-cycle enzyme Ribulose-1,5-Bis-Phosphate Carboxylase/Oxygenase (RuBisCo) and appear as rather large, electron-opaque bodies in the electron microscope. Euglenophyte pyrenoids are always located in the plastid stroma inside all three membranes. Some algae such as the heterokonts have a pyrenoid between the inner and outer pairs of plastid membranes, but the euglenophyte pyrenoid

has never been observed between any of the plastid membranes. All pyrenoids are also penetrated by thyllakoids, however the lamellation tends to change inside the pyrenoid matrix (Leedale 1967, Dragos *et. al.* 1979, Brown *et. al.* 2002).

Euglenophyte Plastid DNA

The plastid DNA of *Euglena gracilis* was one of the first well-studied organellar genomes. The ptDNA of *Euglena* is very AT-rich, giving it a low density and allowing for easy separation from nuclear DNA (Brawerman & Eisenstadt 1964). *Euglena* ptDNA was the first known example of a circular chloroplast genome (Manning *et. al.* 1971). Although the euglenophyte plastid is undoubtedly derived from a green alga (Lee 1977, Gibbs 1978, Leander *et. al.* 2001), there is very little similarity between the plastid genome of the green algae and the euglenophytes. Green algal and plant ptDNA is arranged as a pair of inverted repeats separated by a small single copy region (SSC) and a large single copy region (LSC) (Soltis *et. al.* 1998). The ptDNA of *Euglena* is arranged as a 143 kb circular genome with a 54 bp variable number tandem repeat (VNTR) at the origin of replication and 3 identical ribosomal RNA operons (Hallick *et. al.* 1993). Although the ptDNA of euglenophytes doesn't contain the inverted repeats, it is still approximately the same size as that of green algae or plants (Hallick *et. al.* 1993). The source of the compensating DNA is a rather unique one.

The ptDNA of the angiosperm *Nicotiana* contains only 21 introns (Shinozaki *et. al.* 1986, Shimada and Sugiura 1991), whereas the ptDNA of *Euglena gracilis* contains over 150 introns, totaling approximately 38% of the total ptDNA. *E. gracilis* does possess some group I introns, but the majority are group II and group III introns. Group II introns are self-splicing and characterized by a conserved core secondary structure and

boundary sequences (Copertino *et. al.* 1991). Group III introns are unique to the euglenophytes and are essentially smaller versions of group II introns (Christopher & Hallick 1989). The euglenophytes have another unique intron feature, twintrons. Twintrons are introns within introns (Copertino & Hallick 1991). Since the internal intron disrupts an essential functional domain in the secondary structure of the external intron, the internal intron is spliced out first, the external intron reassembled, and then spliced out itself (Copertino *et. al.* 1991). Twintrons can be either group II within group III or *vice versa* (Copertino & Hallick 1991, Hallick *et. al.* 1993, Thompson *et. al.* 1994).

Recent advances in euglenophyte systematics

The majority of the studies on euglenophyte systematics have been based primarily on comparisons of nuclear ribosomal RNA genes. The rRNA based systematics began with a small data set (Montegut-Felkner 1997) and primarily addressed questions regarding the higher-level systematics of the euglenids as a whole. This was followed by inclusion of many more euglenophytes into a larger data set (Linton *et. al.* 1999) and showed the first molecular evidence that the genus *Euglena* is not monophyletic. This study also demonstrated that some of the apochloric forms (*Astasia* and *Khawkinea*) are clearly derived from photosynthetic ancestors. This was followed by an even larger data set (Linton *et. al.* 2000) and demonstrated further the paraphyly of *Euglena*. The small subunit (SSU) rDNA data was then expanded to include some of the phagotrophic and osmotrophic euglenids (Preisfeld *et. al.* 2000) and confirmed that the euglenophytes arose from phagotrophic anscestors. The SSU rDNA data set has recently been expanded to cover most of the genera within the euglenida (Preisfeld *et. al.* 2000, Leander and Farmer 2001b, Leander *et. al.* 2001, Müllner *et. al.* 2001,) and demonstrated

that not only is *Euglena* paraphyletic, but that *Phacus, Lepocinclis,* and *Astasia* as currently defined are polyphyletic. Most recently, the nuclear data set has been expanded two-fold by the addition of the large subunit rRNA gene (LSU) (Brosnan *et. al.* 2003).

Not all of the molecular systematic studies in the euglenophytes have been from nuclear DNA. Thomson (1994) attempted to apply the plastid RbcL (the large subunit of Ribulose,1-5,Bis-Phosphate Carboxylase/Oxygenase) gene to the euglenophytes with little success. The most recent plastid-based molecular phylogeny was based on plastid-encoded SSU rDNA (Milanowski *et. al.* 2001). While it didn't provide high resolution to higher taxa, Milanowski's study did demonstrate the monophyly of the group that Pringsheim (1956) and Leedale (1967) called 'radiate' and that Zakrys (1986) calls the subgenus *Euglena*.

General Outline and Objectives

The work described in this dissertation was all performed under the auspices of a National Science Foundation PEET (Partnerships for Enhancing Expertise in Taxonomy) grant. There are two fundamental goals of the PEET project 1) to train new taxonomists in areas where the organisms under study are poorly understood and/or for which there are few living experts; and 2) to monograph the organisms under study using modern techniques to determine relationships and provide descriptions. The PEET grant that has funded my research was charged with these two fundamental directives, and were centered around the genus *Euglena*.

As mentioned above, the genus *Euglena* has been the subject of taxonomic research for the last 50 years. In that time numerous authors have divided the genus based on a number of characters, but primarily they have used plastid morphology as the

primary feature used to define their groups. It is understandable that they do this, as the plastid is the most conspicuous feature of any *Euglena* cell. It was with this in mind that I began my work. I initially set out with three goals: 1) to redescribe existing species of *Euglena* and/or to name newly discovered species; 2) to study the plastids of *Euglena* with modern microscopy techniques in order to define and score stable, heritable characters of plastid structure that could be used in morphological phylognetics; and 3) to couple the morphological features of the plastid with a molecular phylogeny generated *via* a plastid-encoded protein gene, and to use this information to generate a stable apomophy-based taxonomy of *Euglena*. Since early on in our PEET project it was shown that the genus *Euglena* is paraphyletic, the data set was expanded to attempt to include as many species of euglenophytes as possible with the hope that plastid-based information might prove useful in describing new taxa.

Chapter 2 is my first contribution to the PEET project. In it I use modern microscopy to redescribe a rare species of *Euglena* heretofore seen only once in a village pond in Austria 60 years ago. In chapter 3 I address the issue of using plastid-based characters in evaluating sister-group relationships between euglenophytes not traditionally called *Euglena*. The work in chapter 3 raised some very interesting questions regarding the stability of plastids, and the usefulness of plastid-based features in taxonomy. In chapter 4 studies of plastid division and development were performed on six taxa from several lineages of euglenophytes, demonstrating that plastids show marked changes in morphology and ultrastructure during the course of the cell division cycle. Finally in chapter 5 the partial sequence of the gene encoding PsaA (Photosystem I apoprotein A) was used in a molecular analysis to determine if traditional plastid-based

taxonomies are useful as well as to determine the legitimacy of the claims made in chapter 3.

References

Brawerman G, Eisenstadt JM (1964) Deoxyribonucleic acid from the chloroplasts of *Euglena gracilis*. Biochim Biophys Acta 91: 477-485

Brosnan S, Shin W, Kjer K, Triemer RE (2003) Phylogeny of the Photosynthetic Euglenophytes Inferred from the Nuclear SSU and Partial LSU rDNA. Intl J Sys Evol Microbiol [in press]

Brown PJP, Zakrys B, Farmer MA (2002) Plastid morphology, ultrastructure, and development in *Colacium* and the loricate euglenophytes (Euglenophyceae). J Phycol 39: 1-8

Cavalier-Smith T (1981) Eukaryote Kingdoms: seven or nine? Biosystems 14: 461-481

Cavalier-Smith T (1982) The origins of plastids. Biol J Lin Soc 17: 289-306

Candolle AP de (1813) Thoerie elémentaire de la botanique. Chez Deterville, Paris

Christopher DA, Hallick RB (1989) *Euglena gracilis* chloroplast ribosomal protein operon – A new chloroplast gene for ribosomal protein L5 and description of a novel organelle intron category designated group-III. Nuc Acid Res 17: 7591-7608

Conrad W (1916) Révision des éspèces indigènes et français du genre *Trachelomonas* Ehrenbg. Annales de Biologie Lacustre 8:193-212

Copertino DW, Hallick RB (1991) Group II twintron – an intron within an intron in a chloroplast cytochrome B-559 gene. Embo J 10: 433-442

Copertino DW, Christopher DA, Hallick, RB (1991) A mixed group II/group III twintron in *Euglena gracilis* chloroplast ribosomal protein S3 gene: evidence for intron insertion during gene evolution. Nuc Acid Res 19: 6491-6497

Dangeard P-A (1901) Recherches sur les Eugléniens. Le Botaniste 8: 297-357

Deflandre G (1926) Sur quelques Euglénacées nouvelles du Vénézuéla. Bull Mus Nat His. Na. 6:421-422

Deflandre G (1930) *Strombomonas* nouveau genre d'Euglénacées (*Trachelomonas* Ehrb. pro parte.). Arch Prot Kund 69: 551-614

Delwiche CF, Palmer JD (1997) The origin of plastids and their spread via secondary endosymbiosis. Pl Sys Evol [suppl] 11: 51-86

Dragos N, Péterfi S, Craciun C (1979) Fine structure of *Euglena.* II. *Euglena stellata* Mainx and *Euglena viridis* Ehrenberg. Nova Hedwigia 31: 223-46

Fritsch FE (1961) Structure and Reproduction of the Algae vol. 1. University Press, Cambridge. 791 pp.

Gibbs SP (1960) The fine structure of *Euglena gracilis* with special reference to chloroplasts and pyrenoids. J Ultrastr Res 4: 127-148

Gibbs SP (1962) The ultrastrcture of the pyrenoids of algae, exclusive of the green algae. J Ultrastr Res 7: 247-261

Gibbs SP (1978) The chloroplasts of *Euglena* may have evolved from a symbiotic green algae. Can J Bot 56: 2882-2889

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

Hallick RB, Hong L, Drager RG, Favreau MR, Monfort A, Orsat B, Spielmann A, Stutz E (1993) Complete sequence of *Euglena gracilis* chloroplast DNA. Nuc Acids Res 21: 3537-3544

Hollande A (1952) Classes de Eugléniens (Euglenoidina Butschli 1884) Traité de Zoologie, vol. I fasc. I. Grasse, P. P. [ed.] Maison et. cie., Paris 238-284

Jahn TL (1951) Euglenophyta in Manual of Phycology, Ed. G. M. Smith, Chronica Botanica Co. Waltham, Massachusetts 69-81

Klebs G (1883) Über die organisation einiger flagellaten-gruppe und ihre beziehungen zu algen und infusorien.Untersuchen aus dem Botanishes Institut zu Tübingen. 1: 233-362

Leander BS, Farmer MA (2000) Comparative morphology of the euglenid pellicle. I. Patterns of strips and pores. J Euk Microbiol 47: 469-479

Leander BS (2001) Evolutionary morphology of the euglenid pellicle. PhD dissertation The University of Georgia

Leander BS, Farmer MA (2001a) Comparative morphology of the euglenid pellicle. II. Diversity of strip substructure. J Euk Microbiol 48: 202-217

Leander BS, Farmer MA (2001b) Evolution of *Phacus* (Euglenophyceae) as inferred from pellicle morphology and small subunit rDNA. J Phycol 37: 143-159

Leander BS, Triemer RE, Farmer MA (2001) Character evolution in the heterotrophic euglenids. Europ J Protistol 37: 337-356

Leander BS, Witek RP, Farmer MA (2001) Trends in the evolution of the euglenid pellicle. Evolution 55: 2215-2235

Lee RE (1977) Evolution of algal flagellates with chloroplast endoplasmic reticulum from the cilliates. S Afr J Sci 73: 179-182

Leedale, G. F. (1967) The Euglenoid Flagellates. Prentice-Hall, Englewood Cliffs, NJ. 220 pp.

Linton E, Hittner D, Lewandowski C, Auld T, Triemer RE (1999) A molecular study of euglenoid phylogeny using SSU rDNA. J Euk Micro 46: 217-223.

Linton EW, Nudelman MA, Conforti V, Triemer RE (2001) A molecular analysis of the euglenophytes using SSU rDNA. J Phycol 36: 740-746.

Manning JE, Wolstenholme DR, Ryan RS, Hunter JA, Richards OC (1971) Circular chloroplast DNA from *Euglena gracilis* (Electron microscopy, density gradient centrifugation/mitochondria). Proc Natl Acad Sci USA 68: 1169-1173

Margulis L (1981) Symbiosis in Cell Evolution. W. H. Freeman and Company, San Francisco, USA. 452 pp

Mayr E, Ashlock PD (1991) Principles of systematic zoology. McGraw-Hill Inc., New York, 475 pp

McFadden GI, Gilson PR (1995) Something borrowed something green: lateral transfer of chloroplasts by secondary endosymbiosis. Trends Ecol Evol 10: 12-17

Milanowski R, Zakryś B, Kwiatowski J (2001) Phylogenetic analysis of chloroplast small-subunit rDNA genes of the genus *Euglena* Ehrenberg. Int J Sys Evol Microbiol 51: 773-81

Montegut-Felkner AE, Triemer RE (1997) Phylogenetic relationships of selected euglenoid genera based on morphological and molecular data J Phycol 33: 512-

519

Mullner AN, Angeler DG, Samuel R, Linton EW, Triemer RE (2001) Phylogenetic analysis of phagotrophic, phototrophic and osmotrophic euglenoids by using the nuclear 18S rDNA sequence. Int J Sys Evol Microbiol 51: 783-791

Preisfeld A, Berger S, Busse I, Liller S, Ruppel HG (2000) Phylogenetic analyses of various euglenoid taxa (Euglenozoa) based on 18S rDNA sequence data. J Phycol 26: 220-226

Pringsheim EG, (1956) Contributions toward a monograph of the genus *Euglena*. Nov Act Leop 125: 1-168

Sagan L. (1967) On the origin of mitosing cells. J Theor Biol 14: 225-275

Senn G (1900) Euglenineae. In Die Natürlichen Pflaztenfamilien Engler, A., & Prantl, K. Eds. 1:173-185

Simpson AGB (1997) The identity and composition of the Euglenozoa. Arch Prot Kund 148:318-328

Soltis DE, Soltis PS, Doyle J (1998) Molecular Systematics of Plants II: DNA sequencing. Kluwer Academic Publishers, Norwell, MA, USA. 574 pp

Stein FR von (1878) Der organismus der infusion theire III. Der Organismus der Flagellaten I. Verlag von Wilhwlm Engelmann, Leipzig.

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chunwongse J, ObokataJ, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence

of tobacco chloroplast genome:its gene organization and expression Embo J 5: 2043-2049

Shimada H, Sugiura M (1991) Fine structural features of the chloroplast genome: comparison of the sequenced chloroplast genomes. Nuc Acid Res 19: 983-995

Tell G, Conforti V (1986) Euglenophyta Pigmentadas de la Argentina. In Biblioteca Phycologica Ed. Cramer, J. Gebr. Borntraeger, Berlin-Stuttgart. 1-201

Thompson MD, Copertino DW, Thompson E, Favreau MR, Hallick RB (1995) Evidence for the late origin of introns in chloroplast genes from an evolutionary analysis of the genus *Euglena*. Nuc Acids Res 23: 4745-4752

Van Den Hoek C, Mann DG, Jahns HM (1995) Algae: An Introduciton to Phycology. University Press, Cambridge. 627 pp

Van Leeuwnhoek A (1674) 6th letter to the royal society of London. Phil Trans 9: 179-182

Zakryś B (1986) Contribution to the monograph of Polish members of the genus *Euglena* Ehrenberg 1830. Nova Hedwigia 42: 491-540

Zakryś B, Walne PL (1998) Comparative ultrastructure of chloroplasts in the Subgenus *Euglena* (Euglenophyta): Taxonomic significance. Cryptogamie Algol 19: 3-18

Zakryś B, Cambra-Sanchez J, Walne PL (2001) Chloroplast ultrastructure of *Euglena cuneata* Pringsheim, *E. deses* Ehrenberg and *E. mutabilis* (Euglenophyceae): Taxonomic significance. Acta Protozool 40: 161-7

CHAPTER 2

REDESCRIPTION OF *EUGLENA RUSTICA* (EUGLENOPHYCEAE), A RARE EUGLENOPHYTE FROM THE INTERTIDAL ZONE[1]

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1 Brown, P.J.P., Leander, B.S., and M.A. Farmer 2001. *Phycologia* 41:445-452
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Abstract:

P.J.P. BROWN, B.S. LEANDER AND M.A. FARMER. 2002. Redescription of *Euglena rustica* (Euglenophyceae), a rare euglenophyte from the intertidal zone. *Phycologia* 41: 445-452. *Euglena rustica*, a rare species of *Euglena*, was discovered growing in patches in the intertidal sand of Nannygoat Beach at the University of Georgia Marine Institute at Sapelo Island, Georgia, USA. A full emended description is given. The cells are ~ 50 μm long and the posterior is never attenuate. The chloroplasts are parietal with an internal pyrenoid. No paramylon is associated with the pyrenoid. The pellicle has 56 or 60 strips at the midpoint of the cell. There are four whorls of strip reduction at the posterior of the cell, and two whorls of exponential strip reduction at the anterior end. *Euglena rustica* also has muciferous bodies located in pockets of the chloroplast between the plastid and the pellicle. The muciferous bodies fluoresce in the 515–555 nm range when excited in the 465–495 nm range. A majority of the cells examined also contained intracellular bacteria in the cytoplasm. We give a formal emended description of *E. rustica* and designate a lectotype and epitype.

Introduction:

The genus *Euglena* Ehrenberg is one of the better-known protistan genera, with just under two hundred species described (Godjics 1953; Huber-Pestalozzi 1955; Pringsheim 1956; Leedale 1967). Although the model organism *Euglena gracilis* Klebs has been well studied for decades (over 2000 references), the euglenids as a whole are only now beginning to be studied intensely (Linton *et al.* 1999, 2000; Leander & Farmer 2000a, b, 2001a, b; Müllner *et al.* 2001; Milanowski *et al.* 2001; Priesfeld *et al.* 2000, 2001) and relatively little is known about phototrophic euglenids from marine

environments. Members of the obligately marine genus *Klebsiella* Pascher have been observed in polluted seawater, in association with macrophytic seaweeds (Leedale 1967), and some species in the genera *Eutreptia* Perty*, Tetreutreptia* McLachlan, Seguel & Fritz and *Eutreptiella* Da Cunha are found in marine environments (Leedale 1967; Walne *et al.* 1986; McLachlan *et al.* 1994; our unpublished observations). There is also evidence of members of the genus *Euglena* in haline mud flats, estuaries and tidal rivers (Palmer & Round 1965; Leedale 1967; Kingston 1997; Serodio *et al.* 1997; Oxborough *et al.* 2000).

The most abundant algal inhabitants of intertidal beaches are usually pennate diatoms, and the beaches of Sapelo Island, Georgia, are no exception (Williams 1962, 1964, 1965; Darley 1979). In addition to the diatom population, there are a number of phytoflagellates present as well on Sapelo Island beaches, including prasinophytes and euglenophytes. There are even large patches of beach that appear green to the naked eye, which, upon closer inspection, are populated almost exclusively by *Euglena rustica* J. Schiller *in* Huber-Pestalozzi. The green patches seem to come and go as the cells migrate through the sand in response to illumination and tides. Vertical migration of euglenids is well documented and has been seen in both freshwater and saline forms, in both sand and mud (Palmer & Round 1965; Kingston 1997, 1999a, b).

The original description of *E. rustica* appears in G. Huber-Pestalozzi's extensive monograph of the euglenids (Huber-Pestalozzi 1955, p. 113). Unfortunately Schiller's description is missing several key features that are indispensable in identifying euglenophytes at the species level, the most notable of which is the presence or absence of pyrenoids. Schiller's description literally says 'pyrenoids?'. Photomicroscopy was still uncommon in 1955 and so the only visual representations of the species in the original

description are three small line drawings (Huber-Pestalozzi 1955, fig. 96), all lacking in detail. This situation is common in euglenophyte taxonomy, making re-evaluation of many taxa necessary. *Euglena rustica* possesses a number of unique ultrastructural features that, together with its unusual environment and vague original description, make a rigorous redescription necessary. This includes features of the pellicle, chloroplasts, intracellular bacteria, and subpellicular muciferous bodies. Features of the pellicle are not discussed in great detail here, owing to previous publication of pellicle data elsewhere (Leander & Farmer 2000a, 2001a; Leander 2001), where the species was referred to as *Euglena* sp. We supply a Latin description for *E. rustica*, although this is not strictly necessary, and clarify the typication of the species.

Material and Methods:

Patches of green-tinted sand were removed from the intertidal zone of Nannygoat Beach on Sapelo Island, Georgia, in May 2000. The sand was placed in plastic storage containers, immersed in seawater, and kept in clear 120 ml plastic cups with opaque lids; these were put in a 20°C incubator on a 14 : 10 light : dark cycle, with an illumination of 15–35 μmol photons m^{-2} sec^{-1} provided by cool-white fluorescent bulbs.

For transmission electron microscopy (TEM), cells were collected from the aqueous layer of the wild sample with a plastic transfer pipette, and the presence of cells was confirmed by light microscopy (LM). The cells were pelleted by gentle centrifugation and fixed in 2% paraformaldehyde plus 0.1% glutaraldehyde, buffered in 0.1 M cacodylate, for 1 h at 4°C. The cells were rinsed twice in 0.1 M cacodylate buffer at 4°C for 10 min each and then postfixed in 1% OsO_4 for 1 h at 4°C and dehydrated in a graded ethanol series. The cells were infiltrated and embedded in epoxy resin

(Polysciences Embed 812, Warrington, Pennsylvania) and light gold–silver sections were cut. The sections were poststained with uranyl acetate and lead citrate, and viewed on a JEOL 100CX II TEM operating at 80 kV.

For scanning electron microscopy (SEM), cells were collected as above and fixed by exposure to OsO_4 vapour for 30 min at room temperature, then with *c.* 0.5% liquid OsO_4 solution for 30 min. They were then collected onto a Millipore^{TM} membrane filter with 8 μm pores. After dehydration using a graded ethanol series, the cells were critical point dried in CO_2, mounted on aluminium stubs, and sputter coated with 15.3 nm of gold. The stubs were viewed on a LEO 982 FEG-SEM operating at 4 kV.

For confocal laser scanning microscopy, cells were collected as above and lightly fixed with 1% glutaraldehyde and 0.5 M cacodylate for 30 min. After fixation, the cells were rinsed twice in Euglena medium (Greenblatt & Schiff 1959), mounted on glass microscope slides, and sealed. The cells were then viewed on a BioRad MRC 600 confocal laser scanning microscope at an excitation wavelength of 568 nm. Living cells immersed in a methylcelluose mixture were observed prior to photomicroscopy in order to ensure that fluorescence patterns were not induced by glutaraldehyde. For conventional LM, cells were prepared as above, except that the fixative was 1% glutaraldehyde in 0.1 M cacodylate buffer; and they were viewed on a Nikon inverted microscope with differential interference contrast (DIC) optics.

Observations:

***Euglena rustica* J. Schiller *in* Huber-Pestalozzi 1955 *emend* P.J. Brown, B.S. Leader & Farmer 2002**

Figs 1–18

Cellula bacilliformis, 46–50 μm longa et 12–14 μm lata; apices nunquam attenuati. Chloroplasti 5–12 per cellulam, parietales, acetabuliformes, discoidei, solo pyrenoide ad centrum sine paramylo. Flagellum cellula 1–1.5 plo longior. Stigma grandis adest in anteriore parte cellulae. Periplastus spiralis, 56 aut 60 striis in media parte cellulae. In posteriore parte cellulae 4 verticilli in quibis striae numero redigunt; in anteriore parte cellulae 2 verticilli in quibus striae numero redigunt. Stria pelliculae cruciatim caesa, similis 'A' apparet. Nucleus amorphus plerumque in media parte cellulae locatus. Marina.

Cells elongate (46–50 μm long, 12–14 μm wide), with a rounded anterior and posterior, and parallel sides; the posterior never tapers. Canal opening visible at anterior of cell. Chloroplasts 5–12, flattened, discoid, distributed over the whole cell, parietally located. Margins irregular to highly lobed. Pyrenoids present, centrally located. No paramylon associated with the plastids. Stigma large and appressed to the reservoir. Paramylon grains small and ovoid. Cells usually motile. Flagellum 1–1.5 × as long as the cell, robust, performing a typical figure eight movement. When not motile, cells become rounded, aflagellate, and adherent to sand grains. Euglenoid movement rare, not pronounced. Pellicle finely and densely striated, with 56 or 60 pellicular strips at the midpoint of the cell. Four whorls of exponential strip reduction at the posterior of the

cell. Two whorls of exponential strip reduction at the anterior end. Frame of pellicle A-shaped in cross section. Nucleus amorphous, centrally located. Marine.

LECTOTYPE (designated here): Huber-Pestalozzi 1955, fig. 96, showing *E. rustica* in different phases of metaboly.

EPITYPE: Fixed in 2% formaldehyde–0.1% glutaraldehyde–0.1 M cacodylate, embedded in resin on a microscope slide [American Museum of Natural History (AMNH Protozoa) 810]. Paratypes fixed as above and prepared for transmission electron microscopy (AMNH Protozoa 811.1, 811.2, 811.3, 812).

TYPE LOCALITY: 31.38936°N, 81.26513°W, End of Beach Road, Nannygoat Beach, Sapelo Island, Georgia, USA.

General morphology and ultrastructure. Euglena rustica is a moderately sized euglenophyte, 50 μm in length. The posterior end remains rounded at all times and has never been observed to become even moderately acute (Figs 1–3). Flagellated cells can be observed swimming in the water column when sand containing *E. rustica* is immersed in seawater. Flagellated cells can also be seen wriggling between the grains via moderate euglenoid movement. Aflagellate, cyst-like cells can often be observed attached to sand grains.

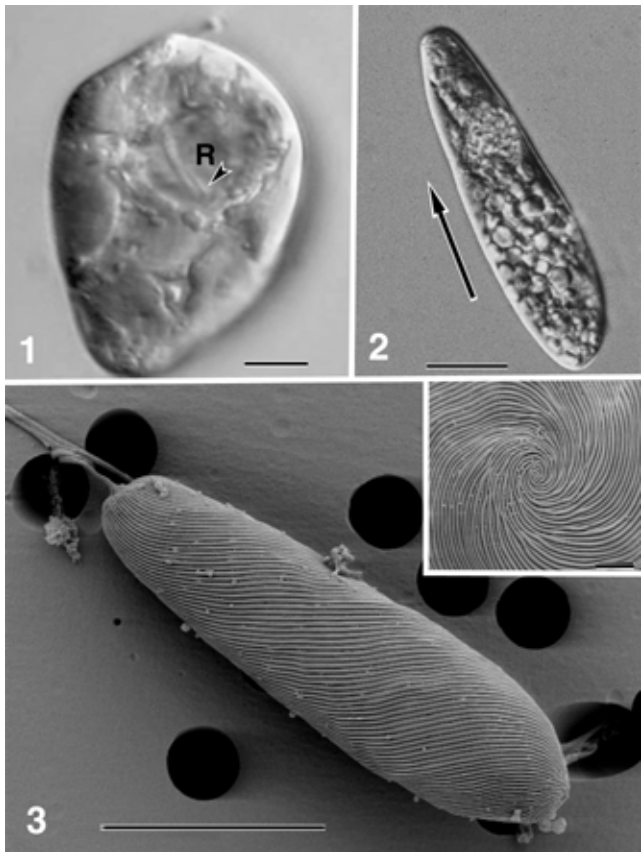
The nucleus is typically located in the central region of the cell, and can be spherical or oblong. Some sections show evidence of the 'lateral lamellar body of unknown function' observed in other euglenids (Leedale 1967). The flagellum is typically 1–1.5 times as long as the cell and moves with the 'figure eight' movement (Gojdics 1953; Leedale 1967) characteristic of photosynthetic euglenids. Aflagellate cells are most commonly found as rounded cyst-like cells attached to sand grains, and are

Figs 2.1–3. *Euglena rustica*: general morphology.

Fig. 2.1. Cell in its most contracted state, LM, DIC. The nonemergent flagellum (arrowhead) is readily apparent inside the reservoir (R). Scale bar = 5 μm.

Fig. 2.2. A fully extended cell, LM, DIC. The arrow points towards the anterior end of the cell. Scale bar = 10 μm.

Fig. 2.3. Whole cell, SEM, highlighting the surface morphology of the pellicle, as well as illustrating the morphology of a cell in its most relaxed state Scale bar = 20 μm. Inset: posterior tip. Scale bar = 2 μm.


R
1
2
3

usually uncommon in the water column. The paramylon is monomorphic and occurs as small ovoid grains (Fig. 2). The reservoir of *E. rustica* is large (*c.* 7 μm in diameter) and the nonemergent flagellum is visible within (Fig. 1). The emergent flagellum bears a very robust flagellar swelling at the point where the flagella can be shaded by the stigma (eyespot), these together comprising the photosensory apparatus (Figs 4, 5). The Golgi apparatus of *E. rustica* is comprised of ~ 10 cisternae, whereas other phototrophic euglenids typically possess 20 or more (Leedale 1967). The morphology of the mitochondrion of *E. rustica* in random TEM sections is consistent with it being a single reticulate body, as is typical of *Euglena*, with several circular cross sections visible in a given micrograph (Figs 6, 7). In several sections the mitochondria was noted to possess unusual cristae (Fig. 7), these being peripheral, which is a morphology not reported in other euglenids, and previously unseen by the authors.

Ultrastructure of plastid morphology. Ultrastructurally, the chloroplasts are similar to those of *E. mutabilis* Schmitz (Zakrys *et al.* 2001), in that they have an internal pyrenoid with no associated paramylon (Figs 9–11). From the surface, the plastids appear discoid, with very obtuse lobes (Fig. 8); in LM, the pyrenoid appears as a refractive region in the centre of each plastid. There are typically 5–12 plastids per cell, which are evenly distributed around the periphery of the cell (Figs 8, 11). In transverse section they can be seen to extend deeper into the cytosol at the site of the pyrenoid (Figs 9, 11). Numerous invaginations of the plastid occur on the face nearest the plasma membrane, and these accommodate small muciferous bodies (Figs 9, 10). The pyrenoid is penetrated by 6–10 thylakoid lamellae (Fig. 10), with the typical euglenophyte arrangement of two thylakoids in each penetrating lamella (not shown).

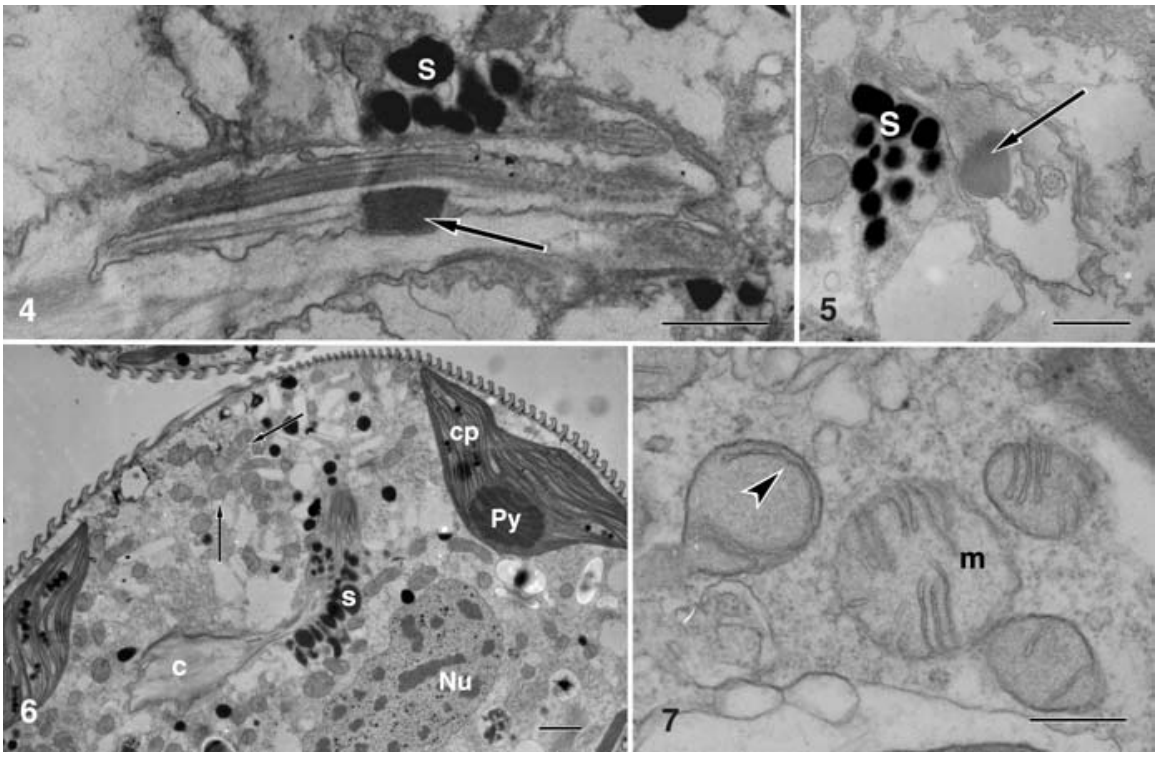
Figs 2.4–7. *Euglena rustica*: general ultrastructure, TEM. Scale bars = 1μm (Figs 4–6) or 0.5 μm (Fig. 7). Abbreviations: s = stigma; c = canal; cp = chloroplast; Py = pyrenoid; Nu = nucleus; m = mitochondrion

Fig. 2.4. Longitudinal section through the photosensory apparatus. The flagellar swelling in *E. rustica* is very robust (arrow), more so than other species of *Euglena* described to date.

Fig. 2.5. Cross section through the photosensory apparatus. The flagellar swelling (arrow) is directly opposed to the stigma.

Fig. 2.6. Anterior portion of the cell. Several sections through the mitochondrial reticulum are visible (arrows). The oblong nucleus and portions of two chloroplasts are also visible.

Fig. 2.7. High magnification of a section through the mitochondrion. The arrowhead indicates the unusual peripheral cristal morphology seen in several sections.


S
4
S
5
cp
Py
s
c
Nu
6
m
7

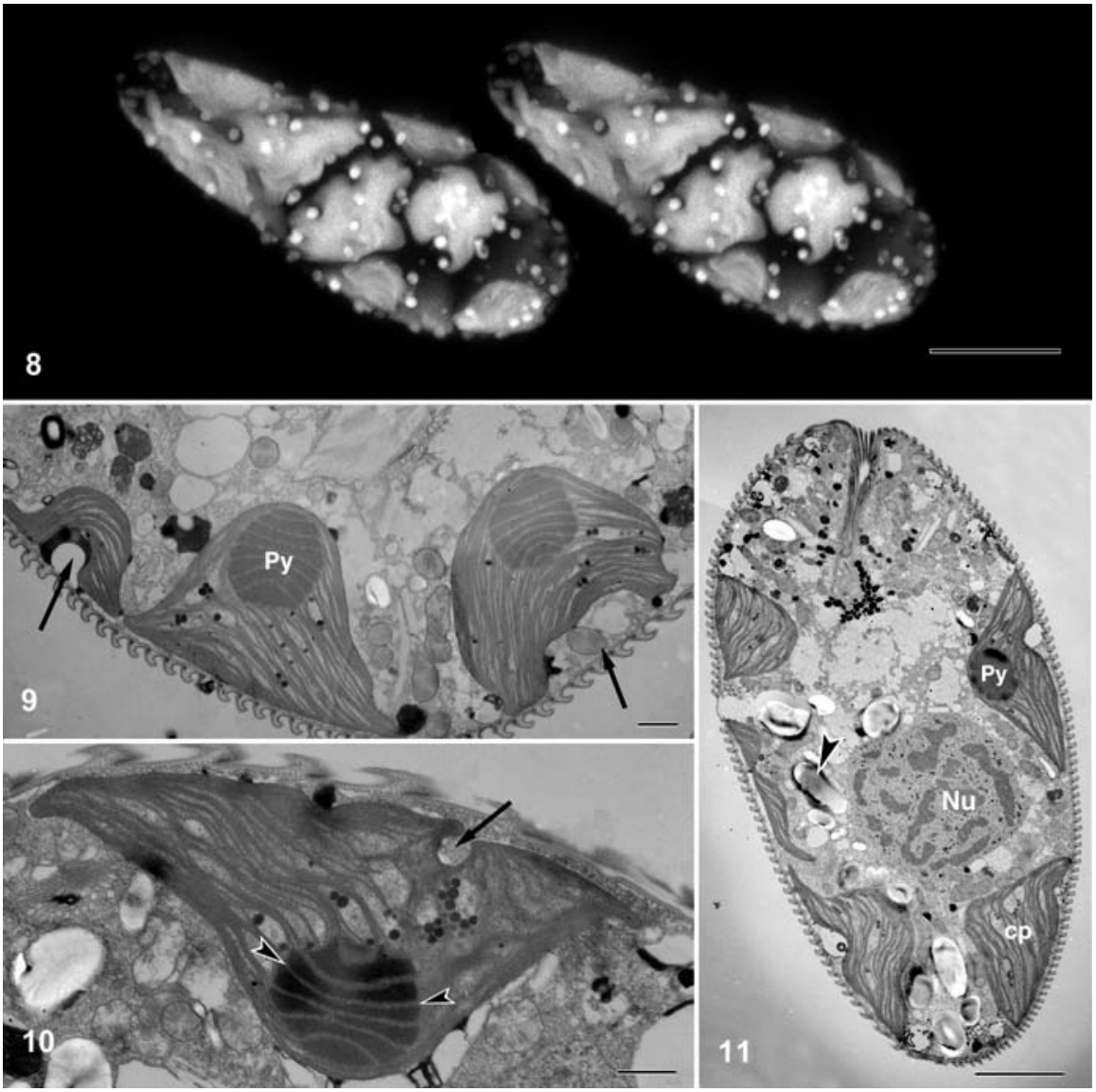
Figs 2.8–2.11. *Euglena rustica*: chloroplast morphology. Abbreviations: Py = pyrenoid; Nu = nucleus; cp = chloroplast.

Fig. 2.8. Confocal stereomicrograph of *E. rustica* autofluorescence at 568 nm excitation. The larger bodies are chloroplasts, and the punctate bodies are the muciferous bodies. Scale bar = 10 μm.

Fig. 2.9. Parietal chloroplasts of *E. rustica*, TEM. The pockets formed to accommodate the muciferous bodies are readily apparent (arrows). Note that the pyrenoid has no associated paramylon. Scale bar = 1 μm.

Fig. 2.10. A single chloroplast, TEM. Thylakoids penetrate the pyrenoid (arrowheads); a muciferous body pocket can also be seen (arrow). Scale bar = 1 μm.

Fig. 2.11. Mid-sagittal section through an entire cell of *E. rustica*. The parietal positions of the plastids are obvious, and the ovoid paramylon morphology can be seen (arrowhead). Scale bar = 5 μm.


8
9
Py
10
11
Py
Nu
cp

Muciferous bodies. Many euglenids possess either muciferous bodies or the more highly modified ejectile organelles known as mucocysts. The muciferous bodies of *E. rustica* are membrane bound bodies located either in invaginated pockets of the plastid or just under the pellicle (Fig. 12). Prior to discharge, they are filled with a moderately osmiophilic, amorphous substance, and are located under the pellicular pores (Fig. 13). After discharge, they appear as empty sacs and are usually distinguishable only if they are located in pockets of the chloroplast (Fig. 12). The muciferous bodies of *E. rustica* are remarkable in that they fluoresce. This phenomenon has not been previously reported in *E. rustica*, and has not been observed by the authors in any other euglenid taxa. The excitation wavelength of the muciferous bodies is around 465–495 nm, with an optimal emission between 515–555 nm. This is quite different from the fluorescence properties of the plastids (excitation = 513 nm, emission = *c.* 568 nm), as can be seen in Figs 14 and 15.

Pellicle Morphology. The pellicle of *E. rustica* has been described already under the name '*Euglena* sp.' (Leander & Farmer 2000a, 2001a). Unlike any other species of *Euglena,* the number of strips around the periphery is either 56 or 60 (Leander & Farmer 2000a). A very useful feature for euglenid systematics is the amount and nature of pellicle strip reduction (Leander & Farmer 2000a; Leander 2001). In most species of *Euglena* and other euglenids, not all of the pellicle strips terminate at the apices of the cell; rather, some terminate in distinct rings or 'whorls' near the apices (Leander & Farmer 2000a; Leander 2001). These whorls can be characterized as exponential, linear, or pseudolinear and appear to result from strip doubling events that have taken place over the evolutionary history of the Euglenophyceae (Leander 2001; Leander & Farmer 2000a,

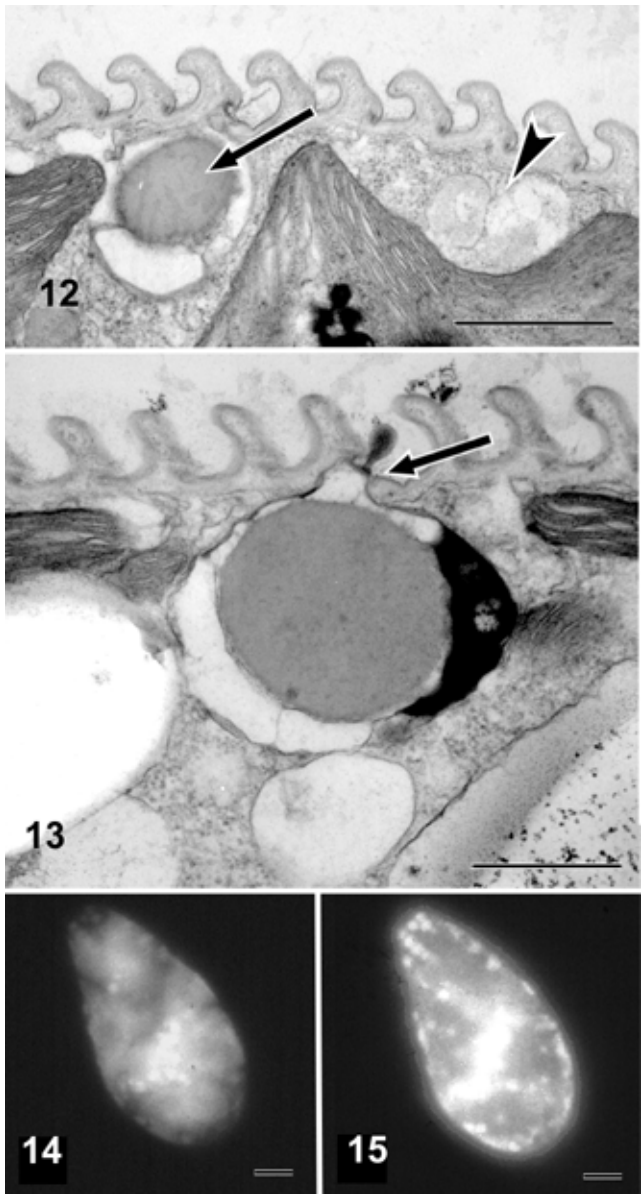
Figs 2.12–15. *Euglena rustica*: muciferous bodies.

Fig. 2.12. A discharged (arrowhead) and an undischarged (arrow) muciferous body, TEM. Note that the discharged muciferous body lies in a pocket of the chloroplast, and would not be readily visible in the cytoplasm proper. Scale bar = 1 μm.

Fig. 2.13. A single undischarged muciferous body immediately underlying a pellicular pore (arrow), TEM. Scale bar = 0.5 μm.

Fig. 2.14. Epifluorescent micrograph of a cell excited at 528–552 nm and imaged at an emission of 578–632. Scale bar = 5 μm.

Fig. 2.15. Epifluorescent micrograph of the same cell as in Fig. 14, but excited at a wavelength of 465–495 nm and imaged at an emission wavelength 515–555 nm. Scale bar = 5 μm.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14
15

2001b). In *E. rustica*, there are four whorls of exponential strip reduction at the posterior end (Fig. 16) and two whorls of exponential strip reduction at the anterior end (Fig. 17) of *E. rustica* (Leander & Farmer 2000a). Also of note is the peculiar 'A-shaped' frame of *E. rustica*'s pellicular strips (Fig. 18; Leander & Farmer 2001a), a state not yet observed in other taxa. These features of the pellicle, when taken together, are sufficient on their own to unambiguously define *E. rustica*.

Endocytic bacteria. Many individual cells host endocytic rod-shaped bacteria. The bacteria are usually surrounded by an electron transparent region and appear to have a central fibrillar core (Fig. 19) similar to that mentioned by Surek & Melkonian (1983). The bacteria were observed dividing in otherwise healthy cells of *E. rustica* (Fig. 19); however, cells that appeared to be unhealthy at the time of fixation hosted several times the bacterial load of their healthy counterparts (Fig. 20). The bacteria were free in the cytosol and were not observed inside any membrane-bound compartment. They were usually located towards the centre of the cell and were most often perinuclear. Not all of our sections showed evidence of bacteria, but cells taken from the same location at different times (nearly a year apart) showed the presence of morphologically similar bacteria.

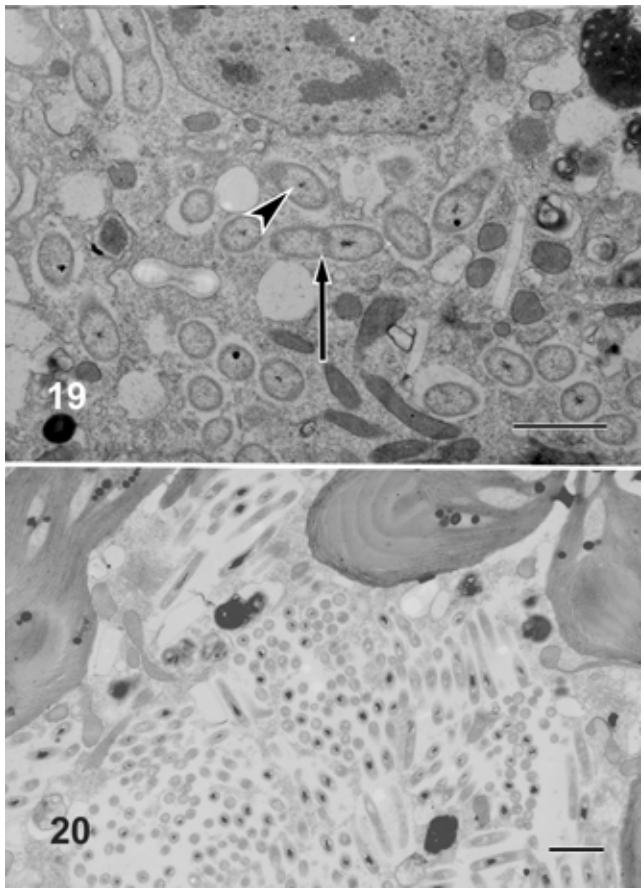
Discussion:

The endocytic bacteria of *E. rustica* cells are something of an enigma. It is not clear at this time whether they have a mutualistic, commensal, or pathogenic relationship with their host. Since our attempts to grow *E. rustica* in pure culture have failed, the exact nature of the association cannot yet be determined. Previous studies on intracellular bacteria in *Euglena* (Leedale 1969; Perterfi *et al.* 1979; Surek & Melkonian 1983; Dragos

Figs 2.19–20. Endocytic bacteria in *E. rustica*, TEM. Scale bars = 1 μm.

Fig. 2.19. Dividing bacteria (arrow) in a healthy cell of *E. rustica*. Many bacteria can be seen to possess a central fibrillar core (arrowhead) like that mentioned by other researchers.

Fig. 2.20. Cell with a high bacterial load. This cell appeared to be unhealthy at the time of fixation.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20

et al. 1990) did not draw any conclusions about the nature of the relationship of the bacteria to the euglenid cell. Surek & Melkonian (1983) reported isolating an axenic strain of *Euglena mutabilis* that contained intracellular bacteria very similar to those seen in *E. rustica*, which seemed to have growth rates that matched those of the host cells. There have been no reports, however, of a profusion of the intracellular bacteria in dead or dying cells. Future investigations with this taxon will attempt to characterize the relationship between the intracellular bacteria and *E. rustica*, once a unialgal culture can be achieved.

Superficially, *E. rustica* bears some resemblance to *Euglena clara* (Skuja 1948) in both its size and shape, but it differs in regard to swimming pattern, speed, and chloroplast structure. The plastid features are very similar to *E. mutabilis* (Zakrys *et al.* 2001), but the pellicle, flagella, cell shape, muciferous bodies, and movement are very different. A search of the literature uncovered a species that is described as having features like those of *E. rustica*, namely *E. retronata* Johnson (1944). However, although these two species are similar, it is apparent that *E. retronata* is significantly different from *E. rustica* in a number of characters, such as cell shape and the tendancy of the paramylon to be packed into the anterior of the cell in *E. retronata*; *E. retronata* is also smaller (Table 1). Some researchers have mentioned *E. obtusa* Schmitz as being present in the intertidal sands of Sapelo Island (Williams 1962). Although we never observed *E. obtusa* in high quantities in the green patches of sand, we did find it at some locations on the beaches of Sapelo Island and so we include it in Table 1 to facilitate identification of these marine or brackish euglenids.

Table 2.1 Comparison of *E. rustica* with other eulgenophytes

	E. rustica **Brown et al. (2002)**	***E. rustica*** **Schiller (1955)**	***E. retronata*** **(Johnson 1944)**	***E. obtusa*** **(Schmitz 1844)**
Dimensions	46-50 µm X 12-14 µm	40-44 µm X 12-14 µm	18-34 µm X 7-15 µm	103.5-123 µm X 17.25-29 µm
Shape	Cells are always rounded front and back. Sides parallel. Canal opening visible in the anterior.	Front tapers to a rounded end, back rounded, sometimes flattened.	Fusiform, broadened in the middle. Posterior is a blunt tip.	Rounded front and back, sides parallel. No visible notch at the canal opening.
Chloroplasts	5-12 flattened discoid bodies, parietally located. Margins range from irregular to highly lobed. Pyrenoids visible in the light microscope; no pyrenoid cap. Chlorplasts distibuted throughout the cell.	6-8 disc-shaped plastids. Pyrenoids might be present. Chloroplasts typically sequestered in the posterior end of the cell.	8-12 elongated concavo-convex bodies, parietally located. Can be forced to the posterior of the cell when paramylon is abundant.	28-40 disc-like plastids with irregular margins. Each plastid bears a single pyrenoid with a paramylon cap. The plastids can be very closely appressed to one another.
Flagellum	1-1.5 X cell length; robust, easily visualized.	0.5 X cell length; very fine.	1-1.23 X cell length.	No emergent flagellum has ever been reported.
Paramylon	Small ovoid granules free in the cytoplasm.	Minute (difficult to see) cytoplasmic granules.	Few to many ovoid grains. If abundant, they are packed into the anterior of the cell.	Numerous cytoplasmic granules in addition to the caps on the pyrenoids.

Acknowledgements:

Financial support was provided by the National Science Foundation PEET (Partnerships for Enhancing Expertise in Taxonomy) grant DEB 4-21348. The authors would like to thank Dr. W.M. Darley for bringing the green patches of sand to our attention and the University of Georgia Marine Institute at Sapelo Island for providing laboratory space and assistance with field work. Special thanks are due to Dr R.I. Curtis for his kind help with the Latin diagnosis.

References:

DARLEY W.M. 1979. Utilization of dissolved organic carbon by natural populations of epibenthic salt marsh diatoms. *Journal of Phycology* 15: 1–5.

DRAGOS N., PETERFI L.S., NEDELCU A., & CRCIUN, V. 1990. Intracellular bacteria in euglenoid flagellates. *Revue Romaine de Biologie. Série de Biologie Végétale* 35: 51–54.

GODJICS M. 1953. *The genus* Euglena. University of Wisconsin Press, Madison Wisconsin. 268 pp.

GREENBLATT C.L. & SCHIFF J.A. 1959. A pheophytin-like pigment in dark adapted *Euglena gracilis*. *Journal of Protozoology* 6: 23 –28.

HUBER-PESTALOZZI G. 1955. *Das Phytoplankton des Süβwassers; Systematik und Biologie: 4 Teil; Euglenophyceen*. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart. 1265 pp.

JOHNSON L.P. 1944. *Euglenae* of Iowa. *Transactions of the American Microscopical Society* 63: 97–135.

KINGSTON M.B. 1997. Vertical migration of *Euglena* sp. in a Piedmont Stream. *Journal of the Elisha Mitchell Scientific Society* 113: 86.

KINGSTON M.B. 1999a. Wave effects on the vertical migration of two benthic microalgae: *Hantzschia virgata* var. *intermedia* and *Euglena proxima*. *Estuaries* 22: 81–91.

KINGSTON M.B. 1999b. Effect of light on vertical migration and photosynthesis of *Euglena proxima* (Euglenophyta). *Journal of Phycology* 35: 245–253.

LEANDER B.S. 2001. *The evolutionary morphology of the euglenid pellicle*. PhD. dissertation, The University of Georgia, Athens, Georgia. 281 pp.

LEANDER B.S. & FARMER M.A. 2000a. Comparative morphology of the euglenid pellicle. I. Patterns of strips and pores. *Journal of Eukaryotic Microbiology* 47: 469–479.

LEANDER B.S. & FARMER M.A. 2000b. Epibiotic bacteria and a novel pattern of strip reduction on the pellicle of *Euglena helicoideus* (Bernard) Lemmermann. *European Journal of Protistology* 36: 405–413.

LEANDER B.S. & FARMER M.A. 2001a. Comparative morphology of the euglenid pellicle. II. Diversity of strip substructure. *Journal of Eukaryotic Microbiology* 48: 202–217.

LEANDER B.S. & FARMER M.A. 2001b. Evolution of *Phacus* (Euglenophyceae) as inferred from pellicle morphology and small subunit rDNA. *Journal of Phycology* 37: 143–159.

LEEDALE G.F. 1967. *The euglenoid flagellates*. Prentice–Hall, Englewood Cliffs, New Jersey. 242 pp.

LEEDALE G.F. 1969. Observations on the endonuclear bacteria in euglenoid flagellates. *Österreichische Botanische Zeitschrift* 116: 279–294.

LINTON E., HITTNER D., LEWANDOWSKI C., AULD T. & TRIEMER R. E. 1999. A molecular study of euglenoid phylogeny using SSU rDNA. *Journal of Eukaryotic Microbiology* 46: 217–223.

LINTON E.W., NUDELMAN M.A., CONFORTI V. & TRIEMER R.E. 2000. A molecular analysis of the euglenophytes using SSU rDNA. *Journal of Phycology* 36: 740–746.

MCLACHLAN J.L., SEGUEL M.R. & FRITZ L 1994. *Tetreutreptia pomquitensis* gen. et. sp. nov. (Euglenophyceae) a quadriflagellate, phototrophic marine euglenoid. *Journal of Phycology* 30: 538–544.

MILANOWSKI R., ZAKRYS B. & KWIATOWSKI J. 2001. Phylogenetic analysis of chloroplast small-subunit rDNA genes of the genus *Euglena* Ehrenberg. *International Journal of Systematic and Evolutionary Microbiology* 51: 773–781

MÜLLNER, A.N., ANGELER D.G., SAMUAL R., LINTON E.W. & TRIEMER R.E. 2001. Phylogenetic analysis of phagotrophic, phototrophic and osmotrophic euglenoids by using the nuclear 18S rDNA sequence. *International Journal of Systematic and Evolutionary Microbiology* 51: 783–791.

OXBOROUGH K., HANLON A.R.M., UNDERWOOD G.J.C. & BAKER N.R. 2000. In vivo estimation of the photosystem II photochemical efficiency of individual microphytobenthic cells using high-resolution imaging of chlorophyll a fluorescence. *Limnology and Oceanography* 45: 1420–1425.

PALMER J.D. & ROUND F.E. 1965. Persistent vertical-migration rhythms in benthic microflora. I. Effect of light and temperature on rhythmic behaviour of *Euglena obtusa*. *Journal of the Marine Biological Association of the United Kingdom* 45: 567–582.

PETERFI L.S., DRAGOS N. & CRACIUN C. 1979. Fine structure of *Euglena* I. *Euglena tristella* Chu. *Nova Hedwigia* 31: 197–221.

PREISFIELD A., BERGER S., BUSSE I., LILLER S. & RUPPEL H.G. 2000. Phylogenetic analysis of various euglenoid taxa (Euglenozoa) based on 18S rDNA sequence data. *Journal of Phycology* 36: 220–226.

PREISFIELD A., BUSSE I., KLINGBERG M., TALKE S. & RUPPEL H.G. 2001. Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the Rhabdomonadales (Euglenozoa). *International Journal of Systematic and Evolutionary Microbiology* 51: 751–758.

PRINGSHEIM E.G. 1956. Contributions toward a monograph of the genus *Euglena*. *Nova Acta Leopoldina* 125: 1–169.

SCHMITZ F. 1884. Beiträge zur Kenntnis der Chromatophoren. *Jahrbuch für wissenschaftliche Botanik* 15: 1–175.

SERODIO J., DA SILVA J.M. & CATARINO F. 1997. Nondestructive tracing of migratory rhythms of intertidal benthic microalgae using in vivo chlorophyll *a* fluorescence. *Journal of Phycology* 33: 542–553.

SKUJA H. 1948. Taxonomie des Phytoplanktons einigen Seen in Uppland, Schweden. *Symbolae Botanicae Upsaliensis*. 9: 1–399.

SUREK B. & MELKONIAN M. 1983. Intracellular bacteria in the Euglenophyceae: prolonged axenic culture of an algal–bacterial system. In: *Endocytobiology II* (Ed. by H.E.A. Schenk & W. Schwemmler), pp. 473–486. Walter de Gruyter, New York.

WALNE P.L., MOESTRUP Ø., NORRIS R.E. & ETTL H. 1986 Light and electron microscopical studies of *Eutreptiella eupharyngea* sp. nov. (Euglenophyceae) from Danish and American waters. *Phycologia* 25: 109–126.

WILLIAMS R.B. 1962. *The ecology of diatom populations in a Georgia salt marsh.* PhD. dissertation, Harvard University, Cambridge, Massachusetts.

WILLIAMS R.B. 1964. Division rates of salt marsh diatoms in relation to salinity and cell size. *Ecology* 45: 877–880.

WILLIAMS R.B. 1965. Unusual motility of tube dwelling pennate diatoms. *Journal of Phycology* 1: 145–146.

ZAKRYS B., CAMBRA-SANCHEZ J. & WALNE P. 2001. Chloroplast ultrastructure of *Euglena cuneata* Pringsheim, *E. deses* Ehrenberg, and *E. mutablis* (Euglenophyceae): taxonomic significance. *Acta Protozoologica* 40: 161–167.

CHAPTER 3

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

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1 Brown, P.J.P., Zakrys, B., and M.A. Farmer (2003) Journal of Phycology 39(1) 1-8
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Abstract:

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

Introduction:

For decades, plastid morphology has been the premier character for classifications within the genus *Euglena*. In her seminal monograph on the genus *Euglena* Gojdics (1953) stated that "Chromatophores are the cytoplasmic features of *Euglena* that are the most conspicuous feature in the cell, and which show such constancy in a given species, that they have special value as taxonomic characters." Gojdics divided the genus *Euglena* into eight groups based primarily on plastid shape and size, and to some extent on pyrenoid features (Gojdics 1953). Pringsheim (1956) also divided *Euglena* into six groups relying primarily on plastid structure. Leedale (1967) formalized Pringsheim's

groups into subgenera, suggesting that *Astasia* is not phylogenetically distinct from *Euglena*, but making no official taxonomic adjustment. The most recent taxonomy of *Euglena* by Zakryś (1986) reduces the number of subgenera to three (*Euglena, Calliglena,* and *Discoglena*), all defined by plastid architecture. Ultrastructural investigations from a variety of euglenophyte taxa (Dragos et al. 1979, Pèterfi et al. 1979, Zakryś and Walne 1998, Zakryś et. al. 2001) have served not only to bolster previous taxonomic schemes using plastid structure but have elucidated ultrastructural details that were absent from earlier analyses (Haller 1959, Mignot 1965 & 1966, Buetow 1968, Leedale 1967 & 1982). In this paper the word 'euglenid' refers to all members of the Euglenida (phagotrophs, phototrophs, and osmotrophs) while 'euglenophyte' refers specifically to those euglenids that posses a plastid (including those that have secondarily lost them e.g. *Astasia Longa*).

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

Three of the five taxa in the current study belong to the only two loricate genera of euglenophytes, *Trachelomonas* Ehrenberg (1833) and *Strombomonas* Deflandre (1930). Most studies of loricate euglenophytes concentrate primarily on lorica morphology, often without mention of other cellular features (Conrad 1916, Deflandre 1926, Conforti et. al. 1993, Conforti & Joo 1994, Conforti 1999, Shi 1999). This emphasis on lorica morphology makes comparison to other euglenophytes difficult. *Colacium* Ehrenberg (1838) is the only colonial euglenophyte genus. During stationary phase growth, the individual cells of *Colacium* are connected by thick bifurcating mucilaginous stalks emanating from the anterior reservoir, resulting in a dendroid colony (Leedale 1967). This colonial habit has prompted some researchers to separate *Colacium* into its own family (Jahn 1951, Christen 1963, Popova and Safonova 1976, Compere 1989), or even a separate order (Bourelly 1970, Tell and Conforti 1986) of equal rank to the other euglenids with a single emergent flagellum. The mucilaginous sheaths surrounding individual cells of *Colacium* are similar in appearance to those preceding the formation of the loricas in *Trachelomonas* and *Strombomonas*. This feature, along with similarities in plastid structure, led us to the hypothesis that *Colacium* might be closely related to the loricates.

Euglena gracilis has long been one of the model organisms for studies of the development and biochemistry of plastids. The genetic continuity of chloroplasts was proven using *E. gracilis* as a model system (Pringsheim & Pringsheim 1952, Schiff & Epstein 1965). While the wealth of information available on *E. gracilis* is significant, there is danger if the studies on *E. gracilis* are extrapolated to include the rest of the euglenophytes. It is often assumed that what is true for *E. gracilis* is also true for the

euglenophytes as a whole, but recent analyses using the nuclear 18S and chloroplast 16S rDNA sequence have shown that *E. gracilis* and its close relatives are potentially the most recently diverged of all euglenophytes (Linton et. al. 1999, 2000 Leander and Farmer 2001, Milanowski at al. 2001, Müllner et. al. 2001). This study not only reports on ultrastructure, but also reexamines some of the fundamental plastid biology of the euglenophytes in an effort to refine some of the basic principles of euglenophyte taxonomy.

Materials and Methods:

Strains and culture conditions:

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

ESSEX Medium:

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

TEM:

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

Confocal Microscopy:

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

Results:

Pyrenoid Structure:

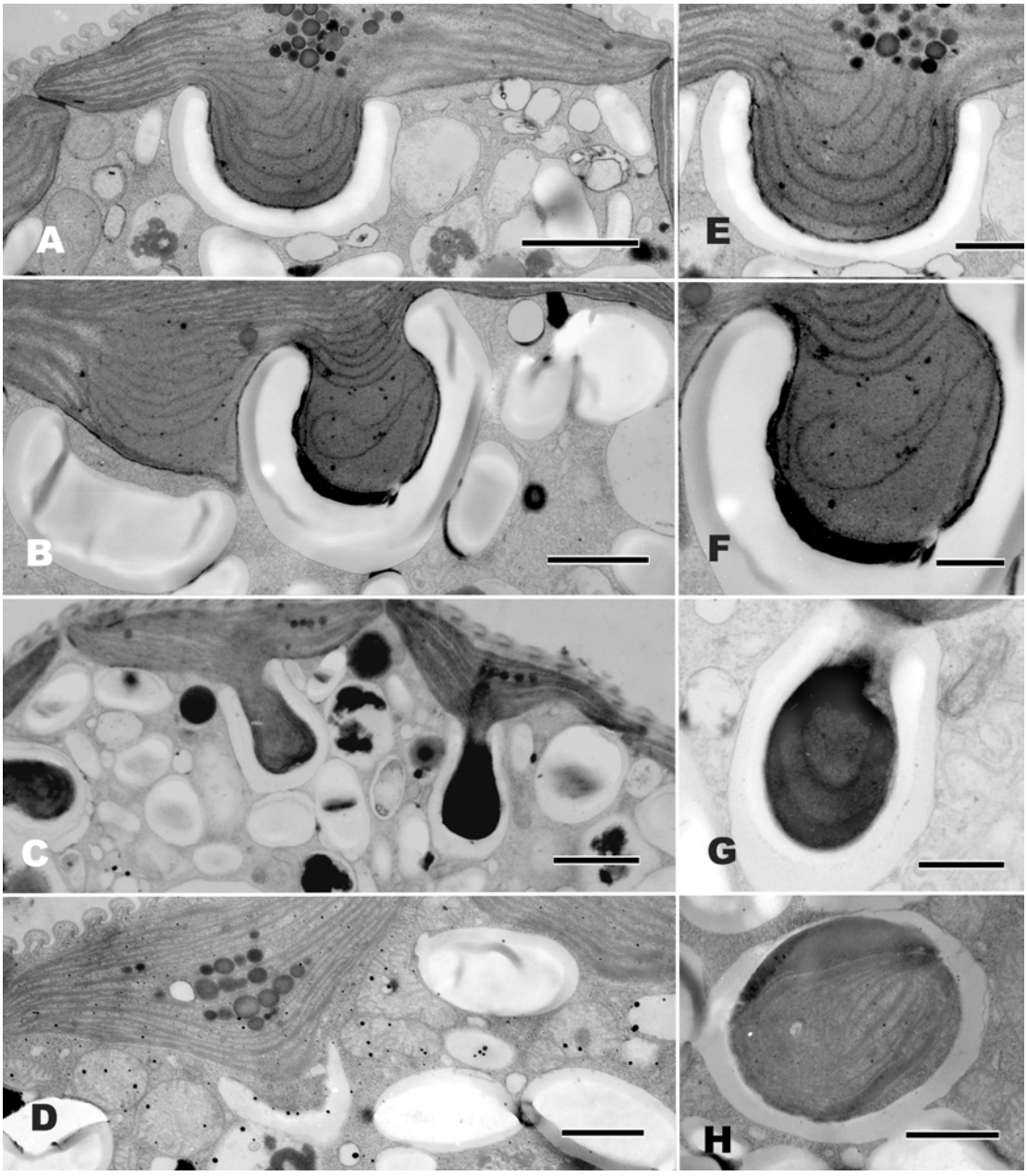
The five species under investigation share a distinct plastid morphology, unlike that of other euglenophytes. In cells undergoing normal growth, each species has 10-15 parietal disk-shaped plastids. Each plastid has a single, large protruding pyrenoid on its cytoplasmic side (Figs. 1 A-D & 2D) which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

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

Plastid development:

The plastids of *S. verrucosa* were studied in an effort to elucidate the progression of pyrenoid development in those plastids with an inwardly projecting pyrenoid. Following cell division the pyrenoid appears as a small electron dense region on the cytoplasmic side of the plastid, and is almost immediately capped with a small grain of

Figure 3.1. Plastid and pyrenoid morphology of *Colacium vesiculosum, Trachelomonas volvocinopsis, Colacium mucronatum,* and *Strombomonas costata*. (A) Chloroplasts of *C. vesiculosum* are characterized by a pyrenoid that protrudes toward the center of the cell. The pyrenoid is not constricted at the proximal end. Bar = 1μm (B) The chloroplasts of *T. volvocinopsis* have similar pyrenoid morphology, but there is a slight constriction at the proximal end, resulting in the paramylon cap curving inward to fit the curve of the pyrenoid. Bar = 1μm (C) The chloroplasts of *C. vesiculosum* are highly constricted at the proximal end, and are much more elongate than those of *C. vesiculosum*. Bar = 1μm (D) The chloroplasts of *S. costata* protrude inward, but do not get as large as those of *S. verrucosa*. Bar = 1μm (E) High magnification of the pyrenoid of *C. vesiculosum* in which the thylakoid lamellae penetrate throughout the pyrenoid matrix and are contiguous through their entire length. Bar = 0.5 μm (F) High magnification view of the pyrenoid of *T. volvocinopsis* with a unique 'recurrent lamella' in the pyrenoid matrix. Bar = 0.5 μm (G) Higher magnification of the pyrenoid of *C. mucronatum* in which the constriction at the proximal end is so pronounced the "neck" of the pyrenoid is absent from this oblique section. Bar = 0.5 μm H. Cross section of the pyrenoid of *S. costata* in which the penetrating lamellae can be easily observed, and are quite numerous. Bar = 0.5 μm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paramylon (Fig. 2A). Initially the pyrenoid is a small homogeneous structure and does not possess thylakoids, but has a robust paramylon cap (Fig. 2B). As material is added to the crystalline matrix of the pyrenoid, it begins to grow primarily along the axis perpendicular to the long axis of the plastid (Fig. 2C). This asymmetric growth 'pulls' nearby thylakoids into the pyrenoid matrix (Fig. 2C). At maturity the pyrenoid can be nearly as large as the plastid body, and protrude inward to the nuclear region (Fig. 2D). This entire progression occurs over the course of 6-8 hours, during the growth (light) phase of the cell cycle.

Using confocal laser-scanning microscopy, ultrastructural events in the development of *S. verrucosa* plastids can be correlated to gross-morphological changes. Immediately after cell division the plastids are small and the pyrenoids are greatly reduced or even absent. During the early periods of the growth phase, the plastids increase in volume and become amorphous (Fig. 3A). Pyrenoids are few, and difficult to distinguish with light microscopy. By the peak of the growth phase (approximately 6-8 hours after cell division) the plastids have nearly doubled in size and pyrenoids are easily visible. At this point the plastids begin to divide, resulting in numerous discoid plastids with prominent pyrenoids (Fig. 3B). Since the thylakoids penetrating the pyrenoid do not fluoresce, pyrenoids appear as dark spots, but only if the pyrenoid is large enough to constitute a significant portion of the entire plastid (Fig. 3B). After cell division, daughter cells are then left with half of the plastids of the parent.

The plastids of the species under study also exhibited a form of plastid adhesion. In nearly every section of growing cells, plastids could be seen adhering to one another, often via an unidentified electron dense material (Fig. 4). In *T. volvocinopsis* adhesion

Figure 3.2. Pyrenoid development follows an ordered series of events in *Strombomonas verrucosa*. (A) The earliest recognizable stage of what will become a pyrenoid is distinguishable by the electron opaque region capped by a small, thin, grain of paramylon (arrow). Bar = 0.5 μm (B) later, a robust paramylon cap is added to the growing pyrenoid. Bar = 0.5 μm (C) As more material is added to the pyrenoid matrix, thylakoid lamellae are "pulled" into the pyrenoid (arrowhead). Bar = 0.5 μm (D) At maturation the pyrenoid is nearly as large as the plastid itself. Bar = 1 μm

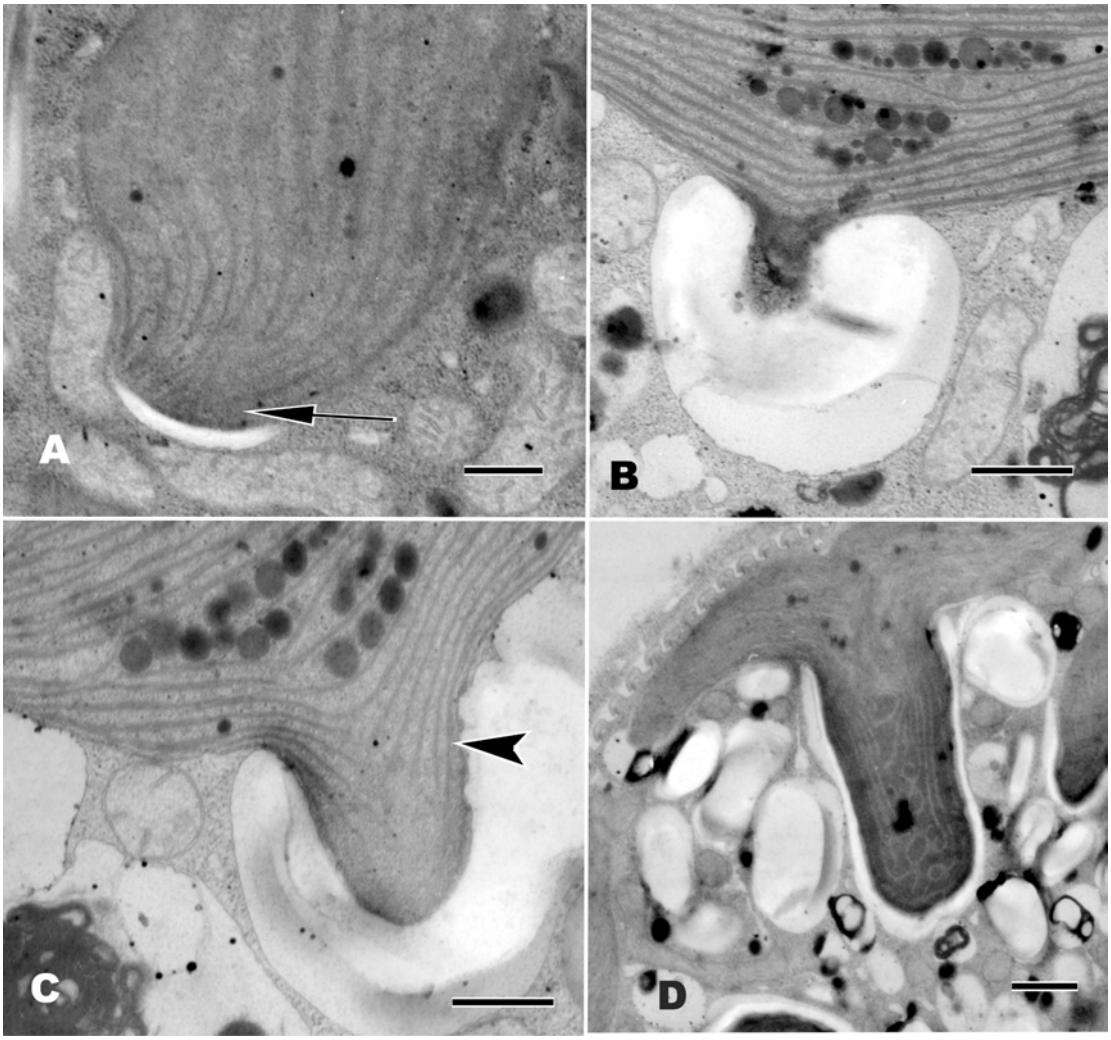
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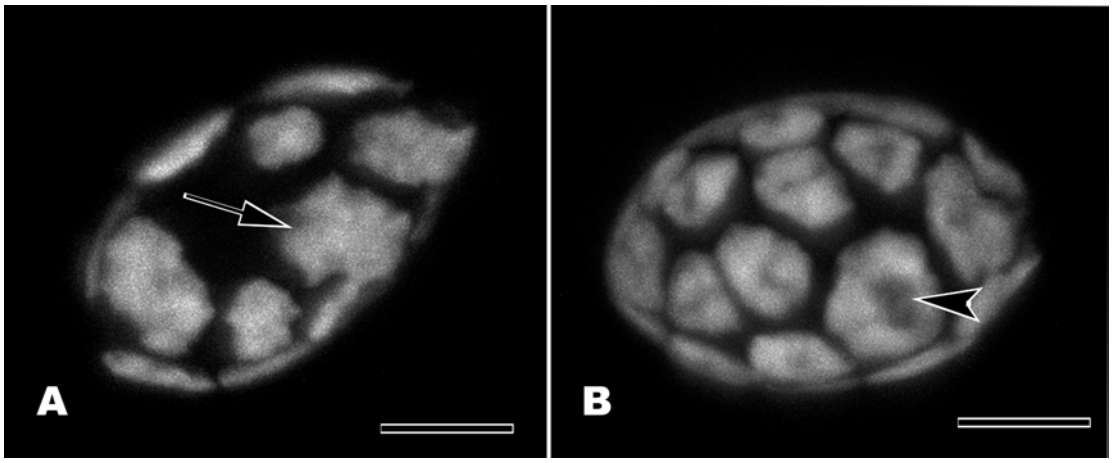
Figure 3.3. (A) At the beginning of the light cycle the plastids of *S. verrucosa* are amorphous. A single large plastid (arrow) can be seen occupying a significant portion of the cell. Bar = 10 μm (B) after 6 hours in daylight, the plastids have divided and a central large pyrenoid can be seen as a fluorescence exclusion zone (arrowhead). Bar = 10 μm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was, more often than not, along the long-axis of the plastid. This results in an overlapping appearance (Fig. 4A). Most plastids in other species were however, adherent end-to end (Figs. 4B & C). This was seen in members of all three genera studied, usually occurring along the long axis of the cell.

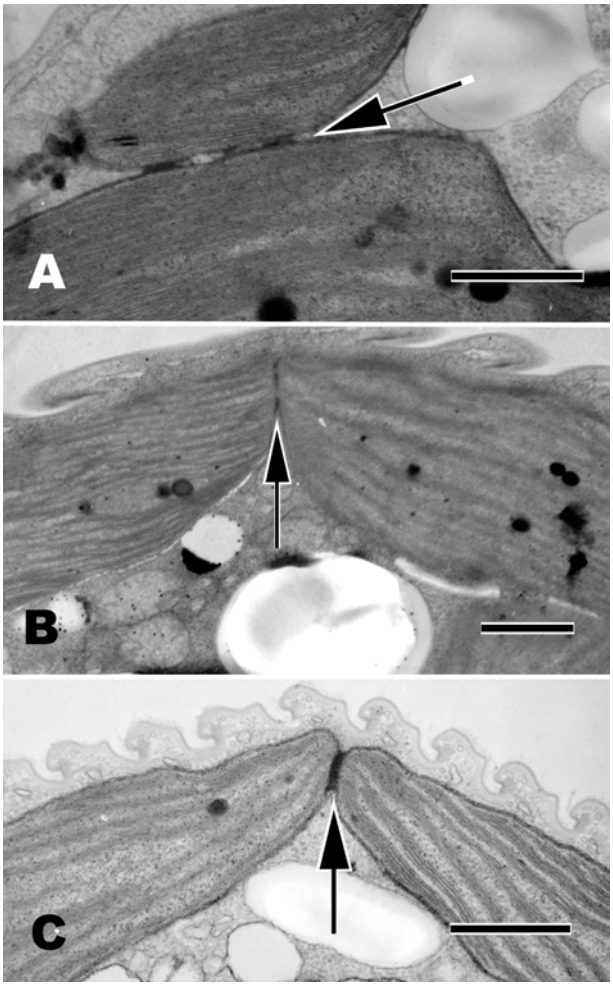
Discussion:

Taxonomic implications:

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

While the species of *Trachelomonas* as well as both species of *Strombomonas* examined in this study possess stalked pyrenoids, there are species of *Trachelomonas* that apparently do not. Pringsheim (1953), while observing several species of *Trachelomonas* grown in culture, noted "the entire number of species of *Trachelomonas* observed can be divided into four groups: one with no pyrenoids, one with naked, one with inner, and one with double-sheathed pyrenoids. The majority of the species of *Trachelomonas* have,

Figure 3.4. Plastid adhesion in *T. volvocinopsis, S. costata,* and *C. vesiculosum*. (A) The plastids of *T. volvocinopsis* are usually found apressed to one another along the overlapping surfaces of adjoining plastids, the adhesion is maintained by an electron opaque material (arrows). Bar = 0.5 μm (B) In *S. costata* the plastids adhere to one another along their margins and are pressed end to end. Bar = 0.5 μm (C) In *C vesiculosum* the plastids adhere much as they do in *S. costata*. Bar = 0.5 μm


A
B
C

however, what I will call ‘inner pyrenoids’ protruding from the center of the concave surface of the chromatophores towards the middle of the cell”. The ‘inner pyrenoids’ and the naked pyrenoids (no associated paramylon) are barely visible in the light microscope and the reported absence of pyrenoids in many species of *Trachelomonas* may not be accurate. Pringsheim’s description of inner pyrenoids accurately describes the pyrenoids we have observed in *Trachelomonas, Strombomonas and Colacium*.

The most recent monographs of the euglenids (Huber-Pestalozzi 1955, Popova 1966, Popova and Safonova 1976) state that *T. volvocinopsis* does not have pyrenoids, while Pringsheim (1953) observed and reported them as ‘inner pyrenoids’. The culture of *T. volvocinopsis* used in this study is identical in every way to Huber-Pestalozzi’s diagnosis adapted from Swirenko’s own (Swirenko 1914). So while there are loricate taxa that probably have “naked” pyrenoids as well as those that definitely have diplopyrenoids (a centrally located pyrenoid capped on either side by a lens-shaped paramylon grain such as those seen in *Euglena agilis* Carter); a lack of pyrenoids altogether is possible but unlikely. Although Pringsheim (1953) states that some species of *Trachelomonas* (those with numerous small chloroplasts like *T. abrupta, T. bulla,* and *T. varians*) have no pyrenoids, the absence of pyrenoids for these taxa should not be accepted as fact until this can be positively documented by electron microscopy.

In addition to the asymmetric pyrenoids, all five taxa in our study exhibit a phenomenon we term plastid adhesion. In which plastids appear to be joined to one another, often by an electron opaque material. This adhesion usually occurs along the lateral borders of the plastid, and when it extends down the length of the cell forms a longitudinal ridge. This is a unique characteristic, not mentioned in previous reports of

plastid ultrastructure, and offers another piece of evidence in support of a *Colacium* – loricate relationship. Unfortunately the function of plastid adhesion in the cell has yet to be discerned.

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

Euglena-Calliglena. Molecular analyses of sequences from additional taxa will allow for the further testing of this hypothesis.

Plastid biology and development:

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

Acknowledgements:

Financial support was provided by the United States National Science Foundation PEET (Partnerships for Enhancing Expertise in Taxonomy) grant no. DEB 4-21348 and a grant from the Polish State Committee for Scientific Research (KBN No 6PO4C01617). The authors also wish to thank Professor Dr U. G. Schlösser, Sammlung von Algenkulturen (SAG), Göttingen, Germany and Dr M. F. Santos, The University of Coimbra, Portugal, for providing euglenophyte strains.

References:

Buetow D. E. 1968. Morphology and ultrastructure of *Euglena. In* Buetow, D. E. [Ed] *The Biology of* Euglena *Vol. 1*. Academic Press, New York. pp 109-84

Bourelly, P. 1970. *Les algues d'eau douce. III: Les algues bleues et rouges. Les Eugléniens, Peridiniens et Cryptomonadines*. Paris. 512 pp.

Conrad, W. 1916. Révision des éspèces indigènes et français du genre *Trachelomonas* Ehrenbg. *Annales de Biologie Lacustre*, 8:193-212

Christen, H. R. 1963. Zur Taxonomie der farblosen Algen. N*ova Hedwig.* 4:437-64

Compere, P. 1989. *Flora pratique des algues d'eau douce de Belgique. I:* Cryptophyceae, Dinophyceae, Raphidophyceae, Euglenophyceae. Jardin Botanique National de Belgique, Meise. 208 pp.

Conforti, V., P. Walne and J. Dunlap. 1993. Comparative ultrastructure and elemental compositions of envelopes of Trachelomonas and Strombomonas (Euglenophyta). *Acta Protozool.* 33:71-8.

Conforti, V. and G. J. Joo. 1994. Taxonomic and ultrastructural study of *Trachelomonas* Ehr. and *Strombomonas* Defl. (Euglenophyta) from oxbow lakes in Alabama and Indiana (U.S.A.). *Cryptogam. Algol.* 15(4): 267-86

Conforti, V. 1999. Taxonomic and Ultrastructural Study of *Trachelomonas* (Ehr.) (Euglenophyta) from Suptropical Argentina. *Cryptogam. Algol.* 20:167-207

Cook, J. R., Haggard, S. S., and Harris, P. 1976. Cyclic changes in chloroplast structure in synchronozed *Euglena gracilis*. *J. Protozool.* 23:368-73

Deflandre, G. 1926. Sur quelques Euglénacées nouvelles du Vénézuéla. *Bull. Mus. Nat. Hist. Nat*. 6:421-2

Dragos, N., Péterfi, S., and Craciun, C. 1979. Fine structure of *Euglena.* II. *Euglena stellata* Mainx and *Euglena viridis* Ehrenberg. *Nova Hedwigia* 31: 223-46

Deflandres, G. 1930. *Strombomonas* nouveau genre d'Euglenacées (*Trachelomonas* Ehrb. pro parte). *Archiv fur Protistenkunde*, 69:551-614

Ehara, T., Tetsuaki, O., and Hase, E 1990. Interactions between the nucleus an cytoplasmic organelles during the cell cycle of *Euglena gracilis* in synchronized cultures. IV. An aggregate form of chloroplasts in association with the nucleus appearing prior to chloroplast division. *Exp. Cell Res.* 190:104-12

Ehrenberg, C. G. 1833. Dritter Beitrung zur Erkenntnis großer Organisation in der Richtung des Kleinsten Raumes, *Physikalishe Abhandlungen Königlichen Akademie der Wissenschaften zu Berlin*. 145-336

García-Ferris, C., de los Rios, A., Ascaso, C., and Moreno, J. 1996. Correlated biochemical and ultrastructural changes in Nitrogen-starved *Euglena gracilis*. *J. Phycol.* 32:953-63

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

Haller D. E. G. 1959. Structure submicroscopique d'*Euglena viridis. Archives des Sciences, Genéve* 12: 309-40

Harrison, P. J., Waters, R. E., and Taylor, F. J. R. 1980. A broad spectrum artificial seawater medium for coastal and open ocean phyotplankton. *J. Phycol.* 16:28-35

Huber-Pestalozzi G. 1955. *Das Phytoplankton des Süβwassers; Systematik und Biologie: 4 Teil; Euglenophyceen*. E. Schweizerbartsche Verlagsbuchhandlung, Stuttgart., 606 pp.

Jahn, T. L. 1951. "Euglenophyta" *Manual of Phycology*, ed. G. M. Smith, 69-81. Waltham: Chronica Botanica Co.

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

Leedale, G. F. 1967. *The Euglenoid Flagellates* Prentice-Hall, Englewood Cliffs, NJ. 242 pp.

Leedale, G. F. 1982 Ultrastructure, in *The biology of* Euglena, vol. 3, ed. D. Buetow, 1-28. New York: Academic Press.

Linton, E., Hittner D., Lewandowski C., Auld T., and Triemer R. E. 1999. A molecular study of euglenoid phylogeny using SSU rDNA. *J Euk Micro.* 46:217-23.

Linton, E. W., Nudelman, M. A., Conforti, V., and Triemer, R.E. 2000. A molecular analysis of the euglenophytes using SSU rDNA. *J Phycol*, 36:740-6.

McLachlan, J. L., Seguel, M. R., and Fritz, L. 1994. *Tetreutreptia pomquetensis* gen. et. sp. nov. (Euglenophyceae): a quadriflagellate, phototropic, marine euglenoid. *J. Phycol.* 30:538-44

Mignot J-P. 1965. Ultrastructure des Eugléniens I. Étude de la cuticule chez différentes espéces. *Protistologica* 1: 5-15

Mignot J-P. 1966. Structure et ultrastructure de quelques Euglénomonadines. *Protistologica* 2: 51-117

Milanowski, R., Zakryś, B., and Kwiatowski, J. 2001. Phylogenetic analysis of chloroplast small-subunit rDNA genes of the genus *Euglena* Ehrenberg. *IJSEM* 51: 773-81

Müllner, A. N., Angeler, D. G., Samual, R., Linton, E. W., and Triemer, R. E. 2001. Phylogenetic analysis of phagotrophic, phototrophic and osmotrophic euglenoids by using the nuclear 18S rDNA sequence. *IJSEM* 51: 783-91

Pelligrini, M. 1980. Three-dimensional reconstruction of organelles in *Euglena gracilis* Z. I. Qualitative and quantitative changes of chloroplasts and mitochondrial reticulum in synchronous photoautotrophic culture. *J. Cell Science* 43: 137-66

Péterfi, L. S., Dragos, N., and Craciun, C. 1979. Fine structure of *Euglena.* I. *Euglena tristella* Chu. *Nova Hedwigia* 31: 197-221.

Popova, T. G. & Safonova, T. A. 1976. Flora plantarum cryptogamarum URSS **9.** Euglenophyta. **2**. Izd. Nauka, Leningrad. 286 pp.

Popova, T.G. 1966. Euglenovyje Vodorosli. [Euglenophyta]. – Flora Sporovyvh Rastenij SSSR. Izd. Nauka, Moskva-Leningrad. 8: 1-412

Pringsheim, E. G. and Pringsheim O. 1952. Experimental elimination of the chromatophores and eyespot in *Euglena gracilis*. *New Phytologist* 51: 65-76

Pringsheim, E. G. 1953. Observations on some species of *Trachelomonas* grown in culture. *New Phytol.,* 52: 93-113, 238-66.

Shi, Z., Wang, Q., Xie, S., Dai, J. & Chen, L. 1999. Flora algarum sinicsrum aquae dulcis. Tomus VI: *Euglenophyta*, pp. 297-414. Ed. Academiae Sinicae: Science Press (in Chinese).

Schiff, J. A. and Epstein, H. T. 1965. The continuity of the chloroplast. *In* Locke, M [ed] *Euglena*. *Reproduction: Molecular, Subcellular, and Cellular*. Academic Press, New York. pp.131-89

Swirenko, D. O. 1914. Zur Kenntnis der russischen Algenflora. I. Die Euglenaceengattung *Trachelomonas*. *Arch. Hydrobiol. Planktonk.* 9: 630-647

Tell, G. & Conforti, V. 1986. Euglenophyta Pigmentadas de la Argentina. *In* Cramer, J. [Ed] *Biblioteca Phycologica* Gebr. Borntraeger, Berlin-Stuttgart. pp. 1-201

Walne, P. L., Moestrup, Ø., Norris, R. E., and Ettl, H. 1986. Light and electron microscopical studies of *Eutreptiella eupharyngea* sp. nov. (Euglenophyceae)

from Danish and American waters. *Phycologia* 25: 109-26

Zakryś, B. 1986. Contribution to the monograph of Polish members of the genus *Euglena* Ehrenberg 1830. *Nova Hedwigia* 42: 491-540

Zakryś B., Walne P. L. 1998 Comparative ultrastructure of chloroplasts in the Subgenus *Euglena* (Euglenophyta): Taxonomic significance. *Cryptogamie Algol.* 19: 3-18

Zakryś B., Cambra-Sanchez J., Walne P. L. 2001 Chloroplast ultrastructure of *Euglena cuneata* Pringsheim, *E. deses* Ehrenberg and *E. mutabilis* (Euglenophyceae): Taxonomic significance. *Acta Protozool*. 40: 161-7

CHAPTER 4

ULTRASTRUCTURE AND COMPARATIVE MORPHOLOGY OF PLASTID DIVISION AND PARTITIONING IN THE EUGLENOPHYTES.

*Brown, P.J.P. and Farmer, M. A., submitted to the journal *Protoplasma*

Summmary:

The euglenophytes are a well-known but understudied group of protists whose plastids are derived from a secondary endosymbiosis with a green alga. Plastid division and development has been characterized in *Euglena gracilis*, but relatively little is known about partitioning mechanisms in other euglenophytes. Plastid morphology and ultrastructure during light period and dark period growth was examined in six species of euglenophytes: *Eutreptiella gymnastica*, *Euglena acus*, *Euglena mutabilis*, *Euglena viridis*, *Trachelomonas volvocinopsis*, and *Phacus pyrum*. We uncovered two basic mechanisms by which euglenophytes partition their plastids during cell division: 1) in cells with numerous (more than 10 plastids), the plastids are partitioned into daughter cells stochastically based on their position in the parent cell at the time of cell division. In cells with relatively few plastids (less than 10) the plastids fragment prior to cell division in order to be partitioned stochastically. The exception is *Phacus pyrum* in which the plastid does fragment prior to division, but partitioning is achieved primarily by division of the plastid along the incipient cleavage furrow. This study demonstrates that the euglenophytes possess a broad diversity of plastid partitioning mechanisms, and elucidates a novel fragmentation method for plastid partitioning.

Keywords: Euglena, Eutreptiella, Trachelomonas, Phacus, chloroplast division, chloroplast morphology.

Introduction:

The euglenophytes are a monophyletic group of protists that acquired a plastid *via* secondary symbiogenesis with a green alga (Gibbs 1978) as early as the 443 MYA (Gray and Boucot 1989, Leander et al. 2001). In that time the euglenophyte plastid has evolved into myriad morphotypes ranging from a single, large, axial, stellate, plastid per cell (as in *Euglena viridis*) to tens of small, discoid, parietal plastids per cell (*e.g. Euglena acus*). Plastids also vary in terms of their pyrenoid morphology. Pyrenoids differ with regard to their size, shape, location within the plastid, associations with the euglenid reserve polysaccharide paramylon, and even in their presence or absence. This vast diversity of plastid forms found in eulenophytes has led numerous researchers to rely heavily on plastid morphology in the definition of groups within taxonomic frameworks (Lemmermann 1913, Chu 1946, Gojdics 1953, Pringsheim 1956, Leedale 1967, Zakrys 1986). Unfortunately, most of what is known about plastid morphogenisis and development is based on studies of a single species; *Euglena gracilis* (Ben-Shaul et al. 1964, 1965, Cook and Harris 1976, Epstein and Schiff 1961, Könitz 1965).

Their diversity of form makes euglenophyte plastids appear quite attractive for use as taxonomic characters. However, very little is known about their basic biology, especially with respect to plastid division and development. Most studies have used bleached *E. gracilis* and followed the development of plastids from 'proplastids' and *vice-versa* (Epstein and Schiff 1961, Schiff et al. 1961, Ben-Shaul et al. 1964, Ben-Shaul et al. 1965, Schiff and Epstein 1965) or on the relationship between nutrient levels and plastid morphology (Garcia-Ferris et. al. 1996, Conforti 1998). Ultrastructural studies of

plastid division and development in synchronized cultures of *E. gracilis* began with the early report of Könitz (1965) in which pyrenoid cycling was first reported. Cook et al. (1976) noted ultrastructural changes in the lamellae and demonstrated that the pyrenoid is transient in actively growing cultures. This was followed by Pelligrini's (1980) exhaustive TEM study of plastid structure during an entire cell cycle. The most recent study by Ehara et al. (1990) noted an interesting aggregation stage during plastid division cycles in *E. gracilis*. While these reports give a fairly complete picture of plastid development in *E. gracilis* they do not mention any other euglenophyte taxa.

Most of the reports on plastid division and development in organisms outside the genus *Euglena* are incidental remarks, without accompanying micrographs or drawings. For instance Leedale (1967) reports that embedded pyrenoids divide with the plastid but stalked pyrenoids begin at their distal end followed by fission. Gojdics (1934) reported on the division of the large plastids in *Euglena deses* in which pyrenoids are persistent throughout the cell cycle, but sorting into daughter cells is random. Of particular interest is the mechanism by which taxa with a single plastid per cell (*e.g*. *Euglena viridis* Ehrenberg) manage to sort that organelle with such high fidelity. The only published comments on the matter are an incidental report that the paramylon center divides synchronously with the cell nucleus at the time of mitosis (Leedale 1967). However, there are no drawings or micrographs accompanying this statement.

We followed plastid division and subsequent development in six euglenophyte species from five different genera. Using both optical and electron microscopy, we investigated plastid morphology in these taxa as well as the relationship between cell

cycle and plastid morphology. These results are described and discussed with relation to evolution of the plastid, taxonomy, and organelle inheritance.

Materials and Methods:

Strains and culture conditions. The following strains were used: *Euglena viridis* Ehrenberg (UTEX LB85), *Euglena acus* Ehrenberg (UTEX LB1316), *Euglena mutabilis* Schmitz (UTEX LB364), *Trachelomonas volvocinopsis* Swirenko (SAG 1283-16), *Phacus pyrum* Ehrenberg ammend. Stein (UTEX LB2354), and *Eutreptiella gymnastica* Da Cunha (CCMP 1594). They were obtained from the following culture collections: CCMP, Provasoli - Guillard National Center for Culture of Marine Phytoplankton; SAG, Sammlung von Algenkulturen Pflanzenphysiologisches Institut der Universität Göttingen; and UTEX, the Culture Center for Algae at the University of Texas. *Euglena viridis*, *Euglena acus*, *Euglena mutabilis*, *Trachelomonas volvocinopsis*, and *Phacus pyrum* were maintained in ESSEX medium (Brown et al. 2002), while *Eutreptiella gymnastica* was maintained in K medium (Keller et al. 1987) all at 20 ± 1°C with a 12:12 h light:dark cycle.

Transmission electron microscopy: Actively growing cultures were sampled at two time points: 2-3 hours after the beginning of the light period and 7-9 hours after the beginning of the dark period. Cells were pelleted in an ultracentrifuge at ~2000 X g and fixed in one of 3 fixatives for 1 hour at 4 °C: Freshwater 1 (*Trachelomonas volvocinopsis*), Freshwater 2 (*Euglena viridis*, *E. acus*, *E. mutabilis*, and *Phacus pyrum*), or Seawater fix (*Eutreptiella gymnastica*). Freshwater 1 is 2% glutaraldehyde in 0.1 M cacodylate; Freshwater 2 is 2% paraformaldehyde/1% glutaraldehyde in 0.1M

cacodylate; and Seawater fix is 2% paraformaldehyde/1% glutaraldehyde, 0.6M sucrose, and 0.1M cacodylate. After primary fixation cells were washed 3X 5 min. in buffer ± sucrose and post-fixed in 1% osmium tetroxide (OsO_4) using the same fixative vehicle for each species. Cells were dehydrated in a graded ethanol series, infiltrated, embedded, and polymerized in Embed 812 epoxy resin (Polysciences, Warrington, PA, USA). Light gold/silver sections were cut and post-stained with saturated aqueous uranyl acetate and lead citrate. The sections were viewed on a transmission electron microscope (JEOL 100 CX II, Peabody, Massachusetts) operating at 80 KeV.

Multiphoton Microscopy: Cells were sampled every 30 min. for 3 hours during two time periods: early growth phase (0-3 hours after beginning of the light period) and during cell division (6-9 hours after the beginning of the dark period). 100 µl of Freshwater 2 fixative and 100 µl of 4',6-Diamidino-2-phenylindole (DAPI) was added to a 500 µl aliquot of cell suspension and incubated at room temperature for 10 min. 2-3 drops of the fixed cells were mounted on Poly-L-Lysine coated coverslips, allowed to settle, and then sealed with VALAP (equal parts Vaseline, lanolin, and paraffin). Slides were viewed on a Leica SP2 confocal microscope with an attached Coherent MIRA femtosecond-pulse Ti-Sapphire laser at a wavelength of 740 nm. Images were collected at emission wavelengths of 390-500 nm (DAPI) and 580-780 nm (Chlorophyll autofluorescence).

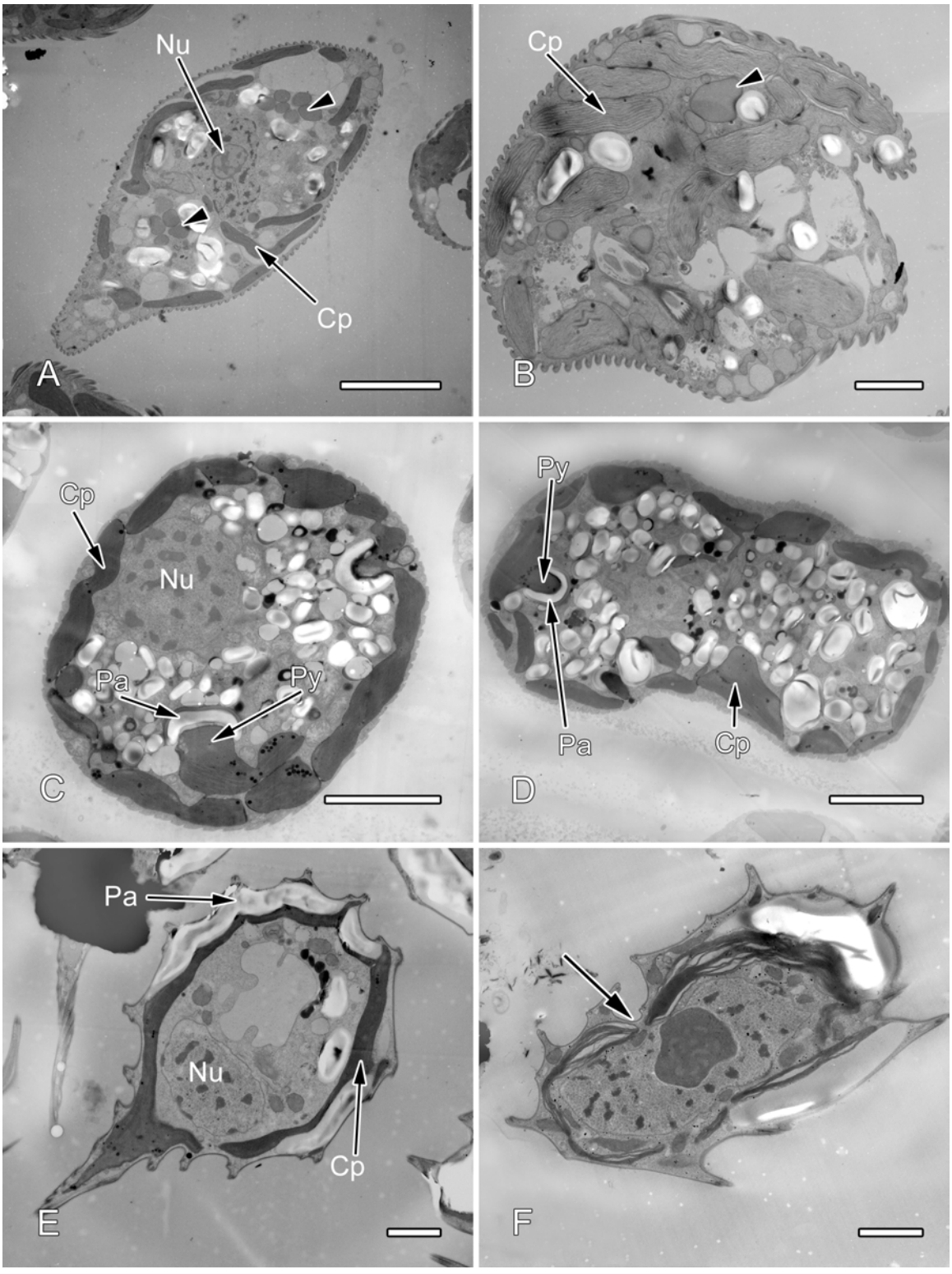
Results:

Ultrastructure of plastid development: The morphology of interphase plastids is typically referred to as the normal plastid morphology for a given species. However, there are marked changes in plastid structure during the cell division cycle. During light

period growth the plastids of *Eutreptiella gymnastica* are arranged as a pair of star-shaped aggregations, one anterior and one posterior to the nucleus (Fig. 1 A). Typically each plastid lobe contains a bulbous pyrenoid at one extremity, and it is at this end that the individual lobes are aggregated (Fig. 1 A). The pyrenoid aggregation is usually surrounded by numerous ovoid grains of paramylon (the euglenid reserve polysachharide) into what is called a 'paramylon center' (Fig. 1 A). During the dark period and prior to cell division, the plastid aggregations break up into numerous small disc-like bodies (Fig. 1 B) located throughout the cell. Although the paramylon center breaks up and the plastids are no longer aggregated, the pyrenoids remain intact, and simply appear as small bulbs on the end of each now sac-shaped or strap-like plastid (Fig. 1 B).

The plastids of *Trachelomonas volvocinopsis* are arranged around the periphery of the cell during light period growth with a large pyrenoid protruding from the inner surface of the plastid toward the nucleus (Fig. 1 C). The plastids of *T. volvocinopsis* are nearly always parietal and in fully mature cells they nearly fill the cortical cytoplasm (Fig. 1 C). During cell division, plastid morphology isn't altered greatly and plastids with light phase morphology are sorted into the daughter cells (Fig. 1 D). Plastid growth and division occurs after cell division and into the early portions of the light period. The pyrenoids form first, often resulting in plastids with two or more pyrenoids before they divide (data not shown) resulting in the normal single pyrenoid morphology.

Fig. 4.1 A-F. Ultrastructure of plastid division in *Eutreptiella gymnastica*, *Trachelomonas volvocinopsis*, and *Phacus pyrum*. **A.** *E. gymnastica* is spindle-shaped during the light period with numerous plastids (*Cp*) aggregated at their pyrenoids into a paramylon center (*arrowheads*) on either side of the nucleus (*Nu*). Bar: 5 μm **B.** During the dark period the plastids of *E. gymnastica* (*Cp*) have become dispersed, although they still retain their pyrenoids (*arrowhead*). Bar: 2 μm. **C.** *Trachelomonas volvocinopsis* has several large, disk-shaped plastids (*Cp*) around the periphery of the cell. The pyrenoids (*Py*) protrude from the inner face of the plastid toward the center of the cell and are capped by a large grain of paramylon (*Pa*). Bar: 5 μm **D.** During the dark period *T. volvocinopsis* retains its pyrenoid (*Py*) along with the paramylon cap (*Pa*) and the plastids (*Cp*) remain distributed only in the periphery of the cell. Bar: 5 μm. **E.** *Phacus pyrum* is a rounded cell with a tapering tip and a posterior nucleus (*Nu*). It has a single large plastid (*Cp*) that occupies the entire periphery of the cell. The cell usually contains a large U-shaped paramylon grain (*Pa*) seen here in cross section. Bar: 2 μm. **F.** During the light period the plastid of *P. pyrum* (*cp*) fragments in the anterior of the cell, but the bulk of the plastid divides parallel to the incipient cleavage furrow (*arrow*). Bar: 2 μm.

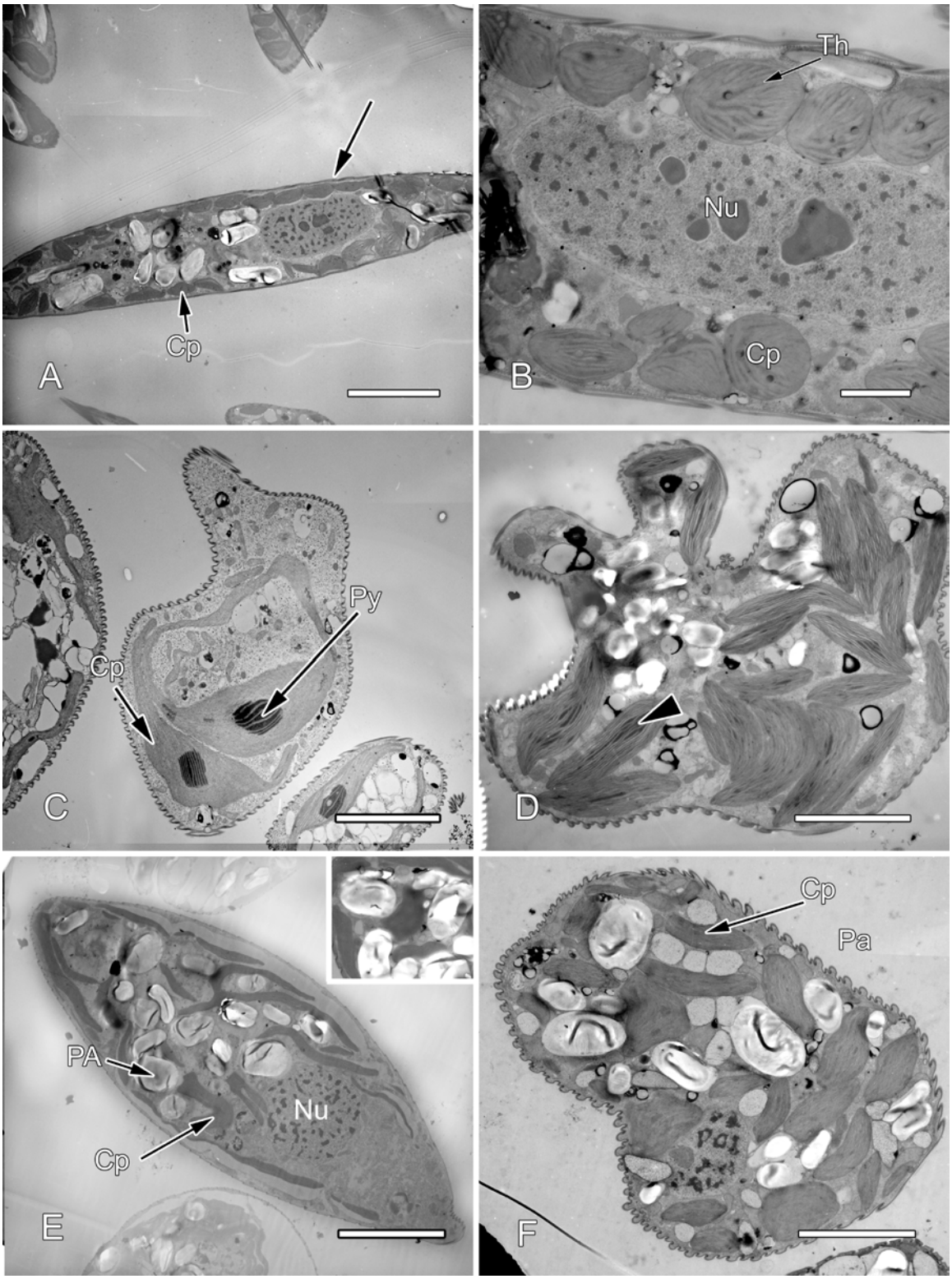

Nu
Cp
A
Cp
B
Cp
Nu
Pa
Py
C
Py
Pa
Cp
D
Pa
Nu
Cp
E
F

The plastids of *Phacus pyrum* are large cup-shaped bodies with a single pyrenoid located at the posterior of the cell (Fig. 1 E). The 'arms' of the plastids wrap around the periphery of the cell, much like the familiar cup-shaped plastids of *Chlamydomonas rheinhartii*(Fig. 1 E). During the dark period and prior to mitosis, the plastid of *P. pyrum* divides along the plane of cell division and appears to fragment to a small degree (Fig. 1 F).

The plastids of *Euglena acus* are arranged as numerous (30-100) small discs located throughout the cell body (Fig. 2 A) and do not bear pyrenoids at any time during their cell cycle (Fig. 2 A & B). Other researchers (Leedale 1967) have noted that the plastids of *E. acus* do not tend to follow a particular division cycle, and that was observed in this case. The plastids of *E. acus* appear to be dividing throughout the light period (Fig. 2 A). During the dark period plastid ultrastructure is nearly identical to that of the light period. However, we did notice that the plastids appear to be swollen and more rounded during the dark period and the thyllakoids appear less lamellate (Fig. 2 B).

The plastids of *Euglena mutabilis* are large shield-shaped structures located around the periphery of the cell (Figs. 2 C). They are typically two in number, but that number varies from one to as many as four in a light-phase cell. Each plastid swells in the central portion around a large, conspicuous pyrenoid (Fig. 2 C). Unlike other euglenophytes with central pyrenoids, the pyrenoid of *E. mutabilis* is not associated with crystalline paramylon, nor does there appear to be more than one

Fig. 4.2 A-F. Ultrastructure of plastid division in *Euglena viridis*, *Euglena acus*, and *Euglena mutabilis*. **A.** During the light period *E. viridis* is characterized by a single stellate plastid (*Cp*) with a center anterior to the nucleus (*Nu*). The center of the plastid typically bears a pyrenoid surrounded by small grains of paramylon (*inset*). Bar: 10 μm. **B.** During the dark period the plastid (*Cp*) has become fragmented, there is no evidence of a pyrenoid, and the paramylon (*Pa*) is distributed randomly. Bar: 5 μm. **C.** *Euglena acus* is a long thin cell with as many as 80 discoid plastids (*Cp*) located throughout the cell. Plastid division occurs throughout the cell cycle and large plastids can be seen dividing into the characteristic smaller ones (*arrow*). Bar: 10 μm. **D.** During the early portions of the dark period, the plastids (*Cp*) of *E. acus* become swollen and the thylakoids (*Th*) become distended. *Nu* nucleus Bar: 2 μm. **E.** During the light period *Euglena mutabilis* has 1-4 large shield-shaped plastids (*Cp*) each bearing a large conspicuous pyrenoid (*Py*) in the center of the plastid. Pyrenoids are never directly associated with paramylon. Bar: 5 μm. **F.** During the dark period the plastids of *E. mutabilis* undergo a reticulated phase, followed by fragmentation of the plastids (*Cp*) into numerous smaller bodies. Although not as large, pyrenoids (*Py*) can still be seen in some of the plastid fragments. Bar: 5 μm.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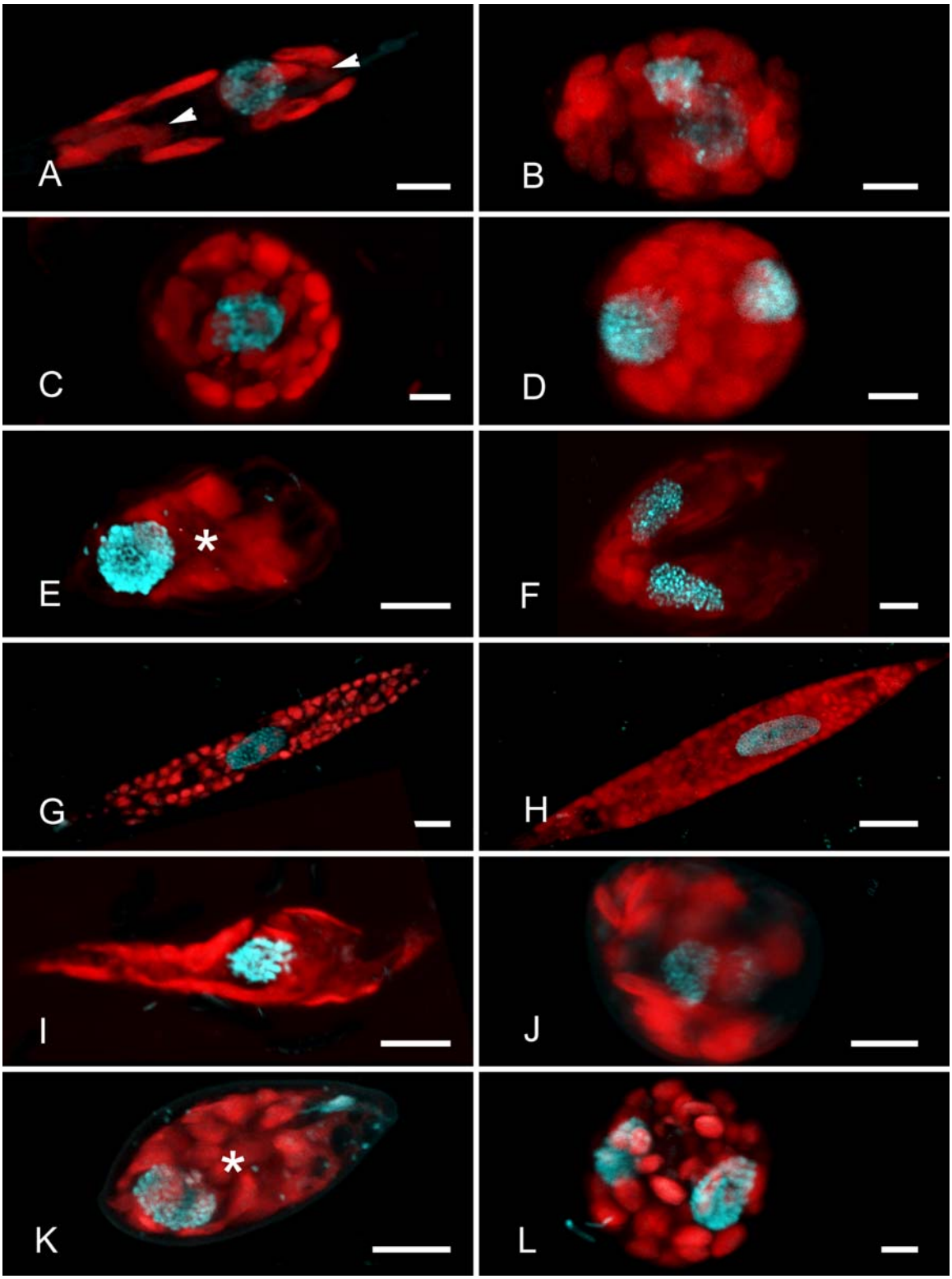
morphotype of paramylon present (Fig. 2 C). During the dark period, plastid ultrastructure is quite different. The plastids divide and fragment into several small disc-shaped structures and are located throughout the cell (Fig. 2 D). The pyrenoids are reduced to small electron-opaque regions in the center of the smaller plastid bodies (Fig. 2 D), and these reduced pyrenoids are no longer visible at all in the light microscope (data not shown).

Euglena viridis has a single star-shaped plastid in each cell (Fig. 2 E). The main body of the plastid is located in the central portion of the cell near the nucleus, and contains the single pyrenoid (Fig. 2 E). Arms radiate from the central portion outward toward the cell cortex forming the familiar stellate arrangement (Fig. 2 E). Prior to mitosis the pyrenoid disappears and the plastid begins to fragment. The result is numerous disc-shaped or round bodies distributed throughout the cell (Fig 2 F). The axial arrangement is compeletely lost, and in no cell that was clearly pre mitotic (possessing a duplicated cytoskeleton and/or two emergent flagella) or mitotic was a pyrenoid observed (Fig. 2 F)

Multiphoton microscopy of plastid division and partitioning. During light-phase growth, *E. gymnastica* is spindle-shaped and the plastids are arranged axially, as two aggregations, one on each side of the nucleus. The paramylon center appears dark in fluorescence microscopy due to the small number of thyllakoids present in a pyrenoid (Fig. 3 A). At the time of nuclear division, the plastids can be seen to have lost their axial orientation and changed their shape to become more sack-like and distributed throughout the cell (Fig. 3 B). There appears to be no indication of the incipient cleavage furrow in regards to plastid location or orientation.

Figure 4.3 A-L. Multiphoton microscopy of plastid division and development in *Eutreptiella gymnastica*, *Trachelomonas volvocinopsis*, *Phacus pyrum*, *Euglena acus*, *E. mutabilis*, and *E.viridis.* All cells are arranged with the posterior end of the cell to the left of the image. The position of the pyrenoids was identified by observation under transmitted light. Blue = DAPI, Red = Chlorophyll autofluorescense. **A.** Light period morphology of *Eutreptiella gymnastica* plastids have a paramylon center (arrowheads) on either side of the nucleus. Bar 8 μm. **B.** Mitotic plastids of *E. gymnastica* are numerous, sack-like, and are no longer axial. Bar 4 μm. **C.** Light period plastid moprhologyof *Trachelomonas volvocinopsis*. Bar 4 μm. **D.** Mitotic stage *T. volvocinopsis* plastids have doubled in number and occupy the entire periphery of the cell. Bar: 4 μm. **E.** *Phacus pyrum* has a posterior nucleus and a single cup-shaped plastid with a large central pyrenoid (asterisk). Bar: 8 μm. **F.** By cytokinesis the anterior portion of the daughter cells of *P. pyrum* contains plastid fragments, but the main portion of the plastid is being divided along the plane of cytokinesis. Bar: 4 μm. **G.** Light period plastids of *Euglena acus*. Bar 8 μm. **H.** During the dark period, prior to mitosis, plastids are so numerous it is difficult to distinguish them as separate. Bar 16 μm. **I.** Light period *Euglena mutabilis* has large shield-shaped plastids occupying most of the periphery of the cell. Bar: 8 μm. **J.** During mitosis the cells become rounded, the plastids fragment, and become located throughout the entire cell. Bar: 8 μm. **K.** Light period *Euglena viridis* has a posterior nucleus, a single large stellate plastid with a central axial pyrenoid (asterisk) and branches radiating outward toward the cell periphery. Bar: 8 μm. **L.** At mitosis the single plastid has become ~ 20 plastid fragments located primarily in the cell periphery. Bar: 4 μm.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T. volvocinopsis is a spherical cell and its plastids are parietal throughout the cell cycle (Figs. 3 C & D) and typically number between 10 and 15 per cell (Fig. 3 C). Plastids grow and divide primarily during the early portions of the light period, but continue throughout. At the time of mitosis the plastids are still arranged parietally around the cell. However, their number doubles over the course of the day and can reach as many as 40 individual plastids crowded around the periphery of the cell (Fig. 3 D). Again, the position of the plastids is uniform, with no apparent direction in regards to the location of the daughter nuclei.

Phacus pyrum has a single cup-shaped plastid located on the periphery of the cell. During the light period the cell is teardrop shaped with the nucleus located in the far posterior of the cell (Fig. 3 E). The bulk of the plastid is located in the central portion of the cell and is characterized by a single large pyrenoid located adjacent to the anterior portion of the nucleus (Fig. 3 E). Prior to mitosis the nucleus moves to a more central location within the cell, the plastid begins to become more irregularly shaped, and the pyrenoid begins to dissipate. By the time of cytokinesis the plastid has divided ahead of the advancing cleavage furrow and is more highly fragmented at the anterior end of the cell than at the posterior (Fig. 3 F).

Euglena acus is a long spindle-shaped cell with many lenticular plastids located throughout the cytoplasm. During the light period plastids are numerous, but there is still sufficient space between them to discern individual plastids (Fig. 3 G). Plastid division occurs throughout the light period. In a premitotic cell (evidenced by the doubling in cell size, and the duplication of the chromosomes) the plastids have become so numerous that it is difficult to discern individual plastids at the light-microscope level (Fig. 3 H). The

plastids remain distributed more or less evenly throughout the cell and there is no indication of directionality (Fig. 3 H).

During light period growth the plastids of *Euglena mutabilis* are shield-shaped and located around the entire periphery of the cell. The plastids follow the cell-cortex so closely that they appear almost tubular in the anterior portion of the cell (Fig. 3 I). During the early portions of the dark period, the plastids grow and take on a reticulate morphology, the pyrenoids become reduced and are no longer visible using optical means (data not shown). By mitosis, the plastids have become fragmented into smaller plastids of varying size scattered throughout the cytoplasm (Fig. 3 J).

Euglena viridis has a single stellate plastid oriented axially in the cell. During the light period the cell is egg-shaped and the nucleus is located in the posterior portion of the cell (Fig. 3 J). The large central pyrenoid is located in the center of the cell immediately anterior to the nucleus with arms that radiate out from the pyrenoid toward the cell periphery (Fig. 3 J). At telophase of mitosis the single plastid has become fragmented into numerous sack-shaped plastids scattered throughout the cell (Fig. 3 K). Again there appears to be no orientation of the plastids with respect to the incipient cleavage furrow.

Discussion:

In order for cell division to be successful, numerous mechanisms exist to ensure that daughter cells receive sufficient cellular material. Some cellular constituents are partitioned into daughter cells by highly regulated and tightly controlled mechanisms (*e.g.* mitotic division of chromosomes). Other cellular constituents are partitioned in a more or less random pattern. For instance, the mitochondria of scorpion spermatozoa

have been shown to be partitioned into daughter cells by a stochastic mechanism during spermatogenesis (Birky 1983). Plastids of algae and plants can be partitioned at random, by tightly controlled mechanisms, or by a stochastic process intermediate between the two extremes.

The marine alga *Ochromonas danica* has a single plastid with two lobes connected by a thin bridge near the nucleus. Prior to cell division the bridge begins to narrow and eventually constricts into two separate plastids. Meanwhile the chloroplast ER in-folds and rejoins the nuclear envelope. The immature plastid then generates a new eyespot *de novo* in the posterior of the plastid, the whole plastid then rotates around so that the eyespot is in the anterior of the cell in preparation for cytokinesis (Slankis & Gibbs 1972). This differs markedly from the plastids of another unicellular marine alga *Olisthodiscus luteus*. *O. luteus* contains from 8 to 34 small discoidal plastids. Hennis (1981) and Hennis and Birky (1984) demonstrated that although there is a tendency towards symmetry due to the even distribution of plastids throughout the cell, plastid partitioning is purely stochastic. In order to understand the mechanisms used by euglenophytes we sampled numerous species demonstrating a great deal of plastid diversity. The results are summarized in Figure 4 and Table 1.

Euglena acus has a plastid morphology familiar to most, in that it resembles the plastids of many other algae and the land plants. In fact its partitioning mechanism is very similar to that of the embryophytes. Plastid growth and division does occur throughout the cell cycle as previously reported (Leedale 1967). However, the bulk of it occurs during the light period and early portions of the dark

Figure 4.4 A-F. Model of plastid division and development and the relation to the cell division cycle. I = Light period (stationary phase) morphology; II = Pre-mitosis; III = Early mitosis; and IV = Late mitosis/cytokinesis. **A.** *Euglena viridis*: I - A single axial plastid with central pyrenoid and paramylon center. II – Pyrenoid is lost and the paramylon center disperses. III – Plastids become reticulated and begin to fragment. IV – Plastid has become fragmented into several smaller sac-like plastids. **B.** *Euglena acus*: I – Cell is elongated with many disk-shaped plastids. II – Plastids grow and divide throughout the light period. III – Cell is much larger and the plastids have doubled in number. IV – Plastid number is returned to 'normal' via stochastic partitioning as a function of cytokinesis. **C.** *Trachelomonas volvocinopsis*: I – Cell is rounded and has 8 – 14 discoidal plastids with inner pyrenoids arranged around the periphery of the cell. II – Plastid division occurs during the light period with pyrenoids being produced *de novo* and sorted via plastid division. III – Plastids are arranged around the entire periphery of the cell with their pyrenoids intact. IV – Plastids are sorted into daughter cells stochastically as a function of their position during cytokinesis. **D.** *Eutreptiella gymnastica*: I - Cell is spindle shaped with a pair of plastid aggregations on either side of the nucleus. II – Paramylon center disperses and the plastid aggregations break up. III – The plastids grow and divide, some maintaining their pyrenoid. IV – By the time of cytokinesis the plastids are sack-like and partitioned into daughter cells stochastically. **E.** *Euglena mutabilis*: I - Cell is usually (*E. mutabilis* demonstrates a high degree of euglenoid metaboly) elongated with 1-4 shield shaped plastids wrapped around the periphery of the cell. II – Plastids become reticulated and begin to fragment, the pyrenoids are no longer visible in the light microscope. III – Cell is rounded and the plastids are fragmented into numerous smaller plastids. IV – Plastids are sorted stochastically into daughter cells at cytokinesis. **F.** *Phacus pyrum*: I – Cells are rounded with a long tapering posterior. The nucleus (dark circle) is in the posterior of the cell and the large plastid occupies most of the cell periphery and contains a large, central pyrenoid (lighter circle). II –The pyrenoid is lost and the plastid begins to fragment. III – During mitosis the plastid is fragmented primarily at the anterior of the cell, while the main portion of the plastid divides down the center. IV – The plastid is divided ahead of the cleavage furrow and is sorted into the daughters *via* a passive mechanism.

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Table 4.1. Comparison of plastid development and partitioning.

Species	Plastid morphology	Plastid number (in light period)	Timing of plastid division	Fragmentation?	Pyrenoid present during cell division
Euglena acus	lenticular	50-100	constant	no	no*
Trachelomonas volvocinopsis	parietal w/ inner pyrenoid	8-15?	after cell division	no	yes
Euglena mutabilis	shield-shaped w/ naked pyrenoid	1-4	before cell division	yes	yes
Phacus pyrum	cup shaped w/ central pyrenoid	1	at cell division	some	no
Eutreptiella gymnastica	aggregate	8-16 (2 aggregations)	before cell division	no	yes
Euglena viridis	stellate	1	before cell division	yes	no

* *E. acus* has never been shown to have a pyrenoid at any stage of the cell cycle.

period prior to mitosis and cell division. This is similar to patterns observed in *Euglena gracilis* (Pelligrini 1980, Cook et. al. 1976, Ehara et. al. 1990), but *E. acus* does not appear to regulate the timing of plastid division as stringently as *E. gracilis*. A very interesting phenomenon was noted in dark period cells of *E. acus*, cyclic plastid swelling. The only other report of this phenomenon was by Cook et. al. (1976) in which they noted that in 80% of cells sampled during the dark period showed marked swelling with widely separated lamellae. This phenomenon is believed to be associated with the age of the cell and not a direct result of the dark. However, since their cultures (as well as ours) are synchronized most cells are of approximately the same age. Cyclic plastid swelling can occur as early as the last 2 hours of the light period, and in some cases persist through the night and for 1-2 hours into the next light period.

Trachelomonas volvocinopsis with its moderate number of chloroplasts with conspicuous pyrenoids arranged around the periphery of the cell is most similar to *Euglena gracilis*. Although it would be expected that given the high degree of similarity between the plastids of these two organisms they would employ similar mechanisms of plastid partitioning, they have some striking differences. Firstly, Ehara et. al. (1990) showed that the plastids of *E. gracilis* undergo two separate aggregation phases, in which they are concentrated around the nucleus, followed by plastid division and fusion. The plastids of *T. volvocinopsis* remain parietal throughout cell division, and were not observed to aggregate around the nucleus. Secondly, it has been shown unequivocally that the pyrenoids of *E. gracilis* are transient and typically persist only through the first 2/3 of the light period (Könitz 1965, Cook et. al. 1976 Pelligrini 1980, Ehara et. al. 1990). In *T. volvocinopsis* however, pyrenoids and their associated paramylon caps were seen

throughout all stages of the cell cycle. After cell division, and during the early portions of the light period, the plastids of *T. volvocinopsis* grow and generate new pyrenoids *de novo* alongside existing ones. As the plastids divide they each receive one pyrenoid. Most striking is that this is very different from what was seen in *Strombomonas verrucosa*, a species closely related to *T. volvocinopsis* and having nearly identical plastid morphology (Brown et. al. 2002). *S. verrucosa* has the same inner pyrenoid morphology as *T. volvocinopsis*, yet possesses transient pyrenoids and does not generate new pyrenoids until after plastid division (Brown et. al. 2002). As with *E. gracilis* and *E. acus* partitioning of the plastids into daughter cells appears to be dependent solely on the location of the plastid at the time of cytokinesis.

Euglena mutabilis with just one to four large plastids per cell could be very susceptible to mistakes in partitioning if partitioning is random. Even if the plastids divide prior to cell division there is still a minimum 12.5% chance that a daughter cell will receive no plastids under a strictly random model. There are two possible mechanisms for ensuring that daughter cell receive plastids: 1) associate the plastid with the cytoskeleton during mitosis and thus partition the plastids with high fidelity in much the same manner as the nucleus; or 2) fragment the plastid to a degree that the probability of a daughter cell receiving no plastids is very low. *E. mutabilis* uses the second strategy and by fragmenting its plastids, ensures that even without some sort of compensatory mechanism each daughter cell will receive at least one plastid.

The only species of *Euglena* to be studied in detail regarding plastid division and partitioning other than *E. gracilis* is *Euglena deses*, often thought to be a close relative of *E. mutabilis* (Gojdics 1953, Pringshiem 1956). Gojdics (1934) noted that the plastids of

E. deses divide after cell division and that their pyrenoids remain intact and divide with the plastid. Gojdics also noted that there was some migration of plastids along the cytoplasmic bridge between two new daughter cells prior to completion of cytokinesis. Although *E. mutabilis* and *E. deses* have different plastid morphologies in light period or stationary phase cells, the similarities between the plastids of dividing cells in these species is striking.

Phacus pyrum unlike many other euglenophytes has a single plastid per cell during the light period, and in all stationary phase cells (unpublished results). Also unlike other euglenophytes is the seemingly regulated manner in which it partitions that plastid. Plastid division in *P. pyrum* is superficially similar to that of the well-known green alga *Chlamydomonas*, in which division of the plastid occurs before cell division and proceeds longitudinally along the plastid ahead of the cleavage furrow (Goodenough 1970, Ettl 1976). That is where the similarity between *P. pyrum* and *Chlamydomonas* ends. *P. pyrum* follows the general tendency of euglenophytes with fewer than 10 plastids to fragment the plastid before division, but because the plastid is more or less divided *via* binary fission, the degree of fragmentation is nominal. Also unlike *Chlamydomonas*, *P. pyrum* does not have a pyrenoid at the time of cell division and regenerates its pyrenoid after the plastid begins to resume its light period morphology.

Eutreptiella gymnastica has a plastid morphology identical to that of *Eutreptiella eupharyngea* and *Tetreutreptia pomquitensis* (Walne 1986, McLachlan 1994) and is apparently unique to members of the order Eutreptiales. The Eutreptiales are the earliest diverging euglenophytes and contain many features that differentiate them from other euglenophytes. This plastid morphology is sometimes referred to as “stellate with a

paramylon center" (Leedale 1967) but it is fundamentally different from the stellate plastids that characterize the genus *Euglena* subgenus *Euglena* (Zakrys 1986). The stellate plastids of *Euglena Euglena* are large star-shaped plastids with a central pyrenoid and radiating arms (Dragos and Perterfi 1979, Zakrys 1986, Zakrys 1998, Zakrys et.al. 2002). *E. gymnastica* does not have two stellate plastids, rather it has 10 – 20 plastids arranged into two aggregations on either side of the nucleus. The paramylon center is, like in *Euglena Euglena*, the site of the pyrenoid, but unlike *Euglena Euglena* there are several pyrenoids aggregated together instead of just one. The presence of 10 – 20 plastids per cell instead of just 2 makes stochastic partitioning much less complicated and more successful. All that is necessary to prepare for cell division is for the pyrenoid aggregation to break up and for the paramylon center to disperse. This results in the plastids spreading out to occupy all portions of the cell and ensures that both daughter cells receive some plastid complement. Unlike *Euglena gracilis*, the disaggregated plastids do retain pyrenoids during cell division.

Euglena viridis presents the most interesting mechanism of plastid partitioning. *E. viridis* is a member of the subgenus *Euglena* and has the characteristic single, axial, stellate plastid morphology that defines the group (Dragos & Perterfi 1979). Although evidence suggests that plastid number can vary in *Euglena Euglena* according to culture media (Zakrys et. al. 2002), our cells were maintained in the same media throughout all experiments. Leedale (1967) stated from light microscopic observations that prior to cell division, the stellate plastid of *E. viridis* divided longitudinally with the pyrenoid intact; however after looking at dozens of cells fixed during the dark period we were unable to see evidence of either a pyrenoid or a paramylon center.

Instead, shortly after the inception of the dark period the pyrenoid begins to dissipate and the paramylon center disperses. The plastid then begins to lose its axial orientation and starts to form a reticulum spreading throughout the cell. The reticulated stage is followed by the pinching-off of the extremities of the reticulum until the plastid has been fragmented into numerous, independent, disk or ball-shaped plastids spread throughout the entire cell. The resultant morphology is very similar to that of dividing cells of *Eutreptiella gymnastica*. The fragmented stage does not last long however, as the plastids fuse together to regain their stellate appearance and axial orientation, often resuming normal morphology before cytokinesis is complete. While we never directly observed plastid fusion in *E. viridis* it has been observed in the fragmented plastids of *Euglena gracilis* (Ehara et. al. 1990)

It is striking, from the viewpoint of comparative cytology, that two species with outwardly similar plastid morphologies such as *E. viridis* and *P. pyrum* use such drastically different mechanisms of plastid partitioning, while maintaining some fundamental similarities. Both have a single plastid per cell, and each plastid has a single centrally located pyrenoid. Yet *P. pyrum* divides and partitions its plastid via a seemingly simple method of binary fission, whereas *E. viridis* goes through a very complex fragmentation and fusion process in order to successfully partition its plastid component. The fact that the plastid of *P. pyrum* does fragment to some degree suggests that the capacity is there, but that perhaps *P. pyrum* has evolved a more high-fidelity mechanism.

One of the striking phenomena seen in several species of euglenophytes is the tendency of the plastid to fragment prior to cell division. This presents a problem, for it

is not enough for each daughter cell to receive adequate photosynthetically active thyllakoids, each must also receive at least one copy of the plastid genome. Plastid DNA is located in the stroma of all chloroplasts, but it is not dispersed as single copies throughout the plastid, rather it is aggregated into structures called nucleoids (Coleman & Nerozzi 1999). There are 2 different conformations of plastid nucleoids; the ring nucleoid and 'scattered' nucleoids (Coleman 1985). Ring nucleoids are a continuous string of multiple genome copies characteristic of algae that possess girdle lamellae. The scattered nucleoid is composed of numerous small aggregates of plastid DNA spread among the thyllakoids and is found in the plastids of plants, green algae, and the euglenophytes. Euglenophytes, once thought to possess only scattered nucleoids, have been shown to have (at least some of the time) single, large, branching nucleoids (Ehara et. al. 1990) as well. This dispersal of plastid DNA throughout the stroma of the plastid insures that even if the plastid is randomly fragmented, each fragment is likely to receive at least one copy of the plastid genome, thus allowing for the high degree of fragmentation seen in some euglenophyte species (*e.g*. *E. viridis,* and *E. mutabilis*).

While fragmentation of the plastid in order to achieve successful partitioning is unusual, there are other organelles, most notably the Golgi apparatus of metazoans in which this occurs. Prior to mitosis, the Golgi apparatus of animal cells breaks down concomitantly with the nuclear envelope into numerous fragments. These fragments have been proposed to be partitioned into daughter cells either by stochastic mechanisms or via active sorting by the cytoskeleton (Shima et. al. 1998). While there have been some challenges to this model (reviewed in Barr 2002), it is apparent that the Golgi apparatus does fragment prior to mitosis and then reforms following cytokinesis.

Although it would seem unlikely to fragment such a complex organelle as the plastid in order to partition it, there is precedent for this phenomenon, and as long as the plastid DNA is scattered throughout the plastid it appears to be an attractive mechanism for euglenophytes.

Finally, there is the problem of incomplete sorting resulting in non-photosynthetic daughter cells. The absolute need for a plastid has been demonstrated in all stably photosynthetic organisms, as well as some organisms that have lost their ability to photosynthesize (eg. *Plasmodium*). In addition to photosynthesis, plastids carry out numerous other physiological processes rendering them invaluable to their host cells. Even though numerous cell lines isolated from yeast, chicken fibroblasts, and humans have been grown successfully without mitochondria or mitochondrial DNA (Coleman & Nerozzi 1999) the only organism successfully grown without a plastid is *Euglena gracilis* (Rawson & Boerma 1976, Conkling et. al. 1993). Coleman & Nerozzi (1999) make the case that *Euglena* is an exception to the rule due to the presence of two pathways for porphyrin synthesis. Recent findings by Hannaert et. al. (2003) could suggest another explanation. They have discovered a number of genes in the nuclear genomes of Kinetoplastids (the sister group to the euglenids) with high affinity to cyanobacterial, green algal, and plant genes. The most telling is the gene encoding sedoheptulose-1,7-bisphosphate (SPBase), an enzyme known only from the Calvin cycle of Chlorophyte plastids. Hanneart et. al. (2003) and Martin & Borst (2003) suggest that this indicates kinetoplastids and euglenophytes both derived their plastid genes from a common ancestor. These recent results suggest that there is more biochemical propensity for aplastidy in the euglenophytes than previously thought. The propensity for euglenophyte

cells to partition their plastids *via* random (or at least stochastic) mechanisms and their unique ability to survive without the products of plastid biosynthesis might explain the origin of several species of euglenids believed to be non-photosynthetic relatives of euglenophytes (eg. *Astasia, Khawkinea, Hyalophacus,* C*yclidiopsis* and *Trachelomonas reticulata*).

Conclusions:

While there is some degree of diversity in the mechanisms euglenophytes employ to partition their plastids during cell division, there are two main themes. The first is found in cells with numerous plastids during light period or stationary phase growth, and they simply let cytokinesis partition the plastids where they lie. The second is found in cells that have a single, or very few plastids during light period or stationary phase growth, and they fragment their plastids prior to cell division, resulting in a transient plastid morphology and number similar to the first group. This allows the plastids to be partitioned stochastically with a good probability of success. Even cells that have a quasi-regulated partitioning mechanism (*i.e*. *P. pyrum*) still fragment their plastids, albeit to a lesser degree, prior to cell division. The euglenophytes are uniquely suited to this apparently 'sloppy' mechanism because of their high tolerance for becoming aplastidic.

Acknowledgements:

Financial support was provided by the U. S. National Science Foundation PEET (Partnerships for Enhancing Expertise in Taxonomy) grant no. DEB 4-21348. We also

thank Dr. Bozena Zakrys for providing some euglenophyte strains and Dr. John Shields for technical assistance.

References:

Barr F (2002) Inheritance of the endoplasmic reticulum and Golgi apparatus. Curr Opin Cell Biol 14: 496-499

Ben-Shaul Y, Schiff JA, Epstein HT (1964) Studies of chloroplast development in *Euglena*. VII. Fine structure of the developing plastid. Plant Physiol 39: 231-240

Ben-Shaul Y, Epstein HT, Schiff JA (1965) Studies of chloroplast development in *Euglena*. 10. The return of the chloroplast to the proplastid condition during dark adaptation. Can J Bot 43: 129-136

Birky CW (1983) The partitioning of cytoplasmic organelles at cell division. Int Rev Cytol Suppl 15: 49-89

Brown, PJP, Zakryś B, Farmer F (2002) Plastid morphology, ultrastructure, and development in *Colacium* and the loricate euglenophytes (Euglenophyceae) J Phycol 39: 1-8

Chu SP (1946) Contributions to our knowledge of the genus *Euglena*. Sinensia 17: 75-134

Coleman, AW (1985) Diversity of plastid DNA configuration among classes of eukaryotic algae. J Phycol 21: 1-16

Coleman AW, Nerozzi AM (1999) Temporal and spatial coordination of cells with their plastid component. Int Rev Cytol 193: 125-164

Conforti V (1998) Morphological changes in the Euglenophyta in response to organic enrichment. Hydrobiologica 369/370: 277-285

Conkling BA, Thomas EJ, Oritz W (1993) Delayed but complete loss of chloroplast DNA in heat-bleaching cultures of *Euglena gracilis*. Plant Physiol 142: 307-311

Cook JR, Haggard SS, Harris P (1976) Cyclic changes in chloroplast structure in synchronized *Euglena gracilis*. J Protozool 23: 368-373

Da Cunha AM (1913) Contribuiçâo para o conhecimento da fauna de protozoários do Brasil Mem Inst Oswaldo Cruz RJ 5:101-122

Dragos N, Perterfi LS, Craciun C (1979) Fine structure of *Euglena*. 2. *Euglena stellata* Mainx and *Euglena viridis* Ehrenberg. Nova Hedwigia 31: 223-246

Ehara T, Osafune T, Hase E (1990) Interactions between the nucleus and cytoplasmic organelles during the cell cycle of *Euglena gracilis* in synchronized cultures. Exper Cell Res 190: 104-112

Ehrenberg CG (1830) Neue Beobachtungen über blutartige Erscheinungnen in Ägypten, Arabien, und Sibirien, nebst einer Übersicht und Kritik der früher bekannten. Pogg Ann Physik Chem 94:477-514

Epstein HT, Schiff JA (1961) Studies of chloroplast development in *Euglena*. 4. Electron and fluorescence microscopy of the proplastid and its development into a mature chloroplast. J Protozool 8: 427-432

Ettl H (1976) Progress of plastid division in *Chlamydomonas*. Protoplasma 88: 75-84

García-Ferris C, de los Ríos A, Ascaso, C, Moreno J (1996) Correlated biochemical and ultrastructural changes in nitrogen-starved *Euglena gracilis*. J Phycol 32: 953-963

Gibbs SP (1978) The chloropalsts of *Euglena* may have evolved from symbiotic green algae. Can J Bot 56: 2883-2889

Gojdics M (1934) The cell morphology and division of *Euglena deses* Ehrenberg. Trans Am Microscop Soc 58: 241-248

Gojdics M (1956) The genus *Euglena*. The University of Wisconsin Press, Madison, 268 pp

Goodenough UW (1970) Chloroplast division and pyrenoid formation in *Chlamydomonas*. J Phycol 6: 1-6

Gray J, Boucot AJ (1989) Is *Moyeria* a euglenoid? Lethaia 22: 447-456

Hannaert V, Saavedra E, Duffieux F, Szikora J-P, Rigden DJ, Michels PAM, Opperdoes FR (2003) Plant-like traits associated with metabolism of *Trypanosoma* parasites. Proc Natl Acad Sci USA 100: 1067-1071

Hennis AA (1981) M.S. Thesis. The Ohio State University

Hennis AS, Birky CW (1984) Stochastic partitioning of chloroplasts at cell division in the alga *Olisthodiscus*, and compensating control of chloroplast replication. J Cell Sci 70: 1-15

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23: 633-638

Könitz W (1965) Elektronenmikroskopische Untersuchengen an *Euglena gracilis* im Tagesperiodischen Light-Dunkel-Wechsel. Planta 66: 345-373

Kuroiwa T (1991) The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. Int Rev Cytol 128: 1-63

Leander BS, Witek RP, Farmer MA (2001) Trends in the evolution of the euglenid pellicle. Evolution 55: 2215-2235

Leedale G (1967) The Euglenoid Flagellates. Prentice Hall, Engelwood Cliffs, 220pp.

Lemmermann E (1913) *Eugleninae*. In: Die Süsswasserflora Deutschlands, Österreichs, und Der Schweiz. G. Fischer, Jena, pp 115-174

Martin W, Borst P (2003) Secondary loss of chloroplasts in trypanosomes. Proc Natl Acad Sci USA 100: 765-767

McLachlan JL, Sequel, MR, Fritz L (1994) *Tetreutreptia pomquitensis* gen. et. sp. nov. (Euglenophyceae): a quadriflagellate, phototrophic, marine euglenoid. J Phycol 30: 538-544

Pelligrini M (1980) Three-dimensional reconstruction of organelles in *Euglena gracilis Z* J Cell Sci 43: 137-166

Pringsheim EG (1956) Contributions toward a monograph of the genus *Euglena*. Nova Acta Leopoldina 125: 1-170

Rawson JRY, Boerma C (1976) Influence of growth conditions upon the number of chloroplast DNA molecules in *Euglena gracilis*. Proc Natl Acad Sci USA 73: 2401-2404

Schiff JA, Lyman H, Epstein HT (1961) Studies of chloroplast development in *Euglena*. III. Experimental separation of chloroplast development and chloroplast replication. Biochim Biophys Acta 51: 340-346

Schiff JA, Epstein HT (1965) The continuity of the chloroplast in *Euglena*. In: Locke M (ed) Reproduction: molecular, subcellular, and cellular. Academic Press NewYork, pp 131-189 (The reproduction of subcellular systems)

Schmitz F (1884) Beiträge zur Kenntnis der Chromatophoren. Jahrb f wiss Bot 15:1-175.

Shima DT, Cabrera-Poch N, Pepperkok R, Warren G (1998) An ordered inheritance strategy for the Golgi apparatus: Visualization of mitotic disassembly reveals a role for the mitotic spindle. J Cell Biol 141: 955-966

Slankis T, Gibbs SP (1972) The fine structure of mitosis and cell division in the chrysophycean alga *Ochramonas danica*. J Phycol 8: 243-256

Stein FR (1878) Der Organismus der Infusionsteire. III. Abt. Der Organismus der Flagellaten. I. Verlag von Wilhelm Engelmann, Leipzig.

Swirenko DO (1914) Zur Kenntnig der russischen Algenflora. I. Die Euglenaceengattung *Trachelomonas*. Arch Hydrobiol Planktktonk 9: 636-647

Walne PL, Moestrup O, Norris RE, Ettl H (1986) Light and electron microscpical observations of *Eutreptiella eupharyngea* sp. nov. (Euglenophyceae) from Danish and American waters. Phycologia 25: 109-126

Zakryś B (1986) Contribution to the monograph of the Polish members of the genus *Euglena* Ehrenberg 1830. Nova Hedwigia 42: 491-540

Zakryś B, Walne P (1998) Comparative ultrastructure of chloroplasts in the subgenus *Euglena* (Euglenophyta): taxonomic significance. Cryptogamie Algol 19: 3-18

Zakryś B, Milanowski R, Empel J, Borsuk, P, Gromadka R, Kwiatowski J (2002) Two different species of *Euglena*, *E. geniculata* and *E. myxocylindracea*

(Euglenophyceae), are virtually genetically and morphologically identical. J Phycol 38: 1190-1199

CHAPTER 5

PHYLOGENY AND INTRON CONTENT OF THE EUGLENOPHYTE PSAA GENE[1]

1 – Brown, P.J.P. and M. A. Farmer, to be submitted to the International Journal of Systematic and Evolutionary Microbiology

Abstract

We determined the partial sequence of the photosystem I apoprotein A gene (PsaA) from the chloroplast genome of 12 euglenophyte species was determined. Distance, maximum likelihood, and Bayesian methods were used to infer the phylogeny of the euglenophyte plastid in relation to other groups of eukaryotic algae. The euglenophytes form a sister group with the green algae. When the prasinophyte green alga *Mesostigma* is included in the analyses it forms the most basal branch of the lineage leading to euglenophyte plastids. These data suggest that the euglenophytes acquired their plastid very early in the green algal radiation. The sequence of psaA was also used to infer the phylogeny of 12 species of euglenophytes from the genera *Euglena*, *Strombomonas*, *Trachelomonas*, *Colacium, Eutreptia,* and *Eutreptiella*. These data suggest that the Eutreptiales are paraphyletic, and that *Euglena anabaena* is a member of the subgenus *Calliglena*. The PsaA gene of euglenophyte plastid DNA contains three novel intron positionse. These include a large intron in the genus *Colacium*, an intron with a possible twintron in the *Discoglena*, and a small group III intron unique to *E. acus*. Results of phylogenetic analyses and intron mapping are discussed.

Introduction

The Euglenozoa diverged shortly after the eukaryotic acquisition of mitochondria, and represent some of the deepest-branching mitochondrial eukaryotes based on studies of nuclear encoded proteins and rDNA (Baldauf *et. al.* 2000). The Euglenozoa is composed of three main lineages, the Diplonemids, the Kinetoplastids, and the Euglenids. Within the euglenids exists a sub-group called the euglenophytes, the only photosynthetic members of the group (Cavalier-Smith 1981, Simpson 1997).

Euglenophytes represent some of the more well-known organisms in the group. Although the genus *Euglena* is familiar to most general biology students, little is known about most of the organisms that make up the group.

Over the past decade or so, there have been great steps taken to expand our knowledge of euglenophyte diversity and systematics. The vast majority of the studies in euglenid molecular systematics have been based on the gene encoding the small subunit ribosomal RNA (Montegut-Felkner and Triemer 1997, Linton *et. al.* 1999, 2000, Leander *et. al.* 2001, Preisfeld *et. al.* 2000, 2001, Müllner *et. al.* 2001). These have been very informative in reshaping our conceptions of euglenid phylogeny but they only include data from nuclear encoded genes. In order to better understand and verify the nuclear phylogenies, it would be useful to have data from a second independent genome. There have been only two systematic studies within the euglenophytes using plastid-encoded genes. The first (Thompson *et. al.* 1995) used the gene for the large subunit of Ribulose 1,5 BisPhosphate Carboxylase/Oxygenase (RbcL). Although RbcL has been used successfully in numerous plastid phylogenies from nearly all groups of photosynthetic eukaryotes (Chase and Albert 1998), it proved to be a poor indicator of euglenid phylogeny. The only grouping with a high degree of branch support was made up of what turned out to be misidentified members of the same species (*E. gracilis*). This was followed by a study using plastid encoded 16S ribosomal RNA (Milanowski *et. al.* 2001). The phylogenies constructed using 16S rDNA proved to be more effective than RbcL at elucidating phylogeny and showed higher overall topological stability, however there was still some degree of uncertainty in parts of the tree. Milanowski *et. al.* (2001) were able

to identify two major clades of euglenophytes, however the internal topology of these clades was unstable.

The euglenophytes are characterized by a plastid believed to be derived from secondary symbiogenesis between a phagotrophic euglenid and a green alga (*sensu lato*) (Lee 1977, Gibbs 1978). Early hypotheses regarding the relationship between the euglenid plastid and the green algae were based largely on biochemistry. The euglenophytes are one of a few groups of eukaryotes whose plastids contain chlorophyll b, the other two being the green algae and the little-known chlorarachniophytes. The presence of a third surrounding membrane led to the theory that the euglenophyte plastid was derived secondarily, not from a cyanobacterium, but through a eukaryotic symbiote (Gibbs 1978). Long before the advent of molecular systematics it was widely assumed that the euglenophyte plastid had its origins in the green algae. That still leaves a major remaining question: from which green alga did the euglenophyte plastid come? Without exception, every high level molecular phylogeny of plastid genes to date contains only a single euglenophyte species, making the addition of more euglenophyte taxa to any analysis paramount to understanding the true relationship between the euglenophyte plastid and the green algae.

The euglenophyte plastid genome is the most intron-rich genome of any organism studied to date (Hallick *et. al.* 1993). Nearly 40% of the entire genome is made up of introns. The euglenophyte plastid genome contains not only group I and group II introns, but a novel group unique to the euglenophyte plastid, group III introns (Drager and Hallick 1993, Copertino and Hallick 1993). Studies of intron content in diverse euglenophyte taxa have contributed a great deal to our knowledge of intron evolution, but

they have all based their evolutionary assumptions on a poorly supported phylogeny (Thompson *et. al.* 1994, Doetsch *et. al.* 1998, 2001).

The goal of this study was to sequence the gene for a separate plastid-encoded protein from a number of diverse euglenophytes for three purposes: 1) To test its utility as a marker for molecular systematics within the euglenophytes and to attempt to provide resolution to the poorly supported sections of other plastid-based phylogenies; 2) to study the intron content of a major photosynthetic reaction center protein gene; and 3) to construct a multi-gene phylogeny of diverse algal plastids with several euglenophyte taxa in order to discern the origin of the euglenophyte plastid from within the chlorophyta. We present here the phylogeny of twelve euglenophytes based on nearly complete sequence of PsaA, the intron content of the gene, as well as a three-gene phylogeny covering the major algal lineages.

Materials and Methods

Strains and culture conditions. The following strains were used: *Eutreptia sp.* (UTEX 2003), *Eutreptiella gymnastica* (CCMP 1594), *Euglena anabaena* (UTEX 373), *Euglena gracilis* strain Z. (UTEX 753), *Euglena viridis* (UTEX 85), *Euglena stellata* (UTEX 372), *Colacium vesiculosum* (UW Lazienki), *Strombomonas costata* (ACOI 2992), *Euglena tripteris* (UTEX LB1311), *Euglena mutabilis* (SAG 1224-9a), *Phacus pyrum* (UTEX LB 2345), and *Trachelomonas volvocina* (AICB 323). All cultures except for *Eutreptia sp.* and *Eutreptiella gymnastica* were grown in 10 ml of ESSEX medium (Brown *et. al.* 2003) in capped test tubes at 20° C on a 12:12 light dark cycle. *Eutreptia sp.* and *Eutreptiella gymnastica* were grown in 20 ml of K medium (Keller *et. al.* 1987)

in 50 ml tissue culture flasks under the same light and temperature regime as above. The cultures were obtained from the following collections: UTEX, The Culture Collection of Algae at the University of Texas; CCMP, the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton; AICB, Culture Collection of Algae at the Institute of Biological Research, Cluj-Napoca, Romania; ACOI, Culture Collection of Algae at the Department of Botany, University of Coimbra, Portugal; SAG, Sammlung von Algenkulteren Göttingen; UW, Culture Collection of Algae at the department of Plant Systematics and Geography at Warsaw University, Poland.

DNA isolation, amplification, cloning, and sequencing. The total cellular DNA was isolated from a 10 ml log-phase culture using the DNeasy plant mini-kit (Qiagen Corp.) according to manufacturer's instructions (with the addition of Proteinase K during lysis). A 50 µl PCR reaction contained 1 U Taq DNA polymerase (Sigma), 100 µM dNTP Mix (Sigma), 2 mM MgCl2, 0.5 µM ea. forward and reverse primer, 1X reaction buffer (Sigma), and 30 – 60 ng genomic DNA. Touchdown PCR (Don *et. al.* 1991) was performed according to table 1 using overlapping combinations of the primers in table 2. PCR products were cloned into pCR4-TOPO using the TOPO-TA cloning kit (Invitrogen) with the following modifications to the manufacturer's protocol: vector was reduced to 0.5 µl, salt solution was reduced to 0.5 µl, and PCR product was 2 µl. All 3 µl of the cloning reaction was used to transfect Top-10 chemically competent cells, and two ampicillin selective plates (With X-Gal) were spread with 50 and 100 µl of 1-hour culture each. three or four white colonies were transferred to liquid selective media and grown overnight. Plasmids were purified using the Plasmid Mini-Kit (Qiagen) according to manufacturer's protocols. Inserts were verified by restriction digestion. In most

Table 5.1. Touchdown PCR reaction conditions[1]

Stage	Number of Cycles	Annealing Temperature
1	1	60 °C
2	2	58 °C
3	3	55 °C
4	3	52 °C
5	3	50 °C
6	9	48 °C
7	15	45 °C

1-All cycles were carried out with a 30 sec. denaturation at 94 °C and extension for 1 min. at 72 °C followed by a final extension of 20 min. at 72 °C

Table 5.2. PsaA PCR primers

Primer name	Position of 5' end[1]	Sequence (5' – 3')
PsaA1F[2]	1	ATGACWATWACWCCTCCNGARC
PsaA43F	129	AARGGNCCWAAWACNACWACTTGGATHTGG
PsaA54F	162	GCTGATGCGCAYGAYTTYGA
PsaA175F	525	TTTGCWGGTTGGTTTCAYTAYCATAA
PsaA213F	639	TGGGCWGGWCATCARATWCAYG
PsaA308F	924	GCAGGTCATATGTATAARACTAATTGG
PsaA441F	1323	AAYTTAACCCAWACRTAWAARRATCC
PsaA220R[3]	660	ACATGAATTTGRTGWCCNGCCCA
PsaA220R-t	660	ATTTGRTGWCCNGCCCARG
PsaA378R	1134	CCATAWGGNGGCATWGMRTACATRTGYTG
PsaA447R	1341	CCTARRAAWATRCAWACCCAYTTYAA
PsaA544R	1632	TGWATNGTAAAWGCRTGWATATGRTGWAC
PsaA581R	1743	CACGWCCWGGWCCATCACAAG

1-Position of 5' end refers to the nucleotide position in *E. gracilis* cDNA, abbreviations follow IUPAC convention.
2- 'F' denotes a forward primer.
3 – 'R' denotes a reverse primer

instances two clones were sequenced, one on either strand using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Mix ver. 3.0 or 3.1, and primed using the T3 and T7 sequences that flank the insert. The readings from the ABI 3100 DNA sequencer were assembled using Gene Runner ver. 3.05 and verified manually. All sequencing was carried out at the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia.

Sequence alignment and analysis. Sequence accession numbers are summarized in Table 3. The 16S rDNA sequences were aligned with Clustal X using the default options. The rough alignment was then checked and edited manually using Se-Al ver. 1.0a1 (Rambaut 1996) and any regions that could not be unambiguously aligned were removed. The PsaA and RbcL nucleotide sequences were aligned manually using Se-Al ver. 1.0a1. Both the euglenophyte-only PsaA and the larger multigene alignment are available online at http://www.uga.edu/caur/alignments-fasta.txt or by contacting the corresponding author. Distance and Likelihood analyses were carried out using PAUP* 4.0b10 (Swofford 1999) and Bayesian analysis was carried out using MrBayes ver. 2.01 (Huelsenbeck & Ronquist 2001) both on a Macintosh G4. Distance analyses were carried out using uncorrected, Jukes-Cantor (Jukes & Cantor 1969), HKY85 (Hasegawa *et. al.* 1985), General Time Reversible (Lanave *et. al.* 1994, Rodriguez *et. al.* 1990), and LogDet (Lockhart *et. al.* 1994) models with equal or varying rates of among-site variation. Distance trees were calculated using the Neighbor-Joining method (Saitou & Nei 1987) and optimized using minimum evolution (ME); bootstrap analysis was performed using heuristic searches, optimizing with ME, and run for 1000 replicates. Maximum Likelihood analysis was carried out using the HKY85 and GTR models with

Table 5.3. Sequence accession numbers used in this study.

Organism	PsaA	RbcL	16S
Mesostigma viride	NC_002186	NC_002186	NC_002186
Synechocystis	NC_000911	NC_000911	NC_000911
Nephroselmis olivacea	NC_000927.1	NC_000927.1	NC_000927.1
Chlorella vulgaris	NC_001865	NC_001865	NC_001865
Chlamydomonas rheinhardii	BK000554	BK000554	BK000554
Zea mays	X86563.2	X86563.2	X86563.2
Spinacea	NC_002202	NC_002202	NC_002202
Chaetosphaeridium globosum	NC_004115	NC_004115	NC_004115
Odontella sinensis	NC_001713	NC_001713	NC_001713
Guillardia theta	NC_004088	NC_004088	NC_004088
Porphyra purpurea	NC_000925	NC_000925	NC_000925
Cyanidium caldarium	NC_001840	NC_001840	NC_001840
Cyanophora paradoxica	NC_001675	NC_001675	NC_001675
Euglena gracilis	X70810	X70810	X70810
Euglena viridis	XXXXXXX[1]	U21010	AF289248
Euglena stellata	XXXXXXX	U21009	AF289244
Euglena anabaena	XXXXXXX	U21004	AF289240
Euglena mutabilis	XXXXXXX	----------[2]	----------
Euglena tripteris	XXXXXXX	----------	----------
Eutreptiella gymnastica	XXXXXXX	----------	----------
Eutreptia sp.	XXXXXXX	----------	----------
Strombomonas costata	XXXXXXX	----------	----------
Trachelomonas volvocina	XXXXXXX	----------	----------
Colacium vesiculosum	XXXXXXX	----------	----------
Phacus pyrum	XXXXXXX	----------	----------

1-These sequences have not been assigned accession numbers yet.
2-These sequences were not used in the analysis, this does not mean that some of these species have not had this gene sequenced.

or without calculation of among-site rate variation. Heuristic searches were carried out with ACCTRAN optimization, TBR branch-swapping, and random addition of taxa (10 reps.). Bootstrap analysis was carried out for 100 cycles using the same options, but without random addition of taxa. Bayesian inference was carried out for 400,000 generations using the GTR and HKY85 models with a discrete gamma distribution for among-site rate variation and a shape factor calculated from previous analyses. Multigene analyses were rooted using the cyanobacterium *Synechocystis* and the euglenophyte-specific trees were rooted using the green algae *Nephroselmis* and *Chlamydomonas*.

Results:

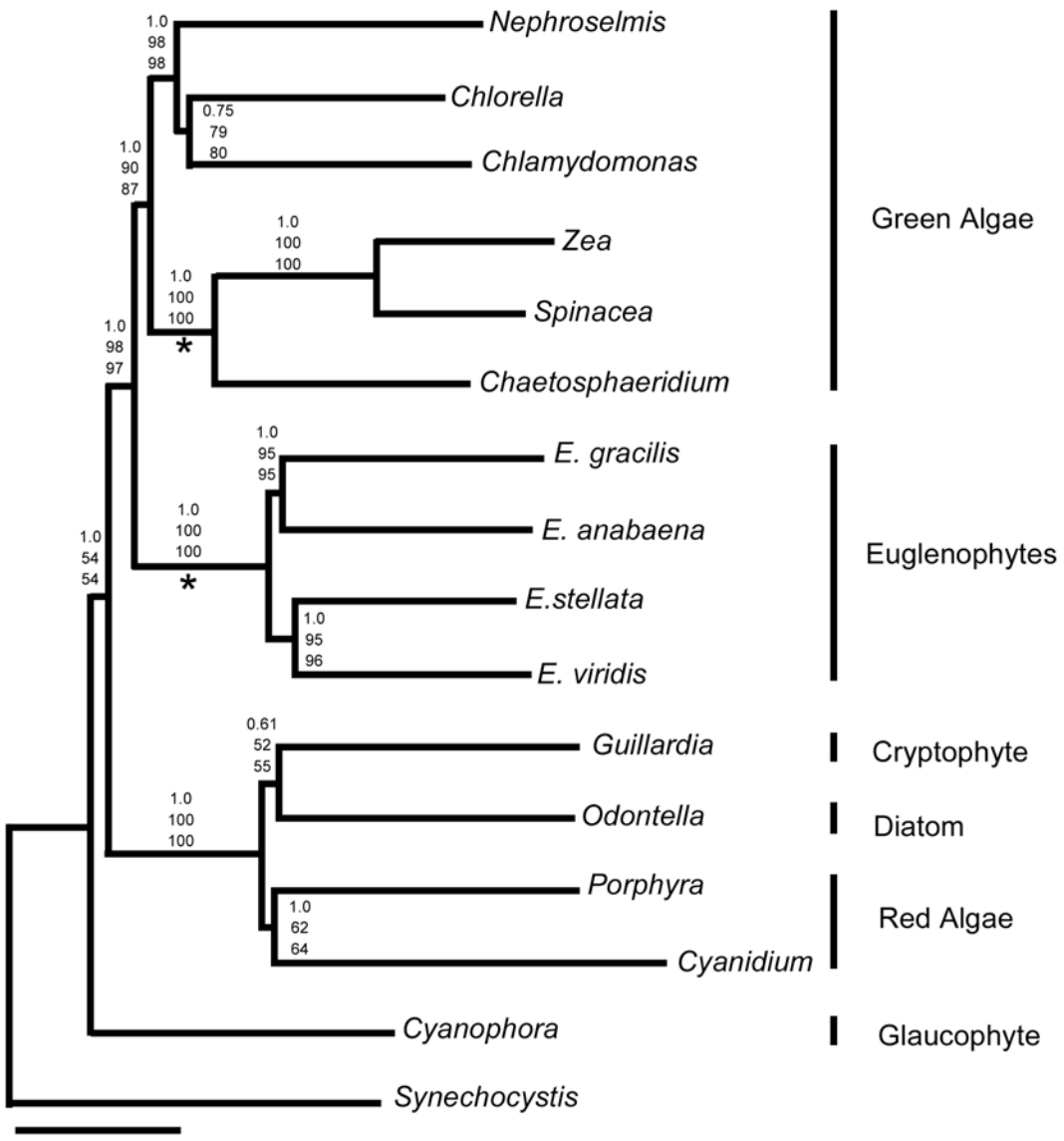
PsaA was sequenced from position 1-581 in *Euglena stellata*, *E. anabaena*, and *E. tripteris*; from position 54-581 in *Eutreptia sp.*, *Eutreptiella gymnastica*, *Euglena viridis*, *Colacium vesiculosum*, *Phacus pyrum*, and *Trachelomonas volvocina*; and from position 175-581 in *Strombomonas costata*, and *Euglena mutabilis*. All positions are relative to the complete amino acid sequence of *Euglena gracilis* (genbank X70810.2). The alignment of PsaA was unambiguous and all positions were included. Ambiguous portions of the 16S rDNA sequences were removed prior to analysis. The PsaA analyses for euglenophyte taxa were performed on the region from residue 175-581 for a total of 1220 bp. The combined, multigene analysis was performed on a concatenated sequence consisting of 1317 bp from RbcL, 1421 bp from chloroplast 16S, and 1668 bp of PsaA; for a combined concatenated sequence of 4406 bp.

Multigene analysis. Due to the high degree of conservation in the protein genes that make up 2/3 of the analyzed sequence, parsimony analysis was very uninformative,

and in some instances misleading. Parsimony did find a monophyletic euglenophyta, but the green algae (a very well-defined group) were polyphyletic. Parsimony analysis failed to unite *Porphyra* and *Cyanidium* which are both red algae. For this reason parsimony analysis was not considered in drawing any conclusions.

Figure 1 shows the phylogram obtained using minimum evolution with LogDet distances; the topologies of the maximum likelihood and Bayesian trees were identical. Support for each branch is indicated at the nodes and are from top to bottom, Bayesian posterior probability, maximum likelihood bootstrap, and minimum evolution bootstrap. The cyanobacterium *Synechocystis* was used to root the tree. The glaucocystophyte *Cyanidium* was shown in all analyses to be the earliest diverging eukaryotic plastid. The next-largest group consisted of the red algae and red-algal related secondary plastids, as sister to the green algae and the euglenophyte plastids, with varying support. Taxa with a red algal plastid were found to be monophyletic in all three analyses. The support for monophyly of the red algae varied, but the grouping was found more than 60% in all analyses. The sister relationship between *Odontella* (a diatom) and G*uillardia* (a cryptophyte) was weakly supported in all analyses and was reduced to a polytomy with the primary red plastids in some distance analyses (data not shown). The euglenophytes were recovered as monophyletic with 100% support, and are clearly a sister group to the green algae. The relationship of the euglenophytes to other taxa was dependant on the inclusion of the enigmatic green alga *Mesostigma viride*. The asterisks indicate the two nodes at which *Mesostigma* branched in different analyses. In all distance analyses except LogDet, *Mesostigma* branched as the sister group to the streptophytes with

Figure 5.1. The phylogram obtained from maximum likelihood analysis (HKY85 + G + I) of a concatenated 3-gene alignment composed of RbcL, PsaA, and 16S rDNA. The topology is identical to trees generated with minimum evolution using LogDet distances and Bayesian inference (GTR + G + I). –lnL = 4097.82119, ti/tv = 1.303006, $_p$inv = 0.323371, α = 0.934050. Bayesian: ngen = 400,000, burn in = 140 trees. Numbers at the nodes represent from top to bottom, Bayesian posterior probabilities, ML bootstrap (100 replicates), and LogDet bootstrap (1000 replicates). Asterisks represent the 2 nodes at which *Mesostigma* branched. Bar = 0.05 substitutions per site.

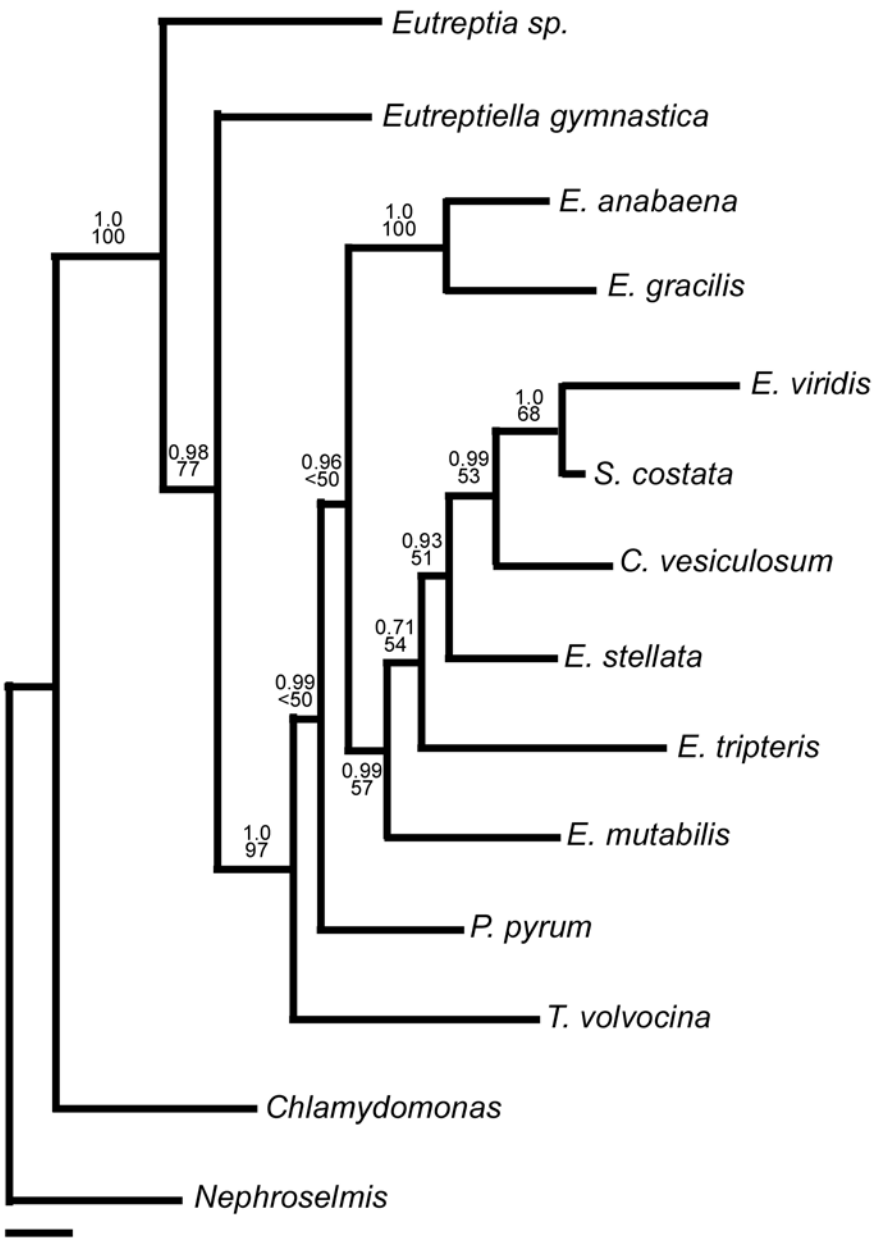

Nephroselmis
Chlorella
Chlamydomonas
Zea
Spinacea
Chaetosphaeridium
E. gracilis
E. anabaena
E.stellata
E. viridis
Guillardia
Odontella
Porphyra
Cyanidium
Cyanophora
Synechocystis
Green Algae
Euglenophytes
Cryptophyte
Diatom
Red Algae
Glaucophyte
1.0
98
98
0.75
79
80
1.0
90
87
1.0
100
100
1.0
100
100
*
1.0
98
97
1.0
95
95
1.0
100
100
*
1.0
54
54
1.0
95
96
0.61
52
55
1.0
100
100
1.0
62
64

bootstrap support ranging from 62-71%. In both Bayesian and maximum likelihood analyses *Mesostigma* branched as the sister group to the euglenophytes, with a Bayesian posterior probability of 1.0 and weak bootstrap support. When *Mesostigma* was removed from the analysis all distance methods, with the exception of LogDet, placed the euglenophytes as sister group to the red-algal plastids, with bootstrap support from 51-62%, however when site to site variation was corrected using the LogDet paralinear distances, the euglenophyte-green algal relationship was recovered and had high bootstrap support (97%).

PsaA analysis within the euglenophytes. Figure 2 is the maximum likelihood (HKY85 +G + I) tree obtained from analysis of 1220 bp of the euglenophyte PsaA gene. The green algae *Nephroselmis* and *Chlamydomonas* were used to root the tree and changing the outgroup with other green algae had no effect on topology. *Eutreptia sp.* is the earliest diverging euglenophyte followed by *Eutreptiella*. This divergence order is found in all analyses with support values that vary from 54 % (distance bootstrap) to 1.0 posterior probability (Bayesian).

The euglenales (*sensu* Leedale 1967) were monophyletic with high bootstrap and posterior probability support (97% and 1.0 respectively). Within the euglenales there is only one branch that shows consistency and a high degree of statistical support; that is the branch consisting of *Euglena gracilis* + *E. anabaena* (100% bootstrap, 1.0 posterior probability). Support for the internal branches varies, but with the exception of posterior probabilities, support values are fairly low. The location of *Trachelomonas volvocina* and *Phacus pyrum* at the base of the euglenales remains constant regardless of the

Figure 5.2. The phylogram obtained from a maximum likelihood analysis (HKY85 + G + I) of the euglenophyte PsaA gene. The topology is identical to a tree obtained using Bayesian inference (HKY85 + G + I). –lnL = 9655.06384, ti/tv = 0.857241, $_p$inv = 0.479850, α = 1.682748. Bayesian: ngen = 400,000, burn in = 150 trees. Numbers at the nodes represent Bayesian posterior probabilities (top) and ML bootstrap percentages (bottom) from 100 replicates. Bar = 0.05 substitutions per site.

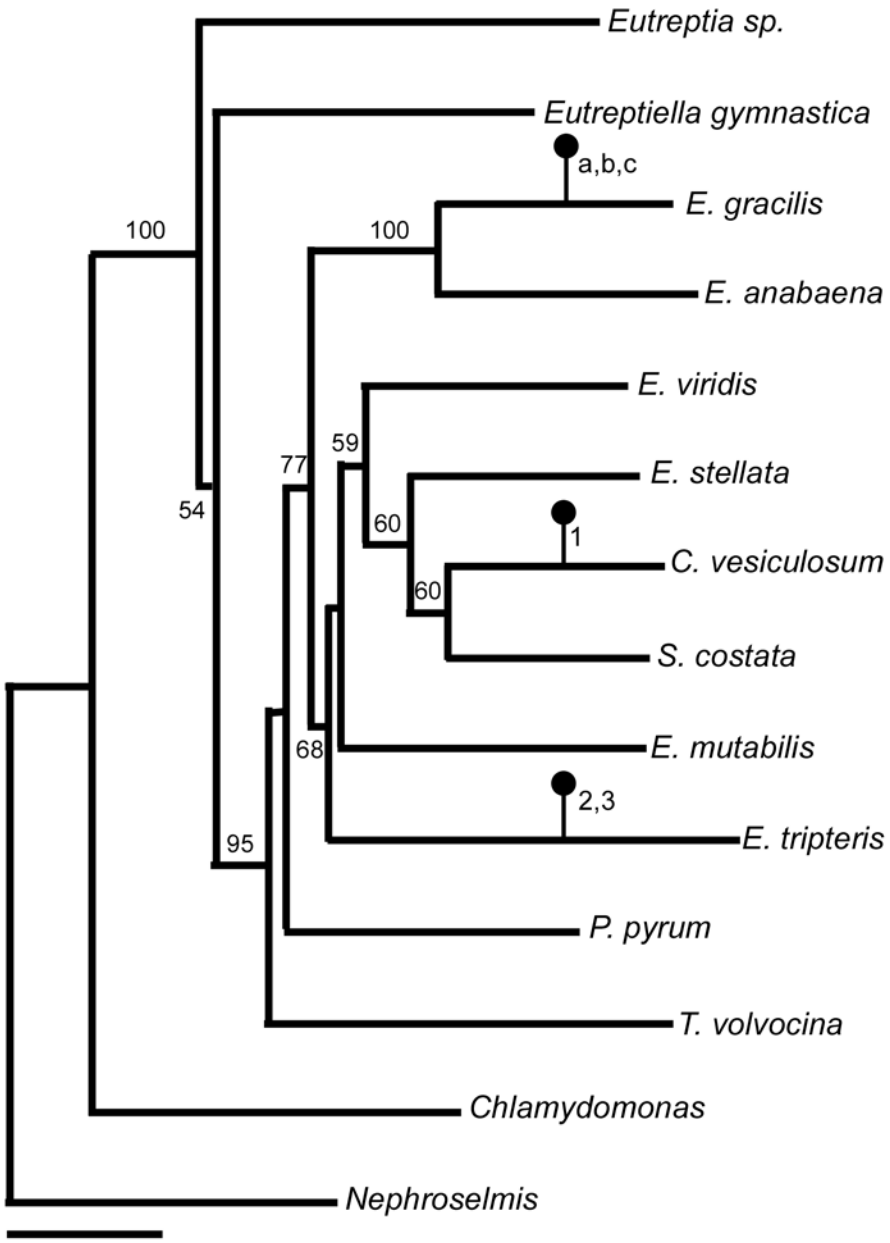

Eutreptia sp.
Eutreptiella gymnastica
E. anabaena
E. gracilis
E. viridis
S. costata
C. vesiculosum
E. stellata
E. tripteris
E. mutabilis
P. pyrum
T. volvocina
Chlamydomonas
Nephroselmis
1.0
100
1.0
100
0.98
77
0.96
<50
1.0
68
0.99
53
0.93
51
0.71
54
0.99
<50
0.99
57
1.0
97

substitution model used, and although ML bootstrap support is low (<50%) posterior probabilities support this position. The clade composed of *Euglena viridis* and *E. stellata* with *Strombomonas costata* and *Colacium vesiculosum* had only moderate support as did the positions of *Euglena mutabilis* and *E. tripteris*.

Figure 3 is a ME LogDet tree of euglenophyte PsaA. The paraphyly of the eutreptiales is preserved, albeit with much weaker bootstrap support (54%) than was seen using likelihood methods. The monophyly of the euglenales is also preserved and supported with 95% bootstrap, as is the sister group relationship between *Euglena gracilis* and *E. anabaena*. The primary difference between the distance tree, and those based on likelihood models (Fig. 2) is relationships between *Euglena viridis*, *E. stellata*, *Colacium vesiculosum*, and *Strombomonas costata*. Instead of being polyphyletic, the Radiate are paraphyletic and include a group composed of *C. vesiculosum* and *S. costata*. Support values for internal branches were higher than those obtained using ML bootstrap, but lower than Bayesian posterior probabilities.

Intron content. PsaA is, in regards to introns, unique among euglenophyte plastid genes to be studied to date. Among the 12 taxa sequenced, and two additional taxa that were amplified by PCR, but not completely sequenced, 3 novel intron sites were discovered, one containing a putative twintron. The first site is found in *Colacium vesiculosum* and its sister species *C. mucronatum* (and possibly in *S. costata*). Figure 4A shows the PCR product from residues 54-220 in *Trachelomonas volvocina*, *C. vesiculosum*, and *C. mucronatum*. Sequencing of this region in *C. vesiculosum* shows a 660 bp insert between codons 204 and 205 in the translated sequence. Unfortunately we

Figure 5.3. The phylogram obtained from a minimum evolution analysis using LogDet distances of euglenophyte PsaA. Score = 1.57882. Numbers at the nodes represent bootstrap percentages from 1000 replicates. Branches with lollipops represent lineages that contain introns, the numbers and letters refer to the intron positions in figure 4 D. Bar = 0.05 substitutions per site.


Eutreptia sp.
Eutreptiella gymnastica
a,b,c
E. gracilis
E. anabaena
E. viridis
E. stellata
1
C. vesiculosum
S. costata
E. mutabilis
2,3
E. tripteris
P. pyrum
T. volvocina
Chlamydomonas
Nephroselmis
100
100
54
77
59
60
60
68
95

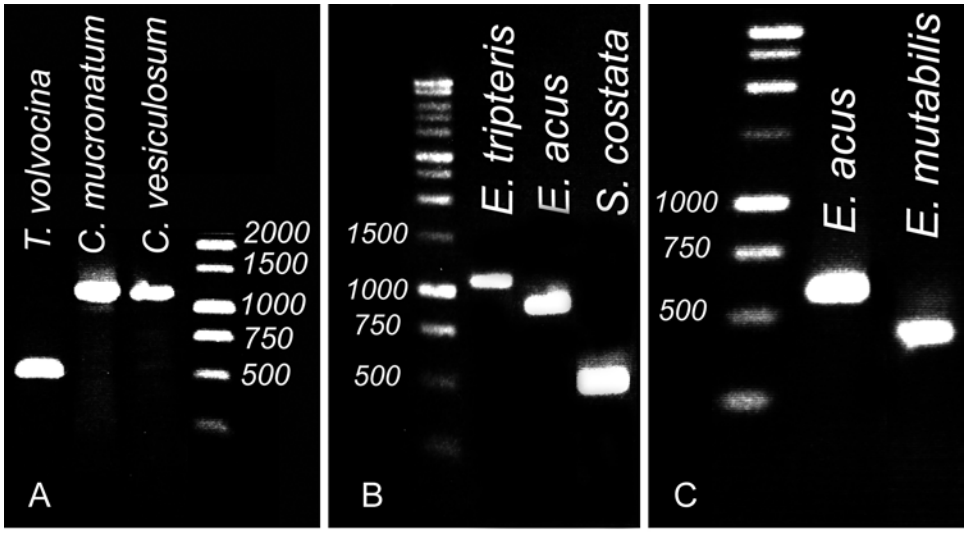
were unable to successfully amplify this region in *S. costata* more than once, so without repeatability it is questionable whether or not it has an intron.

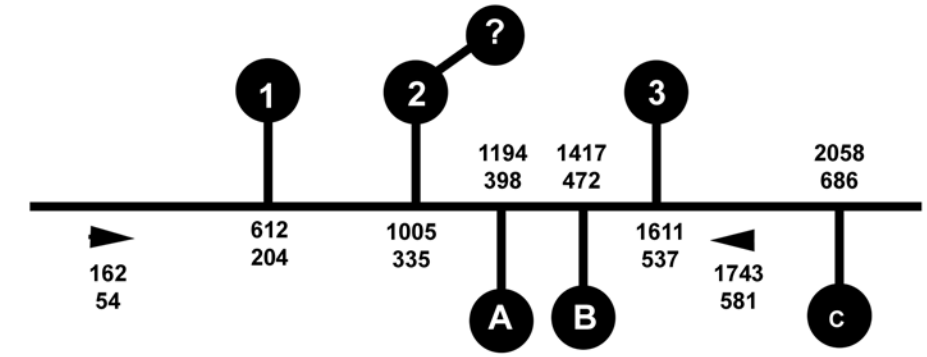
Figure 4B shows the PCR products from residue 308 to 447 in *Euglena tripteris*, *Euglena acus*, and *S. costata*. Both *E. tripteris* and *E. acus* have a substantial insert in this region. When sequenced, both inserts map to a location between codons 335 and 336 in the translated sequence. The insert in *E. acus* is 476 bp long, whereas the insert in *E. tripteris* is approximately 150-200 bp longer (so far we have been unable to successfully sequence across the insert in *E. tripteris*).

Figure 4C shows the PCR product from residues 441-581 in *Euglena acus* and *E. mutabilis*. *E. acus* has a 151 bp insert between residues 537 and 538 that isn't found in any other species of euglenophyte studied. The locations and sizes of all the introns discovered in this study are summarized in Figure 4D. Lollipops above the line represent introns discovered in this study, and lollipops below the line represent introns found in *E. gracilis*. The intron denoted by a small 'c' is outside of the region of the PsaA gene studied here (indicated by the arrows).

Discussion. The relationships between the euglenophytes and the green algae were always close, whether as sister groups or one within the other. Previous studies of plastid-encoded genes have always included *E. gracilis* as the only representative of the euglenophytes, and usually place *E. gracilis* in the same position as we have here; either sister to the green algae or part of the basal green algal radiation (Martin *et. al.* 1992, Martin *et. al.* 1998, Martin *et. al.* 2002). The earliest plastid based molecular phylogenies dealt solely with RbcL (Martin *et. al.* 1992). Although they did show some relationship

Figure 5.4. a) PCR products from primer pair 54F and 220R. The control product from *T. volvocinopsis* is the expected size of 498 bp, while those of *C. mucronatum* and *C. vesiculosum* are 1158 bp. b) PCR products from primer pair 308F and 447R. The control product from *S. costata* is the expected size of 417 bp, while the product from *E. acus* is 893 bp. The product from *E. tripteris* is approximately 1020 bp. c) The PCR product obtained using the primer pair 441F and 581R. The control product from *E. mutabilis* is the expected 420 bp, while that of *E. acus* is 571 bp. d) Intron map of PsaA in euglenophytes. Arrows indicate the 5' most and 3' most ends of PCR contigs. Lollipops above the line represent introns that were discovered in this study, lollipops below the line indicate introns in *E. gracilis*. The numbers at primers and introns are the nucleotide (top) and codon (bottom) position of the intron or primer relative to *E. gracilis* cDNA.


T. volvocina
C. mucronatum
C. vesiculosum
2000
1500
1000
750
500
A
1500
1000
750
500
E. tripteris
E. acus
S. costata
B
1000
750
500
E. acus
E. mutabilis
C


?
1
2
3
1194
398
1417
472
2058
686
612
204
1005
335
1611
537
162
54
1743
581
A
B
c

D

between the euglenophyte plastid and green algae, RbcL has been shown to be prone to rampant horizontal gene transfer, and can give spurious results in large-scale molecular phylogenies (Delwiche & Palmer 1996). Other studies have attempted to use nuclear encoded photosynthetic genes in discerning plastid phylogenies. Durnford *et. al.* (1999) did a study using the nuclear-encoded LHC class I and II genes from a number of algae and land plants. Some of their phylogenies indicated that the euglenophyte plastid diverged within the basal portions of the green algal lineage, and are related to those of the Prasinophyceae, a paraphyletic class of basal green algae. Other analyses placed the euglenophyte LHC genes as sister to a divergent member of the LHC family in *Lycopersicon* (an angiosperm) or came out as the root of all eukaryotic LHC genes.

All studies of plastid phylogeny have been unequivocal about a relationship existing between green algae and the euglenophyte plastid. Unfortunately, no one has been able to elucidate the specific relationship between them with a high level of confidence. Usually the euglenophyte plastid comes out in odd places (*e.g.* with tomato in Durnford *et. al.* 1999) or more often, as the sister group to all the green algae (Adachi *et. al.* 2000, Martin *et. al.* 1992, 2001). All of these studies used *E. gracilis* as the sole representative of the euglenophytes. If taxon sampling is small, especially if a distinct and divergent clade (*e.g.* the euglenophytes) is represented by a single species, no matter how much data is included, the aberrant taxon tends to be placed at the base of the branch to which it is most closely related (Naylor & Brown 1997) regardless of method. This results in possibly erroneous results even if sequences from 45 plastid-encoded proteins are used (Martin *et. al.* 2001).

In this study we attempted to address the bias of taxon sampling within the euglenophytes. Our results confirm those of other researchers in placing the origins of the euglenophyte plastid early in the green algal radiation. Turmel *et. al.* (1999), in an analysis of 37 plastid proteins from numerous algae, placed *E. gracilis* as sister to the higher green algae, but subordinate to the prasinophyte *Nephroselmis*. The addition of sequences from more euglenophytes suggests that the euglenophyte plastid may have been acquired before *Nephroselmis* diverged from the rest of the green algae. Turmel *et. al.* (1999) show gene loss data that are very suggestive of a prasinophyte origin of the euglenophyte plastid.

Our difficulty, and that of others in attempting to uncover the actual position of the euglenophyte plastid in relation to green algal phylogeny, has been one of taxon sampling. This study is the first to use sequence data from more than one euglenophyte species and goes a long way toward addressing part of this difficulty. Here we provide compelling evidence that the euglenophyte green algal symbiosis was ancient, but we are unable to elucidate the specific relationship between the euglenophyte plastid and any major green algal lineage. This difficulty is due in large part to the fact that the lineage we now believe was the plastid donor (the prasinophytes) is remarkably under sampled. Nakayama *et. al.* (1998) demonstrated that the prasinophytes are actually a diverse, paraphyletic series composed of four main clades at the base of the green algal tree. We believe that the next step to understanding the true relationship between the green algae and the euglenophyte plastid will be sampling from some of these more basal greens such as *Halosphaera*, *Pyramimonas*, and *Mantoniella*.

The only major difference in the multigene trees generated by different optimization methods (distance *versus* likelihood-based) was in the position of *Mesostigma*. All distance methods placed *Mesostigma* as the sister-group to the streptophytes, with varying degrees of support (GTR+G+I = 69, HKY85+G = 71, uncorrected = 62, GTR+G ML distance = 71,). Likelihood methods placed *Mesostigma* at the base of the branch leading to the euglenophytes and Bayesian posterior probability for this node is 0.8. This incongruity is not new to this study; in fact the placement of *Mesostigma* has been something of an enigma for several years.

Traditionally classified as a prasinophyte due to its unicellular scaly nature, the placement of *Mesostigma* within the green algal tree has become the subject of some controversy in the last few years. Currently there are two preferred hypotheses regarding the divergence of *Mesostigma*, on very different portions of the green algal tree. One camp places *Mesostigma* as an immediate ancestor to the Streptophytes (Bhattacharya *et. al.* 1998, Karol *et. al.* 2001, Delwiche *et. al.* 2002, Martin *et. al.* 2002) while another places *Mesostigma* as the closest living relative to the green algae as a whole (Lemieux *et. al.* 2000, Turmel *et. al.* 2001, Turmel *et. al.* 2002).

If *Mesostigma* is, as Turmel, Otis, and Lemieux posit, the ancestor to all green algae, then its placement as the sister-group to the euglenophytes in our likelihood analyses makes sense. Numerous analyses place *Euglena gracilis* as either sister to the green algae, or within the prasinophytes. If *Mesostigma* is actually the earliest diverging of the prasinophytes (c.f. Turmel *et. al.* 2001, 2002) then a sister grouping with the euglenophytes here adds weight to the conclusions of Turmel *et. al.* (1999) that suggest that the euglenophyte plastid was acquired from a prasinophyte. This conclusion is

supported further by the high degree of rate heterogeneity among the taxa in the concatenated analysis ($X^2 = 481.49$, df = 48, $p < 10^{-8}$) implying that the distance trees are somewhat suspect regarding the placement of *Mesostigma*.

Euglenophyte PsaA phylogeny. Previous reports using nuclear rDNA genes have placed *Eutreptia* and *Eutreptiella* at the base of the euglenophyte tree (Linton *et. al.* 1999, 2001, Leander *et. al.* 2001, Müllner *et. al.* 2001, Preisfeld *et. al.* 2001). Although represented as monophyletic and sister to the other euglenophytes in Müllner *et. al.* (2001), support for a monophyletic phototrophic Eutreptiales is low. Most other analyses (Linton et. al 2001, Preisfeld *et. al.* 2001, Leander *et. al.* 2001) only use a single photosynthetic Eutreptiallian species. Our data show moderate support for a clade comprised of *Eutreptiella* plus other euglenophytes that is exclusive of *Eutreptia*. These data suggest that, as currently defined, the Eutreptiales are paraphyletic.

The placement of *Euglena anabaena* on the euglenophyte tree has proven to be problematic. Linton *et. al.* (2001), using the sequence of the 18S rDNA gene, showed *E. anabaena* to be the earliest diverging Euglenalian species with high bootstrap support. In a later, larger analysis Müllner *et. al.* (2001) suggested that *E. anabaena* is most closely related to the clade containing *Phacus pyrum* and *Lepocinclis ovata*, but there was poor statistical support for this grouping. In a combined analysis of both 18S rDNA and a large suite of morphological characters Leander *et. al.* (2001) were unable to discern any clear relationship between *E. anabaena* and any other taxon within the euglenales, suggesting that the molecular evidence for a relationship between *E. anabaena* and *P. pyrum* + *L. ovata* was poorly supported. Zakrys (1986) placed *E. anabaena* in the subgenus Calliglena based on morphological characters such as the presence of a

diplopyrenoid (Zakrys 1986, Shin *et. al.* 2000). However in a recent study of plastid SSU rDNA, Milanowski *et. al.* (2001) showed no clear relationship between *E. anabaena* and other members of the subgenus *Calliglena*. In all of our analyses using PsaA the relationship between *E. anabaena* and the only other member of the *Calliglena* (*E. gracilis*) was strongly supported, regardless of optimization criterion or model, suggesting that *E. anabaena* is in fact a member of the *Calliglena*.

This study of PsaA is only the third plastid-based molecular phylogeny of the euglenophytes. The first was performed by Thompson *et. al.* (1995) using RbcL. Thompson *et. al.*'s trees were very poorly supported (bootstrap support for the backbone of the tree ranged from 34-59 with the majority of clades supported by less than 40%), and at least one of the species was misidentified. This was followed by Milanowski *et. al.* (2001) who used the sequence of the plastid encoded small subunit ribosomal RNA. Milanowski *et. al.*'s analysis covered many more taxa, and sampled more extensively outside the genus *Euglena*. The 16S rDNA data showed high levels of support for two main clades of euglenophytes, but support within these clades was minimal. The positions of *C. vesiculosum*, *S. costata*, and *T. volvocina* were unfortunately prone to change depending on the optimization criterion and model of sequence evolution, and their positions were never strongly supported by internal measures of support. Our analysis has slightly higher levels of support for both the backbone and internal nodes than either of the previous analyses, however with the exception of Bayesian posterior probabilities, support values for the PsaA phylogeny were not unequivocal. In spite of the overall poor support for some parts of the PsaA topology, there were some interesting results.

Euglena species with radially arranged plastids have been called both the Radiate (Pringshiem 1956, Leedale 1967) and the subgenus *Euglena* (Zakrys 1986), but in either case they are assumed to be a monophyletic group. Recent molecular (Brosnan *et. al.* 2003) and morphological (Leander & Farmer 2001, Brown & Farmer 2003) analyses have confirmed a strong phylogenetic and developmental relationship between these organisms, yet both this analysis and the 16S rDNA analysis of Milanowski *et. al.* (2001) showed this group to be paraphyletic. The most probable reason for this is the lack of informative characters in the plastid (at this phylogenetic level) and the limited taxon sampling in both this study and that of Milanowski et. al (2001).

PsaA Intron content. *Euglena gracilis* is the only photosynthetic euglenophyte to have its entire plastid genome sequenced (Hallick 1993). The *E. gracilis* plastid genome contains 155 group II and group III introns, making it the most intron rich genome currently known (Thompson *et. al.* 1995). Thompson *et. al.* (1995) showed that the RbcL gene of *E. gracilis* contains more introns than the eight other euglenophytes to which he compared it. Not only does *E. gracilis* contain the most introns, but their posisitons in other euglenophytes appears to be conserved. From this study, Thompson *et. al.* proposed a cumulative model of intron evolution in which basally branching euglenophytes have fewer introns, and *E. gracilis* has the most, with the position of those introns being conserved through time. The implication of this work was that if *E. gracilis* or a close relative has an intron, introns in other species will be found only in those positions.

The positional conservation of introns was supported by Doetsch *et. al.* (1998) in a study of a maturase-encoding group III twintron in the PsbC gene. They demonstrated

the positional conservation of the Mat-1 intron in PsbC, but in a departure from Thompson *et. al.* showed the presence of a unique intron outside of *E. gracilis*. *Lepocinclis buetschlii*, a much more basally branching euglenophyte was shown to possess an additional group III twintron inside of the Mat-1 intron. Therefore Doetsch showed that intron acquisition has occurred since the divergence of the lineages leading to *E. gracilis* and *L. buetschlii*, however, the additional intron was still in the same location as one in *E. gracilis*. More recently Doetsch et. al (2001) studied intron content in the PsbK operon of 12 euglenophyte species. They showed the presence of introns in the PsbK gene in all 12 species (from 3 genera). Most surprisingly was the presence of a group II intron in *Euglena stellata* and *E. sanguinea*, in a position where no other euglenophytes (including *E. gracilis*) have an intron. At the time this made the PsbK operon the most intron-rich region of the euglenophyte genome.

Our study demonstrates the existence of three completely novel intron positions, not found in *E. gracilis* (Stevenson and Hallick 1994) or outside of a small, well-defined taxon. The size of the insert in both species of *Colacium* indicates that it is either a large group II intron or a combination of group II and group III twintrons. All analyses that include both *Euglena tripteris* and *E. acus* have demonstrated a very close relationship between these two taxa (Linton *et. al.* 2001, Müllner *et. al.* 2001, Leander *et. al.* 2001, Brosnan *et. al.* 2003). The intron 2 locus in *E. tripteris* and *E. acus* was probably present in the common ancestor of both, but *E. tripteris* appears to have acquired an additional 150-200 bp of DNA. The size and location of this insert (inside a pre-existing intron) suggest that it is a group III twintron. The intron locus 3 is found only in *E. acus*, and appears to be a solitary group III intron. This makes the PsaA gene the most intron-rich

locus in the euglenophyte genome, and suggests that euglenophyte plastid introns are even more mobile than was previously thought.

References

Adachi, J., Waddell, P. J., Martin, W., & Hasegawa, M. (2000) Plastid genome phylogeny and a model of amino acid substitution for proteins encoded by chloroplast DNA. *J. Mol. Evol.* **50**, 348-358.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I., & Doolittle, W. F. (2000) A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* **290**, 972-975.

Bhattacharya, D., Weber, K., An, S. S., & Berning-Kock, W. (1998) Actin phylogeny identifies *Mesostigma viride* as a flagellate ancestor of the land plants. *J. Mol. Evol.* **47**, 544-550.

Brosnan S., Shin W., Kjer K., Triemer R. E. (2003) Phylogeny of the photosynthetic euglenophytes inferred from the nuclear SSU and partial LSU rDNA. *Intl. J. Sys. Evol. Microbiol.* [in press]

Brown, P. J. P., Zakryś B., & Farmer M. A. (2003) Plastid morphology, ultrastructure, and development in *Colacium* and the loricate euglenophytes (Euglenophyceae) *J. Phycol*. **39,** 115-121

Cavalier-Smith T. (1981) Eukaryote Kingdoms: seven or nine? *Biosystems* **14**, 461-481

Chapman, R. L., & Waters, D. A. (2002) Green algae and land plants-an answer at last? *J. Phycol.* **38**, 237-240.

Chase, M. W., & Albert, V. A. (1998) A perspective on the contribution of plastid rbcL sequences to angiosperm phylogenetics. in *Molecular Systematics of Plants II: DNA sequencing*. [Ed.] Soltis, D. E., Soltis, P. S., & Doyle, J. J., Kluwer Academic Publishers, Norwell, Massachusetts.

Delwiche, C. F., & Palmer, J. D. (1996) Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. *Mol. Biol. Evol.* **13**, 873-882.

Delwiche, C. F., Karol, K. G., Cimino, M. T., & Systma, K. J. (2002) Phylogeny of the genus *Coleochaete* (Choleochaetales, Charophyta) and related taxa inferred by analysis of the chloroplast gene rbcL. *J. Phycol.* **38**, 394-403.

Doetsch, N. A., Thompson, M. D., & Hallick, R. B. (1998) A maturase-encoding group III twintron is conserved in deeply rooted euglenoid species: are group III introns the chicken or the egg? *Mol. Biol. Evol.* **15**, 76-86.

Doetsch, N. A., Thompson, M. D., Favreau, M. R., & Hallick, R. B. (2001) Comparison of psbK operon organization and group III intron content in chloroplast genomes of 12 euglenoid species. *Mol. Gen. Genet.* **264**, 682-690

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., & Mattick, J. S. (1991) Touchdown PCR to circumvent spurious priming during gene amplification. *Nuc. Acids Res.* **19**, 4008

Drager, R. G., & Hallick, R. B. (1993) A novel *Euglena gracilis* chloroplast operon encoding four ATP synthase subunits and two ribosomal proteins contains 17 introns. *Curr. Genet.* **23**, 271-280.

Durnford, D. G., Deane, J. a., Tan, S., McFadden, G. I., Gantt, E., & Green, B.

R. (1999) A phylogenetic assessment of the eukaryotic light-harvesting antenna proteins, with implications for plastid evolution. *J. Mol. Evol.* **48**, 59-68

Gibbs, S. P. (1978) The chloroplasts of *Euglena* may have evolved from symbiotic green algae. *Can. J. Bot.* **56**, 2883-2889

Gockel, G., & Hachtel, W. (2000) Complete gene map of the plastid genome of the nonphotosynthetic euglenoid lagellate *Astasia longa*. *Protist* **151**, 347-351.

Hallick, R. B., Hong, L., Drager, R. G., Favreau, M. R., Monfort, A., Orsat, B., Spielmann, A., & Stutz, E. (1993) The complete sequence of *Euglena gracilis* chloroplast DNA. *Nuc. Acid. Res.* **21**, 3537-3544.

Hasegawa, M., Kishino, H., & Yano, T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **21**, 160-174.

Huelsenbeck, J. T., & Ronquist, F. (2001) MrBayes: a program for Bayesian inference of phylogeny. Program available at http://morphbank.ebc.uu.se/mrbayes/

Jukes, T. H. & Cantor, C. R. (1969) Evolution of protein molecules. in Mammalian Protein Metabolism. [Ed] Munro, H. N. pp. 21-132. Academic Press. New York

Karol, K. G., McCourt, R. M., Cimino, M. T., & Delwiche, C. F. (2001) The closest living relative of land plants. *Science* **294**, 2351-2353.

Keller, M. D., Selvin, R. C., Claus, W., & Guillard, R. R. L. (1987) Media for the culture of oceanic ultraphytoplankton. *J. Phycol*. **23**, 633-638.

Kishino, H., & Hasegawa, M. (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in the hominoidea. *J. Mol. Evol.* **29**, 170-179.

Lanave, C., Preparata, G., Saccone, G., & Serio, G. (1984) A new mothod for calculating evolutionary substitution rates. *J. Mol. Evol.* **20**, 86-93.

Leander, B. S., Witek, R. P., & Farmer, M. A. (2001) Trends in the evolution of the euglenid pellicle. *Evolution* **55**, 2215-2235..

Lee R. E. (1977) Evolution of algal flagellates with chloroplast endoplasmic reticulum from the ciliates. *S. Afr. J. Sci.* **73,** 179-182.

Leedale, G. F. (1967) *The Euglenoid Flagellates*. Prentice-Hall, Englewood Cliffs, NJ. 220 pp.

Lemieux, C., Otis, C., & Turmel, M. (2000) Ancestral chloroplast genome in *Mesostigma viride* reveals an early branch of green plant evolution. *Nature* **403**, 649-652.

Linton, E. W., Hittner, D., Lewandowski, C., Auld, T., & Triemer, R. E. (1999) A molecular study of euglenoid phylogeny using small subunit rDNA. *J. Euk. Micro.* **46**, 217-223.

Linton E. W., Nudelman M. A., Conforti V., Triemer R. E. (2001) A molecular analysis of the euglenophytes using SSU rDNA. *J. Phycol*. **36,** 740-746.

Lockhart, P. J., Steel, M. A., Hendy, M. D., & Penny, D. (1994) Recovering evolutionary trees under a more realistic model of sequence evolution. *Mol. Biol. Evol.* **11**, 604-612.

Moreira, D., Guyander, H. le, & Phillpe, H. (2000) The origin of red algae and the evolution of chloroplasts. *Nature* **405**, 69-72.

Martin, W., Somerville, C. C., & Loiseaux-de Goër, S. (1992) Molecular phylogenies of plastid origins and algal evolution. *J. Mol. Evol.* **35**, 385-404.

Martin, W., Stoebe, B., Goremykin, V., Hansmann, S., Hasegawa, M., & Kowallik, K. V. (1998) Gene transfer to the nucleus and the evolution of chloroplasts. *Nature* **393**, 162-165.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelson, S., Lins, T., Leister, D., Stoebe, B., Hasegawa M., & Penny, D. (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. USA* **99**, 12246-12251.

Milanowski, R., Zakrys, B., & Kwiatowski, J. (2001) Phylogenetic analysis of chloroplast small-subunit rRNA genes of the genus *Euglena* Ehrenberg. *Int. J. Sys. Evol. Microbiol.* **51**, 773-781.

Montegut-Felkner, A. & Triemer, R. E. (1997) Phylogenetic relationships of selected euglenoid genera based on morphological and molecular data. *J. Phycol*. **33**, 512-519.

Müllner, A. N., Angeler, D. G., Samuel, R., Linton, E. W., & Triemer, R. E. (2001) Phylogenetic analysis of phagotrophic, phototrophic and osmotrophic euglenoids by using nuclear 18S rDNA sequence. *Int. J. Sys. Evol. Microbiol.* **51**, 783-791.

Naylor, G. J. P., & Brown, W. M. (1997) Structural biology and phylogenetic estimation. *Science* **388**, 527-528.

Preisfeld, A., Berger, S., Busse, I., Liller, S., & Ruppel, H. G. (2000) Phylogenetic analysis of various euglenoid taxa (Euglenozoa) based on 18S rDNA sequence data. *J. Phycol*. **36**, 220-226.

Preisfeld, A., Busse, I., Klingberg, M., Talke, S., & Ruppel, H. G. (2001) Phylogenetic position and inter-relationships of the osmotrophic euglenids based on SSU rDNA data, with emphasis on the rhabdomonadales (Euglenozoa). *Int. J. Sys. Evol. Microbiol.* **51**, 751-758.

Pringsheim, E. G., (1956) Contributions toward a monograph of the genus *Euglena*. *Nov. Act. Leop.* **12**, 1-168

Rambaut, A. (1996) Se-Al: sequence alignment editor. Ver 1.0a1. Program available via http://evolve.zoo.ox.ac.uk/Se-Al/Se-Al.html.

Rodriguez, R., Oliver, J. L., Marin, A., & Medina, J. R. (1990) The general stochastic model of nucleotide substitution. *J. Theor. Biol.* **142**: 485-501.

Saitou, N., & Nei, M. (1987) The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406-425

Shin, W., Boo, S. M., & Inouye, I. (2000) Ultrastructure of *Euglena anabaena* var. *minor* (Euglenophyceae). *Phycol. Res.* **48**, 19-25.

Simpson, A. G. B. (1997) The identity and composition of the Euglenozoa. *Arch. Protistendk.* **148**, 318-328.

Stevenson, J. K., & Hallick, R. B. (1994) The PsaA operon pre-mRNA of *Euglena gracilis* chloroplast is processed into photosystem I and II mRNAs that accumulate differentially depending on the conditions of cell growth. *Plant J.* **5**, 247-260.

Swofford, D. L. (1999) PAUP*: phylogenetic analysis using parsimony (*and other methods). Ver. 4.0b10 Sinauer Associates, Sunderland, Massachusetts.

Thompson, M. D., Copertino, D. W., Thompson, E., Favreau, M. R., & Hallick, R. B. (1995) Evidence for the late origin of introns in chloroplast genes from an evolutionary analysis of the genus *Euglena*. *Nuc. Acids Res.* **23**, 4745-4752

Turmel, M., Otis, C., & Lemieux, C. (1999) The complete chloroplast DNA sequence of the green alga *Nephroselmis olivacea*: Insights into the architecture of ancestral chloroplast genomes. *Proc. Natl. Acad. Sci. USA* **96**, 10248-10253.

Turmel, M., Otis, C., & Lemieux, C. (2002) The complete mitochondrial DNA sequence of *Mesostigma viride* identifies this green alga as the earliest green plant divergence andpredicts a highly compact mitochondrial genome in the anscestor of all green plants. *Mol. Biol. Evol.* **19**, 24-38.

Turmel, M., Ehara, M., Otis, C., & Lemieux, C. (2002) Phylogenetic relationships among streptophytes as inferred from chloroplast small and large subunit rRNA gene sequences. *J. Phycol.* **38**, 364-375.

Zakryś B. (1986) Contribution to the monograph of the Polish members of the genus *Euglena* Ehrenberg 1830. *Nova Hedwigia* **42**, 491-540.

CHAPTER 6

CONLCUSION

Although the work described in this dissertation was originally intended to be used to construct a phylogeny of the euglenophytes, it has resulted in more of a deconstruction of current taxonomic paradigms within the group. This work began with an alpha taxonomic project, redescribing a rare species of euglenophyte. The plastid-based investigations began with work that described the diversity of plastid morphology and ultrastructure not only between species of euglenophytes, but within certain species. The results of studies on plastid ultrastructure and development in various euglenophytes not only challenge traditional paradigms of euglenophyte taxonomy, but also demonstrate an entirely novel method of plastid division and partitioning. In addition PsaA, the gene encoding a photosystem I core protein was sequenced. The results of the PsaA sequencing gave some insight into relationships between euglenophyte species, as well as provided insight into intron evolution in some major groups of euglenophytes. Finally a multi-gene phylogeny gives some support to a Prasinophyte origin of the euglenophyte plastid.

Alpha Taxonomy:

Before its description here, *Euglena rustica* had only been described from a single observation in a village pond in Austria (Huber-Pestalozzi 1955). The new description of this rare species provides valuable light and electron microscopical data, as well as a Latin diagnosis. It also provides the first evidence of fluorescent muciferous bodies in

the euglenophytes. Finally, epoxy-embedded specimens were deposited in the permanent collection of the American Museum of Natural History, a necessary but often overlooked step in protistan taxonomy.

Loricate Plastids:

The unique plastid morphology of euglenophytes with inner pyrenoids was examined in detail. The presence of such an unusual pyrenoid morphology in the genera *Colacium*, *Strombomonas*, and *Trachelomonas* suggested that there might be a relationship between these three genera. A sister group relationship between *Strombomonas* and *Trachelomonas* is widely accepted among euglenologists, but *Colacium* has traditionally been placed in other groups. The phenomenon of plastid adhesion was also noted in all three genera. Plastid adhesion is believed to be a byproduct of lorica formation in the loricate euglenophytes, although it was not discussed here for the sake of professional courtesy. Based on these results a relationship between *Colacium* and the loricate euglenophytes was postulated. The most striking result of this study was the observation that the pyrenoid of one of these taxa is transient, coming and going over the course of a day. The drastic changes in plastid and pyrenoid morphology in *Strombomonas verrucosa* challenged many notions in euglenophyte taxonomy, namely the stability of these two features as indicators of species designation. This required me to shift the focus of this research towards an understanding of plastid division and development in the euglenophytes as a whole.

Plastid Development:

Using multiphoton microscopy coupled with transmission electron microscopy, plastid development and partitioning was studied in six species of euglenophytes with

diverse plastid and cellular morphologies. From these studies, four major classes of plastid division and partitioning strategies were described.

Class I is found in those organisms with many (*i. e.* hundreds) lenticular plastids. In these organisms, the plastids divide by fission throughout the course of the cell cycle. At the time of cell division the plastids are partitioned into daughter cells stochastically, their fate decided by their location in the parent cell at the time of cytokinesis. This type of plastid partitioning strategy is common not only these euglenophytes, but in many other groups of algae and the land plants.

Class II is found in organisms with moderate numbers of larger plastids (10-20 per cell) with pyrenoids. In these organisms, the plastids are still sorted stochastically, but the timing of plastid division is regulated. In some species plastid division occurs after cell division (*T. volvocinsopsis*, and *S. verrucosai)* while in others it happens before (*E. gracilis* [see Ehara et. al. 1990]) but all of the plastids in a given cell are usually dividing at about the same time. In all organisms with a class II partitioning strategy the pyrenoid is either reduced (*S. verrucosa*) or disappears completely just prior to plastid division. During and immediately after plastid division, new pyrenoids are synthesized *de novo*. In the loricate euglenophytes, it is not uncommon to see more than one pyrenoid on a single plastid just before that plastid divides. This is probably the most common plastid division and partitioning strategy in the euglenophytes.

Class III is found in organisms with a single plastid in a rigid cell. This division and partitioning strategy was found in *Phacus pyrum* in this study, and is present in almost all species of algae with a single plastid. Class III involves division of the single plastid into two half-plastids along the plane of cell division, resulting in a half-plastid on

either side of the incipient cleavage furrow. After cell division, the half plastids develop their normal plastid morphology. It should be noted that unlike other groups of algae that employ a similar strategy, euglenophytes with a class III strategy lose their pyrenoid prior to division, and regenerate it after cell division.

Class IV plastid division and partitioning is found in non-rigid cells with either few (less than 5) plastids or a single plastid. This class of plastid division is unique to the euglenophytes, and reported for the first time here. Class IV division and partitioning begins with the dissolution or reduction of the pyrenoid(s). After pyrenoid reduction, the plastid(s) begin to lose their original spatial orientation and shape. Next, the plastid begins to fragment into numerous smaller pieces so that at the time of cell division the cell is completely filled with as many as 20 or more plastid fragments. These plastid fragments are then sorted stochastically into the daughter cells just like those of class I and II. After cell division, the plastid fragments fuse together and the pyrenoid(s) is/are regenerated. An explanation for this particular plastid division and partitioning strategy can be found in the rigidity of the cell. All organisms that utilize a class IV partitioning strategy exhibit moderate to high levels of euglenoid movement. This peristaltic motion of the cell results in a high rate of cytoplasmic mixing. If a single plastid were to divide only once amid all this mixing, there is a high probability that one of the daughter cells would not receive any plastid material. However, if the plastid is fragmented into numerous small pieces, then no matter how much the cytoplasm is churned up, each daughter cell stands a reasonable chance of getting at least one fragment. Since plastid DNA is scattered throughout the plastid in euglenophytes, most plastid fragments have everything they need to grow into a new large plastid. This plastid partitioning strategy is

found only in the euglenophytes and is intrinsically linked to the degree of euglenoid movement in some cells.

The results of the studies on plastid division and partitioning demonstrate a high degree of plastid morphological diversity not only between species, but also within a species over the course of a cell division cycle. This intraspecific variability raises some concern over the role plastid morphology should play in any future euglenophyte taxonomy. The best possible use for plastid morphology in taxonomy is *a posteriori*, that is to identify clades that have been identified via other means. While not as constant as one would like, the plastids are still "the most conspicuous features of the cell" (Gojdics 1953) and a taxonomy that uses plastid morphology for the sake of identification is still going to be useful to field biologists and other non-experts. For example, many lines of evidence suggest that a clade exists within the euglenophytes comprised of organisms that possess a true stellate plastid (as defined in chapter one) in interphase cells (Priesfield *et. al.* 2000, Leander *et. al*. 2001, Linton *et. al.* 2001, Milanowski *et. al.* 2001). This clade has been named as a subgenus of *Euglena* twice, being called *radiate* by Pringshiem (1956) and Leedale (1967) and *euglena* by Zakrys (1986). The most obvious character of these cells is their stellate plastid, and that character is useful in identification of these organisms. It is not useful in the circumscription of these organisms as Zakrys *et. al.* (2002) points out, because although the core morphology is essentially the same, the number of stellate plastids in the cell can vary due to nutrient content. Plastid morphology is a useful aid in the identification and description of euglenophytes, but it should not "have special value as [a] taxonomic character(s)" (Gojdics 1953).

PsaA Sequencing:

This study also investigated the sequence of PsaA, the gene encoding a core photosynthetic reaction center protein. Study of this gene provided three main results: 1) it gave some insight into euglenophyte phylogeny and the effectiveness of the plastid genome in this regard, 2) it was shown to contain three novel intron loci, all of which fall along well-established taxonomic boundaries, and 3) it helped shed some light on the relationship between the green algae and the euglenophyte plastid.

The PsaA phylogenetic analyses within the euglenophytes also had three main results, the first of which involved the Eutreptiales. The genera in this group have consistently been considered the most basally branching euglenophytes (Leedale 1967) and recent molecular phylogenies using nuclear genes (Preisfeld *et. al.* 2000, Linton *et. al.* 2001, Leander *et. al.* 2001) agree with this assessment. PsaA confirms that the plastid phylogeny in agreement with nuclear phylogenies in this regard, and the Eutreptiales are in fact the earliest diverging euglenophytes. In addition this study also demonstrated some evidence that the Eutreptiales may be paraphyletic. Secondly, this study showed a strong, and biologically reasonable relationship for *Euglena anabaena*. PsaA phylogenies consistently grouped *E. anabaena* with *E. gracilis,* a grouping postulated by Zakrys (1986) in her subgeneric classification of *Euglena*, but questioned in some analyses using either nuclear genes (Linton *et. al.* 2001, Leander *et. al.* 2001) or morphological characters of the cytoskeleton (Leander *et. al.* 2001). Neither nuclear genes nor cytoskeletal morphology uncovered a well-supported relationship between *E. anabaena* and any other major group of euglenophytes, yet neither analysis is inconsistent with the results found using PsaA. Thus it can be surmised with some

confidence that *E. anabaena* is a close relative of *E. gracilis*. Finally, PsaA showed very poor statistical support for most internal branches of the tree, regardless of the optimality criterion used. This along with previous plastid-based phylogenies (Thompson *et. al.*1995, Milanowski *et. al.* 2001) demonstrates the unsuitability of plastid-encoded genes in the inference of euglenophyte phylogeny at this systematic level.

This study also demonstrated the presence of three heretofore unknown intron loci in the PsaA gene of numerous euglenophytes. The first locus is localized between codons 204 and 205 and is found only in the genus *Colacium*. The second locus is found in both *Euglena tripteris* and *Euglena acus*. These species have been shown to be close relatives in numerous morphological (Zakrys 1986, Leander *et. al.* 2001) and molecular phylogenetic (Preisfeld *et. al.* 2000, Linton *et. al.* 2001) analyses. This indicates that this locus probably dates back to before the divergence of these two species, but after their divergence from other euglenophytes. The added ~100 bp in *E. tripteris* indicates the likely presence of a group III twintron in this species. Finally *E. acus* has a 151 bp insert near the 3' end of the gene indicating that this locus was acquired after the divergence of this species from *E. tripteris*.

Finally this study used a large concatenated sequence consisting of not only PsaA, but also the gene encoding the large subunit of rubisco (RbcL) and the plastid encoded SSU rDNA gene (16S rDNA). The results of this study indicate that the relationship between the euglenophyte plastid and the green algae is an ancient one. The euglenophytes always branched as a sister-group to the green algae when the Prasinophyte green alga *Mesostigma viride* was excluded from the analysis. When *M. viride* was included, it branched as either sister to the Streptophytes (Charophytes plus

land plants) or as sister to the euglenophytes. If methods that account for high rate heterogeneity were used to assess phylogeny, *M. viride* branched as the sister to the euglenophytes. These data, along with molecular phylogenetic and gene loss data from other Prasinophytes (Turmel *et. al.* 1999) suggest a Prasinophyte origin of the euglenophyte plastid.

Conclusion:

The results of these studies have laid the groundwork for many years of experimentation and observation. The first step in the continuation of the study of plastid development and partitioning in the euglenophytes is to study in detail the class IV partitioning strategy. In order to do this, a new model system has to be developed and that model should revolve around *Euglena viridis*. *E. viridis* is the organism most well-suited to become the next euglenophyte model species for three reasons: 1) it grows quickly compared to most other euglenophytes with a class IV strategy and is easily synchronized, 2) it can be maintained in a more defined culture medium than other class IV euglenophytes, making biochemical and molecular biological examination more straightforward, and 3) it has one of the easier plastid genomes with which to work. Once a good model is established numerous experiments suggest themselves. There are many molecules that could play a role in plastid fragmentation and subsequent fusion (*e.g.* dynamin, FtsZ, and SNAREs). One of the first courses of action would be to use fluorescent microscopy to look for any interesting localization of these molecules. If these molecules appear to be playing a role, drugs known to inhibit them could be used to investigate their role in plastid development. Mutagenesis could give clues to the ease with which one plastid morphotype could be transformed into another. Finally, it would

be of no small use to develop transformation technology in a euglenophyte in order to really understand the genetic basis of plastid development.

In order to better understand the evolution of the euglenophyte plastid and to gain more insight into its origins there are a number of experiments that need to be done. The first set of experiments is already underway and that is the expansion of the plastid gene data set in more Prasinophytes and Eutreptiales. Currently the PsaA gene of *Pyramimonas parkeae* is being sequenced for addition to the multigene plastid phylogeny. Also, work is underway to completely sequence the plastid genome of *Eutreptia lanowii* (UTEX 2003). The key to understanding the origins of the euglenophyte plastid lies in better understanding the Prasinophytes and the plastids of the Eutreptiales.

In conclusion, although plastid morphology is not only unstable but hypervariable within a species it is still useful for some aspects of euglenophyte taxonomy, especially identification. The amazing diversity of the euglenophyte plastid, not only in regards to morphology but also intron evolution and molecular phylogeny make it an excellent subject of research for many years to come.

References:

Ehara T., Osafune T., Hase E. (1990) Interactions between the nucleus and cytoplasmic organelles during the cell cycle of *Euglena gracilis* in synchronized cultures. *Exper. Cell. Res*. **190**, 104-112.

Gojdics M (1956) The genus *Euglena*. The University of Wisconsin Press, Madison, 268 pp.

Leander, B. S., Witek, R. P., & Farmer, M. A. (2001) Trends in the evolution of the euglenid pellicle. *Evolution* **55**, 2215-2235.

Leedale, G. F. (1967) *The Euglenoid Flagellates*. Prentice-Hall, Englewood Cliffs, NJ. 220 pp.

Linton E. W., Nudelman M. A., Conforti V., Triemer R. E. (2001) A molecular analysis of the euglenophytes using SSU rDNA. *J. Phycol*. **36,** 740-746.

Milanowski, R., Zakrys, B., & Kwiatowski, J. (2001) Phylogenetic analysis of chloroplast small-subunit rRNA genes of the genus *Euglena* Ehrenberg. *Int. J. Sys. Evol. Microbiol.* **51**, 773-781.

Preisfeld, A., Berger, S., Busse, I., Liller, S., & Ruppel, H. G. (2000) Phylogenetic analysis of various euglenoid taxa (Euglenozoa) based on 18S rDNA sequence data. *J. Phycol*. **36**, 220-226.

Pringsheim, E. G., (1956) Contributions toward a monograph of the genus *Euglena*. *Nov. Act. Leop.* **12**, 1-168.

Thompson, M. D., Copertino, D. W., Thompson, E., Favreau, M. R., & Hallick, R. B. (1995) Evidence for the late origin of introns in chloroplast genes from an evolutionary analysis of the genus *Euglena*. *Nuc. Acids Res.* **23**, 4745-4752.

Turmel, M., Otis, C., & Lemieux, C. (1999) The complete chloroplast DNA sequence of the green alga *Nephroselmis olivacea*: Insights into the architecture of ancestral chloroplast genomes. *Proc. Natl. Acad. Sci. USA* **96**, 10248-10253.

Zakryś B. (1986) Contribution to the monograph of the Polish members of the genus *Euglena* Ehrenberg 1830. *Nova Hedwigia* **42**, 491-540.

Zakryś B., Milanowski, R., Empel, J., Borusk, P., Gromadka, R., & Kwiatowski, J. (2002) Two different species of *Euglena*, *E. geniculata* and *E. myxocylindracea* (Euglenophyceae) are virtually genetically and morphologically identical. *J. Phycol.* **38,** 1190-1199.