A Morphological Re-Description of *Ploeotia pseudanisonema*, and Phylogenetic Analysis of the Genus Ploeotia

by

Andrew Buren Brooks

(Under the direction of Mark A. Farmer)

Abstract

The genus *Ploeotia* represents a group of small, colorless, heterotrophic euglenids commonly found in shallow-water marine sediments. Species belonging to this genus have historically been poorly described and studied. However, a Group I intron discovered within the small subunit ribosomal DNA gene of *Ploeotia costata*, makes *P. costata* the only euglenazoan known to possess an actively splicing Group I intron. This intron contains conserved secondary structures that indicate monophyly with introns found in Stramenopiles and Bangiales red algae. This project describes the internal and external morphology of a related species, *Ploeotia pseudanisonema*, using light and electron microscopy, and investigates the possibility of a Group I intron within *P. pseudanisonema*. Using recently obtained SSU rRNA sequences, we also examine the phylogeny of *Ploeotia*, and comment on the relationship of the genus *Keelungia* to *Ploeotia*.

Index words: *Ploeotia pseudanisonema*, *Ploeotia costata*, *Ploeotia vitrea*, *Keelungia pulex*, Transmission electron microscopy, Scanning electron microscopy, Small subunit rRNA, Euglenozoan Phylogeny

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by

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Chapter 1

Introduction

Isolated from shallow-water marine sediments from tropical regions, including Fiji, Hawaii, and Rio de Janeiro, *Ploeotia pseudanisonema* was formally described by Larsen and Patterson (37). *Ploeotia* was described by Félix Dujardin in 1841 (22), and was widely forgotten until being re-described by Farmer and Triemer in 1988 (26). Based upon this work by Farmer and Triemer, Larsen and Patterson describe *Ploeotia* as "organisms which have ingestion devices not exhibiting pumping movements, a rigid body, a posterior flagellum that trails against the substrate, and an anterior flagellum that beats along its entire length" (37). The genus *Ploeotia* currently includes 19 species, all of which are colorless heterotrophs (Table 1.1) (1; 22; 25; 26; 37; 40; 45; 48). Genus *Ploeotia* exists as a basal branch among non-helical, heterotrophic euglenids, which include the genera *Petalomonas*, *Notosolenus*, *Entosiphon*, and the newly recognized genus *Keelungia*.

We documented the external and internal morphology of *P. pseudanisonema* using differential interference contrast light microscopy, scanning electron microscopy, and transmission electron microscopy. We also sequenced the genomic small subunit ribosomal RNA gene of *P. pseudanisonema* for comparison to a complimentary DNA sequence to determine the presence or absence of a Group I intron, like that found in *Ploeotia costata*.
The SSU rRNA sequence was then used to elucidate the phylogenetic relationship between *P. pseudanisonema* and other members of *Ploeotia*, and to determine the placement of *Ploeotia* within Euglenazoa.

1.1 The Feeding Apparatus

Four modes of nutrient acquisition exist within Euglenazoa: Bacteriotrophy, Eukaryotrophy, Phototrophy, and Osmotrophy. The species *P. pseudanisonema* is bacteriotrophic, a specialized form of heterotrophy (38). Bacteriotrophy in euglenazoans is accomplished by use of a microtubule reinforced flagellar pocket called the microtubular root or MTR (6), and a rod and vane supported feeding apparatus (except in the case of *Petalomonas*, which lacks this feature) (38). The other form of heterotrophy found in Euglenida is eukaryotrophy (52). Eukaryotrophy also utilizes the MTR plus rod and vane apparatus (38). The ability to consume eukaryotic prey is believed to have arisen from a change in the number and arrangement of pellicle strips in a bacteriotrophic ancestor, which allowed for euglenoid movement (39). Phototrophy has resulted from a secondary symbiosis between a eukaryotrophic ancestor and an alga (38). This event is associated with another pellicle strip duplication event, an increase in cell size, and an increase in the number of articulation zones between pellicle strips (38). Osmotrophic euglenazoans subsist entirely on nutrients pinocytosed from their environment, and have arisen from both phagotrophic and phototrophic ancestors (9).

Triemer and Farmer (51) describe four types of feeding apparatus associated with heterotrophy in euglenids. The Type I is the simplest apparatus, consisting of a cytoplasmic pocket supported by a microtubule lining. The cytoplasmic pocket is composed of the flagellar pocket merged with a feeding pocket. The microtubule lining derives from the microtubular flagellar roots. This type is found in *Petalomonas*, *Diplonema*, and *Calycimonas*. A Type II feeding apparatus consists of two supporting rods and a series of plicate vanes. The rods consist of a relatively low number of microtubules surrounded by a dense amorphous matrix. A single microtubule may be associated with each vane, and the vanes remain closely appressed to the rods for most of their length. The vanes surround an invagination which becomes the cytosome. This type is found in *Ploeotia* and in some *Diplonema* species. The Type III apparatus consists of two supporting rods and plicate vanes. However, Type III supporting rods are composed of a central cylinder of microtubules surrounded by a matrix, which is itself surrounded by a ring of microtubules. Vanes arise from the rods, but diverge from the rods towards the anterior of the cell. Type III is found in *Peranema*, some *Dinema* species, and *Anisonema*. The Type IV apparatus is composed of supporting rods similar to those found in Type III, but near the base of a Type IV apparatus, one rod bifurcates giving rise to three rods for nearly the entire length of the cell. The number of microtubules within each rod rises dramatically as the rod approaches the anterior of the cell. A Type IV feeding apparatus is capable of extending and withdrawing over a distance of 3-5 μm. This type of apparatus is only found in *Entosiphon*.

1.2 The Pellicle

The pellicle is an extremely diverse structure with a general organization common to all euglenids (39). Pellicle morphology can be quite complex, and varies among taxa (39). The pellicle is composed of four main parts: the plasma membrane; repeating proteinaceous

units or "strips," composed mostly of articulins (a broad group of cytoskeletal proteins); supporting microtubules; and tubular cisternae of endoplasmic reticulum (5; 32). The proteinaceous strips are found immediately beneath the plasma membrane (39). Supporting microtubules and ER cisternae run beneath and parallel to the proteinaceous strips (39). Pellicle strips may be arranged longitudinally or helically (39). The number, arrangement, and substructural details may provide information about the phylogeny and taxonomy of a species (39). Leander and Farmer identify four broad categories of pellicle frame, each of which approximates the observed shape of the frame. S-shaped frames possess a heel and a rounded arch; plateau or π-shaped frames possess a flattened arch; M-shaped frames possess a distinct median depression, or groove within the arch; and A-shaped frames possess an arch surface obliquely oriented to the horizontal region of the heel (39).

1.3 The Flagella and Flagellar Motility

The standard euglenoid flagellar apparatus consists of two basal bodies giving rise to three microtubular roots (27). Each basal body has a microtubular root anchored to the side opposite of the other basal body, which extends anteriorly, and the third root originates from the ventral basal body and proceeds anteriorly between the two basal bodies (27). Each basal body gives rise to a flagellum in all euglenids, with the exception of the phototrophic order Euglenales, which has only one emergent flagellum, with the other flagellum terminating within the flagellar pocket (49). The two flagella are usually designated as anterior or recurrent based on their position in relation to the cell (27). Each flagellum contains an axoneme with the classic "9+2" arrangement of microtubules. Both flagella also contain a paraxial rod, or PAR, which is situated parallel to the axoneme (19). The size and form of the PAR differs between the anterior and recurrent flagellum (19). The exterior of the flagella is covered with hair-like trans-membrane extensions called mastigonemes (43).

The gliding motility observed in heterotrophic euglenids is not fully understood, but is dependent on the flagella remaining in contact with a substrate (46). In 2003, Saito showed that in *Peranema trichophorum*, movement is not generated by the beating of the anterior flagellum, but by movement of the substrate-attached portions of the anterior flagellum and the posterior flagellum (46). Mastigonemes were shown to adhere to polystyrene bead, which were then translocated along the length of the flagellum (46). Therefore it is reasonable to infer that mastigoneme-surface adhesion is necessary for gliding motility in *Peranema*. One could then infer that this model of mastigoneme-surface-mediated gliding is used by other gliding euglenids, albeit in a slightly modified fashion, as in other genera, such as *Euglena* and *Peranema*, the recurrent flagellum adheres to the substrate, rather than the anterior flagellum.

1.4 Genetic Analysis

The small subunit ribosomal RNA (SSU rRNA) gene codes for the 18S ribosomal subunit, which is vital for RNA transcription. In addition to its vital biological function, the SSU rRNA gene is widely used as a molecular phylogenetic marker.

Group I introns are genetic elements that exist in nuclear DNA, but are removed during post-transcriptional processing by a self-splicing mechanism, which results in intron excision and exon ligation (12–14; 35). Group I introns are divided into five classes and several subclasses based on secondary structural elements (41; 50). Introns in SSU rRNA genes are restricted to a small number of insertion sites (34), and phylogenetic groups are established based on these insertion sites (3; 4; 28; 30; 33). Group I introns are widely distributed in multiple branches of the eukaryotic tree of life, including rhodophytes, green algae, ciliates, fungi, filose amoeba, and myxomycetes (3; 4; 10; 18; 47), which likely indicates horizontal

gene transfer as a means of distribution (7). The group I intron found in the SSU rRNA gene of *Ploeotia costata* by Busse and Preisfeld is currently the only known instance of a Group I intron in a euglenazoan.

Keelungia pulex is a recently discovered colorless, heterotrophic euglenoid found in water samples from Taiwan (15). Chan et al. argue that based on unique pellicle morphology that lacks proteinaceous strips, and on phylogenetic distance from *P. costata* that *K. pulex* should be considered the type species of the novel genus *Keelungia*. Using new SSU rRNA sequences from *P. pseudanisonema* and *P. vitrea*, we comment on the relationship between *K. pulex* and *Ploeotia*, and discuss the future of *Ploeotia*.

1.5 Goals

Documentation of behavior, morphology, and phylogeny of *P. pseudanisonema*, for this project was done through light microscopy, electron microscopy, gene amplification and sequencing, and phylogenetic comparison. Differential interference contrast light microscopy was employed to observe the behavior of live *P. pseudanisonema*, as well as to document the external and internal morphology of the organism in a way that is useful for identifying it in environmental samples. Scanning electron microscopy was used to document the full external morphology of the organism. Transmission electron microscopy was used to document the internal morphology of the organism, focusing on the pellicle and feeding apparatus substructure. Gene amplification and sequencing was used to elucidate the sequence of the small subunit ribosomal RNA gene, for comparison to other Euglenid organisms. Phylogenetic analyses were used to elucidate the location of *P. pseudanisonema* within the Euglenid clade.

1.6 Organisms

***Ploeotia costata* Farmer & Triemer, 1988**

Ovate cells, 21-25 μm in length by 18 μm in width. Five longitudinal grooves; two dorsally, two laterally, one ventrally. Grooves are comprised of two bifurcate ridges, and have crenate margins. Anterior flagellum is half the length of the cell, ventral flagellum about 1.5 to twice the cell length. (26)

***Ploeotia oblonga* Larsen & Patterson, 1991**

Cell oblong, 20-30 μm in length by 11-16 μm in width, slightly flattened. Five longitudinal grooves; 2 dorsal, 3 ventral. Two longitudinal ridges along the edges of the central ventral groove. Anterior flagellum about the length of the cell, recurrent flagellum twice the cell length. Recurrent flagellum is approximately 1 μm thick for its entire length. Wart-like projections are visible on the ridges on the posterior of the cell. (37)

***Ploeotia pseudanisonema* Larsen & Patterson, 1991**

Cells oblong to oval, 10-19 μm in length by 6-10 μm in width, flattened. Eight to twelve delicate longitudinal grooves. Anterior flagellum is 1.5 times cell length, recurrent flagellum up to four times cell length. Exhibits *Anisonema*-like reversals of movement. Distinguished from *Anisonema* by the obvious presence of an ingestion organelle when viewed with a light microscope. (37)

***Ploeotia vitrea* Farmer & Triemer, 1988**

Ovate cells, 17-21 μm in length by 12 μm in width. Slightly flattened ventrally. Ten longitudinal ridges; eight dorsal, two ventral. Anterior flagellum is half the cell length, recurrent flagellum 1.8-3.5 times cell length. (26)

Entosiphon sulcatum Dujardin, 1878

Ovoid cells, 15-40 μm in length by 10-18 μm in width. Slightly flattened ventrally. Twelve longitudinal grooves. Anterior flagellum is equal to cell length, recurrent flagellum is 1-1.5 times cell length. Feeding apparatus may be extruded up to 2.5 μm during feeding. (23)

Keelungia pulex Chan et al., 2013

Oblong to oval cells, 8-11 μm in length, 5.5-8 μm in width. Ten delicate longitudinal grooves; five dorsal, five ventral. Anterior flagellum is equal to cell length, recurrent flagellum is 2-3 times cell length. Anterior flagellum is generally kept in a sigmoidal shape. (15)

Table 1.1: Species belonging to *Ploeotia* and heterotrophic euglenids discussed

Species	Cell Shape	Length	Width	Pellicle Structure	Flagella	Unique Features	Author
Ploeotia adhaerens	Rounded, with blunt anterior and posterior protrusions; flattened	20-28 m	16-23 m	8 longitudinal ridges; 4 dorsal, 2 ventral; 2 lateral	Anterior ¡ 1 Cell Length Recurrent = 1 CL	Both flagella possess swollen bases. Retracts flagella in response to bright light.	Larsen & Patterson, 1991
P. azurina	Ovate, tapered posterior end; flattened ventrally	10-16 m		8-10 longitudinal or slightly oblique ridges; 5 dorsal, 1 ventral, 2 lateral	Anterior = 1.5 CL Recurrent = 2 CL	Two common morphs are observed. The smaller is noticably flattened, with a longer recurrent flagellum. Larger is more rounded, has slightly spiral pellicle ridges, and two additional ventral ridges.	Patterson & Simpson, 1996
P. corrugata	Oval, with posterior indention; flattened ventrally	7-14 m	5-12 m	6 longitudinal ridges; 4 dorsal, 2 lateral	Anterior = 1 CL Recurrent = 2 CL	Tilts forward to feed.	Larsen & Patterson, 1991
P. costata	Ovate	21-25 m	18 m	5 longitudinal grooves; 2 dorsal, 1 ventral; 2 lateral	Anterior = 0.5 CL Recurrent = 1.5 - 2 CL	Grooves are comprised of bifurcated ridges, and have crenated margins.	Farmer & Triemer, 1988
P. decipiens	Oval, with blunt posterior	16-20 m	10-13 m	6 longitudinal ridges; 4 dorsal, 2 lateral	Anterior = 1 CL Recurrent = 2 CL	Flat ventrally. Ingestion organelle extends the full length of the cell.	Larsen & Patterson, 1991

P. discoides	Oval; flattened	10-12 m	7-9 m	3 fine longitudinal grooves; 1 dorsal, 2 lateral	Anterior = 1.5 CL Recurrent = 1.5 - 2 CL	Ingestion organelle extends the full length of the cell.	Larsen & Patterson, 1991
P. heracleum	Oblong; flattened	14 m	10 m	8 longitudinal ridges; 4 dorsal, 4 ventral	Anterior = 1 CL Recurrent = 2 CL		Larsen & Patterson, 1991
P. laminae	Oval, with small posterior indention; flattened dorsally, slightly concave ventrally	22-40 m	15-20 m	7 longitudinal ridges; 1 dorsal, 6 ventral. 2 ventral ridges are more prominent	Anterior = 1 CL Recurrent = 3 CL	Flagella emerge subapically. Flagellar pocket and nucleus are both on the left side of the cell.	Al-Qassab, 2002
P. longifilum	Round, or slightly oblong	12-20 m	7-15 m	1 groove, ventral	Anterior = 1 CL Recurrent = 5 CL	Ingestion organelle broad anteriorly, with widely spaced supporting rods.	Larsen & Patterson, 1991
P. obliqua	Obvate, with left margin more convex than right; slightly flattened	10-15 m	7-12 m	4 grooves, ventral	Anterior = 1 CL Recurrent = 2.5 CL	Cell glides with quick trembling motion.	Schroeckh, 2003

P. oblonga	Oblon; slightly flattened	20-30 m	11-16 m	5 longitudinal grooves; 2 dorsal, 3 ventral. 2 longitudinal ridges, ventral.	Anterior = 1 CL Recurrent = 2 CL	Recurrent flagellum approximately 1 m for entire length. Warts or globules adhere to the posterior of the cell.	Larsen & Patterson, 1991
P. plumose	Oblong to ovate; flattened	17-35 m	14 m	9 longitudinal grooves; 4 dorsal, 5 ventral	Anterior = 2 CL Recurrent = 2-3 CL	Feather-like inclusions arise from the grooves. A single large, oval posterior vacuole is often present.	Ekebom, 1995
P. plana	Ovate, with right anterior margin slightly prominent; flattened	9-14 m	5-10 m	5 longitudinal grooves; 4 ventral, 1 dorsal	Anterior = 0.5 - 0.75 CL Recurrent = 2 CL	Dorsal groove is broader than ventral grooves.	Schroeckh, 2003
P. pseu-danisonema	Oblong to oval; flattened	10-19 m	6-10 m	8-12 delicate longitudinal grooves	Anterior = 1.5 CL Recurrent = 4 CL	Exhibits Anisonema-like reversals of movment. Differentiated from Anisonema by the obvious presence of an ingestion organelle.	Larsen & Patterson, 1991
P. punctata	Oblong, slightly flattened	13-16 m	9-11 m	3 fine longitudinal grooves, dorsal.	Anterior = 1 CL Recurrent = 2-3 CL	Grooves contain regularly spaced internal bodies.	Larsen & Patterson, 1991

P. robusta	Obliquely oval, with a lip on the anterior left; flattened	30-40 m	16-21 m	1 longitudinal groove, ventral	Anterior = 1 CL Recurrent = 4 CL	Flagella emerge subapically. Longitudinal groove extends from the anterior lip. Nucleus found on left side of the cell. While gliding, cell regularly retracts with swift, jerking motion and changes direction.	Larsen & Patterson, 1991
P. scrobic-ulata	Obliquely oval, with left side more convex than right; flattened	40 m	20 m	Pellicle is pitted, rather than grooved	Anterior = 0.5 CL Recurrent = 2 CL	Pitted pellicle. Ingestion organelle only about 0.5 cell length.	Larsen & Patterson, 1991
P. tenuis	Obvate, obtuse posteriorly; flattened	18 m	12 m	5 delicate longitudinal grooves; 3 dorsal, 2 ventral	Anterior = 1 CL Recurrent = 2 CL		Larsen & Patterson, 1991
P. tasmanica	Elongate oval, posteriorly rounded; slightly flattened	13-15 m		5 delicate longitudinal grooves; 2 dorsal, 3 ventral	Anterior = 1 CL Recurrent = 2 CL		Lee, 2005
P. vitrea	Ovate; slightly flattened ventrally	17-21 m	12 m	10 longitudinal ridges; 8 dorsal, 2 ventral	Anterior = 0.5 CL Recurrent = 1.8-3.5 CL		Farmer & Triemer, 1988

Entosiphon sulcatum	Ovoid; slightly flattened ventrally	15-40m	10-18 m	12 longitudinal grooves	Anterior = 1 CL Recurrent = 1-1.5 CL	Feeding apparatus may extend up to 2.5 m during feeding.	Dujardin, 1878
Keelungia pulex	Oblong to oval	8-11 m	5.5-8 m	10 delicate longitudinal grooves; 5 dorsal, 5 ventral	Anterior = 1 CL Recurrent = 2-3 CL	Anterior flagllum generally kept in a sigmoidal shape.	Chan, 2013

Chapter 2

Materials and Methods

2.1 Cell Culture

Ploeotia pseudanisonema cultures were obtained from the American Type Cultures Collection, Manassas, Virginia, USA, and maintained on ATCC 802 media at 24°C. During culture flask refreshment, the vigorous employment of a cell scraper on the bottom of flask is important to dislodge thick bacterial plaques which drastically interfere with growth of *P. pseudanisonema*. Within the ATCC, the organism is incorrectly labeled *Ploeotia oblonga*. Based on the Larsen and Patterson description, this researcher has re-identified the culture as *P. pseudanisonema*, and a correction will be sent to the ATCC once this manuscript has been submitted for publication.

2.2 Light Microscopy

Four day old *P. pseudanisonema* cultures were concentrated by centrifugation, and used to make sealed-coverslip wet-mount slides to observe vegetative cells. Slides were examined using a Nikon Ti-U microscope fitted with high contrast DIC prisms. Images and videos were recorded using an Andor iXon2 EMCCD camera. One week old cultures were used to observe encysted cells.

2.3 Scanning Electron Microscopy

Four day old *P. pseudanisonema* cultures were concentrated by centrifugation. Samples were fixed using Osmium Tetroxide (OsO_4) vapor followed by addition of OsO_4 to the media, dehydrated by a stepwise ethanol gradient, critical point dried using a Samdri 780-A from Tousimis (Rockville, MD), mounted on a Aluminum SEM stub, and gold sputter coated at an approximate thickness of 153 Å using a Module Sputter Coater from Structure Probe, Inc. (West Chester, PA). Stubs were examined using a Zeiss 1450EP Environmental scanning electron microscope.

2.4 Transmission Electron Microscopy

Standard TEM fixation

Four day old *P. pseudanisonema* cultures were concentrated by centrifugation, and prepared using a standard Glutaraldehyde/OsO_4 fixation, stepwise ethanol dehydration, and acetone to EPON 812 infiltration. Samples were sectioned to approximately 50nm thickness and collected on Formvar coated Copper slot grids. Grids were stained with Uranyl Acetate and Lead Citrate. Grids were examined using a JEOL 100CX TEM.

Substrate-dependant Flat Embed Fixation

This protocol is modified from the protocol described by D.R. Mitchell (42). Cells were concentration by centrifugation. Before fixation, cells were allowed to settle onto a glass coverslip and begin gliding motility, for approximately 15 minutes. Cells were fixed with 1% gluteraldehyde in the cell culturing media at room temperature for one hour. The fixative was then replaced by ATCC 802 media with 1% low melt agarose. After the agarose had solidified, coverslips were carefully placed into a coverslip staining jar and post-fixed for one hour with 1% OsO_4 at room temperature. Dehydration and embedding with EPON 812 resin were performed as follows: ethanol in 10% increasing increments through 70% and then in 5% increasing increments through 100%. This was followed by two washes in 100% acetone. Infiltration with EPON 812 resin was accomplished through EPON 812:Acetone dilutions of 1:2, 1:1, and 2:1, and 1:0 for one hour each step, and a 1:0 overnight step. EPON 812 was cured overnight at 60°C. After curing, the coverslips were separated from the cured resin by immersion in liquid nitrogen (LN). Individual cells or small groupings of cells were remounted and sectioned either parallel or perpendicular to the coverslip surface to obtain sections capturing ciliary gliding motility. Sections were stained with uranyl acetate and lead citrate, and were viewed using a Tecnai 20 transmission electron microscope at 120KV.

2.5 Primer Design

Ploeotia costata

The gene sequence AF525486, published by Busse and Preisfeld (8), was obtained from NCBI GenBank. Primers were produced by Invitrogen (Carlsbad, CA).

Table 2.1: *Ploeotia costata* table of primers

Primer Name	Sequence
P. costata BP 419 Forward	GGTTACAATGGCAGCGAAA
P. costata BP 1400 Forward	GCTCGTAGTTGGATYCAGAGC
P. costata BP 2300 Forward	GGCTTAATTTGACTCAACACGGG
P. costata BP 1400 Reverse	GCTCTGRATCCAACTACGAGC
P. costata BP 2300 Reverse	CCCGTGTTGAGTCAAATTAAGCC
P. costata BP 3100 Reverse	CCTACAGCTACCTTGTTACGA

Ploeotia pseudanisonema

Primers for *P. pseudanisonema* SSU gene were designed from the Virginia Commonwealth University Euglenazoan Tree of Life database, which includes *P. pseudanisonema*. This database is currently unavailable to the public, but plans for public access are in progress. The BLAST feature of the database was used to identify potential SSU sequence based on comparison to *P. costata*. Ideal primers were designed using the Primer 3 feature of Geneious R 6 6.1.5 from Biomatters Inc. (San Francisco, CA). Primers were produced by Invitrogen (Carlsbad, CA).

Table 2.2: *Ploeotia pseudanisonema* table of primers

Primer Name	Sequence
P. pseudanisonema BP 79 Forward	GCTGAATCTGCGGACGGCTCA
P. pseudanisonema BP 531 Forward	TCCGCGGGTCTCTGACTCGT
P. pseudanisonema BP 1073 Forward	GCAAGGCGCTCGAGGTCCAA
P. pseudanisonema BP 1531 Forward	TGGTGGTGCATGGCCGTTCT
P. pseudanisonema BP 721 Reverse	TCCACGGAAAGGCCGTGTGC
P. pseudanisonema BP 1139 Reverse	CGTACCACCCCGCTTGCTCC
P. pseudanisonema BP 1664 Reverse	CGACCGAAGCCGCTCGAACT
P. pseudanisonema BP 1921 Reverse	AGGCATTCCTCGTTCAAGGCAAA

2.6 Whole Genome Isolation

Four day old cultures of *P. pseudanisonema* and one week old cultures of *P. costata* were concentrated by centrifugation. DNA was collected from pelleted cells using a DNeasy Plant Mini Kit from Qiagen (Valencia, CA). Isolated DNA was stored at -20°C.

2.7 Polymerase Chain Reaction

The 18S SSU gene was amplified by PCR using 10x Buffer, $MgCl_2$, nucleotide, and Platinum Taq Polymerase obtained from Invitrogen (Carlsbad, CA), as well as the primers previously described. Reactions were incubated in a Bio-Rad DNAEngine. The incubation program started with denaturation at 94°C for 3 minutes, followed by 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes; after 50 cycles, a final incubation at 72°C for 10 minutes. PCR products were purified using a QIAquick PCR Purification kit from Qiagen (Valencia, CA). Product final concentration and purity were calculated using a Nanodrop ND-1000 Spectrophotometer. All products were stored at -20°C.

2.8 Gel Electrophoresis

Gel electrophoresis was used to estimate product length, and to check the specificity of the gene amplification. A 2% agarose solution was created by mixing 0.8 g agarose with 40mL TAE buffer. To the solution, 8 μl of GelStar was added to stain nucleic acids. The solution was cast and allowed to solidify in an Owl Separation Systems B1A mold (Portsmouth, NH). 3 μl of PCR product samples were mixed with 4 μL of 5x GelPilot Loading Dye from Qiagen (Valencia, CA), and loaded into the agarose gel. Gels were run at 100 volts for one hour. Gels were imaged using a Canon-1089 camera above a UV light source. Pictures were taken using Doc-ItLS UVP v6.1.1 software.

2.9 PCR Product Sequencing

All PCR products were sequenced by the Georgia Genomic Center using a single-tube Sanger sequencing protocol. Results were uploaded to Geneious R 6 6.1.5 from Biomatters Inc. (San Francisco, CA) for examination and comparison.

2.10 Phylogenetic Analysis

SSU rRNA sequences for *Ploeotia pseudanisonema* was generated by our collaborators at Virginia Commonwealth University, and corroborated by sequences produced in this lab. SSU rRNA sequences for 28 other euglenids were obtained from NCBI Genbank. Sequences were aligned in Geneious R 6 6.1.5 from Biomatters Inc. (San Francisco, CA) using ClustalW (36) alignment algorithms. A ClustalW DNA weight matrix, a Gap Open Cost of 15, and a Gap Extend Cost of 6.66 were used for the ClustalW alignment. After alignment, sequences were uploaded to Phylogeny.fr (20; 21), where they were aligned using MUSCLE 3.7 (24).

Gblocks 0.91b (11) was used to refine the alignment by selecting highly conserved sequences fit for phylogenetic analysis, and eliminate divergent sequences. Maximum likelihood analysis of selected sequences was performed by PhyML 3.0 (29), and arranged into a phylogram by TreeDyn (16). Phylogram branches with maximum likelihood support values below 50% were collapsed. Multiple iterations of phylograms were created with the inclusion or exclusion of various taxa, using the same parameters mentioned above.

2.11 Image Editing

All images were cropped, contrast corrected, and labeled using Photoshop CS5.1 version 12.1 from Adobe Systems Inc. (San Jose, CA).

Chapter 3

Results

3.1 Light Microscopy

Differential interference contrast light microscopy confirms the general description published by Larsen and Patterson in 1990 (37). Vegetative cells are typically between 10 and 16μm in length and between 6 and 10μm wide. These sizes are confirmed by scanning electron microscope images. The feeding apparatus rods originate slightly to the right of center at the posterior of the cell, and proceed forward to a feeding orifice on the right side of the flagellar pocket (Figure 3.1 A.). The nucleus is located above the feeding apparatus support rods, and in the right half of the cell (Figure 3.1 B.). The anterior flagellum is approximately 1.5 times cell length. Posterior flagellum is greater than 2 times cell length, but rarely reaches the 4 times cell length documented by Larsen and Patterson. Numerous feeding vacuoles may be observed in the posterior of the cell (Figures 3.1 A.-C.). During normal gliding behavior, the entire length of the posterior flagellum remains in contact with the substrate, while the posterior of the cell itself may rise up above the substrate. The anterior flagellum does not maintain regular contact with the substrate, but sweeps in front of the cell body in constant motion. During feeding, the entire cell pitches forward to put

the anterior end of the cell in close proximity to the substrate, while the posterior end rises to an almost vertical position. Cells commonly encysted in as few as four days post refreshment. Feeding apparatus support rods remain assembled during the cyst stage, but decrease in length along with the cell body. During the cyst stage, contractile vacuoles have been documented continuously ejecting water from the cell body (Figures 3.1 D.-H.).

Figure 3.1: DIC images of *Ploeotia pseudanisonema*

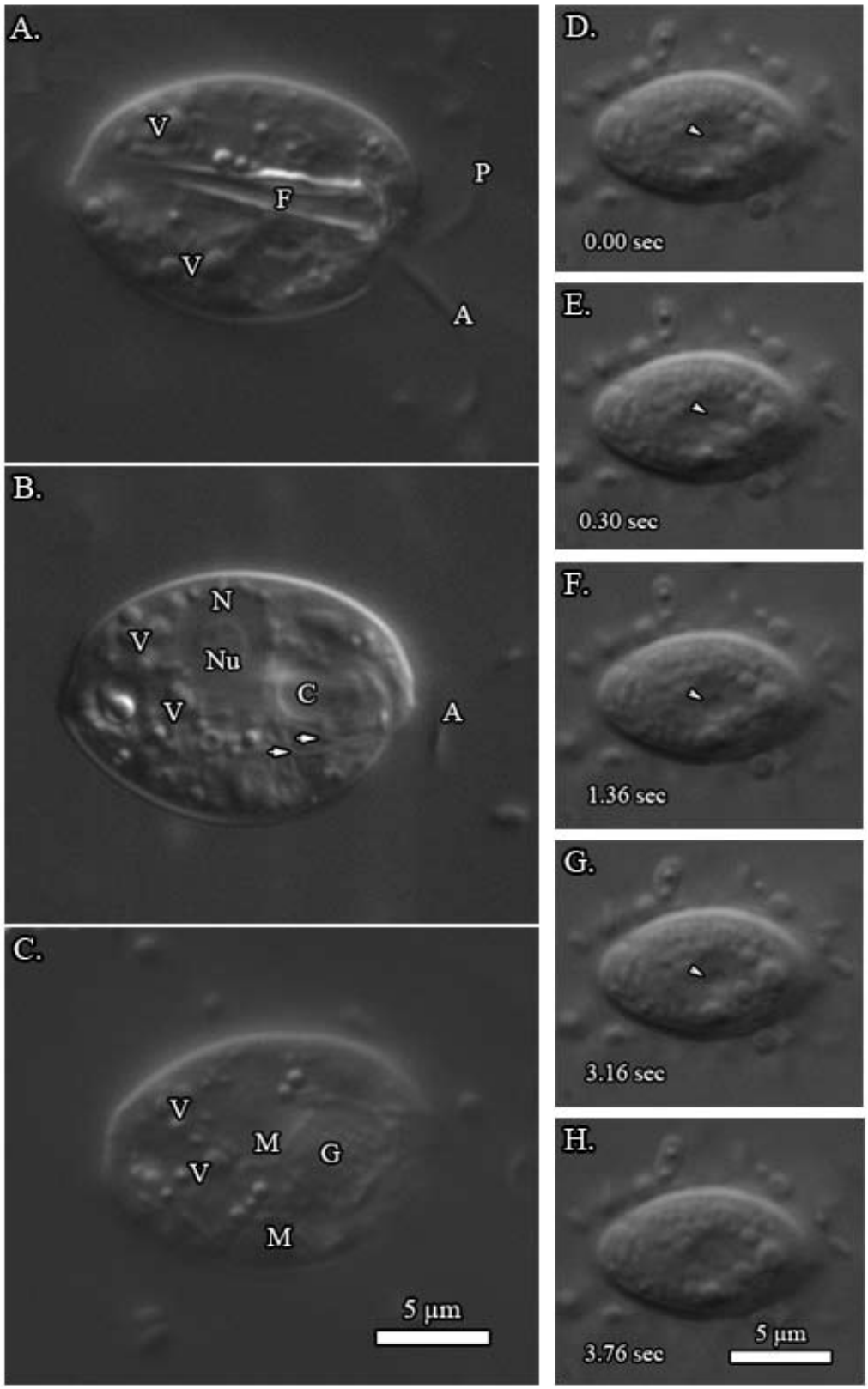
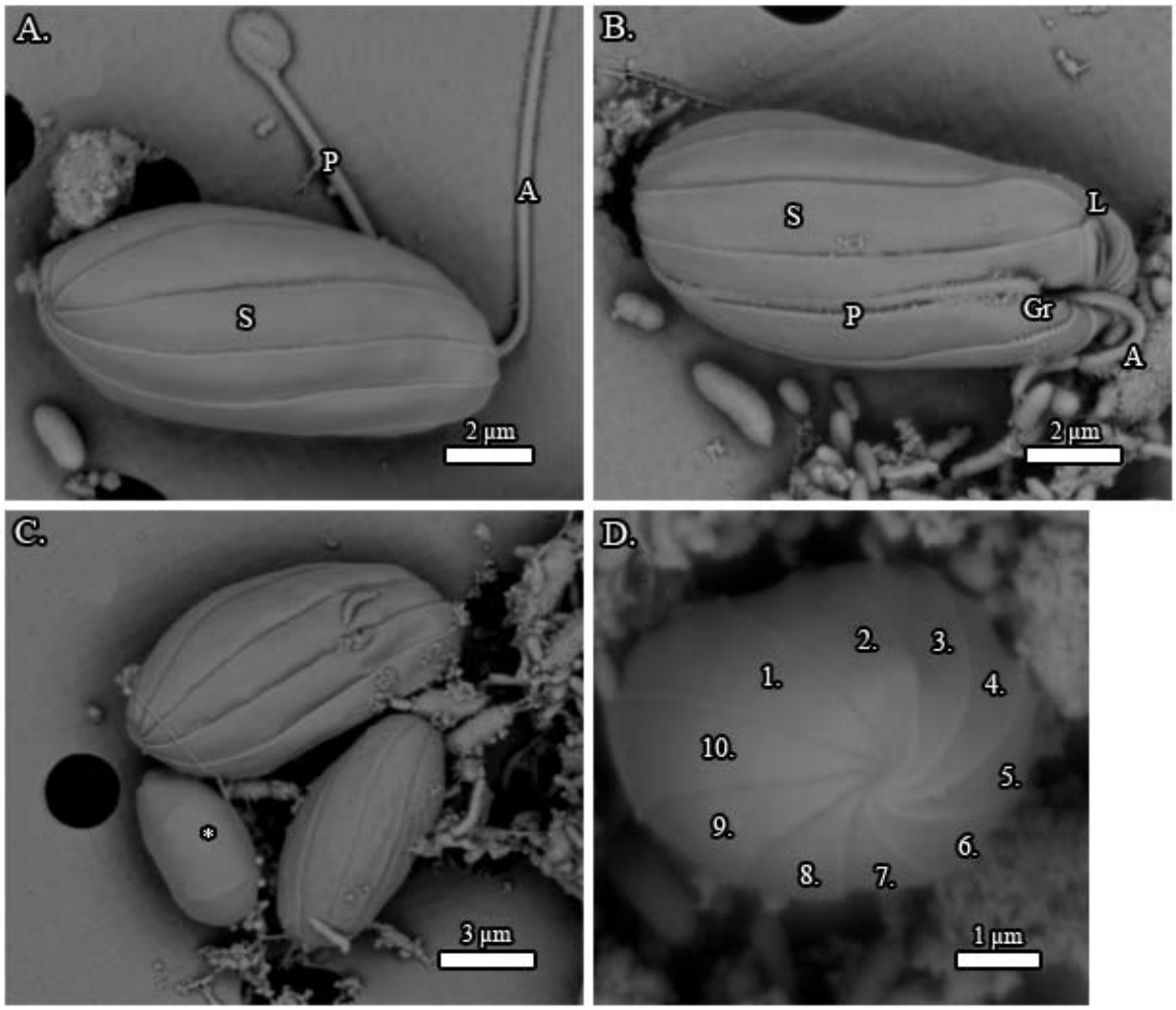


Figure A.-H. Differential Interference Contrast Light Microscopy. **Figures A.-C.** Vegetative *Ploeotia pseudanisonema* seen ventrally advancing from ventral to dorsal in longitudinal section. **Figures D.-H.** Time-lapse series of cyst-state *P. pseudanisonema*. **A.** Both the posterior flagellum (P) and anterior flagellum (A) are seen; posterior is not in the normal position curved beneath the cell. The feeding apparatus is show extending nearly the entire length of the cell, with numerous vacuoles (V) in the posterior half of the cell. **B.** The nucleus (N) and nucleolus (Nu) are visible on the right side of the cell. A contractile vacuole (C) can be seen next to the flagellar pocket, where the internal lengths of both flagella (Arrows) are visible. **Figure C.** Branches of mitochondrial (M) cisternae are visible in the ventral periphery, as are Golgi (G) derived vesicles. **Figures D.-H.** Time lapse showing the filling and emptying of a single contractile vacuole (Arrowhead).

3.2 Scanning Electron Microscopy

Scanning electron microscopy reveals the presence of ten longitudinal grooves without exception (Figure 3.2 D.). Grooves converge in a swirl at the posterior end of the cell (Figure 3.2 D.). The dorsal side of vegetative cells displays five grooves (Figure 3.2 A.), and the ventral side displays the remaining five grooves (Figure 3.2 B.). The central groove on the ventral side is notable as being slightly wider than the others, to accommodate the recurrent flagellum lying within the groove (Figure 3.2 B.). A ventral groove not associated with the pellicle runs at a diagonal from the left side of the flagellar pocket, across the pellicle groove immediately left of center, and terminating at or near the central pellicle groove (Figure 3.2 B.). The recurrent flagellum is commonly seen lying in this groove, and continuing into the central pellicle groove (3.2 B.). Mastigonemes may be observed on both the anterior and recurrent flagella (Figure 3.2 B.). Visible on the right side of the flagellar pocket, the exterior cap of the feeding apparatus is comprised of dorsal and ventral lip structures in an elongated "C" shape (Figure 3.2 B.). A recessed plate exists within the lip structures (Figure 3.2 B.). Individual cells at varying stages of encystation were observed, and exhibit a decrease in size associated with cyst mucosa excretion, and a further decrease in size after full encystation (Figure 3.2 C.). Cyst cells as small as 4μm in length by 3μm in width were observed.

Figure 3.2: SEM Images of *Ploeotia pseudanisonema*

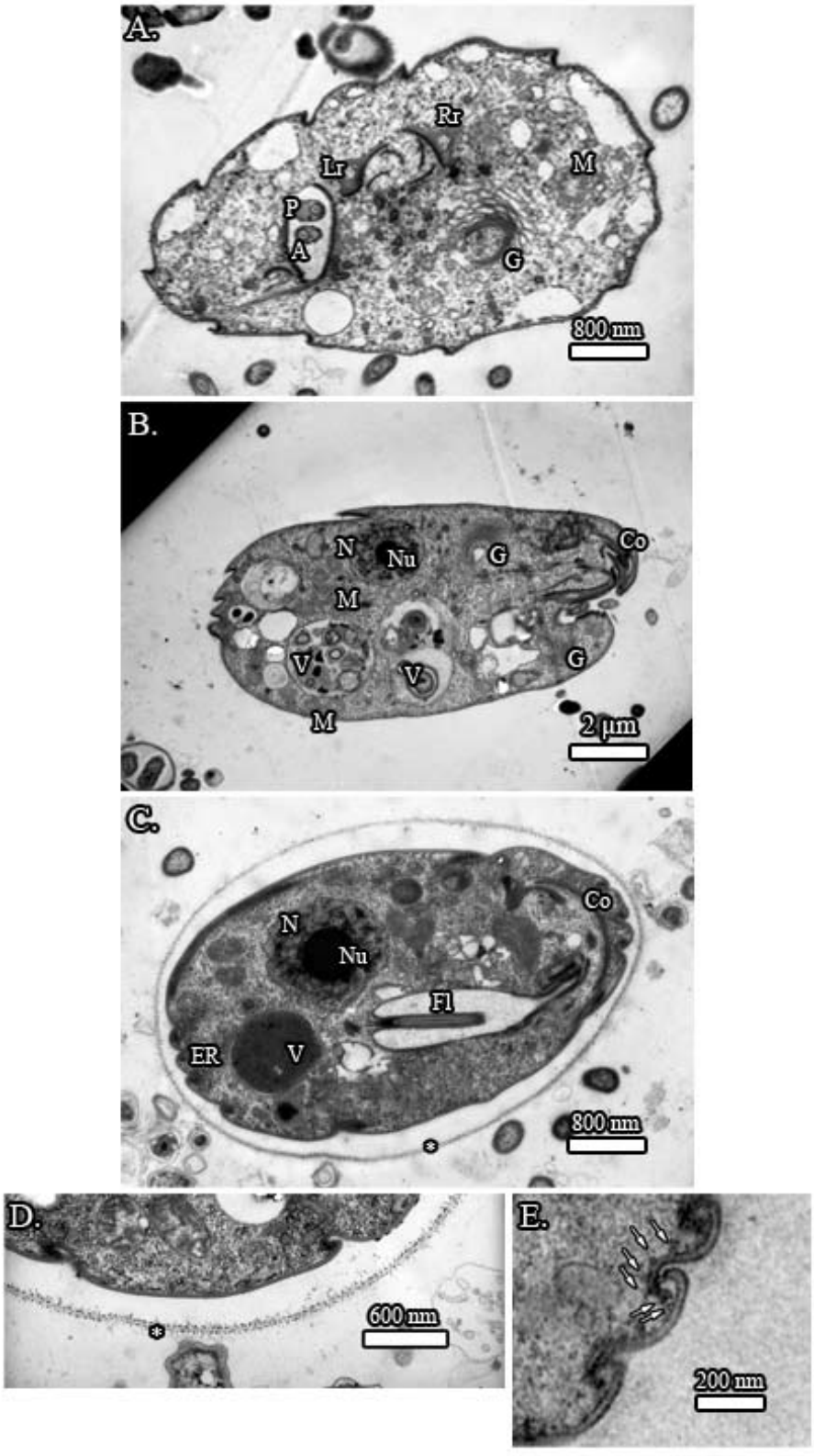


A.-D. Scanning Electron Microscopy. **A.** Dorsal view of *Ploeotia pseudanisonema*, five pellicle strips (S) may be clearly seen, as well as both flagella (P & A). **B.** Ventral view of *P. pseudanisonema*, the recurrent flagellum (P) is seen folded back in the ventral groove (Gr) and between two pellicle strips (S). The external lip (L) structures of the feeding apparatus are seen at the anterior of the cell. **C.** Vegetative cells compared to a cyst stage cell (*). **D.** A posterior view of *P. pseudanisonema* showing the junction of all ten pellicle strips (1.-10.).

3.3 Transmission Electron Microscopy

The flagellar pocket of *P. pseudanisonema* opens into the left half of the cell, and appears to be on the same plane as the feeding apparatus (Figure 3.3 A.). The nucleus is oriented in the right side of the cell body at approximately the mid-point of the body, and superior to the feeding apparatus (Figure 3.3 B.). Stacks of Golgi membrane are observed in the anterior half of the cell, near the flagellar pocket in nearly every case (Figures 3.3 A. & B.). Endoplasmic reticulum cisternae were often observed under the junctions between pellicle strips. Vesicles are observed throughout the cell. Larger vesicles identified as food vacuoles by the occasional presence of bacteria congregate near the base of the feeding apparatus (Figures 3.3 B. & C.). Tubular branches of mitochondria pervade the cell in seemingly random patterns, but it is common to observe at least one branch of mitochondria in close association with the feeding apparatus (Figure 3.3 A.). While encysted, all components of the feeding apparatus remain constructed (Figure 3.3 C.). The flagella do not remain at full length, but the root structure, transition zone, and a small length of flagellum remains intact (Figure 3.3 C.).

Figure 3.3: TEM images of *Ploeotia pseudanisonema*



A.-E. Transmission Electron Microscopy. **A.** Coronal section showing the arrangement of the left (Lr) and right (Rr) supporting rods relative to the flagellar pocket. **B.** Ventral longitudinal section showing the location of the comb (Co) in the extreme anterior of the cell. Food vacuoles (V) containing partially digested bacteria are seen in the posterior of the cell. **C.** Ventral longitudinal section of an encysted cell. The mucous layer (*) of the cyst may be seen around the periphery of the cell. The feeding apparatus comb and flagella (Fl) both remain assembled. **D.** The mucous layer of an encysted cell. **E.** The junction of two pellicle plates. At least six microtubules (Arrows) can be seen supporting the heel and arch along the entire length of the cell. Notice the flattened arch which indicates a π-shaped strip.

Pellicle Structure

TEM images confirm the presence of 10 longitudinal pellicle strips. Each proteinaceous strip is a singlet with a flattened arch, consistent with the plateau (π) shape described by Leander and Farmer (39) (Figure 3.3 E.). The proteinaceous layer of each strip is approximately 37 μm thick (Figure 3.3 E.). Each strip junction is supported by 6 microtubules arranged around the bridge and heel of adjoining strips (Figure 3.3 A.). More microtubules support the arch of the strip, with the number varying based on the width of the pellicle strip. Cisternae of endoplasmic reticulum are often observed beneath the junctions between pellicle strips.

Feeding Apparatus

The feeding apparatus of *P. pseudanisonema* is composed of three parts: a pair of supporting rods, the comb, and the cytopharynx. The comb and cytopharynx exist in the extreme anterior of the cell, while the supporting rods extend for the entire length of the cell. The apparatus emerges on the right side of the flagellar pocket, and runs toward the posterior center of the cell at a slight ventral angle (Figure 3.4 A.).

Both supporting rods are cone-shaped, and extend the entire length of the cell, tapering posteriorly. The bases of both support rods were observed in contact with pellicle at the posterior of the cell (Figure 3.4 B.). Both support rods are comprised of a layer of electron-opaque matrix with two layers of supporting microtubules, termed "cement," by Belhadri et al. (2), and two vanes associated with each rod (Figures 3.4 C.-E.). For at least part of their length, each rod possesses an arch of the electron-opaque matrix. The support rod nearest the flagellar pocket, is hereafter referred to as the left rod, with the other support rod hereafter referred to as the right rod. Posteriorly, the left rod is curled into a tight sigmoid shape, with the right rod in an arch shape, overlapping the left rod (Figure 3.4 C.). For much of the length of the left rod, the arch of electron dense matrix is absent or highly reduced (Figure 3.4 C.). On the right rod, the arch is present, but reduced along much of

the length (Figure 3.4 C.). As the rods continue anteriorly, the sigmoid shape of the left rod relaxes into a hook shape, and the two rods spread apart (Figure 3.4 D.). The arch of the right rod moves from the center of the feeding apparatus to the outer edge of the rod, but remains reduced until after the rod passes the base of the flagellar pocket (Figure 3.4 D.). Near the base of the flagellar pocket, the arches expand; the left arch becomes rounded, while the right arch assumes a triangular shape (Figure 3.4 E.). At the extreme anterior of the cell, both support rods drastically extend and assume a curved shape, which gives rise to the comb (Figure 3.4 F.). A third support rod, we have termed the sub rod, arises from the right rod, and contributes to the structure of the comb (Figure 3.4 F.). The number of microtubules associated with each rod varies along the entire length, but orientation of the two microtubule layers remains constant. The base layer of microtubules is continuous, and conforms to the shape of the electron-opaque matrix (Figures 3.4 C.-E.). The second microtubule layer contains dispersed single microtubules and horizontal clusters of 3 to 6 microtubules (Figures 3.4 C.-E.). One of these clusters is found underneath the arch structures of both support rods. Two gently curving vanes are associated with each feeding apparatus support rod (Figures 3.4 C.-E.). In the posterior end of the cell, the vanes are merged with the rods (Figures 3.4 C. & D.). As the feeding apparatus approaches the anterior end of the cell, the vanes separate from the support rods (Figure 3.4 F.). Each vane is supported by one microtubule at its distal end (Figures 3.4 C.-E.). A fifth microtubule, unassociated with the vanes, arises at the center of the vanes in the anterior half of the cell (Figure 3.4 E.). In the extreme anterior of the cell, the vanes and their microtubules disappear entirely (Figure 3.4 F.).

Arising from the right support rod, the comb is comprised of cement including two slightly separated layers of microtubules (Figure 3.4 F.). At the anterior of the comb, the right supporting rods give rise to the dorsal lip. The left support rod gives rise to the ventral lip. The sub rod gives rise to the recessed plate (Figure 3.4 G.). A proteinaceous scaffold extends

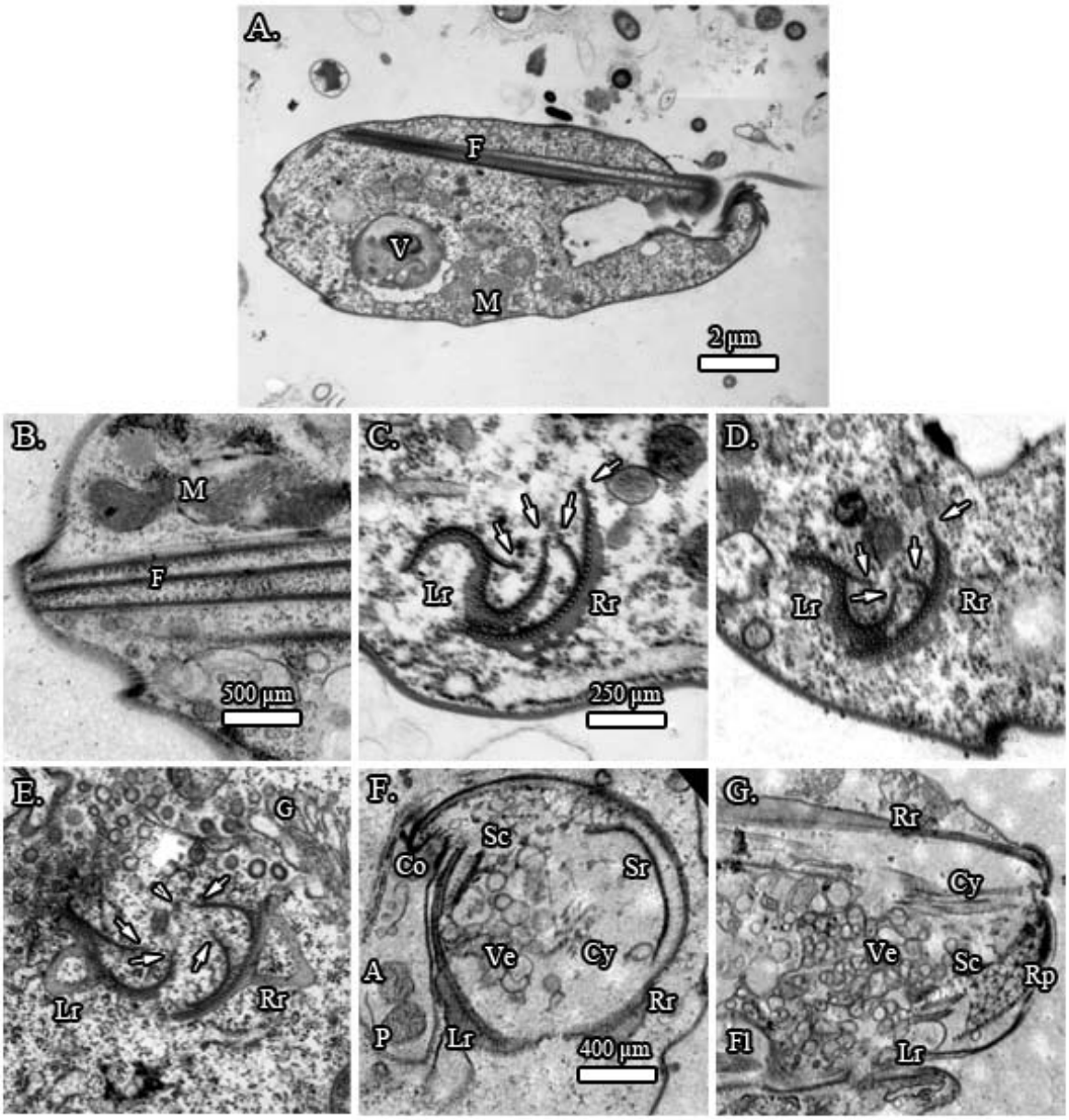
from the sub rod to the left and right supporting rods (Figures 3.4 F. & G.).

The cytopharynx exists behind the comb, and between the two support rods (Figures 3.4 F. & G.). The cytopharynx is a funnel-shaped structure starting at a gap between the recessed plate, and the junction of the dorsal and ventral lips. Small vesicles are present along the length of the cytopharynx (Figures 3.4 F. & G.). The cytopharynx is not seen in association with vanes (Figure 3.4 F.).

Flagellar Structure

Both the anterior and recurrent flagella possess a distinct paraxial rod. The recurrent paraxial rod is arranged in a lattice pattern, and is noticeably wider and thicker than the anterior paraxial rod (Figure 3.3 A.). The anterior paraxial rod appears to be a hollow tube (Figure 3.3 A.). Fine mastigonemes line the flagellar pocket, and both flagella. The flagellar pocket is lined by microtubules, arising from the three microtubular roots associated with the two basal bodies. The two basal bodies are connected by a striated connecting fiber.

Figure 3.4: TEM images of the *Ploeotia pseudanisonema* feeding apparatus



A.-G. Transmission Electron Microscopy. **A.** Ventral longitudinal section showing the length of the feeding apparatus (F). **B.** In longitudinal view, the base of the supporting rods of the feeding apparatus can be seen in contact with the pellicle at the posterior of the cell. **C.-E.** Coronal views at various points along the cell length. The shape and arrangement of the left (Lr) and right (Rr) supporting rods changes from posterior to anterior. Four vanes, each supported by a single microtubule (Arrows), are present for nearly the entirety of the supporting rod length. Near the flagellar pocket, a single microtubule (Arrowhead) appears between the four vanes. **F.** Coronal view at the extreme anterior of the cell. At the extreme anterior of the cell, both supporting rods drastically elongate. A sub rod (Sr) , and the comb (Co) arise from the right rod. Vanes disappear as the cytoproct (Cy) opens; numerous vesicles are seen around the cytoproct. The proteinaceous scaffold (Sc) is seen connecting the right rod, supporting rod, comb and left rod. **G.** Longitudinal view of the exterior feeding apparatus. The recessed plate (Rp) is shown covering the cytoproct. The proteinaceous scaffold seen in the previous figure is seen connecting the recessed plate to the left rod.

3.4 Small Subunit rRNA Genetic Analysis

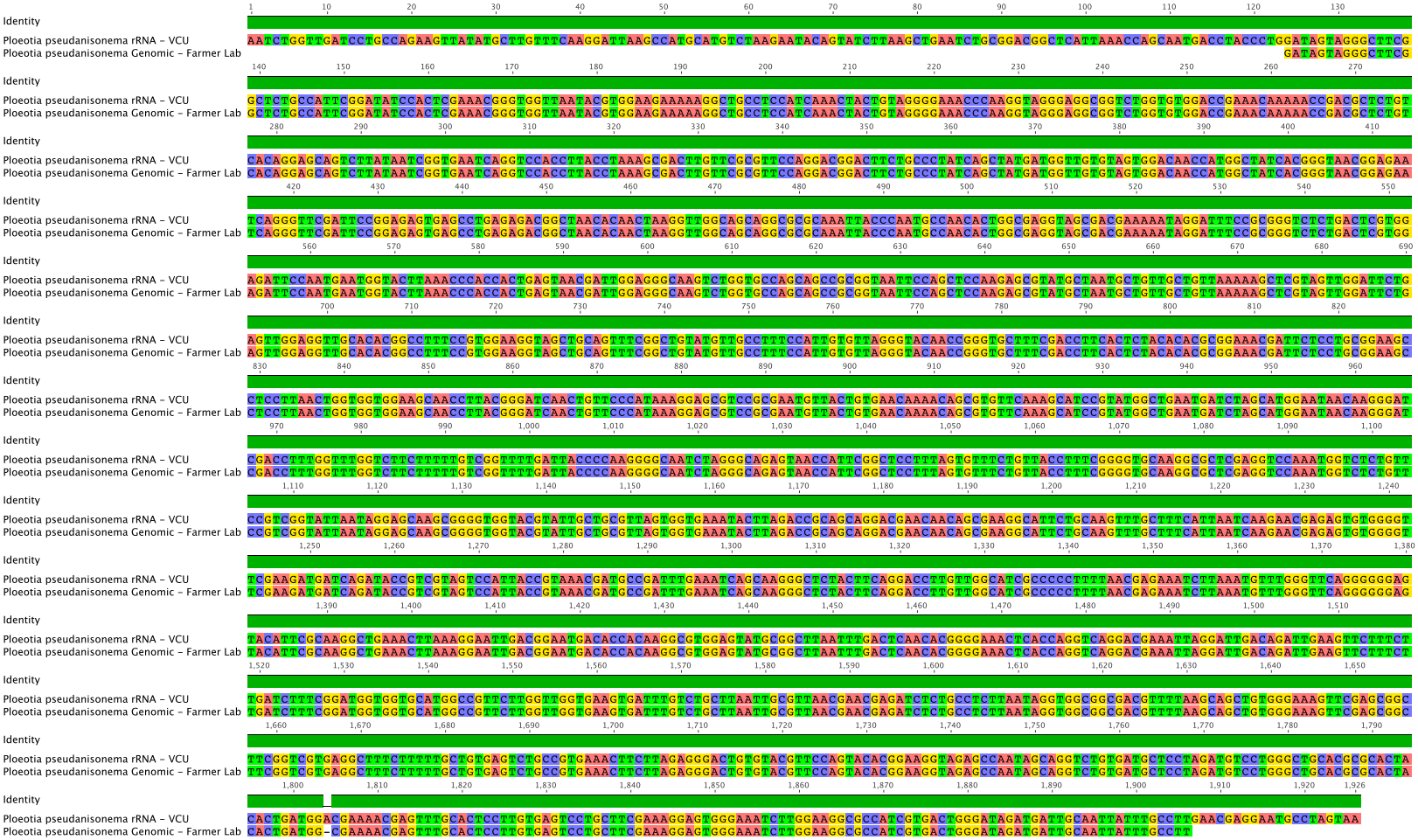
Ploeotia pseudanisonema

Ploeotia pseudanisonema whole genome extraction sequences were compared to complementary DNA sequences obtained by collaborating researchers at Virginia Commonwealth University. These sequences were found to be identical (Figure 3.5). *P. pseudanisonema* sequences were compared to the *Ploeotia costata* sequence AF525486, submitted to NCBI GenBank by Busse and Preisfeld (8). No consensus of sequence was observed between *P. costata* and *P. pseudanisonema* for the duration of the Group I intron sequence between bases 787 and 1280, negating the presence of a related Group I intron within the SSU gene of *P. pseudanisonema* (Figure 3.6).

Ploeotia costata

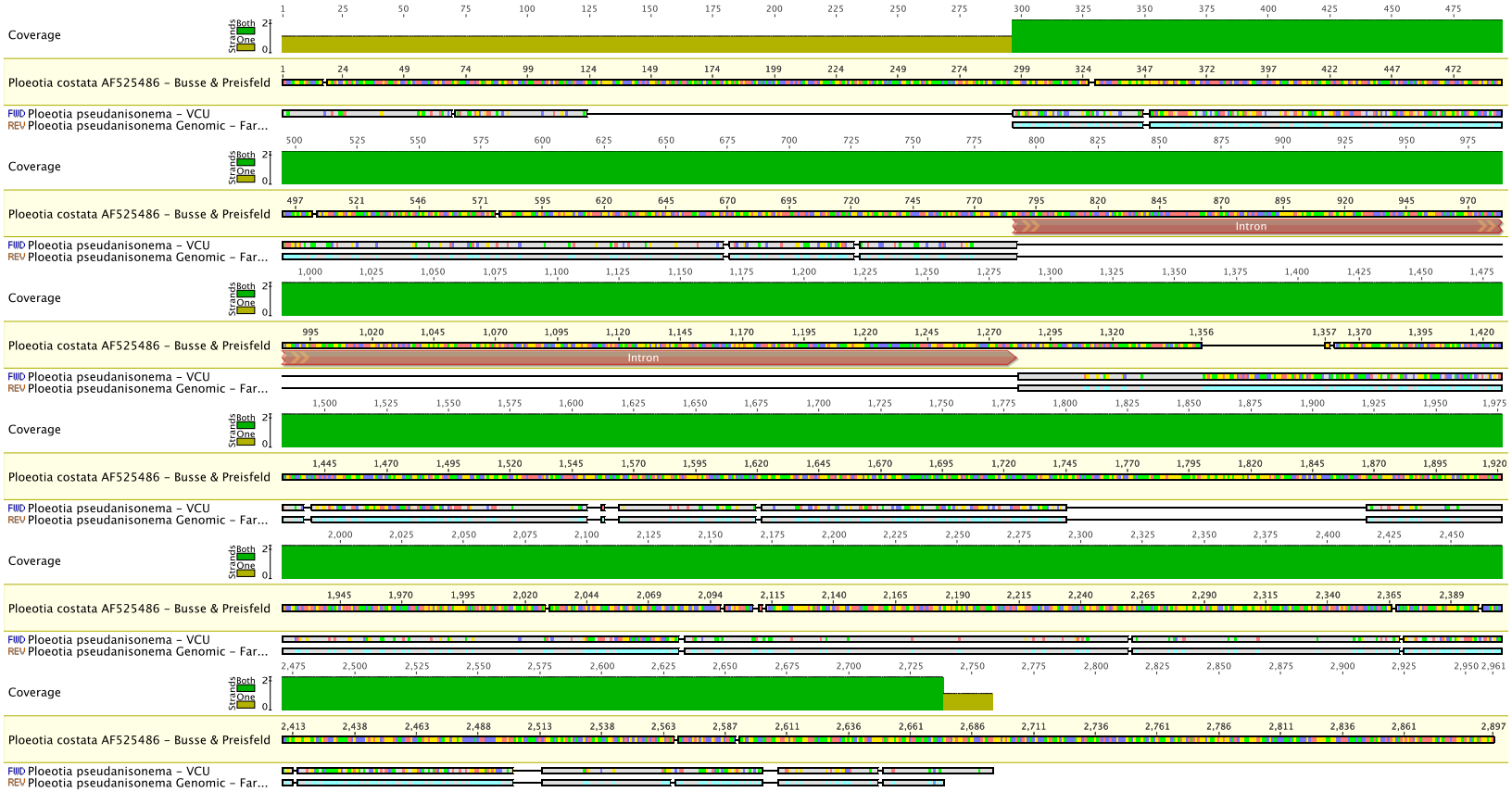
Ploeotia costata whole genome extraction sequences were compared to GenBank sequence AF525486 submitted by Busse and Preisfeld (8), and found to be identical (Figure 3.7). These comparisons confirmed the presence of an intron in the ribosomal SSU gene.

Figure 3.5: Comparison of *Ploeotia pseudanisonema* cDNA and Genomic SSU sequence



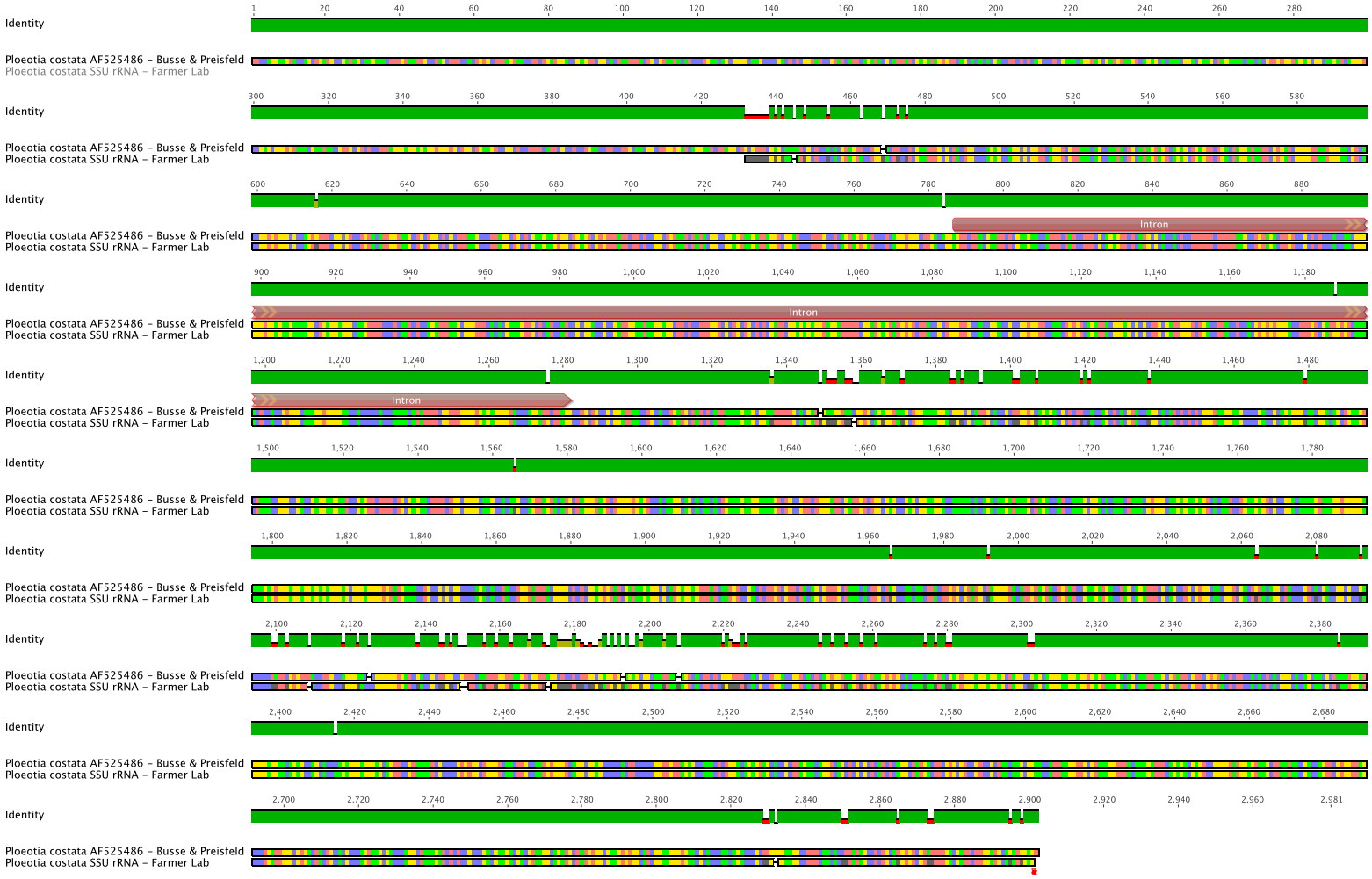
Alignment of Complementary and Genomic SSU rRNA of *Ploeotia pseudanisonema*. Alignment created using Geneious R 6 6.1.5. Complementary DNA created from SSU rRNA isolated from *P. pseudanisonema* by our collaborators at Virginia Commonwealth University is compared to Genomic *P. pseudanisonema* DNA isolated by our lab. The two sequences are shown to have greater than 99% similarity, negating the presence of a self-excising Group I intron in the Genomic SSU rRNA gene.

Figure 3.6: Comparison of *Ploeotia costata* Genomic SSU to *Ploeotia pseudanisonema* Genomic and cDNA SSU sequence



Multiple sequence alignment of *Ploeotia costata* and *Ploeotia pseudanisonema*. Alignment created using Geneious R 6 6.1.5. The Group I intron found in *Ploeotia costata* is observed between bases 787 to 1280 of sequence AF525486. In this figure the intron sequence is annotated by a thick red line. No congruency is shown between *P. costata* sequence AF525486 and *P. pseudanisonema* SSU sequences obtained by this lab or by our collaborators at Virginia Commonwealth University for the duration of the intron sequence.

Figure 3.7: Confirmation of *Ploeotia costata* intron sequence

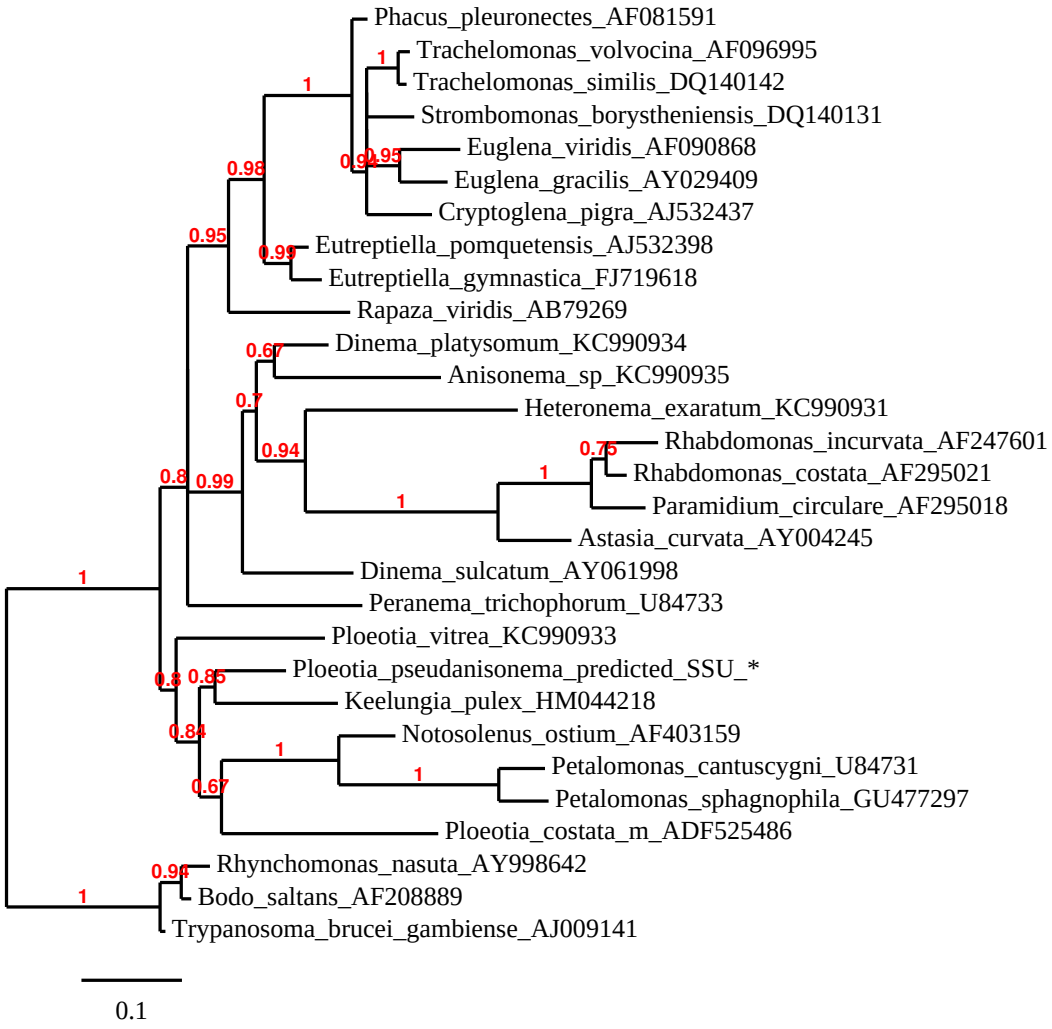


Alignment of GenBank *Ploeotia costata* SSU rRNA gene sequence *Ploeotia costata* SSU rRNA gene sequence isolated by this lab. Created using Geneious R 6 6.1.5. The Group I intron found in *Ploeotia costata* is observed between bases 787 and 1280. In this figure it is annotated by a thick red line labelled "Intron." A consensus sequences obtained by this lab shows strong consensus with *P. costata* sequence AF525486, submitted to GenBank by Busse and Preisfeld (8).

Phylogenetic Analysis

The *Ploeotia pseudanisonema* ribosomal SSU gene was compared to that of 28 related euglenoids using ClustalW, and a maximum likelihood analysis was performed using PhyML available through Phylogeny.fr portal. Sequences averaged 2229 base pairs in length. Our analysis supported four well-documented clades: kinetoplastids, which were designated as the outgroup, and euglenids with osmotrophic, autotrophic, and heterotrophic feeding behaviors. The autotrophic and osmotrophic clades were well supported, with high bootstrap values. *Peranema trichophorum* is heterotrophic, but possesses a helical pellicle, and therefore belongs to the same clade as the osmotrophs and autotrophs, all of which possess a helical pellicle. The *Ploeotia* species were somewhat dispersed within the heterotrophic clade. *Ploeotia vitrea* appears to be a basal member of the heterotrophic clade. *Ploeotia costata* was recovered in a monophyletic group with the genera *Notosolenus* and *Petalomonas*, but with only 67% bootstrap support. In all versions of the phylogram created, the *P. costata*, *Notosolenus*, and *Petalomonas* clade was recovered, but with only modest support (approximately 50-70%). *Ploeotia pseudanisonema* and *Keelungia pulex* formed a clade with a Maximum Likelihood bootstrap support of 85% (Figure 3.8). This clade was recovered with strong support ranging from approximately to 85-95%.

Figure 3.8: Euglenid phylogenetic tree



Maximum likelihood analysis phylogenetic tree based on SSU rRNA from *Ploeotia pseudanisonema* and 28 different euglenoids. Accession numbers of sequences obtained from NCBI Genbank are present. * indicates an currently unpublished complimentary DNA sequence obtained by our collaborators at Virginia Commonwealth University, and corroborated by sequences obtained in this lab. Branches labelled with maximum likelihood bootstrap percentages. Branches with bootstrap values below 50% are collapsed. Modes of nutrition are based on Leander et al. (38).

Chapter 4

Discussion

4.1 Light Microscopy

Differential interference contrast light microscopy observation of *Ploeotia pseudanisonema* confirms most of the description of morphology and movement published by Larsen and Patterson (37). The only major differences observed by this group are the general size range of the cell body, and the length of the recurrent flagellum. The size range observed by this lab was 9-14 μm in length by 6-8 μm in width, for vegetative cells. The recurrent flagellum was consistently observed to be smaller than four times cell body length. Recurrent flagella of approximately three times cell body length were regularly observed. This morphological difference could be a result of long-term culture of a single-cell isolate *P. pseudanisonema* strain.

The presence of a mucoid test-bound cyst stage has not been reported in other members of *Ploeotia*, or in *Keelungia pulex*, though it is well documented amongst autotrophic euglenids, including *Euglena* (31), *Trachelomonas* (17), and *Eutreptiella gymnastica* (44). In culture, a significant portion of *P. pseudanisonema* cells will secrete a mucus coat and enter a cyst stage within one week, with encysted cells remaining viable for at least one month. Formation of these cysts seems to coincide with stationary growth phase of the culture.

Hindak, 2000 indicates three types of cyst for *Euglena*: 1. Protective; 2. Reproductive; 3. Resting or Transient. The low numbers of cyst stage cells before stationary growth phase seems to indicate that the cyst is not involved with reproduction. A lack of predation or exposure to changes in environmental conditions would preclude a protective purpose for the cysts. It seems likely, therefore, that these cysts are simply a resting or transient stage of the *P. pseudanisonema* lifecycle that was not observed in environmental samples.

4.2 Electron Microscopy

The feeding apparatus of *P. pseudanisonema* is comprised of two supporting rods, a comb, and a cytopharynx. Both supporting rods are composed of two layers of microtubules surrounded by an electron-dense matrix, and each has two associated vanes. The vanes are hook shaped, and possess one supporting microtubule each. They do not remain appressed to the rods for the entire length of the feeding apparatus. Based upon these findings, the feeding apparatus of *P. pseudanisonema* is classified as a Type II, in accordance with the four categories described by Triemer and Farmer (51). In the anterior half of the cell, a fifth microtubule appears between the four vanes, but is itself unassociated with a single vane.

P. pseudanisonema possesses a lip structure similar to that of *Ploeotia costata*, *Ploeotia vitrea*, *Keelungia pulex*, and *Entosiphon sulcatum*(15; 26; 53). When viewed by scanning electron microscopy, the lip structure is C-shaped, like that of *P. costata*, *P. vitrea*, *Keelungia pulex*, and *E. sulcatum* (15; 26; 53). Between the lips, is a recessed plate, which arises from the right supporting rod. Beneath the lips is a comb structure made of two layers of microtubules in an electron dense matrix. The lip structure of *P. pseudanisonema* posses a unique structure compared to heretofore studied *Ploeotia* species. Images collected during this study show no signs of microtubular roots connecting the lip structure to the pellicle, as are seen in *P. costata*, *P. vitrea*, and *E. sulcatum* (53).

The feeding apparatus of *P. pseudanisonema* was never observed to protrude like that of *E. sulcatum*, but the recessed plate observed in *P. pseudanisonema* is supported by a proteinaceous scaffold, remarkably similar to that of *E. sulcatum* (53)(Figures 23 & 24). The presence or absence of this scaffold structure has not been documented in *P. costata*, *P. vitrea*, or *K. pulex*.

4.3 Small Subunit rRNA Genetic Analysis

Comparison between *P. costata* SSU sequences obtained in this lab, and those published Busse and Preisfeld (8) confirmed the presence of a Group I intron within the SSU gene of *P. costata*. A comparison between genomic DNA and complimentary DNA of *P. pseudanisonema* found the genomic and rRNA sequences to be identical, negating the possibility of a related Group I intron in *P. pseudanisonema*. Our phylogenetic analysis of the genus *Ploeotia* indicated a greater-than-expected evolutionary distance between *P. costata* and *P. pseudanisonema*.

Phylogenetic Analysis

Our phylogram showed *Ploeotia pseudanisonema* and *Keelungia pulex* form a clade together with greater than 85% Maximum Likelihood support. This clade indicates that *P. pseudanisonema* and *K. pulex* are more closely related to one another than they are to any other member of the heterotrophic euglenid clade. Within the non-helical heterotrophs, *Ploeotia vitrea* is the most basal member in all iterations of this tree. The genera *Notosolenus* and *Petalomonas* form their own strongly supported clade. *Ploeotia costata* forms a clade with *Notosolenus* and *Petalomonas*, but support for this clade is mediocre at best, with Maximum Likelihood bootstrap support never exceeding 70%. This clade may change with the inclusion of more *Ploeotia* SSU sequences.

The clades comprised of euglenids with helical pellicles are well supported. *Peranema trichophorum* represents the basal member of the helical pellicle clade. *Entosiphon sulcatum* is highly divergent from other non-helical heterotrophic euglenids, and as such, it is not included in this iteration of the euglenid phylogenetic tree.

Re-evalutating Genus *Keelungia*

Chan et al. (15) present a convincing argument for the creation of a new species for *K. pulex*, as its overall morphology is unique, and their phylogenetic evidence for the creation of genus *Keelungia* is sound. Our novel phylogenetic data present a strong relationship between *K. pulex* and*P. pseudanisonema*. Several physical similarities also exist between the two species. Both possess 10 delicate longitudinal grooves with a posterior swirl, and while *K. pulex* lacks proteinaceous pellicle strips, it is, thus-far, the only euglenid known to possesses this apomorphy. While unique, the feeding apparatus support rod substructure of both species are more similar to each other than they are to either *P. costata* or *P. vitrea*. The support rod substructure of both *P. vitrea* and *P. costata*, shown in Farmer and Triemer, 1988, Figures 8 & 9 (26), feature a more robust cement layer with openings embedded within the cement layer, and a sigmoidal projection from the left support rod of both species. The support rods of *K. pulex* and *P. pseudanisonema* share a similar morphology in that both have a delicate cement layer; protruding arches of cement, rather than embedded openings; and they both lack the sigmoidal projections (Figure: 3.4); Chan et al., Figures 24 & 26 (15).

Based upon feeding apparatus sub-structure similarities, and more importantly, upon phylogenetic similarity of the ribosomal SSU gene, we recommend that *P. pseudanisonema* be included within the genus *Keelungia*, and renamed *Keelungia pseudanisonema* (Larsen and Patterson) Brooks. However, such a re-description would require a modification of the description of *Keelungia*. The lack of proteinaceous pellicle strips is currently the distinguishing physical feature of *Keelungia*. Including the lack of proteinaceous pellicle strips my be

problematic as a generic descriptor, as *K. pulex* is currently the only euglenid known to exist that lacks proteinaceous pellicle strips, which could lead to the exclusion of future organisms that otherwise closely fit the type description, as is the case with *P. pseudanisonema*. This could lead to the creation of extraneous genera that will further complicate an already complex clade, or the inclusion of new species into the genus *Ploeotia* that have a divergent SSU gene, but are physically similar to the type species, *P. vitrea*.

Future morphological and phylogenetic study of the species currently belonging to the genus *Ploeotia* will help to clarify the complex relations within this genus, possibly leading to the renaming of several species as more phylogenetic data become available for comparison. The further elucidation of the heterotrophic euglenid lineage may also provide insights into the basal characteristics of heterotrophic euglenids.

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