Morphology, Molecular Systematics, and Phylogenetics of a Novel Ciliate, *Scindostoma hanania* (Alveolata: Ciliophora)

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Dedication

The author wishes to dedicate this thesis to his father, Hanania Michael Foox.
Acknowledgments

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Abstract of Thesis

Morphology, Molecular Systematics, and Phylogenetics of a Novel Ciliate, *Scindostoma hanania* (Alveolata: Ciliophora)

Ciliates (Phylum Ciliophora) are the largest group of single-celled organisms, some of the most complex cells known, and are vital to nearly every aquatic ecosystem. Historical understanding of the members of Ciliophora has changed with the development of new methods and can be explained in five “ages.” Today’s age, the “Age of Refinement,” is one in which molecular data have been infused with morphological data in order to reshape the phylum. The purpose of this study is to fully describe a novel ciliate, *Scindostoma hanania*. This study describes the morphology and ultrastructure of this ciliate, elucidates its phylogenetic position through molecular data, and ultimately verifies this phylogeny in a total evidence framework. The findings of this study require taxonomic revision within the order Prorodontida, and the thesis concludes with a formal description of a resurrected family, Cryptocaryonidae, to which *Scindostoma hanania* belongs.

These findings underscore the poor understanding of the phylum Ciliophora and verify the need for “refinement.” Works such as this one ameliorate the relatively small amount of data available, and are another piece in the puzzle of ciliate evolution. This thesis provides the groundwork for future redescriptions of the phylum.

Any names or nomenclatural acts within this thesis are disclaimed for nomenclatural purposes, and the thesis is not issued for any purposes of zoological nomenclature.
Table of Contents

Dedication.........................................................................................................................ii
Acknowledgments.............................................................................................................iii
Abstract of Thesis............................................................................................................iv
List of Figures....................................................................................................................vi
List of Tables.....................................................................................................................vii
Preface...............................................................................................................................viii

Chapter 1: Morphological and Ultrastructural Description of a Novel Ciliate
(Alveolata: Ciliophora) Scindostoma hanania.................................................................1

Chapter 2: Molecular Systematics and Phylogenetic Placement of a Novel Ciliate
(Alveolata: Ciliophora), Scindostoma hanania .................................................................25

Chapter 3: Corroborating Phylogenetic Position of Scindostoma hanania and
Recovering the family Cryptocaryonidae through Total Evidence Analysis.................51

References.........................................................................................................................11, 35, 63

Appendices.........................................................................................................................103
List of Figures

Figure a. Variation in cell morphology in the phylum Ciliophora.................................xviii
Figure b. Schematic of basic ciliate morphology........................................................xx
Figure 1.1-1.8. Light and scanning electron microscopy of *Scindostoma hanaia*..........15
Figure 1.9-1.13. Somatic cortex and ciliature..............................................................17
Figure 1.14-1.16. Somatic and oral dikinetids..............................................................19
Figure 1.17-1.21. Oral organization............................................................................21
Figure 1.22-1.27. Brosse structure and other cellular components............................23
Figure 2.1. Maximum likelihood phylogenetic tree.......................................................41
Figure 2.2. Highest posterior probability Bayesian inference tree..............................43
Figure 2.3. Strict consensus parsimony tree...............................................................45
Figure 2.4. Phylogenies of class Prostomatea based on different alignments..............47
Figure 2.5. Phylogenies of class Prostomatea under same alignment but different optimality criteria.................................................................49
Figure 3.1. Summary cladograms with support matrices at nodes...............................101
List of Tables

Table 1.1. Biometrical data of *Scindostoma hanania*…………………………………13

Table 3.1. Morphological characters and GenBank accession numbers used in total evidence analysis........................................................................................................................................91

Table 3.2. Studies consulted for morphological and molecular data…………………95

Table 3.3. Nucleotide substitution costs for POY analyses. .................................99
Ciliates (phylum Ciliophora) comprise one of the largest groups of single-celled organisms, with over 10,000 described and many more undescribed species. They are among the most diverse unicellular organisms (Fig. a); they range from as small as a few micrometers to as large as two millimeters, inhabit aquatic systems around the world, and exhibit a wide variety of life cycles and strategies, from free-swimming heterotrophs to obligate parasites (Lynn and Small 1991). Ciliates are also among the most complex unicellular organisms, containing locomotory and chemosensory cilia, multiple nuclei, and cytostomes (mouth structures). Many can encyst to survive extended periods of environmental stress. Some harbor endosymbiotic bacteria or algae. By consuming bacteria, microscopic fungi, and other ciliates, they play vital roles in nearly every aquatic ecosystem, ranging from ponds to oceans, lakes to rivers, and even soils and shallow, sandy waters (Lynn and Small 1991).

Several features define a ciliate. These features include: (1) the presence of microtubular hair-like structures called cilia; (2) the presence of a diploid micronucleus
(responsible for storing of genetic information passed on to daughter cells following
reproduction) as well as a polyploid, vegetative macronucleus (responsible for all protein
transcription); (3) the ability to reproduce both by asexual mitosis and by sexual
conjugation, during which individuals will form cytoplasmic connections and exchange
genetic material.

Ciliates are grouped in the phylum Ciliophora and are one of four phyla in the
superphylum Alveolata. The Ciliophora are united with Apicomplexa, Chromerida, and
Dinoflagellata, by possessing flattened, membrane-bound vesicles forming a continuous
system underneath the outer limiting membrane (cortical alveoli). The phylum is
currently divided into two subphyla: the Postciliodesmatophora, with two classes, and the
Intramacronucleata with ten classes (Lynn 2008).

As cellular biological methods have advanced and new data have been
accumulated, the historical understanding of systematics of the Ciliophora has changed.
Corliss (1979) explained that changes in ciliate systematics occurred in four “periods.” In
the Age of Discovery (1880-1930), ciliates were observed and described through light
microscopy without the use of cellular staining techniques, as exemplified by Bütschli
(1887-1889) and Kahl (1930-1935) (Lynn 1991). In the Age of Exploitation (1930-1950),
as understanding of ciliate diversity improved, the number of described taxa more than
doubled. In the Age of Infraciliature (1950-1970), staining procedures such as the
Chatton-Lwoff silver staining protocol (used in this thesis) helped reveal infraciliary
features of cilia, allowing for clearer distinction among ciliates. In the Age of
Ultrastructure (1970-1990), transmission electron microscopy allowed biologists to
explore ultrafine morphology within cells and resolve many of the remaining questions in
systematics and cell biology of ciliates. Greenwood et al (1991a) suggested that the movement to integrate molecular data into phylogenetic studies be named the Age of Refinement. The use of molecular data in several papers (Elwood et al. 1985; Lynn and Sogin 1988; Baroin et al. 1988; Adoutte et al. 1989) both demonstrated tremendous genetic diversity within the phylum and reaffirmed the major clades established based on ultrastructural research. Though the incorporation of molecular data has helped reshape our understanding of ciliate diversity into what it is today, much work remains. The only locus widely amplified within the ciliate genome has been the 18S small subunit ribosomal RNA gene, and it has been widely documented that gene trees are not necessarily concordant with species trees (e.g., Pamilo and Nei 1988; Maddison 1997). Further, taxon sampling must be increased, as neither ultrastructure nor molecular sequences has been explored for a majority of ciliate diversity. By providing new ultrastructural and molecular data, works such as this one help contribute to our understanding of this relatively poorly explored phylum, and will help to resolve the challenge of reconstructing ciliate evolution.

BASIC CILIATE ANATOMY

Ciliates are very complex eukaryotic cells. A description of some of the fundamental components of these cells is provided here to improve the clarity of this thesis (Fig. b). Note that not all of these features are found in every species.

1. Pellicle (sometimes called the cortex)
The pellicle refers to the outer 1µm of the cell and includes secreted structures that might be present on the cell surface. This includes:

a. **Plasma membrane**: The cell surface of ciliates, as in other eukaryotic cells, is limited by a cell membrane that has a layer of glycoproteins (the glycocalyx) associated with its inner and outer surface.

b. **Perilemma**: A secreted layer covering the surface of the cell. Its function is unknown.

c. **Alveoli**: Membrane-bound flattened vesicles forming a continuous system underneath the outer limiting membrane. This alveolar membrane is synapomorphic to the Alveolata, the superphylum containing the phylum Ciliophora.

d. **Epiplasm**: A thin, proteinaceous layer of varying thickness just below the alveolar membrane.

e. **Alveocolysts**: Invaginations of the inner alveolar membrane that penetrate the epiplasm, forming membranous sacs.

2. **Somatic (body) cilia**

a. **Kinety**: The somatic cilia of a ciliate are organized in rows called kineties (singular kinety).

b. **Kinetosome**: Synonymous with “basal body.” A microtubular structure similar in structure to a centriole that gives rise to the cilium.

c. **Kinetid**: The kinetosome, its associated fibrils and the cilium. Fibrils commonly associated with the kinetid are the transverse and postciliary
microtubular ribbons and the striated kinetodesmal fiber. These associated fibrils and the pattern they form is called the infraciliature.

i. Transverse microtubules originate adjacent to the anterior side of the kinetosome and extend up towards the cell surface before bending towards the adjacent kinety.

ii. Postciliary microtubules originate adjacent to the posterior side of the kinetosome and extend up towards the cell surface before bending towards the posterior end of the cell.

iii. Kinetodesmal fibers originate adjacent to the basal body and extend towards the anterior end of the cell.

d. **Parasomal sacs:** Invaginations of the cell membrane adjacent to basal bodies. Sites of nutrient uptake.

e. **Subkinetal microtubules:** Robust microtubular ribbons that run parallel to and underneath kineties, but do not originate from the kinetosome.

f. **Basal microtubules:** A small collection of microtubules that are immediately adjacent to kinetosomes that run parallel to the entire length of kineties just underneath the inner alveolar membrane, but do not originate from the kinetosome.

3. **Oral apparatus** – Most ciliates are phagotrophic, ingesting food through a specialized opening on the surface of the cell.

   a. **Cytostome:** Oral opening into the cell.

   b. **Cytopharynx:** A gullet that extends from the cytostome to the site where food vacuoles are formed.
c. **Oral kinetosomes**: Specialized cilia surrounding the cytostome. The cilia are involved in prey capture and manipulation. The infraciliature of these cilia often support and underlie the cytopharynx.

d. **Nematodesmata**: Dense hexagonal bundles of microtubules that partially or completely surround and support the cytopharynx.

e. **Brosse (also called the Brush)**: Specialized cilia found adjacent to the oral area. Believed to be chemosensory.

4. **Osmoregulation and waste removal**

   a. **Cytoproct**: A structure in the membrane through which undigested residue in the food vacuoles is extruded from the cell.

   b. **Contractile Vacuole**: A membrane-bound, microtubular supported structure that pumps water from the cell and helps maintain ion balance. The pore through which fluid is pumped is a permanent feature of the cell membrane.

5. **Nucleus**

   a. **Macronucleus**: The one or more large, usually polyploid nucleus that regulates metabolism of the cell can have a variety of shapes (oval, elongate, beaded, etc).

      i. **Homomeric**: The condition in which the chromosome fragments are evenly spread out throughout the nucleus.

      ii. **Heteromeric**: The condition in which chromosome fragments are clumped on one end of the nucleus.

   b. **Micronucleus**: The one or more small, oval, diploid nucleus that is primarily responsible for sexual recombination.
c. **Conjugation:** The basic sexual process in which macronuclei are usually resorbed and micronuclei divide by meiosis to form haploid nuclei. One of these haploid micronuclei is exchanged through a cytoplasmic bridge that forms between two individuals. Once exchanged the nucleus that has entered the cell from the mating partner fuses with one from the cell to form a new diploid micronucleus. The new diploid micronucleus will divide by mitosis and one of the daughter nuclei will replicate its DNA to become a polyploidy macronucleus.

6. **Extrusomes** (exocytotic vesicles that discharge their contents outside the cell when subjected to a stimulus)
   
   a. **Mucoeysts:** Ovoid to elongate membrane-bound vesicles that exude mucus-like material.
   
   b. **Toxicysts:** A “tube within a tube” structure that telescopes out when fired. They are used primarily in prey capture and are localized in very specific regions of the cell (most often the oral area).
   
   c. **Trichocysts:** Capped, spindle-shaped vesicles that extrude a long filament.

It is important to understand that this work represents the tip of an iceberg. As more ultrastructural and genetic data become available for an increasing proportion of ciliate diversity, our systemic and phylogenetic understanding of the phylum will continuously change. With regard to molecular phylogenetic inference, not only must genetic data be accumulated, but for more than one locus. The evolutionary history of the 18S gene is not sufficient to reconstruct the evolutionary history of ciliate species. The
lack of ultrastructural data for a large portion of ciliates further limits our understanding of the phylum. This thesis underscores the importance of these limited data; though the phylogenetic position of a novel ciliate is elucidated with multiple lines of evidence, it is clear that any phylogenetic inference can only be tenuously accepted. The class Prostomatea, to which this ciliate belongs, is particularly poorly understood and weakly described. These findings emphasize the need for a holistic revision of this class. The following is a necessary stepping-stone, and is one that will hopefully promote the future work that must be done.

LITERATURE CITED


Figure a. Variation in cell morphology in the phylum Ciliophora. (1) *Paramecium tetraurialia*, an elongant, ovoid oligohymenophorean with midventral oral region. (2) *Chlamydodon mnemosyne*, an ellipsoidal ciliate with a prominent “railroad track” groove. (3) *Stentor coeruleus*, a highly contractile, trumpet-shaped ciliate. Its oral cilia spiral all around its flared-out anterior end. (4) *Euplotes sp.*, an ellipsoidal ciliate with a distinct ciliary lapel projecting from its adoral zone. (5) *Vorticella sp.*, a highly contractile, stalked peritrich that can compress into a tight, helical coil. Feeding nearby is *Coleps sp.*, a barrel-shaped cell armed with calcium carbonate alveolar plates with small, lateral teeth. (6) *Acineta tuberosa*, a triangular trophont, laterally flattened and encased in a calcium carbonate lorica, stalked and with ciliary tentacles. All images by Diana Lipscomb and used with permission.
Figure b. Schematic of basic ciliate morphology, showing somatic kineties (K), oral kineties (O), the cytostome (C), the macronucleus (Mn), and the micronucleus (Mi). Magnification shows basic components of the kinetid (Ki), with the kinetosome (Ks) and its associated fibrils, including the transverse (T) and postciliary (Pc) microtubular ribbons and the kinetodesmal fiber (Kd). A cartoon of a ciliate’s digestion shows ingestion of food through the cytostome (C), into a food vacuole (FV), and eventual excretion through the cytoproct (Cp). Image by Diana Lipscomb and used with permission.
Chapter 1: Morphological and Ultrastructural Description of a Novel Ciliate (Alveola: Ciliophora), *Scindostoma hanania*

*Scindostoma hanania* is a novel ciliate found in marine salt marshes of the Delmarva Peninsula of the United States. The purpose of this study is to describe its morphology and ultrastructure through light microscopy and scanning and transmission electron microscopy.

**MATERIALS AND METHODS**

*Scindostoma hanania* was found in environmental samples, including sediment and water, taken from ponds on Burton’s Island at the Indian River inlet at Rehoboth, Delaware (38° 35’ N, 75° 04’ W) in May and June 2011. These samples were maintained in tightly sealed jars in the lab for up to six months. Subsamples of the environmental samples were examined with a stereomicroscope. *Scindostoma hanania* cells were isolated via micropipette into spot wells containing marine water filtered through Cameo 0.22µ Nitrocellulose syringe filters. For light microscopy, cells were observed live using brightfield and differential interference contrast microscopy on a Leica Leitz DMRB.
Morphological details were further investigated using: (1) Chatton-Lwoff stained cells (Foissner 1991), (2) methylene blue stains of 1 μm semithin sections of cells embedded for transmission electron microscopy (Huber et al. 1968), and (3) Hoechst Fluorescent stained cells (Latt et al. 1975).

For scanning electron microscopy, cells were transferred to BEEM capsule baskets (Hayunga 1977), fixed in Karnovsky’s fixative (2.5% v/v glutaraldehyde, 2.5% v/v paraformaldehyde, 0.1M cacodylate buffer, 0.7M sucrose, 2.5mM CaCl₂), rinsed with 0.1M cacodylate buffer, secondarily fixed with 2% (w/v) osmium tetroxide in cacodylate buffer for 30 minutes, dehydrated, critical point dried, mounted onto an SEM stub and sputter coated. Specimens were examined with a LEO 1430VP scanning electron microscope.

For transmission electron microscopy, cells were transferred to BEEM capsule baskets (Hayunga 1977), fixed in Karnovsky’s fixative, rinsed with buffer, secondarily fixed with 2% (w/v) osmium tetroxide in cacodylate buffer for 30 min, dehydrated, and embedded in an epoxy resin (Epon 812). Ultrathin cellular sections were stained first with 4% (w/v) aqueous uranyl acetate followed by 0.2% (w/v) lead citrate. Sections were examined on a JEOL JEM 1200EX transmission electron microscope.

The terminology used to describe ciliate morphology follows that of Lynn 2008.

RESULTS
Light and scanning electron microscopy. *Scindostoma hanania* is an oval ciliate with a slightly flattened, slit-like oral area (Fig. 1.1). It measures about 113-160 µm in length and 80-120 µm in diameter (Table 1.1).

At the anterior end of the cell is an apical, slit-like cytostome, surrounded by swollen “lips” that have a series of lines (or “ribs”) that extend from the outer margin of the lips inward to the cytostome (Fig. 1.2, 1.3, 1.4). These prominent lips measure 33-41 µm in length (37.2 µm average) and 18-29 µm (24.4 µm average) at their widest point. The cytostome is not surrounded by a ring of paired oral kinetosomes, as would be typical of a prostome (Corliss 1979); instead, the oral ring is split into two, non-connecting, semicircular segments. Each segment originates on either side of what is approximately the widest point of the lips, wraps around the lips in opposite directions, and extends past the end of the lips and down the side of the cell approximately 15 µm (Fig. 1.4). Associated with these oral dikinetids is a row of palps (Fig. 1.3) (see ultrastructure). Three brosse kineties originate posterior to the terminal end of one oral kinetosome segment and extend posteriorly (Fig. 1.4, 1.5). The brosse is aklitoloph, meaning that the adjacent somatic kineties do not abut on either side of the brosse (Hiller and Bardele, 1988); instead, three kineties terminate at the posterior end of the brosse, while neighboring kineties circumvent the brosse and continue towards the edge of the oral kinetids on one side and towards the edge of the lips on the other side (Fig. 1.5).

The cell is holotrichous, with evenly distributed somatic cilia emerging from rectangular pits on the surface of the cell (Fig. 1.6) and running in a range of 108-114 rows called kineties (Table 1.1) (Fig. 1.4, 1.5). The majority of these somatic kineties are bipolar, originating posterior to the oral kinetosomes and terminating at the posterior pole.
The oral dikentid rows do not fully surround the cytostome, leaving two regions absent of dikentids. At these regions, the bipolar somatic rows originate adjacent to the cytostomal ribs, and extend toward the posterior pole akin to the other somatic kineties. There are, however, three exceptions to where the kineties originate: (1) as the oral dikentid rows do not fully surround the cytostome, two regions adjacent to the cytostome are “naked”; as a result, 15-17 (16.1 average) somatic kineties originate at the edge of the cytostomal lips. Three of these rows originate in the space between the edge of the lips and the side of the beginnings of the oral dikinetids; (2) adjacent to the oral dikinetidal row not associated with the brosse, 16-21 (18.0 average) kineties originate at the edge of the cytostomal lips, three of which originate in the space between the edge of the lips and the side of the oral dikinetids; (3) three kineties originate at the posterior end of the brosse (Fig. 1.4, 1.5). All kineties terminate at the posterior pole. Scattered throughout the cell in between kinetal rows are contractile vacuole pores (Fig. 1.8).

A single, large, ovoid to dumbbell-shaped macronucleus is typically found underneath the cytostome, located approximately one-third of the length of the cell down into the cytoplasm (Fig. 1.1, 1.9). The micronucleus was not observed.

*Scindostoma hanania* characteristically swims in an erratic, epicyclical pattern. As it corkscrews through the water, it rotates unevenly, favoring one dorsoventral side. This is perhaps a result of an uneven distribution of sensory cilia, with the three brosse kineties located on just one side of the cell. This may cause the cell’s axis of rotation to be based off of the concentration of cilia rather than the center of the cell. *Scindostoma hanania* was not observed dividing and perhaps replicates in a cyst stage. Green cells that appeared to be algae were observed in food vacuoles.
**Ultrastructure.** The somatic cortex of the cell is composed of a plasma membrane and a well-developed alveolar system which together form a network of longitudinal ridges and shallow furrows that delimit a series of roughly rectangular areas that demarcate an individual kinetosomal territory (Fig. 1.9). A thin, supporting layer of epiplasm underlies the inner alveolar membrane, but no longitudinally directed ribbons of microtubules are found extending within the apex of the cortical ridges (i.e., there are no ridge microtubules; Fig. 1.10). Spindle-shaped mucocysts are found inserted into the cortex throughout the cell.

The somatic cilia of *Scindostoma* arise from rectangular pits on the cell surface (Fig. 1.7). The majority of the somatic kineties are comprised of monokinetids for most of their length; however, the 1-4 anteriormost are dikinetids. All of these cilia have typical eukaryotic morphology (9+2 axoneme, flat axosomal plate, and normal kinetosome) (Fig. 1.10).

The somatic kinetosome has three associated infraciliary fibers: the kinetodesmal fiber, and the transverse and postciliary microtubular ribbons. The kinetodesmal fiber is striated and arises near triplets 5, 6 and 7 (Fig. 1.11). It extends anteriorly from the kinetosome toward the cell surface and terminates without overlapping with the kinetodesmal fibers of other kinetosomes.

The postciliary ribbon arises adjacent to kinetosomal triplet 9 at an angle approximately 90° to the kinety axis (Fig 1.12). These microtubules extend posteriorly and upward towards the cell surface where they end in the longitudinal cortical ridge without overlapping with the microtubules of the adjacent posterior kinetosome (Fig 1.10).
A radial transverse microtubular ribbon originates on the anterior side of the kinetosome (Fig 1.9). These microtubules extend laterally under the inner alveolar membrane and terminate before reaching the adjacent kinety (Fig. 1.13).

A parasomal sac is associated with each kinetosome, found between the postciliary microtubular ribbon and kinetodesmal fiber (Fig. 1.12). Subkinetal microtubules were not observed.

The somatic dikinetids occur at the anterior ends of the kineties. The anterior kinetosome of the pair has a single postciliary microtubule at triplet 9 and a tangential transverse microtubular ribbon adjacent to triples 4 and 5 (Fig. 1.14). These microtubules appear to end at the cell surface anterior to the pair of kinetosomes. The posterior kinetosome has the same infraciliature as the typical somatic monokinetid.

The cytostome is surrounded by oval, alveolated lips that are ribbed by the joining of two alveolar membranes. Both kinetosomes of the circumoral dikinetid are ciliated, with the anterior and posterior kinetosomes having a radial microtubular ribbon (Fig. 1.14). These ribbons neither overlap nor wrap around the cytopharynx. Both kinetosomes of the pair are ciliated and are connected by a striated, electron-dense fiber (Fig. 1.15). Each oral dikinetid has associated with it a toxicyst-bearing palp (Fig. 1.16).

At the base of the oral dikinetids are bundles of nematodesmata (microtubules) that extend into the cytoplasm (Fig. 1.17). These baskets appear highly reduced and are not found prominently around the cytopharynx. Instead, the cytopharynx is surrounded in greater numbers by electron dense fibers that originate near oral dikinetids and their associated toxicyst-bearing palps (Fig. 1.18). These filamentous, non-paracrystalline fibers are elongated, running into the cytoplasm, around the cytopharynx and adjacent to
nematodesmata. They also appear to be contained within membrane-bound capsules, with multiple fibers running concurrently within the same capsule (Fig. 1.19). This electron dense material is distinctly not straight, often bending in all directions within the capsule as it runs anteroposteriorly (Fig. 1.20). There are no obvious X, Y, or Z microtubules lining the cytopharynx. Instead, the electron dense fibers continue to border the lips further down into the cytoplasm (Fig. 1.21).

The brosse consists of three kineties within a depression on the cell surface immediately posterior to the oral opening. Within the depression, the kineties are separated from each other by longitudinal cortical ridges (Fig. 1.22). Each dorsal brosse kinety consists of a row of paired kinetosomes. Both kinetosomes are ciliated but the cilia are short and the axoneme is often distorted (Fig. 1.23). The anterior kinetosome of the pair is associated with a transverse microtubular ribbon on the anterior side. The posterior kinetosome has a single microtubule on its anterior side and a radial ribbon on its posterior side (Fig. 1.24). All of these microtubules extend into the cortical ridges where they terminate (i.e., they do not overlap with microtubules from adjacent kinetosomal pairs).

The endoplasm is separated from the ectoplasm by a tela corticalis which is occasionally pierced by organelles moving from the center of the cell towards the surface (Fig. 1.25). The endoplasm contains an extensive system of rough endoplasmic reticulum, mitochondria, food vacuoles in various stages of digestion, and a homomeric macronucleus. Heterochromatin and several nucleoli appear dispersed in the nucleoplasm of the macronucleus (Fig. 1.26). The micronucleus was not observed. A contractile vacuole pore supported by microtubules is visible near the somatic cortex (Fig. 1.27).
DISCUSSION

The morphology of *Scindostoma hanania* is highly reminiscent of a typical prostome ciliate. The members of the class Prostomatea are ovoid, holotrichous cells with an apical (to subapical) cytostome, bipolar somatic rows comprised of monokinetids (with a few anterior dikinetid rows in some taxa), and circumoral dikinetidal rows with nematodesmata originating from their bases (Lynn 2008). Ultrastructurally, the somatic kinetosomes of prostomes typically include anteriorly directed, non-overlapping kinetodesmal fibers, radial transverse microtubular ribbons, and slightly-to-fully convergent postciliary microtubular ribbons. The prostome somatic dikinetid is comprised of an anterior kinetosome bearing only a tangential transverse ribbon and a posterior kinetosome resembling somatic monokinetids. *S. hanania* possesses all of these traits, strongly suggesting that it is a member of the prostome class. Moreover, it is clear that *Scindostoma* is a member of the order Prorodontida (Corliss 1974), as the only other order (the Prostomatida) is defined by its cells lacking both toxicysts and a brosse. *Scindostoma hanania* possesses these two characters, making it a member of the order Prorodontida.

Though clearly having all the features of a prorodontid, *Scindostoma hanania* also possesses several characters, including autapomorphies, which give it taxonomic identity. Primarily, *S. hanania* lacks several microtubular features that are typical of prostomatid ciliates, such as subkinetal microtubules, ridge microtubules, and X, Y, and Z microtubules lining the cytopharynx. In addition to having these possible secondary
losses, this organism is defined by the presence of two morphological features: its unique oral dikinetid distribution, and its electron dense material supporting the cytopharynx. The oral dikinetids are broken into two, non-connecting segments that follow the outline of the cytostome before continuing posteriorly down either side of the cell surface. This differs from the typical prorodontid oral dikinetids—which form a distinct, closed ring around the mouth—and is not seen in any other known ciliate. The typical prorodontid also has numerous, robust baskets of nematodesmata originating underneath the oral dikinetids and running distally. *S. hanania* appears to have these baskets, but they are significantly reduced, and run adjacent to prominent lanes of electron dense fibers, with multiple fibers often running concurrently in a single membrane-bound capsule. This too is not seen in any other prorodontid, and perhaps not in any other ciliate.

It is from this point that characterizing *S. hanania* becomes a challenge; this organism is a prorodontid, and has autapomorphic features, but its position within the order is unclear. Nine families make up the order Prorodontida, and each is defined by an apomorphy that *S. hanania* does not possess (or *S. hanania* possesses a morphological feature that excludes it from certain families). Members of Balanionidae are not holotrichous and have an inward-facing brosse; the alveoli of members of Colepidae act as a cuirass, with rows of calcium carbonate plates with small lateral teeth; members of Lagynidae have perioral somatic ciliation with trikinetids; members of Placidae have slightly spiraling kineties with striae in between, a single dikinetidal row for their brosse, and extrusomes in a lateral pocket; members of Plagiocampidae have a subapical oral region with a single semicircle of oral dikinetids accompanied by extensible lappets in which toxicysts reside; members of Prorodontidae have ribbon-like macronuclei;
members of Urotrichidae are not holotrichous and have caudal cilia; members of Malacophryidae have quadrangular alveoli and paratenes. *S. hanania* does not have any of these diagnostic features. It is possible that *Scindostoma hanania* may fall into the Holophryidae, defined by the brosse as several to many kinetofragments and somatic kineties parallel to the brosse; however, these features are true of nearly every family in the Prorodontida, and cannot be the basis upon which *S. hanania* would be placed in Holophryidae. Because *S. hanania* lacks any of the aforementioned features, it cannot adequately be placed within any prorodontid family, despite having all the feature characteristic of a prorodontid.

The inability to place *Scindostoma hanania* within even a family—let alone genus or species—necessitates a phylogenetic analysis to elucidate its position within this major ciliate clade. The results of a phylogenetic analysis could provide a strong line evidence as to whether *S. hanania* belongs in any of the nine families established in the prorodontid order. Should this organism fall into one of these families, it will be necessary to revise that family in order to accommodate and more clearly define what comprises every one of its members. Should this organism fall at the base of this clade (e.g. outside of any of the nine families), it may be necessary to establish a new prorodontid family that will include the potentially monotypic *Scindostoma hanania*. Regardless, a simple morphological and ultrastructural description is insufficient to resolve the taxonomy of *S. hanania*, necessitating a phylogenetic investigation.
LITERATURE CITED


Table 1.1. Biometrical data of *Scindostoma hanania* with average (Avg), minimum (Min), and maximum (Max) values, as well as standard deviations (SD) and number of cells examined (N). All dimensions are in micrometers. For whole length and width calculations, 24 cells were stained with the Chatton-Lwoff protocol, while 4 were live cells under cover slips. All other measurements were taken with cells stained with the Chatton-Lwoff protocol. All measurements are in micrometers.
<table>
<thead>
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<th>Characteristics</th>
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<th>Min</th>
<th>Max</th>
<th>SD</th>
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<td></td>
<td></td>
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<td>159.9</td>
<td>11.2</td>
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<tr>
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<td>80.3</td>
<td>123.9</td>
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</tr>
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<td><strong>Kineties</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinetal rows</td>
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<td>108</td>
<td>114</td>
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<td>6</td>
</tr>
<tr>
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<td>15</td>
<td>17</td>
<td>0.8</td>
<td>6</td>
</tr>
<tr>
<td>Rows originating at cytostome to left of oral dikinetidal row not associated with brosse</td>
<td>18.0</td>
<td>16</td>
<td>21</td>
<td>1.8</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
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<td>24.4</td>
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<td>29</td>
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Fig. 1.1-1.8. Light and scanning electron microscopy of *Scindostoma hanania*. Figure 1 is interference contrast microscopy. Figures 1.2, 1.3, and 1.6 are scanning electron microscopy. Figures 1.4, 1.5, and 1.7 are Chatton-Lwoff stains. Figure 1.8 is fluorescent microscopy. All scale bars = 10 µm, except figure 6, which = 1 µm. 1.1. Live cell showing the prominent fold of the oral area (O) and location of the macronucleus (Mn). 1.2. Top-down angle of cell, showing the swollen lips (L) that surround the cytostome (C). 1.3. Lateral angle of oral area, with arrows pointing to toxicysts extruding from near the base of lips. 1.4. Top-down angle of cell, showing the split oral kinetosomes (OK), with the three-row brosse (B) posteriorly adjacent to one oral dikinetidal segment. 1.5. Somatic kineties (K) are bipolar, traveling from the anterior to posterior pole (P). The brosse (B) is aklitaloph, with circumventing neighboring kineties. 1.6. Close-up of rectangular areas that contain cilia. 1.7. Contractile vacuole pores (arrows) reside in between kinetal rows. 1.8. A single, ovoid-to-dumbbell shaped macronucleus (Mn) typically rests approximately one-third of the way into the cell, posterior to the cytostome.
Fig 1.9-1.13. Somatic cortex and ciliature. Bar = 1 µm. **1.9.** Somatic cortex of *Scindostoma hanania*. A network of ridges and furrows demarcate rectangular ciliary pits (arrows). Somatic kinetids (Ki) are visible, as well as mucocysts (Mu). **1.10.** Longitudinal section of a cilium (C) in the somatic cortex. A radial transverse microtubular ribbon is visible (arrow). **1.11.** The kinetodesmal fiber (Kd) is striated and non-overlapping. Mitochondria (M) are abundant in the cell, often pressing right up against the somatic cortex. **1.12.** Cross section of a somatic basal body, showing the kinetodesmal fiber (Kd), postciliary microtubular ribbon (Pc), and parasomal sac (Ps) in between the two. **1.13.** The radial transverse microtubular ribbon (arrow) terminates before reaching the adjacent kinety. This ribbon originates from the nearby basal body (Ba). Mitochondria (M) are seen pressed against the rectangular pit.
Fig 1.14-1.16. Somatic and oral dikinetids. Bar = 0.5 µm. 1.14. A cross section near the oral area of the mouth. Toxicysts (To) embedded in palps are associated with each oral pair (arrows). Both kinetosomes of the somatic dikinetid (SD) are paired. The posterior kinetosome is akin to a somatic kinetosome, with a kinetodesmal fiber (Kd) and a postciliary microtubular ribbon (Pc). Both oral kinetosomes are ciliated and have a radial microtubular ribbon (T). 1.15. The oral kinetosomes are connected by an electron dense fiber (EDF). 1.16. Each pair of oral kinetosomes (OK) has associated with it a toxicyst (To) within a palp. One toxicyst may have extruded and pushed an oral pair out of its row. A somatic dikinetid (SD) is visible adjacent to the oral row.
Fig 1.17-1.21. Oral organization. Bar = 0.5 µm. 1.17. Oral dikinetids (OK) in their row are visible, with postciliary ribbons (Pc) visible. This steeply angled section reveals a bundle of nematodesmata (N) where the next pair would be, implying that the nematodesmata originate at the base of the oral basal bodies. 1.18. Electron dense fibers (EDF) originate near the oral kinetosomes (OK) and toxicst-bearing palps (To). 1.19. The electron dense fibers (EDF) appear to reside within membrane-bound capsules (MC). 1.20. The fibers (arrows) are not straight, often bending in all directions within their capsules. 1.21. Deeper down in the cytopharynx, as the lips join, cross sections of electron dense fibers (arrows) can still be seen, whereas nematodesmata disappear, having terminated more anteriorly.
Fig 1.22-1.27. Brosse structure and other cellular components. Bar = 0.5 μm. 1.22.

Brosse kinetosomes are nestled among longitudinal cortical ridges (LCR). 1.23. A close-up shows short ciliated brosse kinetosomes (BK), many of which are distorted. 1.24.

Brosse dikinetdal kinetosomes. The posterior kinetosomes have radial ribbons (RR) and kinetodesmal fibers (Kd). Brosse microtubules do not overlap with microtubules from adjacent kinetosomal pairs (arrow). 1.25. A tela corticalis (TC) travels through the cytoplasm, separating the endoplasm from the ectoplasm. It is often interrupted by cellular material such as mitochondria (M). 1.26. A homomorphic macronucleus (Mn) with heterochromatin and several nucleoli. 1.27. Contractile vacuole pores (CVP) are found in between rows of somatic kineties; two kinetosomes (arrows) are seen.
Chapter 2: Molecular Systematics and Phylogenetic Placement of a Novel Ciliate
(Alveolata: Cilophora) *Scindostoma hanania*

Our understanding of the phylogeny of the phylum Ciliophora has changed with the advent of new technologies for studying these small organisms. Corliss (1974) has described the history of ciliate systematics by dividing it into four distinct periods: (1) the Age of Discovery (1880-1930), in which ciliates were first observed; (2) the Age of Exploitation (1930-1950) in which living organisms were studied and described using light microscopy; (3) the Age of Infraciliature (1950-1970) in which the development of specialized silver stains reveled many additional details of the ciliary patterns (i.e., the infraciliature); and (4) the Age of Ultrastructure (1970-2000) in which the transmission electron microscope was used to describe the ultrafine structure of the cells. In each period, classification schemes were altered to accommodate the new data. Lynn (2008) has suggested that we are currently in the Age of Refinement (sensu Greenwood et al. 1991), in which molecular data, particularly from the 18S small subunit ribosomal gene, help us to revise and solidify phylogenetic relationships, and to corroborate the taxonomic positions of ciliates (Elwood, Olsen and Sogin 1985; Sogin and Elwood 1986; Sogin et al. 1986a).
A novel ciliate, *Scindostoma hanania*, cannot be taxonomically placed on the basis of only its morphology and ultrastructure. Therefore, in the spirit of the Age of Refinement, a phylogenetic analysis using small subunit ribosomal gene data could shed light on the evolutionary relatedness of *Scindostoma hanania* to other ciliates, and may resolve its taxonomic placement.

MATERIALS AND METHODS

**Specimen preparation, DNA amplification, and sequencing.** *Scindostoma hanania* cells were isolated with a micropipette and placed into spot wells with marine water filtered through Cameo 0.22µ Nitrocellulose syringe filters. With as much water removed as possible, the cells were transferred into 2 ml Eppendorf tubes and held frozen at -20°C until enough cells were collected to be processed (~100 cells). DNA was extracted using the QIAGEN DNEasy® Tissue Kit (Appendix 2.1).

The 18S small subunit rRNA gene was amplified by polymerase chain reaction (PCR) using the universal eukaryotic forward primer EukA: 5′-CTGGTTGATCCTGCCAG-3′ and reverse primer EukB: 5′-TGATCCTTCCGAGGGTTC-3′ (Medlin et al. 1988). For internal fragments, the forward primers used were Gas+600: 5′- CGGTAATTCCAGCTCCAATAG -3′ and Gas+1390: 5′- CTGGTTAATCCGATAACG-3′, and the reverse primers used were L: 5′-CCAACTACGAGCTTTTTAACTG-3′, Gas+1220: 5′- CCTGTTGGTGCCCTTCCGT -3′, and Gas+1540: 5′- GGGCATCACAGACCCTGT-3′ (Jeong et al. 2004). PCR was carried out through a Variable Touchdown PCR under the following conditions: one
cycle of 3 min at 94°C; eleven cycles of 30 sec at 94°C, 1 min at 57°C (subtracting one degree every cycle), and 1 min at 72°C; ten cycles of 30 sec at 94°C, 1 min at 47°C, and 1 min at 72°C; and a final cycle of 20 min at 72°C, then held at 4°C until ready to be processed (Appendix 2.2).

The amplified products were cleaned and prepared for sequencing using an ExoSAP-IT PCR Product cleanup kit (Appendix 2.3). Sequencing was performed with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Foulton, CA) using all aforementioned primers. The fragments produced were assembled into a contiguous sequence using Geneious 5.5.6 (Drummond et al, 2011) (see Appendix 2.4 for sequence). The sequence for Scindostoma hanania will be deposited in GenBank.

**Taxon sampling.** Sequences from 123 species were obtained from GenBank. The taxa chosen are representative of the diversity of ciliates within all classes of the phylum Ciliophora. Eleven members of the subphylum Postciliodesmatophora were included as the outgroup.

Phylogenetic error such as long branch attraction can arise due to poor taxon sampling (Bergsten, 2005); this could be a problem when analyzing prorodontid ciliates, because so few identified species have been sequenced, and their sequences tend to be significantly more divergent than those of ciliates in other classes. In order to reduce the potential for long branch attraction, the sequence for Scindostoma hanania was submitted to the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (NCBI’s BLAST, http://blast.ncbi.nlm.nih.gov). This search returned environmental samples or uncultured eukaryotes that were identified as highly similar to S. hanania, and were thus putative members of the order Prorodontida. The accession numbers for these
samples included in the molecular analyses are AB695501, AM412525, AY642698, AY642703, AY665049, AY821920, DQ103858, DQ103872, DQ120009, DQ244026, DQ244029, DQ244032, EF023164, EF023327, EF024111, EF024295, EF024334, EF526888, EF527104, EF527121, EF527202, EU162617, EU162618, FN690043, FR874483, GQ330633, GU067973, HM135056, HM135060, and JN090894.

**Multiple sequence alignment.** Because phylogenetic results are sensitive to alignment methods (Higgins and Lemey 2009), a variety of alignment criteria and algorithms were explored. Sequences were aligned with: MAFFT’s G-INS-i algorithm with a 200PAM / k=2 scoring matrix, a 1.53 gap open penalty and an offset value of 0.123 (Katoh et al. 2002); CLUSTALW (Larkin et al. 2007) with its IUB cost matrix, with a gap open cost of 15 and a gap extend cost of 6.66; MUSCLE using anchor optimization with eight iterations (Edgar 2004); and on the T-COFFEE web server using the default, exhaustive alignment methods (Notredame et al. 2000), producing a total of four multiple sequence alignments (MSAs). The MSA for CLUSTAL had 1398 aligned characters, for MUSCLE had 1468, for MAFFT had 1407, and for T-COFFEE had 1444.

The four MSAs were then tested for performance under a log likelihood optimality criterion. Phylogenetic trees were constructed with each of the respective MSAs using the phylogenetic analysis program RAxML on the CIPRES web-server portal (http://www.phylo.org). Each tree was generated under the GTRCAT model (see next section for model selection) with the same parameter values. Log likelihood scores were then compared. The CLUSTALW alignment generated the log likelihood score closest to zero and was therefore used for phylogenetic analysis.
To improve optimality, the preferred MSA was edited by eye. The large, phylogenetically uninformative intron in the suctorian ciliate *Acineta sp.* was removed. The first 43 and last 26 characters of the fixed alignment were removed as well due to unreliability of ends of sequences and to remove the sequences of the universal primers used in PCR. Finally, throughout the alignment, individual bases were shifted to improve homology hypotheses.

Two versions of a hand-edited alignment were used for a preliminary analysis. This was done in order to demonstrate the significant difference in derived phylogenetic relationship of major clades from highly similar MSAs, showing the sensitivity of the data. The first alignment was labeled “first draft.” The second alignment, labeled “second draft,” was a slightly further modified version of “first draft,” with all changes coming from “by eye” adjustments. Within the preliminary analysis, these two drafts were analyzed in a maximum likelihood framework. After comparison of the performance of these two “drafts,” the “second draft” was chosen for all phylogenetic analyses. These two “draft” MSAs are available as supplementary material.

**Model selection.** To select a model of nucleotide evolution for the maximum likelihood analysis of the optimized alignment, jModelTest 0.1.1 (Posada 2008) was used. The preferred model was chosen under the Akaike Information Criterion (Posada and Buckley 2004). For the Bayesian inference analysis, MrModelTest was used (Nylander 2004), as it only tests models implemented in MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003). Both programs selected the GTR + I + G model (General Time Reversal with a proportion of invariable sites, I, and a discrete gamma distribution, G). This was done for both the preliminary and final analyses.
Phylogenetic analyses. All maximum likelihood analyses were conducted with
the phylogenetic analysis program RAxML on the CIPRES web-server portal
(http://www.phylo.org) running 50 replicates and one hundred bootstrap
pseudoreplicates. The Bayesian inference analysis was conducted with MrBayes v3.2.1
(Ronquist and Huelsenbeck 2003). The analysis consisted of two runs with four chains,
with a total of 1,550,000 generations, the number required for the average standard
deviation of split frequencies to reach below 0.01. Trees were sampled every 500
generations. The burn-in frequency was 25%.

A parsimony analysis was conducted using Tree analysis using New Technology
(TNT) (Goloboff et al. 2003). This analysis was carried out using a heuristic search of
1,000 random addition sequence replicates followed by tree bisection and reconnection
(TBR), and tree-fusing and sectorial searching (Goloboff 1999). The most parsimonious
trees were searched for using 200 iterations of ratchet searched with 20%
upweight/downweight frequencies. All gaps were treated as missing data.

RESULTS

The preferred maximum likelihood tree has a log likelihood of -46,085.02 (Fig. 2.1).
Of the three thousand trees generated under Bayesian inference, the highest posterior
probability density was summarized in a single consensus tree (Fig. 2.2). The parsimony
analysis returned two equally parsimonious trees with a length of 9853, CI of 0.248, RI of
0.635. The strict consensus tree can be found in figure 2.3.

_Scindostoma hanania_ falls consistently within the class Prostomatea. It is always
sister to the marine fish parasite _Cryptocaryon irritans_, and these two are always sister to
*Paraspathidium* sp. Within the Prostomatea, the families Colepidae, Holophryidae, Urotrichidae, Balanionidae, and Placidae are all monophyletic. The plagiopylids, *Plagiopyla* and *Lechriopyla*, also fall within the Prostomatea. More broadly, all trees generated agree with classifications found in recent literature. In all trees, all classes are monophyletic except the Nassophorea, which is a paraphyletic sister to the Phyllopharyngea. Within the Intramacronucleata, the Litostomatea and Spirotrichea form one clade, while the Colpodea, Oligohymenophorea, Prostomatea, Nassophorea, and Phyllopharyngea form another clade. Although the local position of *S. hanania* and the monophylies of classes are consistent, the relationships of these clades within the Prostomatea are highly sensitive, both to the MSA and to the optimality criterion of the analysis.

As mentioned previously, the preferred CLUSTALW alignment was adjusted by eye and two alignments were generated, labeled “first draft” and “second draft.” Each draft was used for a preliminary analysis, in which each MSA was analyzed phylogenetically under the same optimality criterion (maximum likelihood with a GTR+I+G model). The log likelihood scores generated were similar (-46,091.77 for the first draft and -46,085.02 for the second draft), but the resulting relationships of the prostomes were highly disparate (Fig 2.4a, 2.4b). In the “first draft,” *Scindostoma + Cryptocaryon + Paraspathidium* are sister to the Colepidae and Holophryidae, whereas in the “second draft,” they are sister to the Urotrichidae. And while sisters like Plagiopylid/Balanionidae or Colepidae/Holophryidae stay together, their relationship to the whole class is significantly different between the two drafts. Colepidae + Holophryidae are sister to *Scindostoma + Cryptocaryon + Paraspathidium* in the first
draft and to Placidae in the second draft. Plagiopylida + Balanionidae are sister to Urotrichidae in the “first draft” and to Urotrichidae + *Scindostoma* + *Cryptocaryon* + *Paraspathidium* in the “second draft.” The position of Placidae is also quite different: basal in the “first draft,” versus highly derived and sister to Colepidae + Holophryidae in the “second draft.”

The derived topologies were also sensitive to the optimality criterion. This is seen in the final analysis, in which three phylogenetic analyses were conducted using the “second draft” MSA with three respective optimality criteria (maximum likelihood, Bayesian inference, parsimony). While both the maximum likelihood and Bayesian inference analyses generated the same topology (Fig 2.5a), a parsimony analysis generated a different topology altogether (Fig 2.5b), in which *Scindostoma* + *Cryptocaryon* + *Paraspathidium* are sister to Plagiopylida + Placidae. Other branching patterns differ as well; in the maximum likelihood/Bayesian inference tree, the Balanionidae are sister to Plagiopylida, while in the cladistic analysis, they are sister to the Urotrichidae. Again, sister taxa like Colepidae and Holophryidae and *Scindostoma* + *Cryptocaryon* + *Paraspathidium* stay together, but the relationships of these taxa are quite different between ML/Bayesian and parsimony.

**DISCUSSION**

This study was divided into two components, the first of which compared two phylogenetic trees generated under the same optimality criterion but with two slightly different multiple sequence alignments. Although the tree that produced the better log
likelihood score is the preferred tree (in this case, the tree generated under the “second draft”), we have included these “preliminary” data in order to point out that even slight modifications to the fixed alignment have dramatic effects upon the derived topology. This necessitates skepticism toward any hypothesized relationships of the families within the Prostomatea that are generated by this study alone.

Even with the same MSA, different optimality criteria returned different topological patterns. The difference between the maximum likelihood/Bayesian inference and parsimony trees may be due to long branch attraction within the prostomatid taxa, even with the inclusion of uncultured eukaryotes and environmental samples. This would need to be evaluated by removing wildcard taxa, reanalyzing, and comparing the differences, if any. Another consideration is that gene trees—in this case, the 18S rRNA gene—do not necessarily correspond to species trees (Pamilo and Nei 1988; Maddison 1997). However, the lack of additional molecular data in the literature prevents inclusion and analysis of multiple genes. The resolution of these ambiguities is beyond the scope of this study.

All of the molecular phylogenetic analyses conducted in this study confirm the position of Scindostoma hanania within the class Prostomatea. These results are consistent with the morphology of the ciliate (see chapter 1). Scindostoma is also always sister to Cryptocaryon, and the two of those are always sister to Paraspathidium1. The unwavering clustering of Scindostoma with Cryptocaryon, across all analytical criteria, implies a close evolutionary relationship. Lynn (2008) synonymized the family

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1 It is worth noting that Paraspathidium's consistent sister relationship to Scindostoma and Cryptocaryon is worth investigation. Though it is beyond the scope of this study, Paraspathidium's potential position within the Cryptocaryonidae must be assessed in future studies, both through morphological and molecular analysis.
Cryptocaryonidae (in which exists the monotypic *Cryptocaryon irritans*) with Holophryidae—however, all analyses show that Holophryidae and *Cryptocaryon* are paraphyletic. Further, the family Cryptocaryonidae has never been formally described. Wright and Colorni 2002 proposed the name to pull *Cryptocaryon irritans* out of its historical taxonomic placement within Ichthyophthiriiidae, but gave no formal description, leaving the family as a *nomen nudum*. Though it seems evident that Cryptocaryonidae must be split from Holophryidae and formally described, the ambiguities of the molecular analysis leave room for questioning. In light of the gene tree problem, and due to the unavailability of other molecular data, a total evidence analysis is required, combining the small subunit RNA dataset with a morphological dataset. This may help resolve the ambiguities in the strictly molecular analysis. This will allow us to test the placement of *Scindostoma* and *Cryptocaryon*, and to determine whether they belong within the Holophryidae, or whether the synonymized family Cryptocaryonidae is truly its own family, and must be resurrected. Two tasks have become necessary: (1) to resolve the ambiguities of the analyses above in order to test whether *Scindostoma* and *Cryptocaryon* are separate from Holophryidae. This will be done by conducting a total evidence analysis, which includes both molecules and morphology, thereby hopefully resolving the ambiguity produced by an analysis with only molecular data; (2) if the total evidence analysis supports this hypothesis, then the family Cryptocaryonidae must be resurrected, and formally described, with the novel ciliate *Scindostoma hanania* belonging to it.
LITERATURE CITED


Sogin, M. L., Ingold, A., Karlok, M., Nielsen, H., and Engberg, J. 1986a. Phylogenetic evidence for the acquisition of ribosomal RNA introns subsequent to the divergence of some of the major Tetrahymena groups. The EMBO Journal, 5:3625-3630.

Figure 2.1. The preferred maximum likelihood tree. Numbers at nodes are bootstrap support values. Clades are colored and labeled by class, with the turquoise Postciliodesmatophora as the outgroup and the class Prostomatea and its families in red. *Scindostoma hanania* is within the Prostomatea (arrow), sister to *Cryptocaryon irritans*. 
Figure 2.2. Consensus Bayesian inference tree with highest posterior probability.

Numbers at nodes correspond to clade support values. Clades are colored and labeled by class, with the turquoise Postciliodesmatophora as the outgroup and the class Prostomatea and its families in red. *Scindostoma hanania* is within the Prostomatea (arrow), sister to *Cryptocaryon irritans*. 
Figure 2.3 Strict consensus of two most parsimonious trees generated by cladistic analysis in TNT. This tree has a length of 9853, a consistency index of 0.248, and a retention index of 0.635. Clades are colored and labeled by class, with the turquoise Postciliodesmatophora as the outgroup and the class Prostomatea and its families in red. *Scindostoma hanania* is within the Prostomatea (arrow), sister to *Cryptocaryon irritans*. 
Figure 2.4. The different topologies of the class Prostomatea generated under the same optimality criterion (maximum likelihood) but with different drafts of the multiple sequence alignment. (a) Topology derived from the “first draft,” which returned a log likelihood of -46,091.77. (b) Topology derived from the “second draft”—a slightly modified version of the “first draft”—which returned a log likelihood of -46,085.02. While the tree generated with the lower log likelihood is preferred, the purpose of showing both trees is to demonstrate the radical differences in derived topological patterns from minor modifications to the multiple sequence alignment.
Figure 2.5. The different topologies of the class Prostomatea generated under the multiple sequence alignment ("second draft"), but with different phylogenetic optimality criteria. (a) The topologies generated under maximum likelihood and Bayesian inference, which were identical. ML analysis was conducted with RAxML on the CIPRES web server, while Bayesian inference was conducted on MrBayes v3.2.1. (b) The topology generated under the parsimony criterion, found using TNT. Note that all families (as well as *Scindostoma + Cryptocaryon + Paraspithidium*) are preserved, but their relationships to one another are highly variable.
In many of the eleven classes that make up the phylum Ciliophora, the basic cellular features are elaborated and modified into more complex structures, such as intricate feeding organelles, modified cell shapes, and specialized groups of locomotory cilia. This is not the case for the members of the Class Prostomatea. In these ciliates, the cells are typically ovoid to cylindrical, the cilia usually lie in simple bipolar rows, and the apical cytostome is surrounded by relatively simple ciliary organelles (hence the class name). As a consequence, prostomes have historically been considered by many protistologists to be relatively primitive organisms. In many earlier classification schemes, these ciliates are grouped together and placed at or near the base of the ciliate evolutionary tree (see Corliss, 1979).

During the past decade, 18S small subunit rRNA (ssu-rRNA) sequences of ciliate species have provided new information that has inspired many reviews and revisions of ciliate systematics (see Lynn 2008). Molecular studies of the Class Prostomatea (which are restricted to members of the Order Prorodontida, as no ultrastructural or molecular studies have been conducted of members of the Order Prostomatida) consistently indicate that prostomial ciliates are a more advanced clade, perhaps having become secondarily
morphologically streamlined rather than being evolutionarily primitive. However, the number, description and relationship of families in the group are ambiguous (Lynn, 2003, 2008).

This ambiguity was demonstrated in the research conducted for this thesis based on molecular data alone. A phylogenetic analysis of 18S ssu-rRNA sequences, focusing on the novel ciliate *Scinodostoma hanania*, highlighted how the evolutionary relationships of the prostome families are sensitive to the data and to the analysis. Small changes in sequence alignment, as well as assessment of any alignment under different optimality criteria, greatly altered the inferred relationships (see chapter 2). In contrast, under every analysis, *Scindostoma hananaia* was placed within the prorodontids, and was found to have a sister relationship with the destructive marine fish parasite, *Cryptocaryon irritans*. Although these findings implied *S. hanania* belonging to this order, and a close evolutionary relationship between the two ciliates, no conclusion could be drawn due to the overall ambiguity of the results. Analyzing molecular sequences from multiple genetic loci would provide greater phylogenetic resolution for *S. hanania* and *C. irritans* and the prorodontids as a whole. However, this cannot be explored, as only the small subunit rRNA gene has been sequenced and published for nearly all members of the Phylum Ciliophora.

Though molecular data are limited for ciliates, there is a great amount of cell ultrastructural (e.g., morphological) data available. Combining these data in a simultaneous analysis will provide greater explanatory power than an analysis of any one dataset alone (Nixon and Carpenter 1996). Therefore, a simultaneous (or “total evidence”) analysis, uniting molecular data together with morphological data, will return
a better-supported phylogenetic hypothesis, and will bring more evidence to bear on the question of prostomate phylogeny. This combined analysis is achieved through the dynamic homology protocol under a parsimony criterion, as implemented in POY (Varon et al. 2010).

By uniting these datasets in a total evidence analysis, the phylogenetic position of *Scindostoma hanania* is clarified, as well as the relationships of the families within the Order Prorodontida. The relationship of *S. hanania* to *Cryptocaryon irritans* is also explored, and *S. hanania* is given a formal, taxonomic description.

**MATERIALS AND METHODS**

**Morphological and molecular data.** Only ciliates that have both sequence and morphological data available were selected for the total evidence analysis. Of the 124 taxa represented in the previous molecular analysis, 45 were removed because no ultrastructural data is available. As a result, 78 ciliates were chosen for the study, with representatives from all classes within Ciliophora. Eleven members of the subphylum Postciliodesmatophora served as outgroup taxa.

Morphological data were obtained from the literature and from direct observation of *S. hanania* cells under light, scanning electron, and transmission electron microscopy (see chapter 1), resulting in a matrix with 60 characters. This morphological matrix can be found in Table 3.1. The small subunit ribosomal RNA sequence for *Scindostoma hanania* was sequenced de novo (see chapter 2). Ssu-rRNA sequences for other ciliates were obtained from the National Center for Biotechnology Information’s GenBank
Prior to the phylogenetic analysis, the molecular sequences were visualized in Seaview (Gouy et al. 2010) and stripped of primer nucleotides. In order to identify missing data at the 5’ and 3’ ends, sequences were heuristically aligned using ClustalO (Sievers et al. 2011). The sequences were then fragmented into partitions of missing data and present data. Partitions force the POY-based total evidence analysis (see below) to assume no homologues between fragments, thereby ensuring that missing data cannot be homologized to present data. This was done in order to prevent the introduction of \textit{ad hoc} hypotheses that would lead to false assumptions of indel events (see Wheeler et al. 2009). This also reduced computational time required for the analysis. This revised molecular dataset was then concatenated with the morphological dataset, and is available as Supplementary Material.

**Phylogenetic analysis.** All analyses were run through POY 5.0.1 (Varon et al. 2010) on the \textit{Enyo} cluster server (American Museum of Natural History; see http://research.amnh.org/scicomp/amnh-computer-cluster). The molecular and morphological supermatrix was entered as unaligned data, per the dynamic homology approach of the software. Each analysis ran for a maximum time of one hour and thirty minutes with \textit{Geleia fossata} set as the root taxon.

The direct optimization analysis was repeated in seven iterations. Each iteration used a different combination of alignment costs for transitions, transversions, and indels (see Wheeler 1995). This method, known as a ‘sensitivity analysis,’ evaluates the effects
of parameter variation on the stability of nodes (Giribet 2003). By varying the cost matrix of each iteration, this method tested the sensitivity of the data to the alignment costs. The parameters used for the cost matrix of each iteration can be found in Table 3.3. The phylogenetic tree generated by each analysis was individually visualized using CladeScan (Sanders 2010). The consensus tree of these analyses, with the agreement of each individual analysis noted at every node, is known as a ‘Navajo rug’ plot (Giribet et al. 2006). This Navajo rug plot is shown in Figure 3.1.

RESULTS

Figure 3.1 shows a summary consensus tree for all phylogenetic analyses conducted in this thesis. The tree on the left shows the class level relationships, while the subtree on the right shows the relationships of the prostomial families. Above each node is a tripartite box showing nodal support from the maximum likelihood, Bayesian inference, and parsimony analyses. Below each node is an L-shaped Navajo rug, showing nodal support from each direct optimization iteration.

The sensitivity analysis converged on the same class-level topology in nearly every iteration, with the exceptions of iterations “211” and “221.” Even in those cases, only one topological difference was observed, in which the class Litostomatea moved from the subphylum Intramacronucleata to the subphylum Postciliodesmataphora. All seven analyses returned monophyletic classes, with the exception of the Nassophorea, which was consistently a paraphyletic grade at the base of the Colpodea. The Class Prostomatea was consistently monophyletic, and sister to the Class Oligohymenophorea.
The consensus subtree of the class Prostomatea shows highly variable family relationships. Generally, Colepidae and Holophryidae are sister families, forming a clade with Placidae on one end of the prostome tree. The union of these three families, however, is poorly supported. The reciprocal clade includes the families Balanionidae, Urotrichidae, the plagiopylids, and the genera *Scindostoma* and *Cryptocaryon*. Plagiopylida and Balanionidae form a well-supported monophyly at the base of a clade including Urotrichidae, *Scindostoma* and *Cryptocaryon*. The monophyly of these three taxa is poorly supported, whereas the sister relationship of *Scindostoma* and *Cryptocaryon* is always supported. For the consensus tree, several analyses ("3221" and "8411") show support for no nodes except for *Scindostoma* and *Cryptocaryon*. Under no analysis did *Scindostoma* and *Cryptocaryon* form a monophyly with Holophryidae.

**DISCUSSION**

The main goal of this thesis was to determine the phylogenetic position of the novel ciliate *Scindostoma hanania*. In the analyses generated using strictly 18S gene data, *S. hanania* was consistently placed within the Class Prostomatea, and always as the sister taxon of *Cryptocaryon irritans*. However, the overall results were too ambiguous to justify any phylogenetic conclusion or consequential taxonomic revision. This ambiguity called for further, better-supported evidence through a simultaneous analysis of multiple datasets. After analyzing the molecular sequence data in tandem with all morphological data for every ciliate that had both data types available, the results regarding these two ciliates were the same. *Scindostoma hanania* was always placed within the Class
Prostomatea, and was always sister to *Cryptocaryon irritans*. Despite the ambiguity found phylum-wide and within the prostomes, these relationships remained constant, no matter the alignment, its cost, or the optimality criterion of the analysis.

This result, however, raises more questions because the placement of the genus *Cryptocaryon* has been controversial. The genus *Cryptocaryon* contains a single species, *Cryptocaryon irritans* Brown 1951, and is a destructive parasite of marine fishes in aquaria and in commercial mariculture (Colorni 1985; Diamant et al. 1991).

*Cryptocaryon irritans* is not host specific but is restricted to fishes in tropical environments where the water does not dip below 19°C (Dickerson 2006). It infects surface epithelia (skin, gills, and eyes), causing major lesions and high mortality rates in tropical fishes. Cryptocaryonosis is similar to white spot disease in freshwater fish (ichthyophthiriasis) and, because of this, *Cryptocaryon* was placed by Brown alongside the causative agent of that disease (*Ichthyophthirius multifiliis*) in the Class Oligohymenophora, Subclass Hymenostomatia, Order Tetrahymenida, and Family Ichthyophthiridae (Brown 1951). Cheung et al (1981) examined *Cryptocaryon* with scanning electron microscopy and noted the absence of ciliary membranelles around the cytostome and hence questioned whether *C. irritans* belonged in the Hymenostomatia. Colorni and Diamant (1993) published a limited ultrastructural description of *C. irritans* and suggested a reassessment of the taxonomic position as well. In an unpublished dissertation, Keskintepe (1995) described the ultrastructural anatomy of *C. irritans* in detail and, based on the structure of the kinetid and the oral area, suggested that the genus belonged in the Prostomatea. Because this study remained unpublished, this suggestion was not generally known or acknowledged.
Based on sequence analysis of the 18S ribosomal RNA gene, Wright and Colorini (2002) proposed that Cryptocaryon irritans be placed in the Prostomatea, Order Prorodontida. They proposed a new family name, Cryptocaryonidae, but did not provide a description or diagnosis for the family. Because the family was not properly diagnosed, this name is a *nomen nudum*. Although he did not discuss his reason, this is probably why Lynn (2008) submerged the Cryptocaryonidae into the family Holophryidae within the Prorodontida and synonymized the two names. Thus, the genus Cryptocaryon currently belongs within the family Holophryidae, though there is no taxonomic justification for the synonymization of Cryptocaryonidae with Holophryidae.

This taxonomic designation is undermined by the phylogenetic findings. In every tree, Cryptocaryon irritans is polyphyletic to members of the family Holophryidae. Never under any alignment, of any dataset, or under any optimality criterion does C. irritans form a monophyletic clade with the family Holophryidae. Morphological disagreements further separate C. irritans from the Holophryidae. Members of Holophryidae have caudal cilia; C. irritans does not. Members of Holophryidae have cytoprocts, C. irritans does not. Members of Holophryidae have subkinetal microtubules; C. irritans does not. Members of Holophryidae have radial postciliary microtubular ribbons on both oral kinetosomes; C. irritans only has one on its anterior oral kinetosome. Members of Holophryidae have overlapping circumoral microtubular ribbons; C. irritans has non-overlapping ribbons. These phylogenetic, surface morphological, and ultrastructural differences distinctly separate C. irritans from the family Holophryidae.

All of these same distinctions hold true for Scindostoma hanania. Under no
phylogenetic analysis does *S. hanania* form a clade with Holophryidae. Similarly, the same surface morphological and ultrastructural differences distinguish *S. hanania* from that family. What separates *S. hanania* and *C. irritans* from Holophryidae therefore also provides morphological evidence for their union. Not only are these two ciliates paraphyletic with respect to Holophryidae, they never unite consistently with any established family within the Order Prorodontida. Even the consensus tree, which shows the cladogram with the highest nodal agreement, shows only four of the seven direct optimization iterations (and two of the three static alignment analyses) agreeing with the sister relationship of *S. hanania* and *C. irritans* with the family Urotrichidae.

Morphological distinctions also separate them; members of Urotrichidae have strictly monokinetetal kineties and overlapping kinetodesmal fibers, whereas these two ciliates have a mixture of mono- and dikinetids and non-overlapping kinetodesmal fibers, making them unlikely members of the Urotrichidae. In fact, morphological distinctions make *S. hanania* (and, by association, *C. irritans*) an unlikely member of any of the current families within the Order Prorodontida (see the discussion in chapter 1 for more details).

Multiple lines of evidence support the monophyly of *Scindostoma hanania* with *Cryptocaryon irritans*. Similarly, multiple lines of evidence refute the placement of both *S. hanania* and *C. irritans* within the family Holophryidae. Given the phylogenetic affinity of these two ciliates, their distinction from all prorodontid families, and the lack of taxonomic justification for the synonymization of Cryptocaryonidae with Holophryidae, we are left with these conclusions: (1) that *Scindostoma hanania* and *Cryptocaryon irritans* are monophyletic within the Class Prostomatea, Order Prorodontida; (2) that these two ciliates constitute their own, unique family within the
Order Prorodontida; (3) that the family Cryptocaryonidae sensu Wright and Colorni 2002 must be re-split from Holophryidae, formally described, and include Scindostoma hanania along with the type genus Cryptocaryon as its two members.

Family Cryptocaryonidae

Type genus. Cryptocaryon Brown, 1951.

Diagnosis. Prorodontid ciliate, distinguished by 5’-ACT-3’ base pair sequence of the small subunit ribosomal gene at positions 1687-1689 relative to sequence of Tetrahymena pyriformis (GenBank sequence X56171).

Description. Cell, ovoid to cylindroid; holotrichous; alveoli, often well-developed; perilemma, absent; cytoproct, absent; oral region apical to subapical; cytostome, with oral dikinetids, both ciliated; oral extrusomes, as toxicyst-bearing palps; toxicysts, rod-shaped; nematodesmata originating from bases of circumoral dikinetids; brosse, as one to several kinetofragments, akitoloph and prominent; somatic kineties, bipolar monokineties with 1-4 rows of apical dikinetids; somatic dikinetid kinetosomes, both ciliated; somatic extrusomes as mucocysts; kinetodesmal fiber, running anterior and short with striated fiber;postciliary microtubular ribbon, non-convergent angle and non-overlapping; transverse microtubular ribbon, one radial set and non-overlapping; macronucleus, globular to ellipsoidal and prominent; micronucleus, present; contractile vacuole, as several pores between somatic kinetal rows.

Remarks. Wright and Colorni (2002) proposed the family Cryptocaryonidae based on molecular evidence. However, their work contained no formal description of the group,
creating an invalid *nomen nudum*. Lynn (2008) synonymized the family with another family within the order, Holophryidae, again without explanation. The molecular evidence within this thesis requires a resurrection of the family, and the invalid name proposed by Wright and Colomi necessitates this formal description for the resurrection.

It is unlikely that the members of Cryptocaryonidae belong to other families within the Order Prorodontida, because the conspicuous synapomorphies of those families, such as the calcium carbonate shell of members of Colepidae, are uniformly not present within members of Cryptocaryonidae. The members of this family are united by a molecular synapomorphy found within the sequence of their small subunit ribosomal gene. It is not clear what morphological apomorphy unites the members of this family; however, this is because the order as a whole is poorly described, and contains several polyphyletic families, such as Holophryidae. Any morphological apomorphies of Cryptocaryonidae will not be evident until the order is fully revised. A thorough revision of the Order Prorodontida will help clarify the relationships of its families, and will shed light on the morphological apomorphies that unite members of Cryptocaryonidae.

**Genus *Scindostoma*, gen. nov.**

**Type species.** *Scindostoma hanania*, sp. nov.

**Diagnosis.** Most similar to *Cryptocaryon irritans*, but distinguished by oral dikinetal ring, which is split into two, non-connecting, semicircular fragments that wrap around oral lips and extend down side of the cell approximately 15 µm; cytopharynx surrounded by elongated, filamentous, non-paracrystalline, membrane-bound electron dense fibers
that originate beside oral toxicyst-bearing palps and extend into the cytoplasm; somatic parasomal sacs found between kinetodesmal fiber and transverse microtubular ribbon instead of between kinetodesmal fiber and postciliary microtubular ribbon; ridge microtubules are absent instead of present longitudinally above epiplasm.

**Description.** See Chapter 1.


Table 3.1. Morphological characters and GenBank accession numbers used in total evidence analysis. Modified with permission from Lipscomb et al, 2012.

<table>
<thead>
<tr>
<th>Character</th>
<th>States</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Perilemma</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>2. Alveolocysts</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>3. Ridge or longitudinal microtubules</td>
<td>(0) absent above the epiplasm (longitudinal), (1) embedded in the epiplasm (ridge)</td>
</tr>
<tr>
<td>4. Toxicyst</td>
<td>(0) absent, (1) single type (rod-shaped), (2) two types (large and small), (3) bowling pin shaped, (4) single type (nail-shaped)</td>
</tr>
<tr>
<td>5. Extrusome with stopper-like cap</td>
<td>(0) absent haptocyst, (1) haptotrichocyst</td>
</tr>
<tr>
<td>6. Rhabdocyst</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>7. Trichites</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>8. Trichocysts</td>
<td>(0) absent, (1) nailshaped tip, (2) tip with four arms and polygonal body, (3) small cap-like tip with peripheral longitudinal microtubules</td>
</tr>
<tr>
<td>9. Macronucleus</td>
<td>(0) homomeric, (1) heteromeric</td>
</tr>
<tr>
<td>10. Kinety composed of</td>
<td>(0) monokinetids, (1) dikinetids, (2) mixture of mono- and dikinetids (3) polykinetids are cirri, (4) polykinetid longitudinally linked kinetosomes, (5) telotroch band</td>
</tr>
<tr>
<td>11. Axosomal plate is a cone</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>12. Subkinetal microtubules</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>13. Subkinetals support contractile vacuole canals</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>14. Basal microtubules</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>15. Kd length</td>
<td>(0) short, (1) overlapping</td>
</tr>
<tr>
<td>16. Kd fiber</td>
<td>(0) striated, (1) absent-filament instead, (2) keel-shaped</td>
</tr>
<tr>
<td>17. Kd direction</td>
<td>(0) anterior anterior-laterally laterally, (1) posterior-laterally</td>
</tr>
<tr>
<td>18. Pc angle convergent</td>
<td>(0) no (divergent or 90 degrees), (1) yes</td>
</tr>
<tr>
<td>19. Overlapping Pcs</td>
<td>(0) absent, (1) stacks, (2) triads, (3) longitudinal ribbons adjacent to each other, (4) ribbons parallel to cell surface (disorganized?) , (5) stacks perpendicular to cell surface</td>
</tr>
<tr>
<td>20. Tmts sets</td>
<td>(0) one set, (1) two sets</td>
</tr>
<tr>
<td>21. Tmts orientation</td>
<td>(0) radial, (1) tangential</td>
</tr>
<tr>
<td>22. Dikinetid transverse microtubules</td>
<td>(0) Tangential on anterior kinetosome, radial on posterior kinetosome, (1) Tangential on anterior kinetosome, absent on posterior kinetosome, (2) Tangential on both kinetosomes, (3) Radial on anterior, tangential on posterior kinetosome, (4) Tangential on anterior, group of microtubules on posterior, (5) Absent on anterior, (6) Absent on anterior, radial on posterior, (7) Absent on both</td>
</tr>
<tr>
<td>23. Transversodesma</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>24. Transverse extends anteriorly and overlaps</td>
<td>(0) absent, (1) present</td>
</tr>
<tr>
<td>25. Parasomal sacs</td>
<td>(0) absent, (1) one between Kd and transverse microtubules, (2) one between the Kd and postciliary microtubules (3) three - one anterior to KD, two just behind tmt on each kinetosome (4) two - 1 between Kd and Pc, 1 opposite between Tmt and Pcs, (5) one between Tmt and Pcs, (6) one in the craterlike depression surrounding the two cilia of the dikinetid</td>
</tr>
<tr>
<td>26. Dikinetid kinetosomes</td>
<td>(0) both ciliated anterior kinetosome, (1) ciliated, posterior naked or very short</td>
</tr>
<tr>
<td>27. Cytostome</td>
<td>(0) present, (1) suctorian tentacles</td>
</tr>
<tr>
<td>28. Oral monokinetids</td>
<td>(0) absent with pc and transverse, (1) Oral monokinetid through loss of outer kinetosome, (2) Oral pairs absent oralized somatic monokinetids instead, (3) Oral pairs absent, oralized somatic dikinetids for vestibular kinety</td>
</tr>
<tr>
<td>29. Oral dikinetids</td>
<td>(0) both ciliated one ciliated and other not (or cilia very short) neither ciliated (or cilia very short) Dikinetids, (1) absent</td>
</tr>
<tr>
<td>AZM only</td>
<td></td>
</tr>
<tr>
<td>30. Oral kinetid pattern</td>
<td>(0) radial pc on anterior, tangential tmt on post (opp side from mouth), (1) tangential tmt on anterior, radial pc on post (opp side from mouth), (2) radial pc on anterior, nothing on posterior, (3) radial pc both kinetosomes on side towards mouth, (4) Overlapping circumoral microtubular ribbons running counterclock, (5) radial pc anterior kinetosomes on side towards mouth, single microtubule on posterior kinetosome</td>
</tr>
</tbody>
</table>

91
31. Oral dikinetid position: (0) pair at end of kineties and encircle the cytostome, (1) form distinct paroral membrane, haplokinety or circum-oral kinety, (2) dikinetids absent.
32. Circle of robust nematodesmata rods lining cytopharynx: (0) absent, (1) present.
33. Cytostomial lamellae (Z lamellae) line cytopharynx: (0) absent, (1) present perpendicular to cytopharynx, (2) in concentric circles to form feeding tubes or tentacles.
34. Subcytostomal or accessory lamellae (Y lamelae) line cytopharynx: (0) absent, (1) present.
35. Nematodesmal lamellae (X lamelae) line cytopharynx: (0) absent, (1) present.
36. Nematodesmata paired: (0) no, (1) yes.
37. Polykinetids form AZM: (0) absent, (1) present.
38. Oral polykinetid forms quadrulus: (0) absent, (1) present.
39. Long polykinetid parallel to dikinetid: (0) absent, (1) present.
40. Radial ribbon on polykinetid: (0) absent, (1) present, (2) present and tangential ribbon opposite side.
41. Brosse position: (0) brosse absent, (1) interrupt oral ring outside oral ring, (2) inside oral ring.
42. Brosse kinetid: (0) absent (1) Posterior with postciliaries, anterior with transverse, (2) Posterior with postciliaries, anterior no fibers, (3) Posterior with normal infraciliature., anterior with no fibers, (4) both have normal infraciliature.
43. Lieberkuhn organelle: (0) absent, (1) present.
44. Stalk: (0) absent (1) peduncle podite, (2) suctorian stalk, (3) peritrich stalk.
45. Rosette adjacent to cytostome: (0) absent, (1) present.
46. Supporting Fiber (bound by fibrils and containing a regular arrangement of nodes and fibrils): (0) absent, (1) present.
47. Ciliary plaques: (0) absent, (1) present.
48. Ciliary rosette: (0) absent, (1) present.
49. Longitudinal rows: (0) absent, (1) single particles, (2) double particles.
50. Orthogonal array: (0) absent, (1) present.
51. Macronucleus during division cycle: (0) does not divide divides without microtubules, (1) divides with microtubules.
52. Replication band: (0) absent, (1) present.
53. Cell division: (0) monotomic palintomy strobilization, (1) budding external, (1) budding internal.
54. Plane of cell division is: (0) homothetogenic, (1) enantiotropic, (2) longitudinal.
55. Origin of new oral cilia: (0) apokinetal, (1) parakinetal, (2) buccokinetal, (3) telokinetal, (4) mixokinetal.
56. Apokinetal stomatogenesis: (0) absent, (1) subsurface pouch or tube (hypoapokinetal), (2) on cell surface (epiapokinetal).
57. Parakinetal stomatogenesis: (0) absent, (1) one kinety (monoparakinetal), (2) several kineties (polyparakinetal), (3) amphiparakinetal, (4) teloparakinetal, (5) biparakinetal.
58. Buccokinetal stomatogenesis: (0) absent, (1) ophryobuccokinetal, (2) scuticobuccokinetal.
59. Telokinetal stomatogenesis: (0) absent, (1) Each kinety gives rise to oral pairs, (2) Some kineties give rise to oral pairs, (3) Kineties absent and barren, (4) kinetosomes give rise.
60. Parental oral structures: (0) Retained, (1) Completely reorganized, (2) Partially reorganized.
Ichthyophthirius
Climacostomum
Macropodinium
Dexiotrichides
Condylostoma
Chlamydodon
Cryptocaryon
Chattonidium
Blepharisma
Bresslaua
Bursaria
Cohnilembus
Colpoda
Cophyllum
Cryptozoon
Cyclidium
Dasytricha
Didinium
Dileptus
Enchelyodon
Enchelys
Ephelota
Eutelopula
Eutelsis
Eveline
Favella
Frontonia
Gefersis
Geleia
Glaucophyllum
Halteria
Hartmannula
Heliocystis
Hemichrysalis
Homalozoon
Ichthyophthirius
Isoschiza
Isotricha
Isotrichopus
Lacrymario
Lechriopula
Lembadion
Litonotus
Laxodes
Laxophyllum
Macropodinium
Obertrumia
Ophryoglena
Paramecium
caudatum
Paramecium
tetraurelia
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<td>Placus</td>
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Table 3.2. Studies consulted for morphological and molecular data. Modified with permission from Lipscomb et al, 2013.
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Table 3.3. Summary of nucleotide substitution costs for each analysis executed in POY.

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<td>1</td>
</tr>
<tr>
<td>“3221”</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>“8411”</td>
<td>4</td>
<td>1</td>
<td>32</td>
<td>1</td>
</tr>
</tbody>
</table>
Fig 3.1. Cladograms of hypothesized ciliate phylogenetic relationships with summary support matrices at nodes. Tripartite box above edges corresponds to nodal support in preferred trees under maximum likelihood, Bayesian inference, and parsimony optimality criteria. L-shape boxes under edges corresponds to sensitivity analysis generated in POY. On the left is the phylogeny of the phylum Ciliophora with subphylum Postciliodesmatophora and classes of the subphylum Intramacronucleata. The dotted line for the Class Prostomatea connects with the tree on the right, which is the phylogeny within the Class Prostomatea. Only the sister relationship of *Scindostoma hanania* and *Cryptocaryon irritans* is fully supported by all analyses under all optimality criteria.
Appendices

Appendix 2.1: DNA extraction using the QIAGEN DNEasy® Tissue Kit.

To the 2 ml Eppendorf tube filled with cells of interest, 180 µl of Buffer ATL was added, followed by 20 µl of Proteinase K, which was then incubated at 56°C for one hour. The sample was then vortexed with a Thermolyne Maxi Mix Plus™. 200 µl of Buffer AL was added, the sample was vortexed thoroughly, followed by 200 µl of 95% ethanol (EtOH) and another thorough vortexing. The sample was pipetted into a DNeasy Mini spin column and centrifuged at 8,000 rpm for one minute in an Eppendorf 5417C centrifuge. The spin column was transferred to a new collection tube, with 500 µl of Buffer AW1 added, and centrifuged again at 8,000 rpm for one minute. This step was repeated with Buffer AW2, except centrifuged at 14,000 rpm for three minutes. The spin column was transferred to a new Eppendorf tube, where 200 µl of Buffer AE was added, and centrifuged at 8,000 rpm for one minute. The Eppendorf tube, with the desired nucleic acids, was stored at -20°C.

Appendix 2.2: Protocol for polymerase chain reaction (PCR).

The first item prepared was a “master mix” of reagents to account for 44.75 out of 50 µl in every PCR iteration. This master mix consisted of 29.75 µl dH₂O, 5 µl 10X Taq polymerase buffer, 4 µl MgCl₂, 4 µl dNTPs, 1 µl of the given forward primer, and 1 µl of the given reverse primer (summarized below). These quantities were multiplied by the amount of tubes required for the reaction. Once 44.75 µl of this master mix was added to
each tube, 5 µl of the extracted DNA (see Appendix 3.1) was added, followed lastly by
0.25 µl of Taq polymerase for a 50 µl total. This was then run on variable touchdown
PCR on an MJ Research thermal cycler (PTC-200); the protocol is summarized below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>AACCTGGGTGATCCA</td>
<td>Medlin et al. 1988</td>
</tr>
<tr>
<td>Gas+600</td>
<td>CGGTAATTCAGCTCCAATAG</td>
<td>Jeong et al. 2004</td>
</tr>
<tr>
<td>Gas+1220</td>
<td>CCTGGTGTTGCCCTTCCGTC</td>
<td>Jeong et al. 2004</td>
</tr>
<tr>
<td>L</td>
<td>CCAACTACGAGCTTTTTAACTG</td>
<td></td>
</tr>
<tr>
<td>Gas+1390</td>
<td>CTGGTTAATCCGATAACG</td>
<td>Jeong et al. 2004</td>
</tr>
<tr>
<td>Gas+1540</td>
<td>GGGCATAACAGACCTGT</td>
<td>Jeong et al. 2004</td>
</tr>
<tr>
<td>B</td>
<td>TGATCCTGCCCGGTCTCA</td>
<td>Medlin et al. 1988</td>
</tr>
</tbody>
</table>

Table 2.2.1: Primers used to amplify the 18S gene in *Scindostoma* via polymerase chain
reaction.

<table>
<thead>
<tr>
<th>dH₂O</th>
<th>10X Buffer</th>
<th>MgCl₂</th>
<th>dNTPs</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>DNA</th>
<th>Taq polymerase</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>29.75</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0.25</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 2.2.2: Polymerase Chain Reaction proportions, in microliters.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time (Min:sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>3:00</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>0:30</td>
</tr>
<tr>
<td>3</td>
<td>57°C</td>
<td>1:00</td>
</tr>
</tbody>
</table>
Table 2.2.3: Variable Touchdown PCR protocol, “EXTDC2.” Run on an MJ Research thermal cycler (PTC-200) for PCR.

Appendix 2.3: Cleanup of PCR products and preparation for sequencing reaction.

Following amplification, the samples had to be cleaned of leftover dNTPs and primers. The ExoSAP protocol, with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) being the two hydrolytic enzymes, was used. An initial “master mix” of 52.75 µl dH₂O, 3.525 µl Exonuclease I and 3.525 µl SAP was created, with 23.92 µl aliquoted into each sample. The samples were returned to the thermal cycler, where they underwent an “Exosapit” protocol (see table 3.3.1 below).
Following Exosapit, the samples were ethanol precipitated. 4.64 µl of 3M sodium acetate and 93 of µl cold, 95% EtOH were added and held at room temperature for 15 minutes, then centrifuged at 2°C for 15 minutes at 14,000 rpm on a Sorvall LegendMicro 21R Centrifuge. Following removal of supernatant, 93 µl of cold, 70% EtOH was added, and the samples were centrifuged again at 2°C for five minutes. The supernatant was removed again. This 70% EtOH step was repeated twice more. Following the third removal of 70% EtOH supernatant, the samples were speed vacuumed dry on a Labconco Centrivap Concentrator at room temperature for 25 minutes to remove any remaining liquid. Each sample was then resuspended in 25 µl dH₂O and stored at -20°C for sequencing. In the same way a gel was run after PCR to ensure amplification, a quantification electrophoresis gel was run to ensure continued presence of DNA in the final, “Exosapped” product.

The cleaned product could now be prepared for sequencing. Another “master mix” was prepared, containing 76.5 µl dH₂O, 18 µl 5X buffer (400 mM TBE and 10 mM MgCl₂), and 18 µl development kit dye Terminator Cycle Sequencing starting mix (DTCS) from a Beckman Coulter Quick Start Mix. 12.5 µl of this master mixture was pipetted into new Neptune PCR tubes. A select, diluted primer was added to each sample, depending on the desired region to be sequenced. Stock primers were diluted into 1:5.6 ratios of primer to dH₂O, and 1 µl of those dilutions were used for each sample. Finally, 1.5 µl of appropriate, cleaned DNA was pipetted into its respective, new tube. These samples were run again on the thermal cycler under the “DTSX35” reaction (see table 2.3.2 below).
A “stopping solution” was prepared to eliminate any leftover Exonuclease I and SAP. This consisted of 18 µl 3M Sodium Acetate, 18 µl diluted EDTA, and 9 µl glycogen. 5 µl was added to each tube, all of which were then ethanol precipitated and speed vacuumed dry in the same manner as before. Finally, these dried samples were resuspended in 40 µl of deionized dimethyl-formaldehyde (dDMF), also known as a “Sequence Loading Solution” (SLS).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time (Min:sec)</th>
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<tbody>
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<td>2</td>
<td>80°C</td>
<td>15:00</td>
</tr>
<tr>
<td>3</td>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>

Table 2.3.1: “Exosapit” protocol to allow Exonuclease I and Shrimp Alkaline Phosphatase to remove unwanted, leftover dNTPs and primers.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time (Min:sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96°C</td>
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</tr>
<tr>
<td>2</td>
<td>50°C</td>
<td>0:20</td>
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<tr>
<td>3</td>
<td>60°C</td>
<td>4:00</td>
</tr>
<tr>
<td>4</td>
<td>Go to step 1, 34 times</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>

Table 2.3.2: “DTSX35” protocol, enabling destruction of any remaining reagents and readiness for Sanger sequencing.

Appendix 2.4: Sequence of the 18S small subunit rRNA gene of Scindostoma hanania.
This sequence will be deposited into the National Center for Biotechnology Information GenBank as the small subunit rRNA gene of *Scindostoma hanania*:

5’-

```
TTTCTGGTGATTCTGCCAGTAGTCATATGCTTGTCTTAAGACTAAGCCATGCA
TGTCTAAAGTATAAATAGTATACAGTGAAACTGCGAATGGCTCATTAAAACAG
TTATAGTTTTATTTGATAAATTAAATCTACATGGATAAACCCTAGTAGTAATTCTAGAG
CTAATACATGCTGCAAGACCTGACTTTTTGGAAGGGTTGTATTATTAGATCT
CAAACCAATATTCCTTCCGGGTCTATTGTGATGATTACATAAAACTGGTGAGAT
CGAGACTAGTCTCTCGATATAATCATTCAAGATTGTTCTGCTCCATACAGCTTTCG
GTAGTGTTATGGGACTACCATGGCAGTCACGGTGTAACGGGGAATTAGGGTTCG
ATTCGGGAGAGGAGCCTGAGAAACGGCTACCATTCTAAGAGGGAAGCAGCA
GGCGCTAAATTACCCTACGCTTCAGGAGATGTAACAGAGGAAATCAACACT
TCGGGGCTCACGGCTTCACGAGATATCCATGCAATGAGGAATAATTCCCT
ATCGAGGAACAAATTGGGAAGGCACAGTCTGGTGCCAGGAAATCAACAG
GCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCCTGATGTGAATT
TCTGGCTTTTTTTCTGTACGAGATGATGTTTACTTTGA
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TTAATTAGGACTATGGCTATTTTGGTTGTTATTAGCCTTCTGTAATGAT
TTAATGGGACAGTTGGGGGGCATTAGTTAATTGTCAGAGGATGAAATTCTT
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GGCTGAAACTTAAAGGAATTGACGCAAGGGCACCACCAAGGATGTGAGGCCTG
CGGTGAATTATGGGACAAGGATACTTACCAGTGTCAGACATGGGTGGG
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GGACTATGC GloTTCAAGGAGATTGAGGCAATAACAGGTCAGTGATG
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GATCGATCTCTTGGGAAATTATAGACTGTAAGATAAGTAGCAAG
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CCTACCAGTTTCGAGTGTCCCGGTAAACCTTCTGGGACTGCGCTAGTTTCTAGC
TAGTGCGGGAAGTTAAAGTTAACCACCTCCTAGAGGAAGGAGAAAAGTGCTAA
CAAGGTTTCCGTTAGGTCGCAAGCGAAGGATCAAG-3’