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Sistemática de microeucariotos ciliados (Alveolata, Ciliophora) simbiontes de mamíferos herbívoros no Brasil

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de concentração: Comportamento e Biologia Animal, da Universidade Federal de Juiz de Fora, como requisito parcial para obtenção do grau de Doutor.

Orientador: Prof. Dr. Roberto Júnio Pedroso Dias
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Dedico este trabalho ao meu amado primo Rogério de Castro Cedrola Júnior (in memorian)*, que tanta alegria me trouxe durante a nossa convivência neste mundo*.

"Dizem que antes de um rio entrar no mar, ele treme de medo. Olha para trás, para toda jornada que percorreu, para os cumes, as montanhas, para o longo caminho sinuoso que trilhou através de florestas e povoados e vê à sua frente um oceano tão vasto, que entrar nele nada mais é do que desaparecer para sempre. Mas não há outra maneira. O rio não pode voltar. Ninguém pode voltar. Voltar é impossível na existência. O rio precisa aceitar sua natureza e entrar no oceano. Somente ao entrar no oceano o medo irá se diluir, porque apenas então o rio saberá que não se trata de desaparecer no oceano, mas de se tornar o oceano".

Khalil Gilbran

"*Tenho em mim todos os sonhos do mundo*"

Fernando Pessoa

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RESUMO

A presente tese contribuiu para ampliar o conhecimento sobre a ciliatofauna simbionte de mamíferos herbívoros no Brasil, por meio de estudo acerca da sistemática dos ciliados, principalmente daqueles pertencentes à subclasse Trichostomatia, ordens Entodiniomorphida e Vestibuliferida. Realizou-se, pela primeira vez nas Américas Central e do Sul, amplo invetário taxonômico de microeucariotos ciliados associados a mamíferos herbívoros domésticos (bovinos, equinos e ovinos) e selvagem (capivara) no Brasil. O inventário, realizado por meio de caracterizações multidisciplinares, apontou a presença de aproximadamente 90 espécies de ciliados, distribuídas em duas classes, duas subclasses, três ordens e 10 famílias. A caracterização por meio de abordagem multidisciplinar, disponibilizou novos e importantes dados morfológicos estruturais, principalmente acerca dos padrões infraciliares das espécies, e moleculares (11 novas sequências do 18S rDNA) de ciliados tricostomatídeos, contribuindo para elucidar diversos problemas e inconsistências taxonômicos que há muito permeiam a sistemática da subclasse Trichostomatia. Ainda, grandes revisões sistemáticas unidas a reconstruções filogenéticas moleculares e a revisitação de dados morfológicos pouco explorados, nunca antes realizados para este grupo, contribuíram para melhor compreender a evolução dos caracteres em Trichostomatia, permitindo a elaboração de hipóteses acerca das relações de parentesco no interior deste grupo e o estabelecimento de novas classificações taxonômicas baseadas em divergências evolutivas.

Palavras-chave: Bovinos, Blepharocorythidae, Capivara, Ophryoscolecidae, Ovinos, Sistemática, Taxonomia, Trichostomatia

ABSTRACT

The present thesis contributed with the knowledge on the symbiotic ciliates of herbivorous mammals in Brazil. For the first time in Central and South America, a large taxonomic inventory of ciliated microeucaryotes associated with domestic (bovine, equine and ovine) and wild (capybara) herbivorous mammals in Brazil was conducted. The inventory, performed through multidisciplinary characterizations, demonstrate the presence of ~90 ciliate species, distributed in two classes, two subclasses, three orders and 10 families. The characterization through a multidisciplinary approach has provided important new structural morphological data, especially about species infraciliary bands patterns, and molecular (11 new 18S rDNA sequences) of trichtomatid ciliates, helping to elucidate many long-standing taxonomic problems and inconsistencies in the systematics of the subclass Trichostomatia. Moreover, systematic reviews, molecular phylogenetic reconstructions and revisited underexplored morphological data, never performed before for this group, contributed to a better understanding of the evolution of the characters in Trichostomatia, allowing the elaboration of hypotheses about the relationships within this group and the establishment of new taxonomic classifications based on evolutionary divergences.

Key-words: Blepharocorythidae, Capybara, Cattle, Ophryoscolecidae, Sheep, Taxonomy, Trichostomatia, Systematics

RESUMO PARA DIVULGAÇÃO CIENTÍFICA

Os protozoários ciliados que vivem no estômago e intestino de mamíferos herbívoros são organismos microscópicos, ou seja, que não conseguimos visualizar a olho nu. Eles habitam o sistema digestório destes animais e participam de diversas funções durante a digestão de seus hospedeiros. Há muitos anos, estes microrganismos vem sendo estudados por diversos pesquisadores, no mundo inteiro, os quais tentam compreender, especificamente, quais são as funções que realizam. Hoje, sabemos que eles são importantes, por exemplo, na digestão do alimento consumido pelos seus hospedeiros. É por isso, que os mamíferos, como as vacas e os cavalos são tão grandes e se alimentam quase que exclusivamente de vegetais. Apesar de serem muito investigados, devido ao papel que possuem na digestão dos animais que os abrigam, poucos são os pesquisadores que se destinam a estudar os seus aspectos morfológicos e suas relações de parentesco. Assim, a presente tese de Doutorado possuiu como objetivo ampliar o conhecimento acerca dos protozoários encontrados no sistema digestório de mamíferos no Brasil. Primeiramente, investigou-se quais espécies de protozoários ciliados habitam o sistema digestório de mamíferos herbívoros no Brasil. Esta investigação apontou a presença de aproximadamente 90 espécies de protozoários ciliados, associadas a mamíferos herbívoros domésticos (bovinos, equinos e ovinos) e selvagem (capivara). Muitas destas espécies não possuíam descrições morfológicas detalhadas e sequências gênicas depositadas em bancos de dados. Assim, após a identificação destas espécies, descreveu-se suas características morfológicas e moleculares (sequências gênicas), acrescentando novos dados acerca destes microrganismos na literatura científica e contribuindo para melhor entender as relações de parentesco estabelecidas entre esses ciliados e outras espécies próximas.

APRESENTAÇÃO

A presente tese amplia o conhecimento acerca dos microeucariotos ciliados (Alveolata, Ciliophora) encontrados em associação simbiótica com mamíferos herbívoros no Brasil. Possui pequena *Introdução* e onze *Seções*, cada qual correspondendo a um artigo científico diagramado segundo normas dos periódicos para os quais foram ou serão enviados. A *Seção 1* traz uma revisão sobre a diversidade e sistemática de ciliados tricostomatídeos (Ciliophora, Litostomatea), na qual é apresentado um histórico acerca dos estudos desenvolvidos com este grupo de ciliados, desde a sua descoberta, há 177 anos, até os dias atuais, destacando as incongruências sistemáticas e propondo soluções para esses problemas e perspectivas para estudos futuros; As *Seções 2, 3* e *4* trazem inventários taxonômicos de microeucariotos ciliados associados a mamíferos herbívoros domésticos (bovinos e equinos) e selvagem (capivara), totalizando aproximadamente 90 espécies inventariadas, das quais, várias constituem novos registros para o país e para o continente americano, bem como primeiros registros nos hospedeiros analisados. A *Seção 5* caracteriza morfologicamente quatro espécies pertencentes ao gênero *Metadinium* (Ciliophora, Entodiniomorphida, Ophryoscolecidae) e apresenta notas taxonômicas sobre este gênero, ressaltando as dificuldades da delimitação das espécies que o compõem. A *Seção 6* redescreve por meio de dados morfológicos, a espécie *Polyplastron alaskum* Dehority, 1974 (Ciliophora, Entodiniomorphida, Ophryoscolecidae), uma rara espécie de ciliado encontrada em simbiose com ovinos domésticos e selvagens, incluindo, pela primeira vez, dados sobre o padrão de infraciliatura desta espécie. A *Seção 7* redescreve a espécie *Eremoplastron rostratum* (Fiorentini, 1889) (Ciliophora, Entodiniomorphida, Ophryoscolecidae) com base em informações morfológicas e moleculares, apresenta ampla discussão sobre a sistemática dos gêneros *Eremoplastron* e *Eudiplodinium*, e propõe novo gênero em Ophryoscolecidae, *Paraeremoplastron*. A *Seção 8* reconstroi hipótese para evolução da família Ophryoscolecidae, utilizando dados moleculares contidos no marcador 18S rDNA e dicuste sobre a validade do gênero *Eodinium* (Ciliophora, Entodiniomorphida, Ophryoscolecidae). A *Seção 9* realiza ampla revisão sistemática da família Blepharocorythidae (Ciliophora, Entodiniomorphida) e propõe nova organização para a família, levando em consideração dados morfológicos e moleculares. A *Seção 10* discute, por meio de dados morfológicos e moleculares, se o formato de capacete em ciliados entodiniomorfídeos (Ciliophora, Entodiniomorphida) reflete divergência evolutiva, ou se representa apenas, uma simples homoplasia. Por fim, a *Seção 11* discute, por meio de dados morfológicos e reconstruções filogenéticas moleculares, a validade do gênero *Elytroplastron* (Ciliophora, Entodiniomorphida, Ophryoscolecidae). Destaca-se que, dentre as Seções apresentadas, as Seções 1, 2, 3, 5, 8 e 10 encontram-se publicadas. As Seções 6 e 9 encontram-se submetidas e as Seções 4, 7 e 11 estão em finalização para submissão.

INTRODUÇÃO

Os microeuucariotos ciliados (Alveolata, Ciliophora) incluem mais de 8000 espécies descritas, das quais aproximadamente 3000 são simbiontes ou parasitos, os quais habitam grande diversidade de hospedeiros invertebrados e vertebrados (LYNN, 2008). Entre os ciliados simbiontes de vertebrados, parcela significativa é encontrada em simbiose com o trato gastrointestinal de mamíferos herbívoros, e encontra-se, atualmente, distribuída em duas subclasses, Trichostomatia Bütschli e Suctoria Clapèrede & Lachmann (LYNN, 2008). Esses microeucarariotos participam em diversos processos no metabolismo de seus hospedeiros, atuando, principalmente, na degradação de carboidratos solúveis e insolúveis (DEHORITY, 1986).

Dentre os microeucariotos ciliados simbiontes de mamíferos herbívoros, a subclasse Trichostomatia inclui a maior parcela desta diversidade, sendo atualmente representada por quase 1000 espécies, distribuídas em três ordens, Entodiniomorphida Reichenow in Doflein & Reichenow, caracterizada por reunir ciliados dotados de ciliaturas oral e somática limitadas a tufos ou bandas e organizadas em policinécias; e Macropodiniida Lynn e Vestibuliferida de Puytorac et al., as quais incluem ciliados completamente cobertos por cílios e dotados de vestíbulo densamente ciliado (LYNN, 2008). Recentemente, os ciliados tricostomatídeos tem sido alvo de diversos estudos funcionais, tais como os focados na otimização da eficiência alimentar do hospedeiro (NEWBOLD et al., 2015) e aqueles que investigam seu papel na metanogênese gastrointestinal (MALMUTHUGE & GUAN, 2017). No entanto, apesar deste crescente interesse, questões relativas à sistemática do grupo, bem como suas relações evolutivas são ainda, amplamente negligenciadas.

O grupo foi descoberto na primeira metade do século XIX por Gruby & Delafond (1843). No entanto, esses autores apresentaram apenas um relato breve e sucinto sobre as altas densidades de "animáculos" associados ao estômago e intestino de bovinos e cavalos domésticos. Dessa forma, o primeiro autor a publicar um trabalho taxonômico formal sobre ciliados tricostomatídeos foi F. Stein (STEIN, 1858) que descreveu, superficialmente, várias espécies, incluindo-as na família Ophryoscolecidae (Ciliophora, Trichostomatia). Após estes primeiros relatos, várias outras espécies foram descritas, provenientes de várias localidades geográficas e de diferentes espécies de hospedeiros, totalizando, até o final da década de 1970, mais de 400 espécies descritas. Nos últimos 30 anos, poucos trabalhos taxonômicos foram produzidos com tricostomatídeos e este período foi caracterizado principalmente por inventários taxonômicos em várias espécies de hospedeiros, domésticos e selvagens, e de diferentes localidades geográficas. Assim, uma nova visão sobre a sistemática de ciliados tricostomatídeos surgiu apenas no final dos anos 90, com o advento das técnicas moleculares. As primeiras filogenias moleculares (WRIGHT & LYNN, 1997a, b; WRIGHT et al., 1997) corroboraram vários estudos morfológicos iniciais recuperando os tricostomatídeos como um grupo monofilético no interior da classe Listostomatea. A partir do início dos anos 2000 e com o aumento de sequências de muitos membros da subclasse Trichostomatia disponíveis em repositórios públicos (CAMERON & O'DONOGUE, 2004; CAMERON et al., 2001, 2003; STRÜDER-KYPKE et al., 2007; ITO et al. ., 2010, 2014; SNELLING et al., 2011; MOON-VON DER STAAY et al., 2014; GRIMM et al., 2015; KITTELMANN et al., 2015; ROSSI et al., 2015; BARDELE et al., 2017; CEDROLA et al., 2017, 2020), as relações filogenéticas internas na subclasse começaram a ser elucidadas, causando uma revolução em sua sistemática e indicando várias incongruências taxonômicas, principalmente no que diz respeito às ordens Entodiniomorphida e Vestibuliferida, as quais parecem não constituir grupos naturais.

Assim, a presente tese contribuiu para ampliar o conhecimento sobre a ciliatofauna simbionte de mamíferos herbívoros no Brasil, por meio de estudo acerca da sistemática destes ciliados, principalmente os pertencentes à subclasse Trichostomatia, ordens Entodiniomorphida e Vestibuliferida. Realizou-se, pela primeira vez nas Américas Central e do Sul, amplo invetário taxonômico de microeucariotos ciliados associados a mamíferos herbívoros domésticos (bovinos, equinos e ovinos) e selvagem (capivara) no Brasil, por meio de caracterizações multidisciplinares. Ainda, foram disponibilizados novos e importantes dados morfológicos estruturais, principalmente acerca dos padrões infraciliares, e moleculares (11 novas sequências do 18S rDNA) das espécies inventariadas, contribuindo para elucidar diversos problemas e inconsistências taxonômicos que há muito permeavam a sistemática da subclasse Trichostomatia. Grandes revisões sistemáticas unidas a reconstruções filogenéticas moleculares e a revisitação de dados morfológicos pouco explorados, nunca antes realizados para este grupo, contribuíram para melhor compreender a evolução dos caracteres em Trichostomatia, permitindo a elaboração de hipóteses acerca das relações de parentesco no interior deste grupo e o estabelecimento de novas classificações taxonômicas.

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SEÇÃO 1

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Trichostomatid Ciliates (Alveolata, Ciliophora, Trichostomatia) Systematics and Diversity: Past, Present and, Future

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Vestibuliferida

Abstract

The gastrointestinal tracts of most herbivorous mammals are colonized by symbiotic ciliates of the subclass Trichostomatia, which form a well-supported monophyletic group, currently composed by ~1000 species, 129 genera, and 21 families, distributed into three orders, Entodiniomorphida, Macropodiniida, and Vestibuliferida. In recent years, trichostomatid ciliates have been playing a part in many relevant functional studies, such as those focusing in host feeding efficiency optimization and those investigating their role in the gastrointestinal methanogenesis, as many trichostomatids are known to establish endosymbiotic association with methanogenic Archaea. However, the systematics of trichostomatids presents many inconsistencies. Here, we stress the importance of more taxonomic works, to improve classification schemes of this group of organisms, preparing the ground to proper development of such relevant applied works. We will present a historical review of the systematics of the subclass Trichostomatia highlighting taxonomic problems and inconsistencies. Further on, we will discuss possible solutions to these issues and propose future directions to leverage our comprehension about taxonomy and evolution of these symbiotic microeukaryotes.

1. Introduction

The gastrointestinal tracts of most herbivorous mammals are colonized by symbiotic ciliates of the subclass Trichostomatia Bütschli, 1889 (Supplementary Video 1), which play a central nutritional role in these associations and are required for an efficient fermentative process and also contribute to the degradation process of proteins, lipids, nitrogen compounds and carbohydrates, such as cellulose, hemicellulose and starch (Dehority, 1986; Wright et al., 2015). These microeukaryotes form a well-supported monophyletic group, currently composed by ~1000 species, 129 genera, and 21 families (Supplementary Material 1) that are distributed over three orders: Entodiniomorphida Reichenow in Doflein & Reichenow, 1929, including species with ciliary zones restricted to tufts or bands, and infraciliatures organized as polybrachykinties; and Macropodiniida Lynn, 2008 and Vestibuliferida de Puytorac et al., 1974, including ciliates all covered by cilia and with a densely ciliated vestibulum (Lynn 2008; Cedrola et al., 2015; Gao et al., 2016). In recent years, trichostomatid ciliates have been playing a part in many relevant functional studies, such as those focusing in host feeding efficiency optimization (Newbold et al., 2015) and those investigating their role in the gastrointestinal methanogenesis, as many trichostomatids are known to establish endosymbiotic association with methanogenic Archaea (Embley et al., 2003), which may accounts for up to 60% of methane emissions in the Earth's atmosphere (IPCC;

Malmuthuge and Guan, 2017). However, the systematics of trichostomatids presents many inconsistencies. Here, we stress the importance of more taxonomic works, to improve classification schemes of this group of organisms, preparing the ground to proper development of such relevant applied works. We will present a historical review of the systematics of the subclass Trichostomatia highlighting taxonomic problems and inconsistencies. Further on, we will discuss possible solutions to these issues and propose future directions to leverage our comprehension about taxonomy and evolution of these symbiotic microeukaryotes.

2. Past

Trichostomatid ciliates were discovered in the first half of 19th century by Gruby and Delafond (1843). However, the authors, presented only a brief and succinct report about high densities of "animaculous" inhabiting the stomach and intestine of domestic cattle and horses. The first illustrations of trichostomatid ciliates are attributed to Colin (1854) while the author studied domestic mammals. G. Colin performed live observations of many species, possibly including members of the genera *Blepharocorys* Bundle, 1895, *Bundleia* da Cunha & Muniz, 1928, *Cycloposthium* Bundle, 1896, *Diplodinium* Schuberg, 1888 and *Entodinium* Stein, 1859. The first author to publish a formal taxonomic work on trichostomatid ciliates was F. Stein (Stein, 1858) describing, although superficially, species of the genera *Entodinium*, *Isotricha*, and *Ophryoscolex* and the family Ophryoscolecidae. Following, several novel species were described from many geographic locations and from different host species. In this period, comprehended from the work of F. Stein (Stein, 1858) till late 1970's, more than 400 species were described, indicating that trichostomatid ciliates may constitute diverse group of organisms (Bundle, 1895; Fiorentini, 1889; Poche, 1913; Da Cunha, 1914a,b; Gassovsky, 1919; Crawley, 1923; Buisson, 1923a-c, 1924; Fantham, 1926; Dogiel, 1927, 1928, 1932, 1934, 1935; Becker and Talbot, 1927; Kofoid and MacLennan, 1930, 1932, 1933; Hsiung, 1930, 1935a,b, 1936; Jirovec, 1933; Kofoid and Christenson, 1933; Kofoid, 1935; Wertheim, 1935; Fonseca, 1939; Moriggi, 1941; Sládeček, 1946; Bush and Kofoid, 1948; Lubinsky, 1957, 1958a, 1958b; Latteur, 1966a-d, 1967, 1968, 1969, 1970; Wolska, 1967b, 1968, 1969). Most of these studies were done based only on live observations and in simple ciliatological techniques, such as hematoxylin and iodine staining methods, which were the available tools by that time. Nevertheless, many morphological characters, such as skeletal plates (Dogiel, 1923; Dogiel and Fedorowa, 1925; Schulze, 1924, 1927), contractile vacuoles (Kraschnninikow, 1929; MacLennan, 1933), concretion vacuoles (Dogiel, 1929), and paralabial organelles (Bretschneider, 1962) could be clearly characterized, allowing the inclusion of these microeukaryotes into phylum Ciliophora, orders Entodiniomorphida and Vestibuliferida (for history of classification, see Supplementary Material 2). In this same period, the first works proposing hypotheses about the evolution of these group of organisms were proposed. According to Dogiel (1947) and Lubinsky (1957a, b, c), within the family Ophryoscolecidae, subfamily Entodiniinae could be considered ancestral due to their single ciliary zone, single contractile vacuole, caudal spines poorly developed, and lack of skeletal plates. The Ophryoscolecinae is considered to be the most derived group for presenting two ciliary zones, large number of vacuoles and skeletal plates, and developed caudal projections. Diplodiniinae is considered an intermediate group.

The development of silver impregnation techniques in 1930's (Bodian, 1936, 1937), which can reveal in details infraciliary and other argentophilic structures patterns, represented a great revolution in the systematics of Ciliophora (Lynn, 2008). They were initially applied to trichostomatids by Noirot-Timothée (1956a, b) where the infraciliary band patterns of *Epidinium* Crawley, 1923 and *Ophryoscolex* Stein, 1858 were described, respectively. Further studies were performed by several authors and contributed to the establishment of infraciliary bands pattern in various trichostomatid ciliate species (Noirot-Timothée, 1960; Grain, 1962, 1963a, b, 1964, 1965; Batisse, 1966). However, the greatest contribution was achieved by M. Wolska in a series of seminal works (Wolska, 1963; 1964; 1965; 1966a, b; 1967a, b; 1968; 1969; 1970; 1971a, b; 1978a-d; 1979; 1985; 1986), which described infraciliary band patterns and morphogenetic processes on ciliates of the families Buetschliidae Poche, 1913, Blepharocorythidae Hsiung, 1929, Spirodiniidae Strelkow, 1939, Pseudoentodiniidae Wolska, 1985 (Entodiniomorphida) and Isotrichidae Bütschli, 1889 and Paraisotrichidae Da Cunha, 1915 (Vestibuliferida). According to these descriptions there are several patterns of infraciliary bands in Trichostomatia in which are composed by at least one of these bands: adoral polybrachykinety, dorsal polybrachykinety, dorso-adoral polybrachykinety, kinety loop, paralabial kineties, vestibular polybrachykinety, and vestibular kineties (Supplementary Image 1). As results of these detailed investigations, the author proposed hypotheses on the evolutionary relationship of these organisms within Trichostomatia (Wolska, 1971b).

Ultrastructural works had also a role on the systematics of trichostomatid ciliates. Bonhomme (1989), after collecting data on ultrastructure of many Entodiniomorphina (order Entodiniomorphida) representatives in addition to their own ultrastructural observations, suggested that this suborder could be classified into two groups, according to their cortex ultrastructure information. The first, composed by ciliates with cortex lacking dense longitudinal cords (genus *Cycloposthium* Bundle, 1895; Ophryoscolecidae Stein, 1859 and Troglodytellidae Corliss, 1979); and the second, composed by the ones with dense longitudinal cords (genus *Tripalmaria* and Spirodiniidae Strelkow, 1939).

Further, based on a compilation of structural and ultrastructural data, Small & Lynn (1981) proposed Trichostomatia as a subclass of the class Litostomatea, and sister group of subclass Haptoria Corliss, 1974.

Over the last 30 years, after a long period of scarce taxonomic data being produced, many taxonomic inventories of trichostomatids isolated from several mammalian host species, domestic and wild, from different geographic locations (Supplementary Table S1) started to be produced, leading to the characterization of a series of novel species, including trichostomatids inhabiting the gastrointestinal tract of Australian marsupials (Dehority, 1996; Cameron and O'Donogue, 2001, 2002a-c, 2003a-c, 2004a, b; Cameron et al., 2000a, b, 2001a, b, 2002, 2003). These ciliates present several morphological exclusive features among trichostomatids. For this reason, Lynn (2008) proposed the creation of a new order to include them, Macropodiniida. This period was also characterized by the establishment of specific new silver impregnation techniques for trichostomatid ciliates, such as the adaptations of ammoniacal silver carbonate impregnation proposed by Ito and Imai (1998) and Rossi et al. (2016) and the adaption of Protargol's impregnation for vestibuliferids proposed by Ito and Imai (2000). These techniques allowed the development of several studies describing the infraciliature and morphogenetic process in different trichostomatid species (Ito and Imai, 1998, 2003, 2005, 2006; Ito and Tokiwa, 2018; Ito et al., 1997, 2001, 2002, 2006, 2008, 2010, 2011, 2014, 2017, 2018; Gurelli and Ito, 2014; Gurelli and Akman, 2016; Cedrola et al., 2016, 2017a, b, 2018a, b; Gurelli, 2018, 2019), in which were very important to understand the evolutionary relationships within Trichostomatia.

A novel view on the systematic of trichostomatids ciliates emerged in the late 1990s with the advent of molecular techniques. The first molecular phylogenies (Wright and Lynn, 1997a, b; Wright et al., 1997) corroborated the initial morphological studies placing trichostomatids as a monophyletic group within Listostomatea. Starting from early 2000's and with the increase of sequences from many members of subclass Trichostomatia available in public repositories (Cameron and O'Donogue, 2004b; Cameron et al., 2001b, 2003; Strüder-Kypke et al., 2007; Ito et al., 2010, 2014; Snelling et al., 2011; Moon-Von der Staay et al., 2014; Grimm et al., 2015; Kittelmann et al., 2015; Rossi et al., 2015; Bardele et al., 2017; Cedrola et al., 2017), the internal phylogenetic relationships within the subclass began to be elucidated causing a revolution in their systematics and indicating several taxonomic incongruences, mainly with respect to Entodiniomorphida and Vestibuliferida, in which do not seems to constitute natural groups.

3. Present

Currently, the subclass Trichostomatia consists of three major orders, Entodiniomorphida, Macropodiniida, and Vestibuliferida. Macropodiniida is the only one to which multidisciplinary taxonomic approaches were applied (Cameron and O'Donogue, 2001, 2002a-c, 2003a-c, 2004a, b, Cameron et al., 2000a, b, 2001a, b, 2002, 2003). Their representatives are distributed in three monophyletic families all with well-supported internal nodes (Figure 1, Supplementary Image 2). However, most of the species diversity of Trichostomatia is distributed within Entodiniomorphida and Vestibuliferida, which are extremely neglected groups concerning taxonomic studies. According to 18S rDNA phylogenetic reconstructions (Figure 1, Supplementary Image 2), the order Entodiniomorphida is not monophyletic, emerging in the tree as two independent clades, one containing representatives of the families Blepharocorythidae Hsiung, 1929, Parentodiniidae Ito et al., 2002, Pseudoentodiniidae Wolska, 1986, Cycloposthiidae Poche, 1913, Spirodiniidae Strelkow, 1939, Polydiniellidae Corliss, 1960, Troglodytellidae Corliss, 1979, and Ophrysocolecidae Stein, 1859; and another containing members of the family Buestchiliidae Poche, 1913. Moreover, many of these families do not constitute natural groups, such as Blepharocorythidae, Cycloposthiidae, and Spirodiniidae; and for those that are monophyletic, such as Ophryoscolecidae, internal phylogeny is poorly supported, as detected in previous works (Rossi et al. 2015; Cedrola et al. 2017). Many inconsistencies can also be observed in the order Vestibuliferida with representatives distributed in three distinct clades (Figure 1, Supplementary Image 2), in which the families Balantididae and Paraisotrichidae do not constitute natural groups. Moreover, 18S rDNA sequences are only available from representatives of 16 out of the 21 currently recognized families of Trichostomatia. The families with no molecular data are: Gilchristinidae (Ito et al., 2014), Rhinozetidae Van Hoven et al., 1988, Telamonididae Latteur & Dufey, 1967 (Entodiniomorphida); and Protocaviellidae Grain & Corliss, 1979 and Protohallidae Cunha & Muniz, 1927 (Vestibuliferida). Still, many of the existing families of which molecular data are available, such as Polydiniellidae Corliss, 1960 and Troglodytellidae Corliss, 1979 (Entodiniomorphida) and Pycnotrichidae Poche, 1913 (Vestibuliferida) have only one representative with its 18S rDNA sequenced, limiting the power of phylogenetic reconstructions within the whole group. The scarcity and absence of consistent morphological data from many trichostomatid groups is also of concerns, for example, there are no structural (infraciliary pattern and morphogenesis) and ultrastructural data described for many cycloposthiids, troglodytellids, and spirodiniids, which makes impossible to establish homology hypothesis on trichostomatids. Moreover, the lack of detailed morphological data contributes to generate several taxonomic inconsistencies and create restraints to elaboration novel classification schemes that reflect evolutionary divergences.

4. Future

Despite the great advances obtained after implementing silver staining, ultrastructural and molecular methods, it is clear that huge gaps are still shading our view about the systematics of Trichostomatia, mainly when we compare this data which produced with other Ciliophora groups (Warren et al., 2017). We need, in the forthcoming years to invest more in detailed descriptions and redescriptions of infraciliary band patterns and morphogenesis, on 18S rDNA sequencing, and in depth ultrastructure characterizations from trichostomatids from a wide variety of hosts from many geographical regions and especially those from neglected areas, such as neotropical area, with emphasis to

Entodiniomorphida and Vestibuliferida representatives and to poorly sampled trichostomatid families, such as Protocaviellidae and Protohallidae from domestic and wild rodents and Gilchristinidae, Rhinozetidae, and Telamonididae from elephants, rhinos and wild pigs, respectively. Moreover, improvements to trichostomatid cultivation techniques, which still incipient (Williams and Coleman, 1992; Dehority and Wright, 2014; Newbold et al., 2015; Belzecki et al., 2016), would be of great importance to obtain suitable samples for morphology and molecular characterization approaches. Collectively, this information will contribute to develop more robust phylogenetic hypotheses, to elaborate taxonomic reformulations, contributing to elucidate the many taxonomic incongruences presented above and to establish new classification schemes that reflect evolutionary divergences within Trichostomatia.

Further, it is also time to apply Sanger sequencing to obtain important and informative sequences, such as ITS and 28S ribosomal sequences, in which have been useful to understand the phylogenetic relationships within Litostomatea (Rajter and Vďačný, 2017). In addition, it is possible to obtain new hydrogenosomal sequences, such as 16S and Fe-Hydrogenase and use the next generation sequencing (NGS) to allow for phylogenomic reconstruction works, as already been done for other Ciliophora groups since last decade (Feng et al., 2015; Gentekaki et al., 2017; Jiang et al., 2019). This data could be used in macro-evolutionary approaches to reveal divergence times and the mode of evolution in trichostomatid ciliates. The timescale and evolutionary dynamics of these symbiotic ciliates are yet to be determined (Newbold et al., 2015), molecular dating studies are restricted to Wright & Lynn (1997a), Vďačný et al. (2015) and Vďačný (2018), which employed different molecular dating methods, taxon sampling and calibration data, using mostly the fossil record of hosts and the posterior ages estimated from previous studies as calibration priors for ciliates time tree. Since Baele et al. (2006), provided evidences for the presence of numerous heterotacheous sites (sites in which its substitution rates can vary with time) within the 18S rDNA of ciliates, which can potentially produce phylogenetic artifacts, further improvements to the resolution of tricostomatid phylogenies could also be possible through the use of evolutionary models, such as the mixture of branch lengths (MBL) (Zhou et al., 2007) able to recognize and deal with heterogeneous rates.

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7. Author Contributions Statement

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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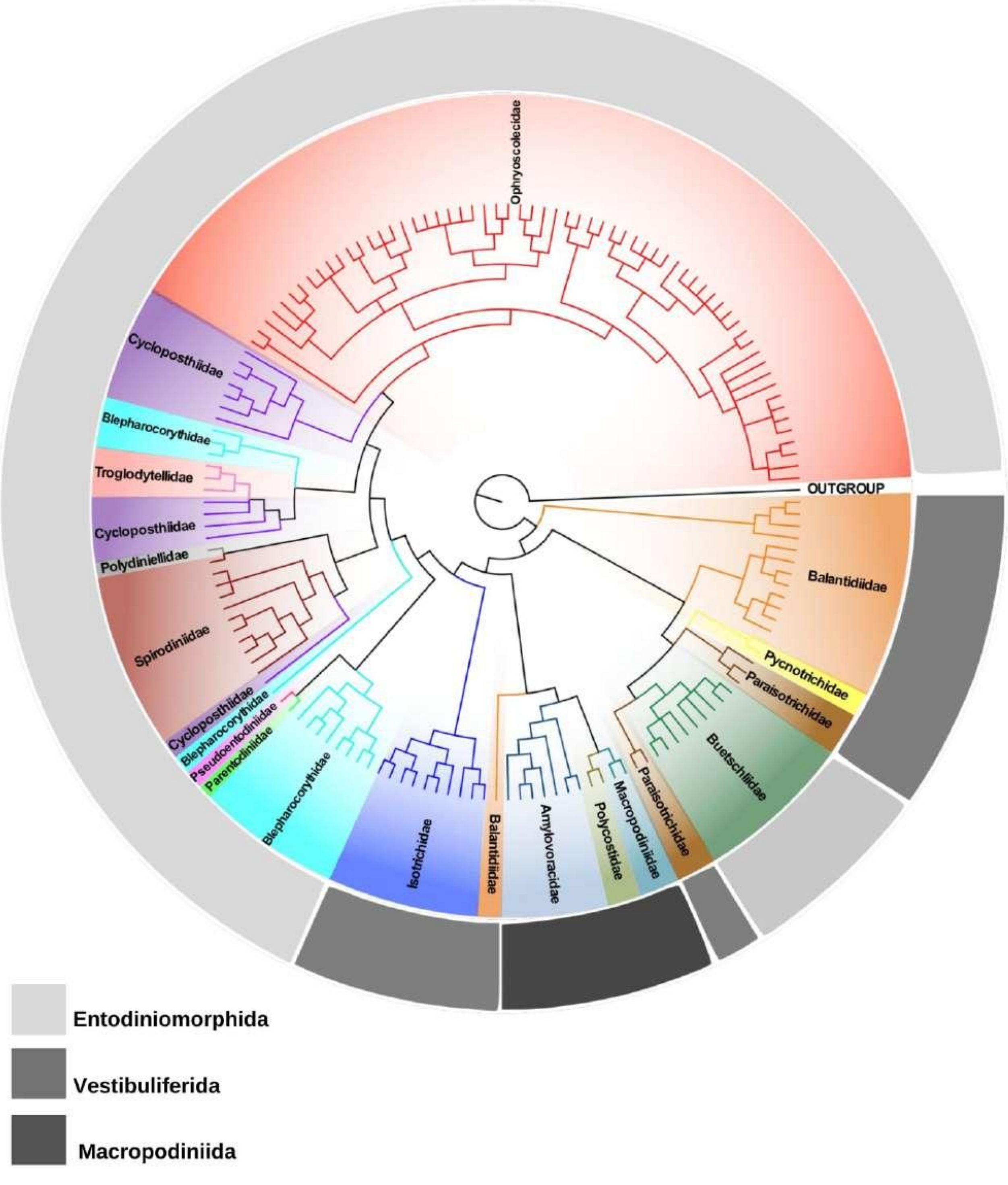
7. Figures

Figure 1. Phylogenetic tree of trichostomatid ciliates (Ciliophora, Litostomatea, Trichostomatia) estimated by Bayesian Inference and based on 18S rDNA data. *Spathidium papiliferum* was chosen as out group.


Ophryoscolecidae
OUTGROUP
Balantidiidae
Pycnotrichidae
Paraisotrichidae
Buetschliidae
Paraisotrichidae
Macropodiniidae
Polycostidae
Amylovoracidae
Balantidiidae
Isotrichidae
Blepharocorythidae
Parentodiniidae
Pseudoentodiniidae
Blepharocorythidae
Cycloposthiidae
Spirodiniidae
Polydiniellidae
Cycloposthiidae
Troglodytellidae
Blepharocorythidae
Cycloposthiidae
Entodiniomorphida
Vestibuliferida
Macropodiniida

SEÇÃO 2

(Seção publicada no periódico *Acta Protozoologica*, A3, FI: 1.491)

Intestinal ciliates of Brazilian Capybara (*Hydrochoerus hydrochaeris* L.)

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Abstract

In our study, we aimed to identify and quantify the intestinal ciliates in a Brazilian capybara and to compare the obtained data with previous studies on the capybara ciliate community in other geographic locations within the American continent. We identified 20 species belonging to four families and ten genera. This is the first study on intestinal ciliates in Brazilian capybara since the last reports for the country in the 1960s. Among the identified species, *Anacharon gracilis*, *A. lepturus*, *Cycloposthium bursa*, *Monoposthium cynodontum*, *Ogimotopsis pumila*, *Paracunhamunizia calocoma*, *Protohallia nana* and *Uropogon urai* were recorded for the first time in Brazil, and the giant ciliate *Muniziella cunhai* was observed for the second time in a symbiotic association with capybaras in the country. The present study highlighted the importance of knowing the gastrointestinal ciliate communitycommunity associated with wild hosts in order to better understand their geographic distribution and host specificity.

Keywords: Capybara, Entodiniomorphida, rodents, symbiotic ciliates, Trichostomatia, Vestibuliferida.

Introduction

Many herbivorous mammals, such as Artiodactyla, Perissodactyla, Proboscidea, Rodentia, Primates, and Marsupialia have established symbiotic associations with ciliated protozoa in their gastrointestinal tract, and these microeukaryotes can digest cellulose and starch, contributing significantly to the digestive metabolism of their hosts (Dehority 1986).

In rodents, endosymbiotic ciliates were detected in association with Brazilian-guinea-pigs (*Cavia aperea*) (Cunha et al. 1914, Neiva et al. 1914, Hasselmann 1918), guinea-pigs (*Cavia porcellus*) (Alves et al. 2007, Cunha et al. 1914, Hasselmann 1918), coypus (*Myocastor coypus*) (Silva et al. 2007), gundis (*Ctenodactylus gundi*) (Chatton and Pérard 1919) and capybaras (*Hydrochoerus hydrochaeris*) (Table 2).

The capybara is native to South America, more precisely to the east of the Andes, from Colombia and Venezuela south to Northern Argentina. It is the largest living rodent in the world (106-134 cm head-body length, 50-62 cm shoulder height, and 35-66 kg weight) inhabiting savannas and dense forests, living near bodies of water. It is a highly social species and can be found in groups as large as 100 individuals (Emmons 1997).

Endosymbiotic ciliates in Brazilian capybaras were first recorded by Cunha (1915), which described five species belonging to genera *Cycloposthium* (Entodiniomorphida) and *Paraisotricha* (Vestibuliferida). After this first report, several species have been described within the national territory (Cunha 1915, Cunha and Muniz 1925, Cunha and Muniz 1927a, b, c, Hollande and Batisse 1959, Batisse 1965, 1966), with only a few reports on these ciliates in other geographical locations (McLure 1976, Dehority 1987, Ito and Imai 2000a, b, Rodríguez-Durán et al. 2015).

In our study, we aimed to identify and quantify the intestinal ciliates in a Brazilian capybara and to compare the data obtained with previous studies on its ciliate communitycommunity in different geographical locations.

Materials and Methods

We studied a single capybara (*Hydrochoeris hydrochaeris*). The specimen was provided by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), Juiz de Fora, Minas Gerais, Brazil (218470S, 438220W) after being hit by a car. Samples were collected manually from the cecal mass, consisting of 20 cm^3 of cecal content, which was fixed in 18.5% formalin (v/v) (Dehority 1984).

Species identification was based on the descriptions made by Cunha (1915), Hasselmann (1918), Cunha and Muniz (1925, 1927a, b, c), Fonseca (1939), Hollande and Batisse (1959), Batisse (1965, 1966) and Ito and Imai (2000a, b). To identify the ciliates, we used an Olympus BX-51 light microscope (600 X and 1000 X magnification) equipped with an Olympus Evolt E-330 digital camera. We used the Image-Pro Plus 6.0 software to perform morphometry on 20 specimens of each species stained with the Lugol's solution (D'Agosto and Carneiro 1999) and impregnated by silver carbonate (Rossi et al. 2016).

The average relative abundance was estimated from a sample of 300 cells (Ito et al. 1994, Mishima et al. 2009) and the density was determined in a Sedgewick-Rafter counting chamber as in methods of to Dehority (1984) and D'Agosto and Carneiro (1999).

Results and Discussion

In this study, we identified 20 species of trichostomatid ciliates, distributed over two orders (Entodiniomorphida and Vestibuliferida), four families (Cycloposthiidae, Protocaviellidae, Protohallidae and Pycnotrichidae) and ten genera (*Anacharon*, *Cycloposthium*, *Enterophrya*, *Hydrochoerella*, *Monoposthium*, *Muniziella*, *Ogimotopsis*, *Paracunhamunizia*, *Protohallia* and *Uropogon*) (Table 1, Fig. 1). This is the first study on the Brazilian capybara's intestinal ciliates since the previous reports for the area in the 1960s (Batisse 1965, 1966, Holande and Batisse 1959). The ciliate community inventoried in the present work is similar to that of other capybara check-lists in other geographic locations within the American continent (Table 2). The exception is the ciliate community associated with capybaras sampled at Columbus Zoo in Columbus, USA, which presented ciliates belonging to the family Ophryoscolecidae (*Entodinium* spp., *Elytroplastron bubali* and *Eudiplodinium magii*) (Dehority 1987).

According to Dehority (1987) and Ito and Imai (2000a, b) the capybara's cecal ciliate community is highly specific, and few of their symbiotic ciliates species are found in association with other herbivorous mammals. Outstandingly are the species belonging to the genus *Enterophrya* (*E. elongata* and *E. piriformis*), which are originally described as guinea-pig symbionts (*Cavia porcellus*) (Hasselmann 1918) and ophryoscolecid ciliates, sampled in capybaras in the USA (Dehority 1987).

In fact, ophryoscolecid ciliates colonizing the capybara's gastrointestinal tract is an atypical finding since it has not been previously reported. Since the animals sampled by the author were kept in enclosures shared with other animals in the Columbus Zoo, patagonian cavies (*Dolichotis patagonu*) and llama (*Lama glama*), a transfaunation process could be favored and therefore the capybaras could be harbor ciliates typically found in other mammalian species. The referred author also points out that the examination of the cecal content of capybaras housed at the Luiz de Queiroz School of Agriculture in Piracicaba, Brazil has demonstrated the occurrence of ciliated protozoa similar to those described as typical of capybara, another indication that the ciliate community in Columbus Zoo capybaras was unusual.

Studies on the capybaras gastrointestinal tract physiology show that the pH of the cecal region of these animals remains close to neutrality (6.5 to 7) (Gonzalez-Jimenez 1977, Borges et al. 2014), very similar values to those found in the ruminal environment and very favorable to the establishment of ophryroscolecid species (Cedrola et al. 2016).

Among the identified species, *Anacharon gracilis*, *A. lepturus*, *Cycloposthium bursa*, *Monoposthium cynodontum*, *Ogimotopsis pumila*, *Paracunhamunizia calocoma*, *Protohallia nana* and *Uropogon urai* were recorded for the first time in Brazil. The species *Muniziella cunhai* was observed for the second time in symbiotic association with capybaras in Brazil.

These first records may be related to the use of the silver impregnation technique for the identification of the ciliates. These preparations allowed the visualization of details of the oral and somatic infraciliatures (Fig. 1), essential for the specific identification of these ciliates, especially regarding species of the order Vestibuliferida.

Muniziella cunhai was described by Fonseca (1939) in the cecal contents of Brazilian capybaras. On this occasion, the author described the morphology of the species based on live observation and specimens fixed in 70º GL alcohol. Later, Batisse (1965), re-examining the material obtained by Fonseca (1939), redescribes the species based on information obtained on sections stained with ferric hematoxylin, eosin, and light green. According to both authors (Fonseca 1939 and Batisse 1965), this species is characterized mainly by large body dimensions (> 1 mm), by the body completely covered by cilia and by the presence of a wide body groove where the vestibular opening is located. After these first reports, McLure (1976) and Ito and Imai (2000a) recorded the species in capybaras in Venezuela and Bolivia, respectively. McLure's report (1976) did not involve the morphological characterization of the specimens found, not allowing the comparison of the specimens with those characterized in Brazil. Ito and Imai (2000a), however, pointed out the complexity of describing the morphology of specimens due to the difficulty of impregnating them with silver. Ito and Imai (2000a) then presented only the measures of their body dimensions (227.3 ± 34.5 μm). Although Ito and Imai (2000a) provided morphometry for their specimens, they were much lower in body size than those observed by Fonseca (1939) and Batisse (1965). The specimens of *Muniziella cunhai* isolated in the present study are in the process of morphological and molecular characterization and will be, as soon as possible, compare with the species' previous records.

The total ciliate density was 405.9 × 10^{4} ml^{-1} of cecal content. The Cycloposthiidae family dominated the relative abundance and density, followed by the families Protocaviellidae, Protohallidae, and Pycnotrichidae (Table 1). *Cycloposthium* and *Cycloposthium bursa* were respectively the most abundant genus and species in higher density (Table 1). The total ciliate density observed in the present study was lower than that found by Ito and Imai (2000a, b), 702.^{4} × 104 ml^{-1} of cecal contents. Such variations are difficult to explain but are probably related to inherent characteristics of the hosts' individual metabolisms, as well as the type of diet consumed by the animal and geographic and climatic factors, as suggested by Gürelli and Göçmen (2012).

The present study highlights the importance of knowing the gastrointestinal ciliate community associated to wild hosts, in order to obtain more precise information about the geographic distribution and specificity of the ciliate species to certain groups of hosts. In addition, this work may contribute to future studies on the phylogenetic relationships within the subclass Trichostomatia, since none of the symbiotic ciliates species typical to capybaras have molecular descriptions and many of them have not been described based on suitable ciliatological techniques.

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Figure and Tables

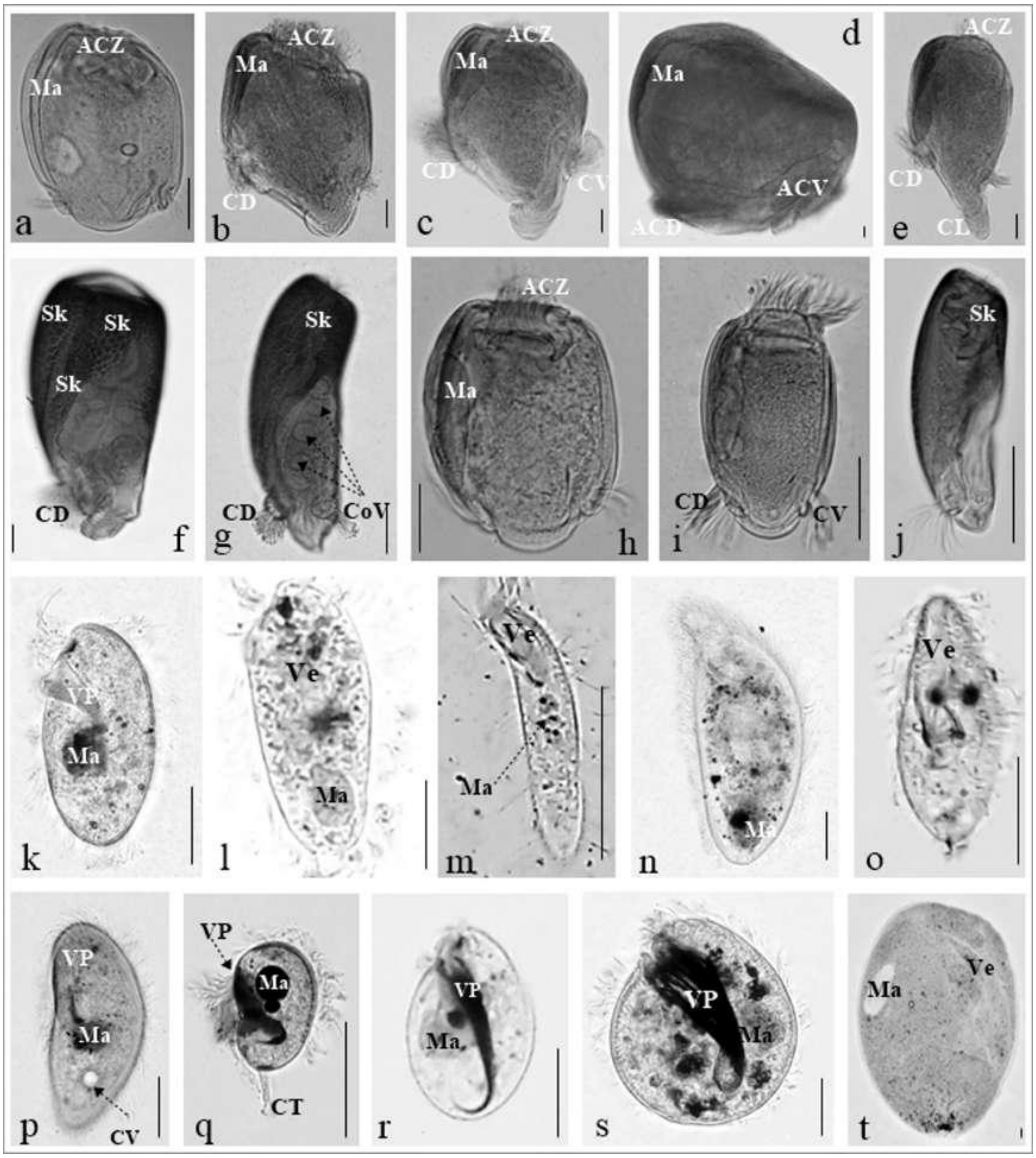

Figure 1. Intestinal ciliates recorded in Brazilian capybara (*Hydrochoerus hydrochaeris*). a-j. family Cycloposthiidae (ciliates after Lugol's solution). a. *Cycloposthium bursa*, b. *Cycloposthium caudatum*, c. *Cycloposthium compressum*, d. *Cycloposthium cristatum*, e. *Cycloposthium elongatum*, f. *Cycloposthium hydrochoeri*, g. *Cycloposthium incurvum*, h. *Cycloposthium lenticularis*, i. *Cycloposthium minutum*, j. *Monoposthium cynodontum*. k-q. family Protocaviellidae (ciliates after silver carbonate impregnation). k. *Anacharon gracilis*, l. *Anacharon lepturus*, m. *Enterophrya elongata*, n. *Hydrochoerella intestinalis*, o. *Ogimotopsis pumila*, p. *Uropogon urai*. r-s. family Protohalidae (ciliates after silver carbonate impregnation). r. *Protohallia nana*, s.

Protohallia uncinata. t. family Pycnotrichidae (live observation). s. *Muniziella cunhai*. ACZ. Adoral ciliary zone, CD. caudalia dorsal, CV. caudalia ventral, CL. caudal lobe, CoV. contractile vacuole, CT. caudal tail Ma. macronucleus, Sk. skeletal plate Ve. Vestibulum, VP. vestibular polybrachykinety. Bars: 20 µm.

Table 1. Relative abundance (%) and density ($\times 10^4$ ciliates/ ml of cecal contents) of ciliate species recorded in Brazilian capybara (*Hydrochoerus hydrochaeris*)

Order / Family / Species	**Relative Abundance**	**Density**
Order Entodiniomorphida Reichenow, 1929		
Family Cycloposthiidae Poche, 1913	58.99	239.1
Cycloposthium bursa Ito and Imai, 2000*	8.33	33.8
Cycloposthium caudatum Cunha and Muniz, 1927	6.66	27
Cycloposthium compressum Cunha, 1915	6	24.3
Cycloposthium cristatum Cunha and Muniz, 1927	6.33	25.7
Cycloposthium elongatum Holande and Batisse, 1959	6	24.3
Cycloposthium hydrochoeri Cunha, 1915	5	20.2
Cycloposthium incurvum Cunha, 1915	5.67	23
Cycloposthium lenticularis Holande and Batisse, 1959	5.67	23
Cycloposthium minutum Cunha and Muniz, 1927	7	28.4
Monoposthium cynodontum Ito and Imai, 2000*	2.33	9.4
Order Vestibuliferida de Puytorac *et al.*, 1974		
Family Protocaviellidae Grain in Corliss, 1979	26.33	120.2
Anacharon gracilis Ito and Imai, 2000*	4.00	16.2
*Anacharon lepturus*Ito and Imai, 2000*	3.33	13.5
Enterophrya elongata Hasselmann, 1918	3.33	13.5
Hydrochoerella intestinalis Cunha and Muniz, 1925	7	28.4
Ogimotopsis pumila Ito and Imai, 2000*	2.67	10.8
Paracunhamunizia calocoma Ito and Imai, 2000*	4.00	16.2
Uropogon urai Ito and Imai, 2000*	5.33	21.6
Family Protohallidae Cunha & Muniz, 1927	10	40.4
Protohallia nana Ito and Imai, 2000*	5	20.2
Protohallia uncinata (Cunha and Muniz, 1927)	5	20.2
Family Pycnotrichidae Poche, 1913	1.33	5.4
Muniziella cunhai Fonseca, 1939	1.33	5.4

*First record in Brazil.

Table 2. Distribution of intestinal ciliates in symbiotic association with Capybara (*Hydrochoerus hydrochaeris*) around the world

	Geographic locations										
	Brazil (1)	**Brazil (2)**	**Brazil (3)**	**Brazil (4)**	**Brazil (5)**	**Brazil (6)**	**Venezuela (7)**	**EUA (8)$^+$**	**Bolívia (9)**	**Bolívia (10)**	**Brazil (11)**
Order/ Family/ Species											
Order Entodiniomorphida Reichenow in Doflein and Reichenow, 1929											
Family Blepharocorythidae Hsiung, 1929											
Blepharocorys hydrochoeri Cunha and Muniz, 1925		+									
Family Buetschliidae Poche, 1913											
Protolutzia hydrochoeri Cunha and Muniz, 1925		+									
Family Cycloposthiidae Poche, 1913											
Cycloposthium bursa Ito and Imai, 2000										+	+
Cycloposthium caudatum Cunha and Muniz, 1927				+		+	+			+	+
Cycloposthium compressum Cunha, 1915	+		+			+	+			+	+
Cycloposthium cristatum Cunha and Muniz, 1927				+			+			+	+
Cycloposthium elongatum Holande and Batisse, 1959						+	+			+	+
Cycloposthium hydrochoeri Cunha, 1915	+					+	+			+	+
Cycloposthium incurvum Cunha, 1915	+						+			+	+

Cycloposthium lenticularis Holande and Batisse, 1959				+				+	+
Cycloposthium magnum Cunha & Muniz, 1927			+		+				
Cycloposthium minutum Cunha and Muniz, 1927		+		+	+			+	+
Cycloposthium vorax Cunha and Muniz, 1927		+							
Monoposthium cynodontum Ito and Imai, 2000								+	+
Family Ophryoscolecidae Stein, 1959									
Elytroplastron bubali (Dogiel, 1928)						+			
Entodinium bimastus Dogiel, 1927						+			
Entodinium caudatum Stein, 1858						+			
Entodinium dubardi Buisson, 1923						+			
Entodinium longinucleatum Dogiel, 1925						+			
Eudiplodinium maggii (Fiorentini, 1889)						+			
Order Vestibuliferida de Puytorac *et al.*, 1974									
Family Protocaviellidae Grain in Corliss, 1979									
Anacharon gracilis Ito and Imai, 2000							+		+
Anacharon lepturus Ito and Imai, 2000							+		+
Cunhamunizia batissei Ito and Imai, 2000							+		
Enterophrya elongata Hasselmann, 1918	+						+		+

Enterophrya piriformis Hasselmann, 1918		+				
Eriocharon accuminatus Ito and Imai, 2000	+	+			+	+
Hydrochoerella intestinalis Cunha and Muniz, 1925		+		+	+	+
Ogimotoa trichoradiata Ito and Imai, 2000					+	
Ogimotopsis campanulata Ito and Imai, 2000					+	
Ogimotopsis pumila Ito and Imai, 2000					+	+
Paracunhamunizia calocoma Ito and Imai, 2000					+	+
Uropogon urai Ito and Imai, 2000					+	+
Family Paraisotrichidae Cunha, 1917						
Paraisotricha hydrochoeri Cunha, 1915	+	+				
Family Protohallidae Cunha and Muniz, 1927						
Protohallia uncinata (Cunha and Muniz, 1925)		+		+	+	+
Protohallia nana Ito and Imai, 2000					+	+
Family Pycnotrichidae Poche, 1913						
Muniziella cunhai Fonseca, 1939			+	+	+	+

[1] Cunha (1915), [2] Cunha and Muniz (1925), [3] Cunha and Muniz (1927a), [4] Cunha and Muniz (1927b), [5] Fonseca (1939), [6] Holande and Batisse (1959), [7] McLure (1976), [8] Dehority (1987), [9] Ito and Imai (2000a), [10] Ito and Imai (2000b), [11] Present study.

$^+$: animal in captivity

SEÇÃO 3

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Intestinal ciliates (Alveolata, Ciliophora) in Brazilian domestic horses (*Equus caballus* L.) and a review on the ciliate communities associated with horses around the world

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ABSTRACT. The aims of this study were to identify and quantify the gastrointestinal ciliates from Brazilian domestic horses, to compare the data obtained with previous studies and to review data on ciliate communities associated with horses around the world. We used eight domestic horses, kept under extensive management. Fecal samples were obtained immediately after defecation of animals and fixed in 18.5% formaldehyde. We identify 24 species of endosymbiotic ciliates, distributed in two subclasses, two orders, five families and 13 genera. The ciliate composition was similar among the animals analyzed. The family Blepharocorythidae presented the higher prevalence and the family Buetschliidae was observed in greater abundance and density. Among the species identified, *Cycloposthium dentiferum* was observed for the first time in Central and South America and *Bundleia elongata*, *B. inflata*, *B. piriformis*, *B. vorax* and *Cycloposthium psicauda*, were observed for the first time in the American continent. In addition, the species *Buissonella tapiri*, which was originally discovered in fecal contents of the American tapir (*Tapirus terrestris*) in Brazil was observed for the first time associated to *Equus caballus*. The review on the ciliate communities associated with horses around the world demonstrated that the equine ciliates are a very diversified group with representatives in two subclasses, Suctoria and Trichostomatia, totaling 78 species distributed in three orders and six families, having been registered in different equine hosts (*Equus asinus*, *Equus caballus* and *Equus quagga*) and geographic locations (USA, China, Russia, Taiwan, Mexico, Cyprus, Turkey, Kyrgyzstan and Brazil).

Key-words. Entodiniomorphida, Suctoria, symbiotic ciliates, Trichostomatia

INTRODUCTION

Protist ciliates (Alveolata, Ciliophora) include more than 8000 described species, of which approximately 3000 are symbionts or parasites, inhabiting a wide range of invertebrate and vertebrate hosts. Among the symbiotic ciliates of vertebrates, a significant portion is found in symbiosis with herbivorous mammals, and are mainly distributed in two subclasses, Trichostomatia Bütschli and Suctoria Clapèrede & Lachmann (Lynn 2008).

In equines, almost 80 species of these ciliates have been recognized, and the role they play in host metabolism is still controversial. According to Dehority (1986) they participate in the digestion of soluble carbohydrates and are indispensable to the health of the host. However, French *et al*. (1996), observed ciliates of the species *Polymorphella ampulla* (Dogiel) and *Cycloposthium* sp. in the intestinal mucosa of horses causing persistent cramps and diarrhea.

The composition of the intestinal ciliate communities of equids is known in various geographic locations (Hsiung 1930, 1935 a, b, 1936; Ike *et al*. 1981, 1983 a, b, 1985; Imai 1999, Ito *et al*. 1996; Strelkow 1939; Kornilova 2004; Tung 1992; Aladro-Lubel *et al*. 2010; Göçmen *et al*. 2012; Gürelli 2012; Gürelli & Göçmen 2010, 2011, 2012; Gürelli *et al*. 2015). However, until the present moment, no investigations have been conducted on the symbiotic ciliates of horses in Central and South America.

Thus, the aims of this study were to identify and quantify the gastrointestinal ciliates from Brazilian domestic horses (*Equus caballus* L.), to compare the data obtained with previous studies and to review data on ciliate communities associated with domestic and wild horses around the world.

MATERIALS AND METHODS

We studied the feces of eight individuals of domestic horses (*Equus caballus*), six males and two females, kept under extensive management, feeding only on pasture in Juiz de Fora, Minas Gerais, Brazil (21º 41’ S 43º 20’ O). Fecal samples were obtained immediately after defecation of animals and consisted of 20 cm^3 fixed in an equal volume of 18.5% formaldehyde (Dehority 1984).

The taxonomy of the generic names was based on Lynn (2008) and the identification of species was based on descriptions of Cunha & Muniz (1925); Hsiung

(1930), Strelkow (1939), Levine (1961) and Wolska & Piechaczek (1970). To identify the ciliates, we used an Olympus BX-51 light microscope (600 X and 1000 X magnification) equipped with an Olympus Evolt E-330 digital camera.

The prevalence of each species was determined according to Bush *et al*. (1997), the average relative abundance was estimated from a sample of 300 cells from each animal (Ito *et al*. 1994; Mishima *et al*. 2009) and the density was determined in a Sedgewick-Rafter chamber according to Dehority (1984) with modifications of D'Agosto & Carneiro (1999).

RESULTS AND DISCUSSION

Ciliate community in Brazilian domestic horses

Across all eight equids analyzed, two subclasses, two orders, five families, 13 genera and 24 species (22 trichostomatids and two suctorians) of ciliates were identified (Table 1). There are no new species and new morphological data on the species inventoried. In the Figure 1, we present some ciliate species reported in this work.

The ciliate composition was similar among the animals analyzed, being entodiniomorphids (subclass Trichostomatia) the most representatives, in all animals evaluated. The family Blepharocorythidae Hsiung presented a prevalence of 100% (Table 1), and the family Buetschliidae Poche was observed in greater abundance (44.67) and density (2.9×10^4 ciliates/ ml fecal contents) (Table 1).

Among the trichostomatid ciliates identified, *Cycloposthium dentiferum* was observed for the first time in Central and South America and *Bundleia elongata*, *B. inflata*, *B. piriformis*, *B. vorax* and *Cycloposthium psicauda*, were observed for the first time in the American continent. In addition, the species *Buissonella tapiri*, which was originally discovered in fecal contents of the American tapir (*Tapirus terrestris* L.) in Brazil (Cunha & Muniz 1925) was observed for the first time associated to *Equus caballus*.

Buissonela tapiri was described by Cunha & Muniz (1925) as a symbiont of American tapir in Brazil, and was redescribed in detail by Wolska & Piechaczek (1970), who isolated it from tapirs kept at the Łódź Zoo, Poland. This is the third record of this species and the first record in symbiosis with *Equus caballus*. Taking into account that the species was described in Brazil and the tapir specimens used by Wolska & Piechaczek

(1970) also came from this country, new studies are needed to understand if *B. tapiri* may be a Brazilian endemic species.

Suctorian ciliates (Phyllopharyngea, Suctoria) associated with the digestive tract of herbivorous mammals belong to the family Allantosomatidae Jankowsky. They are represented, currently, by six genera (*Allantosoma* Gassovsky, *Allantoxena* Jankowsky, *Arcosoma* Jankowsky, *Brevitentaculum* Gurelli, *Strelkowella* Kornilova and *Vanhovenia* Dovgal) and nine species (Dovgal 2002; Kornilova 2004; Lynn 2008; Gurelli 2016). They present the body in cylindrical shape with tentacles in capitate or stick shape, arranged in fasciculi, at the poles or evenly distributed in the body (Dovgal 2002; Lynn 2008; Gurelli 2016). Two species of suctorians were observed in the present study, *Allantosoma intestinale* Gassovsky and *Arcosoma dicorniger* Hsiung. While the species *A. intestinale* has been widely recorded as a symbiont of equines in several geographical locations (Table 2), *A. dicorniger* seems to be a rare species, since it was observed only in horses in the USA (Hsiung 1930) and in Mexico (Aladro-Lubel *et al.* 2006; Güiris *et al.* 2010). In this way, this is the first record of this species in Central and South America (Table 2).

These new records for Central and South America as well as for the American continent may be related to the scarcity of studies involving the characterization of gastrointestinal ciliates associated with herbivorous mammals around the world, since this group of ciliates has been neglected, which highlights the importance of the present study.

The fecal ciliate composition in this study is similar to other records around the world, but more similar to the ciliate communities associated with horses from Taiwan, Mexico and Cyprus, due to the absence of ciliates belonging to the order Vestibuliferida (Table 2).

A review on the ciliate communities associated with horses around the world

The first report of microeukaryotes ciliates associated to the intestinal tract of horses was performed by Gruby & Delafond (1843), who analyzed the gastrointestinal content of several mammalian species, reporting the presence of small organisms associated with the intestine of the domestic horse, *Equus caballus*. On this occasion, the authors made a brief scientific report, and therefore did not delineate and named the organisms found, and it is not possible for subsequent authors to recognize them.

Eleven years later, Collin (1854) made a new report of these microeukaryotes, when he made drawings of eight morphotypes found in the gut of *Equus caballus*, among which the ciliates currently known as *Blepharocorys uncinata*, *Bundleia* spp. and *Cycloposthium* spp.

After these first reports, several taxonomic studies were performed (Bundle 1895, Fiorentini 1890, Gassovsky 1919, Buisson 1923, Strelkow, 1939, Hsiung 1930, 1935a, b, 1936, Chavarria 1933) and many taxa of symbiotic ciliates of equines have been described succinctly, using simple ciliatological techniques, in different geographical locations (Table 2). Among these works, we highlight those of Buisson (1923), Strelkow (1939) and Hsiung (1930, 1935a, b, 1936), who organized the biodiversity of these ciliates, proposing new classifications, creating new genera, families and associating them, evolutionarily, with the symbiotic ciliates of other herbivorous mammals, such as ruminants (i.e., family Ophryoscolecidae Stein).

Despite the great relevance of these studies, only from the year 1960, with the use of silver impregnation techniques and scanning and transmission electron microscopy, that these ciliates began to be studied in detail. Fernández-Galiano (1959), Wolska (1969, 1971, 1978a, b, 1979, 1980, 1981) and Imai *et al*. (1979), in detailed morphological studies on the species *Blepharocorys* spp., *Bundleia posticiliata*, *Circodinium minimum*, *Cochliatoxum periachtum*, *Cycloposthium affine*, *Cycloposthium edentatum*, *Didesmis quadrata*, *Gassovskiella galea*, *Holophryoides ovalis*, *Ochoterenaia appendiculata*, *Spirodinium equi*, *Tetratoxum parvum*, *Tetratoxum unifasculatum*, *Triadinium caudatum* and *Tripalmaria dogieli*, extended the understanding of the morphological complexity of these organisms, describing the oral and somatic infraciliary patterns, as well as the ultrastructural organization of the body, which opened the way for new approaches in the study of these microeukaryotes, mainly ecological studies.

The years 1980, 1990 and 2000, therefore, were characterized by numerous ecological studies and inventories on the ciliates associated with different equine hosts, especially the studies conducted in Japan by Ike *et al*. (1981, 1983a, b, 1985), Tung (1992), Ito *et al*. (1996) and Imai (1999), who studied the dynamics and distribution of ciliates in the intestinal tract of hosts, as well as experiments to understand the process of intestinal colonization by these ciliates.

Subsequent inventories were performed by Kornilova (2003, 2006) in Russia, by Aladro-Lubel *et al.* (2006) and Güiris *et al.* (2010) in Mexico, by Gürelli (2012) and Gürelli & Göçmen (2011, 2012) in Turkey, Göçmen *et al.* (2012) and Gürelli & Göçmen (2010) in Cyprus, Gürelli *et al.* (2015) in Kyrgyzstan and the present study in Brazil.

The years 2000 were characterized by studies involving the molecular characterization of equine ciliates with the purpose of inferring the phylogenetic relationships into subclass Trichostomatia. Strüder-Kypke *et al.* (2007) were the first to use this approach, sequencing the 18S rDNA of the species *Cycloposthium edentatum*, *Cycloposthium ishikawai*, *Tripalmaria dogieli*, *Cochliatoxum periachtum*, and *Paraisotricha colpoidea*. After, Snelling *et al.* (2011), Moon-Van der-Staay *et al.* (2014) and Ito *et al.* (2014) provided sequences of the species *Alloiozona trizona*, *Blepharoconus hemiciliatus*, *Blepharocorys angusta*, *Blepharocorys curvicula*, *Blepharocorys jubata*, *Blepharocorys microcorys*, *Blepharocorys uncinata*, *Bundleia benbrooki*, *Bundleia nana*, *Bundleia posticiliata*, *Circodinium minimum*, *Cycloposthium bipalmatum*, *Didesmis ovalis*, *Gassovskiella galea*, *Hemiprorodon gimnoprosthium*, *Ochoterenaia appendiculata*, *Paraisotricha minuta*, *Prorodonopsis coli*, *Spirodinium equi*, *Sulcoarcus pullucidulus*, *Tetratoxum excavatum*, *Tetratoxum parvum*, *Tetratoxum unifasciculatum* and *Triadinium caudatum*, currently totaling 29 18S rDNA sequences of symbiotic ciliates of equines available in molecular databases.

Although several studies with different approaches have been performed with equine ciliates, few are the data on the physiology of these microorganisms, and, consequently, it is difficult to determine what role they play in their hosts, because to better understand these issues are necessary *in vitro* experiments, which requires the maintenance of cultures. However, a protocol for this purpose was proposed only recently (Belzecki *et al.* 2016).

Table 2 compiles information on the intestinal ciliate fauna composition of domestic and wild horses around the world. The analysis of these data shows that the ciliate communities are similar among the different geographical locations sampled. However, some unusual records are observed, such as the species *Buissonela tapiri* in this study. In addition, some species were described and never later recorded, for example, *Wolskana tokarensis*, described in Natives Tokara Pony in Japan (Ito *et al.* 1996). These reports demonstrate that the equine ciliates are a very diversified group with representatives in two subclasses, Suctoria and Trichostomatia, totaling 78 species distributed in three orders and six families, having been registered in different equine hosts (*Equus asinus*, *Equus caballus* and *Equus quagga*) and geographic locations (USA, China, Russia, Taiwan, Mexico, Cyprus, Tukey, Kyrgyzstan and Brazil).

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Figure and Tables

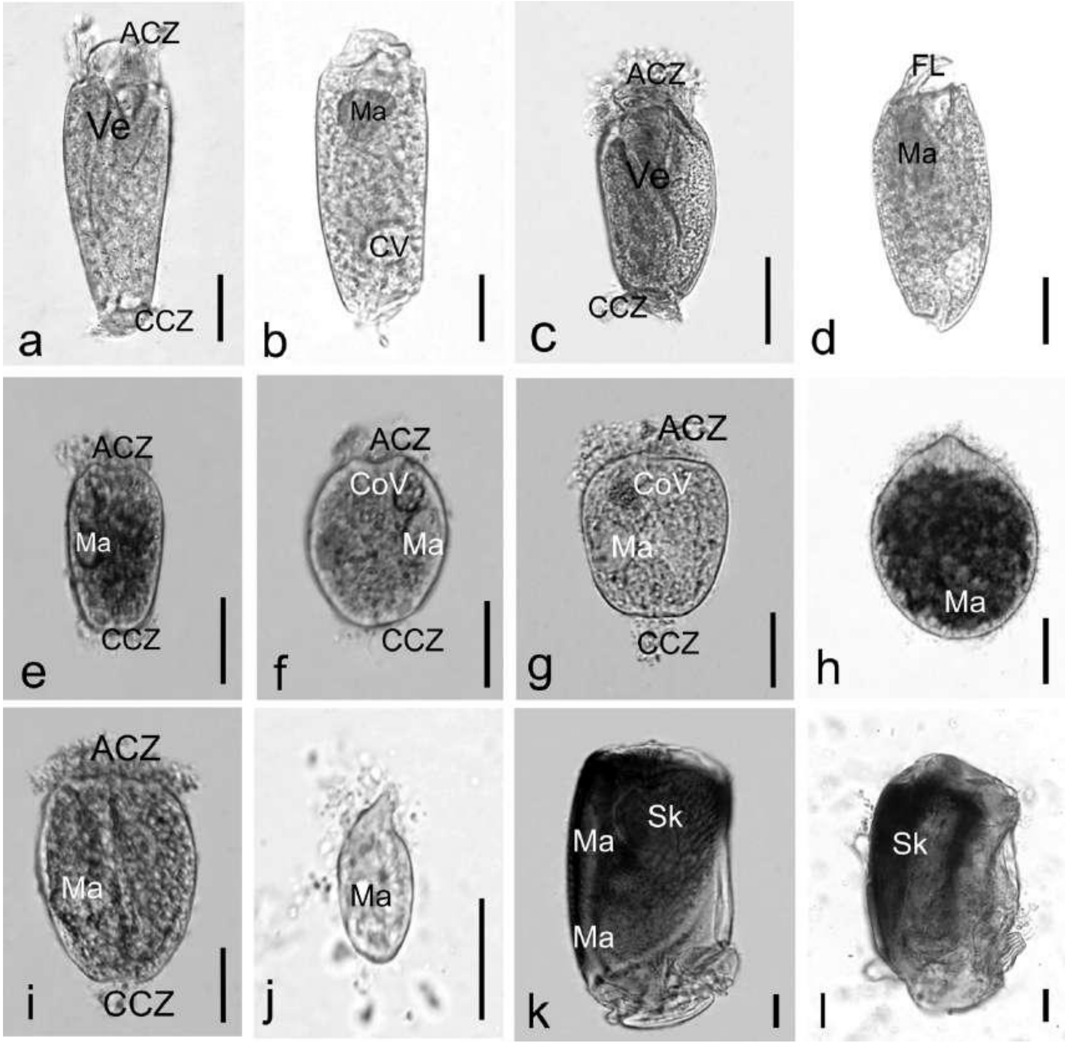

Figure 1. Intestinal ciliates recorded in Brazilian domestic horses (*Equus caballus*, L.), after staining by Lugol's Solution. a-d. Family Blepharocorythidae Hsiung; e-j. Family Buetschliidae Poche; k.l. Cyclosposthiidae. a. *Blepharocoys angusta*, b. *Blepharocorys cardionucleata*; c. *Blepharocorys curvicula*; d. *Blepharocorys valvata*; e. *Bundleia elongata*; f. *Bundleia posticiliata*, g. *Bundleia vorax*, h. *Buissonela tapiri*, i. *Didesmis quadrata*, j. *Polymorphella ampula*, k. *Cycloposthium edentatum*, l. *Tripalmaria dogieli*. ACZ. Adoral ciliary zone, CCZ. caudal ciliary zone, CV. Contractile vacuole, CoV: Concrection vacuole, FL: frontal lobe, Ma. macronucleus, Sk. skeletal plate, Ve. Vestibulum. Scale bars: 10 µm.

Table 1. Prevalence, average relative abundance and average density of intestinal ciliate species recorded in Brazilian domestic horses (*Equus caballus* L.) (n=8)

Subclass/Order/Family/Genus/Species			
Subclass Trichostomatia Bütschli			
Order Entodiniomorphida Reichenow in Doflein and Reichenow	**Prevalence**	**ARA**[a]	**Density**
Family Buetschliidae Poche			
Blepharosphaera intestinalis Bundle	37.5	0.13	122.67
Buissonela tapiri Wolska & Piechaczek**	12.5	0.08	54.67
Bundleia elongata Strelkow*	100	13.46	7605.33
Bundleia inflata Strelkow*	100	4.42	2739.33
Bundleia piriformis Strelkow*	100	5.33	3098.00
Bundleia posticiliata (Bundle)	100	15.21	8630.00
Bundleia vorax Strelkow*	75	6.25	3912.00
Didesmis quadrata Fiorentini	100	1.67	1045.33
Polymorphella ampulla (Dogiel)	100	2.83	1577.33
Sulcoarcus pellucidulus Hsiung	87.5	1.29	748.00
Family Blepharocorythidae Hsiung, 1929			
Blepharocorys angusta Gassovsky	100	9.79	5587.33
Blepharocorys cardionucleata	100	1.71	913.33
Blepharocorys curvicula Gassovsky	100	3.17	1868.67
Blepharocorys jubata Bundle	100	3.08	1864.00
Blepharocorys valvata (Fiorentini)	100	7.63	4072.67
Ochoterenaia appendiculata Chavarria	100	3.13	1543.33
Family Cycloposthidae Poche, 1913			
Cycloposthium bipalmatum (Fiorentini)	100	7.21	3400.00
Cycloposthium dentiferum Gassovsky	87.5	2.75	1798.00
Cycloposthium edentatum Strelkow	50	1.04	930.00
Cycloposthium psicauda Strelkow*	25	0.13	108.67
Tripalmaria dogieli Gassovsky	37.5	0.96	349.33
Family Spirodiniidae Strelkow			
Tetratoxum spp.	75	1.42	832.67

Subclass Suctoria Clapèrede & Lachmann			
Ordem Exogenida			
Family Allantosomatidae Jankowski			
Allantosoma intestinale Gassovsky	100	7.00	4047.33
Arcosoma dicorniger (Hsiung)	12.5	0.21	270.00

* First record in the Central and South America

** First record in *Equus caballus* L.

[a] Average Relative Abundance (ARA)

Table 2. Review on the ciliate communities associated with horses around the world

Subclass/Order/Family/Genus/Species	**USA [1]**	**China [2]**	**Japan [3]**	**Russia [4]**	**Taiwan [5]**	**Mexico [6]**	**Cyprus [8]**	**Turkey [9]**	**Kyrgyzstan [10]**	**Brazil [11]**
Subclass Trichostomatia Bütschli										
Order Vestibuliferida de Puytorac *et al.*										
Family Paraisotrichidae da Cunha										
Paraisotricha beckeri Hsiung	+	-	+	+	-	-	-	-	-	-
Paraisotricha colpoidea Fiorentini	+	-	+	+	-	-	-	+	+	-
Paraisotricha minuta Hsiung	+	+	+	+	-	-	-	+	+	-
Order Entodiniomorphida Reichenow in Doflein & Reichenow										
Suborder Archistomatina de Puytorac *et al.*										
Family Buetschliidae Poche										
Alloiozona trizona Hsiung	+	-	+	+	+	+	-	+	+	-
Ampullacula ampulla Hsiung		-		-		-		-	-	-
Blepharoconus benbrooki (Hsiung)	+	+	+	+	+	+	+	+	+	-
Blepharoconus cervicalis Hsiung		-		-		+	-	-	-	-
Blepharoconus hemiciliatus Gassovsky	-	-	+	+	+	-	-	+	+	-
Blepharoprosthium pireum Bundle	+	+	+	+	+	+	-	+	+	-
Blepharoprosthium polytrichum Strelkow	-	-	+	+	-	-	-	+	+	-
Blepharosphaera citriformis Strelkow	-	-	+	+	-	-	-	-	-	-
Blepharosphaera ellipsoidalis Hsiung	+	+	+	+	+	+	-	+	+	-
Blepharosphaera intestinalis Bundle	+	+	+	+	-	+	+	-	-	+
Blepharozoum zonatum Gassovsky	-	-	+	+	-	-	-	-	-	-
Buissonella tapiri Cunha & Muniz	-	-	-	-	-	-	-	-	-	**+**
Bundleia asymetrica Strelkow	-	-	+	+	-	-	-	-	-	-

Bundleia dolichosoma Strelkow	-	-	+	+	-	-	-	+	-	-
Bundleia elongata Strelkow	-	-	+	+	-	-	-	+	+	+
Bundleia inflata Strelkow	-	-	+	+	-	-	-	+	+	+
Bundleia nana Strelkow	-	-	+	+	-	-	-	+	+	-
Bundleia piriformis Strelkow	-	-	-	+	-	-	-	+	-	+
Bundleia posticiliata (Bundle)	+	+	+	+	+	+	+	+	+	+
Bundleia triangularis Strelkow	-	-	-	+	-	-	+	+	-	-
Bundleia vorax Strelkow	-	-	+	+	-	-	-	-	-	+
Didesmis ovalis Fiorentini	+	+	+	+	+	+	-	+	-	-
Didesmis quadrata Fiorentini	+	-	+	+	+	+	-	-	-	+
Didesmis spiralis Hsiung	+	-	+	+	+	-	-	-	-	-
Hemiprorodon gymnoprosthium Strelkow	-	-	+	+	-	-	-	+	-	-
Holophryoides macrotricha Strelkow	-	-	+	+	+	-	-	+	-	-
Holophryoides ovalis (Fiorentini)	-	-	+	+	+	-	+	+		-
Paraisotrichopsis composita Gassovsky	-	+	+	+	-	-	-	+	-	-
Polymorphella ampula (Dogiel)	+	+	+	+	+	+	+	+	+	+
Prorodonopsis coli Gassovsky	-	+	+	+	-	-	+	+		-
Sulcoarcus pellucidulus Hsiung	-	+	-	+	-	+	-	-	-	+
Wolskana tokarensis Ito *et al.*	-	-	+	-	-	-	-	-	-	-
Suborder Blepharocorythina Wolska										
Family Blepharocorythidae Hsiung										
Blepharocorys angusta Gassovsky	+	-	+	+	+	+	+	+	-	+
Blepharocorys cardionucleata Hsiung	+	-	+	+	+	+	-	-	-	+
Blepharocorys curvicula Gassovsky	+	+	+	+	+	+	+	+	+	+
Blepharocorys jubata Bundle	+	-	+	+	+	+	-	-	-	+
Blepharocorys microcorys Gassovsky	-	-	+	+	+	-	+	+	+	-

Blepharocorys uncinata (Fiorentini)	+	+	+	+	-	+	-	+	+	-
Blepharocorys valvata (Fiorentini)	+	+	+	+	+	+	+	-	-	+
Charonina equi Hsiung	+	+	+	+	+	+	+	-	-	-
Circodinium minimum Wolska	+	+	+	+	+	+	+	+	-	-
Ochoterenaia appendiculata Chavarria	-	-	+	+	+	+	-	+	-	+
Suborder Entodiniomorphina Reichenow in Doflein & Reichenow										
Family Cycloposthidae Poche										
Cycloposthium affinae Strelkow	+	-	+	+	-	-	-	-	-	-
Cycloposthium bipalmatum (Fiorentini)	+	+	+	+	+	+	-	+	+	+
Cycloposthium corrugatum Hsiung	+	-	+	+	-	-	-	-	-	-
Cycloposthium dentiferum Gassovsky	+	-	+	+	+	-	-	-	-	+
Cycloposthium edentatum Strelkow	+	+	+	+	+	+	+	+	+	+
Cycloposthium hemioni Kornilova	-	-	-	+	+	-	-	+	-	-
Cycloposthium plicatocaudatum Gassovsky	-	-	-	+	-	-	-	-	-	-
Cycloposthium ponomareyi Kornilova	-	-	-	+	-	-	-	-	-	-
Cycloposthium psicauda Strelkow	-	-	-	+	-	-	-	-	-	+
Cycloposthium scutigerum Strelkow	+	-	+	+	+	+	-	-	-	-
Tripalmaria dogieli Gassovsky	+	-	+	+	+	+	-	+	-	+
Family Spirodiniidae Strelkow										
Cochliatoxum periachtum Gassovsky	+	+	+	+	+	-	-	+	+	-
Ditoxum brevinucleatum Strelkow	-	+	+	+	-	-	+	-	-	-
Ditoxum funinucleum Gassovsky	+	-	+	+	-	+	-	-	+	-
Gassovskiella galea (Gassovsky)	+	+	+	+	+	+	-	+	-	-
Spirodinium confusum Hsiung	-	+	+	+	-	-	+	+	-	-

Spirodinium equi Fiorentini	+	+	+	+	+	+	-	-	+	+
Spirodinium magnum Ike *et al.*	-	-	+	+	-	-	-	-	-	-
Spirodinium nanum Strelkow	-	-	+	-	-	-	-	-	+	-
Spirodinium uncinucleatum Hsiung	-	+	-	+	-	-	+	-	-	-
Tetratoxum excavatum Hsiung	+	-	+	+	+	+	+	+	+	?
Tetratoxum unifasciculatum (Fiorentini)	+	+	+	+	+	+	+	+	+	?
Tetratuxum parvum Hsiung	+	+	+	+	+	+	-	+	+	?
Triadinium caudatum Fiorentini	+	+	+	+	+	+	-	-	-	-
Triadinium magnum Hsiung	-	+	-	+	-	-	-	-	-	-
Subclass Suctoria Clapèrede & Lachmann										
Order Exogenida Colin										
Allantosoma intestinale Gassovsky	+	-	+	+	+	+	+	+	+	+
Allantosoma cucumis Strelkow	-	-	+	+	-	-	-	-	-	-
Allantoxena biseriale (Strelkow)	-	-	+	+	-	-	-	-	-	-
Allantoxena japonensis (Imai)	-	-	+	+	-	-	-	-	-	-
Arcosoma brevicorniger (Hsiung)	+	-	+	+	+	+	-	-	-	-
Arcosoma dicorniger (Hsiung)	+	-	+	+	+	+	+	-	-	+
Arcosoma lineare (Strelkow)	-	-	+	+	-	-	-	-	-	-
Strelkowella urunbasiensis Kornilova	-	-	-	+	-	-	-	-	-	-

SEÇÃO 4

(Seção a ser submetida no periódico *Tropical Animal Helth and Production*, A3, FI: 1.089)

Rumen ciliates (Ciliophora, Trichostomatia) in Brazilian domestic cattle: taxonomic inventory and taxocenosis structure

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Abstract

Since their discovery, rumen ciliates of domestic cattle were reported in various geographic locations and several host species. However, until now there is only one taxonomic inventory of ciliates associated to Brazilian cattle, including specific identifications. Thus, the present study aimed to access the ciliate composition, relative abundance, richness, and density of rumen ciliates of Brazilian cattle feeding diets supplemented with crescent urea levels. Across all treatments analyzed, one subclass, two orders, four families, 11 genera and 32 species of ciliates were identified. The composition and species richness varied among the four treatments evaluated. However, the total ciliate density was not affect by the experimental diets used ($p>0.01$). The species *Entodinium dalli* was identified for the first time in Brazil and the species *Holophryozoon bovis*, described by Jirovec in domestic cattle in Russia was morphologically characterized in the present study based on live observations and staining by Lugol's and MFS solution.

Keywords: *Holophryozoon bovis*, Trichostomatid ciliates, ruminants, symbiotic ciliates, urea-based diets

Introduction

Ciliates in the subclass Trichostomatia were found in large amounts colonizing the gastrointestinal tracts of several herbivorous mammals (Cedrola et al. 2019a). In ruminants they are found in the rumen and participates in various processes in digestive metabolism of their hosts, degrading structural and non-structural carbohydrates, and acting as bioindicators, showing the balance and proper functioning of ruminal activities (Williams and Coleman 1992; Wright 2015).

Since their discovery by Gruby and Delafond (1843), rumen ciliates of domestic cattle were reported in various host species and several geographic locations (Cedrola et al. 2019a). However, until now there is only one taxonomic inventory of ciliates associated to Brazilian cattle, including specific identifications (Dehority 1986). In this way, it is extremely necessary to perform new trichostomatid ciliates taxonomic surveys in the country, since the gastrointestinal ciliatofauna inventoried in Brazil are very peculiar and include several new reports and new species (Dehority 1979, 1986; Cedrola et al. 2016, 2017a, b, 2018a, b, 2019b). The present study aimed to access the ciliate composition, relative abundance, richness, and density of rumen ciliates of Brazilian cattle feeding diets supplemented with crescent urea levels.

Material and methods

We used four Holstein x Gir cattle (*Bos primigenius taurus* L. x *Bos primigenius indicus* L.), females, kept in individual pens in the experimental field José Henrique Bruschi, Embrapa Gado de Leite, Coronel Pacheco, Minas Gerais, Brazil. During the experimental period, the animals received diet composed by grass hay and crescent levels of urea (**T1** grass hay; **T2** grass hay + 100% protein source from protein concentrate; **T3** grass hay + 50% protein source from protein concentrate + 50% N from urea; **T4** grass hay + 100% N from urea). The experimental design used is a Latin square 4 x 4.

Samples were obtained manually, via rumen fistula and consisting of 20 cm^3 of rumen content, which was fixed in 18.5% formalin (Dehority 1984). Identification of species was based on the descriptions of Dogiel (1927), Kofoid and MacLennan (1930, 1932), Kofoid and Christenson (1933), Dehority (1974), Ogimoto and Imai (1981), and Williams and Coleman (1992). To identify the ciliates, we used an

Olympus BX-51 light microscope (600 X and 1000 X magnification) equipped with a Olympus Evolt E-330 digital camera and Image-Pro Plus 6.0 software, to perform morphometry on 20 specimens of each species stained with the Lugol's solution (Cedrola et al. 2015). The orientation of the ciliates for description was adopted from Dogiel (1927): the side closest to the macronucleus was termed the dorsal side, and the opposite one, the ventral side, thus, defining the right and left sides.

The prevalence of each species was determined according to Bush et al. (1997), the average relative abundance was estimated from a sample of 300 cells from each animal (Ito et al. 1994; Mishima et al. 2009) and the density was estimated in a Sedgewick-rafter chamber according to Dehority (1984).

The data on the ciliate density were accessed by Kruskal-Wallis test followed by Dunn test, which are the nonparametric analyses equivalent to ANOVA and Tukey test, respectively. The total and specific densities were compared among the different treatments, considering the effects to $p<0.01$. All the analyses were performed in the car package version 3.0 (Fox and Weisberg 2019), implemented in R Studio software version 3.5.1 (R Development Core Team, 2018).

Results and Dicussion

Taxonomic inventory

Across all treatments analyzed (T1, T2, T3, and T4), one subclass, two orders, four families, 11 genera and 32 species of ciliates were identified (Table 1).

This is the first study on the rumen ciliates from Brazilian domestic cattle, with specific identifications, since the previous reports in the 1986 by Dehority (Dehority 1986). The ciliatofauna inventoried by Dehority (1986) was similar to that observed in this study. However, the author noticed a greater species richness, 57 species, 25 species more than on that work. This differences may be related to the animal's management (Goçmen et al. 2002). The animals in Dehority's (1986) experiment were maintaining exclusively on pasture, diet that favors the establishment of rumen ciliates, as on pasture the animals consume several types of grasses (Williams and Coleman 1992; Cedrola et al. 2016). Although the diets used in the present study were composed in great amount by hay, it comes from only one species of grass and were supplemented by protein concentrate and/or urea (T2, T3 and T4), which can promote deleterious effect on some rumen ciliate species.

Among the species identified, the species *Entodinium dalli* was identified for the first time in Brazil and the species *Holophryozoon bovis*, described by Jirovec (1932) in domestic cattle in Russia was morphologically characterized in the present study based on live observations and staining by Lugol's and MFS solutions.

Holophryozoon bovis Jirovec, 1932 (Figure 2) displays the body oval shaped (length: 20.43 ± 1.07; width: 9.67 ± 0.94, *n* = 10), small, laterally compressed, and totally recovered by cilia. The cilia are long and widely spaced. The vestibulum is located in the anterior body portion, and is long and very conspicuous, occupying ~1/3 of the total body length. The cytoplasm is clear and translucent and contain several inclusions, probably starch granules and bacteria. The macronucleus is rounded shaped, without defined position in the body. The micronucleus is elliptical shaped, and is located in the surface of the macronucleus. A small contractile vacuole is located in the posterior end of body.

These two first records may be related to few rumen ciliates surveys performed in Brazil, using specific identifications, since most studies in Brazil are generic inventories. In addition, the first record of *Holophryozoon bovis* may be due to their small body dimensions, which difficult it detection in samples with a large amount of ciliates.

The species *Holophryozoon bovis* was described by Jirovec (1932) in the rumen contents of domestic cattle in Russia. Subsequently, was registered by Dehority et al. (1983) in association with domestic cattle in Tenessee, EUA, as *Oligoisotricha bubali* (Dogiel, 1928). Later, the species was described again, under a new name, *Microcetus lappus* by Orpin and Matissen (1986). Thus, according to International Code of Zoological Nomenclature (2000), *M. lappus* is a junior synonymous of *H. bovis*. Jirovec (1932) in the original description, pointed out that *H. bovis* displays several morphological characters uncommon with other gastrointestinal ciliates and classified it into family Buetschliidae (order Entodiniomorphida). However, the author highlights the need for further morphological data to better understand it taxonomic placement. On the other hand, Orpin and Matissen (1986) do not classified the species in family level, but suggest it taxonomic placement into order Vestibuliferida, due to it long and conspicuous vestibulum. However, the family Buetschliidae and the order Vestibuliferida do not constitutes monophyletic groups (Ito et al. 2014; Kittelmann et al.

2015; Cedrola et al. 2019a). In this way, new morphological and molecular data will be necessary to better understand this issue.

Taxocenosis structure

The ciliate species composition and richness varied among the four treatments evaluated (Table 1; Figure 1). However, the total ciliate density was not affect by the experimental diets used ($p>0.01$) (Table 1).

These variations in species composition and richness are in agreement with several literature reports (Kittelmann and Janssen 2011; Newbold et al. 2015; Cedrola et al. 2016, 2017; Freitas et al. 2017; Santana-Neto et al. 2017; Reis et al. 2019), which pointed out that the rumen ciliates respond specifically to different treatments. Even so, the number of unique species in each treatment was small (T1 = 4; T2 = 0; T3 = 2 and T4 = 1), what may be explained by the experimental diets compositions, which are constituted in great amount by grass hay. The hay has the capacity to stimulate rumen ciliates when we compare to other kind of feeding systems, using only energetic or protein concentrates, which may promote an intense deleterious effect in the ciliaofauna as observed by Cedrola et al. (2016) in domestic sheep.

The experimental diets composition may explain, also, the absence of effects on total ciliate density, which remained constant even in T4, treatment with 100% urea. Thus, we suppose that the total replacement of the protein by non-protein nitrogen (urea) in the conditions performed in the present experiment can be advantageous, as this supplement is affordable and effective in ruminant production systems, since the ciliatofauna stability may indicate an equilibrium in the ruminal functions and consequently in both animal performance and efficiency (Khiaosa-ard and Zebeli 2014).

Although the total ciliate density was not affect in the present experiment, we observed effects on densities of some species. *Entodinium dalli* exhibited an increase in density in T4 ($p<0.01$), which may be related to a possible urea stimulation on bacterial populations (Wang et al. 2018), which are food source for some *Entodinium* species (Williams and Coleman 1992; Wright 2015). *Entodinium ovinum*, *Eodinium bilobosum*, and *Polyplastron multivesiculatum* exhibited a decrease in densities in T2, T3, and T4 ($p<0.01$), which suggest a significant intolerance of these species to both concentrate and urea-based diets, when we compare to other ciliate species inventoried in this work. Moreover, several experimental studies highlight that some *Polyplastron* *multivesiculatum* populations were negatively affected by energetic and protein concentrate inclusions (Williams and Coleman 1992), which may explain these effects. In addition, the species *Diplodinium tetratacanthum* showed lower densities in T3 and T4, the treatments with urea inclusions, suggesting a possible intolerance of this species to urea supplementations, but not to the protein concentrate. Lastly, *Eremoplastron rostratum* showed an increasing in density in T2 and a subsequent decrease in T3 and T4. Possibly, this species can be stimulated by the protein concentrate, however is poorly tolerant to the urea inclusions. (Table 1).

Entodinium exiguum displayed the largest relative abundance in this experiment, totalizing 26,08; 18,77; 25,58 and 27 % of the total abundance observed in T1, T2, T3 and T4, respectively (Figure 1), which are in accord with the classical literature, that pointed out species belonging to genus *Entodinium* as the most abundant in domestic ruminants (Williams and Coleman 1992).

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Figures and Table

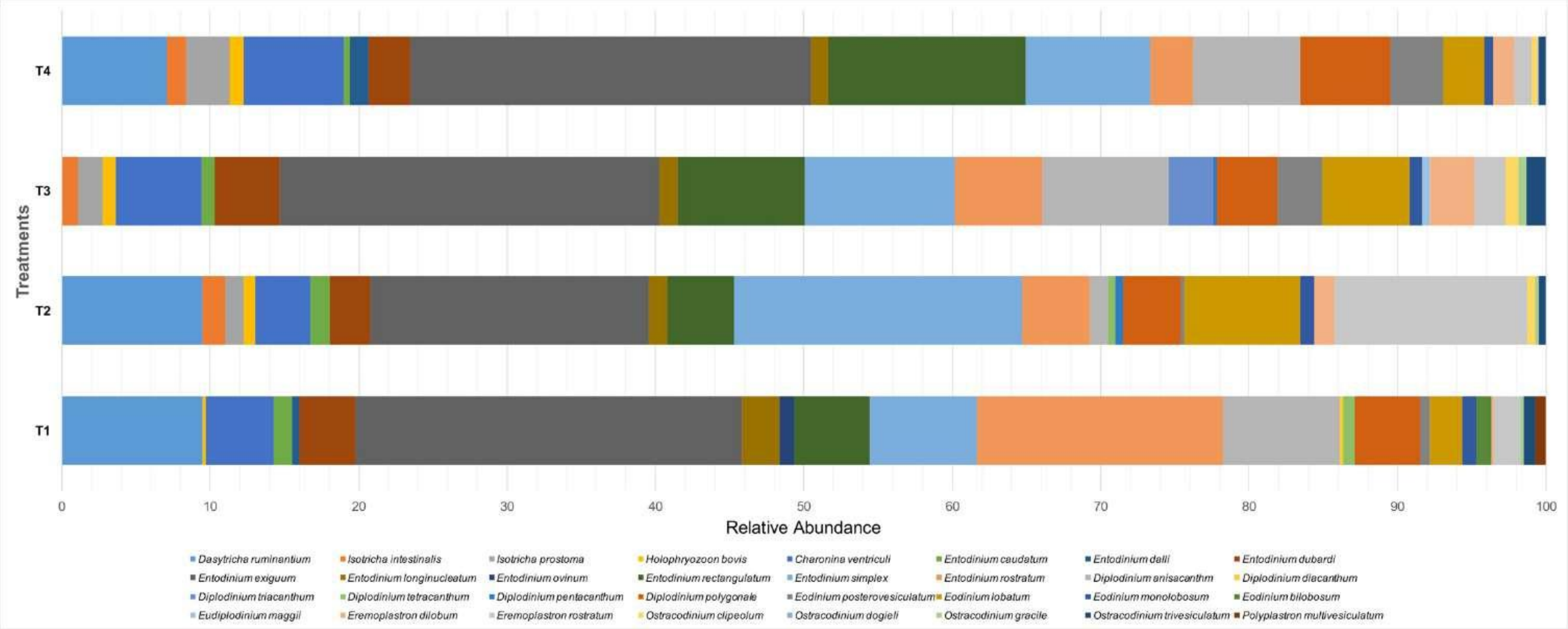

Figure 1. Relative abundance of rumen ciliate species in four experimental diets. T1: treatment 1; T2: treatment 2; T3: treatment 3; and T4: treatment 4.

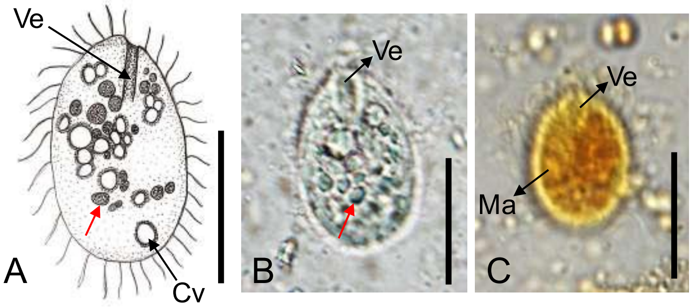

Figure 2. *Holophryozoon bovis* Jirovec, 1932 recorded in Brazilian domestic cattle. Cv: contractile vacuoles; Ma: Macronucleus; Ve: Vestibulum; Red arrows: intracytoplasmatic granules. Bars: 20 µm.

Table 1. Averages of rumen ciliate species densities (x 10^4) ciliates /ml rumen contents of domestic cattle fed diets with crescent urea levels.

Ordem Vestibuliferida	**T1**	**T2**	**T3**	**T4**	***p***
Família Isotrichidae					
Dasytricha ruminantium	2.82^{a}	2.23^{a}	2.27^{a}	1.50^{a}	0.4321
Isotricha intestinalis	0.00^{a}	0.35^{a}	0.19^{a}	0.26^{a}	0.2409
Isotricha prostoma	0.00^{a}	0.29^{a}	0.30^{a}	0.63^{a}	0.1895
Ordem Entodiniomorphida					
Família Buetschliidae					
Holophryozoon bovis	0.07^{a}	0.17^{a}	0.16^{a}	0.19^{a}	0.4836
Família Blepharocorythidae					
Charonina ventriculi	1.33^{a}	0.88^{a}	1.06^{a}	1.43^{a}	0.0934
Família Ophryoscolecidae					
Entodinium caudatum	0.37^{a}	0.29^{a}	1.69^{a}	0.08^{a}	0.1951
Entodinium dalli	0.14^{a}	0.00^{a}	0.00^{a}	0.26^{b}	0.0045
Entodinium dubardi	1.11^{a}	0.64^{a}	0.79^{a}	0.60^{a}	0.4317
Entodinium exiguum	7.76^{a}	4.41^{a}	4.71^{a}	5.74^{a}	0.5822
Entodinium longinucleatum	0.74^{a}	0.29^{a}	0.23^{a}	0.24^{a}	0.9607
Entodinium ovinum	0.29^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.0001
Entodinium rectangulatum	1.51^{a}	1.05^{a}	1.58^{a}	2.81^{a}	0.2449
Entodinium simplex	2.15^{a}	2.21^{a}	1.85^{a}	1.79^{a}	0.8469
Entodinium rostratum	4.93^{a}	1.05^{a}	1.09^{a}	0.62^{a}	0.0231
Diplodinium anisacanthum	2.33^{a}	0.29^{a}	0.82^{a}	1.52^{a}	0.3100
Diplodinium diaacanthum	0.74^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.0245
Diplodinium triacanthum	0.00^{a}	0.00^{a}	0.18^{a}	0.00^{a}	0.0245
Diplodinium tetracanthum	0.22^{a}	0.11^{a}	0.00^{b}	0.00^{b}	0.0005
Diplodinium pentacanthum	0.00^{a}	0.11^{a}	0.04^{a}	0.00^{a}	0.0811
Diplodinium polygonale	1.31^{a}	0.92^{a}	0.75^{a}	0.12^{a}	0.8531
Eodinium posterovesiculatum	0.19^{a}	0.05^{a}	0.18^{a}	0.76^{a}	0.3697

Eodinium lobatum	0.64^{a}	0.64^{a}	0.53^{a}	0.58^{a}	0.9183
Eodinium monolobosum	0.29^{a}	0.23^{a}	0.33^{a}	0.12^{a}	0.8541
Eodinium bilobosum	0.29^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.0056
Eudiplodinium maggii	0.00	0.00	0.09	0.00	0.0245
Eremoplastron dilobum	0.04	0.17	0.18	0.30	0.2429
Eremoplastron rostratum	0.52^{a}	0.86^{b}	0.38^{a}	0.24^{a}	0.0010
Ostracodinium clipeolum	0.00	0.11	0.16	0.08	0.0406
Ostracodinium dogieli	0.00	0.00	0.00	0.01	0.0248
Ostracodinium gracile	0.07	0.05	0.09	0.00	0.2966
Ostracodinium trivesiculatum	0.22	0.11	0.18	0.10	0.2178
Polyplastron multivesiculatum	0.22^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.0001
Total de ciliados	29.76^{a}	17.65^{a}	18.43^{a}	21.28^{a}	0.9763

Means followed by the same letter in the same row do not differ by the nonparametric Kruskal-Wallis with $p < 0.01$. T1: Treatment 1, T2: Treatment 2, T3: Treatment 3, T4: Treatment 4.

SEÇÃO 5

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Morphology and description of infraciliary bands pattern in four *Metadinium* Awerinzew & Mutafowa, 1914 species (Ciliophora, Entodiniomorphida, Ophryoscolecidae) with taxonomic notes on the genus

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ABSTRACT

The genus *Metadinium* includes ophryoscolecid ciliates, with two retractile ciliary zones in the anterior body portion, two broad skeletal plates, a lobed macronucleus and two contractile vacuoles. Species belonging to this genus were recorded in several geographical locations and in different host species. However, they were mostly described based on a single ciliatological technique, and until now, there is only one description of the infraciliary pattern in *Metadinium* ciliates (*Metadinium medium*). The present study performs the morphological and infraciliature characterization of four species belonging to *Metadinium*: *M. esalqum, M. minorum*, *M. rotundatum* and *M. ypsilon*; and presents taxonomic notes on the genus. The oral infraciliature in these four *Metadinium* species corresponds to the Diplodinium-type, pattern shared with genera *Diplodinium*, *Diploplastron*, *Eudiplodinium*, *Eremoplastron* and *Polyplastron*. A brief review conducted highlights the difficulty of delimiting *Metadinium* species due to the morphological similarity among them.

Key-words: Brazil, *Metadinium*, ophryoscolecid ciliates, rumen ciliates, sheep

INTRODUCTION

Silver impregnation techniques are essential in the taxonomy of several groups of ciliates (Foissner 2014), since they reveal the oral and somatic infraciliatures, a taxonomic character of extreme relevance in this group.

The family Ophryoscolecidae (Ciliophora, Entodiniomorphida) is found in symbiosis with herbivorous mammals, mainly ruminants and pseudoruminants, domestic and wild (Williams & Coleman 1992). The oral infraciliary pattern in these ciliates is important and has helped to clarify the evolutionary relationships within the family (Ito & Imai 2006, Rossi *et al.* 2015).

The genus *Metadinium* Awerinzew & Mutafowa, 1914 includes 12 species of ophryoscolecid ciliates, which present as sinapomorphic characters two retractile ciliary zones in the anterior body portion, two broad skeletal plates, a lobed macronucleus and two contractile vacuoles (Kofoid & MacLennan 1932; Williams & Coleman, 1992). Species belonging to this genus were recorded in several geographical locations and in different species of domestic and wild ruminant hosts (Williams & Coleman 1992). However, they were mostly described based on a single ciliatological technique, basically semipermanent preparations based on iodine or methyl green (Deority 1975, 1979; Dogiel 1927; Lubinsky 1963) and until now, there is only one description of the infraciliary pattern of *Metadinium* ciliates (*Metadinium medium*) (Gurelli & Akman 2017).

Thus, the present study performs the morphological and infraciliature characterization of four species belonging to *Metadinium*: *M. esalqum* (Dehority, 1979); *M. minorum* (Dehority, 1975); *M. rotundatum* Kofoid and Christenson, 1933 and *M. ypsilon* (Dogiel, 1925); and also presents taxonomic notes on the genus.

MATERIALS AND METHODS

Samples of rumen contents were obtained from 16 sheep (*Ovis aries*, L.) kept in the Setor de Caprino-ovinocultura, Departamento de Zootecnia, Universidade Federal Rural de Pernambuco, Pernambuco, Brazil (for details, see Cedrola *et al.* 2016) and of a sheep kept in the Fazenda Experimental da Embrapa – Gado de Leite, Minas Gerais, Brazil (for details, see Rossi *et al.* 2015). Each sample consisted of 20 cm^3 of rumen contents fixed in formalin 18.5% (Dehority 1984). The species identification was based on previous descriptions of Dogiel (1927); Kofoid & MacLennan (1932), Kofoid & Christenson (1933) and Dehority (1975, 1979). Morphometry was based on specimens stained with Lugol's solution (D'Agosto & Carneiro 1999), using the software Image-Pro Plus 6.0. The orientation of ciliates for description followed Dogiel (1927), i.e. the side near the macronucleus is the dorsal side; this defines together with the anterior body end, the ventral, right, and left body sides. The impregnation of infraciliary bands was made by silver carbonate (Rossi *et al*. 2016). The term polybrachykinety refers to infraciliary bands composed of numerous, small and parallel kineties (Fernandez-Galiano 1979). The drawing of *Metadinium* ciliates was made after free hand sketching during observation of the specimen under an Olympus BX51 microscope equipped with differential interferential contrast. The diagrams of the oral infraciliature are made with aid of a drawing device, and were based on silver impregnated specimens.

RESULTS AND DISCUSSION

Morphology and infraciliary bands pattern of four *Metadinium* species (Fig. 1)

General morphology. The four *Metadinium* species, characterized in this study, were identified based on descriptions and redescriptions of Dogiel (1927), Kofoid & MacLennan (1932), Kofoid & Christenson (1933) and Dehority (1975, 1979).

Metadinium esalqum has a rectangular body (body length 81.45 ± 7.72 µm [mean ± SD] and body width 57.51 µm ± 6.25), *M. minorum* has an oval body (body length 89.33 µm ± 1.82 and body width 61.21 µm ± 5.39), *M. rotundatum* present an oval body (body length 78.70 µm ± 5.69 and body width 53.80 µm ± 3.88) and *M. ypsilon* has an elliptical body (body length 90 µm ± 5.21 and body width 59.31 µm ± 5.92). The anterior portion of the body is strongly flattened in the four species. The external pellicle presents prominent longitudinal striations. The adoral and dorsal retractile ciliary zones lie in the same plane at the anterior body portion. The ciliary zones are separated by a slight protuberance, the operculum. The vestibulum is short and wide. The cytopharynx is large and contains many longitudinal fibers that support the structure to allow the ingestion of large food particles. These fibers were designated "esophageal fibers" by Kofoid & MacLennan (1932). The cytoproct opens at the posterior body portion and it is wide and rugged. The macronucleus is rod-shaped in the four species. However, in *M. esalqum* (macronuclear length 53.06 µm ± 7.88 and macronuclear width 12.11 µm ± 1.35) and *M.* y*psilon* (macronuclear length 58.96 µm ± 2.40 and macronuclear width 9.49 µm ± 1.17) the macronucleus presents two lobes in the dorsal surface and in *M. minorum* (macronuclear length 58.48 µm ± 10.11 and macronuclear width 11.19 µm ± 2.47) and *M. rotundatum* (macronuclear length 41.08 µm ± 1.98 and macronuclear width 12.11 µm ± 0.87) the macronucleus presents only one lobe in the dorsal surface. The micronucleus is elliptical in the four species and is located in the median dorsal portion of the macronucleus. Two contractile vacuoles are positioned in the dorsal side of the macronucleus and presents conspicuous excretory pores. One vacuole is located in anterior end of the macronucleus and other are located in the posterior end of the macronucleus. The four species present two large skeletal plates on the left side of the body. In *M. esalqum*, *M. minorum* and *M. ypsilon*, one of the skeletal plates, departs from the base of the adoral ciliary zone and the other departs from the base of the operculum. In *M. rotundatum* both depart from the base of the adoral ciliary zone. In all four species the plates fuse in the median portion of the body and remain attached until the posterior third. Skeletal plates are the characters that most vary in *Metadinium* ciliates and therefore will be considered in more detail in the topic “taxonomic notes on the genus”.

Infraciliary bands. The oral infraciliature in these four *Metadinium* species corresponds to the Diplodinium-type (Ito and Imai 2006). This pattern consists of an adoral polybrachykinety (AP), vestibular polybrachykinety (VP), dorsal polybrachykinety (DP) and paralabial kineties (PK). AP involves the buccal opening and VP is long and extends into the vestibule, if originating internally by the dorsal side of AP. DP extends laterally along the dorsal side of the body and PK, ranging in number from four to five, are located close to the ventral portion of AP.

Morphological analysis of the oral and somatic infraciliature is extremely useful in taxonomic descriptions of microeukaryotes ciliates (Foissner 2014). In ophryoscolecids are recognized several infraciliary patterns (Noirot-timothée 1960; Fernández-Galiano 1958; Ito *et al*. 1997, 2001, 2002, Ito & Imai 1998, 2003, 2005, 2006; Mishima *et al*. 2009) and some taxa in this family have specific infraciliary bands pattern, which facilitates the taxonomic identification in the group (Ito & Imai 2003; Ito *et al*. 2001). However, until the present study, only the species *Metadinium medium* had characterized as to oral infraciliature.

The *Metadinium* species, characterized in this work, presents the infraciliary bands that differ from that observed in *Metadinium medium*. *Metadinium medium* presents the infraciliary bands similar to Ostracodinium gracile-type and Ostracodinium mammosum-type (Gurelli & Akman 2017). Our four species, on the other hand, presents infraciliary bands organized according to the Diplodinium-type (Ito & Imai 2006). This pattern is composed of *Diplodinium* Schuberg, 1888, *Diploplastron* Kofoid & MacLennan, 1932, *Eremoplastron* Kofoid & MacLennan, 1932, *Eudiplodinium* (Dogiel, 1927) and *Polyplastron* species (Noirot-Timothée 1960; Ito & Imai 2006).

All these taxa belong to the subfamily Diplodiniinae. In recent molecular studies, Diplodiniinae ciliates species group together monophyletically (Rossi *et al*. 2015; Cedrola *et al*. 2017) and these species share various morphological characters, such as: number and position of the ciliary zones and position of the nuclear apparatus (Kofoid & MacLennan 1932). It is possible that such morphological similarity demonstrates that Diplodiniinae species may be closely related phylogenetically. However, studies using molecular information have not yet answered this question, due to few nucleotide sequences of Diplodiniinae representatives in databases (Cedrola *et al*. 2017, Rossi *et al*. 2015) and the low resolution of the 18S rDNA to define the phylogenetic relationships within the family Ophryoscolecidae (Cedrola *et al*. 2017).

Thus, new morphological and molecular studies involving a larger number of sequences of this group, as well the use of other molecular markers in the recovery of the phylogenetic analyzes and approaches using reconstruction of ancestral character are important to investigate if the oral infraciliature patterns may reflect evolutionary divergence in the family Ophryscolecidae.

Taxonomic notes on the genus *Metadinium* (Fig. 2; Table 1)

The genus *Metadinium* was established by Awerinzew & Mutafowa (1914) to include a single species of ophryoscolecid ciliate, *Metadinium medium* Awerinzew & Mutafowa, 1914, at the time characterized by the presence of two separate ciliary zones. These authors suggested that this morphological feature was a novelty in relation to the other ciliates of the group already described, because it was believed that *Diplodinium* presented the ciliary zones connected by the left side.

Dogiel (1927), however, in a monograph on the family Ophryoslolecidae, found that the ciliary zones in both *Metadinium* and *Diplodinium* were identical and considered Awerinzew & Mutafowa (1914) proposal unjustified. Also on this occasion, the author broadly reformulated the taxonomy of the family Ophryoslocidae proposing the division of the genus *Diplodinium* into four subgenera: *Anoplodinium* (no skeletal plates and two contractile vacuoles); *Eudiplodinium* (one or two skeletal plates and two contractile vacuoles); *Polyplastron* (five skeletal plates and numerous contractile vacuoles) and *Ostracodinium* (one broad skeletal plate and two to six contractile vacuoles). Thus, *M. medium* and other similar species became the subgenus *Eudiplodinium*.

Kofoid & MacLennan (1932) reviewed the taxonomy of the family Ophryoslolecidae and observed that *M. medium* [*Diplodinium* (*Eudiplodinium*) *medium*], as well as other similar species, shared several morphological characteristics, such as: two skeletal plates, lobed macronucleus, two contractile vacuoles, broad ectoplasm and hard external surface, which allowed the creation of a new genus to include them. As *M. medium* had been described by Awerinzew & Mutafowa (1914) in the genus *Metadinium*, the authors chose to reestablish the genus and proposed a new diagnosis for it: "Ophryoscolecidae with two dorsal and adoral zones at anterior end of the body, two skeletal plates beneath right surface, occasionally fused at posterior end, large macronucleus with two or three prominent dorsal lobes, two contractile vacuoles lying close to macronucleus, cuticle and ectoplasm heavy, conspicuous esophageal fibries beneath dorsal and right lateral surface".

Following the diagnosis proposed by Kofoid & MacLennan (1932), the genus *Metadinium* is represented by 12 species (Fig. 2; Table 1), differentiated mainly by body size and shape, macronucleus shape and skeletal plates morphology and position. However, due to the morphological similarity among them, the specific identification of *Metadinium* ciliates becomes difficult.

The species *M. caudatum* Lubinsky, *Metadinium fissilaminatum* (Dogiel) and *M. sablei* (Van Hoven *et al.*) (Fig. 2c, d and j) presents very similar general morphology, which makes identification difficult. All three have very large and long skeletal plates, which cover a large part of the left body surface. The plates depart approximately from the base of the operculum and fuse in the anterior third of the body, following fused until the final third of the body. These species also have the elliptical body shape with small lobe in the posterior region. However, they differ in relation to the host and the geographical location where they were described and later recorded. *M. caudatum* was described in musk ox (*Ovibos moschatus* Blainville) in Ottawa, Canada (Lubinsky 1963) and again recorded in the same host in Banks Island, Canada (Dehority 1985). *M. fissilaminatum* was described in domestic and wild ruminants in Uganda, Africa (*Kobus kob thomasi* (Sclater), *Redunca redunca* (Pallas), *Syncerus caffer* Sparrman and *Bos taurus indicus* L. (Dogiel 1932) and subsequently registered in *Kobus leche kafuensis* Haltenorth in Zambia, Africa (Imai *et al.* 1992). *M. sablei* was described in the *Hippotragus niger antelope* (Harris) in Rhodesia, Africa (Van Hoven *et al.* 1979). In fact, the similarity between *M. fissilaminatum* and *M. sablei* was detected by Imai *et al*. (1992), which investigated the possibility of such species being synonymy.

The species *Metadinium banksi* (Dehority) and *Metadinium esalqum* (Dehority) (Fig. 2b and e) are also very similar to each other and differ mainly in the body dimensions and morphology of the skeletal plates. *M. banksi* was described in *Ovibos moschatus* in Banks Island, Canada (Dehority 1985) and according to the original description, it has larger body dimensions than *M. esalqum* (Table 1). However, *M. banski* was recorded on other occasions in hosts and in different geographic locations in the Australian red deer (*Cervus elaphus* L.) (Dehority 1997) and in domestic goats (*Capra hircus* L.) (Göçmen 2002). The specimens recorded in the Australian red deer were significantly smaller than those isolated in the original description, showing the possibility of polymorphism in this species, making it difficult to distinguish *M. esalqum*. Also, the second character used to differentiate them, the morphology of the skeletal plates, also causes doubt, since *M. esalqum* may present significant polymorphism in its skeletal plates (Dehority 1979).

The other species belonging to the genus are less difficult to identify, since they have markedly different morphological characters. *M. medium* (Fig. 2a) is the only species to present unfused skeletal plates; *M. magnum* (Fig. 2f) has large body dimensions and trilobed macronucleus with little prominent lobes; *M. ossiculi* (Fig. 2h) shows very thin skeletal plates and highly developed cytopharyngeal fibers; *M rotundatum* (Fig. 2i) is the only species to present small body dimensions and in strongly rounded shape; *M. tauricum* (Fig. 2k) has curved skeletal plates and inclined toward the dorsal portion of the ciliate body. Finally, *M. ypsilon* (Fig. 2l) has a trilobed macronucleus and wide skeletal plates that almost completely cover the left side of the body, which is strongly elliptical.

This study characterized, for the first time, the infraciliature in four species of the genus *Metadinium* (*M. esalqum*, *M. minorum*, *M. rotundatum* and *M. ypsilon*), classifying them in the *Diplodinium*-type (Ito & Imai 2006). It also demonstrated, through a brief taxonomic review on the genus, the importance of the detailed morphological characterization of *Metadinium* spp., during the specific delimitation, considering the great similarity observed between the species that compose this genus.

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Figures and Table

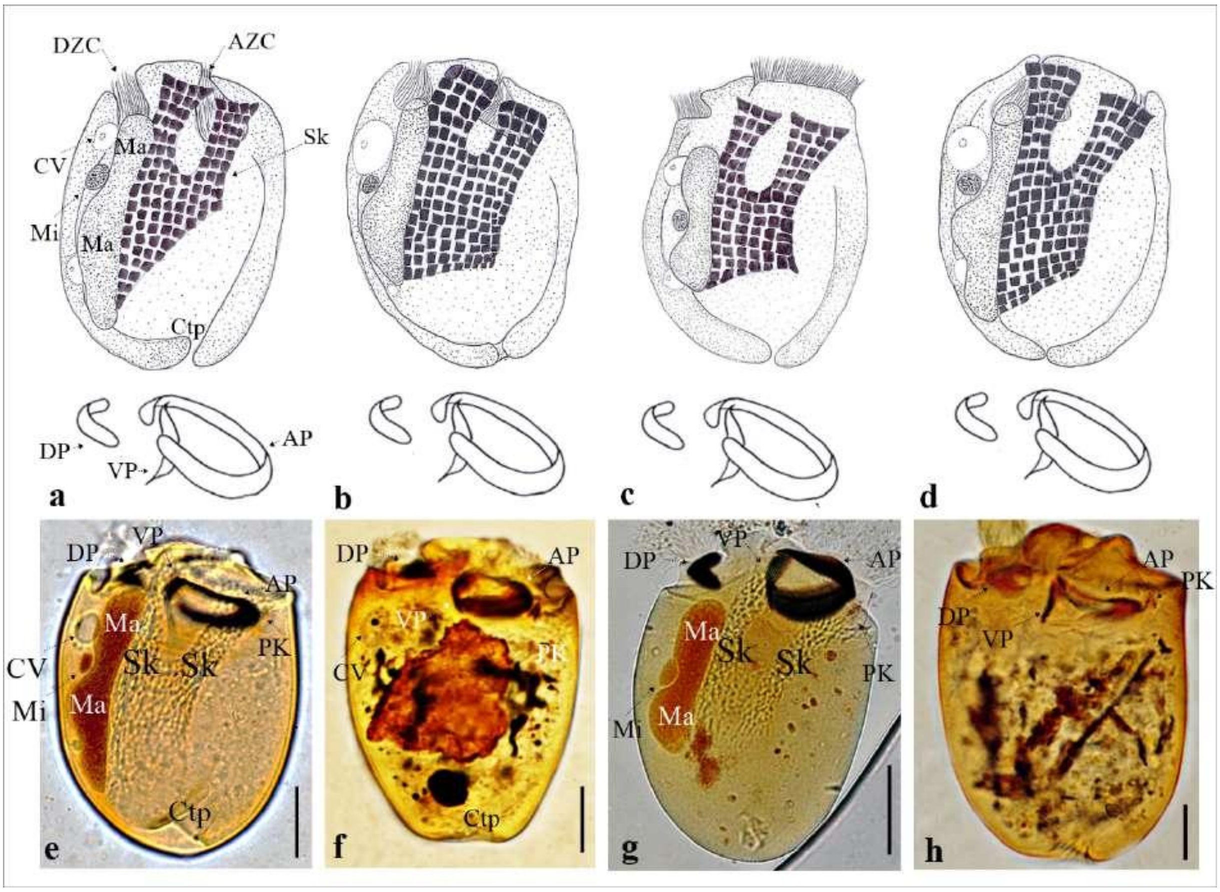

Figure 1. Four species of *Metadinium* ciliates reported in Brazilian domestic sheep (*Ovis aries* L.) a-d schematic drawings based on specimens stained with Lugol's solution and impregnated with silver carbonate; e-h specimens after silver carbonate impregnation. a and e. *Metadinium esalqum* (Dehority, 1979); b and f. *Metadinium minorum* (Dehority, 1975); c and g. *Metadinium rotundatum* Kofoid & Christenson, 1933; d and h. *Metadinium* aff. *ypsilon* (Dogiel, 1925). AP: adoral polybracykynety; ACZ: adoral ciliary zone; CV: contractile vacuole; Ctp: Citoproct; DCZ: dorsal ciliary zone; DP: dorsal polybrachykynety; Ma: macronucleus; Mi: micronucleus; PK: paralabial kineties; Sk: skeletal plates; VP: vestibular polybrachykynety. Bars: 20 µm.

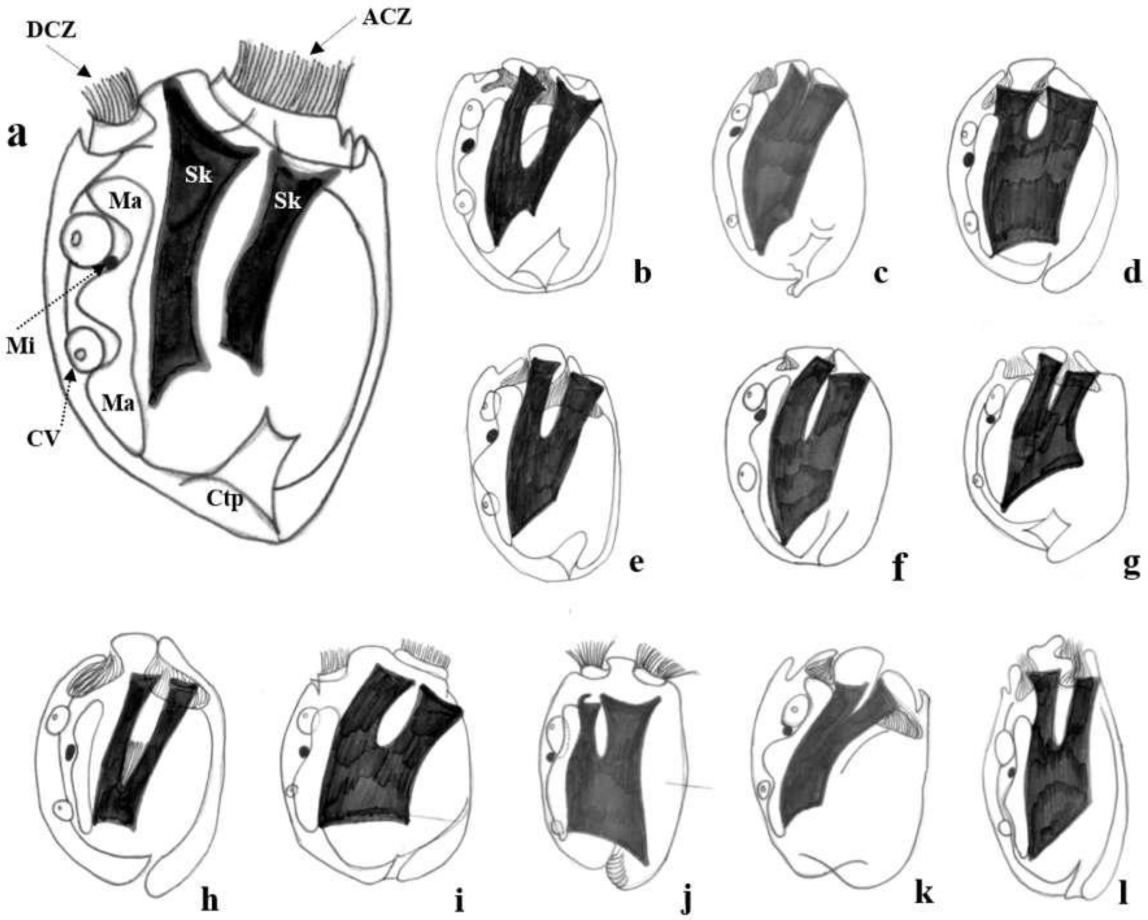

Figure 2. Schematic drawings of the species described in genus *Metadinium* Awerinzew & Mutafowa, 1914 based on the original description. a. *Metadinium medium*, type species of the genus; b. *M. banksi* c. *M. caudatum*; d. *M. fissilaminatum;* e. *M. esalqum*; f. *M. magnum* g. *M. minorum*; h. *M. ossiculi*; i. *M. rotundatum*; j. *M. sablei*; k. *M. tauricum*; l. *M. ypsilon*. ACZ: adoral ciliary zone; DCZ: dorsal ciliary zone; Ma: macronucleus; Mi: micronucleus; CV: contractile vacuole; Ctp: Cytoproct; Sk: Skeletal plates. Figures prepared out of scale. For morphometric data, see Table 1.

Table 1. Morphological and morphometrics characteristics of the *Metadinium* Awerinzew & Mutafowa, 1914 species*.

Species	**Body lenght (µm)**	**Body width (µm)**	**Body shape**	**Macronuclear shape**	**Macronuclear lenght (µm)**	**Macronuclear width (µm)**
Metadinium banksi (Dehority, 1975)	118-162	77-118	Eliptical	Trilobed	86	9-11
Metadinium caudatum Lubinsky, 1963	175-250	115-180	Eliptical	Trilobed	140	4
Metadinium fissilaminatum (Dogiel, 1932)	97-140	55-84	Eliptical	Trilobed	*	*
Metadinium esalqum (Dehority, 1979)	65-96	47-66	Retangular	Trilobed	51	6-9
Metadinium magnum (Dogiel, 1925)	156-231	92-162	Eliptical	Trilobed	106	5-14
Metadinium medium Awerinzew & Mutafowa, 1914	180-272	92-170	Eliptical	Bilobed	128	7-14
Metadinium minorum (Dehority, 1975)	95-131	65-89	Eliptical	Bilobed	67	5-11
Metadinium ossiculi Imai *et al*. 1992	60-90	40-60	Eliptical	Bilobed	*	*
Metadinium rotundatum Kofoid & Christenson, 1933	52-73	35-61	Oval	Bilobed	38	5-7
Metadinium sablei Van Hoven, 1979	76-102	52-69	Eliptical	Triboled	58	8
Metadinium tauricum (Dogiel & Fedorowa, 1925)	185-288	70-160	Triangular	Trilobed	146	7-23
Metadinium ypsilon (Dogiel, 1925)	110-152	60-72	Eliptical	Trilobed	68	4-10

*Data obtained from original descriptions and redescriptions: Awerinzew & Mufafowa (1914), Dehority (1975, 1979), Dogiel (1925, 1927), Dogiel & Fedorowa (1925), Imai *et al.* (1992), Kofoid & Christenson (1933), Kofoid & MacLennan (1932), Lubinsky (1963) and Van hoven (1979).

SEÇÃO 6

(Seção submetida no periódico *Acta Protozoologica*, A3, FI: 1.491)

Redescription of *Polyplastron alaskum* Dehority, 1974 (Ciliophora, Entodiniomorphida), a rare rumen ciliate species from domestic and wild sheep

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Abstract. The present study redescribe the species *Polyplastron alaskum* based on morphological characterization and provides, for the first time, data on its infraciliary bands pattern. *Polyplastron alaskum* was described in Alaskan dall montain sheep (*Ovis dalli*), and registered again only in the present study, 45 years later, inhabiting the rumen contents of domestic sheep (*Ovis aries*) in northeastern Brazil. All the taxonomic characters observed in the original description was observed in the present study. The infraciliary pattern in *Polyplastron alaskum* is slightly different of Diplodinium-type, observed in some ophryoscolecids belonging to the subfamily Diplodiniinae.

Key-words: Trichostomatia, Brazilian domestic sheep, ophryoscolecid ciliate

INTRODUCTION

The genus *Polyplastron* was established by Kofoid & MacLennan (1932) and includes six ophryoscolecid species (Ciliophora, Trichostomatia, Ophryoscolecidae):

Polyplastron multivesiculatum (Dogiel & Fedorowa), *P. alaskum* Dehority, *P. arcticum* Lubinsky, *P. californiense* Bush & Kofoid, *P. fenestratum* (Dogiel), and *P. monoscutum* (Dogiel). They are taxonomically characterized by two retractable ciliary zones in the anterior end of body, four to five skeletal plates and four to five contractile vacuoles (Williams and Coleman 1992). Among those species, only *Polyplastron multivesiculatum* has a broad geographic distribution and low host specificity (Williams and Coleman 1992). The other five ones are rare and were never found again after the original description. Here, we performed the second report of *Polyplastron alaskum*, 45 years later, and redescribe this species based on morphological characterization and provides, for the first time, data on its infraciliary bands pattern.

MATERIAL AND METHODS

Rumen samples were obtained from 16 domestic sheep (*Ovis aries* L.) housed in individual stalls and distributed in a completely randomized design with four treatments and four replicates, in the Universidade Federal Rural de Pernambuco, Pernambuco, Brazil. The animals received water and mineral salts *ad libitum*, and a diet of corn, soybean meal, and vegetable oil. The basal diet consisted of Tifton hay (*Cynodon dactylon*) (for details see Medeiros *et al.* 2009). Samples were obtained manually, at slaughter of animals, from the rumen mass center, consisting of 20 cm^3 of rumen content, which was fixed in 18.5% formalin (Dehority 1984). Species identification was based on the descriptions of Dogiel (1927), Kofoid and MacLennan (1932), Bush and Kofoid (1948), Lubinsky (1958), Dehority (1974), and Williams and Coleman (1992). To identify the ciliate, we used an Olympus BX-51 light microscope (600 X and 1000 X magnification) equipped with an Olympus Evolt E-330 digital camera and Image-Pro Plus 6.0 software to perform morphometry on 20 specimens stained with the Lugol's solution (D'Agosto and Carneiro 1999; Cedrola *et al.* 2015). The orientation of the ciliates for description was adopted from Dogiel (1927): the side closest to the macronucleus was termed the dorsal side, and the opposite one, the ventral side, thus, defined the right and left sides. The infraciliary bands were stained using the silver carbonate impregnation technique (Cedrola *et al.* 2015; Rossi *et al.* 2016). The term polybrachykinety denotes infraciliary bands composed of numerous, short and parallel kineties (Fernandez-Galiano 1979). Free-hand drawings were created for observation of the specimens stained with Lugol's solution and after silver carbonate impregnation, under a microscope (Olympus BX-51). All drawings were obtained so that the anterior side of the body was at the top of the page. The species prevalence was determined according to Bush *et al*. (1997) and the relative abundance was estimated according to Mishima *et al*. (2009).

RESULTS

Polyplastron alaskum Dehority, 1974 (Fig. 1, Table 1)

Elliptical body shape, laterally compressed, and rounded posteriorly. Two retractable ciliary zones, one adoral and one dorsal, at anterior end of body, separated by an inconspicuous and tapered operculum. Complex skeletal apparatus, composed by five skeletal plates. Two plates are located on the right surface of body, primitiva and carina. Primitiva is juxtaposed to the macronucleus, and is wide and long, extending from the anterior portion of the operculum to the posterior body portion; carina is located near the ventral surface of body and present the same length than primitiva, extending from the base of the adoral ciliary zone to the posterior body portion. Three plates are located on the left body surface, tergum, anticarina and scutum. Tergum is located in the dorsal surface of body, and are wide and long; anticarina is located in the middle portion of body, and are wide and short; and scutum is located in the ventral surface of body, and are short and inconspicuous. The anterior end of the left plates was connected by transverse polysaccharide bars. Ectoplasm extend beyond of the body and forms an inconspicuous small lobe. Endoplasm with many food particles, essentially vegetal fibers and starch granules. Long macronucleus, club-shaped, located in the dorsal surface of body, extending from the base of the ciliary zone to the posterior body portion. The elliptical micronucleus is in a depression in the dorsal anterior surface of the macronucleus. Five contractile vacuoles, are located in a line in the dorsal surface of body, each with a conspicuous opening excretory pore. Rectum is a large, rigid and tubular structure located in the posterior end of body, where cytoproct opens.

Oral infraciliature. Oral infraciliature resembles the Diplodinium-type (Ito and Imai 2006), however present some morphological differences. There is composed by an adoral polybrachykinety (AP), vestibular polybrachykinety (VP), a dorsal polybrachykinety (DP) and paralabial kineties (PK). AP involves the buccal opening; VP is long and extends spirally into the vestibule, originating internally by the dorsal side of AP. DP extend laterally along dorsal anterior side of body. Four or five PK close to ventral portion of AP.

Remarks. *Polyplastron alaskum* slightly resembles *Polyplastron arcticum*, a rumen ophryoscolecid ciliate described by Lubinsky (1958), inhabiting the rumen contents of Canadian reindeer (*Rangifer tarandus* Smith). However, *P. arcticum* displays only four skeletal plates, four contractile vacuoles, and largest body dimensions.

Habitat and hosts. *Polyplastron alaskum* was originally described in the rumen contents of wild sheep, dall montain sheep (*Ovis dalli* Nelson) in the vicinity of Cantwell, Alaska (Dehority 1974) and found again only in the present study, in low prevalence (6.25%) and relative abundance (0.8 ± 1.6), inhabiting the rumen contents of domestic sheep (*Ovis aries* L.) in Recife, Pernambuco, Brazil.

Voucher slides: Type material. Voucher slides with Brazilian population of *Polyplastron alaskum* (C_Dd_0002_01) were deposited in the collection of the Laboratório de Protozoologia (LabProto), Universidade Federal de Juiz de Fora, Minas Gerais, Brazil.

DISCUSSION

The genus *Polyplastron* currently includes six ophryoscolecid ciliate species, in which only *Polyplastron multivesiculatum* was observed in several host species, domestic and wild, and present a broad geographic distribution (Williams and Coleman 1992). The other five species were not observed again since original description, and should be considered rare species.

There are now a total of at least 137 species of rumen trichostomatid ciliates associated with domestic and wild sheep around the world (Booyse and Dehority 2011; Cedrola *et al*. 2016). Most of them present a broad geographic distribution. However, some species are rare and have a restricted geographical distribution. *Polyplastron alaskum* was described by Dehority (1974) in Alaskan dall montain sheep (*Ovis dalli*), and registered again only in the present study, inhabiting the rumen contents of domestic sheep (*Ovis aries*) in northeastern Brazil. The Brazilian domestic sheep were introduced in the country after the colonization process, from different geographical locations. Thus, it is likely that *P. alaskum* were introduced in Brazil along with domestic sheep.

In the morphological and morphometric characterizations performed in the present study, we observed all taxonomic characters present in the original description (Fig. 1; Table 1) and we described for the first time, the oral infraciliature in *Polyplastron alaskum*. The pattern in this species is slightly different of Diplodinium-type (Ito and Imai 2006), observed in some ophryoscolecids belonging to the subfamily Diplodiniinae, such as *Diplodinium* Schuberg, *Eremoplastron* Kofoid & MacLennan, *Eudiplodinium* (Dogiel) and in *Polyplastron multivesiculatum*.

Diplodinium-type infraciliary pattern present the VP straight into the vestibulum (Ito and Imai 2006), instead, in *P. alaskum* we observed a spiral VP. The infraciliary bands in other *Polyplastron* species was described only for *Polyplastron multivesiculatum* (Fernadez-Galiano 1958). According to the author, this species does not display spiral VP. New data on infraciliature of *Polyplastron* species is necessary to understand whether spiral VP is a automomophic character of *Polyplastron alaskum*.

The present study performed the second report of the species *Polyplastron alaskum* and redescribed it based on morphological data and description, for the first time, of the infraciliature.

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Figure, Legend and Table

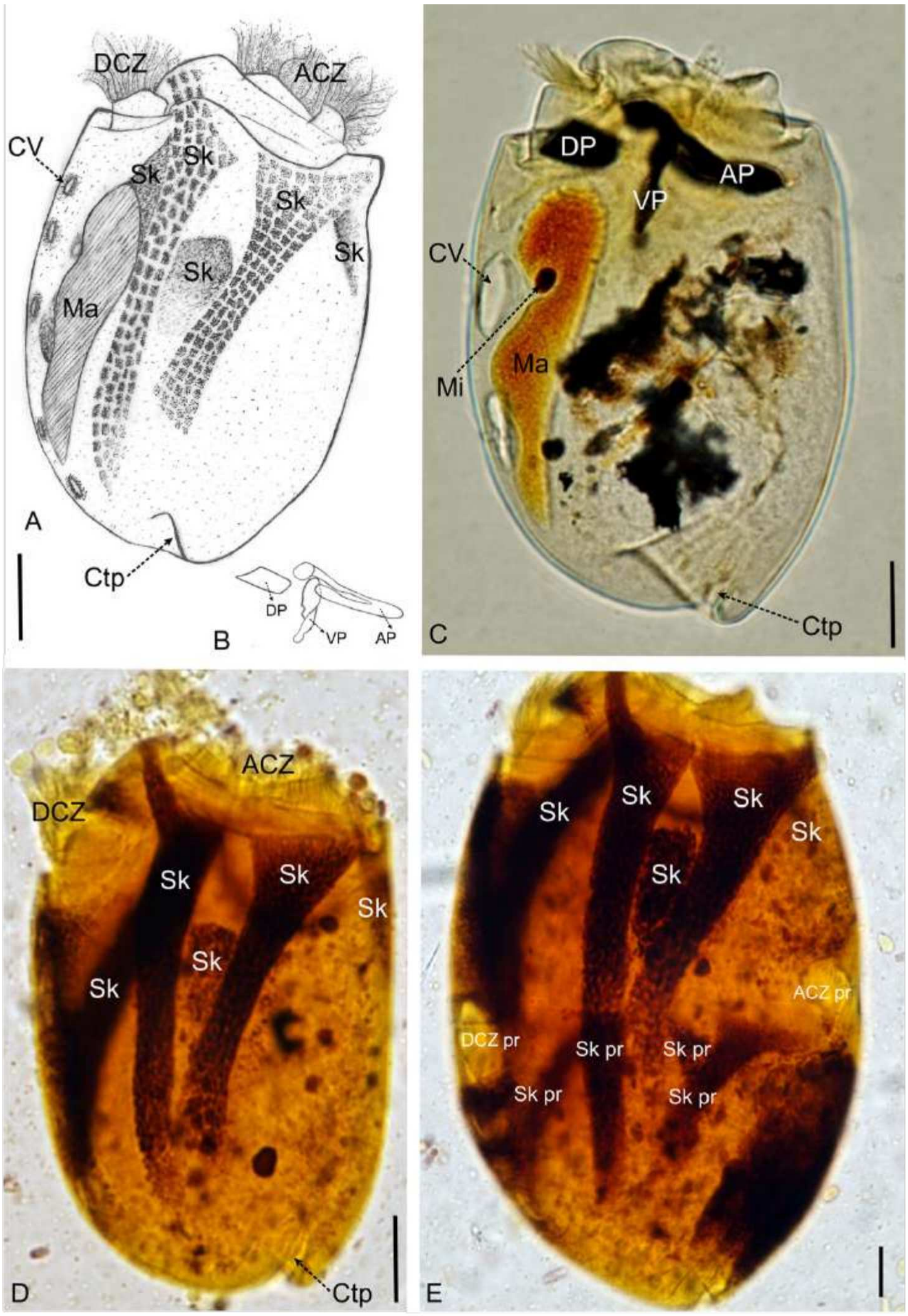

Figure 1. *Polyplastron alaskum* Dehority, 1974 recorded in Brazilian sheep. **A.** Schematic drawing based on specimens after Lugol's solution; **B.** Schematic drawing of infraciliary bands based on specimens after silver carbonate impregnation; **C.** Specimen after silver carbonate impregnation; **D.** Specimen after Lugol's solution staining; **E.** Specimen after Lugol's solution staining showing the morphogenetic pattern of skeletal plates and ciliary zones. Ma: macronucleus; Mi: micronucleus; CV: contractile vacuole; Ctp: citoproct; Sk: skeletal plate; ACZ: adoral ciliary zone; DCZ: dorsal ciliary zone;

AP: adoral polybrachykinety; DP: dorsal polybrachykinety; VP: vestibular polybrachyninety; Sk pr: skeletal plate primordium; ACZ pr: adoral ciliary zone primordium; DCZ pr: dorsal ciliary zone primordium. Scale bars: 20 µm.

Table 1. Morphometric characterization of *Polyplastron alaskum* Dehority, 1974 recorded in Brazilian sheep

Characters[*]	**Mean**	**SD**	**Minimum**	**Maximum**	**CV (%)**	***n***
Body length	101.86	3.20	98.05	109.88	3.15	20
Body width	76.54	2.19	74	81.05	2.86	20
Body length/ body width ratio	1.33	0.02	1.30	1.37	1.45	20
Ma length	63.10	1.88	60.55	66.41	2.97	20
Ma width	12.40	1.08	10.87	15.01	8.73	20
Ma length/ Ma width ratio	5.12	0.45	4.35	5.81	8.78	20
Mi diameter	3.06	0.26	2.74	3.74	8.42	20

[*] Based on specimens stained with Lugol's solution. All dimensions in micrometers. Ma: macronucleus; Mi: micronucleus; SD: Standard deviation; CV: Coefficient of variation; *n*: sample size.

SEÇÃO 7

(Seção a ser submetida no peiódico *European Journal of Protistology*, A4, FI: 2.626)

Molecular phylogeny and systematic review support a new genus in Ophryoscolecidae (Ciliophora, Entodiniomorphida)

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Abstract

The family Ophryoscolecidae constitute a monophyletic group of gastrointestinal ciliates of herbivorous mammals, which its systematics are poorly understood. The genus *Eremoplastron* includes ophryoscolecid ciliates with two retractable ciliary zones in the anterior end of body, two contractile vacuoles, rod-shaped macronucleus and one slender skeletal plate. The validity of this genus has been widely challenged, due to similarities with *Eudiplodinium*, ophryoscolecid genus which also possesses one slender skeletal plate. Although these inconsistencies generate a number of taxonomic problems, these incongruences have never been discussed appropriately. The present study redescribe, using a multidisciplinary approach, the species *Eremoplastron rostratum* and discusses aspects regarding the systematics of genera *Eremoplastron* and *Eudiplodinium*. We observed that the genera *Eremoplastron* and *Eudiplodinium* are not synonymous and that *Eremoplastron* species do not constitute a monophyletic group. These findings strongly suggest the existence of a new genus into Ophryoscolecidae, *Paraeremoplastron* gen. n.

Key-words. Litostomatea, Symbiotic ciliates, Herbivorous mammals, *Paraeremoplastron*, Trichostomatia

Introduction

The family Ophryoscolecidae Stein, 1858 constitute a monophyletic group of gastrointestinal ciliates of herbivorous mammals, including ~300 species, distributed in three subfamilies, Entodiniinae Lubinsky, 1957, Diplodiniinae Lubinsky, 1957, and Ophryoscolecinae Lubinsky, 1957, and 16 genera (Williams and Coleman 1992). Several studies with ophryoscolecids were focused in the role which they play in the metabolism of their hosts. However, questions about its systematics are poorly understood (Cedrola et al. 2017a, 2018, 2019).

Among the three ophryoscolecid subfamilies, Diplodiniinae includes the largest number of species (~150) and is permeated by several taxonomic inconsistences. Recent systematic reviews performed with diplodiniines (Cedrola et al. 2017, 2018) pointed out the need for a wide taxonomic reorganization within the genera in this subfamily.

The genus *Eremoplastron* Kofoid & MacLennan, 1932 includes diplodiniine ciliates with two retractable ciliary zones in the anterior end of body, two contractile vacuoles, rod-shaped macronucleus and one slender skeletal plate (Kofoid and MacLennan 1932). *Eremoplastron* species were reported in several ruminant host species and displays a wide geographic distribution (Williams and Coleman 1992). The validity of this genus has been widely challenged, due to similarities with *Eudiplodinium* Dogiel, 1927 (Dogiel 1927; Kofoid and MacLennan 1932; Noirot-Timothée 1960; Latteur 1966), ophryoscolecid genus which also possesses one slender skeletal plate.

Although these inconsistencies generate a number of taxonomic incongruences, these problems have never been discussed appropriately. The present study redescribe, using a multidisciplinary approach, the species *Eremoplastron rostratum* and discusses aspects regarding the systematics of genera *Eremoplastron* and *Eudiplodinium*, using morphological information and phylogenetic reconstructions based on 18S rDNA. We observed that the genera *Eremoplastron* and *Eudiplodinium* are not synonymous and that *Eremoplastron* species do not constitute a monophyletic group. These findings strongly suggest the existence of a new genus into Ophryoscolecidae.

Materials and Methods

Sampling, species identification and morphological characterization

Rumen samples containing the species *Eremoplastron rostratum* (Fiorentini, 1889) were obtained via rumen fistula, for one Holstein x Gir cattle (*Bos primigenius taurus* L. x *Bos primigenius indicus* L.) kept in the experimental field José Henrique Bruschi, Embrapa Gado de Leite, Coronel Pacheco, Minas Gerais, Brazil. Each sample consists of 20 cm^3 of rumen content fixed in MFS solution (Cedrola et al. 2015) for the morphological analyses and in absolute ethanol (Cedrola et al. 2015) for the molecular techniques. Identification of *E. rostratum* was based on the redescriptions of Kofoid and MacLennan (1932) and Kofoid and Christenson (1933), instead the original description, are more details. The morphometric analyses were performed on 20 specimens fixed with MFS solution (Cedrola et al. 2015). Staining of skeletal plates and infraciliary bands impreganation was made by Lugol's solution and silver carbonate impregnation, respectively (Cedrola et al. 2015; Rossi et al. 2016). The term polybrachykinety refers to infraciliary bands composed of numerous, short and parallel kineties (Fernandez-Galiano 1979). The drawing of *Eremoplastron rostratum* was made after free hand sketching during observation of the specimen under an Olympus BX51 microscope equipped with differential interferential contrast. The diagrams of the oral infraciliature are made with aid of a drawing device, and were based on silver impregnated specimens. The orientation of the ciliates for description was described as follows: the side near the macronucleus lies was termed the dorsal side; the opposite one the ventral side, these defined the right and left sides (Dogiel 1927).

DNA extraction, PCR and DNA sequencing

Total DNA extractions were performed from 10 absolute ethanol fixed specimens of *E. rostratum* using the DNeasy Blood and Tissue kit (Qiagen Biotecnology Brazil Ltd., São Paulo, SP, Brazil) and following the animal tissue protocol. The 18S rDNA were amplified using a Nested-PCR approach. The universal primer set, eukA and eukB (Medlin et al. 1988), was used in the first round of PCR, and a specific primer set for the subclass Trichostomatia Büetschli, 1889, 87F and 1412R (Cedrola et al. 2017) was used in the second round of reaction. The amplification conditions used in both reactions were: 1 cycle at 94 ºC for 2 min; 35 cycles at 94 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 2 min; 1 cycle at 72 ºC for 7 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Biotecnology Brazil Ltd., São Paulo, SP, Brazil) and sequenced using the 87F and 1412R (Cedrola et al. 2017) and the internal ciliate-specific primer set 384F and 1147R (Dopheide et al. 2008).

Phylogenetic analyses

Our data sets were generated using both data obtained in the present work and sequences from previously characterized organisms (n=28). The data were available from the Silva database release 123 (Pruesse et al. 2007), including representatives of the class Litostomatea Small and Lynn, 1981. The alignments were performed using the SINA (Pruesse et al. 2007). Followed by manual inspection, regions of ambiguous nature and primers sequences were removed from the alignment in which was subsequently trimmed producing an equi-length data set. Subsequently, the data sets were analyzed using JModel-Test 2.1.4 (Guindon and Gascuel 2003; Darriba et al. 2012) for selection of the optimal model of sequence evolution. Bayesian inference (BI) analyses were performed using MrBayes v. 3.2 (Ronquist et al. 2012) and the evolutionary model was the GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were performed using three heated chains and one cold chain (N chains = 4) with a sample and print frequency of 500 and a diagnostic frequency of 5,000, for 1,000,000 generations or until the average standard deviation of the split frequencies was below 0.01 indicating the convergence of the two independent runs. All remaining trees after discarding the burn-in (25%) were used in calculation of posterior probabilities using a majority rule consensus. Maximum likelihood (ML) analysis was carried out using the PhyML v 3.0 software (Guindon et al. 2010) applying a GTR + G + I nucleotide substitution model and the reliability of internal branches were assessed using the non-parametric bootstrap method with 1,000 replicates.

Results and Discussion

Multidisciplinary characterization of *Eremoplastron rostratum* from Brazilian cattle (Figure 1; Table 1)

The specimens isolated in present study, have an oval and laterally compressed body. Two retractable ciliary zones, one adoral and one dorsal, at anterior body portion, are separated by a conspicuous operculum. Ectoplasm extending beyond of the body and forms one caudal spine, which vary in length and curvature. The macronucleus is rod-shaped. The elliptical micronucleus is located in a depression in the dorsal anterior surface of the macronucleus. A single thin skeletal plate, is located on the right side of the body, juxtaposed to the macronucleus. Two contractile vacuoles, each with a dorsally opening excretory pore, are located dorsally to the macronucleus. Rectum is a small and tubular structure located in the posterior end of the body, where cytoproct opens.

Oral infraciliature corresponding to the Diplodinium-type (Ito and Imai 2006). This pattern consists of an adoral polybrachykinety (AP), vestibular polybrachykinety (VP), a dorsal polybrachykinety (DP) and paralabial kineties (PK). AP involves the buccal opening; VP is long and extends into vestibule, if originating internally by the dorsal side of AP. DP extending laterally along dorsal side of the body. Four to five PK close to ventral portion of AP.

The new sequence of the species *E. rostratum*, obtained in this study, was deposited in GenBank database under the following accession number: number. The sequence presents 1435 pb, 41.81% of GC (guanine-cytosine) and 99% of similarity with another sequence, of the same species, isolated from domestic sheep (*Ovis aries* L.) in Poland (Moon-Van der Staay 2014).

Eremoplastron rostratum was described by Fiorentini (1889) as *Diplodinium rostratum* and presents a wide geographic distribution, and has already been registered in several host species, domestic (Cedrola et al. 2016; Clarke et al. 1964; Dogiel 1927; Gurung et al. 2002; Imai 1985; Imai et al. 1989; Kofoid and MacLennan 1932; Mishima et al. 2009, Gürelli 2018) and wild (Booyse et al. 2014; Dogiel 1927; Ishaq and Wright 2014; Kofoid and Christenson 1933; Kubesy and Dehority 2002; Towne et al. 1988).

The body dimensions in this species is similar to those observed by Dogiel (1927) in specimens isolated in Russian domestic cattle, since the specimens isolated by Kofoid and MacLennan (1932) and Kofoid and Christenson (1933) were slightly smaller. Morphological and morphometric polymorphism in rumen ciliates were poorly reported (Kofoid and Christenson 1933; Cedrola et al. 2017), and could be associated to host metabolic variations, geographic features (Göçmen et al. 2002) and ecological relationships established among ciliates, such as competition, predation and cannibalism, as observed by Cedrola et al. (2017) in *Diplodinium anisacanthum* and Martinele and D'Agosto (2008) in *Elytroplastron bubali*.

Oral infraciliature in *E. rostratum* was described by Noirot-Timothée (1960) and redescribed by Gurelli (2018). The oral infraciliature in this species comprises a AP, a VP, a DP, and four to five PK, organized according to Diplodinium-type proposed by Ito and Imai (2006). This type of infraciliary band is also shared by other ciliate species belonging to the subfamily Diplodiniinae (Ito and Imai 2006).

Taxonomic history of *Eremoplastron* and *Eudiplodinium* (Figure 2; Table 2)

Dogiel (1927), in his monograph on the family Ophryoscolecidae, established four subgenera for *Diplodinium*: *Anoplodinium* (absence of skeletal plates and two contractile vacuoles), *Eudiplodinium* (one or two skeletal plates and two contractile vacuoles), *Polyplastron* (five skeletal plates and numerous contractile vacuoles) and *Ostracodinium* (broad skeletal plate and two to six contractile vacuoles).

Later, in a systematic review of Ophryoscolecidae, Kofoid and MacLennan (1932) raised the subgenera proposed by Dogiel (1927) at the genus level, and distributed *Eudiplodinium* species in four genera: *Eudiplodinium* (one slender skeletal plate, hook-shaped macronucleus and two contractile vacuoles), *Eremoplastron* (one slender skeletal plate, rod-shaped macronucleus and two contractile vacuoles), *Diploplastron* (two slender skeletal plates, rod-shaped macronucleus and two contractile vacuoles) and *Metadinium* (two broad skeletal plates, lobed macronucleus and two contractile vacuoles). Several species classified in *Eremoplastron* by Kofoid and MacLennan (1932) had been redescribed in Dogiel's monography (Dogiel, 1927) as subspecies of *Eudiplodinium neglectum* (*Eudiplodinium neglectum neglectum, Eudiplodinium neglectum bovis, Eudiplodinium neglectum spectabile, Eudiplodinium neglectum impalae, Eudiplodinium neglectum insigne, Eudiplodinium neglectum giganteum, Eudiplodinium neglectum monolobum, Eudiplodinium neglectum rugosum,* and *Eudiplodinium neglectum dilobum*). Hence, Kofoid and MacLennan (1932) raised them as species level.

Noirot-Thimothée (1960), in a structural and ultrastructural study on ophryoscolecid ciliates, considered the proposal of Kofoid and MacLennan (1932) unjustified because, according to her, the infraciliary arrangement of the species belonging to the genus *Diplodinium* sensu Dogiel, 1927 is the same. Thus, the author proposed to maintain Dogiel's systematic classification (Dogiel 1927).

Latteur (1966) reviewing the systematic of the family Ophryoscolecidae points out inconsistencies in the classifications proposed by Dogiel (1927) and Kofoid and MacLennan (1932), mainly concerning the number of skeletal plates. According to the author, the number of these structures is not so important in the genera delimitation in Ophryoscoledidae. Thus, in their taxonomic proposal, Latteur (1966) considers as synonyms the genera with the same number and morphology of skeletal plates and invalidate the genera *Eremoplastron* and *Diploplastron*, proposed by Kofoid and MacLennan (1932), maintaining them as *Eudiplodinium* and *Metadinium*, respectively.

Although the studies carried out by Dogiel (1927) and Noirot-Timothée (1960) are extremely relevant to the knowledge about the diversity of ophryoscolecid ciliates, the taxonomic classification models proposed by such authors are not used by researchers in the area. According to subsequent authors, ciliates belonging to the genus *Diplodinium* sensu Dogiel (1927) are very diversified morphologically, presenting important differences in nuclear apparatus and skeletal plates, which distinctly separate them.

In this context, the taxonomy of the genera *Eremoplastron* and *Eudiplodinium* presents several incongruences due to the different classifications. According to Kofoid and MacLennan (1932), these genera have 17 and three species, respectively and according to Latteur (1966) the genus *Eremoplastron* is not valid and the genus *Eudiplodinium* contains 20 species. In addition, Göçmen et al. (2001) decribed a ophryoscolecid species with one to five skeletal plates, *Eudiplodinium dehorityi*. However, the placement of this species in this genus should be better evaluated, since this species shows variable number of skeletal plates (one to five), which are not taxonomic characterist of genus *Eudiplodinium*.

Molecular phylogeny and taxonomic reorganizations (Figure 2 and 3; Table 2)

In the present study, two phylogenetic inference methods (Maximum likelihood and Bayesian inference) using information from 18S rDNA sequences of trichostomatid ciliates were used to reconstruct the evolutionary history of the family Ophryoscolecidae (Figure 3). According to our analyses, ciliates belonging to genera *Eudiplodinium* and *Eremoplastron* does not constitute a monophyletic group (Figure 3).

Eudiplodinium maggii emerged in a clade as sister-group of *Metadinium medium* and *M. minorum* species (0.98 BI; 85 ML) (Figure 3, clade B). In fact, the morphological characters of *E. maggii* support this phylogenetic placement. The body of *E. maggii* is large and displays a heavy and resistant pellicle, with an ectoplasm thick, which occupies a large proportion of body. Also, this species displays large contractile vacuoles with conspicuous excretory pores and large and robust cytopharyngeal and cytoproct openings, characters also found in *Metadinium* species, and very different from those observed in *Eremoplastron* species (Kofoid and MacLennan 1932).

However, *Eremoplastron* ciliates sequences also do not form a monophyletic group, since the sequences corresponding to the species *E. neglectum* and *E. dilobum* constituted a monophyletic group with *Diploplastron affine* (0.99 BI; 80 ML) (Figure 3, clade A), and *E. rostratum* sequences emerged as sister-group of all diplodiniids ciliates (1.00 BI; 98 ML) (Figure 3, clade C). These findings raise discussions about the systematics of the genera *Eremoplastron* and *Eudiplodinium*.

The species *E. dilobum* and *E. neglectum* present several morphological characters in common, such as: the rounded shape of the body; the skeletal plate "primitiva" juxtaposed to the ventral side of macronucleus; and an inclined rod-shaped macronucleus. These characters are also shared by a group of species belonging to the genus *Eremoplastron*: *E. bovis*, *E. rotundum*, *E. brevispinum*, *E. monolobum*, *E. spectabile*, *E. magnodentatum*, *E. tarandi*, and *Eremoplastron trilobatum* (Figure 2, Table 2) and are very similar from those found in *Diploplastron* species, *D. affine*, and *D. dehorityi*, supporting our phylogenetic analyses (Figure 3). In fact, according to Kofoid and MacLennan (1932) several *Eremoplastron* ciliates displays similar morphological characters with *Diploplastron affine*, despite the distinct number of skeletal plates.

On the other hand, *E. rostratum*, as discussed above, does not cluster to the sequences of *E. dilobum*, *E. neglectum* and *D. affine*. Indeed, *E. rostratum* is morphologically distinct from such species and has morphological characters supporting its phylogenetic placement in our analyses (Figure 3). This species presents an elliptical body shape, the skeletal plate "primitiva" not juxtaposed to the ventral surface of the macronucleus; and rod-shaped macronucleus. These morphological characters are also shared by a group of species of the genus *Eremoplastron*: *E. impalae*, *E. rugosum*, *E. kenyensis*, *E. bubalus*, *E. giganteum*, and *E. insigne* (Figure 2, Table 2).

These findings strongly suggest that the genus *Eremoplastron* should be dismembered in two genera. The first one, composed by the species *E. impalae*, *E. rostratum*, *E. rugosum*, *E. kenyensis*, *E. bubalus*, *E. giganteum*, and *E. insigne* which remains as *Eremoplastron*; and the second one, composed by the species *E. bovis*, *E. rotundum*, *E. brevispinum*, *E. monolobum*, *E. spectabile*, *E. magnodentatum*, *E. tarandi*, and *Eremoplastron trilobatum*, which needs a new name.

Genus *Eremoplastron* sensu novo

Diagnosis. Body elliptical shaped, recovered by a delicate pelicle. Two retractile ciliary zones at anterior end of body separated by a rounded operculum. A single and slender skeletal plate, "primitiva" beneath the middle of body. Macronucleus rod-shaped. Micronucleus elliptical shaped, located dorsally to the macronucleus. Two contractile vacuoles, with inconspicuous excretory pores lies dorsally to the macronucleus. The cytoproct is thin and lies at posterior end of body.

Type species. *Eremoplastron rostratum* (Fiorentini, 1889)

Establishment of a new genus in Ophryoscolecidae, *Paraeremoplastron* gen. n.

Diagnosis. Body rounded shaped, recovered by a delicate pelicle. Two retractile ciliary zones at anterior end of body separated by a flattened operculum. A single and slender skeletal plate, "primitiva", juxtaposed to the ventral side of the macronucleus. Macronucleus rod-shaped, inclined ventrally. Micronucleus elliptical shaped, located dorsally to the macronucleus. Two contractile vacuoles, with inconspicuous excretory pores lies dorsally to the macronucleus. The cytoproct is thin and lies at posterior end of body.

Type species. *Paraeremoplastron neglectum* (Dogiel, 1925)

Etymology. The name *Paraeremoplastron* reflects the similarities among the new genus and the genus *Eremoplastron*.

Eudiplodinium sensu Kofoid & MacLennan, 1932

Diagnosis. Body rounded shaped, recovered by a tick pelicle. Two retractile ciliary zones at anterior end of body separated by a flattened operculum. A single and slender skeletal plate, "primitiva", juxtaposed to the ventral side of the macronucleus. Macronucleus hook-shaped. Micronucleus elliptical shaped, located anteriorly and dorsally to the macronucleus. Two contractile vacuoles, with conspicuous excretory pores lies dorsally to the macronucleus. The cytoproct is wide and lies at posterior end of body.

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Figures and Tables

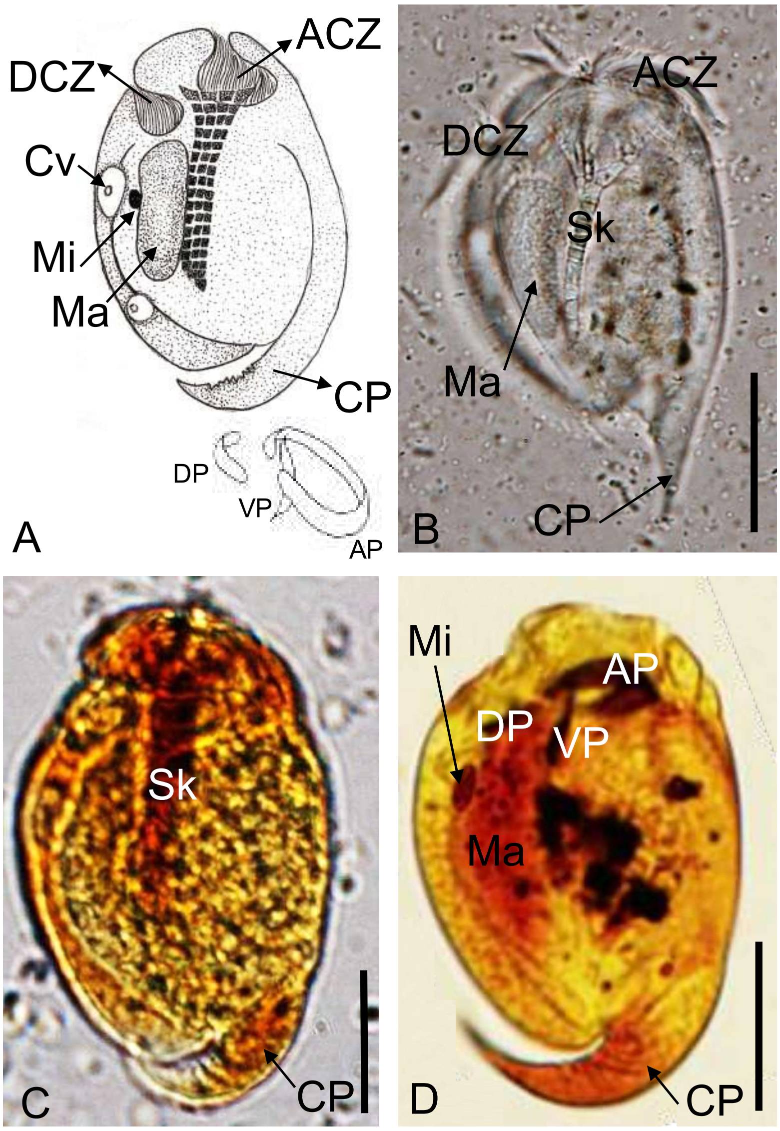

Figure 1. *Eremoplastron rostratum* (Fiorentini, 1889) recorded in Brazilian cattle. A. Schematic drawings of specimen in vivo and schematic drawing of infraciliry bands pattern, after silver impregnation; B. Specimen in vivo; C. Specimen after Lugol's solution staining; D. Specimen after silver carbonate impregnation. ACZ: adoral ciliary zone; AP: adoral polynrachykinety; CP: caudal process Cv: contractile vacuole; DCZ: dorsal ciliary zone; DP: dorsal polybrachykinety; Ma: macronucleus; Mi: micronucleus; Sk: skeletal plate. Bars: 20 µm.

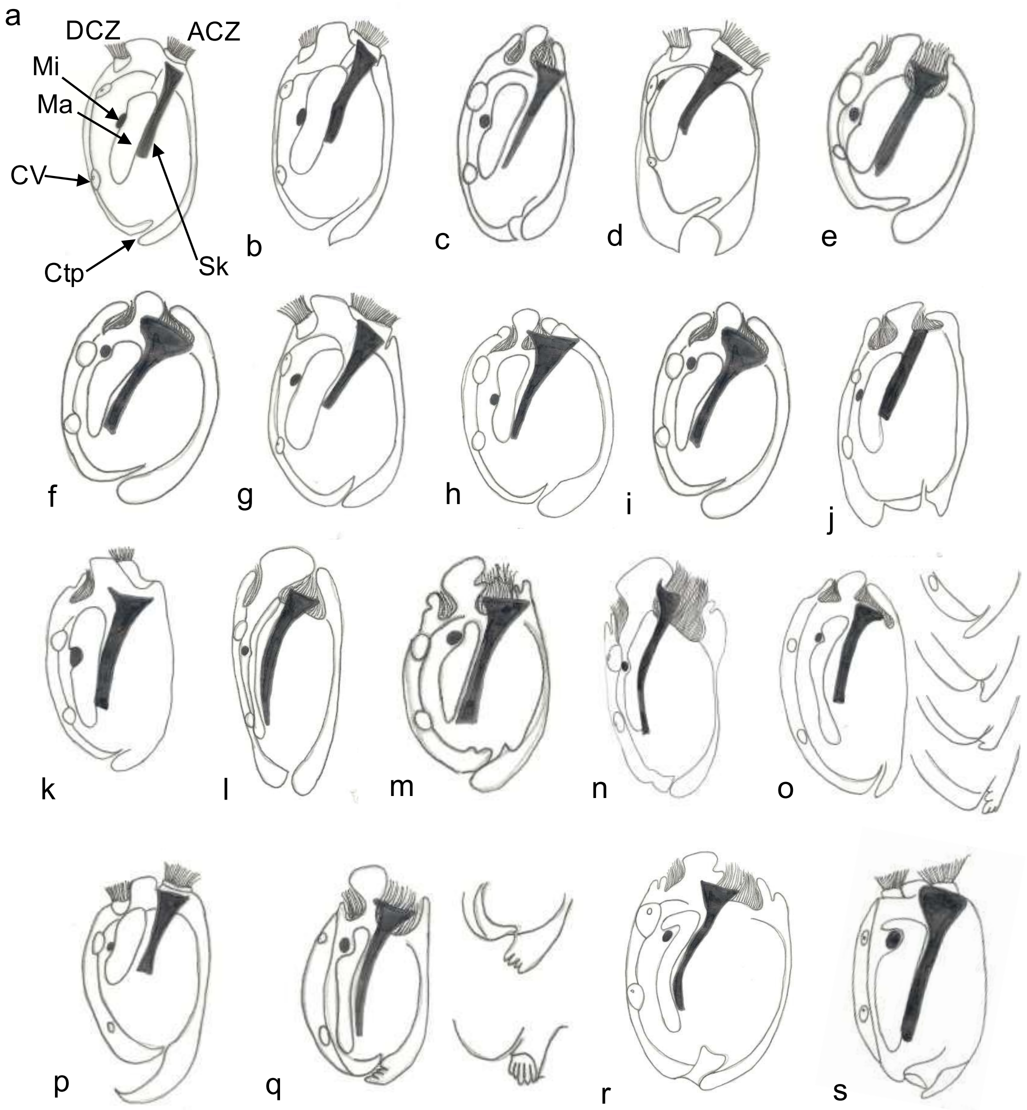

Figure 2. *Paraeremoplastron* gen. n. (A-J), *Eremoplastron* Kofoid and MacLennan, 1932 (K-Q), and *Eudiplodinium* Dogiel, 1927 (R-S) species. A. *Paraeremoplastron bovis*, B. *Paraeremoplastron brevispinum*, C. *Paraeremoplastron dilobum*, D. *Paraeremoplastron magnodentatum*, E. *Paraeremoplastron monolobum*, F. *Paraeremoplastron neglectum*, G. *Paraeremoplastron rotundum*, H. *Paraeremoplastron spectabile*, I. *Paraeremoplastron tarandi*, J. *Paraeremoplastron trilobatum*, K. *Eremoplastron bubalus*, L. *Eremoplastron giganteum*, M. *Eremoplastron impalae*, N. *Eremoplastron insigne*, O. *Eremoplastron kenyensis* and the variatons in its caudal projections, P. *Eremoplastron rostratum*, Q. *Eremoplastron rugosum* and the variations in its caudal projections, R. *Eudiplodinium maggii*, and S. *Eudiplodinium microlateralis*. ACZ: Adoral ciliary zone; Ctp: Citoproct; CV: Contractile vacuole; DCZ: Dorsal ciliary zone; Ma: Macronucleus; Mi: Micronucleus; Sk: Skeletal plate. Figures prepared out of scale. For morphometric data, please see Table 2.

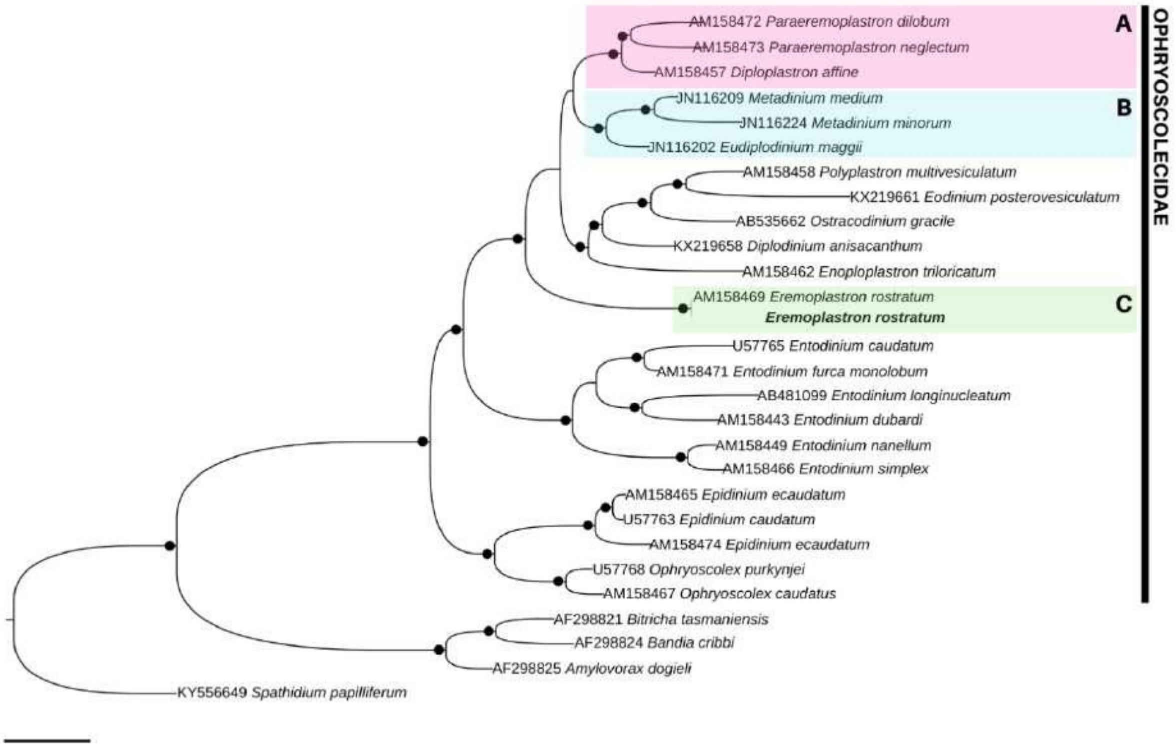

Figure 3. Phylogenetic tree of ophryoscolecid ciliates (Ciliophora, Trichostomatia, Entodiniomorphida) based on 18S rDNA data. Other trichostomatid ciliates were chosen as out group. The new sequence of Holstein-Gyr cattle *Eremoplastron rostratum* population obtained in this study are highlighted in bold. The black dots on the nodes represent maximum likelihood (ML) bootstrap and posterior probability (BI) values > 80/0.8.

Table 1. Measurements (µm) of *Eremoplastron rostratum* (Fiorentini, 1889) recorded in cattle of different geographical locations.

Characters	**Russia**[1]	**India**[2]	**India**[3]	**Brazil**[4]
Body length	54 (46-63)	45 (40-52)	39 (34-48)	51.33 ± 5.57 (40.02-60.65)
Body width	34 (29-47)	25 (22-26)	27 (24-29)	33.59 ± 2.42 (27.73-39.11)
Macronuclar length	-	24 (18-30)	21 (17-30)	26.59 ± 3.81 (20.8-36.8)
Body length / Body width	1.6	-	-	1.52 ± 0.10 (1.37-1.75)
Macronuclar length / Body length	-	-	-	0.51 ± 0.04 (0.42-0.06)

[1] Dogiel (1927); [2] Kofoid and MacLennan (1932); [3] Kofoid & Christenson (1933); [4] present study.

Table 2. Characteristics of the *Eremoplastron*, *Paraeremoplastron* and *Eudiplodinium* species

Species	**Length (µm)**	**Width (µm)**	**Body shape**	**Number of caudal appendages**	**Macronuclear length (µm)**	**Macronuclear width (µm)**	**Macronuclear shape**
Eremoplastron bubalus (Dehority 1979)	35-56	24-37	Ovoid	1	28	6	Rod
Eremoplastron giganteum (Dogiel 1925)	256-500	250-240	Elongated	0	190	10	Rod
Eremoplastron impalae (Dogiel 1925)	74-105	50-72	Subspherical	0	38	10	Rod
Eremoplastron insigne (Dogiel 1925)	124-222	65-125	Elongated	0	87	11	Rod
Eremoplastron kenyensis (Imai 1988)	195-290	125-175	Semi-ovoid	1 variable	148	16	Rod
Eremoplastron rostratum (Fiorentini, 1889)	46-63	29-47	Ovoid	1	24	6	Rod
Eremoplastron rugosum (Dogiel 1927)	69-90	44-63	Semi-ovoid	1	45	5	Rod
Paraeremoplastron bovis (Dogiel 1927)	52-100	36-57	Ovoid	0	41	10	Rod/inclined
Paraeremoplastron brevispinum (Kofoid and MacLennan 1932)	72-92	42-53	Ovoid	2	37	9	Rod/inclined
Paraeremoplastron dilobum (Dogiel 1927)	68-102	40-70	Ovoid	2	41	12	Rod/inclined
Paraeremoplastron magnodentatum (Kofoid and MacLennan 1932)	58-82	30-50	Rectangular	2	37	10	Rod/inclined
Paraeremoplastron monolobum (Dogiel 1927)	58-83	41-70	Subspherical	1	37	10	Rod/inclined
Paraeremoplastron neglectum (Dogiel 1925)	81-124	40-68	Ovoid	1	46	10	Rod/inclined
Paraeremoplastron rotundum (Kofoid and MacLennan 1932)	50-95	51-63	Broadoval	0	43	10	Rod/inclined
Paraeremoplastron spectabile (Dogiel 1925)	115-150	83-101	Subspherical	1	59	12	Rod/inclined
Paraeremoplastron tarandi (Kofoid and MacLennan 1932)	74-105	54-77	Subspherical	1	*	*	Rod/inclined
Paraeremoplastron trilobatum (Shangai and Kshirsagar, 2015)	80-121	64-96	Ovoid	3	44-76	3-8	Rod/inclined
Eudiplodinium magii (Fiorentini, 1889)	104-198	63-125	Ovoid	0	*	*	Hook
Eudiplodinium microlateralis Van Hoven 1983	51-64	30-39	Ovoid	1	*	*	Hook

*: data absent

SEÇÃO 8

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Phylogenetic Analyses Support Validity of Genus *Eodinium* (Ciliophora, Entodiniomorphida, Ophryoscolecidae)

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Running head: **Phylogenetic Analyses Support Validity of Genus *Eodinium***

ABSTRACT The validity of the genus *Eodinium* has been historically disputed due to morphological similarities with *Diplodinium* (absence of skeletal plates as well as adoral and dorsal ciliary zones at the same body level). To address this issue, the 18S rDNA of four *Eodinium posterovesiculatum* morphotypes and four *Diplodinium anisacanthum* morphotypes were sequenced and phylogenetically analyzed. The different inference methods suggest the existence of a last common ancestor of *Eodinium* and *Ostracodinium* that is not shared with *Diplodinium*, strongly supporting the validity of the genus *Eodinium*. Since skeletal plates are present in all members of the genus *Ostracodinium*, the most parsimonious is a secondary loss of skeletal plates in *E. posterovesiculatum*. This work represents a breakthrough in the taxonomy and phylogeny of the family Ophryoscolecidae indicating that the skeletal plates may not reflect evolutionary divergence within this group of ciliates as traditionally proposed.

Keywords. Litostomatea; 18S rDNA phylogeny; ophryoscolecid ciliates; Trichostomatia; Rumen

INTRODUCTION

Classification of symbiotic ciliates (Ciliophora, Trichostomatia) of herbivorous mammals is traditionally based on morphological characters, such as body and nuclear shapes, oral infraciliature, number of contractile vacuoles and number and morphology of skeletal plates. However, recently, molecular data, in particular 18S rDNA sequences, have been used to review the phylogenetic relationships within this group (Grim et al. 2015; Kittelmann et al. 2015; Rossi et al. 2015). Although some phylogenetic analyses corroborate phylogenetic hypothesis based on morphological data (Wright and Lynn 1997a, b; Wright et al. 1997), incongruent results are presented by others (Ito et al. 2010, 2014; Kittelmann et al. 2015; Moon-Van der Staay et al. 2014; Rossi et al. 2015; Strüder-Kypke et al. 2007).

The genus *Eodinium* Kofoid and MacLennan, 1932 comprises species of ophryoscolecid ciliates with two ciliary zones in the anterior body portion, a rod-shaped macronucleus, two contractile vacuoles, but lack skeletal plates (Kofoid and MacLennan 1932). However, the validity of this genus is questioned given the morphological similarities between *Eodinium* and *Diplodinium* Schuberg, 1888 (Ito and Imai 2003; Latteur 1966, 1970; Lubinsky 1957; Noirot-Timothée 1960). Both genera have an ovoid, laterally compressed body with the dorsal ciliary zone at the same level as the adoral zone, and both lack the skeletal plates (Ito and Imai 2003).

Since phylogenetic studies on the family Ophryoscolecidae are still incipient (Rossi et al. 2015), the phylogenetic placement and validity of the genus *Eodinium* remains unclear. The present work investigates the phylogenetic relationship of the genus *Eodinium* based on molecular data (18S rDNA) and expands the current knowledge about the systematics of the family Ophryoscolecidae.

MATERIALS AND METHODS

Sampling and species identification

Samples of four *Eodinium posterovesiculatum* (Dogiel, 1927) Kofoid and MacLennan, 1932 morphotypes (posterovesiculatum-type, lobatum-type, monolobosum-type, and bilobosum-type) and four *Diplodinium anisacanthum* da Cunha, 1914 morphotypes (anisacanthum-type, diacanthum-type, triacanthum-type, and pentacanthum-type) were obtained from four fistulated Holstein x Gir cattles (*Bos primigenius taurus* L. x *Bos* *primigenius indicus* L.) kept in the experimental field José Henrique Bruschi, Embrapa Gado de Leite, Coronel Pacheco, Minas Gerais, Brazil. For the morphological analyses, the rumen content (20 cm^3) was fixed in formalin 18.5% (Dehority 1984), while for the molecular analyses, in 100% ethanol (Cedrola et al. 2015). *Eodinium posterovesiculatum* morphotypes and *Diplodinium anisacanthum* morphotypes identifications were based on the redescriptions (Ito and Imai 2003; Kofoid and Christenson 1933; Kofoid and MacLennan 1932), instead of the original descriptions they provide more details. Subfamily names, within the family Ophryoscolecidae, are in accordance with Lubinsky (1957) proposition, while the generic names follow the proposition of Kofoid and MacLennan (1932). The terminology of the cell sides follows Lubinsky (1957), i.e., the side near the macronucleus is the ventral side; this defines together with the anterior cell end, the dorsal, right, and left cell sides.

DNA extraction, PCR and DNA sequencing

Total DNA extractions were performed from pools of 10 ethanol-fixed ciliates of each morphotype, using the DNeasy Blood and Tissue kit (Qiagen Biotechnology Brazil Ltd., São Paulo, SP, Brazil) and following the animal tissue protocol. The 18S rDNA was amplified, using a Nested PCR approach in which the universal primer set eukA and eukB (Medlin et al. 1988) was used in the first round of PCR reaction, and a specific primer set for the subclass Trichostomatia Bütschli, 1889, 87F (5'- GCA TGT CTA AGT ATA AAT AAC TAC - 3') and 1412R (5' - CGG ATC ACC CGG TAT CGG TAG - 3'), in the second round of the reation. This primer set was designed as described elsewhere (Senra et al. 2016) and tested *in silico*, using the TestPrime 1.0 tool (Klindworth et al. 2012). The aim was to reduce the risk of contamination by other eukaryotic organisms from the rumen environment, such as flagellates, fungi, and plant particles. The PCR cycle was the same in both rounds of amplification, consisting in: 1 step at 94 ºC for 2 min; 35 cycles at 94 ºC for 30 s, 50 ºC for 30 s, 72 ºC for 2 min; and 1 step at 72 ºC for 7 min. The PCR products were purified, using the QIAquick PCR Purification Kit (Qiagen Biotechnology Brazil Ltd., São Paulo, SP, Brazil) and sequenced, using the primers 87F and 1412R (this work) and the internal ciliate-specific primer set 384F and 1147R (Dopheide et al. 2008).

Phylogenetic analyses

Our data set was generated, using both data obtained in the present work (consensus sequences) and sequences from previously characterized organisms (n=28) available from the Silva database release 123 (Pruesse et al. 2007), including representatives of the class Litostomatea Small and Lynn, 1981, subclass Trichostomatia Bütschli, 1889, order Entodiniomorphida Reichenow in Doflein and Reichenow, 1929, family Ophryoscolecidae Stein, 1859 (n=26) as ingroup and family Buetschliidae Poche, 1913 (n=1); order Vestibuliferida de Puytorac et al., 1974 (n=3) and order Macropodiniida Lynn, 2008 (n=4) as outgroups. The alignments were performed, using the ARB software package (Ludwig et al. 2004). After manual inspection, regions of ambiguous nature and primers sequences were removed from the alignment which was subsequently trimmed, producing an equi-length data set. Different nucleotide composition filter masks as implemented in the ARB software package (Ludwig et al. 2004) were then applied to the final alignment. Subsequently, the data sets were analyzed, using the software JModel-Test 2.1.4 (Darriba et al. 2012; Guindon and Gascuel 2003) was used for selection of the optimal model of sequence evolution. Bayesian inference (BI) analyses was performed, using MrBayes v. 3.2 (Ronquist et al. 2012), and the evolutionary model was the GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were performed, using three heated chains and one cold chain (N chains = 4) with a sample and print frequency of 500 and a diagnostic frequency of 5,000, for 1,000,000 generations or until the average standard deviation of the split frequencies was below 0.01, indicating the convergence of the two independent runs. All remaining trees after discarding the burn-in (25%) were used for calculating the posterior probabilities, using a majority rule consensus. Maximum likelihood (ML) analysis was carried out, using the PhyML v 3.0 software (Guindon et al. 2010), applying a GTR + G + I nucleotide substitution model. The reliability of internal branches were assessed, using the non-parametric bootstrap method with 1,000 replicates.

Tree topology test

Three statistical tests, the AU (approximately unbiased), SH (Shimodaira–Hasegawa) KH (Kishino–Hasegawa) tests, were conducted in the computer software CONSEL (Shimodaira and Hasegawa 2002), use the likelihood-based δ statistic to compare the best ML topology (unconstrained tree), obtained in our phylogenetic analysis with an alternative phylogenetic hypothesis which assumes the monophyly of *Diplodinium* and

Eodinium (constrained tree) as suggested by some authors (Noirot-Timothée 1960; Latteur 1966, 1970). The constrained tree was created with PhyML (Guindon et al. 2010), applying the same dataset and parameters used to generate the best ML tree.

RESULTS

The identification of *Diplodinium anisacanthum* and *Eodinium posterovesiculatum* morphotypes was acquired after morphometric analyses of 20 Lugol stained (Cedrola et al. 2015; D'Agosto and Carneiro 1999) specimens of each morphotype (Cedrola et al. unpubl. data; Fregulia et al. unpubl. data).

Eodinium posterovesiculatum (Fig. S1A-D) displays a slightly ovoid and laterally compressed body. The two retractile ciliary zones are separated by a protuberance, the operculum, and are located in the anterior body portion. The vestibulum is prominent. The rod-shaped macronucleus is longitudinally orientated in the ventral cell portion. The micronucleus is close to the midle of the macronucleus, on ventral side. The contractile vacuoles are adjacent to the anterior and posterior ends of the macronucleus. Caudal process could be present, differing in size and number according to the morphotype: without process (posterovesiculatum-type), one process (lobatum-type), one distinct process (monolobosum-type), or two processes (bilobosum-type). *Diplodinium anisacanthum* (Fig. S1E-H) possesses a slightly posteriorly tapered and laterally compressed ovoid body. Two retractile ciliary zones separated by a slight protuberance, the operculum, located at the anterior end. The vestibulum has a third of the body length. The rod-shaped macronucleus is longitudinally orientated in the left ventral quarter of the cell, is anteriorly enlarged, and inclined (angle of 90 °). The ellipsoidal micronucleus and two contractile vacuoles are close to the ventral side of the macronucleus. The morphotypes could displays up to six caudal processes. In this study, four morphotypes were isolated: with two (diacanthum-type), three (triacanthum-type), five (pentacanthum-type), or six (anisacanthum-type) caudal processes.

The eight new *Eodinium* and *Diplodinium* 18S rDNA sequences obtained in the present study, using the designed primer set (87F/1412R), were deposited in GenBank under the following accession numbers: *E. posterovesiculatum* morphotypes [posterovesiculatum-type (KX219691); lobatum-type (KX219659); monolobosum-type (KX219660); and bilobosum-type (KX219655)] and *D. anisa canthum* mophotypes [anisacanthum-type (KX219656); diacanthum-type (KX219657); triacanthum-type

(KX219658); and pentacanthum-type (KX219654)]. *In silico* tests (see methods for details) have shown that these primers are extremely specific for the class Litostomatea and able to amplify 75.8%-98.4% (respectively 0 or 2 mismatches allowed to the binding site) of all of the 18S rDNA sequences that are available in the Silva-ARB database r123 from the subclass Trichostomatia. This analysis also indicates that this primer set cross-reacts with sequences from Haptoria Corliss, 1974, however its usage was experimentally adequate for the purposes of this study.

The observed genetic identity of *Eodinium posterovesiculatum* morphotypes (99.9%) and of *Diplodinium anisacanthum* morphotypes (99.8%) is high (Table S1), suggesting that they constitute single species.

Both phylogenetic analyses, (maximum likelihood and bayesian inference) (Fig. 1) recovered similar evolutionary hypotheses with all *E. posterovesiculatum* morphotypes sequences form a strongly supported monophyletic clade and sister-group to *Polyplastron multivesiculatum* (Dogiel and Fedorowa, 1925) and thus apart of *Diplodinium* morphotypes (Fig. 1; S2). This cluster is the adelphotaxon to *Ostracodinium* (Dogiel, 1927); thus, a synonymy of *Diplodinium* and *Eodinium* is unlikely which is supported by the tree topology tests rejecting a monophyletic relationship of the genera (Table 1).

DISCUSSION

Most of the polymorphism of some ophryoscolecid ciliates (Imai et al. 2002; Latteur 1966; Martinele and D'Agosto 2008; Poljansky and Strelkow 1938; Williams and Coleman 1992) are in the morphology of its posterior body portion i.e. variations in their caudal processes. Because of that, many authors use these variations as distinguishing features, causing a number of taxonomic inconsistencies (Kofoid and Christenson 1933; Kofoid and MacLennan 1932), since these processes could change as influence of presence of predators, the morphology of the posterior body portion is not a valid taxonomic character (Cedrola et al. unpubl. data). This might be cases for *Eodinium posterovesiculatum* and *Diplodinium anisacanthum* morphotypes as well in which are solely distinguishable based on variations in their caudal processes. Our data have shown that *Eodinium posterovesiculatum* and *Diplodinium anisacanthum* morphotypes displays almost identical 18S rDNA sequences (99.8% and 99.9%, respectively), suggesting that they constitute a single species. However, further analysis using other molecular markers are required to test this hypothesis.

The genus *Eodinium* was established by Kofoid and MacLennan (1932), however, its validity has been historically questioned due to the morphological similarities between *Diplodinium* and *Eodinium*: both genera have an ovoid, laterally compressed body with the dorsal ciliary zone at the same level as the adoral zone and they both lack skeletal plates (Kofoid and MacLennan 1932). Lubinsky (1957) considered the genus valid, because *Eodinium* presents a rod-shaped macronucleus, a prominent vestibulum and a ventral nuclear apparatus (macronucleus and micronucleus); whereas the macronucleus of *Diplodinium* is rod-shaped, enlarged in its anterior third, and obliquely orientated in the left body half and its vestibulum has is a third of the body length. According to Noirot-Timothée (1960) and Latteur (1966, 1970), however, the genus is not valid, because the characters mentioned by Lubinsky (1957) are were considered insufficient to separate the two genera. Ito and Imai (2003) studied the oral infraciliature in *Eodinium posterovesiculatum*, and demonstrating that this species displays an exceptional infraciliary arrangement in the family Ophryoscolecidae, namely *Eodinium posterovesiculatum*-type. Besides the 18S tree (this study), this apomorphy supports the validity of the genus *Eodinium*. Hence, the phylogenetic position of the four *Eodinium posterovesiculatum* morphotypes in a monophyletic clade together with *Ostracodinium* spp. and *Polyplastron multivesiculatum* and distinctly apart from *Diplodinium* supports Lubinsky (1957) and Ito and Imai (2003) hypothesis.

Our data also supports Lubinsky`s idea (Lubinsky, 1957) that the absence of skeletal plates in *Eodinium* was a consequence of a secondary loss; and thus represents a plesiomorphic character state. This hypothesis allow discussions about the taxonomy and phylogeny of the family Ophryoscolecidae. According to the traditional classification (Dogiel 1927; Kofoid and MacLennan 1932, 1933), the number and morphology of the skeletal plates are extremely important and the presence of skeletal plates is considered a derived character state. However, our findings suggest that the ancestor of the Ophryoscolecidae had skeletal plates, since are present Entodiniinae, Ophryoscolecinae, and in most of the Diplodiniinae (Fig. 1), and the absence may represents independent evolutionry events of secondary loss within the family. Our analyses suggest that other characters, such as the oral infraciliature, as proposed by Rossi et al. (2015), might be more relevant in the family Ophryoscolecidae.

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FIGURE LEGEND AND TABLE:

Fig. 1. Bayesian inference tree of the family Ophryoscolecidae (Ciliophora, Trichostomatia) based on 18S rDNA sequences. Further trichostomatid ciliates were chosen as outgroup. The values on the branches represent the posterior probability (BI) and maximum likelihood (ML) bootstrap values. The colors represent the number of skeletal plates in each taxon. The symbol (-) indicates posterior probability and/or

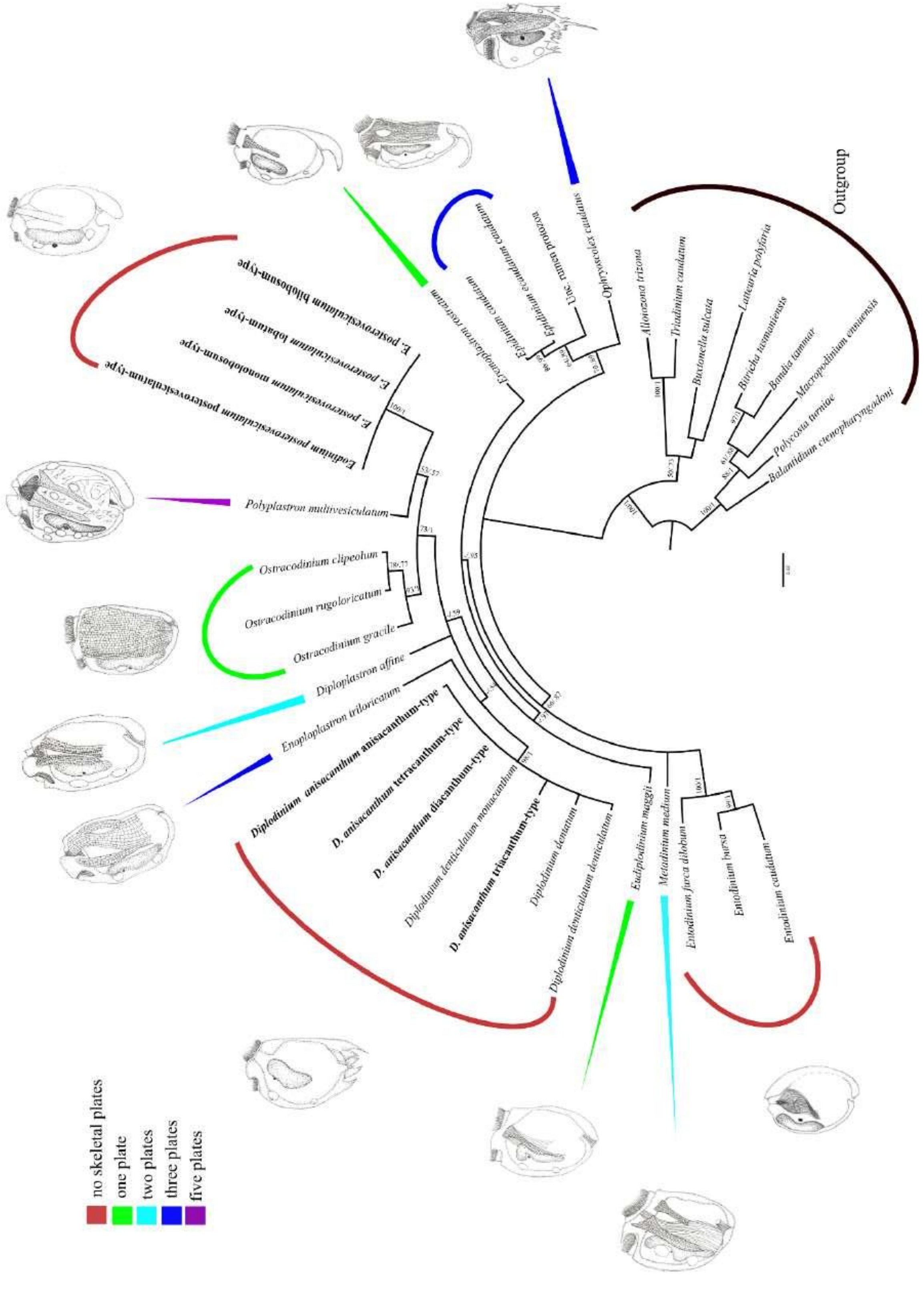
bootstrap values of <0.5/50%. The new sequences of Holstein-Gyr rumen ciliates obtained in this study are highlighted in bold. The scale bar corresponds to two substitutions per 100 nucleotides.

Table 1. Tree topology tests. Log likelihoods (l), Delta Log likelihoods (Δl) and P-values of the AU (approximately unbiased), the SH (Shimodaira-Hasegawa), and the KH (Kishino-Hasegawa) tests.

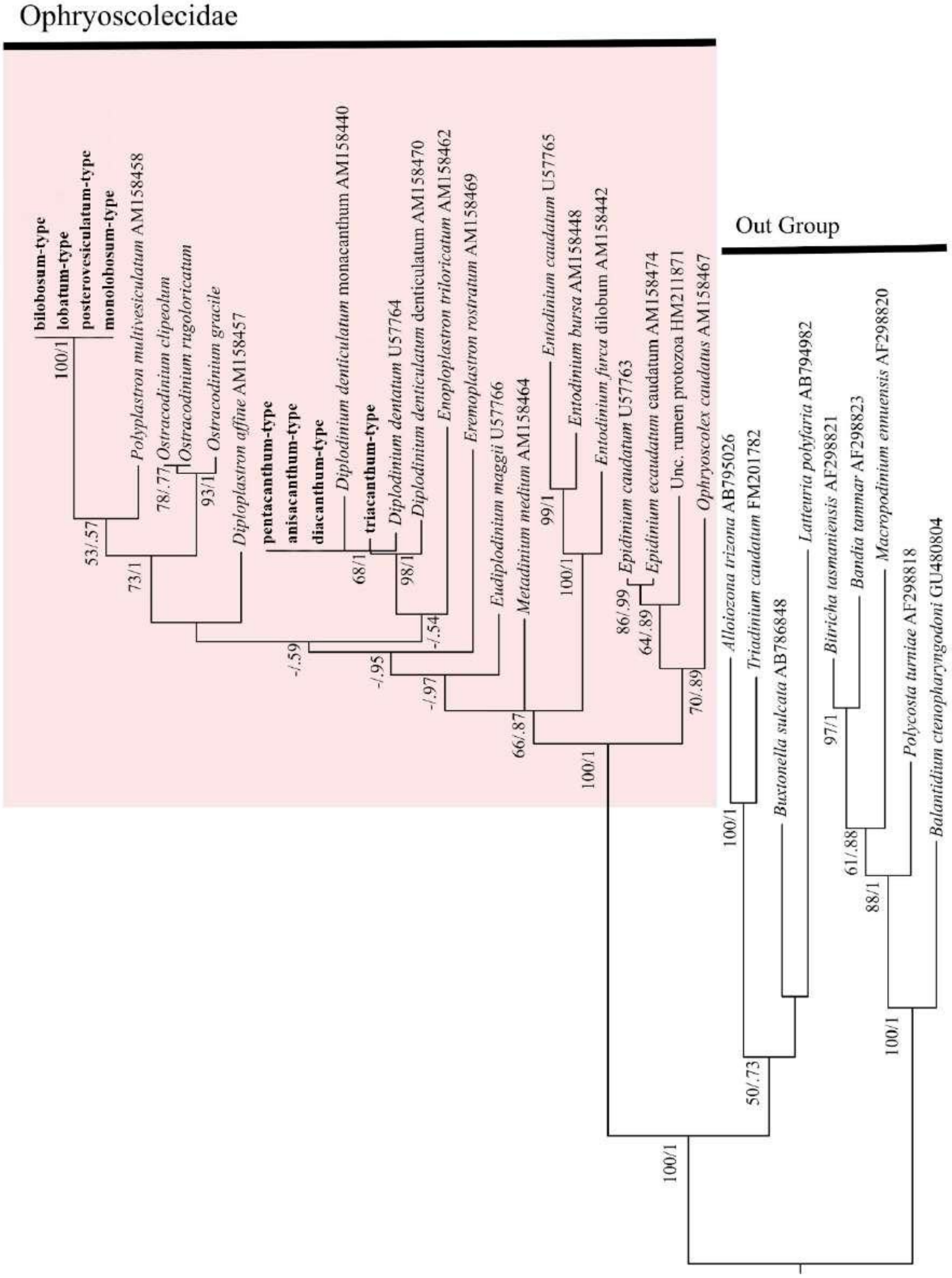
SUPPORTING INFORMATION

Fig. S1. Lateral views of Lugol-stained cells. **A-D.** *Eodinium posterovesiculatum* (Dogiel, 1927) (**A.** posterovesiculatum-type; **B.** lobatum-type; **C.** monolobosum-type; **D.** bilobosum-type) and **E-H.** *Diplodinium anisacanthum* da Cunha, 1914 (**E.** diacanthum-type; **F.** triacanthum-type; **G.** pentacanthum-type and **H.** anisacanthum-type). CP: caudal processes; CV: contractile vacuole; CZ: ciliary zone; Ma: macronucleus; Ve: vestibulum. Scale bars: 20 µm.

Fig. S2. Bayesian inference tree of the family Ophryoscolecidae (Ciliophora, Trichostomatia) based on 18S rDNA sequences. Further trichostomatid ciliates are chosen as outgroup. The values on the branches represent the posterior probability (BI) and maximum likelihood (ML) bootstrap values. The symbol (-) indicates posterior probability and bootstrap values of <0.5/50%. The new sequences of Holstein-Gyr rumen ciliates obtained in this study are highlighted in bold. The scale bar corresponds to 4 substitutions per 100 nucleotide positions.


Outgroup
Alloiozona trizona
Triadinium caudatum
Buxtonella sulcata
Latteuria polyfaria
Bitricha tasmaniensis
Bandia tammar
Macropodinium enniuensis
Polycosta turniae
Balantidium ctenopharyngodoni
Ophryoscolex caudatus
Unc. rumen protozoa
Epidinium ecaudatum caudatum
Epidinium caudatum
Eremoplastron rostratum
E. posterovesiculatum bilobosum-type
E. posterovesiculatum lobatum-type
E. posterovesiculatum monolobosum-type
Eodinium posterovesiculatum posterovesiculatum-type
Polyplastron multivesiculatum
Ostracodinium clipeolum
Ostracodinium rugoloricatum
Ostracodinium gracile
Diploplastron affine
Enoploplastron triloricatum
Diplodinium anisacanthum anisacanthum-type
D. anisacanthum tetracanthum-type
D. anisacanthum diacanthum-type
Diplodinium denticulatum monacanthum
D. anisacanthum triacanthum-type
Diplodinium dentatum
Diplodinium denticulatum denticulatum
Eudiplodinium maggii
Metadinium medium
Entodinium furca dilobum
Entodinium bursa
Entodinium caudatum
🟥 no skeletal plates
🟩 one plate
🟦 two plates
🟦 three plates
🟪 five plates

Topological scenario	**l**	**Δl**	**AU**	**SH**	**KH**
Best ML tree (unconstrained)	-5987.035	-	0.998	0.997	0.997
Monophyly of *Diplodinium* and *Eodinium* (constrained)	-6036.112	49.077	0.002	0.003	0.003


Ophryoscolecidae
Out Group
bilobosum-type
lobatum-type
posterovesiculatum-type
monolobosum-type
100/1
Polyplastron multivesiculatum AM158458
53/.57
Ostracodinium clipeolum
78/.77
Ostracodinium rugoloricatum
Ostracodinium gracile
93/1
73/1
Diploplastron affine AM158457
pentacanthum-type
anisacanthum-type
diacanthum-type
Diplodinium denticulatum monacanthum AM158440
triacanthum-type
68/1
Diplodinium dentatum U57764
Diplodinium denticulatum denticulatum AM158470
98/1
Enoploplastron triloricatum AM158462
-/.54
-/.59
Eremoplastron rostratum AM158469
-/.95
Eudiplodinium maggii U57766
-/.97
Metadinium medium AM158464
66/.87
Entodinium caudatum U57765
99/1
Entodinium bursa AM158448
Entodinium furca dilobum AM158442
100/1
Epidinium caudatum U57763
86/.99
Epidinium ecaudatum caudatum AM158474
64/.89
Unc. rumen protozoa HM211871
Ophryoscolex caudatus AM158467
70/.89
100/1
Alloiozona trizona AB795026
Triadinium caudatum FM201782
100/1
Buxtonella sulcata AB786848
Latteuria polyfaria AB794982
50/.73
100/1
Bitricha tasmaniensis AF298821
Bandia tammar AF298823
97/1
Macropodinium ennuensis AF298820
61/.88
Polycosta turniae AF298818
88/1
Balantidium ctenopharyngodoni GU480804
100/1
0.04

SEÇÃO 9

(Seção aceita para publicação no periódico *Systematics and Biodiversity*, Qualis A1, FI: 2.02)

Molecular phylogeny, systematic review, and revisited unexplored morphological data shed light in the taxonomy of Blepharocorythidae (Ciliophora, Entodiniomorphida)

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Running title: Molecular phylogeny and systematic review of Blepharocorythidae

Abstract

The family Blepharocorythidae includes 26 species of entodiniomorphid ciliates, which are distributed in nine genera and found as symbionts of a wide variety of hindgut fermenting mammals. Currently, the characters used to group the species within this family are a long vestibulum, a single contractile vacuole and ciliary zones at anterior and posterior ends of their bodies; and, as indicated by previous molecular phylogenetic studies this family might not be monophyletic. Here, a molecular phylogeny and a systematic review was performed aiming to elucidate inconsistencies and shed some light into the systematics of this family of ciliates. Although many representatives of this family could not be included in our phylogenetic analysis because their 18S rDNA sequences are not yet available in public repositories, after revisiting many morphology and ultrastructure works, we were able to identify morphological and morphogenetic features that allowed us to suggest a new organization scheme for this family which is in congruence with the molecular phylogenetic data. Moreover, one of the main outcomes of this work is to highlight the importance of revisiting erstwhile literature to improve the systematic of Blepharocorythidae ciliates as is for any other group of organisms.

Introduction

Over their evolutionary history, many herbivorous mammals, such as Artiodactyla Owen, 1848 and Perissodactyla Owen, 1848, have established, in their gastrointestinal tract, symbiotic associations with ciliated protists (Alveolata, Ciliophora), guaranteeing an efficient fermentative digestion and allowing them to survive on diets based, almost exclusively on plant material (mainly cellulose, hemicellulose, lignin and starch) (Dehority, 1986; Lynn, 2008).

These symbionts form a monophyletic and diverse group of organisms distributed in three orders of the subclass Trichostomatia Bütschli, 1889 (Litostomatea): Entodiniomorphida Reichenow in Doflein and Reichenow, 1929, characterized by the presence of somatic ciliature limited to tufts or bands and oral ciliature arranged in polybrachykineties; and Macropodiniida Lynn, 2008 and Vestibuliferida de Puytorac et al., 1974 (Dehority, 1986, Lynn, 2008), which are holotrichous (body completely covered by cilia) with a densely ciliated vestibulum (Lynn, 2008).

The order Entodiniomorphida is currently divided in three suborders. One of those is Entodiniomorphina Reichenow in Doflein and Reichenow, 1929 and is composed by ten families. The two others are Archistomatina Puytorac et al., 1974 and Blepharocorythina Corliss, 1975, represented by the families Buetschliidae Poche, 1913 and Blepharocorythidae Hsiung, 1929, respectively (Lynn, 2008). Ciliates of the family Blepharocorythidae Hsiung, 1929 are found associated with fore- and hindgut fermenting mammals, such as elephants, horses, ruminants and rodents (Dehority, 1986), and there are 26 accepted species distributed into nine genera: *Blepharocorys* Bundle, 1895, *Circodinium* Wolska, 1971, *Charonina* Strand, 1928, *Gorilloflasca* Ito *et al.* 2017, *Ochoterenaia* Chavarria, 1933, *Pararaabena* Wolska, 1968, *Raabena* Wolska, 1967, *Spirocorys* Wolska, 1969 and *Troglocorys* Tokiwa *et al.* 2010 (Hsiung, 1930; Strelkow, 1939; Thurston & Grain, 1971; Wolska, 1971a; Gilchrist *et al.* 1994; Tokiwa *et al.* 2010; Ito *et al.* 2017, 2018; Gurelli, 2019). Most of them sharing a long vestibulum, a single contractile vacuole, and ciliary zones at anterior and posterior ends of their bodies.

Although molecular phylogenetic studies aiming to elucidate the evolutionary history of blepharocorythid ciliates are scarce from the literature, two recent works (Ito et al., 2014; Kittelmann et al., 2015), based on 18S rDNA data, congruently found that this family is polyphyletic, with their members emerging in three distinct clades within Entodiniomorphida. However, besides this observation implies inconsistency between morphological and molecular data used for the systematics of this group, none of these authors proposed detailed explanations to clarify this taxonomic issue.

In the present work, we performed a molecular phylogeny and a systematic review of the family Blepharocorythidae. Our finds highlight the importance of revisiting old literature to better comprehend the systematics of this family of ciliates, and as well as to any other groups of extant organisms.

Material and Methods

Systematic review

The systematic review of the family Blepharocorythidae was carried out based on previous taxonomic works (Fiorentini, 1890; Bundle, 1895; Gassovsky, 1919; Cunha & Muniz, 1925; Jamenson, 1925; Hsiung, 1929, 1930; Chavarria, 1933; Strelkow, 1939; Wolska, 1967a, b, 1968, 1969a, b, 1970, 1971a, b, 1978a, 1979; Thurston & Grain, 1971; Gilchrist *et al.* 1994; Tokiwa *et al.* 2010; Ito *et al.* 2017, 2018; Gurelli, 2019) and data on infraciliary bands were obtained in Wolska (1967a, b, 1968, 1970, 1971a, b, 1978a), Tokiwa *et al.* (2010), and Ito *et al.* (2017, 2018). The terminology for the infraciliature followed Ito & Imai (1998) and Ito *et al.* (2002). The term polybrachykinety denotes infraciliary bands composed of numerous, short and parallel kineties (Fernandez-Galiano, 1979).

Infraciliary bands redescription

Data on infraciliary bands of blepharocorythid ciliates were obtained from Wolska (1967a, b, 1968, 1970, 1971a, b, 1978), Ito et al. (2018, 2019) and Gurelli (2019). The terminology adopted is according to Ito et al. (2018, 2019).

Molecular phylogenetic analyses

Our data sets were generated using data from previously characterized organisms (n=54), available from the Silva database release 123 (Pruesse *et al.* 2007), including representatives of the class Litostomatea Small and Lynn, 1981, subclass Trichostomatia Bütschli, 1889. The dataset was aligned using the ARB software package (Ludwig *et al.* 2004). The alignment was manually inspected, regions of ambiguous nature and primers sequences were removed. Finally, the ends of the dataset were trimmed producing an equal-length alignment. Subsequently, we used JModel-Test 2.1.4 (Guindon & Gascuel, 2003; Darriba *et al.* 2012) for the identification of the best model of sequence evolution. Bayesian inference (BI) analyses were performed using MrBayes v. 3.2 (Ronquist *et al.* 2012) with GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were running until the average standard deviation of the split frequencies went below 0.01, indicating the convergence of the two independent runs. All remaining trees after discarding the burn-in (25%) were used in the calculation of posterior probabilities using a majority rule consensus. Maximum likelihood (ML) analysis was carried out using the PhyML v 3.0 software (Guindon *et al.* 2010) applying a GTR + G + I nucleotide substitution model and the reliability of internal branches was assessed using the non-parametric bootstrap method with 1,000 replicates. Maximum parsimony (MP) analysis was performed using TNT (Goloboff *et al.* 2008) as implemented in the Mesquite v 3.51 (Maddison & Maddison, 2018a) module Zephyr v 2.11 (Maddison & Maddison, 2018b). Heuristic searches with multiple random addition sequences (1000 replicates) saving up to 1000 trees per cycle, swapping trees with TBR. The branch-support was evaluated by bootstrap (Felsenstein, 1985) of 1000 pseudoreplicates. Characters were treated as unordered and equally weighted and gaps was treated as missing data.

Results and Discussion

Systematic review

The family Blepharocorythidae was proposed by Hsiung (1929) and at this time, the family was composed by two genera, *Blepharocorys* Bundle, 1895 and *Charonina* Jamenson, 1928. In the first one, Bundle included the species *Entodinium valvatum* and *Diplodinium uncinatum* (Fiorentini, 1890), however with new designations, *Blepharocorys valvata* and *B. uncinata*, respectively; and proposed a new species, *Blepharocorys jubata*. At this time, in the genus *Charonina*, existed only one species, *C. ventriculi* Jamenson, 1925. Later, Gassovsky (1919), Cunha & Muniz (1925), and Hsiung (1930) proposed four new *Blepharocorys* species (*B. angusta*, *B. cardionucleata*, *B. curvicula*, and *B. microcorys*); and Hsiung (1930) proposed two new *Charonina* species (*C. equi* and *C. nuda*). Chavarria (1933) described the species *Ochoterenaia appendiculata* and Wolska (1967, 1968) proposed the species *Raabena bella* and *Pararaabena dentata*, and later, the same author (Wolska, 1969) redescribe the species *Triadinium minimum* (Gassovsky, 1919) as *Circodinium minimum*, including it in Blepharocorythidae. The species *Spirocorys indicus* was described by Wolska (1969), and two years later, Thurston & Grain (1971) proposed a new *Charonina* species, *C. hippopotami*. Several years later, Gilschrist *et al.* (1994) describe five new *Charonina* species (*C. dicerotis*, *C. odontophora*, *C. tenuis*, *C. tetragona*, and *C. tortuosa*) and Gurelli (2019) reported a new *Charonina* species, *Charonina elephanti*, from the hindgut of Asian elephants. Tokiwa *et al.* (2010) proposed a new species and a new genus, *Troglorys cava*; and Ito *et al.* (2017, 2018) proposed a new genus with two new species, *Gorilloflasca africana* and *G. longior*.

In this way, the family Blepharocorythidae currently includes 26 species of trichostomatid ciliates distributed in nine genera. Among them, the species *Blepharocorys uncinata*, described by Fiorentini (1890) as *Diplodinium uncinatum* is the type species.

Most of blepharocorythid ciliates possess a long vestibulum, a single contractile vacuole and ciliary zones at anterior and posterior ends of the body (Figure 1; Table 1). The only exception is the species *Spirocorys indicus* which has bands of ciliature covering the body (Figure 1).

Infraciliary bands redescription

The infraciliary patterns of the most blepharocorythid ciliates were originally described by Wolska (1966; 1967a, b; 1968; 1970; 1971a, b; 1978; 1979). However,

Tokiwa et al. (2010), Ito et al. (2018, 2019), and Gurelli (2019) describe the species *Troglocorys cava*, *Gorilloflasca africana*, *G. longior* and *Charonina elephanti*, based on general morphological features and infraciliature characterization. The authors used the terminology recently employed in infraciliature description in entodiniomorphid (Entodinimorphida) species, proposed by Ito and Imai (1998). Thus, a redescription of this character in the genera *Blepharocorys*, *Circodinum*, *Ochoterenaia*, *Pararaabena*, *Raabena*, and *Spirocorys* is necessary. The infraciliary patterns varies among these different taxa of blepharocorythid ciliates. *Blepharocorys* displays an adoral polybrachykinety (AP); a dorso-adoral polybrachykinety (DAP); a set of vestibular kineties (VK); a dorsal polybrachykinety (DP); four to five paralabial kineties (PK) and a caudal polybrachykinety (CDP). *Circodinium* displays an AP, a set of VK; a DP; four to five PK; a posterior dorsal polybrachykinety (PDP) and a CDP. Ochoterenaia displays an AP; a DAP; a set of VK; a DP; four to five PK; a PDP and a CDP. Pararaabena displays an AP; a set of VK; a dorsal polybrachykinety (DP); four to five PK and two CDP. *Raabena* displays an AP; a set of VK; four to five PK and two CDP. *Spirocorys* displays atypical infraciliary bands among blepharocorythids showing an AP and several ciliary tufts covering the body.

The AP encircle the vestibular opening; the DAP, if present, is located on the ventral surface of the frontal lobe. In *Raabena*, the VK encircles the left side of the frontal lobe and extends inside the vestibulum. In other genera, the VK are located inside the vestibulum, starting to the posterior portion of the AP or DAP. The VK morphology follows the vestibulum morphology, and thus, are a species-specific character among the different taxa. The PK, according to genera, vary in position, and comprises kinety rows of the "paralabial organelle" (Bretschneider, 1962). The DP lies in the dorsal side of the body; the PDP, if present, is in the dorsal side in the posterior portion of the body and the CDP are located near the cytoproct in the posterior body portion.

Evolutionary relationships within the family Blepharocorythidae

Two main apparent taxonomic inconsistencies, suggesting the polyphyly of the family, could be noticed from molecular phylogenetic reconstructions based on 18S rDNA sequences information (Figure 2; Ito *et al.* 2014; Kittelmann *et al.* 2015). First, *Charonina ventriculi* and *Raabena bella* emerge as sister groups of a set of entodiniomorphid ciliates and then apart from other blepharocorythid ciliates such as *Blepharocorys* spp., *Circodinium minimum* and *Ochoterenaia appendiculata*, in which are grouped in a monophyletic clade with *Parentodinium* sp. Ito *et al.* 2002 (Parentodiniidae) and *Pseudoentodinium elephantis* Wolska, 1986 (Pseudoentodiniidae) as sister groups. Second, *Circodinium minimum* and *Ochoterenaia appendiculata* positioned within *Blepharocorys* clade. These observations were the stimulus to revisit old morphology works searching for detailed information that could shed some light into the phylogenetic relationship and taxonomy of blepharocorythid ciliates.

Members of the family Blepharocorythidae have been historically classified based on superficial morphological features such as the presence of a single contractile vacuole and ciliary zones at anterior and posterior ends of their body, in which are characters also found in many unrelated groups of Trichostomatia ciliates (such as families Cycloposthiidae and Spirodiniidae) and based on the presence of a long characteristic vestibulum (also present in some Ophryoscolecidae), thus, there are not synapomorphies of Blepharocorythidae. Nonetheless, profound divergences concerning the vestibular infraciliature organization across members of the family Blepharocorythidae are observed (Figure 3). Most of the ciliates of the suborder Entodiniomorphina plus their sister groups (*Charonina ventriculi* and *Raabena bella*) (Figure 2) present a similar densely ciliated vestibulum that is distinct from the pattern observed in other blepharocorythids, such as *Blepharocorys* spp., *Circodinium minimum* and *Ochoterenaia appendiculata* (Figure 2), and also present in *Parentodinium* spp. (Parentodiniidae) and *Pseudoentodinium elephantis* (Pseudoentodiniidae) which composed by a few kineties (~5) in their vestibulum.

Wolska (1971b) believed that the morphology of the oral infraciliature could be used to infer evolutionary relationships in blepharocorythid ciliates. According to her insights, the plesiomorphic state would be a wide and densely ciliated VK band, such as in *Raabena bella*, that has undergone into fragmentation to form new polybrachykineties (DP, DAP and narrow VK with reduced numbers of vestibular kineties, ~5), such as observed in *Blepharocorys* spp. and *Ochoterenaia appendiculata* (Figure 4). Moreover, her hypothesis also predicts the existence of an intermediary state as observed in *Charonina ventriculi* and *Pararaabena dentata*, with a vestibulum composed by VK with a considerable number of vestibular kineties (> 20).

However, as mentioned above, this evolutionary relationship hypothesis is in conflict with molecular phylogenetic analyses (Figure 2; Ito *et al.* 2014; Kittelmann *et al.* 2015). In addition to this, it is becoming a consensus among ciliatologists (Lynn, 2008) that information on the morphology of infraciliature is not sufficient to infer evolutionary relationships within Ciliophora, and that morphogenetic and molecular data should also be considered to produce reliable classifications. Moreover, the data available on morphogenesis of blepharocorythid ciliates (Wolska, 1967a, b, 1968, 1970, 1971a, b, 1978; Ito *et al.* 2002, 2017) indicate that VK, DP, and DAP may have independent origins, and so, again refuting Wolska's hypothesis and the monophyly of the group. DAP is originated by the elongation and break of the ventral side of the adoral polybrachykinety (AP), and DP and VK have both independent morphogenetic primordia. With that, four patterns of oral infraciliary bands are present in the long vestibulum of Blepharocorythidae ciliates: Wide VK (*Raabena bella*); DP with moderate VK (*Charonina ventriculi*); DP, DAP and narrow VK (*Blepharocorys* and *Ochoterenaia*); and DP and narrow VK (*Circodinium minimum*).

Considering the morphological differences in the vestibulum (Figure 3), the morphogenetic and the molecular phylogenetic data (Figure 2, Ito *et al.* 2014; Kittelmann *et al.* 2015), we suggest the removal of *Charonina ventriculi* and *Raabena bella* from the family Blepharocorythidae, which should retain species that are morphologically and phylogenetically related to the type species, *Blepharocorys uncinata*. Meanwhile, according to the International Code of Zoological Nomenclature (ICZN, 2000), *C. ventriculi* and *R. bella* should be termed as *incertae sedis* until a proper designation were be proposed.

Regarding the second taxonomic incongruence presented, different phylogenetic analyses indicate that *Circodinium minimum* and *Ochoterenaia appendiculata* are close related species (Figure 2; Ito *et al.* 2014; Kittelmann *et al.* 2015). Wolska (1979) observations on the morphological similarities of these two species (presence of ciliated caudal process, contractile vacuole and cytoproct at same positions, and the shape and position of the nuclear apparatus), and by ultrastructure characters (presence of an epiplasm with a thin layer, cortex constituted by discontinuous microtubular layer, presence of aciliated kinetosomes scattered in the cytoplasm and absence of reticular fibers in the vestibular ultrastructure), also suggest their close phylogenetic relationship.

O. appendiculata was originally described and classified in the family Blepharocorythidae by Chavarria (1933). According to him, this species displayed distinct morphological features (mainly the ciliated caudal process) from those observed in other blepharocorythid genera, that justify the establishment of the genus *Ochoterenaia*. Strelkow (1939) rediscovered the species and pointed out that the characters proposed by Chavarria (1933) were insufficient to establish a new genus, and included the species in the genus *Blepharocorys* (*Blepharocorys appendiculata*). Wolska (1971a, 1978a) reestablished the genus *Ochoterenaia*, because, according to her, the characteristic formation of the later ciliated caudal process and the oral infraciliary pattern present in this species, differentiate this from the pattern observed in the genus *Blepharocorys*.

Circodinium minimum was originally described as *Triadinium minimum* by Gassovsky (1919) and classified into the genus *Triadinium* (Fiorentini, 1889) with other “helmet-shape” body entodiniomorphid ciliates. However, according to Wolska (1969, 1979) the classification of *T. minimum* in *Triadinium* was done based only in superficial aspects of its morphology and proposed the creation and inclusion into a new genus, *Circodinium* of the family.

Indeed, the oral infraciliary arrangement in *O. appendiculata* and *C. minimum* differ from *Blepharocorys* spp. However, the differences are with respect to the position of the infraciliary bands and not in the morphology nor morphogenesis of these bands (Figure 1 and 3). Thus, they might be merely reflection of the body surfaces variation of these species. However, beside these strong evidences indicating these genera should be synonymized, we suggest to keep them separated since their phylogenetic relationship (Figure 2; Ito *et al.* 2014; Kittelmann *et al.* 2015) are not clearly established and could change as more taxa were added or new molecular markers were established.

Unfortunately, the data on infraciliature, morphogenesis and molecular (18S rDNA) for most of the members of the family Blepharocorythidae are greatly fragmented limiting the extension of our systematic reorganization. Nonetheless, based solely on infraciliary and morphogenetic data available we can confidently infer that members of the genera *Troglocorys* and *Gorilloflasca* might be real Blepharocorythidae ciliates because the great similarities shared with *Blepharocorys* spp., *Ochoterenaia appendiculata* and *Circodinium minimum* (Wolska, 1971; Tokiwa *et al.* 2010; Ito *et al.* 2017).

On the other hand, *Pararaabena dentata* presents an infraciliary pattern and morphogenesis highly related to *Raabena bella* (Wolska, 1967a, b, 1968), possibly indicating that this taxon might not be a Blepharocorythidae. However, we believe would be reasonable wait until molecular data become available before proposing the exclusion of this genus of the family.

Further, the genus *Charonina* is represented by ten species (Table 1) of which only *C. ventriculi* has morphological and molecular data available (Wolska, 1971a; Kittelmann *et al.* 2015). Thus, it is not possible to evaluate whether they constitute a natural group or not with Blepharocorythidae, and so, until proper characterization, these *Charonina* spp. should be retained within the family Blepharocorythidae.

Wolska (1969) described the species *Spirocorys indicus* and established the genus *Spirocorys* to include it. According to the author, even though showing profound differences in morphology with other members, it was classified as Blepharocorythidae because, among all described ciliates found in association with mammals till 1969, the Blepharocorythidae ciliates were the most related. Since the lack of molecular phylogeny and more detailed morphological data, we decided keep this species in Blepharocorythidae.

Conclusion

In this study, we presented a systematic review and a reexamination of morphological data from members of the family Blepharocorythidae. We demonstrated that classification based uniquely in superficial and infraciliary bands morphology may not reflect evolutionary divergence and so, we believed that for reliable taxonomical characterizations of members of this family, and for other trichostomatid taxa, it is highly recommended to include information from molecular phylogenetics and on infraciliary morphogenesis data.

Another significant outcome of this work is to highlight the critical importance of revisiting old morphology works to shed some light and help to solve incongruences between morphological taxonomy and molecular phylogenetics.

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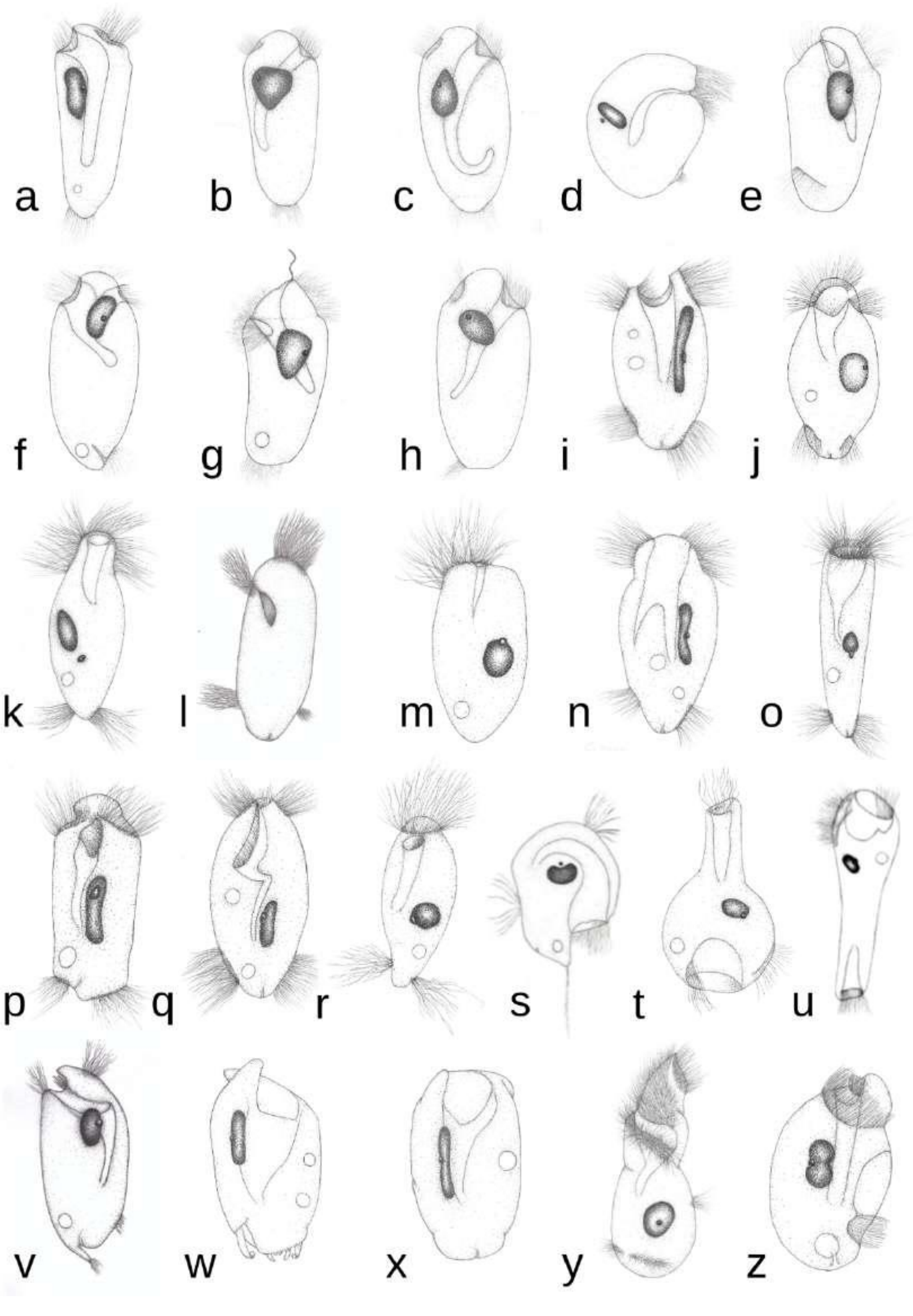

Figure 1. Schematic drawings of the species described in the family Blepharocorythidae (Ciliophora, Trichostomatia, Entodiniomorphida). a. *Blepharocorys angusta*, b. *Blepharocorys cardionucleata*, c. *Blepharocorys curvicula*, d. *Blepharocorys hydrochoeri*, e. *Blepharocorys jubata*, f. *Blepharocorys microcorys*, g. *Blepharocorys uncinata*, h. *Blepharocorys valvata*, i. *Charonina dicerotis*, j. *Charonina elephanti*, k. *Charonina equi*, l. *Charonina hippopotami*, m. *Charonina nuda*, n.

Charonina odontophora, o. *Charonina tenuis*, p. *Charonina tetragona*, q. *Charonina tortuosa*, r. *Charonina ventriculi*, s. *Circodinium minimum*, t. *Gorilloflasca africana*, u. *Gorilloflasca longior*, v. *Ochoterenaia appendiculata*, w. *Pararaabena dentata*, x. *Raabena bella*, y. *Spirocorys indicus*, z. *Troglocorys cava*. For morphometric data see **Table 1**.

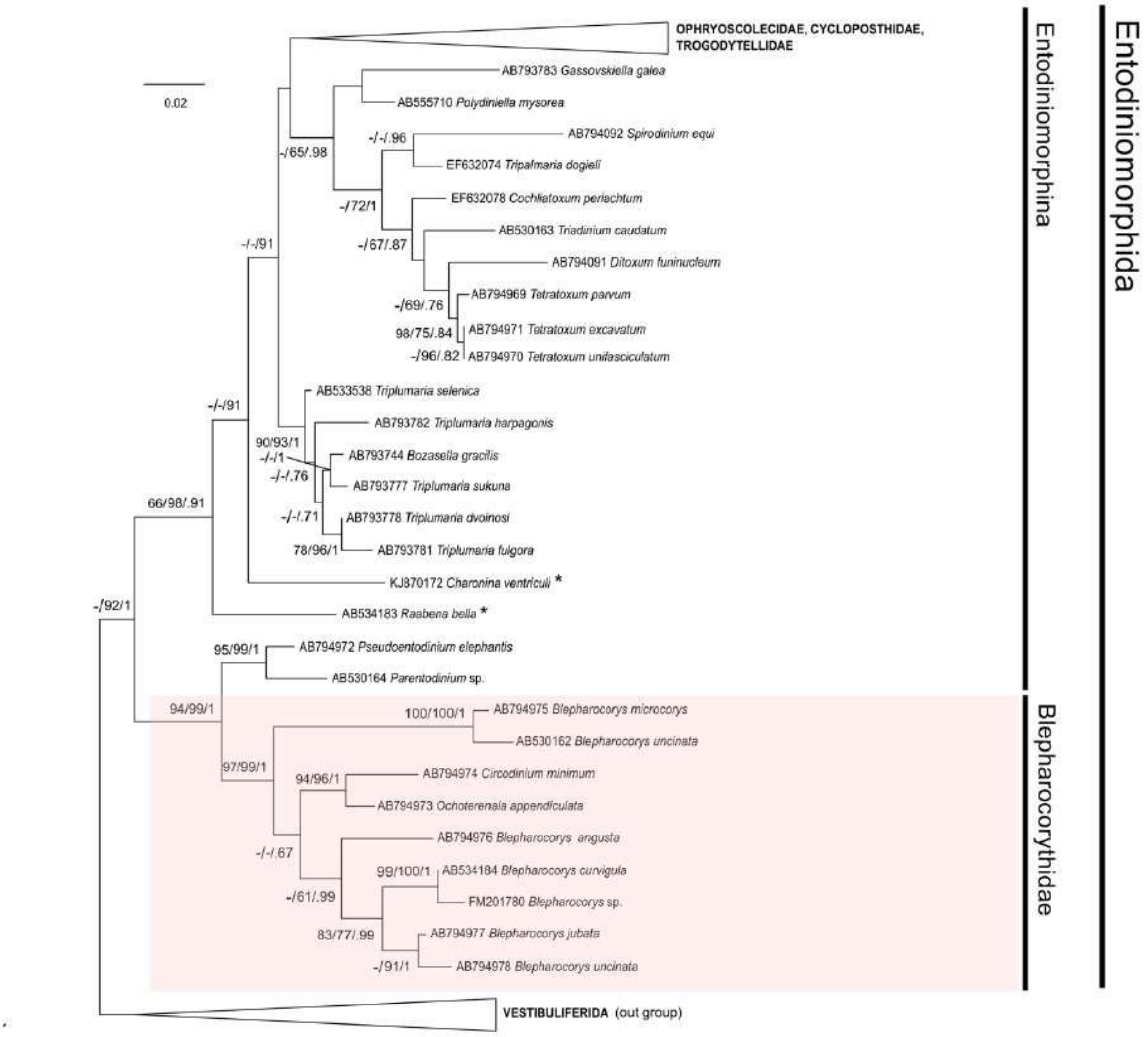

Figure 2. Phylogenetic tree of the family Blepharocorythidae (Ciliophora, Trichostomatia, Entodiniomorphida) based on 18S rDNA sequences. Further trichostomatid ciliates are chosen as outgroup. The values on the branches represent the maximum parcimony (MP) bootstrap, maximum likelihood (ML) bootstrap and posterior probability (BI) values. The symbol (-) indicates bootstrap or posterior

probability values of <0.5/50%. The asterisk (*) meaning species currently classified into the family Blepharocorythidae. The scale bar corresponds to 2 substitutions per 100 nucleotide positions.

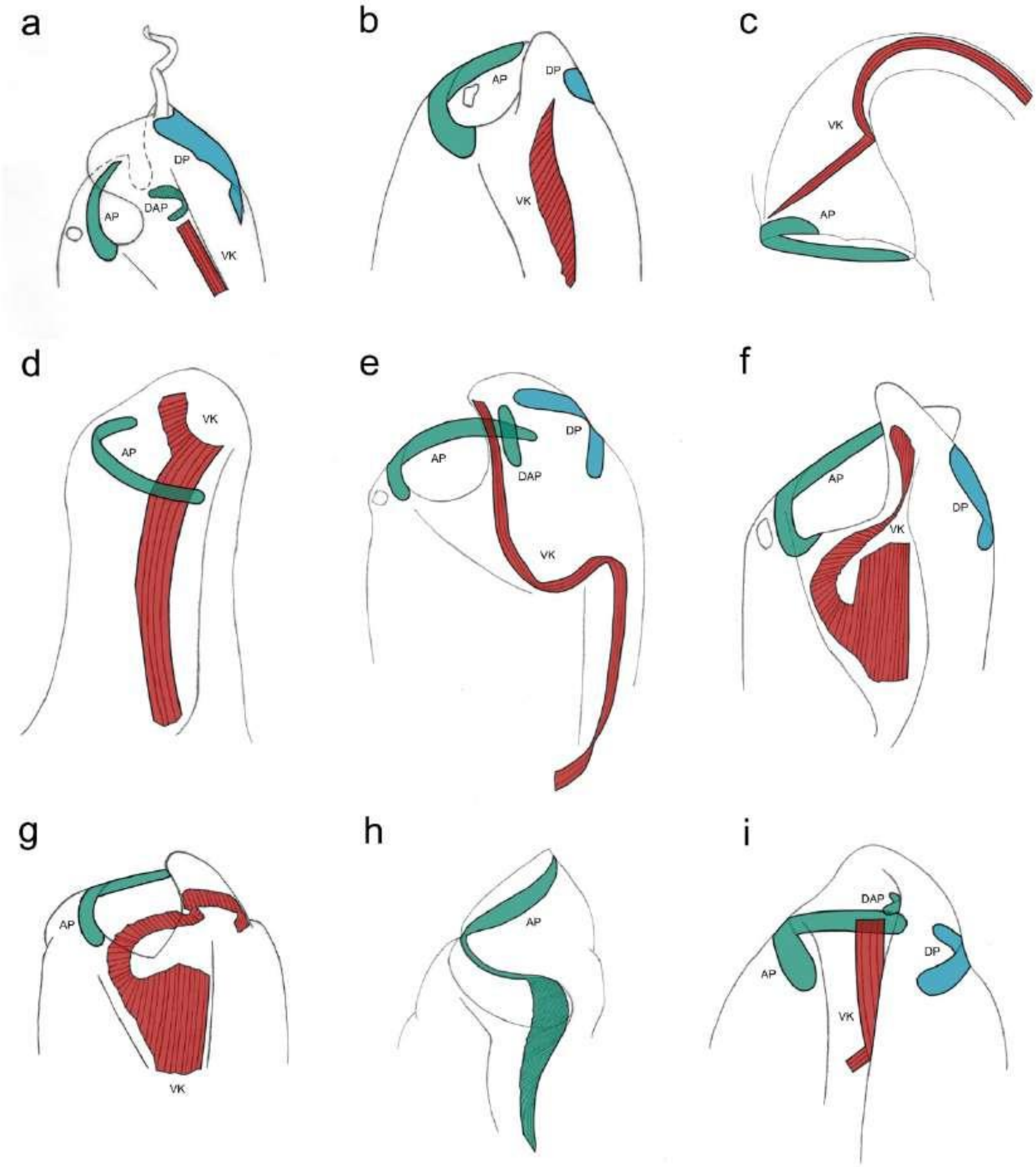

Figure 3. Schematic drawings representing the oral infraciliature in blepharocorythid ciliates. a. *Blepharocorys uncinata*, b. *Charonina ventriculi*, c. *Circodinium minimum*, d. *Gorilloflasca africana*, e. *Ochoterenaia appendiculata*, f. *Pararaabena dentata*, g. *Raabena bella*, h. *Spirocorys indicus*, i. *Troglocorys cava*. Equal colors represent the same morphogenetic origins. AP. adoral polybrachykinety, DAP. dorso-adoral

polybrachykinety, DP. dorsal polybrachykinety, VK. vestibular kineties. Adapted from Wolska (1971a, 1978) and Ito et al. (2002, 2017).

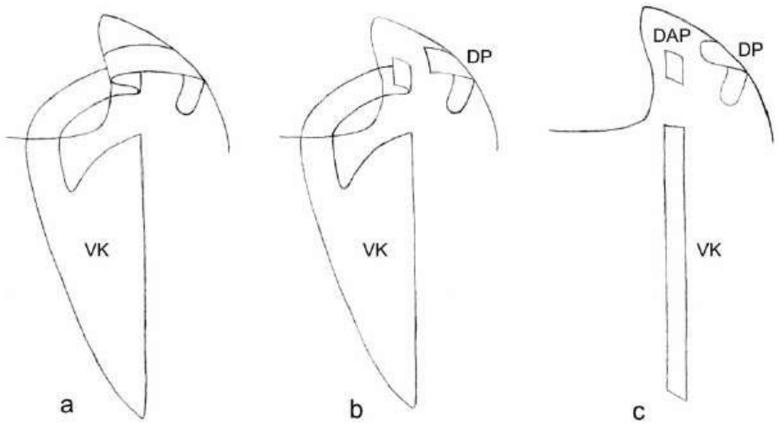

Figure 4. Schematic drawing representing Wolska's evolutionary hypothesis. a. *Raabena bella*, b. *Pararaabena dentata*, c. *Blepharocorys* sp. DP. dorsal polybrachykinety, DAP. Dorso-adoral polybrachykinety, VK. vestibular kineties. Adapted from: Wolska (1971b).

TABLE 1. Morphological and morphometric characteristcs of blepharocorythid species

FAMILY/ GENUS/ SPECIES	LENGTH	WIDTH	BODY SHAPE	FRONTAL LOBE	VESTIBULAR SHAPE	MA LENGTH	MA WIDTH	MA SHAPE	HOST
Blepharocorythidae Hsiung, 1929									
Blepharocorys Bundle, 1895									
Blepharocorys angusta Gassovsky, 1919	58-78	20-25	Elliptical-laterally flatened	Present	Tubular	*	*	Oval	Horse (*Equus caballus*)
Blepharocorys cardionucleata Hsiung, 1930	48-62	17-23	Elliptical-laterally flatened	Present	Tubular	*	*	Heart-shape	Horse (*Equus caballus*)
Blepharocorys curvicula Gassovsky, 1919	69.88 ± 0.84	27.80 ± 0.25	Elliptical-laterally flatened	Present	Tubular-curved	*	*	Oval	Horse (*Equus caballus*)
Blepharocorys hydrochoeri Cunha & Muniz, 1925									Capybara (*Hydrochoerus hydrochoeri*)

Blepharocorys jubata Bundle, 1895	33-60	17-23	Elliptical-laterally flatened	Present	Tubular	*	*	Oval	Horse (*Equus caballus*)
Blepharocorys microcorys Gassovsky, 1919	71.14 ± 0.78	23.28 ± 0.67	Elliptical-laterally flatened	Present	Tubular	*	*	Oval	Horse (*Equus caballus*)
Blepharocorys uncinata (Fiorentini, 1890)	55-74	22-30	Elliptical-laterally flatened	Present	Tubular	*	*	Oval	Horse (*Equus caballus*)
Blepharocorys valvata (Fiorentini, 1890)	52-68	20-27	Elliptical-laterally flatened	Present	Tubular	*	*	Heart-shape	Horse (*Equus caballus*)
Charonina Strand, 1928									
Charonina dicerotis Gilchrist et al., 1994	67 ± 8.6	37 ± 4.0	Oval-lanceolate	Absent	Funnel	32 (28-38)	*	rod-shape	White rhinoceros (*Ceratotherium simum*)
Charonina elephanti Gurelli, 2019	39.6-55.5	20-33.7	Oval-lanceolate	Absent	Tubular	5.4-14.6	5.4-11.1	Oval	Elephant (*Elaphas maximus*)
Charonina equi Hsiung, 1930	39.5 ± 0.30	11.8 ± 0.004	Oval-lanceolate	Absent	Funnel	*	*	Oval	Cattle (*Bos taurus*), Horse (*Equus caballus*)

Charonina hippopotami Thurston and Grain, 1971	18-32	8-14	Oval-lanceolate	Absent	Funnel	*	*	Oval	Hippopotamus (*Hippopotamus amphbius*)
Charonina nuda Hsiung, 1930	35-45	15-19	Oval-lanceolate	Absent	Funnel	*	*	Oval	Horse (*Equus caballus*)
Charonina odontophora Gilchrist et al., 1994	70 ± 5.7	32 ± 7.2	Oval-lanceolate	Present	Funnel	12-21	*	rod-shape	White rhinoceros (*Ceratotherium simum*)
Charonina tenuis Gilchrist et al., 1994	56 ± 10	16 ± 4.1	Oval-lanceolate	Present	Funnel	9	6	Oval	White rhinoceros (*Ceratotherium simum*)
Charonina tetragona Gilchrist et al., 1994	58 ± 4.7	26 ± 3.2	Oval-lanceolate	Absent	Funnel	16-28	*	rod-shape	Black rhinoceros (*Dicerus bicornis*)
Charonina tortuosa Gilchrist et al., 1994	87 ± 9.3	42 ± 5.3	Oval-lanceolate	Absent	Funnel	19-31	*	rod-shape	Black rhinoceros (*Dicerus bicornis*)
Charonina ventriculi (Jamenson, 1925)	28-46	9-15	Oval-lanceolate	Absent	Funnel	*	*	Oval	Ruminants (*Bos taurus*, *Ovis aries*), Horse

									(*Equus caballus*)
Circodinium Wolska, 1971									
Circodinium minimum (Gassovsky, 1919)	31-40	22-36	Helmet-shape	Absent	Curved	*	*	Kidney-shape	Horse (*Equus caballus*)
Gorilloflasca Ito et al., 2017									
Gorilloflasca africana Ito et al., 2017	80.3 ± 15.6 (63.8-137.7)	51.1 ± 9.2 (40.8-84.2)	Flask-shape	Absent	Tubular	12.1 ± 2.7 (7.7-17.9)	*	Oval	Gorilla (*Gorilla beringei*)
Gorilloflasca longior Ito et al., 2018	139.7 ± 18.4 (165.8-227)	57.4 ± 7.4 (45.9-71.4)	Flask-shape	Absent	Tubular	17.5 ± 3.9 (12.8-25.5)	*	Oval	Gorilla (*Gorilla beringei*)
Ochoterenaia Chavarria, 1933									
Ochoterenaia appendiculata Chavarria, 1933	66 (58-72)	28 (24-33)	Elliptical-laterally flatened	Present	Curved	*	*	Kidney-shape	Horse (*Equus caballus*)
Paraaabena Wolska, 1968									
Paraabena dentata Wolska, 1968	50-60	28-36	Oval-laterally flatened	Present	Funnel	*	*	rod-shape	Indian elephant

									(*Elaphas maximus*)
Raabena Wolska, 1967									
Raabena bella Wolska, 1967	44-67	27-36	Oval-laterally flatened	Present	Funnel	*	*	rod-shape	Indian elephant (*Elaphas maximus*)
Spirocorys Wolska, 1970									
Spirocorys indicus Wolska, 1969	68-104	27-47	Pear-shape	Absent	Funnel	*	*	Oval	Indian elephant (*Elaphas maximus*)
Troglocorys Tokiwa et al., 2010									
Troglocorys cava Towika et al., 2010	38.3 ± 3.1 (32.5-42.5)	29.1 ± 3.6 (25-35)	Spherical	Absent	Tubular	8.5 ± 1.3 (7.5-10)	*	Oval or Peanut-shape	Chimpanzee (*Pan troglotydes*)

SEÇÃO 10

(Seção publicada no periódico *The Journal of Eukaryotic Microbiology*, Qualis A4, FI: 2.361)

Helmet-shaped Body of Entodiniomorphid Ciliates (Ciliophora, Entodiniomorphida), a Synapomorphy or a Homoplasy?

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Short running title: Polyphyly of Helmet-Shaped Ciliates

ABSTRACT

Triadinium was created to include *Triadinium caudatum*. Further, four other species were included, *T. minimum*, *T. galea*, *T. elongatum* and *T. magnum*, all sharing a characteristic helmet-shaped body. Wolska and Grain, argued that the inclusion of *T. minimum* and *T. galea* into *Triadinium* was done based on superficial morphological aspects, and established two new genera to accommodate these species: *Circodinium* and *Gassovskiella.* Although the phylogenetic relationships within Entodiniomorphida have been investigated by multiple authors, none of them discussed the evolutionary relationship of helmet-shaped entodiniomorphids. We performed molecular phylogenetics and revisited old literature digging for morphological data to explain our results. According to our analyses the helmet-shaped body is homoplastic and may have evolved from at least three different entodiniomorphid ancestors. *Circodinium minimum* is phylogenetically related to members of Blepharocorythidae, *T. caudatum* emerged within Spirodiniidae and *G. galea* within Polydiniellidae. This phylogenetic hypothesis is partially supported by information on infraciliature and ultrastructure of *C. minimum* and *T. caudatum*. However, such morphological information is not available for polydiniellids. In order to shed some light into the evolution of the helmet-shaped ciliates, future works should focus to collect information on the infraciliature and the ultrastructure of *P. mysorea* and of other *Triadinium* species.

Keywords. 18S rDNA phylogeny; herbivorous mammals; symbiotic ciliates, Litostomatea, Trichostomatia

INTRODUCTION

The symbiotic ciliates of herbivorous mammals constitute a diverse and monophyletic group, distributed in three orders of the subclass Trichostomatia Bütschli, 1889 (Litostomatea): Entodiniomorphida Reichenow in Doflein and Reichenow, 1929, characterized by the presence of somatic ciliature limited to tufts or bands and the oral ciliature arranged in polybrachykineties, and Macropodiniida Lynn, 2008 and Vestibuliferida de Puytorac et al., 1974 (Dehority 1986; Lynn 2008), which have holotrichous somatic ciliature and a densely ciliate vestibulum (Lynn 2008).

The genus *Triadinium* (Entodiniomorphida) was created by Fiorentini (1890) to include *Triadinium caudatum*. Further, four other species were included, *T. minimum* Gassovsky, 1918, *T. galea* Gassovsky, 1918, *T. elongatum* Strelkow, 1931 and *T. magnum* Hsiung, 1935, all sharing the characteristic helmet-shaped and twisted body, with the anterior and posterior regions facing to each other. Wolska (1969, 1971b, 1979) and Grain (1994) argued that the classification of *T. minimum* and *T. galea* in *Triadinium* was done based on superficial aspects of morphology. Therefore, they proposed two new genera to include them: *Circodinium* Wolska, 1971 and *Gassovskiella* Grain, 1994, respectively.

Although the phylogenetic relationships within the order Entodiniomorphida have been thoroughly investigated in a number of recent molecular works (Bardele et al. 2017; Cedrola et al. 2017; Ito et al. 2010, 2014; Kittelmann et al. 2015; Moon-Von Der Staay et al. 2014; Rossi et al. 2015; Snelling et al. 2011; Strüder-Kypke et al. 2007; Vd'acny 2018; Wright and Lynn 1997a, b; Wright et al. 1997), none of them discussed the evolutionary relationship of helmet-shaped entodiniomorphid ciliates. Here, we discuss whether or not this character reflects evolutionary divergences based on molecular phylogenetic analyses and on morphological data available from the literature.

MATERIAL AND METHODS

Our dataset was generated using previously molecularly characterized organisms (n=42), available from the Silva database release 123 (Pruesse et al. 2007), including representatives of the subclass Trichostomatia Bütschli, 1889. The alignment was generated using SINA v. 1.2.11 (Pruesse et al. 2007), subsequently analyzed and edited in GBlocks v. 0.91b (Castresana 2000), and accessed by JModel-Test 2.1.4 (Darriba et al. 2012; Guindon and Gascuel 2003) to select the optimal model of sequence evolution.

Bayesian inference (BI) analyses were performed using MrBayes v. 3.2 (Ronquist et al. 2012) with GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were running until the average standard deviation of the split frequencies went below 0.01, indicating the convergence of the two independent runs. All remaining trees, after discarding the burn-in (25%), were used in the calculation of the posterior probabilities. Maximum likelihood (ML) analysis was carried out using the PhyML v. 3.1 (Guindon et al. 2010) performed in the Seaview v. 4.7 (Gouy et al. 2010) applying the GTR + G + I nucleotide substitution model and the reliability of internal branches was assessed using the non-parametric bootstrap method with 1,000 replicates. Three statistical tests, the AU (approximately unbiased), SH (Shimodaira-Hasegawa), and KH (Kishino-Hasegawa) tests, were conducted in the computer software CONSEL (Shimodaira and Hasegawa 2001). The likelihood-based ∂-statistic we use to compare the best ML topology (unconstrained tree), obtained in our phylogenetic analysis with alternative phylogenetic hypotheses assuming ^{1}the monophyly of *Gassovskiella galea* and *Triadinium caudatum* and ^{2}the monophyly of all helmet-shaped entodoniomorphids. The constrained tree was created with PhyML (Guindon et al. 2010), applying the same dataset and parameters used to generate the best ML tree.

RESULTS AND DISCUSSION

According to our phylogenetic reconstructions (Figure 1) and tree topology tests (Table S1), the helmet-shaped body in entodiniomorphid ciliates is a homoplastic character, that may have arisen at least three times and thus may not reflect evolutionary divergences. The three known entodiniomorphid ciliates with helmet-shaped body in which 18S rDNA sequences are available in public repositories are *C. minimum*, *G. galea* and *T. caudatum*. *C. minimum* emerged in a monophyletic clade within *Blepharocorys* spp. and *Ochoterenaia appendiculata* (Blepharocorythidae), with high support values (100 ML/1.0 BI) (Figure 1, clade C), corroborating the phylogenetic relationship hypothesis proposed by Wolska (1971b) based on their infraciliature.

According to Wolska (1971b), *C. minimum* has an oral infraciliature composed from adoral polybrachykinety, encircling the vestibular opening and five vestibular kineties, organized longitudinally inside the vestibulum (Figure 1 clade C). A similar infraciliary pattern is present in blepharocorythid species, such as: *Blepharocorys* spp. and *Ochoterenaia appendiculata*. Blepharocorythids exhibit an adoral polybrachykinety, encircling the vestibular opening; a dorso-adoral polybrachykinety located next to the frontal lobe; five vestibular kineties organized longitudinally or spirally inside the vestibulum; and a dorsal polybrachykinety located in the anterior dorsal portion of the body (Figure 1 clade C) (Wolska 1971a, 1978).

T. caudatum groups with spirodiniid ciliates with high support values (94 ML/1.0 BI) (Figure 1, clade B), which is also corroborated by Wolska's (1971b) data on infraciliature of *T. caudatum* and spirodiniids. *T. caudatum* shows an oral infraciliature composed from an adoral polybrachykinety, encircling the ventral side of the vestibulum, a perivestibular polybrachykinety, around the dorsal side of the vestibulum, a dorsal polybrachykinety located in the anterodorsal portion of the body. Spirodiniids display an oral infraciliature composed from an adoral polybrachykinety surrounding the ventral side of the vestibulum, a perivestibular polybrachykinety which encircles the dorsal side of the vestibulum, and a dorsal polybrachykinety located in anterodorsal portion of the body (Figure 1, clade B).

Finally, as shown in Figure 1, clade A, *G. galea* emerged as a sister taxon of *Polydiniella mysorea* (Polydiniellidae) (72 ML/0.80 BI). However, according to Grain (1994), *G. galea* has an oral infraciliature composed from an adoral polybrachykinety, a wide vestibular polybrachykinety located at the left wall of the vestibulum, and a dorsal polybrachykinety in the posterodorsal side of the body (Figure 1 clade A). Therefore, *G. galea* should be classified in the Spirodiniidae. *P. mysorea* was described without detailed characterization of its oral ciliature (Kofoid 1935). Therefore, data on infraciliature and ultrastructure of *P. mysorea* as well on other *Triadinium* species are indispensable for better understanding of this issue and will help to elucidate the evolution of the helmet-shaped bodied entodiniomorphid ciliates.

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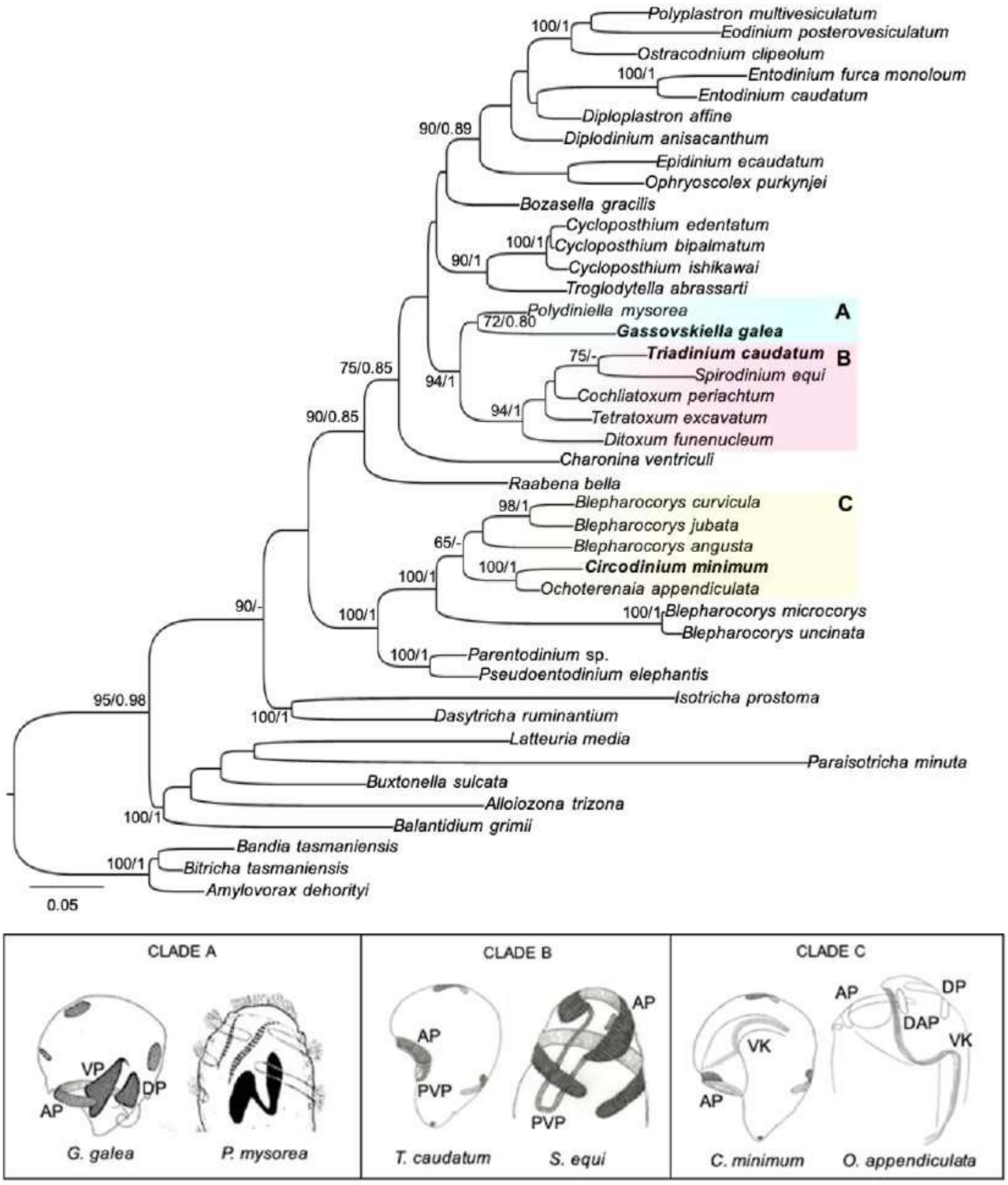
FIGURE

Fig. 1. Phylogenetic tree of entodiniomorphid ciliates (Ciliophora, Trichostomatia, Entodiniomorphida) based on 18S rDNA data. Other trichostomatid ciliates were chosen as out group. The values on the nodes represent the maximum likelihood (ML) bootstrap and posterior probability (BI) values. The symbol (-) indicates bootstrap or posterior probability values of <50/0.50. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. **A.** *Gassovskiella galea* clade, **B.** *Triadinium caudatum* clade, **C.** *Circodinium minimum* clade. AP: Adoral polybrachykinety, DP: Dorsal polybrachykinety, VP: Vestibular polybrachykinety, VK: Vestibular kineties, PVP: Perivestibular polybrachykinety.


Polyplastron multivesiculatum
Eodinium posterovesiculatum
Ostracodnium clipeolum
Entodinium furca monoloum
Entodinium caudatum
Diploplastron affine
Diplodinium anisacanthum
Epidinium ecaudatum
Ophryoscolex purkynjei
Bozasella gracilis
Cycloposthium edentatum
Cycloposthium bipalmatum
Cycloposthium ishikawai
Troglodytella abrassarti
Polydiniella mysorea
Gassovskiella galea
A
Triadinium caudatum
B
Spirodinium equi
Cochliatoxum periachtum
Tetratoxum excavatum
Ditoxum funenucleum
Charonina ventriculi
Raabena bella
Blepharocorys curvicula
C
Blepharocorys jubata
Blepharocorys angusta
Circodinium minimum
Ochoterenaia appendiculata
Blepharocorys microcorys
Blepharocorys uncinata
Parentodinium sp.
Pseudoentodinium elephantis
Isotricha prostoma
Dasytricha ruminantium
Latteuria media
Paraisotricha minuta
Buxtonella sulcata
Alloiozona trizona
Balantidium grimii
Bandia tasmaniensis
Bitricha tasmaniensis
Amylovorax dehorityi
100/1
100/1
90/0.89
100/1
90/1
72/0.80
75/-
75/0.85
94/1
94/1
90/0.85
98/1
65/-
100/1
100/1
90/-
100/1
100/1
100/1
95/0.98
100/1
100/1
100/1
0.05
CLADE A
VP
DP
AP
G. galea
P. mysorea
CLADE B
AP
PVP
AP
PVP
T. caudatum
S. equi
CLADE C
AP
DP
DAP
VK
VK
AP
C. minimum
O. appendiculata

SUPPORTING INFORMATION

Table S1. Tree topology tests

Topological Scenario	**OBS**	**log L**	**AU**	**NP**	**BP**	**PP**	**KH**	**SH**
Best ML tree (unconstrained)	-	-8279.25	1.00	1.00	1.00	1.00	1.00	1.00
Monophyly of *G. galea* and *T. caudatum*	1752.6	-10031.87	3×10^{-50}	9×10^{-19}	0	0	0	0
Monophyly of all helmet-shaped entodiniomorphids	1740.4	-10049.64	5×10^{-28}	2×10^{-13}	0	0	0	0

OBS: the observed log-likelihood differences between unconstrained and constrained trees.
log L: log likelihood
AU: the p-value of the approximately unbiased test calculated from the multiscale bootstrap.
NP: the bootstrap probability calculated from the multiscale bootstrap.
BP: the bootstrap probability calculated in the usual manner.
KH: the Kishino-Hasegawa test.
SH: the Shimodaira-Hasegawa test.

SEÇÃO 11

(Seção a ser submetida ao periódico *The Journal of Eukaryotic Microbiology*, Qualis A4, FI: 2.361)

Is *Elytroplastron* synonym of *Polyplastron* (Ciliophora, Entodiniomorphida, Ophryoscolecidae)?

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Short running title: Is *Elytroplastron* Synonym of *Polyplastron*?

ABSTRACT

The genus *Elytroplastron* displays several taxonomic characters in common to *Polyplastron*, which suggest that these two genera are synonymous. The present study dicusses this taxonomic incongruence by means of systematic review and morphological and molecular data on *Polyplastron* and *Elytroplastron* species. The nuclear apparatus in these genera are the same and the vacuolar apparatus and skeketal plates are very similar both in structure and morphogenesis, differing only in few details. The infraciliary bands pattern and morphogenesis in some *Polyplastron* ciliates are identical to that found in *Elytroplastron*. Moreover, the species *Polyplastron multivesiculatum* and *Elytroplastron bubali*, sequenced in this work, displays 99.8% identity in 18S rDNA sequences. Thus, our data strongly reject the validity of *Elytroplastron*, pointing out that the morphological parameters used to erect this genus are homoplastic and do not reflect evolutionary divergence.

Key-words: entodiniomorphids, rumen ciliates, molecular phylogeny, systematic review, Trichostomatia

INTRODUCTION

The family Ophryoscolecidae Stein, 1858 constitute a monophyletic group of gastrointestinal ciliates of herbivorous mammals, including ~300 species, distributed in three subfamilies and 16 genera (Williams and Coleman 1992). Several studies with ophryoscolecids were focused in the role which they play in the metabolism of their hosts. However, questions about its systematics are poorly understood (Cedrola et al. 2017a, 2018, 2019).

Among the ophryoscolecid genera, *Polyplastron* Dogiel, 1925 and *Elytroplastron* Kofoid & MacLennan, 1932 displays several overlapping taxonomic characters, which suggests that these genera are synonymous (Lubinsky 1958). However, these taxonomic incongruence was never discussed appropriately. The present study discusses these problems in the taxonomy of the family Ophryoscolecidae by means of systematic review, and morphological and molecular data on *Polyplastron* and *Elytroplastron* species.

MATERIAL AND METHODS

Systematic review

The systematic review of genus *Polyplastron* was carried out based on previous taxonomic works (Dogiel and Fodorowa 1925; Dogiel 1927, 1928; Kofoid and MacLennan 1932; Bush and Kofoid 1948; Lubinsky 1958; Dehority 1974).

Sampling and species identification

Samples of *Polyplastron multivesiculatum* (Dogiel & Fedorowa, 1925) were obtained from four fistuled Holstein x Gir cattle (*Bos primigenius* L. x *Bos primigenius indicus* L.) kept in Coronel Pacheco, Minas Gerais, Brazil and samples of *Elytroplastron bubali* (Dogiel, 1928) were obtained from Nellore cattle (*Bos taurus* L.) kept in Dois Vizinhos, Paraná, Brazil. For morphological analyses, the rumen samples (20 cm^3) were fixed in 18.5% formalin (Dehority 1984), while for the molecular analyses, in 100 % ethanol (Cedrola et al. 2015). Identification of *Polyplastron* and *Elytroplastron* species were based on the descriptions and redescriptions of Dogiel (1927, 1928) and Kofoid &

MacLennan (1932). The terminology of the cell sides follows Dogiel (1927), i.e. the side near the macronucleus is the dorsal side; this defines together with the anterior cell end, the ventral, right, and left cell sides.

DNA extraction, PCR, and DNA sequencing

Total DNA extractions were performed from pools of 10 ethanol-fixed ciliates of each species, using DNeasy Blood and Tissue kit (Qiagen Biotechnology Brazil, Ltd., São Paulo, SP, Brazil) and following the animal tissue protocol. The 18S rDNA was amplified, using Nested PCR approach, according to Cedrola et al. (2017).

Phylogenetic analyses

Our dataset was generated using both data obtained in the present study (consensus sequences) and sequences from previously molecularly characterized organisms, including representatives of the class Litostomatea Bütschli, 1889 (n= 37). The alignment was generated using SINA v. 1.2.11 (Pruesse et al. 2007), subsequently analyzed and edited in GBlocks v. 0.91b (Castresana 2000), and accessed by JModel-Test 2.1.4 (Darriba et al. 2012; Guindon and Gascuel 2003) to select of the optimal model of sequence evolution. Bayesian inference (BI) analyses were performed using MrBayes v. 3.2 (Ronquist et al. 2012) with GTR + G + I. Two simultaneous and independent Markov Chain Monte Carlo simulations were running until the average standard deviation of the split frequencies went below 0.01, indicating the convergence of the two independent runs. All remaining trees, after discarding the burn-in (25%), were used in the calculation of the posterior probabilities. Maximum likelihood (ML) analysis was carried out using the RAxML v. 8 (Stamatakis 2014) applying the GTR + G + I nucleotide substitution model and the reliability of internal branches was assessed using the rapid bootstrap method with 1,000 replicates.

RESULTS AND DISCUSSION

Taxonomic history

Polyplastron was established as subgenus of *Diplodinium* by Dogiel (1927) to include ophryoscolecid ciliates with two ciliary zones in the anterior body portion; five skeletal plates, two in the right side, primitiva and carina, and three in the left side of body, tergum, anticarina and scutum; and five contractile vacuoles. In this occasion, there were three species in this subgenus: *Diplodinium* (*Polyplastron*) *multivesiculatum* (Dogiel and Fedorowa, 1925), *Diplodinium* (*Polyplastron*) *fenestratum* (Dogiel, 1927) and *Diplodinium* (*Polyplastron*) *monoscutum* (Dogiel, 1927) (Table 1).

Dogiel (1928), in a study on the symbiotic ciliates of Russian water buffalo (*Bubalus bubalis* L.), found a species morphologically close to *Polyplastron* species, however with four skeletal plates, two in the right side, primitiva and carina, and two in the left side, tergum and scutum and four contractile vacuoles, naming this new species as *Diplodinium* (*Polyplastron*) *bubali*.

Later, Kofoid and MacLennan (1932), in a wide systematic review of the family Ophryoscolecidae, raised *Polyplastron* to the genus level, proposing the following diagnosis "Ophryoscolecidae with dorsal and adoral membranelle zones at anterior end of body; two skeletal plates beneath the right surface, either separate or fused together; three longitudinal plates beneath left surface with the anterior ends connected by transverse bars; a line of vacuoles beneath the dorsal surface, with additional vacuoles beneath the other surfaces".

In the same work, the authors discussed the placement of *Diplodinium* (*Polyplastron*) *bubali* in the genus *Polyplastron*, highlighting some morphological differences between this species and the remained *Polyplastron* species. According to the authors, the number and morphology of skeletal plates and the transverse bars connecting the left plates are very important taxonomic characters, and these are different in *D.* (*P.*) *bubali*. *D.* (*P.*) *bubali* displays the left skeletal plates larger than in *Polyplastron* spp. and not connected by transverse bars. Thus, the authors proposed a new genus to include the species, *Elytroplastron*, with the following diagnosis: "Ophryoscolecidae with dorsal and adoral membranelle zones at the anterior end of body; two skeletal plates beneath right surface, a small plate beneath ventral surface, and a long plate beneath the left surface; cuticle and ectoplasm relatively heavy; conspicuous fibrils beneath dorsal and right lateral surfaces".

During the following decades, new *Polyplastron* species were described, *Polyplastron californiense* Kofoid, 1948; *Polyplastron arcticum* Lubinsky, 1958 and *Polyplastron alaskum* Dehority, 1974 (Table 1), however, those species displays intermediate morphological characters between *Polyplastron* and *Elytroplastron*, suggesting that these two genera might be, instead, a single one.

Molecular phylogeny and taxonomic reorganizations

Molecular (Figure 1) and morphological data (Table 1) strongly reject the validity of *Elytroplastron*, and pointing out that the morphological parameters used to erect this genus are homoplastic and do not reflect evolutionary divergences.

Contradicting Kofoid and MacLennan (1932) hypothesis, our results, showed that the skeletal apparatus of *Polyplastron* spp. is not drastically different from *Elytroplastron bubali*. Indeed, Lubinsky (1958) have pointed out that Kofoid and MacLennan (1932) overestimated the value of this character for the taxonomy of these ciliates.

The plates on the right surface of *Polyplastron* spp. and *Elytroplastron bubali* are identical in position and morphogenetic origin, only superficially differing in *P. monoscutum* and *P. fenestratum*, which they are posteriorly fused. Moreover, Kofoid and MacLennan (1932) emphasize that the most important character supporting the split of the two genera, is the left plates (tergum, scutum and anticarina), which, according to these authors, is joined by transverse bars in *Polyplastron*, a character not observed in *Elytroplastron*. However, the species *P. arcticum* does not have this character (Dehority 1974; Cedrola et al. unpublished data). Moreover, the authors point out that the tergum would be large in *Elytroplastron* and that this was not observed in *Polyplastron*. However, the species *Polyplastron californiense*, *P. arcticum* and *P. alaskum* have broad and long tergum, as in *Elytroplastron bubali* (Bush and Kofoid 1948; Lubinsky 1958; Dehority 1974; Cedrola et al. unpublished data).

Moreover, the species Elytroplastron bubali and Polyplastron multivesiculatum, sequenced in the present work, display 99.8% identity in 18S rDNA gene sequences, which suggest a very close relationship, challenging the vality of *Elytroplastron*.

According to Cedrola et al. (2017), the number of skeletal plates in Ophryoscolecidae ciliates does not reflect evolutionary divergences, since species with the same number of plates do not constitute monophyletic groups. This previous observation corroborates our data, and also challenges the validity of genus *Elytroplastron*.

Still, several studies highlight the significance of infraciliary bands and morphogenetic patterns to understand the evolution in Ciliophora (Lynn 2008), including in the family Ophryoscolecidae (Rossi et al. 2015; Cedrola et al. 2019). In fact, *Elytroplaston bubali* and *Polyplastron* multivesiculatum and *P. alaskum* have the same infraciliary and morphogenetic patterns (Cedrola et al. unpublished data), challenging even more the validity of *Elytroplastron*.

Polyplastron sensu novo

Diagnosis. Body rounded shaped, with a small lobe at posterior end, and recovered by a tick pelicle. Two retractile ciliary zones at anterior end of body separated by a rounded or flattened operculum. Four to five skeletal plates. Two at the right side (primitiva and carina) and two or three at the left side of body (anticarina, scutum, and tergum). Anticarina absent in *Polyplastron bubali* comb. n. and in *Polyplastron arcticum*. Macronucleus rod-shaped, wide anterriorly. Micronucleus elliptical shaped, located anteriorly and dorsally to the macronucleus. Four to five contractile vacuoles, with conspicuous excretory pores lies dorsally to the macronucleus. Other vacuoles are present in the right and left body surfaces, however without a precise location. The cytoproct is wide and lies at posterior end of body.

Type species. *Polyplastron multivesiculatum* Dogiel & Fedorowa, 1925

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FIGURE AND TABLE

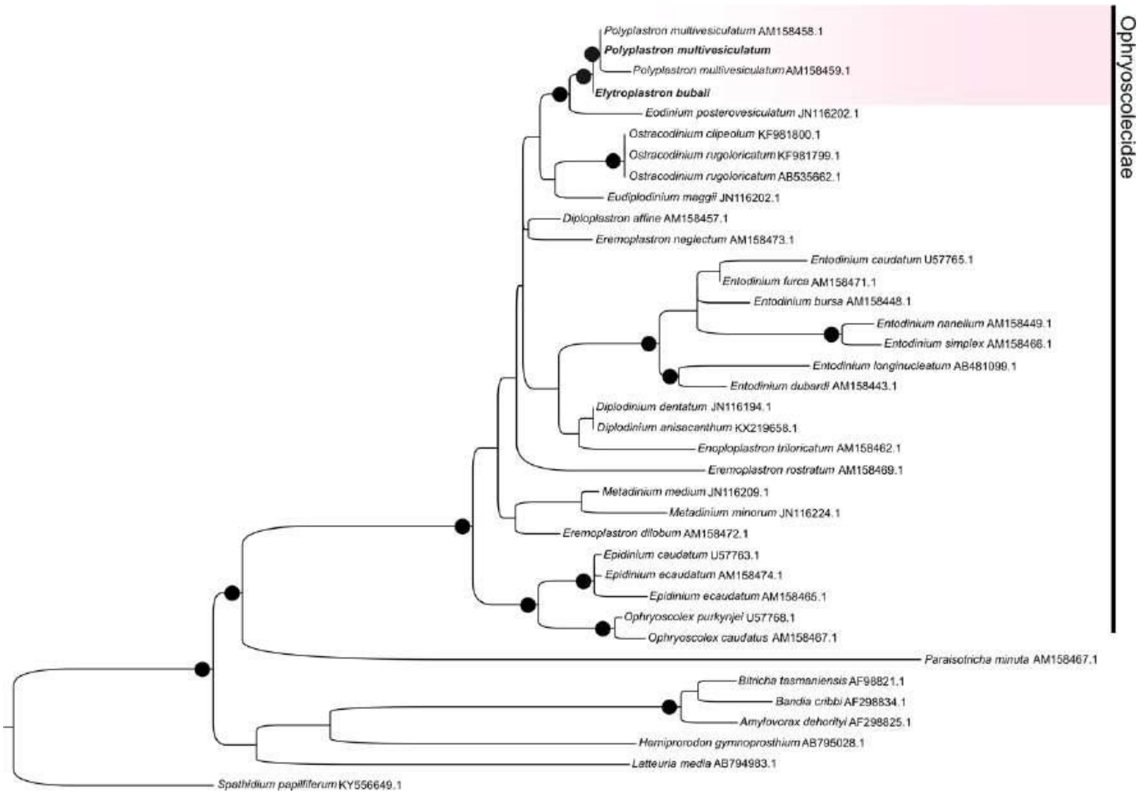

Fig. 1. Phylogenetic tree of ophryoscolecid ciliates (Ciliophora, Trichostomatia, Entodiniomorphida) based on 18S rDNA data. Other trichostomatid ciliates were chosen as out group. The black dots on the nodes represent maximum likelihood (ML) bootstrap and posterior probability (BI) values > 80/0.8.

Table 1. Characteristics of the *Polyplastron* and *Elytroplastron* species

				Skeletal plates	
Species	**Length**	**Width**	**Body shape**	**T**	**A**
Polyplastron alaskum Dehority, 1974	127.3	94.8	oval	broad and long	narrow and short
Polyplastron arcticum Lubinsky, 1958	183	141	oval	narrow and long	absent
Polyplastron californiense Bush and Kofoid, 1948	162	133	oval	broad and long	narrow and short
Polyplastron fenestratum (Dogiel, 1927)	*	*	oval	narrow and short	absent
Polyplastron monoscutum (Dogiel, 1927)	*	*	oval	narrow and	absent

Polyplastron multivesiculatum (Dogiel & Fedorowa, 1925)	120	78	oval	short narrow and	narrow and short
Elytroplastron bubali (Dogiel, 1928)	132	88	oval	short broad and long	absent

T: tergum

A: anticarina

CONSIDERAÇÕES FINAIS

O presente estudo contribuiu significativamente para ampliar o conhecimento acerca dos ciliados simbiontes pertencentes à subclasse Trichostomatia. Os inventários taxonômicos realizados em diferentes hospedeiros domésticos e selvagem (Seções 2, 3 e 4), por meio de caracterizações multidisciplinares, trouxeram à luz novos dados sobre estes microeucariotos, o que, em conjunto com vastas reviões sistemáticas e revistação de dados morfológicos pouco explorados, presentes na literatura clássica, contribuiram para melhor compreender a sistemática desta subclasse, sobretudo no que diz respeito às ordens Entodiniomorphida e Vestibuliferida, que eram, até então, grupos muito negligenciados em estudos desta natureza. Como consequência, os novos dados obtidos permitiram a elaboração de novas classificações taxonômicas, todas baseadas em divergências evolutivas (Seções 5, a 11) e permitiram identificar diversas lacunas ainda existentes no estudo de ciliados tricostomatídeos, as quais poderão ser preenchidas nos próximos anos por meio de estudos semelhantes aos que foram desenvolvidos ao longo deste trabalho. Sendo assim, a presente tese compila diversos modelos para a realização de estudos taxonômicos com foco na ciliatofauna simbionte pertencente à subclasse Trichostomatia.