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Marcos Trindade da Rosa

Dissertação de mestrado:

Stenostomum leucops (Dugès 1828) (Platyhelminthes, Catenulida) reprodução, auxílio no esclarecimento filogenético e transformação genética.

Santa Maria-RS
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Orientador: Prof. Dr. Élgion Lúcio da Silva Loreto.

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Resumo

Stenostomum leucops é um platelminto pertencente à classe Catenulida, apresentando grande plasticidade morfológica e reprodução preponderantemente assexuada, por paratomia. O tempo necessário para a formação completa dos zooides é de aproximadamente 42,5 horas a 28°C. Eles apresentam uma grande capacidade regenerativa, e facilidade de cultivo, tornando este organismo um excelente candidato a organismo modelo para estudos de regeneração, como bioindicador ou para outros estudos que envolvam reprodução assexual, como a transformação genética em células somáticas. Neste estudo, além da estimativa da taxa e velocidade da reprodução assexual, foi quantificado o número de células deste organismo. O número de células em zooides, logo após a paratomia, é aproximadamente 2.500. Empregando DNA *barcoding* adicionamos evidências à hipótese de que este taxa corresponde a um complexo de espécies. O primeiro mitogenoma de um Catenulida foi descrito neste estudo e mostrou algumas diferenças com respeito os de outros platelmintos já anotados. Os genes no mitogenoma de *S. leucops* são codificados em ambas fitas, enquanto em outros platelmintos são codificados em apenas uma fita. A ordem gênica encontrada em *S. leucops* é muito divergente da observada em outros platelmintos. O gene *atp8* esta ausente em outros platelmintos, mas um gene atp8 hipotético altamente divergente foi encontrado em *S. leucops*. Adicionalmente, é sugerido que o anticodon encontrado no RNA transportador K (*trnK)* é uma condição plesiomorfica que pode explicar as diferenças no código genético dos catenulideos. A análise do padrão de regeneração permitiu-nos observar quatro diferentes rotas de regeneração relacionadas com os estágios do desenvolvimento dos zoóids. A transformação genética é obtida facilmente nesta espécie, eletroporada com plasmídeos, ao menos para o gene repórter proteína fluorescente verde (GFP).

Abstract

Stenostomum leucops is a platyhelminthe belonging to class Catenulida, presenting great morphological plasticity and preponderantly asexual reproduction, by paratomy. The time required for a complete formation of the zooids of approximately 42.5 hours at 28 ° C. A variety of energy production features and systems, making this organism an excellent candidate for model organism for regeneration studies, as a bioindicator or for others Studies involving asexual reproduction, such as a genetic transformation in somatic cells. In this study, in addition to estimating the rate and speed of asexual reproduction, the number of cells in the organism was quantified. The number of cells in the zooids, soon after a paratomy, is approximately 2,500. Employing DNA bar code added evidence the hypothesis that this rate corresponds to a complex of species. The first mitogenome of a Catenulide was described in the study and showed some of the related articles in other already noted planlmints. The genes are not mitogenic of S. leucops are encoded in both ribbons, while in other flatworms are encoded in only one strand. The gene order found in S. leucops is very divergent from observation in other flatworms. The atp8 gene is absent in other flatworms, but a highly divergent hypothetical atp8 gene has been found in S. leucops. Additionally, it is suggested that the anticodonate found not RNA transporter K (trnK) is a plesiomorphic condition that may explain how there is no genetic code of catenulide. An analysis of the regeneration pattern allowed us to observe four different regeneration routes related to the developmental stages of zoonoids. A genetic transformation is easily obtained in this species, with a fairly efficient expression transition, at least for the green fluorescent protein (GFP) reporter gene. GFP expression rapidly found shortly after 24 hours was observed when the lengths were electroporated with plasmids, as well as, although less effectively.

Abreviaturas:

3xP3- promotor artificial contendo três locais de ligação para homodímeros Pax-6 na antecedendo uma caixa TATA

cm²- centímetro quadrado

DNA- ácido desoxirribonucleico

GFP- Proteína de fluorescência verde

µg- micrograma

mg- miligrama

µl- microlitro

ml- mililitro

mm- milímetro

CDS- região de DNA codificante

ORF- Quadro aberto de leitura

PB- *piggybac*

PCR- Reação de polimerização em cadeia

TEs- Elemento(s) de transposição

TIRs- Região(es) terminal(s) invertida(s)

12S - Subunidade ribossomal menor

16S- Subunidade ribossomal maior

ATP6- ATP sintase subunidade seis

ATP8- ATP sintase subunidade oito

Cytb- Citocromo b

CO1- Citocromo oxidase subunidade um

CO2- Citocromo oxidase subunidade dois

CO3- Citocromo oxidase subunidade três

ND1- NAD desidrogenase subunidade um

ND2- NAD desidrogenase subunidade dois

ND3- NAD desidrogenase subunidade três

ND4- NAD desidrogenase subunidade quatro

ND4L- NAD desidrogenase subunidade quatro L

ND5- NAD desidrogenase subunidade cinco

ND6- NAD desidrogenase subunidade seis

Sumário

Introdução

1-Caracterização da espécie estudada.

1.1 Posição de Catenulida dentro dos Platyhelminthes

O filo Platyhelminthes é composto de vermes achatados, acelomados, com ausência de órgãos circulatórios e excretores. A excreção e osmorregulação ocorre por protonefrídeos (Marcus 1945). Platyhelminthes são divididos em dois grandes clados, os Rhabditophora onde estão quase todas classes que compõe este filo e a classe Catenulida, como clado mais basal (Telford et al. 2003, Wallberg et al. 2007).

Rhabditophora é composto por Turbellaria, organismos de vida livre e epitélio coberto por cílios e capas de secreção mucosa, e os Neodermata, grupo monofilético, composto pelos organismos parasitas (classes: Trematoda, Monogenea e Cestoda) (Schockaert et al. 2008).

A classe Catenulida (Meixner 1924) é composta por organismos de vida livre, com poucos caracteres morfológicos diagnósticos, tendo de 0,2 a 3 milímetros de tamanho (Larsson et al. 2008).

Catenulida já foi considerado grupo basal, como atualmente aceito, mas contendo em sua composição Acoela e Nemertodermatida (Ehlers 1985)(Figura1). Outra proposta colocava Catenulida sendo grupo irmão dos Bilateria, em vez de grupo irmão de Rhadbitophora e agrupava Acoela com Nemertodermida (Haszprunar 1996). Já para Carranza et al. (1997) Catenulida é considerado grupo basal aos demais Bilateria e formava grupo parafilético com os Platyhelminthes.

Estudos mais recentes, utilizando dados moleculares (COI e 18S) coloca Catenulida sendo basal dos demais Platyhelminthes (Telford et al. 2003, Wallberg et al. 2007), e exclui Acoela e Nemertodermida deste filo.

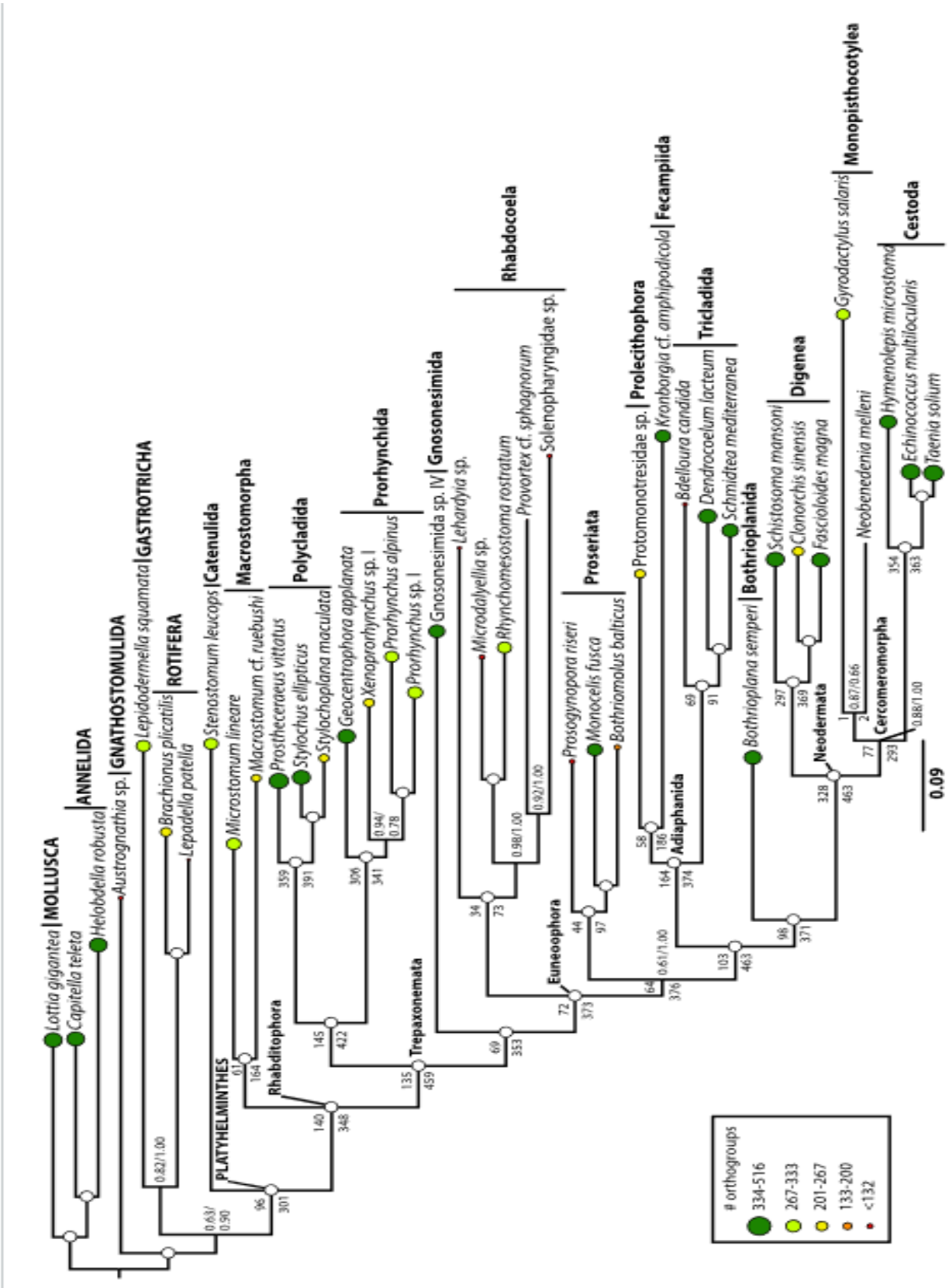

Figura 1: Filogenia de Laumer et al. (2015), resultante de filograma de máximo verossimilhança (ML) com 120,527 sítios de aminoácidos, contendo 25 espécies de Platyhelminthes.

1.2- “*Stenostomum leucops”*

Stenostomum leucops Dugès 1828, pertence a classe Catenulida, sendo uma espécie de vida livre, apresentando 0,5-2mm de comprimento, com distribuição cosmopolita. Os caracteres morfológicos diagnósticos para esta espécie são os gânglios faríngicos em forma de bastonetes ou ovalados, distribuídos pela faringe (Norena et al. 2005). Este organismo tem potencial organismo experimental, por ser facilmente cultivado em laboratório, possuí reprodução preponderantemente assexuada, formam dois zooides, onde ocorre o crescimento corpóreo em sentido axial, seguido do desenvolvimento de uma região cefálica na região mediana do corpo, e após esta estar plenamente formada é seguida da fissão em dois novos organismos, resultando em gerações isogênicas em curto prazo de tempo (Hyman 1951). Um vídeo mostrando o processo de formação de zooides e o processo de paratomia (processo de reprodução por fissão, seguida a formação dos zooides) pode ser acessado em http://w3.ufsm.br/labdros/permanente/paratomy.mp4.

Outra característica que o torna potencial organismo modelo é o fato de possuí uma alta taxa regenerativa (Van Cleave 1929), devido à presença de células tronco, chamadas de neoblastos. Entretanto, a descrição dos padrões regenerativos desta espécie apresenta resultados contraditórios. Ritter e Congdon (1900) descrevem que, quando um organismo formando zooide é seccionado removendo sua região cefálica anterior, a parte cefálica posterior pode migrar pelo corpo até assumir a posição anterior. Child (1903) descreve que em um verme ao ser seccionado há formação de uma região cefálica anterior e degeneração da posterior. Nuttycombe e Waters (1938) apontam que ocorre uma nova formação cefálica anterior e formação de uma região corporal após a região cefálica posterior. Além disso, apontam que as divergências dentre os resultados observado por diferentes autores pode ser associada a pesquisas com diferentes espécies, mas todas sendo nomeadas de *S. leucops*. Nuttycombe e Waters (1938) apontam que *S. leucops* não seria uma espécie válida.

Posteriormente, estudos feitos por Borkott (1970) e Noreña et al (2005) validaram esta espécie, embora dados moleculares recentes utilizados por Larsson et al. (2008) apontem que *S. leucops* pode se tratar de um complexo de espécies.

Estes vermes apresentam poucos caracteres morfológicos diagnósticos, o

que dificulta a identificação, podendo haver confusão entre estudos usando estes organismos.

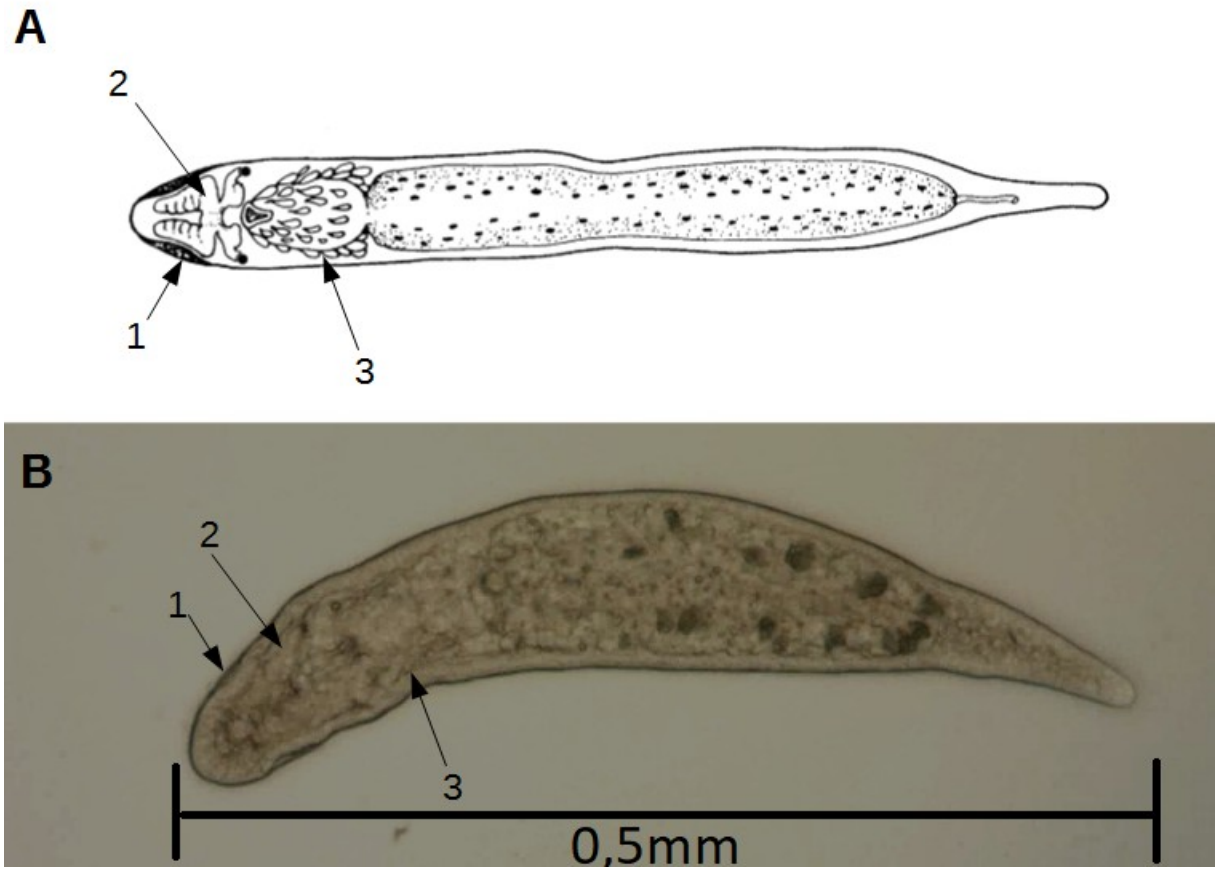

Figura 2: A) Representação esquemática de estruturas morfológicas de *S. leucops* segundo Noreña (2005), B) Fotografia no aumento de 100x de *S. leucops;* 1: Fossos ciliares, 2: Gânglios cerebrais e 3: Glândulas da faringe.

Para auxiliar na identificação e evitar classificações errôneas, além da chave morfológica apresentada por Noreña et al. (2005), se faz necessária a utilização de ferramentas moleculares como DNA *barcoding*, como os estudos de Larsson et al. (2008) e Yamazaki et al. (2012). Estes autores utilizaram sequências de rDNA 18S e do gene CO1, e encontraram divergência genética compatível com aquela normalmente observada entre diferentes espécies, quando compararam sequências, de amostras de *S. leucops* coletadas em diferentes regiões, sugerindo que seriam um complexo de espécies. Para resolver o a questão se *S. leucops* é uma espécie

ou um complexo se faz necessário caracterizar, por DNA *barcoding*, populações cobrindo a distribuição descrita para a espécie.

2-Genoma mitocondrial

Genomas mitocondriais ou mitogenomas apresentam, no Reino Animalia, de 15 a 20Kb, contendo 22 genes de RNA transportadores, dois genes de rRNAs ribossômicos (12S- subunidade ribossômica menor e 16S- subunidade ribossômica maior) e 13 genes codificadores de proteínas (ATPsintase, subunidades seis e oito; citocromo b; citocromo oxidase, subunidades um, dois e três; NAD dehydrogenase, subunidades um, dois, três, quatro, quatro L, cinco e seis). Estes genes são essenciais para síntese do ATP e portanto, têm papel central no metabolismo celular (Boore 1999).

O DNA mitocondrial evolui rapidamente por sofrer mutações em uma taxa 30 vezes mais elevada que a do DNA nuclear. Entretanto, o mitogenoma é altamente estável em relação a rearranjos gênicos, além de sofrer alta pressão seletiva devido a sua importância na fosforilação oxidativa, podendo ser estável durante grandes períodos evolutivos. Essas características podem ser usadas tanto para diferenciação e reconstrução da origem dos taxas, como para a estimativa aproximada da divergência intra-específica (Boore 1999).

Em Platyhelminthes, entretanto, o número de genes codificadores descritos no mitogenoma é de 12, pela ausência do gene A*tp8*. Outra característica do mitogenoma de Platyhelminthes é que todos os já descritos apresentam as CDSs localizadas um uma única fita do DNA, portanto sendo transcritas em um único sentido. Além disso a ordem gênica é altamente conservada nos grupos que compõem os Platyhelminthes (Figura3) (Boore 1999, Le et al. 2002, Johnston 2006, Vallès and Boore 2006, Waeschenbach et al. 2012, Aguado et al. 2015, Golombek et al. 2015, Solà et al. 2015, Ross et al. 2016). Nenhum genoma mitocondrial de Catenulida foi descrito para verificar se estas características também estão presentes neste grupo.

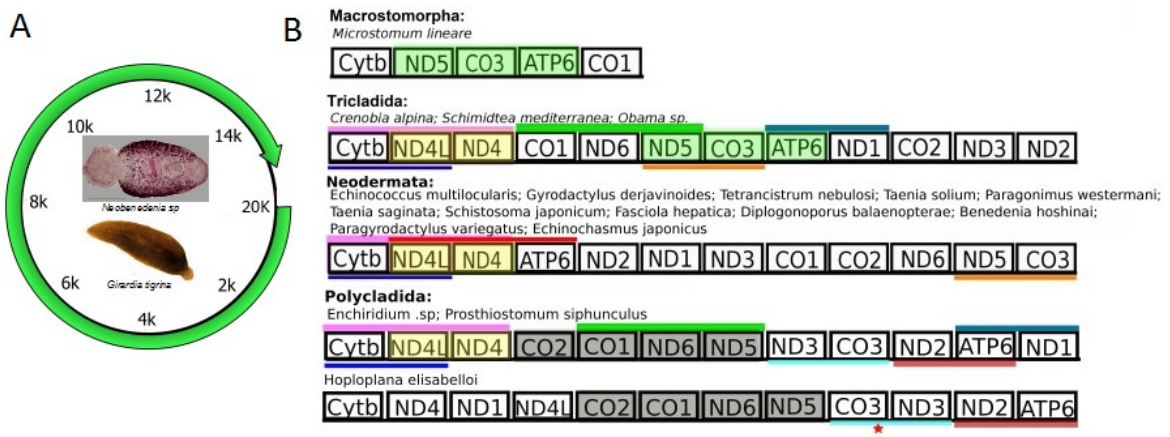

Figura 3: A) Desenho esquemático representando tamanho e sentido transcricional do genoma mitocondrial de Platyhelminthes. B) Sintenia mitocondrial dentre os grupos de Platyhelminthes. Cores semelhantes representando as semelhanças encontradas entre os mitogenomas. Estrela vermelha significa inversão genica.

3- Genoma nuclear de *S. leucops*

O sequenciamento dos genomas nucleares produz um conjunto de dados muito importante para entendermos a biologia e a evolução dos organismos, desde que anotados e descritos adequadamente. Os genomas de Platyhelminthes tem grande atratividade para estudos, visto que parte dos organismos deste grupo têm importância médica, como parasitas ou pela presença de células totipotentes para estudos regenerativos. Um exemplo é a planária *Schmidtea mediterranea* que tem um banco de dados exclusivo Genomic Database (SmedGD) criado em 2007 (Robb *et al.* 2008). Este organismo é a principal referência para estudos sobre regeneração dentre os Platyhelminthes.

Alguns estudos atuais começam a explorar com maior ênfase uma diversidade de Platyheminthes que apresentam diferenças em suas capacidades regenerativas, como, por exemplo, *Microstomum lignano*, que é capaz de formar partes perdidas em três dias, devido à presença de células totipotentes (Egger et al. 2006). Para entender esta capacidade regenerativa se fazem necessários estudos moleculares de vários modelos (Wasik et al. 2015), para entendermos as diferentes capacidades regenerativas.

Os genomas de Platyhelminthes de vida livre descritos até o momento são ricos em sequências repetitivas e elementos de transposição. Por exemplo, em

Macrostomum lignano 75% do genoma é composto por sequências repetitivas e elementos de transposição (Wasik et al. 2015). Mesmo que até o momento não se tenha estudos suficientes sobre o material genético transcricional que é gerado destas regiões repetitivas, os autores levantam a hipótese que elementos de transposição se “aproveitam” que o sistema de silenciamento destes Platyhelminthes tenha como função principal a manutenção de neoblastos e regeneração, abrindo falhas no silenciamento de elementos transponíveis, permitindo assim a sua proliferação nestes genomas (Palakodeti et al. 2008, Zhou et al. 2015).

4-Elementos de transposição (TEs)

Elementos de transposição (TEs) são sequências de DNA com capacidade de mudar sua localização dentro do genoma (Loreto e Moura 2017). Quando os TEs possuem informação genética para produção da maquinaria enzimática para sua própria transposição são denominados autônomos. Quando utilizam a maquinaria de outro elemento são denominados não-autonomos (Loreto e Moura 2017). Os TEs foram descobertos por McClintock em 1950, no milho, por causarem alterações no padrão de coloração dos grãos. Desde então, muitos estudos mostram que os genomas são dinâmicos (Varani et al. 2015), e que a transposição destas sequências de DNAs, podem ter grande influência nos aspectos biológicos e evolutivos dos organismos hospedeiros como, por exemplo, ser uma fonte de variabilidade genética (Dolgin e Charlesworth 2006)

Finnegan (1989) propôs um sistema de classificação para os TEs dividindo-os em Classe I ou retrotransposons que seriam elementos que se movem por um intermediário de RNA, usando uma enzima transcriptase reversa homóloga as usadas por retrovírus. O elemento é transcrito para RNA e copiado reversamente para DNA, sendo posteriormente inserido em uma nova região do genoma. Já os elementos de Classe II, também conhecidos como transposons, agrupam elementos que se transpõem usando um intermediário de DNA, normalmente usando uma enzima chamada transposase que “corta” o elemento da sua posição inicial e o “insere” em uma nova posição do genoma. Com os avanços das técnicas moleculares e de sequenciamento, a diversidade de TEs descritos vem aumentando, como por exemplo o banco de dados de transposons no **Repbase** já apresenta

56.000 sequências (até o dia 20/04/2016), subdivididas em 65 superfamílias (Bao et al. 2015). Com o aumento da diversidade de sequências descritas, novos padrões classificatórios também estão sendo propostos, com mais subdivisões. Alguns pesquisadores da área sugeriram a criação de um comitê para organizar, ajudar a padronizar e melhorar estas classificações (Arensburger et al. 2016).

Além da diversidade genética que os transposons podem ajudar a promover, estes podem causar muitos outros efeitos em células somáticas: como promover plasticidade, efeitos neutros quando estes não interferem beneficamente e nem prejudicial ao funcionamento celular, ou negativo provocando que a célula entre em apoptose ou câncer (revisão em Loreto e Moura 2017).

4.1 *piggyBac*

O elemento *piggyBac* (PB) é um TE que faz parte da classe II, possuindo 2475 pb, com TIRs (repetições terminais inversas) de 13 pb idênticas e repetições internas adicionais de 19 pb assimétricas (Zhao et al. 2016). As TIRs são reconhecidas pela transposase do elemento, que é codificada por uma ORF (*open reading frame*) localizada entre as TIRs do elemento. Este transposon insere-se preferencialmente em sítios com TTAA, onde sua transposição para esses sítios apresentam uma frequência de 30 a 50 % (Zhao et al. 2016).

Estudos usando “construções” de plasmídeos contendo a sequência do marcador GFP, e mecanismos de inserção através de um “*helper*” contendo a sequência da transposase vêm ajudando a entender a invasão dos genomas pelos TEs, os mecanismos de defesas da célula contra a sua proliferação e silenciamento de TEs, além de proporcionar um método menos genotóxico que vetores virais para produção de organismos transgênicos. Estudos empregando o elemento *piggyBac* conseguiram integrar um fragmento de 18Kb em células T humanas (Adelle 2011). O elemento *piggyBac* também se mostrou muito eficaz para a criação de organismos transgênicos, como leveduras, Platyhelminthes, artrópodes e mamíferos (Zhao et al. 2016).

5-Transformação genética e expressão transiente.

A transformação genética consiste na incorporação de uma molécula de DNA exógena, para o genoma da célula outro organismo. Dentre as maneiras mais simples e eficientes de transformação, está a eletroporação que consistes da despolarização da membrana celular, possibilitando a entrada de moléculas de DNA para o interior da célula, que pode ou não ser incorporado ao genoma, produzindo um organismo transgênico (Adelle 2011, Deprá et al. 2004, Estévez et al. 2003, Matsuoka et al. 2010).

Nem todo DNA exógeno acaba anexando-se ao genoma, e este pode ou não ser expresso, mas quando expresso sem ocorrer incorporação, este processo é conhecido como expressão transiente (Wolff e Budker 2005). Exemplos de expressão transiente *in vivo* foram demonstrados pela transferência de material genético entre a bactéria *Escherichia coli* e *Acheta domesticus (*espécie de grilo). Estes após ingerirem água contaminada com *E. coli* contendo plasmídeo contendo eGFP, apresentaram fluorescência em músculos e exoesqueletos após 24 horas (Fauce e Owens 2012).

A expressão de DNA exógeno também já foi descrita em mamíferos, por injeção de plasmídeos por via intravascular em primatas não-humanos e ratos, as células que apresentaram maior facilidade na absorção deste material e expressão foram as de músculo esquelético (Wolff et al. 1990, Danko et al. 1997, Wolff e Budker et al. 2005).

O processo de produção de organismos transgênicos em Platyhelminthes já foi descrito em planárias *Girardia tigrina* (González-Estévez 2003). Para esta transformação foram utilizados dois plasmídeos. Um deles contendo a informação gênica 3xP3-GFP, a região 3xP3 atua como promotor contendo três sítios de ligação do gene 6-Pax, responsável pela diferenciação celular na formação de olho ou estruturas fotorreceptoras, neste promotor esta ligado a sequência codificadora da proteína GFP (green fluorescent portein), sendo usada como marcador de expressão gênica. Estas sequências são flanqueadas pelas TIRs do elemento p*iggyBac* (Horn et al. 2000). Para a transformação genética empregaram também um segundo plasmídeo *helper* pBΔSac que contém a ORF do elemento, mas sem as TIRs, que produz a enzima para a transposição do fragmento contendo o gene GFP, flanqueado pelas TIRs do elemento *piggyBac* do primeiro plasmídeo. Em *G. tigrina*,

foi observado, como esperado fluorescência nos ocelos.

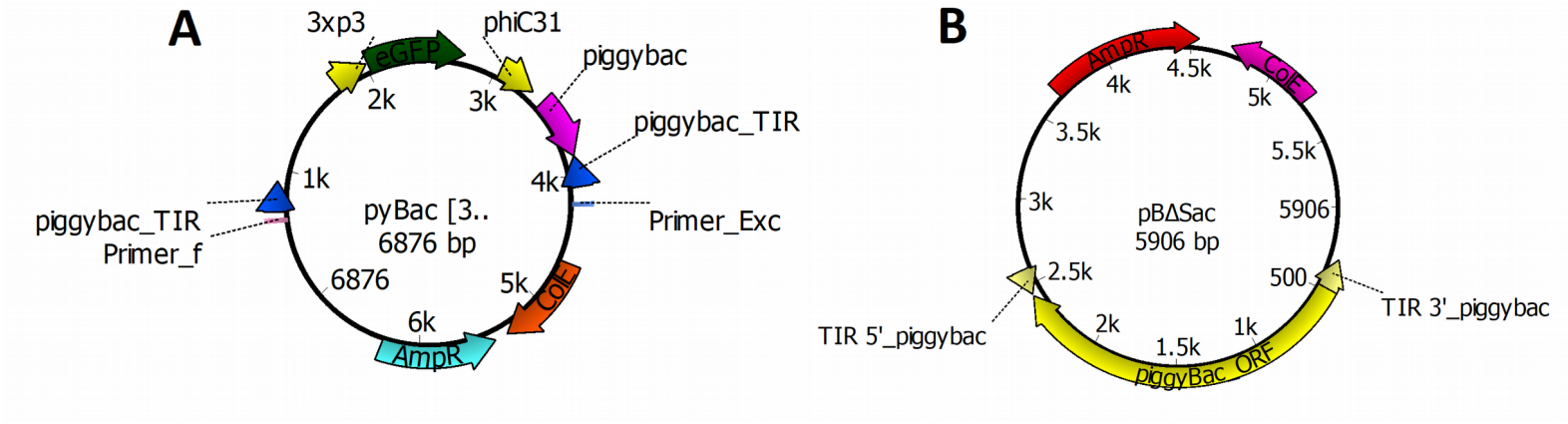

Figura 5: A) Plasmídeo pyBac [3xP3-EGFPafm], em que estão indicando as localizações dos primers (Primer F e Primer Exc). Este conjunto de primers amplifica um fragmento de aproximadamente 3,5 kb, contendo as TIRs do elemento p*iggyBac,* o CDS do gene GFP e região promotora.; B) Plasmídeo pBΔSac, onde o elemento piggyBac teve uma de suas TIRs invertidas, impossibilitando o reconhecimento pela transposase.

Objetivos gerais.

Contribuir com dados biológicos básicos e moleculares da espécie ou complexo de espécie *S. leucops*, para sua utilização como modelo em estudos futuros.

Objetivos específicos

- Descrição das características biológicas de uma linhagem monoclonal de *S. leucops* cultivada em laboratório, em relação a sua plasticidade morfológica, tempo do desenvolvimento de zooide, e quantificação da taxa de proliferação celular.

- Analisar a posição filogenética da linhagem estudada, empregando marcadores moleculares, em relação a outras linhagens da espécie estudada e de outros Catenulidas.

- Sequenciar e anotar o mitogenoma de *S. leucops* para compará-lo com os de outros Platyhelminthes.

Capítulo 1:

Os estudos que resultaram neste mestrado tiveram início na graduação, pela oportunidade oferecida pelo professor Élgion Lúcio da Silva Loreto, proporcionando a minha participação no seu grupo de pesquisa e laboratório LabDros, como aluno de iniciação científica, oportunidade de uma complementação na formação como licenciado em ciências biológicas. Além disso, ajudou a contribuir um pouco sobre o conhecimento da espécie *Stenostomum leucops.*

O trabalho executado na graduação intitulado: “Revisiting the regeneration of *Stenostomum leucops* (Catenulida, Platyhelminthes), now carrying a green lamp" foi submetido para Journal of Experimental Zoology Part B: Molecular and Developmental Evolution e, foi recusado. Porém, as críticas feitas pelos revisores proporcionaram melhorias significativas e amadurecimento. As críticas permitiram gerar os dois artigos produzidos durante o mestrado, assim como o desenvolvimento de outras pesquisas que estão em andamento e serão também apresentadas nesta dissertação

Nossa intenção em iniciar esta dissertação apresentando um trabalho recusado é mostrar como se deu o processo do desenvolvimento das idéias aqui apresentadas, com seus tropeços, incompletudes e avanços. Entendemos que é dessa forma que o conhecimento científico avança.

Revisiting the regeneration of Stenostomum leucops
(Catenulida, Platyhelminthes), now carrying a green lamp

Autores:

Marcos Trindade da Rosa: Revisão bibliográfica, auxílio na formação do texto, manutenção das culturas, anotar o tempo para o ciclo reprodutivo por zooide, contagem celular, extração do material genético, amplificação do fragmento CO1 por PCR, preparação da amostra de sequenciamento, gerar a sequência para análises, cortes e análise do padrão regenerativo, promover transformação genética por eletroporação e anotação dos dados.

Camila de Moura Pereira: Revisão bibliográfica, auxílio na formação do texto e contagem celular.

Geovani Tolfo Ragagnin: Revisão bibliográfica, auxílio na formação do texto e contagem celular.

Élgion Lucio da Silva Loreto: Revisão bibliográfica, auxílio na formação do texto, análises filogenéticas e dados gerados, autor correspondente.

Revisiting the regeneration of Stenostomum leucops (Catenulida, Platyhelminthes), now carrying a green lamp

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Keywords:	Regeneration, stem cells, planarians, GFP, electroporation

Revisiting the regeneration of *Stenostomum leucops* (Catenulida, Platyhelminthes), now carrying a green lamp

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Abbreviated title = *Stenostomum* GFP regeneration patterns

Number of text figures = four (4)

Abreviatures:

GFP – Green Fluorescent Protein
COI - *"cytochrome oxidase c subunit I*
nm- nanometer
Pax-6 - Paired box protein ^
V - Volts

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Abstract

Stenostomum are small flatworms that live in freshwater and normally reproduce by paratomy. They are basal in the platyhelminthes' phylogeny. For more than a century species of this genus, in special *S. leucops*, have been used in regeneration studies. However, some contradictory results remain, as concerns what body part regenerate or the routes of regeneration. We repeated several of these studies, now transforming the worms with vectors carrying an eye-specific GFP marker. *Stenomum leucops* does not possess pigmented eye-spot or ocelli, but two putative photoreceptors structures. We did not find GFP expression in those structures but in the ciliated pits, suggesting these can be involved in light sensibility and function as a primitive eye. This GFP expression pattern allowed us to observe various regeneration routes related with the developmental stage of zooids. A remarkable result obtained was the facility and efficiency that *Stenostomum* can be transformed by electroporation. These worms are easily cultured, have a small number of cells and are transparent, allowing a direct visualization of its organs. These characteristics point this species as a good candidate for animal model in comparative studies with planarians or in expression studies aiming to test genes and promoters isolated from other worms.

Key words

Regeneration, stem cells, planarians, GFP, electroporation.

Introduction

The order Catenulida Meixner, 1924, is represented by small flatworms, generally ranging from 0.5 to 2 mm long. Approximately 100 species have been described across the world, the majority of which live in freshwater. The sexually mature stage is rarely found, since they normally reproduce by paratomy, an asexual reproduction form in which a fission perpendicular to the antero-

posterior axis occurs, being preceded by the development of structures typical of anterior region, in the half of body, forming zooids (see Suppl. Movie 1). In some species, chains with until nine zooids can occur (Hyman, '51). One of the most studied Catenulida is *Stenostumum leucops* (Dugès, 1828), a cosmopolitan species exhibiting some characteristics that make it an excellent experimental organism. For example, it can be easily maintained in culture in which they reproduce quickly, regenerate extensively and have internal organs that can be readily observed (Nuttycombe and Waters, '38).

For more than a century the *Stenostumum* genus and, in special *S. leucops,* have been used in studies about regeneration. However, these studies have been marked by contradictory results. Ritter and Congdon (1900) described that if the cephalic structures in formation in the half of a worm when there are two zooids were located in the posterior position in a sectioned worm, they will migrate to an anterior position. Child ('03) did not found migration of these structures. Instead, he reported a reduction and a resorption of the "ganglionic masses" in the posterior position of a regenerating worm, depending on the developmental stage of these structures. The resorption is complete in a young cephalic structure. However, according to this author, the resorption does not occur when the structures are well developed. Other contradictory result concerns the question if the anterior and posterior halves of a zooid can be able to reconstitute completely. Hartmann ('22) and Ruhl ('27) described that both parts regenerate, while Ritter and Congdon (1900) and Child ('02) reported that only the anterior part reconstitutes. Van Cleave ('29) in a comparative study about regeneration in *S. grande, S. tenuicauda* and *S. leucops,* found that the latter species differs from the former two, because its regeneration is less common, slower and the resorption of the cephalic structures does not occur. However, Van Cleave ('29) and Nuttycombe and Waters ('38) have pointed that at least part of these contradictory results, could be associated to problems of species identification.

The validity of *S. leucops* as a species has been questioned by Nuttycombe and Waters ('38) and Marcus (45). For the first authors "… it seems likely to us that the descriptions of *S. leucops* are

so unreliable as to invalidate completely that species in any fauna; we rather strongly suspect that is a synonym for *Stenostomum*". Indeed, it seems that in the early studies, many different species were used as *S. leucops*. However, further studies done by Borkott ('70) and Noreña et al. (2005) have validated this species. More recently, Larsson et al. (2008) and Yamazaki et al. (2012) used DNA sequences of 18 S rDNA and COI to show that *S. leucops* consists in a monophyletic group. Nevertheless, the genetic divergences observed in these sequences suggest it would correspond to a species complex. To solve the problem if *S. leucops* is one species or a species complex it is necessary to characterize populations worldwide. DNA barcoding can be useful for this task.

Planarians have made up a powerful model for studies of regeneration, stem cells and aging due to their regenerative properties, combined with the development of new molecular technologies and sequencing. *Schmidtea mediterranea, Dugesia (Girardia) japonica* and *D. (G) tigrina* are the species most commonly used in planarians' researches (Oviedo et al., 2008, Sanches-Alvarado, 2012). Due to the basal position of Catenulida in the Platyhelminthes' phylogeny (Rohde et al., '93; Campos et al., '98), it can be a powerful tool for comparative studies with planarians. *S. leucops,* which is a species very easily maintained, could be a candidate for this model organism.

González-Estévez et al. (2003) produced transgenic planarians using electroporation of transposons vectors carrying an eye-specific GFP marker. We hypothesized that a similar approach could be applied to *S. leucops*, which also posses photoreceptors in the anterior end (Palmberg and Reuter, '92). The GFP marker can be a guide to a more detailed analysis of *S. leucops* regeneration, trying to solve the contradictory results reported in the classical studies. Moreover, the molecular characterization of additional *S. leucops* lineages can help to elucidate if it encompasses a single species or a real species complex. In this study we have produced a genetically transformed 3xP3-EGFP line and characterized their regenerating pattern. We show that the transformation of *Stenostomum* is easy, efficient and quick. Others aspects also studied are the time of zooids development, the number of cell in each zooid and the taxonomic nature of this taxon, in order to

collaborate to transform this species in a model for comparative studies with the planarians and even other worms.

Material and Methods

Animals

The worms were collected in a dam at Federal University of Santa Maria, Santa Maria, Brazil (53º17' W; 29º28' S) in 2009, March. These specimens were identified following Noreña et al. (2005). Since then, a stock has been maintained in 25 ml culture flask, with 10 ml of reconstituted water (Knakievicz et al., 2006) at 28ºC. Each 3 days, half of the water volume is changed and about 2 mg of powder milk is added to the medium.

To access the time spent in the asexual reproduction by paratomy, the worms were put in a drop of water in Kline concavity slides, with one worm by concavity. The slides were maintained in a wet chamber and the water was replenished every day. The worms were observed in a stereo microscope each 24 hours, during a week. After fission, each "new" worm was transferred to a new well, and time of process was registered.

Estimating the number of cells

Soon after paratomy, worms were put in a slide with a drop of distillate water and 2.5 µl of ethidium bromide (0.5 mg/ml), covered with a coverslip and observed in an Olympus BX41 fluorescence microscope. Pictures were take using absorption filter 518 nm and emission filter 605 nm. A second estimation was performed using worms with two zooids, apparently mature to perform paratomy. The nucleus were counted directly in a computer screen, marking those that had already been counted.

A second procedure was done using acetic orcein (2%) stain. The worms were photographed and the number of cells was estimated as previously described.

Genetic transformation

For the genetic transformation essays, plasmids pBac [3xp3-EGFPafm] (Horn et al., 2000) containing the inverted terminal repeats (ITRs) of the *piggyBac* transposon and the GFP gene marker under the control of an artificial promoter containing three Pax-6 homo-dimer binding sites were used. This promoter drives strong expression of the GFP protein in the eye-tissues (Gehring, 2002). The *piggyBac* transposase helper-plasmid used was the pBΔSac which has its 5' terminus deleted (Handler and Harrell, '99).

The worms were electroporated by a single pulse of 80V for 1 msec using a Gene Pulser Xcell Electroporation System (BIO RAD) apparatus. Fifty worms were put in a 2mm cuvette with 200 µl of reconstituted water, 35 µl of PBS and 1.5 µg of each plasmid. The experiment was replicated four times. After electroporation, the worms were rinsed in reconstituted water and maintained individually in wells of plastic cell culture plate with reconstituted water (3 ml for well), and fed with milk powder. As control, worms were exposed to the same solutions and plasmids but did not receive the electric pulse.

Regeneration assay

The worms were cut with glass needles, made of capillary tubes by hand-pulling these capillaries over the flame of an alcohol lamp. The worms were selected in two zooid phase under visualization in an inverted microscope and sectioned in three parts. The first cut was done in the half of the pharyngeal glands region, and the second was positioned after the developing fission plane and the ciliated pits that develop in the half of the body (Fig 3, 4). The three parts were

maintained in a drop of water in the slide, in a wet chamber, and were observed in an inverted microscope each three hours.

DNA barcoding

Genomic DNA was isolated from roughly 100 worms following the method described by Oliveira et al. (2009). The primers and PCR conditions used to amplify Cytochrome c Oxidase I (COI) were described by Telford et al. (2000). A fragment of 497 bp was sequenced using a MegaBace 500 automatic sequencer. The dideoxy chain-termination reaction was performed using the DYEnamicET kit (GE Healthcare). The obtained sequence was deposited in GenBank (Accession numbers: KJ476143).

For phylogenetic analysis other COI sequences of *Stenostomum* were obtained in genBank (Accession numbers: AB665116 until AB665124; FJ384873 until FJ38910; AJ405975; AJ405976). Thesenucleotide sequences were aligned using Clustal W (Thompson et al., '94) according to the program default parameters. The phylogenetic analyzes were implemented in Mega 5.0 software (Tamura et al., 2011), by maximum likelihood and maximum parsimony, with 1,000 bootstrap replications.

Results

Life cycle and biological characterization

During the four years in which we have maintained this culture of *S. leucops*, none sexually mature form was observed. In our laboratory conditions, paratomy occurs in an average time of 42.5 hours. However, 45% of the evaluated individuals (n = 98) reproduced in 24 hours and only 4% spent 96 hours to proceed the fission (Suppl. Fig. 1). In this culture only two zooids were observed. The average size of worms with two zooids, preceding paratomy, was 0.98 +/- 0.09 mm, and after fission, 0.49 +/- 0.07 mm. The estimation of cell number soon after paratomy is 2,400 +/- 300 cells. The number of cells in worms preceding the fission is around 5,000.

DNA Barcoding

The phylogenetic analysis conducted through Maximum Likelihood, using the available *Stenostomum* COI sequences , showed that the specimens morphologically identified as *S. leucops* collected in Sweden, London and south of Brazil are paraphyletic in regard to a sequence of *S. grande* from Japan. A similar tree was obtained by Maximum Parsimony (Suppl. Fig. 2). Estimates of evolutionary divergence, using P distance showed very small values among the sequences of *S. leucops* from Sweden (zero to 0.002), although larger distances were observed between these sequences and those collected in London and south of Brazil (around 0.14), and even between the samples from Brazil and London (0.127) (Suppl. Table 1).

Genetic transformation

Genetic transformation of *Stenostomum* by electroporation is an easy and efficient method. Although only 54+/-8% of worms survived the electric pulse and 30+/-7% were be maintained alive 24 hours after electroporation, it is not a significant loss because hundreds of worms can be simultaneously electroporated. Only 24 hours after electroporation, 7 +/-5% of the worms began to fluoresce. The number of worms expressing fluorescence increased in the next four days, when 64+/-2% proved to be genetically transformed (Suppl. Fig. 3). Those worms failing to fluoresce in the first five days, will no more express GFP. The control group, which had not been electroporated, also did not show fluorescence. For the transformed lines, 25% showed a transient expression, losing the GFP expression after few weeks. However, 75% showed stable expression patterns. Our first transformed worms are now with more than six months, andhave maintained the same GFP expression.

The pattern of GFP expression was similar in all lines, although the intensity of expression was variable. GFP expression could be seen spread in the whole body of the worm, in cells below the ciliated epidermis. A more intense expression was observed in the ciliated pits (Fig. 2, Suppl.

Movie 2). The same fluorescence could be observed in the ciliated pits of the zooid, although its fluorescence is less intense until paratomy occurs.

Regeneration

After a mechanical sectioning, diverse routes of regeneration could be observed in worms of this culture. The specific route appears to depend on the planes of fission and the developmental stage of the zooids (Fig. 3 and 4). No regeneration was observed when the section excluded the intestinal portion. However, if some intestinal portion was present, regeneration was always obtained (Fig 3-I, A; II, F, G; Fig 4 C, C1 and C2). A similar situation was observed in both, head and tail regions. No regeneration was observed when the section excluded the intestinal portion but a complete regeneration occurred when some intestinal tissues were present (Fig 3- I, F; II, G, H; Fig 4 A, A1 and A2). In these cases, in 6 to 8 hours after fission, the fluorescent ciliated pits were formed, but the complete regeneration spent 12 to 30 hours. However, when cephalic or tail regions failed to regenerate, the degeneration occurred in 6 to 12 hours.

A more complex regeneration pattern was observed for the median body portion, when the zooid's “cephalic” structures were maintained in the posterior portion of fissioned body (Fig 3-I, B, C, D and E; Fig 4 B, B1 to B5). A possible outcome was the moving of the zooid's ciliated pits and cephalic structures in development to the anterior region. This process occurred in around 10% of the observed cases and was associated with photosensitive structures showing little fluorescence, indicating that the zooid was not well developed when the mechanical fission was done (Fig 3 IB; Fig 4, B4). The most frequent outcome was the degeneration of posterior ciliated pits and cephalic structures with the formation of new ones in the anterior region. This process occurred in around 60% of the cases and was associated with zooids in advanced stages of development, showing a more intense fluorescence in the “zooid ciliated pits” (Fig 3 IC; Fig 4 B3). In 27% of the observed regenerations, the zooid maintained the ciliated pits and cephalic structures in the posterior region,

regenerating the tail. At same times, a new "head" was formed in the anterior region, forming two zooids that will proceed the paratomy. We were not able to find a phenotypic mark, even in fluorescence, for these cases (Fig 3 ID, Fig 4 B2). Three percent of worms did not perform regeneration. In these cases, the ciliated pits and cephalic structures of zooid were well formed, with intense fluorescence. The worms swam for few days without showing signs of regeneration, and eventually died (Fig 3 IE, Fig 4 B5).

Discussion

Stenostomum are very small, fragile and simple worms. The presence of few diagnostic characters and the difficulties to conserve specimens result in identification issues. The contradictory results described in the literature about the regeneration of *Stenostomum leucops* is, at least partially, due to problems in species identification, as suggested by Van Cleave ('29) and Nuttycombe and Waters ('38). For example, Van Cleave ('29) and Child (1900) described that the specimes they have used had until five zooids, which is in discordance with the described characteristics for this species, which has only two zooids. DNA barcoding can help to overcome these problems, giving support to correct species identification and allowing comparisons between researches conducted in different laboratories, with different cultures. The phylogenetic analysis implemented by us with the available *Stenostomum* COI sequences showed that *S. leucops* individuals from Europe and Brazil were grouped in a unique clade. However, these sequences were paraphyletic in regard to *S. grande* from Japan. Considering the genetic divergences observed among these sequences, our analysis supports *S. leucops* as a species complex, as previously pointed by Larsson et al. (2008).

Genetic transformation of *Caenorhabditis elegans*, the main worm model organism, as of other nematodes, involves biolistics, microinjection or transfection by calcium-mediated permeabilization (Lok, 2012). These procedures are, generally, laborious and time-consuming. For

platyhelminthes, González-Estévez et al. (2003) had shown that plasmid injection, followed by electroporation, can produce genetically transformed planarians. However, for this organism, the GFP expression begins two months after transformation. Our data showed that genetic transformation by electroporation of *Stenostomum* is so simples as the use of this methodology for transforming bacteria. Also, the results are fast. Twenty four hours after electroporation the worms begin to express GFP. The fact that some of our culture lost the GFP expression after some weeks, while others showed a stable expression, implies that further studies need to be done to clarify the nature of this transformation.

The GFP gene we have used in our study is under control of the Pax6 dimeric binding sites, in a construct developed by Berghammer et al. ('99). This marker is expressed specifically in eyes tissues of various arthropod taxa. In transformed planarians, GFP expression is detected in the eyes (González-Estévez et al., 2003). *Stenostomum leucops* does not posses pigmented eye-spot or ocelli, the typical photoreceptors for some worms. However, *S. leucops* is photosensitive, and Palmberg and Reuter ('92) described two types of presumptive photoreceptors: a pair of ciliary lamellate bodies, found between the ciliated pits and the anterior brain lobes, measuring 7 by 8 μm; and the light-refracting bodies that are in connection to the posterior brain lobes. These structures did not show fluorescence in our transformed worms. On the other hand, we observed a strong GFP expression in the ciliated pits. This suggests these structures could have photosensitive cells, functioning as a primitive eye or, alternatively, could have been the sensorial structure involved in the eye origin.

We have observed, in our culture, all patterns of regenerations described by Ritter and Congdon (1900), Child ('02, '03), Hartmann ('22), Ruhl ('27) and Van Cleave ('29). The route followed in the regeneration depends on the part of the organism and the stage of development of the zooids. To the anterior and posterior ends, it is fundamental for the sectioned part to contain a small portion of the endodermal digestive sac in order to the regeneration occur. This fact has been previously pointed by Child (1900). Van Cleave ('29) suggested that the stage of zooid

development is important for regeneration. The GFP marker, expressing in the ciliated pits, allowed us to show that in early stages cephalic structures can migrate from posterior to anterior region. In latter stages of regeneration, when the ciliated pits express GFP more intensely, resorption of posterior cephalic structures and development of new ones in the anterior region normally occur.

Planarians are the principal animal model in researches concerning the molecular process that guide the regenerative phenomena. At this time, understanding these processes has gained special importance to support stem cell researches, which are promises of valuable therapies to biomedical sciences. Many genes have been found in transcriptome researches associated with various regenerative processes in planarians (Kao et al., 2013, Sandmann et al., 2011, Onal et al., 2012, Resch et al., 2012). As we have shown in this paper, it is very fast and easy to obtain genetically transformed *S. leucops*. Additionally, this species is easy to cultivate, anatomically simple and has few number of cells. For this reason, we suggest this organism can be useful to assay the genes or regulatory regions of genes involved in regeneration in planarians. Even genes or promoters related to other biological processes and belonging to other platyhelminthes such as *Taenia*, *Echinococcus*, *Schistosoma* and other, could, perhaps, be easily tested in *Stenostomum*.

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Figure legends

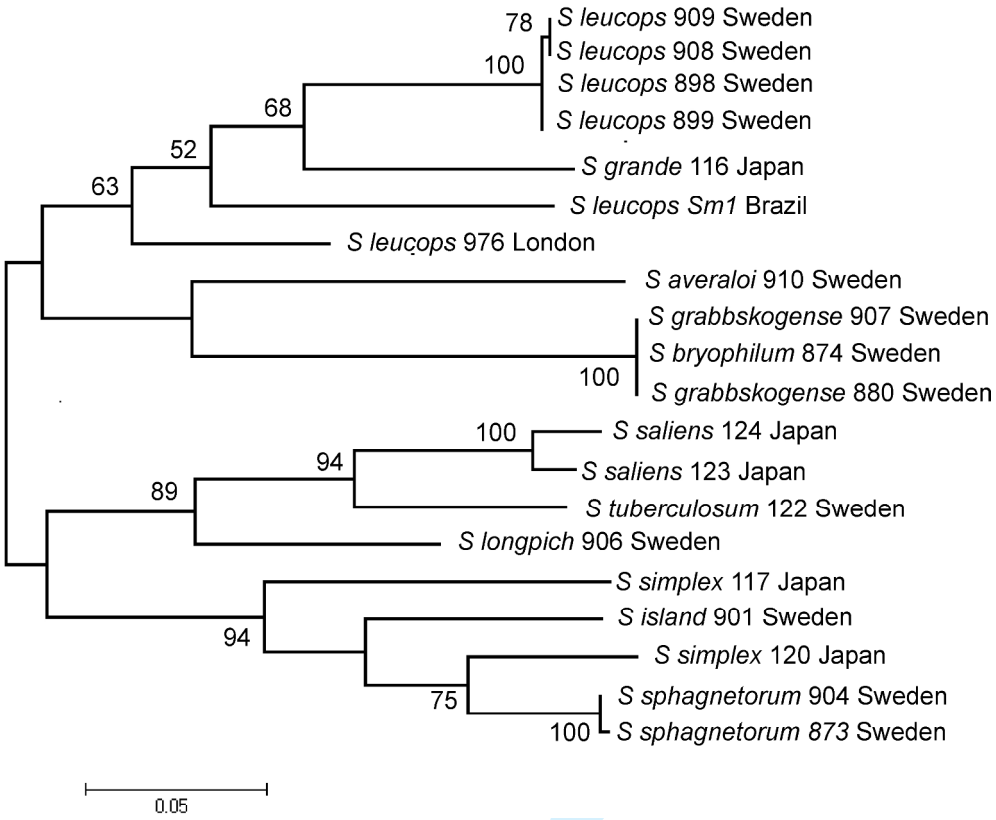
Fig 1. Molecular phylogenetic analysis by Maximum Likelihood method, for Cytochrome c Oxidase (COI) gene, using the T92+G+I nucleotide substitution model. The tree with the highest log likelihood (from 3191.2379) is shown. Bootstrap values are showed in the branches.

Fig 2 – I- *Stenostomum leucops* showing two zooids; II- *S. leucops* expressing GFP florescence. A- first zooid, B- second zooid, 1- ciliated pits, 2-cerebral ganglia, 3- pharyngeal glands, 4- opening the pharynx, 5- digestive tube, 6- zooid ciliated pits in development

Figure 3- For regeneration assay, the worms were cut in three parts (I). The anterior part (A) never regenerates. The posterior part always regenerates (F). For the median part, four different ways can be followed: (B) the head structures that are developing in the fission plane can move to the anterior part and occurs the regeneration; (C) those structures can degenerate and the new ones arising in the anterior region; (D) news structures arise in anterior part, a posterior part is also regenerated forming a worm with two zooids; (E) after some days without differentiation, the structures degenerate. (II) When in the anterior part remains part of intestine (F), its part regenerate (G); if the posterior part do not receive part of intestine, its not able to regenerate (H)

Figure 4 - Florescent ciliated pits in the regeneration process of *Stenostomum leucops*. Worms of two zooid phases are fissioned in three parts A-B-C. The florescent ciliated pits are indicated by circles. The posterior region (A) soon after fission (A1) and completely regenerated (A2), 12 to 30

hours after fission. The intermediary region (B) soon after fission (B1) can developed news structures in anterior part and in the posterior region forming a worm with two zooids (B2); new head is formed in the anterior region and occurs resorption of the posterior head structures (B3); occurs migration of ciliated pits for anterior region (B4); new ciliated pits are formed in the anterior region, but the regeneration to not complete and the worms die (B5).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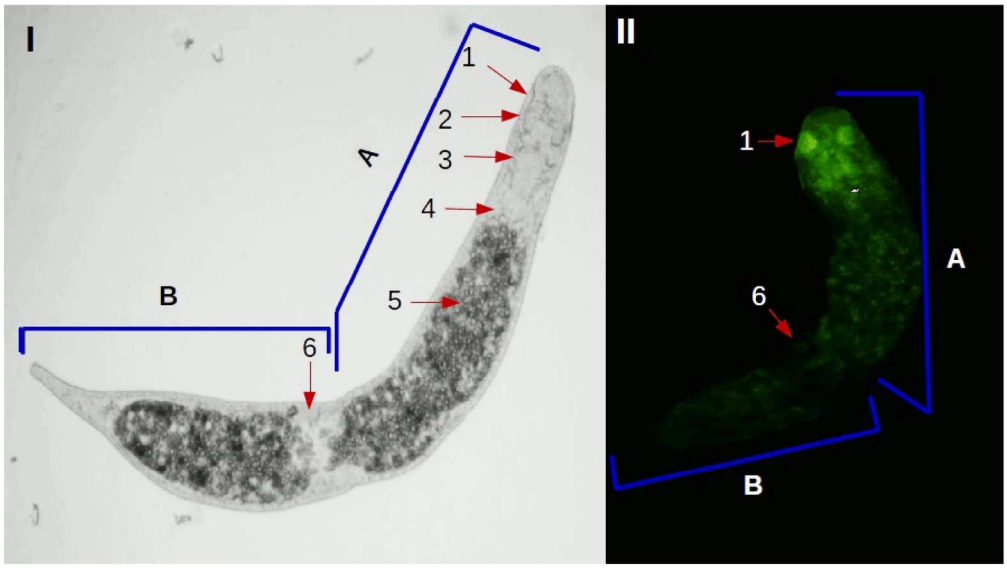

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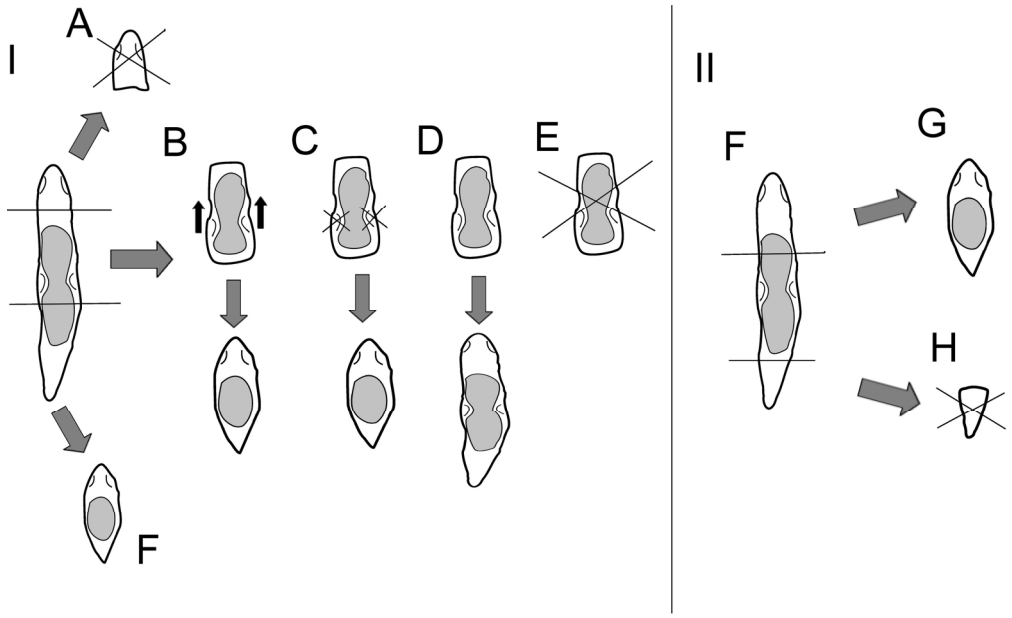

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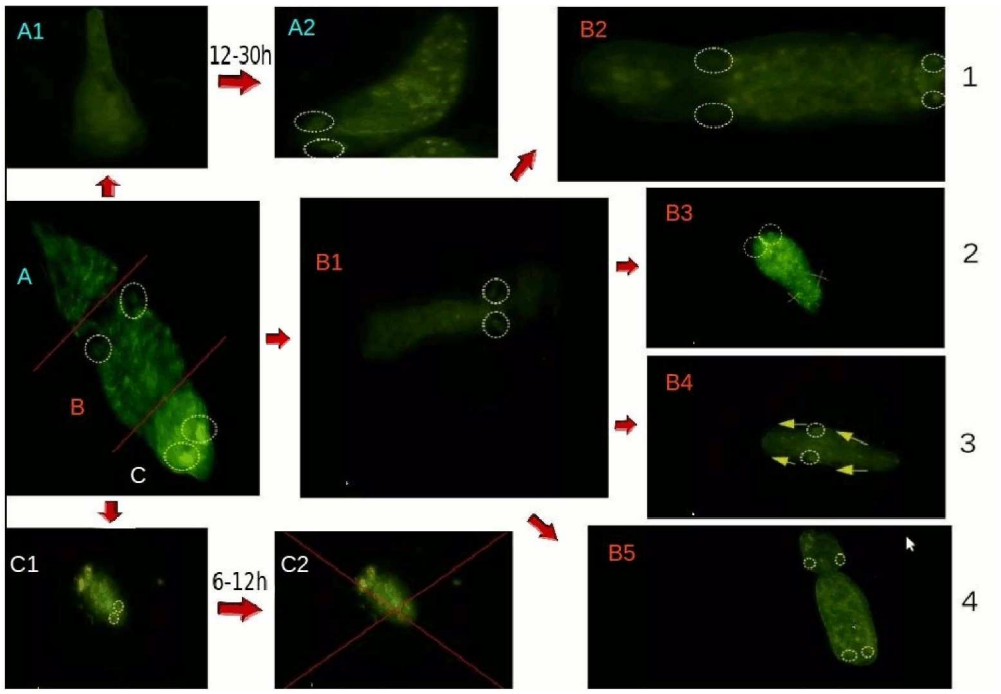

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Parecer:

Decision Letter (JEZ-B-2014-02-0018)

From:gunter.wagner@yale.eduTo:elgion.loreto@pq.cnpq.br, marcostrindadebio@gmail.comCC:andreas.wanninger@univie.ac.atSubject:JEZ Part B: Molecular and Developmental Evolution – JEZ-B-2014-02-0018 Body:Dear Dr. Loreto:

Thank you for submitting your manuscript entitled: Revisiting the regeneration of Stenostomum leucops (Catenulida, Platyhelminthes), now carrying a green lamp to the JEZ Part B: Molecular and Developmental Evolution.

Below you will see the comments of the reviewers. I share the substantial concern and reservation, and am unable to accept the manuscript in its present form. However, if you resubmit the manuscript after making the suggested changes, I will reconsider it for publication. If you decide to re-submit a revised paper it will be assigned a new number, and will be considered a new submission. Please reference the previous submission in your cover letter with the reworked manuscript.

Kindly submit your revision(s) in an acceptable file format. The main document should be in Microsoft Word ".doc" file. The table(s) should also be in Microsoft Word ".doc" and submitted in individual file(s). All figures should be in "tiff "or "eps" format and submitted in individual file(s). PDF format is un-usable by the Production Department because the file cannot be manipulated. These files are required if the manuscript is accepted for publication.

We want to thank you for sending your manuscript to us for consideration. We do hope to see your work again, and look forward to perhaps publishing it at some point in the future. Sincerely, Prof. Gunter Wagner Editor-in-Chief JEZ Part B: Molecular and Developmental Evolution Comments to Author: Associate Editor: Wanninger, Andreas

Reviewer: 1 Comments to the Author The paper by Rosa et al. deals with the characterization of the regeneration process in the platyhelminth genus Stenostomum. It has the potential of being a novel and interesting addition to the literature on flatworm regeneration. However, there are several problems with the paper, both formal and experimental, which I detail below.

1- The writing is, in several places, somewhat strange (the use of English verb tenses etc. is not accurate). I think the authors need to have the paper thoroughly revised by a native English speaker. This is essential for them to get their message across. (AJE)

2- In some cases, the concepts are used without a previous introduction. For instance, in the Abstract, the authors mention "...routes related with the developmental stage of the zooids.", but zooids have not been mentioned before, so we have to assume that everybody knows about the development of zooids.

3- In the experimental procedures some things are unclear. For instance, the authors suggest they have sequenced DNA from 100 worms, I assume that these are the worms kept in the lab (the ones that are transformed). Is there any indication of polymorphism in this sample? Is the population they use a complex mix?

4- The DNA barcoding is interesting but not necessarily informative with respect to the regeneration studies. It just tells us that the Brazilian population seems different from the others; and perhaps regenerate differently.

5- When it comes to the transformation, they use a well-characterized construct (used by other authors before). While we see some good staining, we are not told of the data from the controls. Is there any autofluorescence in these animals? The pictures in Figure 4 are too small and difficult to see.

6- The authors mention that they observe stable expression of the GFP for more than six months. Have they observed whether the signal is passed on to regenerating daughters? Have they performed any test of integration? Do the authors know if the construct has been integrated into the genome?

7- A cryptic sentence on page 8 states that the "GFP expression could be seen spread in the whole body of the organism, in cells below the ciliated epidermis". Does this mean that there is a general background problem?

8- The most interesting part of the paper (in the opinion of this referee) is that devoted to the regeneration mechanisms. This, in itself, needs a thorough discussion; but the authors just give a description in writing plus one diagram of the putative outcomes of the regeneration process. There are no figures showing what is happening (besides, as mentioned, the diagram in Fig. 3 and a few, unclear, panels in Fig. 4). I think the process is interesting enough to merit a better

anatomical/histological description.

9- We see that the patterns of regeneration are very varied, depending on the developmental stage and site of excision. At the same time we know that these regeneration patterns have been described by many authors with clearly contradictory results. One possibility is that the differences come from the use of different populations (maybe different species). How do the authors know that their culture is homogeneous? (This goes back to my point number 3 above.)

Overall, this paper hints at some important issues, both technical and biological, that are relevant to the regeneration biology of platyhelminthes. However, none of these issues is explored very extensively, and the reader is left with a mixture of results that would naturally develop into three different papers: one on the taxonomy of the group; another on the generation of catenulida transgenic lines; and a third on the microanatomy of the regeneration process. None of these topics is explored (or reported) with sufficient detail. I am therefore afraid that the authors should develop the study further before publication.

Reviewer: 2

Comments to the Author **The presented manuscript deals with several topics: the Stenostomum leucops species complex, the genetic transformation of Stenostomum "leucops brasiliense" with an eye-specific GFP marker, and the regeneration capacity of Stenostomum "leucops brasiliense". These are three independent topics, and it is not clear why the topics are molded together into one manuscript. I suggest to publish the greatly improved results in separate papers. With a partial sequence of the 18S ribosomal (!!!!) subunit, a tree was calculated including several published Stenostomum sequences.** The authors show that Stenostomum leucops is paraphyletic. Consequently, the name should not be used anymore, but it is still used in the manuscript and it is even proposed to make this apparently paraphyletic taxon a model species. While it is worthwhile to resolve the Stenostomum leucops species complex, this single new sequence will not provide an answer to this question. The transformation of adult

animals by electroporation with a vector containing an eye-specific GFP marker is a method that was used in several other animals and also another flatworm, Girardia tigrina. It is interesting that it works with great success rate and very fast in Stenostomum. To make this technique generally useful, I encourage to try to couple the GFP marker with other genes of interest, and study the resulting transformed animals. If this is challenging, then a greater focus could be laid on finding out why the eye-specific GFP marker is expressed in the ciliary pits, as no traces of structures hinting at eyes could be found there so far. The transformed animals are not needed or useful in the following part, the regeneration study. Proper bright field images would have provided enough information to follow their experiments. As it is, especially Figure 4? is mostly unhelpful in understanding the results. As pointed out by the authors of the manuscript and several authors before, different results concerning the migration or elimination of head parts in the zooids after artificial cuts are likely due to having used different species, and provide no particularly useful new insights. The English is quite poor with many small mistakes, and some long sentences that are very hard to parse. A native speaker or a professional editor is required to revise the manuscript. In the following part are some more detailed points: The title is vague (“revisiting regeneration”) and too fancy (“carrying a green lamp”). I suggest to use a title that conveys the important scientific findings at a glance. The authors write that the species they deal with “has organs that can be readily observed”. If this is correct, what need is there to use GFP-transformed animals for regeneration studies? Some minor historical disputes about the regeneration processes in Stenostomum leucops and several related species of the same genus are highlighted, and it is pointed out that some authors attributed different results to different species that were used by different authors, as Stenostomum leucops is a species described from many parts of the world and may very well be a cryptic species complex and several authors have labeled Stenostomum leucops a nomen nudum. Different to what Rosa et al. claim on page 4, line 6-8, Borkott (1970) did not validate Stenostomum leucops as a species, but, quite to the contrary, called it “impossible to identify” and named three species in its place (Borkott 1970, page 185, and many more). Attempts have been made to reintroduce the species name Stenostomum leucops (Norena et al. 2005), although

the cosmopolitan distribution of this species renders its validity rather dubious. The phylogenetic tree of Rosa et al. using 18S fragments is missing many published Stenostomum sequences already used in Larsson et al. 2008. Stenostomum “longpit” is mislabeled as Stenostomum “longpitch” in the Rosa et al. tree. Interestingly, the Rosa et al. tree shows Stenostomum leucops as a non-monophyletic group. Page 4, line 25: correct the species names to Dugesia japonica Ichikawa & Kawakatsu, 1964 and Girardia tigrina (Girard, 1850). Citing a rather old systematic study from 1993 is questionable, as there are more recent studies available on flatworm phylogeny. In particular, citing Rohde et al. 1993 is rather strange, as these authors state in the abstract: “the exact position of the Catenulida and Nemertini in relation to the Platyhelminthes has not been resolved”. The other study cited to show that Catenulida occupy a basal position within the Platyhelminthes is Campos et al. 1998, who have a peculiar and rather unlikely sister group relationship between Catenulida and Fecampiida in their tree based on 18S. Page 5, estimating the number of cells: The method described seems flawed, as the worms are not formed of a monolayer where all labeled nuclei are readily observable, but three-dimensional beings. I strongly suggest to repeat the cell counting with macerated animals or counting from confocal stacks. However, it is not even clear why this experiment has been performed at all for the presented study. The figures are not numbered in the submitted file, but provided they are shown in chronological order, they are still weirdly labeled with Roman numbers in addition to N-X. Figure 4? is very hard to understand, as the orientation of animals is arbitrary in different panels and the size and quality of the pictures are rather poor. Decent bright field images would have shown much better what's going on than using transformed animals that show glowing ciliary pits. Supplementary tables and figures are not available for download and could therefore not be checked.Date Sent:11-Apr-2014

Capítulo 2:

Artigo I (publicado):

Stenostomum leucops Dugés, 1828 (Platyhelminthes, Catenulida):

A putative species complex with phenotypic plasticity

Autores:

Marcos Trindade da Rosa: Revisão bibliográfica, auxílio na formação do texto, manutenção das culturas, anotar o tempo para o ciclo reprodutivo por zooide, contagem celular, extração do material genético, amplificação do fragmento CO1 por PCR, preparação da amostra de sequenciamento e gerar a sequência para análises.

Camila de Moura Pereira: Revisão bibliográfica, auxílio na formação do texto e contagem celular.

Geovani Tolfo Ragagnin: Revisão bibliográfica, auxílio na formação do texto e contagem celular.

Élgion Lucio da Silva Loreto: Revisão bibliográfica, auxílio na formação do texto, análises filogenéticas e dados gerados, autor correspondente.

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Stenostomum leucops Dugès, 1828 (Platyhelminthes, Catenulida): a putative species complex with phenotypic plasticity

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Camila. M. Pereira[1]
Geovani. T. Ragagnin[2]
Elgion. L.S. Loreto[1,3,4]

ABSTRACT

Species of Stenostomum *are small flatworms that live in freshwater and normally reproduce asexually by paratomy. They are basal in the phylogeny of Platyhelminthes. For more than a century, species of this genus, especially* S. leucops, *have been used in regeneration and other biological studies. However, some basic aspects of their biology are poorly known. Here, we characterized a strain of* S. leucops *that has been maintained in the laboratory for five years and a recent strain of* S. grande. *The time required for complete formation of zooids of* S. leucops *by asexual reproduction is approximately 42.5 hours at 28°C. The number of cells in the zooids, soon after paratomy, is approximately 2,500. The number of zooids formed in the chain is a plastic characteristic and is dependent on the conditions for cultivation. In some cultivation conditions of* S. leucops, *only worms with two zooids are formed. However, in other conditions, worms with up to five zooids are observed. Phylogenetic analyses of a fragment of the Cytochrome C Oxidase I (COI) sequence showed* S. leucops *and* S. grande *species constitute a species complex, the lineages of which having high intraspecific divergences.*

Key-Words: *Stenostomum grande;* Paratomy; Zooids; Number of cells; Microturbellaria.

INTRODUCTION

The order Catenulida Meixner, 1924, is represented by small flatworms that generally range from 0.5 to 2 mm long. They have unpaired, dorsomedially located protonephridium, anterodorsal testes and male genital pore, and aciliary nonmobile sperm. Normally they are white in reflected light, and translucent in transmitted light. The intestine varies in color depending on their food content (Larsson, 2008). Approximately 100 species have been described worldwide, and the majority lives in freshwater. The

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sexually mature stage is rarely found because Catenulida normally reproduce by paratomy, *an* asexual form of reproduction in which structures typical of the anterior region develop followed by a fission perpendicular to the antero-posterior axis, which forms zooids (a movie of this process can be seen in http://w3.ufsm.br/labdros/permanente/paratomy.mp4). In some species, chains with up to nine zooids can occur (Hyman, 1951). One of the most studied Catenulida is *Stenostomum leucops* (Dugès, 1828), a cosmopolitan species exhibiting characteristics that make it an excellent experimental organism. For example, it can be easily maintained and reproduces quickly in culture, regenerates extensively and has internal organs that can be readily observed (Nuttycombe & Waters, 1938). For more than a century, *Stenostomum,* primarily *S. leucops,* have been used for several experimental biology studies, such as studies of fission and regeneration (Ritter & Congdon, 1900; Child, 1990, 1902; Hartmann, 1922; Ruhl, 1927; Van Cleave, 1929), stem cells (Palmberg, 1990), ultrastructure of sensory organs (Reuter *et al.,* 1993; Palmberg & Reuter, 1992; Ruppert & Schreiner 1980), ultrastructure of the digestive tract (Antoniazzi & Silveira, 1996), senescence (Martínez & Levinton, 1992), neuropeptides (Grahn *et al.,* 1995; Wikgren & Reuter, 1985) and ecology (Nandini & Sarma, 2013; Nandini *et al.,* 2011). However, some basic aspects of the biology of these worms, such as the time required for paratomy or the number of cells constituting the body, are not well known.

The validity of *S. leucops* as a species has been questioned by Nuttycombe & Waters (1938) and Marcus (1945). However, further studies by Noreña *et al.* (2005) have validated this species. More recently, Larsson *et al.* (2008) used DNA sequences of 18 S rDNA and COI to show that *S. leucops* constitutes a monophyletic group. Yamazaki *et al.* (2012) used the same molecular markers and also included species collected in Japan; in their analysis, *S. grande* was placed in a cluster with representatives of *S. leucops.* Furthermore, the genetic divergences observed among the sequences of *S. leucops* collected in different places correspond to that expected for species, suggesting that it is a species complex. In order to solve the problem of whether *S. leucops* is one species or a species complex, it is necessary to morphologically and biologically characterize populations worldwide, and DNA barcoding can be useful for this task.

The basis for DNA barcoding is that short nucleotide sequences can be used to distinguish species, because the genetic variation between species is normally higher than that observed within species. For animals, the sequence used the most is a 650-base fragment of the 5' end of the mitochondrial gene Cytochrome C Oxidase I (COI, cox1) (Hebert *et al.,* 2003). Although several shortcomings have been associated with DNA barcoding, the methodology is now well established and has been shown to be useful in various fields of biological research, including the identification of cryptic species or species complexes (Collins & Cruickshank, 2013; Albu *et al.,* 2010).

The aim of this study was to characterize some morphological and biological characteristics, such as the time required for paratomy and the number of cells in each zooid of a strain maintained in the laboratory for 5 years. A morphological plasticity was observed in the number of zooids formed, which depended on the growing conditions. We also performed a DNA barcoding and phylogenetic analysis. We found that *S. leucops* collected in Sweden, London and Brazil are paraphyletic with regard to *S. grande,* from Japan and Brazil, supporting the hypothesis that this taxon corresponds to a species complex.

MATERIALS AND METHODS

Animal sampling and cultures

The worms were collected in a pond at the Federal University of Santa Maria, Santa Maria, Brazil (53°17'W; 29°28'S). *Stenostomum leucops* was collected in March of 2009 and *S. grande* Child, 1902, in November of 2012. These specimens were identified following Noreña *et al.* (2005) and Damborenea *et al.* (2011). The diagnostic characteristics of *S. leucops* are the light-refracting bodies with disc shaped, pharyngeal glands rounded shape, without a sphincter between pharynx and intestine, and the pharyngeal glands are on the entire surface of the pharynx. They present an elongated posterior end, free of intestine. *Stenostomum grande* have as characteristics the light-refracting bodies disc shaped with more than ten small spheres, pharyngeal glands small rounded shaped and only in the anterior half of the pharynx. They present sphincter between pharynx and intestine, oral pore circular. Culture, for both species, was initiated from a single worm, and the stocks were maintained in a 25 ml culture flask with 10 ml of reconstituted water (Knakievicz *et al.,* 2006). Two different culture conditions were used: *i)* Condition 1 – the worms were grown in a chamber with controlled temperature at 28 ± 2°C and constant luminosity of 33 cd. Every 3 days, half of the water volume was changed, and approximately 2 mg of powdered milk was added to

the medium; *ii)* Condition 2 – the culture flasks were maintained at room temperature under indirect solar illumination. In these conditions, cyanobacteria belonging to the Chroococcales order become abundant in the medium and promote differential growth in the worms.

Vouchers of the used strains were deposited in UFSM Department of Biology collection under numbers SL01-sm01 and SL01-sm01.

Estimation of the time required for asexual reproduction of *S. leucops*

To assess the time required for asexual reproduction by paratomy, a total of 98 worms under Condition 1 were analyzed. They were put in a drop of water in Kline concavity slides with one worm per concavity. The slides were maintained in a wet chamber, and the water was replenished every day. The worms were observed through a stereo microscope every 12 hours. After fission, each "new" worm was transferred to a new well, and the time for the process was registered.

Estimating the number of cells of *S. leucops*

Worms soon after paratomy or showing the constriction between zooids, which characterize that the fission will occur shortly, were put on a slide with a drop of distilled water and 2.5 µl of ethidium bromide (0.5 mg/ml). For each preparation, one worm was put on the slide, covered with a coverslip and squashed, and they were observed through an Olympus BX41 fluorescence microscope. Pictures were taken using a 518 nm absorption filter and a 605 nm emission filter. Additionally, estimates were performed for worms with two, three and four zooids. The nuclei were counted directly from a computer screen, which marked the nuclei that had already been counted.

A second procedure was performed using an acetic orcein (2%) stain. The worms were photographed, and the number of cells was estimated as previously described.

DNA barcoding

Genomic DNA was isolated from approximately 100 worms of each strain following the method described by Oliveira *et al.* (2009). As previously highlighted, the strains were made from a single worm. Thus, as only asexual reproduction has been observed, the worms in each culture are clones, therefore, homogeneous mitochondrial sequences are expected. The primers and PCR conditions used to amplify Cytochrome C Oxidase I (COI) were described by Telford *et al.* (2000). A fragment of 497 bp was sequenced using a MegaBace 500 automatic sequencer. The dideoxy chain-termination reaction was performed using the DYEnamicET kit (GE Healthcare). The sequences obtained were deposited in GenBank (accession numbers: *S. leucops* KJ476143 and *S. grande* KM056359).

For the phylogenetic analyses, other *Stenostomum* COI sequences were obtained from GenBank (accession numbers: AB665116 to AB665124, FJ384873 to FJ38910, AJ405975, and AJ405976). These nucleotide sequences were aligned using Clustal W (Thompson *et al.,* 1994) according to the default program parameters. The phylogenetic analyses were implemented in Mega 5.0 software (Tamura *et al.,* 2011) by neighbor-joining and maximum likelihood with 1,000 bootstrap replications. The genetic distances among different taxa were estimated by P distance also using Mega 5.0 software.

RESULTS

Life cycle and biological characterization of *S. leucops*

The morphological characteristics of *S. leucops* here studied correspond to those described by Noreña *et al.* (2005) and Damborenea *et al.* (2011). The same occurs for *S. grande.*

During the five years that we maintained the culture of *S. leucops,* the sexually mature form was not observed. In cultures maintained under the Condition 1, paratomy occurred after an average time of 42.5 hours. However, 45% of the individuals evaluated (n = 98) reproduced in 24 hours, while 4% required 96 hours to proceed to fission (Fig. 1). During the time that the cultures were maintained in Condition 1, only worms with two zooids were observed. The average size of worms with two zooids preceding paratomy was 0.98 ± 0.09 mm and 0.49 ± 0.07 mm after fission (Fig. 2, I and II). In culture using the Condition 2, maintained under indirect solar illumination, some cyanobacteria grew and the worms developed three, four or five zooids (Fig. 2, III and IV). In this analysis, a total of 53 specimens were observed. Following this observation, we also characterized the development of this strain in a medium supplemented with cyanobacteria. The larger worms were those with

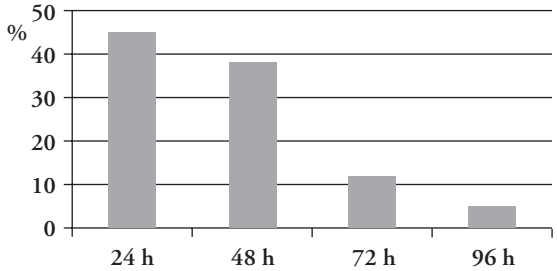

FIGURE 1: The frequency of fission in *Stenostomum leucops* under Conditions 1. The percentage of zooids formed is shown on the Y-axis. The X-axis shows the time (in hours) required for fission to occur.

five zooids and an average size of 1.03 ± 0.05 mm. After paratomy, the size of the worms with one zooid, which were formed from chains of multiple zooids, was 0.45 ± 0.03. An absence of perturbation in the culture flasks is a necessary condition for the growth of more than two zooids. When the culture is agitated, such as when collecting worms using a pipette, the development of multiple zooids is interrupted, and as a consequence the two zooid pattern of paratomy returns. For this reason, we were not able to determine the time necessary for paratomy with multiple zooids, once the transferring to a Kline slide promotes a change of the reproduction pattern of multiple zooids to two zooids.

The cell number estimate for worms with one zooid growing in Condition 1, soon after paratomy, is approximately 2,500. The number of cells in two zooid worms preceding the fission is approximately 5,000. As seen in Table 1, for worms growing in medium supplemented with cyanobacteria (Condition 2), the number of cells in animals with one, two, three and four zooids increases by 2,000 cells for each zooid formed. This suggests that the number of cells necessary to form one zooid is approximately 2,000.

DNA Barcoding

Using the available *Stenostomum* COI sequences, the phylogenetic analysis conducted through neighbor-joining showed that both *S. leucops* and *S. grande,* constitute a complex of species sincespecimens of *S. leucops,* from Sweden, grouped with a specimen of *S. grande,* from Japan, while specimens of *S. leucops,* from Brazil and London, grouped in a clade including a representative of *S. grande,* from Brazil (Fig. 3). A similar tree was obtained by maximum likelihood (data not shown). These analyses indicate that the two nominal species *S. leucops* and *S. grande* are polyphyletic. The estimates of evolutionary divergence using P distance were very small within the sequences of *S. leucops* from Sweden (zero to 0.004). However, larger distances were observed between these sequences and those collected in Sweden and the south of Brazil (approximately 0.14) as well as between the samples from Brazil and London (0.127) (Table 2). The distance found between the *S. grande* from Brazil and Japan is remarkable (0.154), but the distance observed between *S. grande* from Brazil and *S. leucops* from London is lower (0.110). Similar distances were observed between *S. grande* from Japan and the *S. leucops* samples from Sweden. The P distances observed among the twelve species here studied ranged from 0.076 to 0.213.

TABLE 1: Estimates of cell number in worms with 1, 2, 3 and 4 zooids growing in cultivation Procedure 1 and Procedure 2 (average ± standard deviation).

Number of Zooids	Procedure 1	Procedure 2
1	2.400 ± 280	2.158 ± 112
2	4.850 ± 325	3.964 ± 365
3	—	5.166 ± 91
4	—	7.003 ± 207

DISCUSSION

The easy cultivation, fast asexual reproduction, and regeneration are characteristics that make *Stenostomum,* particularly *S. leucops,* a potential model organism for studies of regeneration, stem cells, aging and other biological topics. However, there are very few records in the literature that describe the time necessary for the worms to proceed the natural fission. Hyman (1951) described the time necessary for *Stenostomum* to undergo paratomy as around two days. Reuter & Kuusisto (1992) noted that the time required for a new zooid development in both *S. leucops* and *Microstomum lineare* Müller OF, 1773 varied from 20 to 45 h owing to environmental factors. These studies do not describe the conditions under which the time required for paratomy was determined. Our results showed that the worms required 42.5 hours to carry out the fission at 28°C.

No records were found in the literature for the number of cells that make up *Stenostomum.* For Catenulida, Simanow *et al.* (2012) reported that *Macrostomum lignano* consists of approximately 25,000 cells. This worm is approximately 1.5 mm long, which is about three times larger than the worms used in our study. The number of cells found in *S. leucops* soon after paratomy is approximately 2.500 and, if an adjustment to the length of the body is applied between

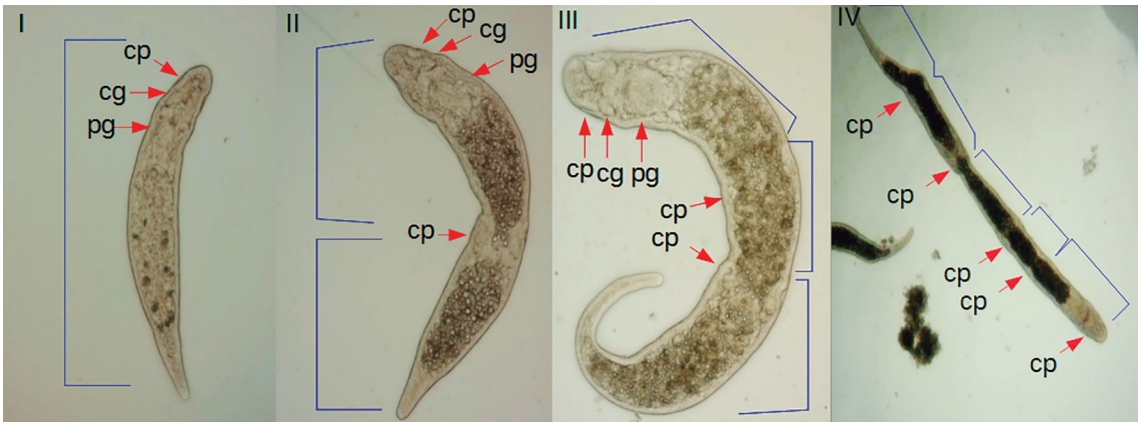

FIGURE 2: General view of *Stenostomum leucops* showing one zooid **(I)**, two zooids **(II)**, three zooids **(III)** and five zooids **(IV)**. cp = ciliated pits; cg = cerebral ganglia; pg = pharyngeal glands.

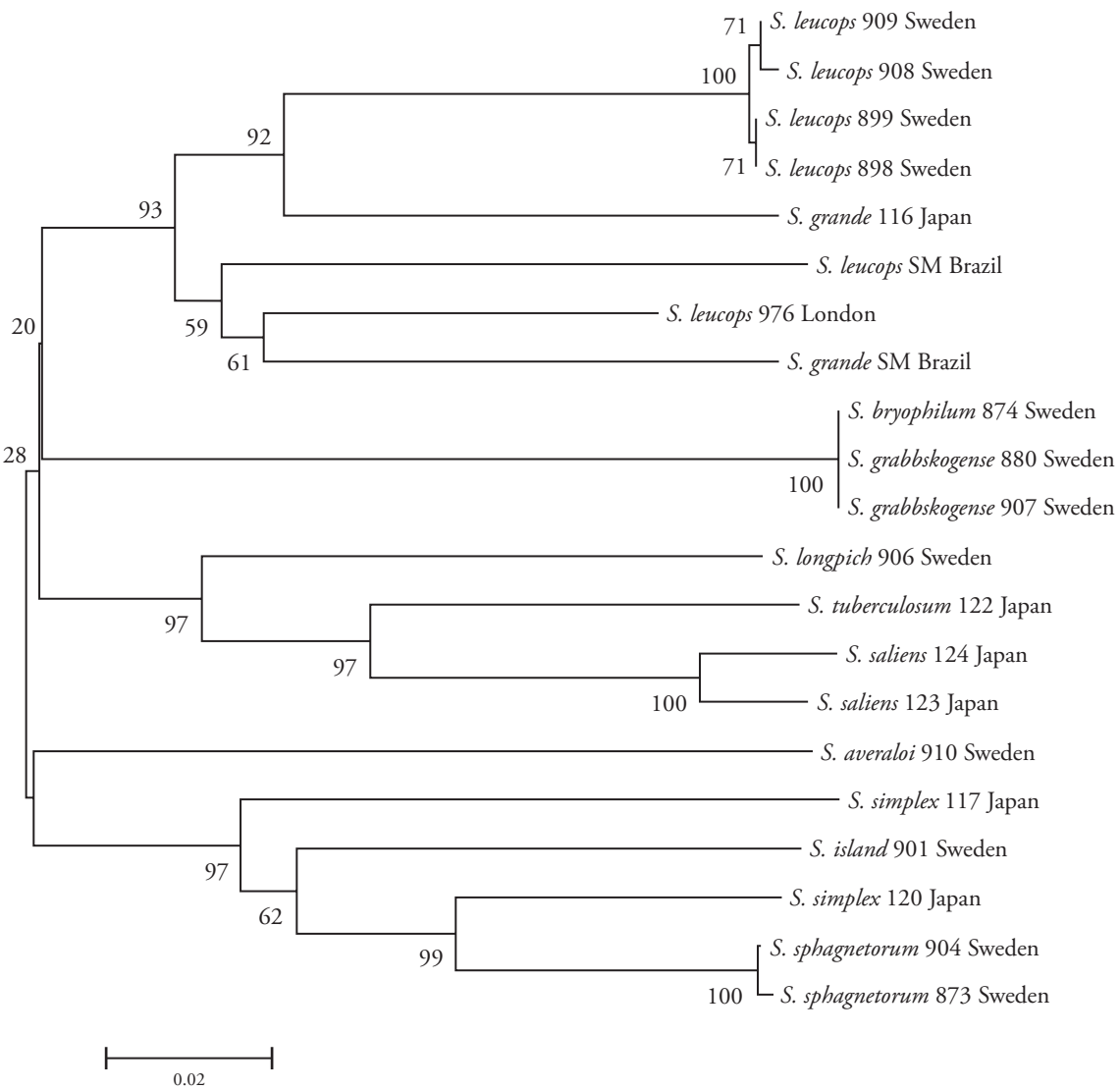

FIGURE 3: Molecular phylogenetic analysis by the neighbor-joining method for Cytochrome C Oxidase (COI) gene using P distance. Bootstrap values for 1000 replications are shown in the branches. Gap/missing data treatment: complete deletion. Sites analyzed: 474.

TABLE 2: Estimates of Evolutionary divergence between sequences, using P distance, conducted in MEGA 5.0. The number of base differences per site between sequences are shown. The analysis involved 20 nucleotidesequences. All positions containing gaps and missing data were eliminated. There were a total of 473 positions in thefinal dataset. The number after the species name correspond the last three number, of GenBank accession number, informed in Materials and Methods.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 *S. sphagnetorum* 904 Sweden																				
2 *S. sphagnetorum* 873 Sweden	0,002																			
3 *S. simplex* 120 Japan	0,076	0,078																		
4 *S. island* 901 Sweden	0,114	0,116	0,122																	
5 *S. simplex* 117 Japan	0,139	0,141	0,148	0,133																
6 *S. saliens* 124 Japan	0,190	0,190	0,207	0,188	0,194															
7 *S. saliens* 123 Japan	0,186	0,186	0,198	0,188	0,181	0,030														
8 *S. tuberculosum* 122 Japan	0,175	0,177	0,188	0,173	0,188	0,105	0,108													
9 *S. longpich* 906 Sweden	0,179	0,179	0,177	0,186	0,194	0,148	0,143	0,137												
10 *S. leucops* SM Brazil	0,181	0,184	0,181	0,184	0,198	0,173	0,165	0,181	0,190											
11 *S. leucops* 976 London	0,156	0,158	0,152	0,167	0,177	0,169	0,162	0,171	0,152	0,127										
12 *S. leucops* 909 Sweden	0,184	0,186	0,173	0,181	0,177	0,196	0,192	0,196	0,175	0,143	0,124									
13 *S. leucops* 908 Sweden	0,186	0,188	0,175	0,184	0,179	0,198	0,194	0,198	0,177	0,146	0,127	0,002								
14 *S. leucops* 899 Sweden	0,184	0,186	0,173	0,181	0,177	0,194	0,192	0,196	0,175	0,143	0,124	0,002	0,004							
15 *S. leucops* 898 Sweden	0,184	0,186	0,173	0,181	0,177	0,194	0,192	0,196	0,175	0,143	0,124	0,002	0,004	0,000						
16 *S. grande* 116 Japan	0,181	0,184	0,184	0,177	0,184	0,186	0,184	0,171	0,173	0,150	0,133	0,118	0,120	0,116	0,116					
17 *S. grande* SM Brazil	0,179	0,181	0,169	0,188	0,175	0,179	0,171	0,192	0,175	0,135	0,110	0,146	0,148	0,143	0,143	0,154				
18 *S. bryophilum* 874 Sweden	0,184	0,181	0,177	0,181	0,213	0,194	0,196	0,190	0,181	0,177	0,171	0,186	0,188	0,186	0,186	0,190	0,194			
19 *S. grabbskogense* 880 Sweden	0,184	0,181	0,177	0,181	0,213	0,194	0,196	0,190	0,181	0,177	0,171	0,186	0,188	0,186	0,186	0,190	0,194	0,000		
20 *S. grabbskogense* 907 Sweden	0,184	0,181	0,177	0,181	0,213	0,194	0,196	0,190	0,181	0,177	0,171	0,186	0,188	0,186	0,186	0,190	0,1994	0,000	0,000	
21 *S. averaloi* 910 Sweden	0,179	0,161	0,196	0,207	0,179	0,192	0,198	0,181	0,186	0,203	0,177	0,188	0,190	0,186	0,186	0,181	0,184	0,190	0,190	0,190

S. leucops and *M. lignano,* it can be estimated that *S. leucops* has 30% of the cells found in *M. lignano*. Nevertheless, *Caenorhabditis elegans,* a nematode similar in size to *S. leucops,* has fewer cells, 959 in the adult hermaphrodite and 1031 in the adult male (Sulston & Horvitz, 1977). A small number of cells was a useful characteristic for transforming *C. elegans* into one of the most prestigious animal models, as it allows precise descriptions of development; for example, it was the first organism to have its connectome (neural wiring diagram) completed (White *et al.,* 1986). The small number of cells observed in *Stenostomum* may also be important to the usefulness of these worms as models for biological studies.

Although the number of zooids is not a diagnostic characteristic for *Stenostomum* species identification, this characteristic is always cited in the descriptions of species of this genus (Van Cleave, 1929; Nuttycombe & Waters 1938; Noreña *et al.,* 2005; Damborenea *et al.,* 2011; Gamo & Leal-Zanchet, 2004). Van Der Land (1965), Gamo & Leal-Zanchet (2004) and Noreña *et al.* (2005) describe *S. leucops* as having only two zooids. On the other hand, Van Claeve (1929) and Palmberg (1990) report that the worms they studied showed multiple zooids (up to five), but do not described the conditions under which these animals were cultivated. Our data show that zooid number is a plastic phenotype and is highly dependent on environmental conditions. We have maintained our cultures for four years and have only observed two zooids. During this time, we were led to think that it was a characteristic of the species, but along the fifth year, the alteration of culture conditions resulted in worms with multiple zooids. This phenotypic plasticity could explain the differences in the descriptions from various authors. Our results suggest that the reproductive pattern with multiple zooids occurs only when the worms are in Condition 2 of maintenance. Even in this condition, disturbance in the cultures, as pipetting the worms, promote the changes of reproductive patterns from multiple for two zooids.

The number of cells in each zooid in worms with multiple zooids is smaller than that observed in animals with only two zooids, approximately 2,000 for the former and 2,500 for the latter on average. Furthermore, the size of worms with two zooids, originated from the fission process of two zooids, is similar to that observed in worms with four or five zooids produced, approximately 1 mm long. Nevertheless, the number of cells in these animals is very different. The two-zooid worms have approximately 5,000 cells compared to 7,000 cells in animals with four zooids. It is likely that some of the cells present in the four or five zooid worms are smaller. This could indicate a faster reproductive process in animals producing multiple zooids. However, as we are not able to measure the time required in paratomy in multiple zooids process, this hypothesis needs additional assays to be clarified.

Stenostomum leucops is distributed worldwide, with registers for North America, Europe, Africa (Larsson, 2008 and references therein) and Japan (Yamazaki *et al.,* 2012). In South America, it has been recorded from Surinam (Van der Land, 1965), Argentina (Noreña *et al.,* 1995), Peru (Damborenea *et al.,* 2011) and the South Brazilian State Rio Grande do Sul (Gamo & Leal-Zanchet 2004, Braccini & Leal-Zanchet, 2013 and in this study). As mentioned previously, the validity of this species has been questioned by Nuttycombe & Waters (1938) and Marcus (1945) who consider the descriptions of this species ambiguous and broad to make its recognition difficult. Molecular analyses performed by Larsson *et al.* (2008) for Swedish Catenulida showed that the nominal species *S. leucops* is strongly supported as monophyletic group. However, the authors noted that variation in the branch lengths may be evidence for ongoing cladogenesis of some *S. leucops* populations, which makes it a candidate for a species complex. Yamazaki *et al.* (2012) performed a phylogenetic analysis for Japanese *Stenostomum* and included sequences of *S. leucops* from Europe. They found that a sequence of *S. grande* from Japan was included in the cluster of *S. leucops* from Europe. Our phylogenetic analysis of the available *Stenostomum* COI sequences showed that *S. leucops* and *S. grande* constitute a species complex. This is supported by by the high genetic divergence observed among sequences from same species. In *S. leucops,* the P distance among samples from Sweden, London and Brazil have the same range as observed among the species here sampled, strongly suggesting it is *a species complex,* as previously noted by Larsson *et al.* (2008). Although the status of *S. grande* as a valid species *has* never been questioned, the results of our phylogenetic analyses and the large genetic distance observed between the COI sequences of *S. grande* from Japan and Brazil suggest that it may also be a candidate for a species complex.

Since it was proposed, DNA Barcoding has allowed the increase of cryptic species discovery, even for species that are not distinguishable morphologically. For some examples see Gill *et al.* (2014), Crawford *et al.* (2012), Clare *et al.* (2011), Hebert *et al.* (2004).

We were not able to find morphological features distinguishing the internal groups in the species

complex and thus we did not propose any taxonomic change for *S. leucops* and *S. grande.*

Stenostomum leucops, while putatively a species complex, has many characteristics that make it an excellent organism for these comparative studies, mainly with planarians. However, as these worms have a simple anatomy and few diagnostic characters to allow the species identification, DNA barcoding can be a good supplementary tool for the characterization of these species for those who are thinking in using these animals as experimental models.

RESUMO

Stenostomum *são pequenos vermes que vivem em água doce e normalmente se reproduzem assexualmente por paratomia. Eles estão na base da filogenia dos platelmintes. Por mais de um século, espécies desse gênero, especialmente* S. leucops, *vêm sendo empregadas em estudos biológicos, principalmente sobre regeneração. Entretanto, alguns aspectos básicos da biologia destes vermes são ainda pobremente conhecidos. Neste estudo, caracterizamos uma linhagem que vem sendo mantida no laboratório por cinco anos. O tempo necessário para reprodução assexuada e completa formação de zoóides, a 28°C, é de aproximadamente 42,5 horas. O número de células nos zoóides, logo após a paratomia, é de aproximadamente 2.500. O número de zoóides presentes nos vermes é uma característica variável e depende das condições de cultivo. Em alguns procedimentos de cultivo de* S. leucops, *apenas cadeias com dois zoóides são formadas. No entanto, em outras condições de cultivo, cadeias de até cinco zoóides podem ser observadas. Análise filogenética empregando sequência do gene de Citocromo C Oxidase (COI) mostrou que* S. leucops *e* S. grande *constituem um complexo de espécies cujas linhagens mostram altas divergências intraespecíficas.*

Palavras-Chave: *Stenostomum grande;* Paratomia; Zooides; Número de células; Microturbellaria.

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Characterization of the first mitochondrial genome of a catenulid flatworm: Stenostomum leucops (Platyhelminthes).

Autores:

Marcos Trindade da Rosa: Revisão bibliográfica, auxílio na formação do texto, manutenção das culturas, preparação da amostra para construção das bibliotecas, montagem e anotação do mitogenoma, análises da sintenia dos genes mitocondriais dentre Platyhelminthes, estruturas dos tRNAs, análises da predição do gene ATP8.

Daniel Siqueira de Oliveira: Revisão bibliográfica, auxílio na formação do texto, montagem e anotação do mitogenoma.

Élgion Lucio da Silva Loreto: Revisão bibliográfica, auxílio na formação do texto, análises dos dados gerados, autor correspondente.

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Short Communication

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Characterization of the first mitochondrial genome of a catenulid flatworm: *Stenostomum leucops* (Platyhelminthes)

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Abstract

The first complete mitochondrial genome of a catenulid, *Stenostomum leucops,* was characterized. Illumina sequencing and 90 813 reads were utilized in the assembly, producing one contig with an average coverage of 1118×. The length of this genome is 15 742 bp with 12 protein-coding, two rRNA and 22 tRNA genes. Although the *atp8* gene is absent in other Platyhelminthes, a highly divergent putative *atp8* gene was found in *S. leucops*. In contrast to other Platyhelminthes, the mitochondrial genes of *S. leucops* are encoded on both strands. The gene order in the *S. leucops* mitogenome is very divergent from those observed in other Platyhelminthes, showing only small blocks of synteny. With AAA as the codon for lysine *S. leucops* shows the probable plesiomorphic condition, whereas Rhabditophora possess the derived GAA. This evolutionary transition is correlated with changes in the respective anticodons in *trn*K. It remains unclear whether the absence of the D arm and loop in *trn*S1 is a convergence in Catenulida and Neodermata.

Key words: Mitochondrial genome – Catenulida – *atp8* – mitochondrial gene order – mitochondrial genetic code - planarian

Introduction

Catenulida (Meixner 1924) is a class of Platyhelminthes represented by small flatworms that generally range from 0.5 to 2 mm in length. Catenulids live preponderantly in freshwater and have a simple anatomy and intraspecific variability, both factors that complicate species identification. Many species are reported as cosmopolitan, possibly due to the scarceness of diagnostic morphological characters (Larsson et al. 2008). The sexually mature stages are rarely found and normally the animals reproduce by paratomy, an asexual reproduction characterized by the formation of anterior structures in the median region of the body that form zooids and after maturation detach to form new organisms. Chains with up to nine zooids can be observed in some species (Hyman 1951). *Stenostomum leucops* (Dugès 1828) is a cosmopolitan species and one of the most studied Catenulida that is used mainly in regeneration research (Ritter and Congdon 1900; Child 1903a,b; van Cleave 1929; Moraczewski 1977; Palmberg 1990). Recently, we have characterized a strain of *S. leucops* with an average size of 0.98 ± 0.09 mm for worms with two zooids, preceding paratomy, and 0.49 ± 0.07 mm for each worm after fission. The time spent in paratomy is approximately 42.5 h at 28°C, producing zooids containing approximately 2400 cells. Two zooids commonly form; however, it is a plastic phenotype capable of producing up to five zooids and subsequently new worms depending on the environmental conditions (Rosa et al. 2015). Phylogenetic studies, using different genes, suggest that this taxon corresponds to a species complex (Larsson et al. 2008; Rosa et al. 2015).

According to Ehlers (1985), Catenulida is a monophyletic group. However, the phylogenetic position of Catenulida within Bilateria was the centre of intense debate (see e.g., Larsson and Jondelius 2008). The actual evidence supports, with a strong support, that Platyhelminthes is formed by two clades, Catenulida and Rhabditophora. More recently, Egger et al. (2015) and Laumer et al. (2015a,b) using hundreds of orthologous genes corroborated the proposition of Platyhelminthes formed by two sister groups, Catenulida and Rhabditophora. Additionally, Laumer et al. (2015a,b) showed that the orders Macrostomorpha and Polycladida are more basal in respect to planarians (Tricladida) and parasitic flatworms (Neodermata).

Animal mitochondrial genomes are useful tools for many studies of systematics, taxonomy and biogeography, from population analyses to resolution on a deep phylogenetic level, even if it comes to phylum-level relationships (Boore 2006). The main characteristics that make mitochondrial genomes very suitable phylogenetic markers include their small size, abundance in tissues, the strict orthology of genes, the presence of genes or regions under different evolutionary rates, their uniparental inheritance and the absence of recombination. Beyond widely used gene sequences, other features of mitochondrial genomes can be phylogenetically informative, including gene content and order of rearrangements, changes in genetic code or the secondary structure of tRNAs and rRNAs (Gissi et al. 2008). These features are more informative when their origins are complex and rare and, therefore, unlikely to have arisen from convergences. For example, mitochondrial gene order has been effectively used in investigating deep-level phylogenetic relationships across the animal kingdom (Bernt et al. 2013; Wey-Fabrizius et al. 2013).

Some features of platyhelminth mitogenomes have been described as applicable in phylogenetic analysis. The alteration in genetic code is an example. In Rhabditophora, the AAA codon codes for asparagine instead of lysine, and AUA codes for isoleucine and not methionine as in the standard invertebrate mitochondrial genetic code (Telford et al. 2000). These features can be interpreted as synapomorphies of Rabditophora and are not shared by Catenulida (Telford et al. 2000). Other potentially informative aspects of mitochondrial architecture are the absence of the *atp*8 gene and the fact that all genes are located on the same strand. These characteristics, however, are shared by other invertebrates (Gissi et al. 2008; Ross et al. 2016).

In this study, we report the first complete mitochondrial genome for a member of the class Catenulida, the species *Stenostomum leucops.* The gene order in the *S. leucops* mitogenome is

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very divergent from those observed in other platyhelminths. In addition, contrary to mitogenomes reported from other platyhelminths, mitochondrial genes, in *S. leucops,* are coded on both DNA strands. Comparison of tRNA genes with other platyhelminths showed the absence of the D arm and loop in *trn*S1 a character shared with Neodermata and some Polycladida. The *trn*K anticodon found in *S. leucops* is different from that observed in Rhabditophora, possibly representing the retained plesiomorphic condition use of the general invertebrate genetic code by Catenulida. While it has been generally assumed that the *atp8* gene has been lost from the mitochondrial genome of Platyhelminthes (Solà et al. 2015), we report the presence of a highly divergent putative *atp8* gene, similar to the one found in other invertebrates.

Materials and Methods

Biological samples and molecular genetic procedures

In this study, *S. leucops* strain SL01-sm01 was used and maintained in laboratory cultures for more than 6 years. This strain was characterized by Rosa et al. (2015). Vouchers of the strains used were deposited in the UFSM Department of Biology collection under numbers L01-sm01 and SL01-sm01.

Genomic DNA was isolated from a pool of 100 individuals using a protocol described by Oliveira et al. (2009) and, posteriorly, amplified using the Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare). A genomic DNA library was constructed from 200 ng of DNA using Illumina TruSeq Nano DNA kit. A MiSeq Reagent Kit v3 (600 cycles) was used for sequencing to produce paired-end reads (2 × 300). Sequencing was carried out using an Illumina MiSeq platform by Unidade de Genômica Computacional Darcy Fontoura de Almeida/LNCC/Brazil.

Genome assemblage and analysis

The FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) was used within the Galaxy web server (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010) to filter and eliminate the low-quality reads. The quality cut-off value applied was 20 and the percentage of bases that should possess a quality value of equal to or higher than the cut-off value of 90.

The assemblage of the mitogenome was performed using the MITObim package (Hahn et al. 2013). The *coI* gene from *S. leucops* (KX553929) was used as the seed for the assembly. MITObim uses an *in silico* baiting approach implemented in the MIRAbait module of the MIRA assembler (v3.4.1.1) (Chevreux et al. 1999). The characterization of the assembled mitochondrial genome of *S. leucops* was performed on the MITOS Web Server (Bernt et al. 2013) using default parameters, and the annotation was performed using UGENE software (Okonechnikov et al. 2012). The *S. leucops* mtDNA sequence was deposited in GenBank under accession number KX553929.

The base composition of nucleotide sequences was described by skewness and was measured using the following formulas:

$$\text{AT skew} = [A - T]/[A + T]),\ \text{GC skew} = [G - C]/[G + C]$$

Searches for the putative highly divergent *atp8* gene in the *S. leucops* mitogenome, the counterpart to those occurring in Tricladida, as described by Ross et al. (2016), were performed by identifying open reading frames (ORFs) using UGENE, and the transmembrane domain and signal peptides were predicted by SMART (Letunic et al. 2014).

Comparative mitochondrial gene order and RNA structures

The mitochondrial gene order in *S. leucops* was compared with those observed in other Platyhelminthes with available complete genomes (Table 1): Three species belonging to Tricladida, three to Polycladida and two to Macrostomorpha (free-living worms), and twelve species of parasitic clades (Neodermata) belonging to Cestoda, Trematoda and Monogenea. The partial mitochondrial genome of *Macrostomum lignano* was assembled using MITObim (Hahn et al. 2013) from PacBio and Illumina raw reads available in GenBank (Bioproject number PRJNA284736). Details of this assemblage can be found in Data 1. A partial sequence with five protein-coding genes available for *Microstomum lineare* (AY228756) was also used. The gene synteny among all species was analysed using the software CREx (Bernt et al. 2007) on the CREx web server (http://www.pacosy.informatik.uni-leipzig.de/crex).

The secondary structures of transfer RNAs were predicted using MITOSWEB (Bernt et al. 2013). For prediction, all secondary structures were determined using MITOSWEB and, for those organisms with sequence available on GenBank, the MITOSWEB outputs were confirmed by comparison with the GenBank annotations. The secondary structures of *S. leucops* tRNAs were compared with those of species listed in Table 1.

Results

The *S. leucops* mitogenome

The sequenced length of the mitogenome of *S. leucops* is 15 742 bp. A total of 90 813 reads were used in the assemblage, corresponding to 0.37% of the total reads. This mtDNA assemblage showed an average coverage of 1118× with a maximum coverage of 2571×. The *S. leucops* mitogenome contains 12 protein-coding genes (PCGs), two rRNA and 22 tRNA genes (Table 2). An A + T-rich region covering 1758 bp is located between the *cox2* and *trn*G genes and has a nucleotide composition of A: 32.1%, T: 38.9%, C: 14.9% and G: 14.1%, corresponding to 71% of the A + T content (Fig. 1). This content is similar to those observed in other Platyhelminthes (Solà et al. 2015). The negative AT skew (−0.095) indicated the occurrence of more Ts than As.

The protein-coding genes showed a length of 10 800 bp, containing 3600 codons in total and accounting for 66.27% of the entire mitogenome. The sizes of these genes are similar to those observed in other free-living Platyhelminthes. Six PCGs showed overlaps of nucleotides with tRNA genes (*nad6*/*trn*S1 −35 bp, *nad4L/trnM* −5 bp, *atp6/nad5* −56 bp, *nad5/trnF* −26 bp, *nad2/trnG* −2 bp and *nad3/trnV* −50 bp). The complete name of these genes is presented in Table 2.

While a conventional search for the *atp8* PCG did not result in the detection of a *atp8* gene, the presence of putative ORFs in a region devoid of annotated genes between *trnC* and *rrnL* led us to search for sequences representing signal peptides and transmembrane domains, which are characteristics of metazoan *atp8* genes (Ross et al. 2016). A hypothetical ORF encoding a protein of 295 amino acids was identified with ATG and TAA as start and stop codons, respectively. The SMART software used for motif prediction suggested the presence of four transmembrane domains and a conserved signal peptide (Fig. 2). It is noteworthy that the first four amino acids in this putative protein (MNQF) are similar to a well-conserved sequence (MPQL) in *atp8* (Hoffmann et al. 1992).

The *S. leucops* mitogenome has genes located on both strands. The protein-coding genes *cox2* and *nad3*, the *rrnS* and *trnG* and *trn*S1 genes are on the minority strand (Fig. 1). The other genes are located on the majority strand. The start codon used by five protein-coding genes is ATT and ATA by seven genes. The stop codon used in this genome is TAG for five PCGs and TAA for seven (Table 2).

The two rRNA genes have a total length of 1713 bp. The large ribosomal gene (*rrnL*) has a length of 1033 bp and is located between tRNA genes *trnI* and *trn*N. The small ribosomal gene (*rrnS*) has a length of 680 bp and is located between tRNA genes *trn*H and *trn*V.

Table 1. List of a Platyhelminthes species included in the present study

Species	Classification	Access GenBank	Life cycle	References
Stenostomum leucops	Catenulida/Stenostomidae	KX553929	Free-Living	This work
Benedenia hoshinai	Monogenea/Capsalidae	NC_014591	Parasitic	
Crenobia alpina	Tricladida/Planariidae	KP208776	Free-living	Solà et al. (2015)
Diplogonoporus balaenopterae	Cestoda/Diphyllobothriidae	NC_017613	Parasitic	Yamasaki et al.(2012)
Fasciola hepatica	Trematoda/Echinostomatoidea	NC_002546	Parasitic	Le et al. (2002)
Echinococcus multilocularis	Cestoda/Taeniidae	NC_000928	Parasitic	Nakao et al. (2002)
Echinochasmus japonicus	Trematoda/Echinostomatidae	NC_030518	Parasitic	
Enchiridium sp.	Polycladida/Prosthiostomidae	NC_028199	Free-living	Aguado et al. (2015)
Gyrodactylus derjavinoides	Monogenea/Gyrodactylidae	NC_010976	Parasitic	Huyse et al. (2008)
Hoploplana elisabelloi	Polycladida/Leptoplanidae	NC_028200	Free-living	Aguado et al. (2015)
Macrostomum lignano	Macrostomorpha/Macrostomidae		Free-living	Supplementary material
Microstomum lineare	Macrostomorpha/Macrostomidae	AY228756	Free-living	Jondelius et al. (2002)
Obama sp.	Tricladida/Geoplanidae	KP208777	Free-living	Solà et al. (2015)
Paragonimus westermani	Trematoda/Troglotrematidae	AF219379.2	Parasitic	
Paragyrodactylus variegatus	Monogenea/Gyrodactylidae	NC_024754.1	Parasitic	Ye et al. (2014)
Prosthiostomum siphunculus	Polycladida/Prosthiostomidae	NC_028201	Free-living	Aguado et al. (2015)
Schistosoma japonicum	Trematoda/Schistosomatidae	NC_002544	Parasitic	Le et al. (2002)
Schmidtea mediterranea	Tricladida/Dugesiidae	NC_022448	Free-living	
Taenia saginata	Cestoda/Taeniidae	NC_009938.1	Parasitic	Jeon et al. (2007)
Taenia solium	Cestoda/Taeniidae	AB086256	Parasitic	Nakao et al. (2003)
Tetrancistrum nebulosi	Monogenea/Ancyrocephalidae	NC_018031	Parasitic	

The 22 tRNA genes present in the *S. leucops* mitogenome occupy a total length of 1352 bp. The size of the tRNA genes ranges from 55 to 66 bp. The A + T content of the 22 tRNAs was 76.55%, with an AT skew of −0.012.

The comparative mitochondrial gene order

We compared the synteny of mitochondrial protein-coding genes among twelve parasitic Platyhelminthes species (Neodermata), belonging to orders Cestoda, Trematoda and Monogenea, three species of Tricladida (free-living platyhelminthes), two of Polycladida, two species of Macrostomorpha, *Microstomum lineare* and *Macrostomum lignano* and the Catenulida *Stenostomum leucops*.

All of the parasitic species (Neodermata) share the same gene order (GO) in their mitogenomes (Fig. 3). In addition, the mitochondrial genomes of all Tricladida analysed are syntenic. This suggests that the mitochondrial gene order is conserved inside of these major clades of Platyhelminthes. However, Tricladida and Neodermata share only two syntenies: one with three genes, *cytb, nad4L* and *nad4,* and the other with two genes *nad5* and *cox3*.

Stenostomum leucops showed an exclusive gene order. This unique synteny includes the sequence of genes *nad4L, nad4* and *atp6,* which is syntenic with regard to parasitic flatworms. However, *nad4L* is located on the minority strand instead of on the majority strand as in Neodermata. The *nad4L* and *nad4* genes are also syntenic with Tricladida, but in this free-living Platyhelminthes these genes are located on the same strands as in Neodermata.

The gene order of two species of Polycladida analysed showed large blocks of synteny. However, comparing these GO with the observed GO in other platyhelminths revealed only little shared synteny. *Macrostomum lignano* also showed an exclusive mitochondrial gene order and does not share any synteny with the partial sequence of *Microstomum lineare,* which is a member of the same clade, Macrostomorpha.

As a general rule, there is conserved synteny inside the clades Tricladide and Neodermata, but little conservation of gene order between them. The samples of Macrostomorpha and Polycladida showed specific gene orders. The same was observed for *S. leucops*, suggesting that this characteristic is very variable among Platyhelminthes major taxa.

Comparative secondary structures of transfer RNAs

The predicted secondary structures of *S. leucops* tRNAs showed that nine tRNAs do not have the typical cloverleaf secondary structures (Fig. 4). In three tRNAs (*trnI*, *trn*N, *trn*S1), the D arm (stem and loop) is absent. In two other tRNAs (*trn*A and *trn*R), the T arm is completely absent (TΨC loop and stem). The *trn*S2 tRNA has the D arm, but the loop is absent. Both *trn*D and *trn*F have the TΨC stem without the loop, while *trn*L1 have the TΨC loop without the stem. U-G base pairings (mismatches) were identified in 17 tRNA secondary structures, found principally in the first and last pairing bases of the D or T stems.

The predicted secondary structures of tRNAs found in *S. leucops* were compared with those of other flatworms. These structures are diversified among Platyhelminthes, but some patterns are conserved among different taxa, mainly within Neodermata and Tricladida (Table S2). For example, the D loop and stem are absent in *trn*S2 of Tricladida, *Macrostomum lignano* and also in *Fasciola hepatica,* which belongs to Neodermata. The D loop and stem are also absent on *trnS1* in all Neodermata and this is shared by *S. leucops.* Also, in this tRNA the arm D is absent in two Polycladida species, *H. elisabelloi* and *P. siphunculus.*

Stenostomum leucops has alterations in some secondary tRNA structures that are not found in other Platyhelminthes analysed. For example, the D loop and stem are absent in *trn*I, *trn*A and *trn*N only in this species. The same was observed for the TΨC loop and stem for *trn*R.

Discussion

The size and gene content of the *S. leucops* mitochondrial genome fits into the range described for Platyhelminthes, which vary in size from 13 to 17 kb, containing 36.0 ± 0.2 genes, with 22.0 ± 0.2 tRNAs (Gissi et al. 2008; Solà et al. 2015). The *atp8* gene is not detected, in *S. leucops,* using conventional tools for mitogenome gene search in. This gene is absent in other Platyhelminthes analysed (Solà et al. 2015). Beyond flatworms, this gene is also not detected in other metazoans including Nematoda, Rotifera, Chaetognatha and Bivalvia. The loss of this gene in five evolutionary-distant taxa led Gissi et al. (2008) to suggest that *atp8* is dispensable. However, recently Ross et al. (2016) identified a putative ORF in Tricladida, with two characteristics

Table 2. Annotation and gene organization of the *Stenostomum leucops* mitogenome

Gene		Start	End	Start codon	Stop Codon	Size (bp)
-co2	*Cytochrome oxidase subunit 2*	963	304	ATA	TAA	660
-trnM	*Methionine tRNA*	1044	1109			66
-nad4L	*NADH dehydrogenase subunit 4L*	1362	1105	ATA	TAA	258
trnW	*Tryptophan tRNA*	1370	1433			64
-trnQ	*Glutamine tRNA*	1436	1498			63
-trnT	*Threonine tRNA*	1501	1562			62
nad4	*NADH dehydrogenase subunit 4*	1586	2797	ATA	TAA	1212
-trnS2	*Serine 2 tRNA*	2813	2874			62
trnD	*Asparagine tRNA*	2878	2940			63
atp6	*ATP synthase subunit 6*	3136	3711	ATT	TAG	577
nad5	*NADH dehydrogenase subunit 5*	3656	5236	ATT	TAG	1581
trnF	*Phenylalanine*	5211	5270			60
nad2	*NADH dehydrogenase subunit 2*	5342	6265	ATA	TAA	924
trnG	*Glycine tRNA*	6264	6324			61
-trnR	*Arginine tRNA*	6330	6388			59
co1	*cytochrome oxidase subunit 1*	6472	8028	ATT	TAA	1557
trnA	*Alanine tRNA*	8046	8102			57
trnK	*Lysine tRNA*	8103	8166			64
trnE	*Glutamate tRNA*	8181	8244			64
trnL2	*Leucine 2 tRNA*	8268	8332			65
-trnH	*Histidine tRNA*	8342	8402			61
-rrnS	*Small subunit ribosomal RNA*	8462	9141			680
-trnV	*Valine tRNA*	9166	9225			60
-nad3	*NADH dehydrogenase subunit 3*	9574	9176	ATT	TAA	399
-trnL1	*Leucine 1 tRNA*	9642	9702			61
trnI	*Isoleucine tRNA*	9783	9841			59
-rrnL	*Large subunit ribosomal RNA*	11 221	9836			1386
-trnC	*Cysteine tRNA*	11 828	11 887			60
-trnY	*Tyrosine tRNA*	11 898	11 959			62
-nad1	*NADH dehydrogenase subunit 1*	12 912	12 013	ATA	TAG	900
-co3	*cytochrome oxidase subunit 3*	13 692	12 928	ATT	TAA	765
-trnS1	*Serine 1tRNA*	13 708	13 762			55
-nad6	*NADH dehydrogenase subunit 6*	14 225	13 728	ATA	TAG	498
-trnP	*Proline tRNA*	14 232	14 295			64
-cytb	*Cytochrome b*	15 375	14 302	ATT	TAG	1074
-trnN	*Asparagine tRNA*	15 397	15 456			60

The minus (−) symbol before genes means these are encoded on the minus strand.

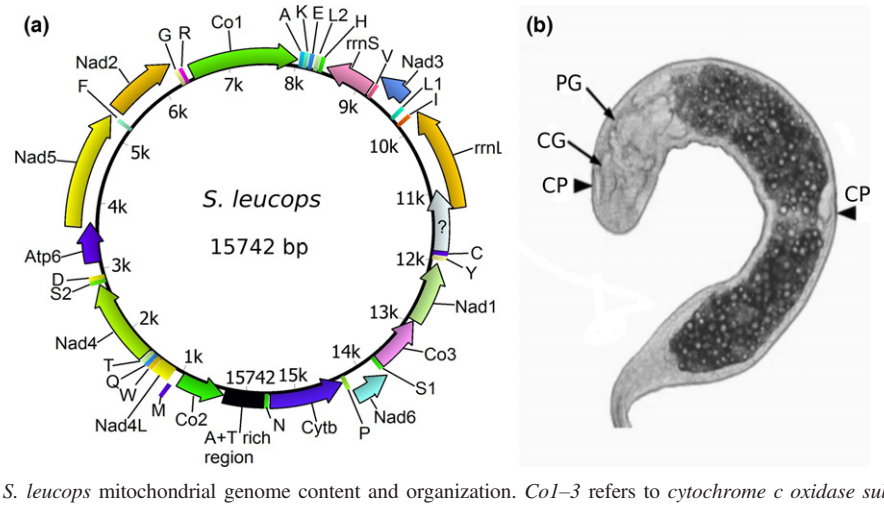

Fig. 1. (a) Summary of *S. leucops* mitochondrial genome content and organization. *Co1–3* refers to *cytochrome c oxidase subunits* 1–3, *Atp*6 refer to *Atpase subunits* 6, *Nad*1–6 and 4 l refer to *NAD dehydrogenase subunits* 1–6 and 4 l and *Cyt b* refers to *cytochrome b*. *rrn*S and *rrn*L refer to ribosomal RNA genes. Letters are the respective tRNA genes. Arrows indicate gene direction. (b) General view of *Stenostomum leucops* showing two zooids. CP, ciliated pits; CG, cerebral ganglia; PG, pharyngeal glands

of the ATP8 protein including the transmembrane domain and signal peptide. Previously, two putative *atp8* genes were identified in species belonging to taxa in which this gene was described as absent. For example, *atp8* was reported by Steinauer et al. (2005) in rotifers and by Lavrov and Brown (2001) in nematodes. We have identified a hypothetical ORF and its predicted protein containing four putative transmembrane domains and a signal peptide. Furthermore, metazoan ATP8 contains the

MPQL amino acid signature conserved at the N-terminus (Gissi et al. 2004). The amino acid residues MNQF were identified at the N-terminus of the putative ATP8 protein of *S. leucops*, which represents a 50% conservation regarding the typical metazoan signature. These results suggest that the highly divergent *atp8* may be more widely distributed in flatworms given its presence in both Tricladida and Catenulida. A broader occurrence of divergent *atp8* is also supported by the presence of similar putative sequences in rotifers and nematodes. As an alternative hypothesis to the one that *atp8* is a 'dispensable' gene, we postulate that many organisms have divergent genes not yet detected by the available annotation tools.

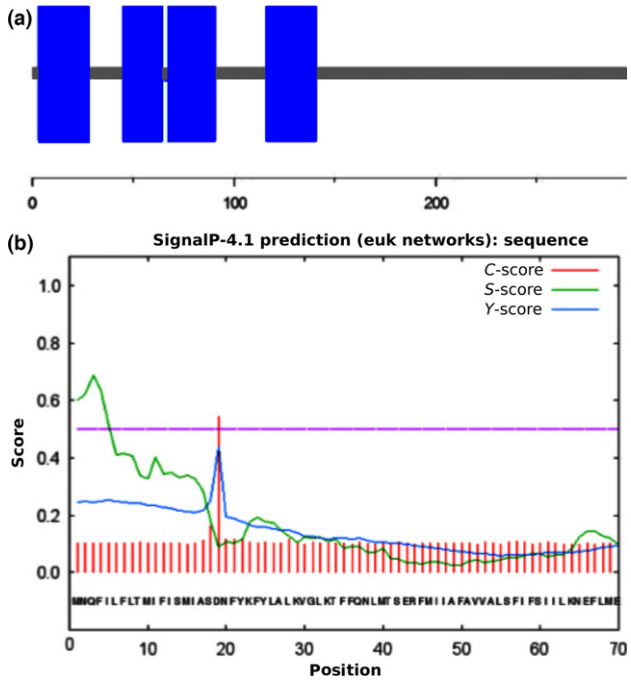

Fig. 2. Sequence domains predicted by SMART for the putative ATP8 protein from *S. leucops*. (a) Four transmembrane domains; (b) twenty amino acids of signal peptides

A characteristic of Platyhelminthes mitochondrial genomes is that all genes are encoded on a single strand of DNA (Le et al. 2002; Johnston 2006; Aguado et al. 2015). However, genes are encoded on both DNA strands in the *S. leucops* mitogenome, which is a unique characteristic among Platyhelminthes. Recently, Ross et al. (2016) showed that all mitochondrial transcription in triclads originates from a single DNA strand, producing lncRNAs. The presence of genes of all Rhabditophora so far described on the same strand suggests that Rhabditophora and Catenulida differ in their mitochondrial transcription mechanisms.

The occurrence of all genes on the same strand is also observed in many other invertebrates including Tunicata, Annelida, Rotifera, Bivalvia, Cnidaria and Porifera, leading the 'theory of ancestral one-coding strand' (Gissi et al. 2008). In this theory, the ancestral pattern is that all genes are encoded on the same strand, as a derived character, and genes are moved to the other strand, initially maintained as a co-oriented gene cluster on the same strand and finally have diverse patterns with genes not more clustered (Gissi et al. 2008). The mitogenome of *S. leucops* is a representative of the clustered pattern, including only two clusters. It is noteworthy that the derived pattern is observed in Catenulida, which is basal in Platyhelminthes phylogeny, and not in Rhabditophora as a more derived clade.

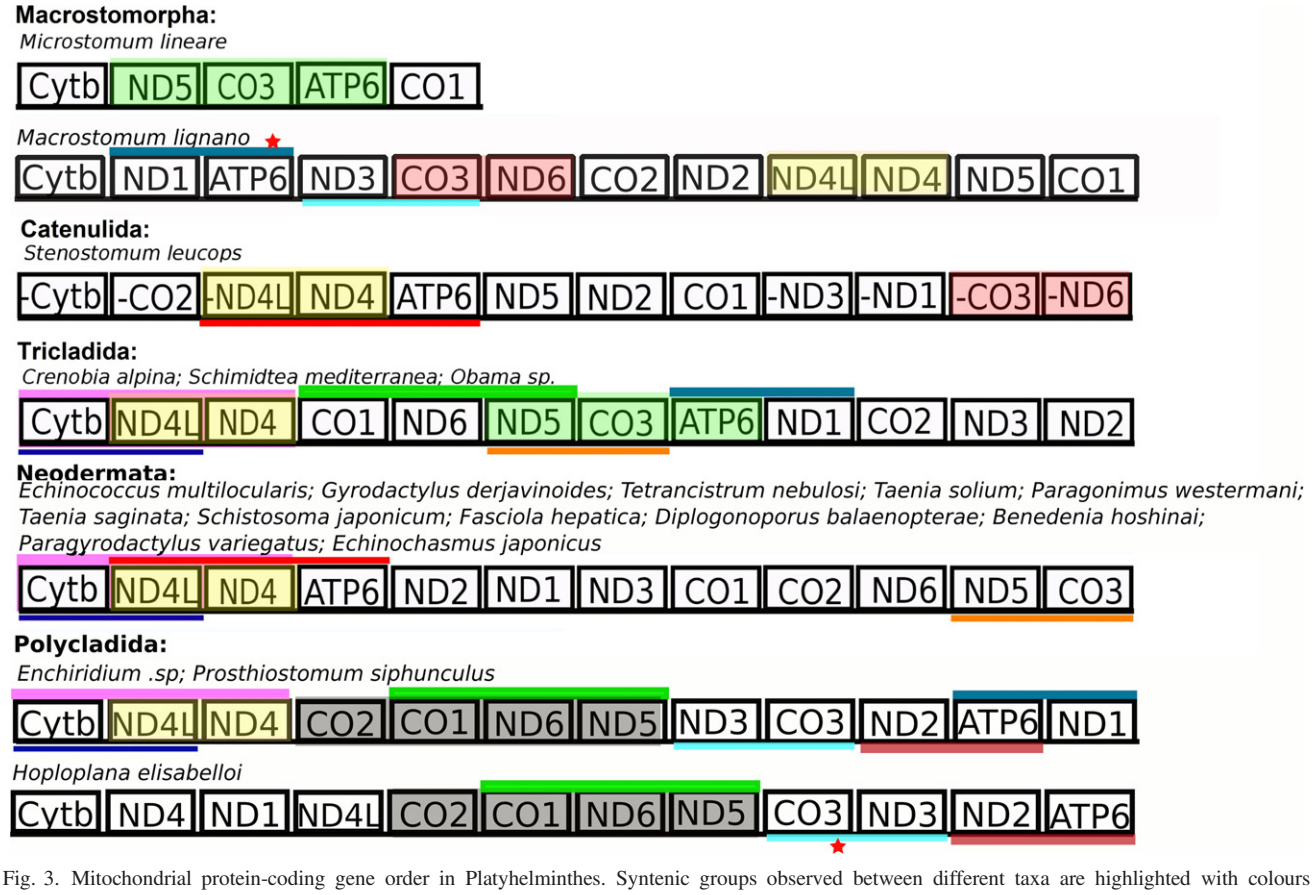

Fig. 3. Mitochondrial protein-coding gene order in Platyhelminthes. Syntenic groups observed between different taxa are highlighted with colours inside or outside of squares representing genes. Red stars represent putative inversions.

Neodermata and Tricladida are the Platyhelminthes with more available mitochondrial genomes, and several studies have documented the conserved gene order characteristic for each of these groups (Le et al. 2002; Vallès and Boore 2006; Waeschenbach et al. 2012; Golombek et al. 2015; Ross et al. 2016). Some cases of gene rearrangements were reported in Monogenea, such as *Aglaiogyrodactylus forficulatus* (Bachmann et al. 2016) and *Paragyrodactylus variegatus* (Ye et al. 2014). Several species in the genus *Schistosoma* (Trematoda) have the typical Neodermata gene order and others show rearranged patterns. These differences are phylogenetically informative (Webster and Littlewood 2012). Polycladida, which is a basal group within Rhabditophora, shows significant differences in mitochondrial synteny from the other Platyhelminthes (Golombek et al. 2015). However, a conserved pattern was observed within three species belonging to the cotyleans suborder, even if several rearrangements were identified in comparison with *S. maculata*, a species of the acotyleans suborder (Aguado et al. 2015). The results obtained in the present study comparing the gene order of *S. leucops* with other flatworms are consistent with the primary conclusions presented by Aguado et al. (2015) and (Golombek et al. 2015). These authors established that 'similitudes between the three analysed groups of Platyhelminthes (Polycladida, Tricladida and Neodermata) are not significantly higher than similitudes between any of them with other relatively distant groups, such as annelids, molluscs, rotifers or acanthocephalans'. The Catenulida can be added to this row of Platyhelminthes groups.

Stenostomum leucops have a mitochondrial gene order very divergent from the putative ground pattern of Lophotrochozoa, showing synteny only for block 4 (*nad*6-*cyt*b) and a partial synteny (*nad*2-*co*1) with block 1 of the conserved blocks of mitochondrial genes of Bilateria (Bernt et al. 2013).

Flatworms have an exclusive genetic code showing four main differences regarding the invertebrate genetic code (Bessho et al. 1992; Telford et al. 2000). However, from these four differences, Catenulida have only two, maintaining the plesiomorphic invertebrate pattern in the remaining two. The codon AAA codes for lysine as in the general invertebrate code and not asparagine as in other flatworms. Also, ATA codes for methionine as the general invertebrate code instead of isoleucine as in the remaining flatworms. Compared to data from other flatworms, the tRNA analysis performed in the present study identified a difference in the anticodon of trnK that can be associated with the observed divergence regarding the lysine/asparagine genetic code between Rhabditophora and Catenulida. The anticodons of all *trn*K analysed from Rhabditophora carry the sequence CUU, while *S. leucops* contain UUU as the anticodon (Table S3). The *trn*K CUU anticodon from Rhabditophora may pair more efficiently with the GAU and GAC asparagine codons than the UUU anticodon found in

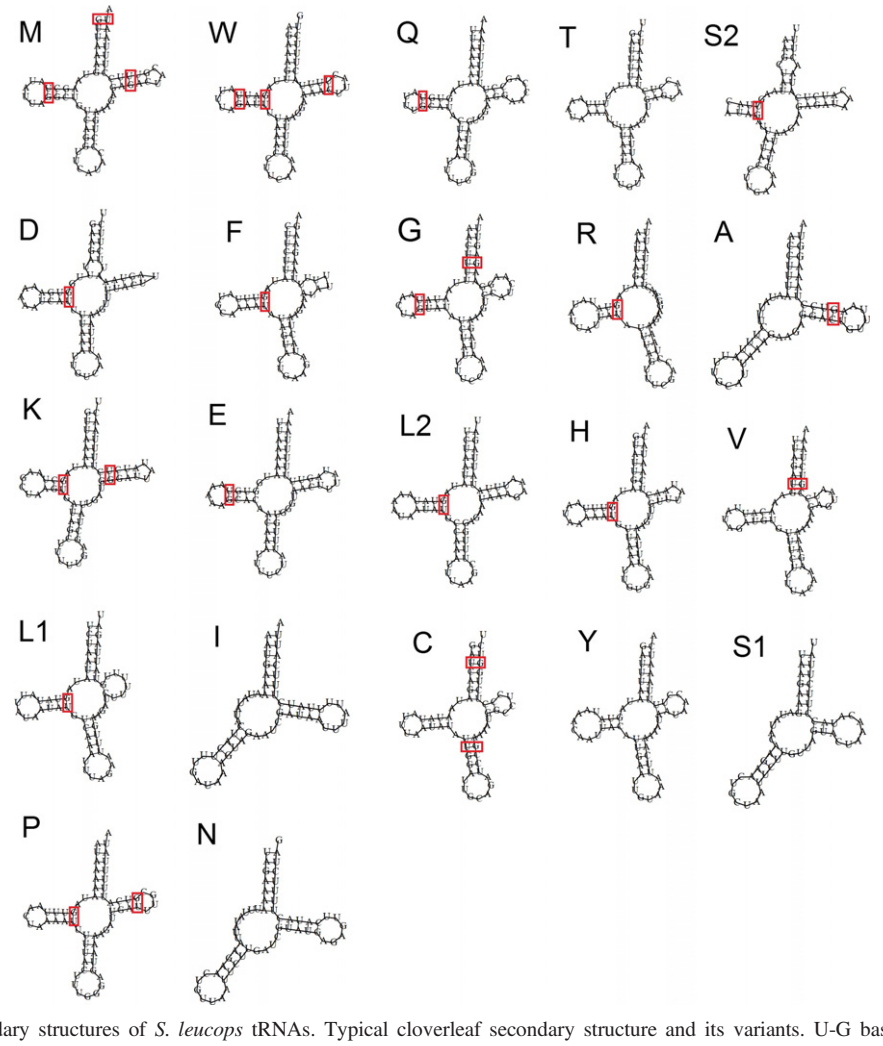

Fig. 4. Predicted secondary structures of *S. leucops* tRNAs. Typical cloverleaf secondary structure and its variants. U-G base pairings (mismatches) are indicated by red boxes.

Catenulida. The UUU anticodon may pair more efficiently with the lysine codons (AUU, AUC and AUA).

The absence of the D arm and loop in *trn*S1 have been reported in all parasitic flatworms, including Acoela and many other Metazoa (Le et al. 2002; Lavrov and Lang 2005; Mwinyi et al. 2010). The same was observed for *S. leucops*. However, it is remarkable that all Tricladida analysed maintain the typical cloverleaf structure. We suggest this characteristic correspond to an apomorphy in Tricladida.

In summary, the *S. leucops* mitochondrial genome has the following main differences from the other Platyhelminthes: (1) it is transcribed on both DNA strands; (2) the gene order is very divergent, showing only small blocks of synteny; (3) *trn*K uses a different anticodon than that observed in Rhabditophora, possibly associated with the plesiomorphic use of the general invertebrate genetic code by Catenulida; (4) the absence of the D arm and loop in *trn*S1 is a shared character of Platyhelminthes, except Tricladida; and (5) the potential presence of a divergent *atp*8 gene.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Description of partial genome of *Macrostomum lignano* (Table S1 and Fig. S1).

Table S2. Predicted secondary structures of tRNAs of the Platyhelminthes analysed.

Table S3. The anticodons of *trn*K of the Platyhelminthes analysed.

**G3: Gen Genom Genet

4-Discussão:

As críticas recebidas pelo trabalho não publicado (Capítulo 1) proporcionaram amadurecimento, visto que tínhamos pouco conhecimento sobre o grupo dos Catenulidas a que pertence *Stenostomum leucops,* e sobre trabalhos relacionados. Graças a elas, pudemos melhorar e construir os dois artigos resultantes nesta dissertação. As críticas também proporcionaram uma melhor visão dos próximos caminhos que ainda serão tomados, após os dois trabalhos já publicados (apresentados no capítulo 2 e 3 desta dissertação).

Platyhelminthes são excelentes modelos para estudos de processos biológicos básicos tais como: sinalização e regulação de células tronco, diferenciação celular, envelhecimento e, principalmente sobre regeneração (Roberts-Galbraith e Newmark, 2015). Além disso, alguns estudos dos mecanismos celulares fundamentais de platelmintos podem ser extrapolados para espécies parasitas (Wheler et al. 2015). As principais espécies de planárias estudas são *Schmidtea mediterranea* e *Girardia japonica* (Robb et al. 2008). Recentemente, uma microplanária filogeneticamente distante dos Tricladidos (planárias), *Microstomum lignano* teve seu genoma sequenciado e vem sendo apontado como um excelente modelo para estudos comparativos com as demais planárias (Wazik et al. 2015). Nesta dissertação estudamos alguns aspectos da biologia básica, e além disso, começamos a descortinar algumas peculiaridades, como os dados moleculares apresentados no mitogenoma de *S. leucops*, pertencente a um grupo ainda mais distante filogeneticamente que os Triclatidos. Sugerimos que esta espécie tem potencial de ser um modelo alternativo.

Macrostomum lignano é um organismo composto por 25.000 células em seu estágio adulto, de reprodução exclusiva hermafrodita. Estudos de Egger et al. (2006) apontam que, quando este organismo, faz uso de seus neoblastos, tem seu período de vida aumentado. Tricladidas como *Schmidtea mediterranea* e *Dugesia dorotocephala* (Baguña 1976, Baguña e Romero 1976), também apresentam determinada capacidade regenerativa, fator que pode ser delimitado pela obrigatoriedade da reprodução ser sexuada. Em *S. leucops* contendo 2.500 células, e reprodução preponderante assexuada, possuem capacidade aparente de manutenção de seus neoblastos com alta eficiência. Em *M. lignano* com danos

constantes, acaba tendo seu tempo de vida aumentado em dois meses, devido uma possível renovação de seus neoblastos (Egger et al. 2006). O pequeno número de células e o processo reprodutivo assexual, quase que obrigatório (em sete anos de cultivo em laboratório, e visualização diária destes vermes, ainda não vimos formas sexuais), fazem *S. leucops* atrativo para estudos comparativos com planárias.

Em restrição alimentar o tamanho do corpóreo, perda de estruturas morfológicas como gônadas e o número de células neoblastos, diminuem em *Macrostomum lignano, Schmidtea mediterranea* e *Dugesia dorotocephala,*(Nimeth et al. 2004, Baguñà 1976, Baguñà e Romero 1981, Oviedo et al. 2003). À medida que os animais recebem recurso alimentar em quantidades necessárias, estes recuperam suas estruturas e tamanho normal, além de seus neoblastos voltam a proliferar, levando a um possível rejuvenescimento dos animais (Lillie 1900, Child 1914, Newmark e Sánchez Alvarado 2002). Em laboratório já submetemos alguns vermes *S. leucops* culturas ricas em nutrientes (cianobactérias), causando mudanças morfológicas, causando aumento no tamanho corporal e formação no número de zooides. Já restrição alimentar, estes tiveram uma drástica redução corpórea, e nenhum destes apresentaram desenvolvimento de zooides, além da perda de um dos poucos carácteres morfológicos para sua identificação, que seria seus gânglios faríngicos.

O cultivo em laboratório possibilita uma descrição bem mais ampla das variações morfológicas, onde uma pré classificação utilizando a chave morfológica de Noreña et al. (2005) se torna ferramenta importante. Mas, pela alta plasticidade morfológica de *S. leucops*e, se faz necessário a utilização da ferramenta DNA Barcode e mesmo outros marcadores moleculares. Estes marcadores são fundamentais para maior precisão na identificação, e para tentar minimizar discussões sobre as espécies usadas em estudos envolvendo o verme "*S. leucops"*, como sugerido por Nuttycombe e Waters (1938). Estudos de Borkott (1970) e Noreña et al (2005) utilizando caracteres morfológiocos, validam a espécie "*S. leucops*". Dados moleculares de Larsson et al. (2008) e do primeiro trabalho publicado desta dissertação (capítulo 2), apontam se tratar de um complexo de espécies. As ferramentas moleculares junto a chave morfológica, se torna algo mais consistente na descrição do organismo usado no estudo. Entretanto, dado ser uma

“espécie” cosmopolita, para se caracterizar este conjunto de espécies e estabelecer os taxas válidos, se faz necessário um conjunto de dados ainda maior, cobrindo a distribuição geográfica deste conjunto de espécies.

O uso apenas de caracteres morfológicas de Catenulida e Rhabditophora, também não se mostrou eficaz para estudos filogenéticos dos Platyhelminthes (Larsson e Jondelius 2008). Estudos mais recentes, usando marcadores moleculares, mostraram com boa confiança que Platyhelminthes são um grupo monofilético, formados por dois clados principais, Catenulida e Rhabditophora, com Catenulida como o clado basal (Larsson e Jondelius 2008, Egger et al. 2015). A análise filogenômica comparativa utilizando os genomas mitocondriais dos Catenulidas e Rhabditophora, podem se tornar mais uma ferramenta para comprovar a basalidade de Catenulida dentro de Platyhelmynthes. Além disso, esses mitogenomas podem ser ferramentas mais confiáveis, para montarmos o quadro evolutivo e filogenético deste filo. Análises filogenéticas mais confiáveis utilizando mitogenomas não puderam ser feitas até a publicação do artigo do capítulo 3, pois era o único mitogenoma de Catenulida sequenciado até aquele momento era o de *S. leucops*.

Para se ter mais suportes dos fenômenos de regeneração e transformação genética previamente observados e descritos no capítulo 1, não possuem dados o suficiente para formação de artigos, se fazendo necessárias outras análises experimentais, moleculares e processamento de dados via ferramentas computacionais. Algumas ideias seguem em perspectivas, onde há uma grande quantidade de dados a serem analisadas, e ainda a serem gerados. Estudos que proporcionaram conhecimento e complementaram a formação que se iniciados na graduação, se torna hoje um leque de perguntas, e respostas levando a cada vez mais questionamentos.

5 – Perspectivas

- Estudos envolvendo os aspectos morfológicos e moleculares de *S. leucops* em processo regenerativo.
- Análise mais profunda dos estudos envolvendo transformação genética utilizando plasmídeos derivados de elementos transponíveis.

- Descrição do mobiloma (conjunto de elementos transponíveis presente em um genoma) através de ferramentas de bioinformática.
- Análises dos dados moleculares já obtidos para metagenômica.
- Montagem e descrição do genoma de *S. leucops.*

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