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## *STENOSTOMUM LEUCOPS* DUGÈS, 1828 (PLATYHELMINTHES, CATENULIDA):

A PUTATIVE SPECIES COMPLEX WITH PHENOTYPIC PLASTICITY

MARCOS T. ROSA<sup>1</sup>

CAMILA. M. PEREIRA<sup>1</sup>

GEOVANI. T. RAGAGNIN<sup>2</sup>

ELGION. L.S. LORETO<sup>1,3,4</sup>

### 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

<sup>1</sup> Universidade Federal de Santa Maria (UFSM), Programa de Pós-Graduação em Biodiversidade Animal. Avenida Roraima, 1.000, Camobi, CEP 97105-900, Santa Maria, RS, Brasil.

<sup>2</sup> Universidade Federal de Santa Maria (UFSM), Curso de Ciências Biológicas. Avenida Roraima, 1.000, Camobi, CEP 97105-900, Santa Maria, RS, Brasil.

<sup>3</sup> Universidade Federal de Santa Maria (UFSM), Departamento de Bioquímica e Biologia Molecular. Avenida Roraima, 1.000, Camobi, CEP 97105-900, Santa Maria, RS, Brasil.

<sup>4</sup> E-mail to corresponding author: [elgion@base.ufsm.br](mailto:elgion@base.ufsm.br)  
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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 (Nutting & 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 (Grahm *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 Nutting & 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  $\mu$ l 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).

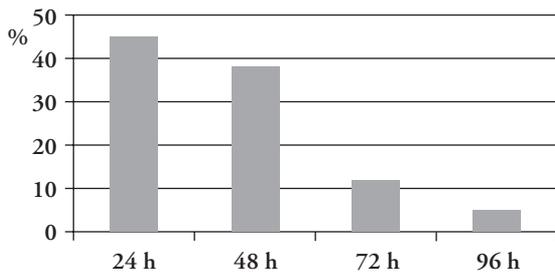
For the phylogenetic analyses, other *Stenostomum* COI sequences were obtained from GenBank (accession numbers: AB665116 to AB665124, FJ384873 to FJ38910, AJ405975, and AJ405976). These nucleotide sequences were aligned using Clustal W (Thompson *et al.*, 1994) according to the default program parameters. 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  $0.98 \pm 0.09$  mm and  $0.49 \pm 0.07$  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  $1.03 \pm 0.05$  mm. After paratomy, the size of the worms with one zooid, which were formed from chains of multiple zooids, was  $0.45 \pm 0.03$ . 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 since specimens 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 us-

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

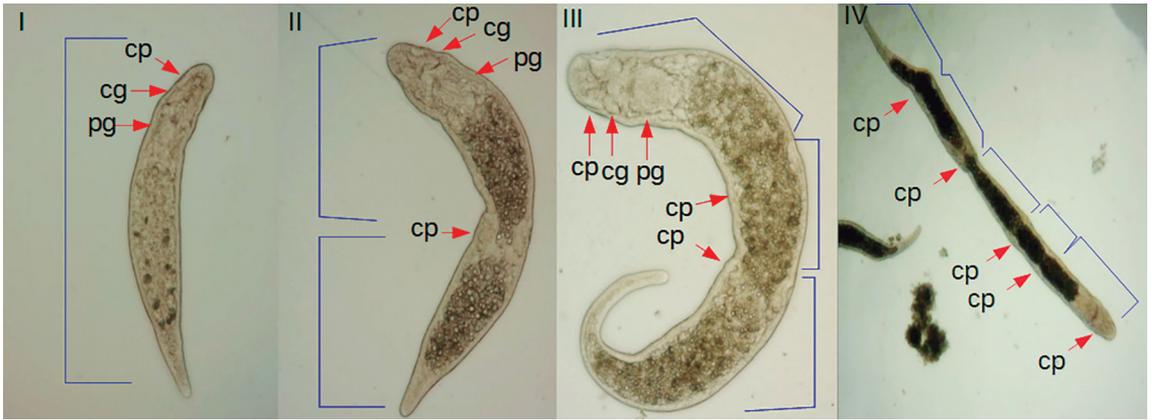
Number of Zooids	Procedure 1	Procedure 2
1	2.400 $\pm$ 280	2.158 $\pm$ 112
2	4.850 $\pm$ 325	3.964 $\pm$ 365
3	—	5.166 $\pm$ 91
4	—	7.003 $\pm$ 207

ing 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.

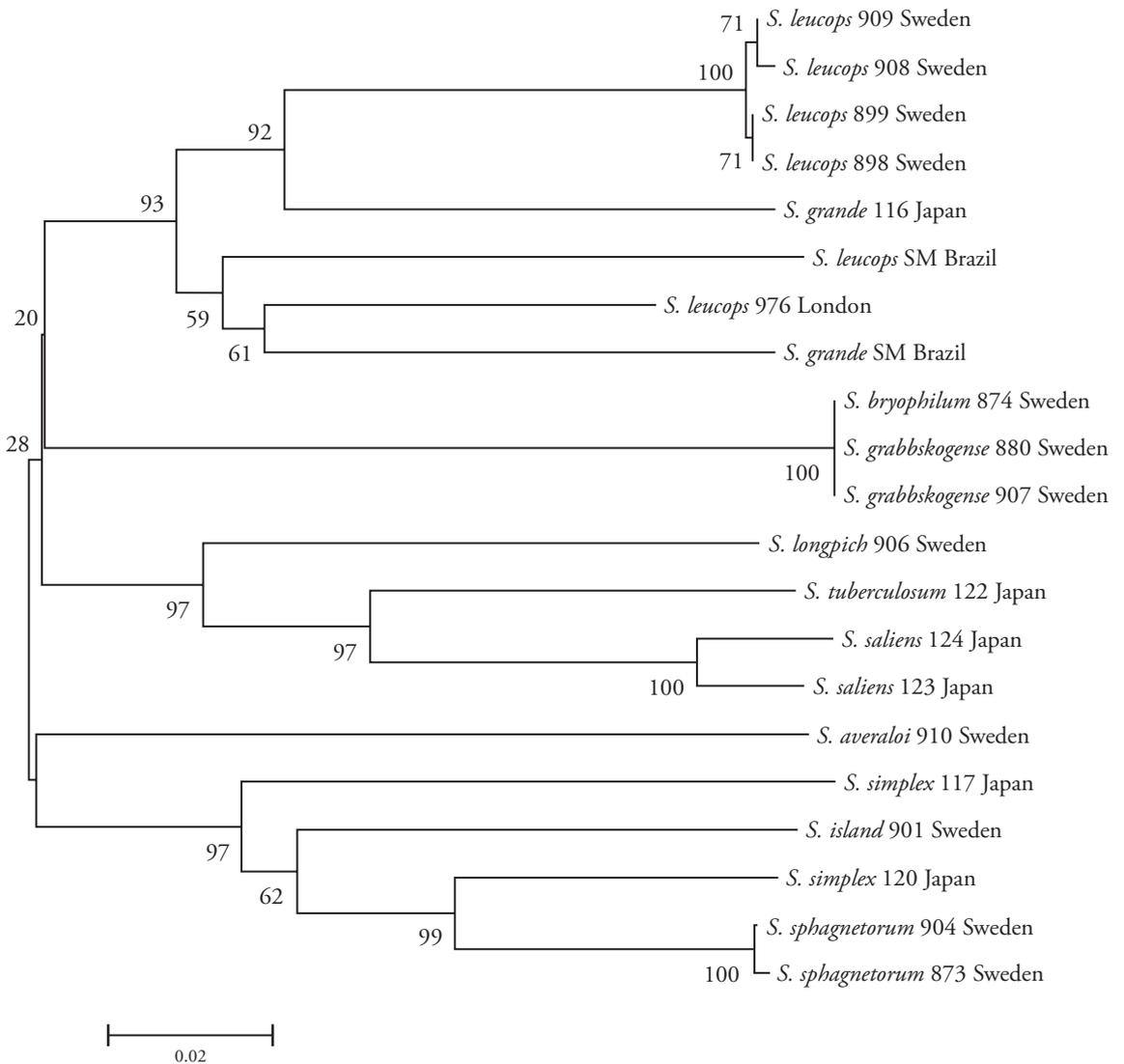
### DISCUSSION

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 the final 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. <i>S. sphagnetarum</i> 904 Sweden																				
2. <i>S. sphagnetarum</i> 873 Sweden	0,002																			
3. <i>S. simplex</i> 120 Japan	0,076	0,078																		
4. <i>S. island</i> 901 Sweden	0,114	0,116	0,122																	
5. <i>S. simplex</i> 117 Japan	0,139	0,141	0,148	0,133																
6. <i>S. saliens</i> 124 Japan	0,190	0,190	0,207	0,188	0,194															
7. <i>S. saliens</i> 123 Japan	0,186	0,186	0,198	0,188	0,181	0,030														
8. <i>S. tuberculosum</i> 122 Japan	0,175	0,177	0,188	0,173	0,188	0,105	0,108													
9. <i>S. longich</i> 906 Sweden	0,179	0,179	0,177	0,186	0,194	0,148	0,143	0,137												
10. <i>S. leucops</i> SM Brazil	0,181	0,184	0,181	0,184	0,198	0,173	0,165	0,181	0,190											
11. <i>S. leucops</i> 976 London	0,156	0,158	0,152	0,167	0,177	0,169	0,162	0,171	0,152	0,127										
12. <i>S. leucops</i> 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. <i>S. leucops</i> 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. <i>S. leucops</i> 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. <i>S. leucops</i> 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. <i>S. grande</i> 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. <i>S. grande</i> 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. <i>S. bryophitum</i> 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. <i>S. grabbiskogense</i> 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. <i>S. grabbiskogense</i> 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. <i>S. atevaloi</i> 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 Cleave (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 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 platelmintos. 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 pouco 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; Zoóides; Número de células; Microturbellaria.

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