Filamentous fungi in the digestive tract of *Phylloicus* larvae (Trichoptera: Calamoceratidae) in streams of the Brazilian Amazon

Fungos filamentosos no trato digestório de larvas de *Phylloicus* (Trichoptera: Calamoceratidae) em igarapés da Amazônia brasileira

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Abstract: Cultivable filamentous fungi were found for the first time in the digestive tract (DT) of larvae of *Phylloicus* (Trichoptera: Calamoceratidae). *Phylloicus* larvae (n=137) were collected in low-order streams in the Brazilian Amazon (Roraima, Pará, and Tocantins states) and dissected to obtain DT contents. Filamentous fungi were cultivated from each individual DT. Filamentous fungi presented different morphologies (geometric mean ± standard deviation of morphospecies per DT = 6.2 ± 6.4), as well as significant variation in population size (colony forming units per DT = 8.5 ± 47.0 x 10^1), among ecological landscapes and among larvae from the same locality. The fact that *Phylloicus* larvae commonly harbor filamentous fungi in their DT (94.9%) indicates that these microorganisms play important roles in the interaction interface with their hosts, which may be related to the degradation of lignocellulosic substrates. From this perspective, the DT of *Phylloicus* may represent a source of fungi with biotechnological potential.

Keywords: Aquatic insects. Fungus-insect interactions. Symbiosis.

Resumo: Esse é o primeiro estudo sobre a ocorrência de fungos filamentosos cultiváveis em associação com o trato digestório (TD) de larvas de *Phylloicus* (Trichoptera: Calamoceratidae). Larvas de *Phylloicus* (n=137) foram coletadas em igarapés de baixa ordem da Amazônia brasileira (estados de Roraima, Pará e Tocantins) e dissecadas para se obter o conteúdo do TD. Fungos filamentosos foram cultivados a partir de cada TD. Eles apresentaram diferentes morfologias (média geométrica ± desvio padrão de morfoespécies por TD = 6,2 ± 6,4), bem como variação significativa no tamanho das populações (unidades formadoras de colônias por TD = 8,5 ± 47,0 x 10^1), tanto entre paisagens ecológicas como entre larvas de uma mesma localidade. O fato de larvas de *Phylloicus* comumente abrigarem fungos filamentosos em seu TD (94,9%), como verificado neste estudo, indica que, potencialmente, esses microrganismos exercem papéis importantes na interface de interação com seus hospedeiros, que podem estar relacionados com a degradação de substratos lignocelulósicos. A partir dessa perspectiva, o TD de *Phylloicus* sp. poderia representar uma fonte de fungos com potencial biotecnológico.

Palavras-chave: Insetos aquáticos. Interações fungo-inseto. Simbiose.

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INTRODUCTION

Fungi and insects are very diverse biological groups (Blackwell, 2011; Stork *et al*., 2015) that can interact with each other, resulting in a variety of associations (Zacchi & Vaughan-Martini, 2002; Douglas, 2015), ranging from parasitism to mutualistic symbiosis (Caldera *et al*., 2009; Schigel, 2012; Six, 2012). In these interactions, both the external surfaces and the internal organs of an insect can be micro-habitats for the colonization of fungi (Zacchi & Vaughan-Martini, 2002; Ricci *et al*., 2011; Douglas, 2015).

In recent years, the digestive tract (DT) of a large variety of insects has been investigated regarding the associated fungal populations (León *et al*., 2016; Stefani *et al*., 2016). Most of the studies carried out so far have focused on the interaction of fungi with terrestrial insects, with few studies involving the fungal microbiota of aquatic insect (White, M. & Lichtwardt, 2004; Siri & Lastra, 2010; Misra *et al*., 2014).

Detritivorous aquatic insects (shredders), such as the larvae of *Phylloicus* (Trichoptera: Calamoceratidae), are of recognized importance in the decomposition of allochthonous organic matter in streams (Cornut *et al*., 2010; Gimenes *et al*., 2010). There is evidence that microbial conditioning of plant debris in streams, promoted mainly by fungi, influences the performance and feeding preferences of shredders in aquatic habitats (Arsuffi & Suberkropp, 1989; Chung & Suberkropp, 2009). Despite this, little is known about the interaction between those shredders and the fungi associated with their digestive tracts.

The present study reports a pioneer investigation on the occurrence of cultivable filamentous fungi in association with the DT of shredder insects of the genus *Phylloicus* from streams in different ecological landscapes in the Brazilian Amazon.

MATERIAL AND METHODS

CHARACTERIZATION OF STUDY AREAS

Sampling was carried out in low-order streams (n=33) with natural riparian vegetation, in different ecological landscapes (Amazon forest, *cerrado*, and *lavrado* [savanna]), in the Brazilian Amazon (Figure 1 and Table 1). The sampling of streams in Amazon forest landscapes was carried out in the Tapajós Nacional Forest (TNF), a conservation unit in Pará state (n=10), and in nearby Santarém municipality, Pará (n=01). The sampling of streams in *cerrado* landscapes occurred in the Lajeado State Park (LSP), a conservation unit in Tocantins state (n=10), and in the surroundings of the Santarém municipality (n = 02). Lastly, the sampling of streams under *lavrado* landscape, a savanna landscape typical of Roraima state, was carried out in the *Serra do Tepequém* (STQ) (n = 10).

Collections carried out in conservation units were authorized by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) (*Sistema de Autorização e Informação em Biodiversidade* - SISBIO, license number 53301 and 55136).

COLLECTION OF *PHYLLOICUS* LARVAE

In each stream, a 50 m stretch was selected where the available substrate (especially foliage) was collected at five points separated by 10 m, with the aid of an aquatic net (0.500 mm mesh and 0.465 m^2 area). At each point, three subsamples were collected and inspected in the field to collect *Phylloicus* shelters (Figure 2). Larvae were carefully removed from the shelters and transferred to tubes containing 1.0 mL of 70% ethyl alcohol where they remained for 30 seconds and immediately transferred to new tubes containing 1.0 mL of sterile distilled water and stored for 2 to 4 hours in isothermal boxes until laboratory processing.

ISOLATION, PURIFICATION, AND MORPHOLOGICAL CHARACTERIZATION OF FUNGI

Under aseptic conditions, the larvae were dissected, using a stereoscopic microscope, and DT content was diluted in 1.0 mL of sterile distilled water. An aliquot of 100 μL of preparation of the DT contents was inoculated in triplicate

in Petri dishes (90 mm diameter) containing Potato Dextrose Agar (PDA) culture medium (potato extract: 4.0 g; dextrose: 20.0 g; agar: 15.0 g) plus chloramphenicol at 0.1 $\mu g.mL^{-1}$ that were incubated at room temperature (25 ± 3 °C) and inspected for up to ten days. As fungal colonies grew on the plate, characterization of all morphological species (morphospecies) was performed. The determination of morphospecies was performed according to criteria proposed by Lacap *et al*. (2003) and Ibrahim *et al*. (2017), that include growth rate, shape, and coloration (reverse of Petri dish and aerial mycelium) of colonies and effects of the isolates in the culture medium. After pure fungal cultures were obtained, preservation was carried out by the Castellani method (Capriles *et al*., 1989). The micro-culture technique was used to identify microscopic structures, following Kern & Blevins (1999). Conidia production was observed microscopically with lactophenol cotton blue staining.

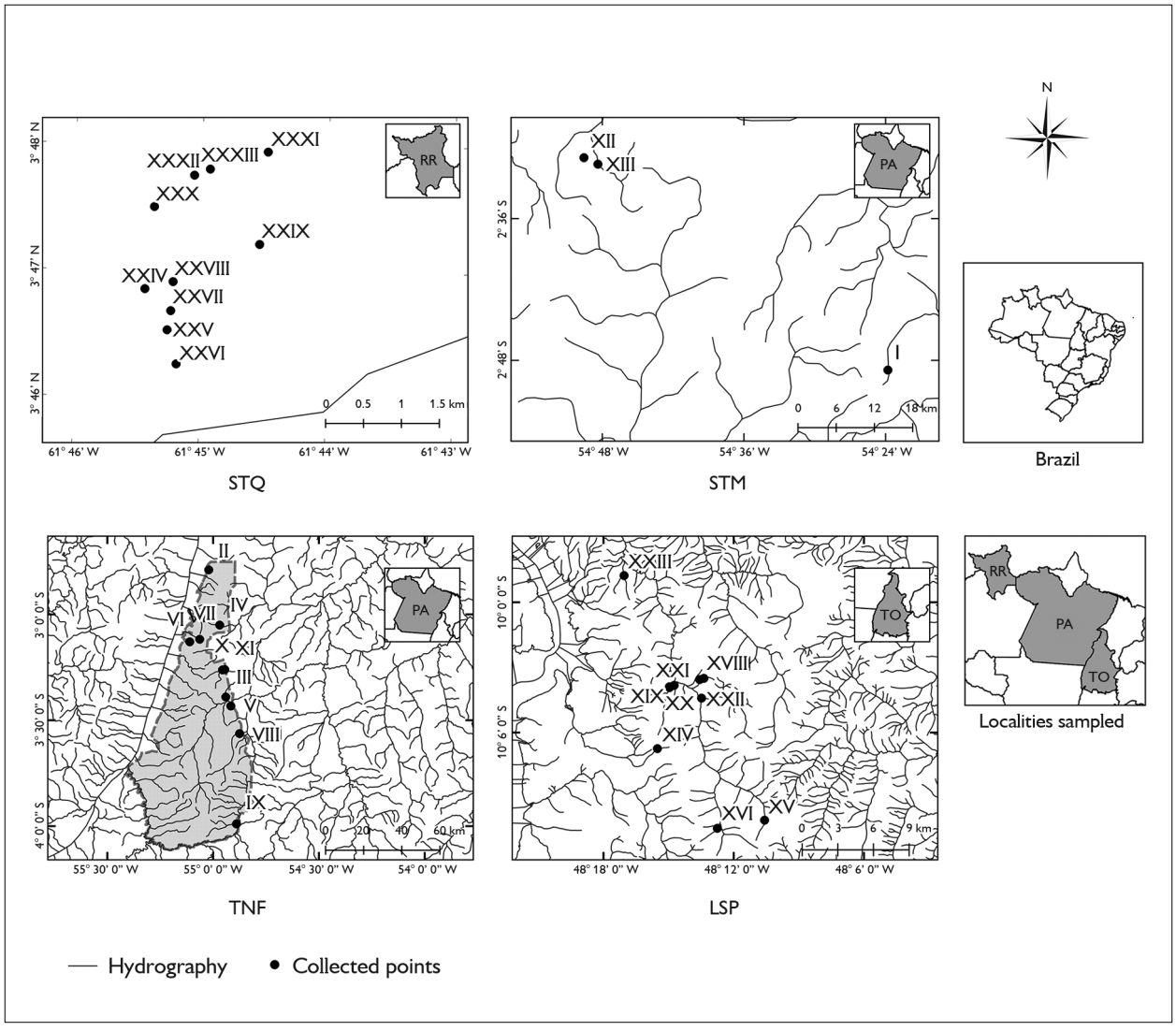

Figure 1. Map of the study localities. Abbreviations: STQ = *Serra do Tepequém*, Roraima; STM = Santarém municipality, Pará; TNF = Tapajós Nacional Forest, Pará; LSP = Lajeado State Park, Tocantins; I to XXXIII indicate the sampled streams.

Table 1. Occurrence of filamentous fungi in the digestive tract of *Phylloicus* (Trichoptera: Calamoceratidae), geometric mean and standard deviation of CFU.DT^{-1} and morphospecies of fungi per stream and ecological landscape sampled. Legends: * = averages followed by the same letter are not statistically significantly different according to Tukey's test, at $p < 0.05$; ** = seven from the 137 larvae did not result in fungal isolation; STM = Santarém municipality, Pará state; TNF = Tapajós Nacional Forest, Pará; LSP = Lajeado State Park, Tocantins; STQ = *Serra do Tepequém*, Roraima.

(Continue)

Ecological landscapes	Location	Streams and geographical coordinates	Total insects collected	% of insects with occurrence of fungi	Geometric mean of CFU.DT^{-1} ± standard deviation*	Geometric mean of morphspecies ± standard deviation
Amazon forest	STM	I (02° 48' 49.6" S; 54° 23' 38.2" W)	n = 3	100.0%	$8.0 \pm 1.8 \times 10^{1}$	7.9 ± 3.1
	TNF	II (02° 47' 23.0" S; 55° 01' 14.9" W)	n = 3	100.0%	45.0 ± 9.0	6.8 ± 2.0
	TNF	III (03° 23' 25.2" S; 54° 56' 26.3" W)	n = 3	100.0%	$7.8 \pm 40 \times 10^{1}$	5.1 ± 3.1
	TNF	IV (03° 03' 02.6" S; 54° 58' 09.3" W)	n = 3	66.6%	$3.9 \pm 5.2 \times 10^{1}$	8.0 ± 8.5
	TNF	V (03° 25' 59.1" S; 54° 54' 59.6" W)	n = 3	100.0%	$8.3 \pm 2.0 \times 10^{1}$	8.1 ± 5.3
	TNF	VI (03° 07' 47.6" S; 55° 06' 39.0" W)	n = 3	100.0%	$13 \pm 7.5 \times 10^{2}$	7.6 ± 1.5
	TNF	VII (03° 07' 04.3" S; 55° 03' 49.5" W)	n = 3	100.0%	$10 \pm 3.1 \times 10^{1}$	4.8 ± 1.7
	TNF	VIII (03° 33' 48.2" S; 54° 52' 30.90" W)	n = 3	100.0%	$1.8 \pm 2.0 \times 10^{2}$	6.6 ± 4.2
	TNF	IX (03° 59' 24.1" S; 54° 53' 24.6" W)	n = 3	100.0%	$2.4 \pm 1.9 \times 10^{2}$	7.3 ± 4.4
	TNF	X (03° 15' 44.7" S; 54° 57' 22.0" W)	n = 10	100.0%	$5.6 \pm 7.9 \times 10^{2}$	7.6 ± 2.1
	TNF	XI (03° 15' 38.7" S; 54° 56' 42.8" W)	n = 4	100.0%	$1.7 \pm 7.4 \times 10^{2}$	5.9 ± 4.3
Subtotal 1			n = 41	97.6%	$1.9 \pm 7.1 \times 10^{2}$ a	6.9 ± 3.2
Cerrado	STM	XII (02° 30' 50.8" S; 54° 49' 33.3" W)	n = 15	86.7%	$5.7 \pm 4.6 \times 10^{1}$	6.0 ± 3.4
	STM	XIII (02° 31' 23.8" S; 54° 48' 22.7" W)	n = 48	91.7%	$3.5 \pm 2.6 \times 10^{1}$	5.2 ± 4.7
	LSP	XIV (10° 06' 44.50" S; 48° 15' 31.10" W)	n = 0	-	-	-
	LSP	XV (10° 10' 02.30" S; 48° 10' 34.70" W)	n = 3	100.0%	$4.2 \pm 3.6 \times 10^{2}$	8.8 ± 2.0
	LSP	XVI (10° 10' 24.80" S; 48° 12' 45.40" W)	n = 3	100.0%	$1.7 \pm 5.7 \times 10^{2}$	5.5 ± 1.5
	LSP	XVII (10° 03' 33.60" S; 48° 13' 34.30" W)	n = 3	100.0%	$11 \pm 8.9 \times 10^{1}$	3.8 ± 2.5
	LSP	XVIII (10° 03' 33.40" S; 48° 13' 49.30" W)	n = 3	100.0%	$2.0 \pm 1.2 \times 10^{2}$	9.5 ± 3.5
	LSP	XIX (10° 03' 53.60" S; 48° 14' 58.00" W)	n = 3	100.0%	$11 \pm 2.8 \times 10^{1}$	9.2 ± 3.5
	LSP	XX (10° 03' 55.90" S; 48° 14' 57.70" W)	n = 3	100.0%	$2.1 \pm 4.1 \times 10^{1}$	3.9 ± 4.4
	LSP	XXI (10° 03' 49.80" S; 48° 14' 44.80" W)	n = 3	100.0%	$15 \pm 3.3 \times 10^{1}$	8.3 ± 1.2
	LSP	XXII (10° 04' 25.00" S; 48° 13' 29.10" W)	n = 3	100.0%	$2.1 \pm 7.3 \times 10^{2}$	3.8 ± 2.5
	LSP	XXIII (09° 58' 46.30" S; 48° 17' 03.20" W)	n = 3	100.0%	66.0 ± 7.0	4.1 ± 2.5
Subtotal 2			n = 90	93.3%	$5.7 \pm 24.9 \times 10^{1}$ b	5.5 ± 4.1
Lavrado (Savanna)	STQ	XXIV (03° 46' 39.90" N; 61° 43' 41.90" W)	n = 0	-	-	-
	STQ	XXV (03° 48' 22.50" N; 61° 42' 32.10" W)	n = 0	-	-	-
	STQ	XXVI (03° 46' 10.60" N; 61° 45' 27.00" W)	n = 0	-	-	-
	STQ	XXVII (03° 46' 43.90" N; 61° 45' 29.30" W)	n = 0	-	-	-
	STQ	XXVIII (03° 46' 43.90" N; 61° 45' 29.10" W)	n = 0	-	-	-
	STQ	XXIX (03° 47' 00.80" N; 61° 44' 51.80" W)	n = 0	-	-	-
	STQ	XXX (03° 47' 16.90" N; 61° 45' 38.40" W)	n = 3	100.0%	$2.3 \pm 2.5 \times 10^{2}$	13.8 ± 2.6
	STQ	XXXI (03° 47' 41.70" N; 61° 44' 47.90" W)	n = 0	-	-	-
	STQ	XXXII (03° 47' 31.60" N; 61° 45' 17.90" W)	n = 0	-	-	-

Table 1. (Conclusion)

Ecological landscapes	Location	Streams and geographical coordinates	Total insects collected	% of insects with occurrence of fungi	Geometric mean of CFU.DT^{-1} ± standard deviation*	Geometric mean of morphspecies ± standard deviation
Lavrado (Savanna)	STQ	XXXIII (03° 47' 32.50" N; 61° 45' 12.70" W)	n = 3	100.0%	5.7 ± 8.9 x 10^{1}	17.9 ± 31.2
Subtotal 3			n = 6	100.0%	1.1 ± 2.2 x 10^{2} c	15.7 ± 21.3
Total (Subtotal 1 + subtotal 2 + subtotal 3)			n = 137	94.9%**	8.5 ± 47.0 x 10^{1}	6.2 ± 6.4

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Isolates were grown in 5% Malt Extract Broth. A maximum of 40 mg of mycelium was collected after seven days of growth in a rotary shaker (100 rpm) at room temperature and used for DNA extraction using a Wizard™ Genomic DNA Purification Kit protocol (Promega, USA), following a slightly modified protocol from that of Burghoorn *et al*. (2002). After the extractions, DNA was analyzed in a NanoDrop 2000 spectrophotometer (Thermo Scientific, Brazil). The oligonucleotide primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White, T. *et al*., 1990) were used to amplify the internal transcribed spacer (ITS) regions of the rDNA (~600 pb), following the amplification conditions proposed by Santos *et al*. (2015). The amplified ITS fragments were electrophoresed on a 1.0% (w/v) agarose gel containing GelRed™ (Biotium Inc., USA) and visualized under ultraviolet light in a photodocumentation system (Loccus Biotechnology, Brazil). The 1 Kb DNA Ladder (Promega, USA) was used as a molecular weight marker.

The amplified products were sequenced in both directions using the same PCR primers in an ABI 3500 XL automated sequencer (Life Technologies, USA) according to the dideoxy or chain termination method (Sanger *et al*., 1977) using a BigDye Terminator sequencing kit v3.1 (Life Technologies, USA). All sequences were compared with sequences deposited in the GenBank Database using a local alignment algorithm for nucleotide sequences (Blastn) (Altschul *et al*., 1990) and in the CBS Database (s.d.).

Figure 2. Photomicrograph of *Phylloicus* (Trichoptera: Calamoceratidae) inside its shelter. Photo: Ana Maria Oliveira Pes.

DESCRIPTIVE STATISTICS

Using Excel2013 (Microsoft™), the percent of DT with fungi was determined. The geometric mean and standard deviation of the Colony-forming Units per DT (CFU.DT^{-1}) and morphospecies per DT (MSP.DT^{-1}) was calculated in relation to all DTs analyzed, DTs of a same stream, and DTs of the same ecological landscape (Amazon forest, *cerrado* or *lavrado* [savanna]).

Analysis of variance (ANOVA) of CFU.DT^{-1} and MSP.DT^{-1} in the ecological landscape (Amazon forest, *cerrado*, and *lavrado* [savanna]) was performed, at $p < 0.05$, using Statistica ver. 10. When there was a significant difference, Tukey's test was performed, using the same software.

RESULTS

The percent fungal occurrence, geometric mean, and standard deviation of CFU.DT^{-1} of fungi and morphospecies are given in Table 1, in relation to total DTs of analyzed *Phylloicus* larvae, as well as in relation to total DTs of each ecological landscape (Amazon forest, *cerrado*, and *lavrado* [savanna]), and each stream.

The population sizes, expressed in CFU.DT^{-1}, showed large variation both among sampled landscapes and among DTs from the same stream, where the total geometric mean of fungi per DT equals 8.5 ± 47.0 x 10^1 CFU.DT^{-1}. Comparing the fungal populations among ecological landscapes, the geometric means of fungi per DT are statistically significantly different according to ANOVA (Figure 3) and Tukey's test, at $p < 0.05$. Population counts varied from 5.7 ± 24.9 x 10^1 CFU.DT^{-1} (in *cerrado*) to 1.1 ± 2.2 x 10^2 CFU.DT^{-1} (in *lavrado* [savanna]) and 1.9 ± 7.1 x 10^2 CFU.DT^{-1} (in Amazon forest).

Different morphological characteristics were observed among the fungal strains associated with *Phylloicus* larvae DTs and resulted in a high number of morphospecies per sampled DT (MSP.DT^{-1} = 6.2 ± 6.4) (Table 1). According to the ANOVA, variation in richness of morphospecies among the sampled landscapes was not significant (Figure 4).

Preliminary identification efforts based on the association between classical and molecular methods (Kern & Blevins, 1999; White, T. *et al*., 1990) allowed the identification of 21 isolates to the genus level (*Penicillium*) and 4 isolates were identified to the species level associated with the larvae collected in streams of *cerrado* in Santarém (STM, Pará State) (Table 2).

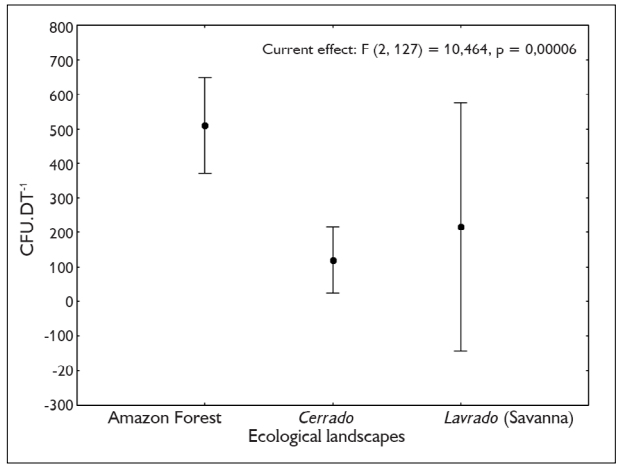

Figure 3. Analysis of variance (ANOVA) of colony-forming units per DT (CFU.DT^{-1}) among ecological landscapes (Amazon forest, *cerrado*, and *lavrado* [savanna]), at $p < 0.05$, using Statistica v.10, n = 137 DTs.

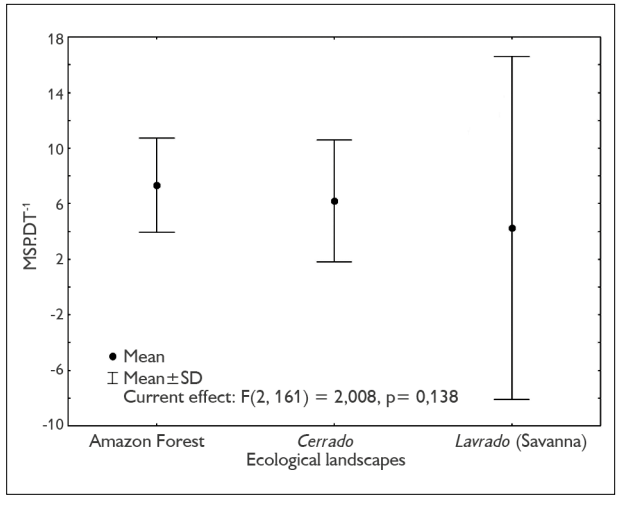

Figure 4. Analysis of variance (ANOVA) of the morphospecies per DT (MSP.DT^{-1}) among landscapes (Amazon forest, *cerrado*, and *lavrado* [savanna]), at $p < 0.05$, using Statistica v.10, n = 137 DTs.

Table 2. Identification of isolates associated with the digestive tract of *Phylloicus* larvae (Trichoptera: Calamoceratidae) collected in streams of *cerrado* in Santarém (STM, Pará State) based on the sequencing of the ITS regions of the rDNA. Legend: * = percentage of similarity between the nucleotide sequences obtained in that study with sequences available in the National Center for Biotechnology Information (NCBI) database.

Isolate code	Fungal species	% ID*	GenBank acession numbers
LAG 8.6	*Penicillium simplicissimum* (Oudem.) Thom, 1930	99	KU059955.1
PON 3.6	*Paraphaeosphaeria arecacearum* Verkley, Göker & Stielow, 2014	99	KM873041.1
PON 9.1	*Paraphaeosphaeria arecacearum* Verkley, Göker & Stielow, 2014	100	JX496100.1
PON 15.1	*Penicillium sclerotiorum* Beyma, 1937	99	KX664361.1

DISCUSSION

The present study is the first report on the occurrence of cultivable filamentous fungi in association with the DT of aquatic insects from the genus *Phylloicus* (Trichoptera: Calamoceratidae). The larvae were shown to harbor filamentous fungi in their DT, since these microorganisms were obtained from most larvae sampled from streams in the Brazilian Amazon.

There was significant variation in the size of the fungal populations (in CFU.DT^{-1}) among the landscapes, with the largest populations found in larvae from Amazon forest streams. These differences may be related to the availability of organic matter in streams, which is usually higher in Amazonian forest streams than in *cerrado* or *lavrado* (savanna) streams (Wantzen, 2003; França *et al*., 2009).

In aquatic ecosystems, fungi play important roles in the breakdown of allochthonous plant detritus, a key ecological process in aquatic environments that ensures the input of organic matter to various other organisms (Cornut *et al*., 2010; Gimenes *et al*., 2010). Moreover, fungi, through their sophisticated enzymatic apparatuses, degrade highly recalcitrant organic compounds such as lignin (Abdullah & Taj-Aldeen, 1989), that can be present in high concentrations in submerged plant materials that are food for larvae of leaf-shredding aquatic insects (Chung & Suberkropp, 2009; Cornut *et al*., 2015). The fact that *Phylloicus* larvae consistently harbored filamentous fungi in their DT indicates that, potentially, these microorganisms play important roles in the interaction with their hosts, which may be related to the degradation of lignocellulosic substrates. This hypothesis also has support in other interactions between fungi and insects that occur in nature, such as among xylophagous insects and yeasts of DT (Grünwald *et al*., 2010).

In this study, a large species richness of morphospecies was isolated from 130 *Phylloicus* larvae, a large collection of insects of the genus, comprising 94.9% of the DTs sampled. Although morphological species are not a perfect proxy for taxonomic species, high richness indicates potential high species richness associated with DT of *Phylloicus*. The DT of other insects is known to harbor a great diversity and a source of new species of fungi and bacteria (Suh *et al*., 2005; Tegtmeier *et al*., 2016), and this collection of fungi may bring species new to science.

Future taxonomic efforts should be undertaken to elucidate the fungal diversity associated with the DT of *Phylloicus*. Preliminary identification efforts based on molecular methods allowed the identification of four isolates associated with *Phylloicus* larvae DTs collected in *cerrado* streams near Santarém. Among these species, two isolates were identified as *Paraphaeosphaeria arecacearum*, which was first isolated from soil under *Elaeis guineensis* Jacq., in Suriname, and recently described by Verkley *et al*. (2014). This is the first occurrence record of this species in association with the DT of an aquatic insect in the tropics. Also one strain of *P. sclerotiorum* and one of *P. simplicissimum* were identified. Interestingly, strains of *P. sclerotiorum* are known as xylanolytic (Knob & Carmona, 2010) and *P. simplicissimum* produces cellulases (Zeng *et al*., 2006). This may be evidence for the role of at least some fungal species in degrading plant

materials in the DT of *Phylloicus* larvae in tropical aquatic ecosystems. According to Knob & Carmona (2010), xylanase showed interesting characteristics for biotechnological processes, such as in feed and food industries. Other cellulases from *Penicillium* spp. also showed a potential for industrial application (Dutta *et al*., 2008; Bomtempo *et al*., 2017). Investigations are on course to characterize the cellulolytic potential of the strains isolated herein.

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