Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Diversity of cellulolytic and xylanolytic fungi associated with the digestive tract of aquatic insect larvae in streams of the Amazon Forest and Cerrado in Brazil

Diversidade de fungos celulolíticos e xilanolíticos associados ao trato digestivo de larvas de insetos aquáticos em riachos de Floresta Amazônica e Cerrado no Brasil

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Abstract

The study of the relationship between fungi and insects brings important contributions to the knowledge of fungal biodiversity and to the understanding of mutualistic ecological interactions. This study reports the occurrence of a community of filamentous fungi in the digestive tract (DT) of mining insect larvae belonging to genus *Stenochironomus* in streams of two Brazilian biomes. Fungi were obtained from the digestive tract of larvae found on trunks and leaves of low-order streams in the Amazon Forest and Cerrado in the north of Brazil. The fungal community was screened for xylanolytic and cellulolytic activities. The diversity of fungal species in the DT of larvae is possibly related to the diversity of diets of species of that genus and the diversity of substrates in the ecosystems. The diversity and richness of fungal species were influenced by ecological differences between locations more than by the types of substrates in which they were collected (trunk and leaf). Most fungi in the DT of *Stenochironomus* larvae sampled in leaves exhibited cellulolytic enzyme activity. Such results stress that the mycobiomes of the DT of *Stenochiromonus* larvae produce enzymes that contribute to the process of breaking down plant remains in their hosts.

Keywords: biodiversity, fungus-insect interaction, *Stenochironomus*, enzymatic activity.

Resumo

O estudo da relação entre fungos e insetos traz importantes contribuições para o conhecimento da biodiversidade fúngica e para o entendimento das interações ecológicas mutualísticas. Este estudo relata a ocorrência de uma comunidade de fungos filamentosos no trato digestivo (TD) de larvas minadoras de insetos do gênero *Stenochironomus* em riachos de dois biomas brasileiros. Os fungos foram obtidos do trato digestivo de larvas encontradas em troncos e folhas de riachos de baixa ordem na Floresta Amazônica e Cerrado no norte do Brasil. A comunidade fúngica foi triada para atividades xilanolíticas e celulolíticas. A diversidade de espécies fúngicas no TD de larvas possivelmente está relacionada à diversidade de dietas das espécies desse gênero e à diversidade de substratos nos ecossistemas. A diversidade e riqueza de espécies fúngicas foram influenciadas mais pelas diferenças ecológicas entre os locais do que pelos tipos de substratos em que foram coletados (tronco e folha). A maioria dos fungos no TD de larvas de *Stenochironomus* amostradas em folhas exibiu atividade enzimática celulolítica. Tais resultados reforçam que os micobiomas do DT de larvas de *Stenochiromonus* produzem enzimas que contribuem para o processo de decomposição de restos vegetais em seus hospedeiros.

Palavras-chave: biodiversidade, interação fungo/inseto, *Stenochironomus*, atividade enzimática.

1. Introduction

Filamentous fungi are microorganisms that colonize virtually all possible substrates in ecosystems and degrade many types of organic substrates and some inorganic ones (Graça et al., 2016). They actively take part in biodegradation processes and nutrient cycling, thus helping to keep the ecosystem working (Vera-Ponce de León et al., 2016). Larvae of insects of the genus *Stenochironomus* Kieffer (1919) belong to the order Diptera of the family Chironomidae, rich in species with worldwide distribution, occurring in all biogeographic regions except for Antarctica (Parise and Pinho, 2016). These aquatic larvae are found mining submerged leaves and trunks in freshwater habitats and,

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Received: July 5, 2022 – Accepted: August 25, 2022

for this reason, are considered true xylophages (Pinho and Pepinelli, 2014).

Many studies have sought to understand the relation between fungi and insects. Some, for example, have investigated the endosymbiotic relations among thermites, ants, and fungi (Nygaard et al., 2016; Biedermann and Vega, 2020). Others work to understand the intestinal environment of insects and how the associated fungal communities are structured in that environment (Stefani et al., 2016; Wu et al., 2020). However, little is known on the structure of the mycobiomes of larvae of aquatic insects, as well as the factors that influence those communities. Although symbiotic relations are difficult to define, a study has recently demonstrated that the DT of larvae of aquatic shredder insects of the genus *Phylloicus* spp. (Trichoptera: Calamoceratidae) in regions of the Amazon Forest host a community of filamentous fungi (present in 94.9% of the DTs analyzed) with some evidence of important interactions with their host (Santos et al., 2018). Studies argue that the community of microorganisms that colonize the DT of insect larvae may help complement the nutritional capacity of the host by producing enzymes that break down plant cell wall (Calderón-Cortés et al., 2012). Since the plant material ingested by the larvae is highly refractory, it is expected that microorganisms, such as fungi, play an important nutritional role for the xylophages (Ali et al., 2017).

The understanding of the nature and the component groups in the symbiosis between fungi and aquatic insects is of great interest not only as it contributes to better understanding the biodiversity and the ecologic role of interacting organisms, but also from the biotechnological standpoint. Filamentous fungi represent a source of metabolically versatile biocatalyzers in the discovery of new products and drugs (Gomes et al., 2018; Alves Junior et al., 2019). Thus, this study aimed to identify the diversity of fungi associated with the digestive tract of larvae of insects of the genus *Stenochironomus* that occur in low-order streams of the Amazon Forest and Cerrado biomes and their potential production of xylanases and cellulases.

2. Material and Methods

2.1. Sample collection and processing

The first phase of the study was conducted in low-order streams in the Amazon Forest (Adolpho Ducke Forest Reserve – ADFR; Amazonas) in mid-August, 2016, and Cerrado (Lajeado State Park – LSP; Tocantins) in mid-June, 2016, in Brazil (Figure 1). Ten sampling spots were delimited in each site in 200 m stretches of each stream. Insect larvae occurred in only five streams of the Amazon Forest and eight of the Cerrado. These larvae were collected

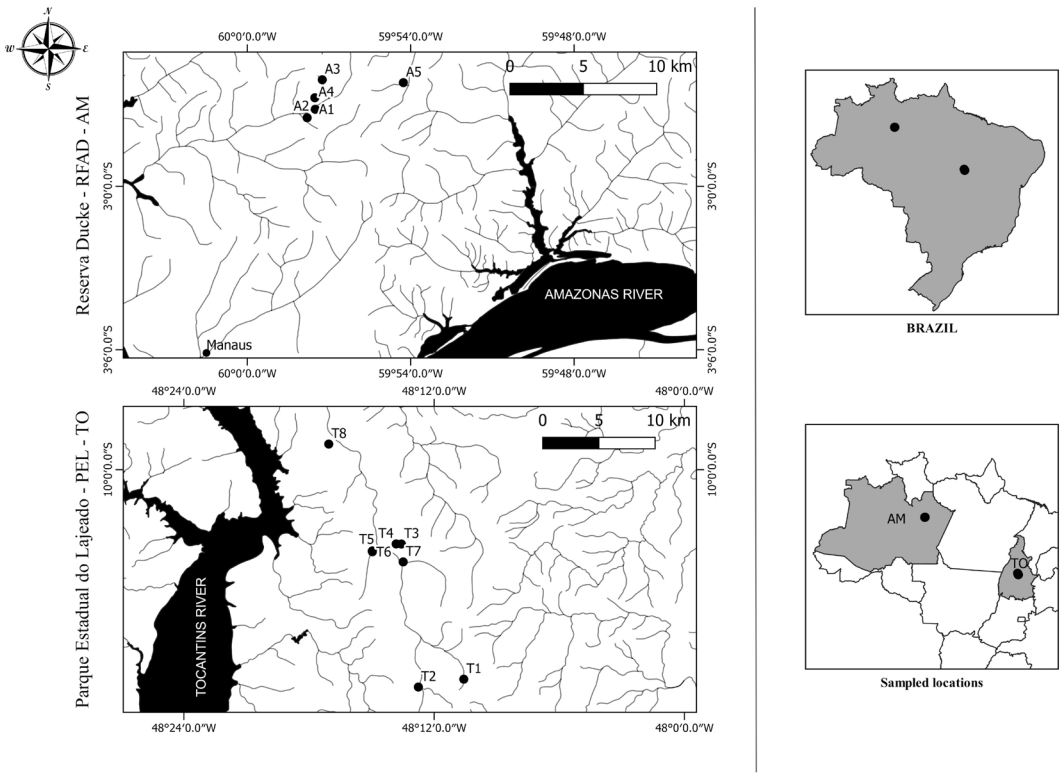

Figure 1. Map of the sampling sites of *Stenochironomus* (Diptera: Chironomidae) in low-order streams in the Adolpho Ducke Forest Reserve in the state of Amazonas (Amazon Forest Biome) and at the Lajeado State Park (LSP) in the state of Tocantins, (Cerrado Biome) Brazil.

in the substrate available (submerged leaves and trunks). A D-shaped dip net (0.500 mm mesh and 0.465 m^2 area) was used to collect leaves from the benthonic stock or trunks at an advanced stage of decomposition, which were triaged in the field for the collection of larvae miners of aquatic insects of the genus *Stenochironomus* (Diptera: Chironomidae). Larvae were identified according to Trivinho-Strixino (2014) by specialists in the field. Each individual larvae was transferred to a sterilized Eppendorf flask containing 1 mL sterile saline solution and stored for 2 to 4 h in ice until processing at the laboratory.

2.2. Fungus isolation and purification

At the laboratory, the larvae were submitted to disinfection of the surface with 70% ethanol for 30 s and washed in sterile water. Dissection was performed using sterilized instruments and a stereo microscope, and the digestive tract (DT) of the mining insect was carefully removed, transferred to a tube and fragmented with the help of a previously sterilized Teflon grinder. Sterile saline solution was added and then a 0.1 mL aliquot was seeded in culture medium on a Petri dish (90 mm diameter) containing potato-dextrose agar (PDA) supplemented with 100 µg/mL chloramphenicol in triplicates. The dishes were incubated at 25 °C for up to 60 days. The fungal isolates obtained were individually transferred to the Petri dishes containing PDA and incubated at 25±3 °C for seven days for purification. After the pure fungal cultures were obtained, they were preserved using the Castellani method (Capriles et al., 1989) and then deposited in the Coleção de Culturas Microbianas Carlos Rosa of the Universidade Federal do Tocantins, Tocantins, Brazil.

2.3. Fungal identification

Each individual isolated was cultivated in a Petri dish containing PDA for 24-48 h and then transferred to malt extract broth (3%), which is a richer medium that stimulates growth, in a rotating shaker (150 rpm) at room temperature for seven days. Next, approximately 40 mg of mycelium were collected for DNA extraction with the Wizard™ Genomic DNA Purification Kit (Promega, USA), following the modified protocol by Burghoorn et al. (2002).

The DNA was analyzed in a NanoDrop 2000 (Thermo Scientific, Brazil) spectrophotometer. Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990) were employed for amplification of the ITS (Internal Transcribed Spacer) region of rDNA (~600 bp) following the amplification conditions proposed by (Santos et al. 2016). The amplified ITS fragments were submitted to electrophoresis in 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 molecular weight marker.

The amplified products were sequenced in both directions using the same PCR starters in an ABI 3500 XL (Life Technologies, USA) automated sequencer according Sanger et al. (1977) using a BigDye Terminator v3.1 sequencing kit (Life Technologies, USA). Sequencing was performed by the company Myleus Biotechnology (http://myleus.com/). Additionally, the amplification of the genes β-tubulin (Bt2a and Bt2b) was used for species of fungi with low intraspecies variation according to the protocols established by Gonçalves et al. (2013).

All sequences were compared with sequences deposited at the GenBank database using a local alignment algorithm for nucleotide sequences BLAST (Basic Local Alignment Search) (Altschul et al., 1990) of the NCBI (National Center for Biotechnology Information) and at the CBS (*Centraalbureau voor Schimmelcultures* Fungal Biodiversity Centre) database (Westerdijk Fungal Biodiversity Institute, 2022). The alignment of the sequences of possible new species with phylogenetically close species and the construction of phylogenetic trees for confirmation of species were carried out in the software MEGA 3.1 (Kumar et al., 2004) using the Neighbor Joining method with 1,000 times bootstrap.

2.4. Diversity, richness, and distribution

Diversity was measured via the indices of Simpson (1-D), Shannon (H '), Margalef, and Chao-1, which were calculated for the number of sampled larvae from streams in the Amazon Forest and Cerrado. The larvae were considered the sampling unit, being the biomes, and not the streams, the variable of interest. β-diversity was calculated by the Whittaker index, which measures the substitution in composition of species between substrates and/or biomes. The indices were calculated with 95% confidence using the software PAST version 4.01 (Hammer et al., 2001).

The abiotic variables (altitude, temperature, dissolved oxygen, pH, conductivity, turbidity, width, depth, and current velocity) of each stream were measured using a multiparametric probe and a principal component analysis (PCA) was generated, revealing the most influential environmental variables in streams in each biome.

2.5. Xylanolytic and cellulolytic screening of the fungal community

The entire fungal community of the DT of *Stenochironomus* larvae was tested for the production of xylanase and cellulase through screening in solid medium containing xylan or carboxymethylcellulose (CMC) as the only carbon source. Enzyme production was assessed via the growth of the strain on a dish and the revelation of hydrolysis halo using Congo red stain.

The strains were reactivated in PDA and then repeated in triplicate in medium with xylan (Xylan, Beechwood purified) or carboxymethylcellulose (CMC) and trace compounds composed of $C_6H_8O_7 \cdot H_2O$ 50; $ZnSO_4 7H_2O$ 50; $Fe(NH_4)_2(SO_4)26H_2O$ 10; $CuSO_4 \cdot 5H_2O$ 2.5; $MnSO_4 \cdot H_2O$ 0.05; H_3BO_3 0.05; $Na_2MO\ 42H_2O$ 0.05; Salt solutions: $Na_3C_6H_5O_7 \cdot 5H_2O$ 150; KH_2PO_4 250; NH_4NO_3 100; $MgSO_4 \cdot 7H_2O$ 10; $CaC_{l2} \cdot 2H_2O$ 5 $g.L^{-1}$ and biotin (0.1 mg mL^{-1}) 5 mL; 0.2 mL chloroform (Vogel, 1956). The hydrolysis halos were revealed according to Maijala et al. (1991), where the dishes were inundated with 10 mL aqueous solution of 0.3 g/L Congo red stain (30 min) and discolored with a 1.0 mol/L sodium chloride solution (15 min).

After staining with Congo red, the fungi that exhibited lighter coloration in the selective medium (hydrolysis halo) were considered as producers of xylanase or cellulase. A digital caliper was used to measure the diameter of the colonies and of the halos and thus calculate the enzymatic index (EI) given by the ratio between those two measures.

3. Results

3.1. Fungal communities

Thirty-five *Stenochironomus* larvae were collected (12 in the Amazon Forest and 23 in the Cerrado) (Table 1). Of the larvae collected in the Cerrado, 13 dwelled in

Table 1. Number of insects sampled, richness, and diversity of species of filamentous fungi in the digestive tract of *Stenochironomus* (Diptera: Chironomidae) larvae in streams of the Amazon Forest and Cerrado in the north of Brazil.

Biome	Substrate	Stream	Geographical coordinates	Total Insects Collected	Richness (S)	Margalef	Simpson (1 – D)	Shannon (H)	Chao – 1
Amazon Forest	Trunk	A1	02°57'10.30"S; 59°57'30.10"W	N=3	4	6.06	0.95	2.96	54
		A2	02°57'29.00"S; 59°57'48.00"W	N=3	4				
		A3	02°56'05.30"S; 59°57'14.79"W	N=2	8				
		A4	02°56'44.97"S; 59°57'31.16"W	N=3	3				
		A5	02°56'11.67"S; 59°54'16.00"W	N=1	1				
				N=12	**20***				
Cerrado	Trunk	T1	48°10'34.70"O; 10°10'02.30"S	N=3	4	4.15	0.9	2.44	35.5
		T2	48°12'45.40"O; 10°10'02.30"S	N=2	4				
		T4	48°13'49.30"O; 10°03'33.40"S	N=1	3				
		T5	48°14'58.00"O; 10°03'53.60"S	N=1	1				
		T6	48°14'57.70"O; 10°03'55.90"S	N=1	1				
		T7	48°13'29.10"O; 10°04'25.00"S	N=2	3				
				N= 10	**13***				
	Leaf	T1	48°10'34.70"O; 10°10'02.30"S	N=3	5	5.3	0.93	2.75	68.5
		T2	48°12'45.40"O; 10°10'02.30"S	N=1	1				
		T3	48°13'34.30"O; 10°03'33.60"S	N=1	1				
		T4	48°13'49.30"O; 10°03'33.40"S	N=2	2				
		T6	48°14'57.70"O; 10°03'55.90"S	N=2	2				
		T7	48°13'29.10"O; 10°04'25.00"S	N=1	1				
		T8	48°17'03.20"O; 09°58'46.30"S	N=3	4				
				N=13	**16***				
			Total richness	**N= 35**	**41***				

* Total number of taxa-excluding repetitive counting of each taxon

leaves of plants fallen into the streams and ten lived in submerged trunks. In Amazonian streams, larvae were only collected from submerged trunks. The presence of fungi was verified in 100% of the individuals collected in both sites and the mean fungal population ranged from 1.7 to 79 CFU/DT between the biomes. The fungi found in the DT of *Stenochironomus* larvae in larvae in the sampled sites belonged to 41 taxa (total richness, Table 1).

The communities of fungi in DT of *Stenochironomus* larvae showed similar values of species richness (S) and diversity (Table 1), although the number of larvae sampled were nearly twice as much in the Cerrado than in the Amazon Forest. The Shannon diversity index was lower in the DT of larvae in the Cerrado than in the Amazon Forest (Table 1). Given the two different substrates of the Cerrado, it was observed that the richness (S) was higher in the DT of larvae from leaves than from trunks, which also held true for the diversity index. The Chao - 1 index, which reveals sampling sufficiency, was greater than the actual richness and higher in the DT of larvae from leaves in the Cerrado than in trunks and Amazon Forest.

3.2. Distribution and diversity

From the DT of *Stenochironomus* larvae sampled, 58 fungi were isolated belonging to 41 taxa and 13 genera (Table 2). The genera that exhibited greater richness (S) of taxa were *Penicillium* (S=11), *Cladosporium* (S=10), *Trichoderma* (S=6), and *Aspergillus* (S=4), accounting for 75% of the number of taxa in the samples. Those genera, with the exception of *Trichoderma*, occurred in both areas. Among the species, only *Cladosporium endophytica* occurred in all environments and substrates (Table 2).

The DT of larvae from trunks in streams of the Amazon Forest hosted 20 fungal taxa belonging to 11 genera (Table 2). In this biome the genera with the highest number of taxa were *Trichoderma* (S=6) and *Cladosporium* (S=4), the former occurring exclusively in the DT of larvae in the Amazon Forest, besides six other genera (*Acremonium*, *Alternaria*, *Clonostachys*, *Neopestalotiopsis, Pyrrhoderma and Talaromyces*). *Trichoderma* was represented by six taxa, displaying high richness in that environment. The most frequent species in Amazon Forest streams were *Cladosporium endophytica*, *Cladosporium kenpeggii*, and *Penicillium shearii*. Other genera exclusive to samples in Amazon Forest streams were represented by singletons (Table 2). Although most taxa occurred exclusively in that environment, two were shared with the fungal community in the DT of larvae from trunks in streams of the Cerrado: *Cladosporium kenpeggi* and *Penicillium shearii*.

From the DT of larvae from trunks in streams of the Cerrado, 13 fungal taxa were identified belonging to three genera: *Aspergillus* (S=3), *Cladosporium* (S=3), and *Penicillium* (S=7), hence, the genus *Penicillium* was the most abundant (Table 2). From the DT of leaf mining larvae in stream in the Cerrado, 16 fungal taxa were identified belonging to six genera. The most frequent genera were *Cladosporium* (S=6) and *Penicillium* (S=6). Of those, four taxa also occurred in the DT of larvae from trunks in streams in the Cerrado: *Cladosporium endophytica*, *Aspergillus sydowii*, *Penicillium camponotum*, and *Penicillium paxilli*. The most frequent species in that substrate and environment was *Penicillium citrinum*.

Of the species found in larvae collected in the streams of both sites (Amazon Forest and Cerrado), *Cladosporium endophytica*, *Cladosporium kenpeggii*, and *Penicillium shearii* showed the highest total frequency of occurrence (6.9%, 5.2%, and 5.2%, respectively). Irrespective of the substrate, 22 taxa occurred only in the DT of larvae from the Cerrado and 15 as singletons. *Penicillium mallochii* and *Penicillium paxilli* had higher frequency of occurrence (6.9% each). Seventeen taxa were isolated only in the DT of larvae from trunks in the Amazon Forest, all occurring as singletons.

Seven taxa of *Cladosporium* could not be identified by ITS, two of which from the DT of larvae in the Amazon Forest and five in the Cerrado (Table 2). One taxon of *Trichoderma* and one of *Penicillium* isolated in the DT of larvae on trunks in Amazon Forest streams and trunks in the Cerrado, respectively, also could not be identified at the species level. The β-diversity calculated using the Whittaker index was greater between the DT of larvae from Cerrado leaves and Amazonian trunks, characterizing higher heterogeneity between those biomes and substrates (Table 3).

Differences in the physicochemical parameters of the water in the streams sampled were detected between sites in Amazon Forest and-sites in Cerrado (Table 4). The mean altitude of Amazon Forest streams was around 54 m, while those in the Cerrado had a mean altitude of 506 m. The mean temperature of the streams in the Amazon Forest was higher than in Cerrado by about 2 °C. The waters in the Amazon Forest streams were acidic and had higher electric conductivity and depth compared to the Cerrado streams.

The PCA showed that the streams in each biome were grouped and, together, components 1 and 2 explained 73.99% of the variation (Figure 2). The most influential environmental parameters in the Amazonian streams were temperature and conductivity, whereas the streams of the Cerrado were most influenced by altitude, pH, turbidity, DO, and current velocity.

3.3. Enzymatic potential

The fungal isolates from the DT of *Stenochironomus* larvae showed an enzymatic profile responsive to xylan and cellulose. Among the isolated from the Amazon Forest trunks, 60.9% exhibited xylanolytic activity and 52.2% exhibited cellulolytic activity (CMCase) (Figure 3A). In this biome, the isolates that had the highest enzymatic indices (EI) when tested for xylanase were *Cladosporium endophytica* MN577266, *Acremonium fusidioide* MN577262 and *Cladosporium kenpeggii* MN577256 (Table 2). For cellulase, the isolate with the highest EI was *Cladosporium kenpeggii* MT508668.

Among the isolates from the DT of *Stenochironomus* larvae on trunks of Cerrado streams, the same percentage of enzyme activity was observed both for cellulase and xylanase (77.8%) (Figure 3B). In this biome, the following isolates stood out for having the highest EI for xylanase: *Cladosporium endophytica* MN577273, *Cladosporium* sp4 T9STC2, and *Penicillium shearii* MN577260 (Table 2).

Table 2. Fungal taxa obtained from the digestive tract of *Stenochironomus* (Diptera: Chironomidae) larvae of the trunks and leaves from streams of the Amazon Forest and Cerrado in the north of Brazil identified by comparison with the corresponding BLASTn sequences from the NCBI GenBank database and their respective enzymatic indices (EI).

BIOME	CODE*	Taxa	EI (Xylanase)	EI (Cellulase)	No. GenBank Accession	Number of basis pars analyzed	Query cover %	Identity (%)	My GenBank number
			Mean ± Dev	Mean ± Dev					
TRUNK AMAZON FOREST	A5STA1	*Acremonium fusidioide*	2.42 ± 0.05	1.61 ± 0.08	NR_130687.1	385	100	98.7	MN577262
	A4STB1	*Alternaria alstroemeriae*	0	0	NR_163686.1	508	98	100	MN577251
	A2STA3	*Aspergillus costaricaensis*	1.12 ± 0.12	0.00	MH862988.1	476	100	100	MN954942
	A4STA5	*Cladosporium endophytica*	1.89 ± 0.3	1.96 ± 0.3	NR_158360.1	468	100	100	MN577241
	A2STB3	*Cladosporium endophytica*	3.6 ± 0.23	1.73 ± 0.38	NR_158360.1	446	100	99.1	MN577266
	A4STB7	*Cladosporium kenpeggii*	0.67 ± 0.03	2.18 ± 0.03	KY646222.1	411	100	99.27	MT508668
	A5STA4	*Cladosporium kenpeggii*	2.21 ± 0.27	1.75 ± 0.03	KY646222.1	456	100	99.56	MN577256
	A1STA4	*Cladosporium* sp1***	1.46 ± 0.33	1.34 ± 0.00					
	A5STB2	*Cladosporium* sp2***	1.41 ± 0.59	1.92 ± 0.66					
	A4STA4	*Clonostachys rosea*	1.15 ± 0.04	1.03 ± 0.01	MH854911.1	410	99	99.76	MN577252
	A1STC2	*Fusarium luffae*	0	1.03 ± 0.03	MK280807.1	433	100	100	MN577248
	A4STB2	*Neopestalotiopsis ellipsospora*	0	1.09 ± 0.01	KM199343.1	434	99	100	MN577253
	A1STA6	*Penicillium chermesinum*	1.82 ± 0.03	0.00	MH861332.1	259	100	100	MN577265
	A2STC3	*Penicillium shearii*	1.29 ± 0.28	0.00	AF033420.1	493	100	99.6	MN577249
	A2STC2	*Penicillium shearii*	1.42 ± 0.2	1.16 ± 0.07	AF033420.1	477	100	99.58	MN577250
	A5STC4	*Pyrrhoderma* sp***	0	0					
	A2STA1	*Talaromyces stolii*	1.6 ± 0.1	1.14 ± 0.06	NR_111781.1	473	100	100	MN577254
	A4STA1b	*Trichoderma asperellum*	0.86 ± 0.34	0	MH021852.1	259	100	100	MN954939
	A4STB5	*Trichoderma breve*	0	0	NR_154574.1		99	99.57	MT508669
	A1STB1	*Trichoderma inhamatum*	0	0	MH861135.1	370	100	100	MN954947
	A8STA1	*Trichoderma* sp***	0	0					
	A4STA1a	*Trichoderma spirale*	0	0	NR_077177.1	505	100	99.6	MN577239
	A4STA2	*Trichoderma yunnanense*	0	0	NR_134419.1	309	100	100	MN954943
TRUNK CERRADO	T5STA2	*Aspergillus niger*	1.16 ± 0.01	1.09 ± 0.03	AY373852.1	615	100	100	MN577245
	T6STA2	*Aspergillus niger*	1.51 ± 0.04	0.00	AY373852.1	469	100	100	MN954948
	T9STA2	*Aspergillus nomius*	0.77 ± 0.27	0.00	NR_121218.1	432	100	100	MN954946
	T5STA5	*Aspergillus sydowii*	1.09 ± 0.03	1.9 ± 0.08	NR_131259.1	448	100	100	MN577244
	T3STC1	*Cladosporium endophytica*	3.12 ± 0.02	1.88 ± 0.12	NR_158360.1	435	100	100	MN577273
	T2STA1	*Cladosporium kenpeggii*	1.98 ± 0.1	1.71 ± 0.02	KY646222.1	433	100	100	MN577259
	T9STC2	*Cladosporium* sp4***	2.56 ± 0.33	1.51 ± 0.11					
	T9STC3	*Penicillium mallochii*	1.41 ± 0.08	1.34 ± 0.04	NR_111674.1	465	99.57	99	MN954945
	T3STC2	*Penicillium cairnsense*	1.59 ± 0.2	1.61 ± 0.23	NR_121508.1	341	100	99.12	MN577242
	T2STB4	*Penicillium camponotum*	1.4 ± 0.24	1.39 ± 0.07	NR_158823.1	449	100	98.66	MN577268
	T7STA1	*Penicillium exsudans*	1.24 ± 0.03	1.39 ± 0.02	NR_153273.1	305	100	100	MN577247
	T3STA5	*Penicillium mallochii***	0	1.42 ± 0.06					
	T3STA4	*Penicillium mallochii***	0	1.54 ± 0.04					
	T5STA8	*Penicillium paxilli*	0	1.31 ± 0.09	MH856391.1	305	100	100	MN577267
	T2STB5	*Penicillium paxilli*	0	0	MH856391.1	472	100	100	MN577255
	T3STA3	*Penicillium paxilli*	1.38 ± 0.19	1.5 ± 0.13	MH856391.1	165	100	100	MN577261
	T2STC1	*Penicillium shearii*	2.14 ± 0.02	0	MH856346.1	252	100	100	MN577260
	T3STA2	*Penicillium* sp***	1.21 ± 0.25	1.16 ± 0.06					

*Code of the Collection of Microbial Cultures Carlos Rosa – Laboratory of Environmental Microbiology and Biotechnology of the Federal University of Tocantins; **Identified with β-tubulin and not deposited; *** Probable new species. Identification to species levels is not possible using the bar-coding primers.

Table 2. Continued...

BIOME	CODE*	Taxa	EI (Xylanase)	EI (Cellulase)	No. GenBank Accession	Number of basis pars analyzed	Query cover %	Identity (%)	My GenBank number
			Mean ± Dev	Mean ± Dev					
LEAF CERRADO	T2SA1	*Aspergillus sydowii*	2.69 ± 0.39	1.92 ± 0.08	NR_131259.1	413	100	99.76	MN577240
	T2SB6	*Cladosporium endophytica*	1.98 ± 0.31	1.82 ± 0.29	NR_158360.1	423	100	99.05	MN577269
	T2SB5	*Cladosporium halotolerans*	2.26 ± 0.25	1.95 ± 0.26	KJ596569.1	266	100	100	MN577258
	T7SC1	*Cladosporium* sp3***	0	2.06 ± 0.07					
	T9SB1	*Cladosporium* sp5***	1.56 ± 0.27	1.89 ± 0.03					
	T10SB2	*Cladosporium* sp6***	1.31 ± 0.03	1.37 ± 0.08					
	T10SB3	*Cladosporium* sp7***	0	1.39 ± 0.09					
	T10SA1	*Curvularia lunata*	0	1.1 ± 0.04	LT631353.1	358	100	100	MN577272
	T5SB7	*Fusarium pseudonygamai*	0	0	MH862656.1	385	100	100	MN577270
	T5SA4	*Paraphaeosphaeria arecacearum*	0	0	NR_145166.1	379	100	99.74	MN954941
	T2SB4	*Penicillium camponotum*	1.09 ± 0.17	0	NR_158823.1	512	100	98.63	MN577257
	T4SC1	*Penicillium citrinum*	1.12 ± 0.07	1.54 ± 0.02	MH856132.1	239	100	100	MN577264
	T2SC6	*Penicillium mallochii*	1.07 ± 0.1	0.00	NR_111674.1	470	100	99.58	MN954940
	T7SB1	*Penicillium multicolor*	1.13 ± 0.3	1.22 ± 0.13	JN799647.1	422	100	99.29	MN577246
	T3SB2	*Penicillium paxilli*	0	1.39 ± 0.1	MH856391.1	366	100	100	MN954944
	T10SC1	*Penicillium quebecense*	1.11 ± 0.03	1.11 ± 0.04	NR_121507.1	304	100	100	MN577271
	T5SA3	*Pennicillium citrinum*	1.94 ± 0.27	1.54 ± 0.29	MH856132.1	302	100	100	MN577243

*Code of the Collection of Microbial Cultures Carlos Rosa – Laboratory of Environmental Microbiology and Biotechnology of the Federal University of Tocantins; **Identified with β-tubulin and not deposited; *** Probable new species. Identification to species levels is not possible using the bar-coding primers.

Table 3. β-diversity of the filamentous fungi communities in the DT of *Stenochironomus* (Diptera: Chironomidae) larvae in streams of the Amazon Forest and Cerrado in the north of Brazil.

	Trunk Forest	Leaf Cerrado	Trunk Cerrado
Trunk Forest	-	0.944	0.818
Leaf Cerrado		-	0.655
Trunk Cerrado			-

Table 4. Physicochemical parameters of sampled streams of Amazon Forest and Cerrado in the north of Brazil.

Parameters*	Amazon Forest	Cerrado
Altitude (m)	54 ± 18.05	506 ± 99.5
Temperature (°C)	25.32 ± 0.4	23.02 ± 0.75
Turbidity	0.87 ± 0.64	1.77 ± 0.87
Dissolved oxygen (mg L^{-1})	5.95 ± 0.36	7.6 ± 1.83
pH	4.54 ± 0.12	6.03 ± 0.61
Electrical conductivity (mS cm^{-1})	19.42 ± 3.68	5.99 ± 3.43
Width (m)	1.98 ± 0.65	2.23 ± 0.87
Depth (m)	0.28 ± 0.15	0.20 ± 0.11
Current velocity (m s^{-1})	0.11 ± 0.05	0.22 ± 0.16

*Arithmetic mean ± standard deviation.

For cellulolytic activity, *Aspergillus sydowii* T5STA5 and *Cladosporium endophytica* MN577273 stood out with the highest EI (Table 2).

Of the fungi found in the DT of *Stenochironomus* larvae on leaves of streams in the Cerrado, 62.5% exhibited enzyme activity for xylanase and 81.2% exhibited cellulolytic activity (Figure 3C). In this biome and substrate, *Aspergillus sydowii* T2SA1 and *Cladosporium halotolerans* MN577258 stood out for having the highest EI for xylanase (Table 2). In the production of cellulase, *Cladosporium* sp3 T7SC1, *Cladosporium halotolerans* MN577258 and *Aspergillus sydowii* MN577240 reached the highest enzymatic indices (Table 2).

4. Discussion

4.1. Fungi from the DT of Stenochironomus larvae

A diverse community of fungi dominated by ascomycetes and occurring as singletons was found in the DT of *Stenochironomus* larvae in streams of the Amazon and Cerrado in Brazil. In both biomes, the genera *Penicillium*, *Cladosporium*, and *Aspergillus* were prevalent in the fungal community. They differ from the fungal community found in the DT of larvae of *Phylloicus* in streams of the same locations in Cerrado and Amazon Forest biomes, in which the genera *Penicillium*, *Pestalotiopsis*, and *Trichoderma* prevailed (Santos et al., 2018).

Several authors have reported the abundance of those genera associated with the DT of insects (Belmont-Montefusco et al., 2020a; Teixeira et al., 2022; Romão et al.,

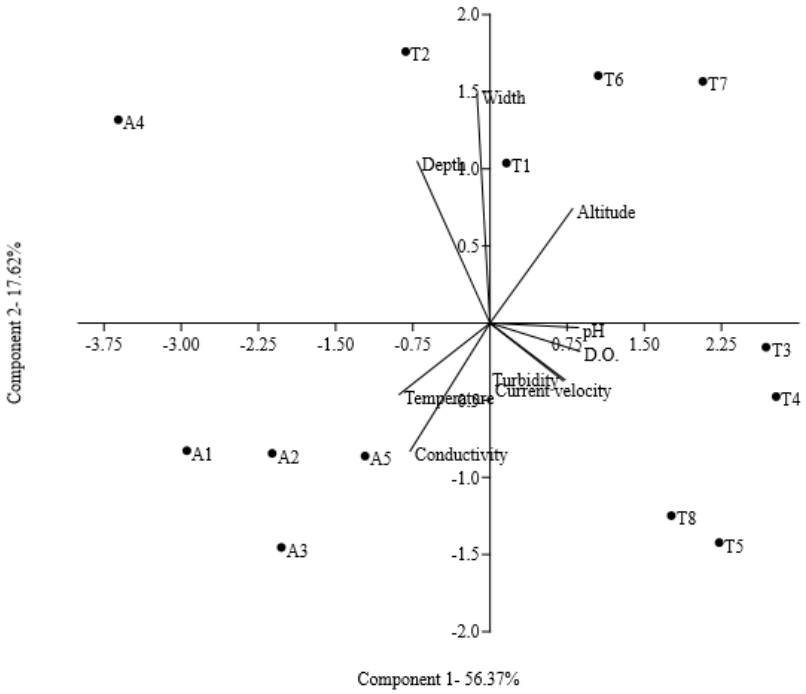

Figure 2. Principal component analysis (PCA) of the physicochemical parameters of the streams sampled in the Amazon Forest - Amazonas (A) and Cerrado - Tocantins (T) in the north of Brazil.

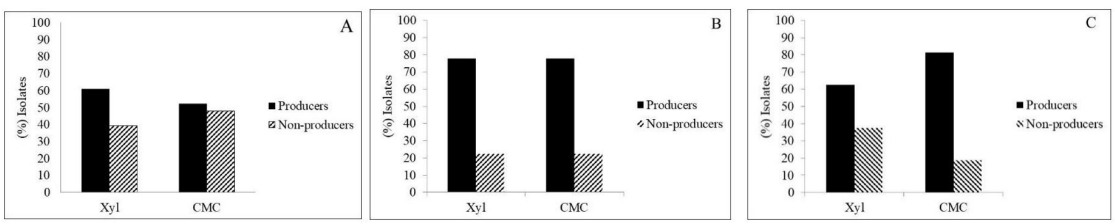

Figure 3. Percentage of fungal isolates from the DT of *Stenochironomus* (Diptera: Chironomidae) from trunks in Amazon Forest (A), trunks in Cerrado (B) and leaves in Cerrado (C) producers and non-producers of xylanase (Xyl) and cellulase (CMCase).

2024). *Penicillium* and *Aspergillus*, which had the highest occurrence in the DT of larvae from trunks in Cerrado streams, were also frequent in association with mosquito larvae (Diptera: Culicidae) of several types of breeding grounds in municipalities of the Brazilian Amazon (Pereira et al., 2009). Fungi of the genus *Penicillium* have been reported in *Dactylopius* (Hemiptera: Dactylopiidae) as one of the most frequent genera (Vera-Ponce de León et al., 2016).

Diptera larvae may acquire some of their endosymbionts from the environment itself and the structure of the mycobiome may vary greatly due to the specialization of the diet, life cycle, location, and substrate of occurrence (Alves Junior et al., 2019). It is possible that fungi hosted in the DT of *Stenochironomus* larvae in rivers of the Amazon Forest and Cerrado may be more related to the plant material ingested by those larvae, considering the genera that were repeatedly found in their DTs and their potential for enzyme production, e.g., *Penicillium*, *Aspergillus*, and *Cladosporium*.

4.2. Influence of the biome on species distribution

Although the results have indicated a common mycobiome between the biomes and substrates studied, some differences were made evident. For example, the DT of larvae on leaves in Cerrado streams had the genus *Cladosporium* as the most frequent while fungi of the genus *Penicillium* were the most frequent in the DT of larvae on trunks in the same environment. The genus *Trichoderma* was exclusively found in the DT of larvae on trunks in Amazon Forest streams. Such result indicates that, although there may be a mycobiome typical of the DT of aquatic larvae that feed on plant material, the occurrence of certain taxa may be influenced both by the differences

between biomes and the substrates that are sources of fungi for the DT of larvae.

β-diversity was high among the fungal community of the DT of *Stenochironomus* in the two biomes sampled, reinforcing the idea that biome has a major influence on fungus composition in the diet of those larvae. In caterpillars of the species *Mamestra brassicae* (Lepidoptera: Noctuidae), Hannula et al., (2019) showed that microbiomes reflect their environment (soil) and, since soil microbiomes vary temporally and spatially, that may also impact the microbiome of the caterpillar.

The vegetation of each biome has a differentiated phytochemical composition, and it is also likely that the endophytic and epiphytic mycobiota of such plant species is geographically diverse. Thus, knowing the variations in physiognomy of the riparian vegetation in the Amazon Forest and the Cerrado, and that different plant species make up such riparian areas, the mycobiome is expected to reflect those differences. Other work has shown that there are different fungal communities on the leaves of different plant species in streams (Medina-Villar et al., 2015). Therefore, it should be expected that biogeographical factors may determine the intestinal microbiota of the larvae since larvae depend on plant substrates, that provide not only the nutrients but also part of the mycobiome for them.

Another hypothesis that explains the differences in the fungal community between biomes is that the contribution to the food stocks available to the larvae in streams may vary geographically and seasonally. Tonin et al. (2017) found consistent evidence of seasonality in the fall of leaf litter in the Amazon and Cerrado. According to those authors, the time of input of plant material is different for each of those biomes, which certainly influences the availability of food for larvae in those streams and, consequently, the structure of the mycobiome associated.

Although there was a greater sampling effort in Cerrado streams, the richness index (Margalef) and diversity indices (Shannon and Simpson) were higher in the DT of larvae in Amazonian streams (Table 1). The principal component analysis (PCA) showed differences in the environmental parameters that influenced each stream, which may have favored the distinction between the diversity and richness indices. The most influential environmental parameters in the Amazonian streams were temperature and conductivity. Leal et al. (2016) when studying multiscale assessment of human-induced changes to instream habitats in Amazonia found that catchment deforestation resulted in consistently warmer streams and conductivity may suffer from influences of road crossings. The Adolpho Ducke Forest Reserve is a neighbor to the city of Manaus, which may be a driver of water characteristics of streams. As Lima et al. (2022) showed, water temperature and other environmental variables affect functional feeding groups of aquatic insects. The streams of the Cerrado were most influenced by altitude, pH, turbidity, DO, and current velocity. In this biome, streams are prone to intermittence and DO and current velocity changes may drive aquatic insect patterns (Valente-Neto et al., 2020). Differences in ecological variables in the biomes, besides contributing to a distinct plant composition, may influence the structures of microbial communities as they interfere on the chemical composition of soils and water (Shi et al., 2011).

4.3. Influence of the substrate on species distribution

In Cerrado streams, differences were found in the composition of the fungal community in the DT of *Stenochironomus* mining larvae of leaves and trunks (Table 1). That indicates the substrate may influence the composition of the mycobiome in the DT of larvae of *Stenochironomus*. If the larvae acquire the DT mycobiome through their diet, that difference may be due to the different fungal community on leaves and trunks. Different plant tissues are usually colonized by different fungal communities (Martins et al., 2016), suggesting that some endophytic species may preferably develop in certain types of tissue.

The *Stenochironomus* larvae have adaptations that favor their survival under the poor nutritional conditions of wood (Anderson and Cummins, 1979). However, evidence in field observations shows that the diversity of foliar substrates is greater than of trunks and that its availability is greater in all stretches of tropical streams. Although there is seasonal variation, some works have shown that, in lotic ecosystems, particularly in low-order streams, leaf detritus are the main form of energy input (Abelho, 2009; Fiori et al., 2016). It was seen that the fungal community associated with larvae on trunks in the Amazon Forest was more xylanolytic than cellulolytic (XYL=60.9% and CMC=52.2%) (Figure 3A). The fungal community associated with larvae on leaves in the Cerrado was more cellulolytic (XYL=62.5% and CMC=81.2%) (Figure 3C). Corroborating such result, Belmont-Montefusco et al. (2020b), who assessed the cellulolytic activity of fungi associated with the DT of larvae of *Stenochironomus* from leaves in Amazonian streams, found that 86.6% of the isolates were producers of that enzyme. It is supposed, therefore, that one of the adaptations of *Stenochironomus* larvae is related to obtaining fungi that aid in the digestion of more refractory materials such as trunks or labile such as leaves, according to their availability. It is possible that such adaptations help obtain food from the different substrates available in the environment.

Another variable that may influence the fungal composition is the phase of the cycle and larval age. Although information on details of the life cycle of *Stenochironomus* is still scarce, it is known that the duration of the development stages of chironomids varies as a function of environmental characteristics. Short periods occur mainly when the temperatures are higher, in the range of 25 °C, when the duration of the larval period is, on average, ten days (Trivinho-Strixino, 2014). That condition is very close to those found at the sites sampled, which favor a shorter cycle and results in not enough time for the colonization of fungal species in the DT.

The number of singletons was significant, both in the Amazon Forest and in the Cerrado, indicating that few species had actually colonized the DT of that host. Although there is no study on food preferences of larval instars of Diptera, one may suppose that different instars may have preferences significant enough to explain the

diversity in taxa as well as the low frequencies of fungal species. Teixeira et al. (2022), when studying the DT fungal community of aquatic shredder larvae, recorded a high occurrence of singletons, consequently resulting in a high diversity of fungal species. The authors attributed this to factors such as the influence of different environmental conditions, diets of species, and life cycles of the insects.

The results point to a core mycobiome common to different biomes and substrates, suggesting that, although there are differences in substrates, some fungal taxa may be selected by the larvae. It is possible that the intestine of the host selects its own mycobiome according to its needs. Shukla et al. (2018) observed that larvae of the *Nicrophorus vespilloides* beetle (Coleoptera: Silphidae) may turn an initially toxic food into a nutritive one by selecting fungi. Thus, despite the influence of the biome and substrates in the mycobiome structure, it is possible that the DT of larvae of *Stenochironomus* established an environmental filter that selects its own mycobiome, opting for some fungal taxa in detriment of others, resulting in a core community independent of sites or dietary substrates.

4.4. Role of mycobiome in the DT of larvae of Stenochironomus

Many studies have focused on understanding the several roles of microbiome in insect larvae and adult insects. It is known that the composition of intestinal microbiomes in adult insects is structured via the diet and innate immune systems (Stefani et al., 2016; Vera-Ponce de León et al., 2016; Wu et al., 2020). The present results show a diverse fungal community with potential for the breakdown of xylan and cellulose. Although most of the isolates had exhibited activity for the production of both enzymes, the evaluation of enzyme activity per substrate showed that the isolates from the DT of larvae on trunks in the Cerrado were more active for the production of xylanase while the isolates from the DT of larvae on leaves head greater enzyme activity for cellulase. It is known that the production of enzymes that break down the cell wall is regulated at the transcriptional level of filamentous fungi, which ensures the enzymes are produced under conditions in which the fungus needs to use plant polymers as sources of energy and carbon (Aro et al., 2005). In addition, evidence from extensive works indicate that the intestinal environment of Diptera larva is abundant in endosymbiont organisms and that some produce enzymes capable of metabolizing lignocellulosic material ingested as food (Pennington et al., 2016; Chen et al., 2016). Such result may indicate that the fungi, besides being part of the diet of the larvae, are adapted to the substrate since they can produce enzymes required to the processing of the food of larvae, as other works have shown (Aro et al., 2005; Santos et al., 2018; Shelomi et al., 2019).

5. Conclusions

The present study showed a significant diversity of the fungal community associated with the DT of *Stenochironomus* in streams of the Amazon and Cerrado in the north of Brazil. Evidence indicates that both the biomes and the substrates may influence the structure of the mycobiome of *Stenochironomus* larvae and for this reason, the fungal diversity was higher in larvae from Amazonian streams. This study contributes to the knowledge of the ecological roles in the interactions that occur between fungi and larvae of insects of the genus *Stenochironomus*, as we provide evidence of the production of enzymes by the fungi that aid in digestion of the substrates used as food by those larvae. The presence of little-explored fungal species, which herein exhibited interesting results for the production of enzymes such as xylanase and cellulase, indicates that there are good chances that new studies further the knowledge in the biotechnological application of the enzymatic potential of those species.

Acknowledgements

The authors thank the National Council for Scientific and Technological Development (CNPq) for funding the project Edital Chamada MCTI/CNPq/FNDCT Ação Transversal - Redes Regionais de Pesquisa em Ecossistemas, Biodiversidade e Biotecnologia No. 79/2013; the staff of the Laboratory of Environmental Microbiology and Biotechnology of the Federal University of Tocantins for the technical support; Dr. Taídes Tavares dos Santos, who helped us with sequencing the fungal samples; the staff of the Laboratory of Citotaxonomy and Aquatic Insects – LACIA of the Instituto Nacional de Pesquisas da Amazônia - INPA, especially Dr Neusa Hamada, Dr Ruth Leila Ferreira-Kepler and Dr Gizelle Amora Gusmão for the fieldwork on collection and identification of *Stenochironomus* larvae.

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