UNIVERSIDADE FEDERAL DO TOCANTINS
CAMPUS UNIVERSITÁRIO DE PALMAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E BIOTECNOLOGIA DA REDE BIONORTE

TAIDES TAVARES DOS SANTOS

FUNGOS ASSOCIADOS AO TRATO DIGESTÓRIO DE *Phylloicus* spp. (TRICHOPTERA: CALAMOCERATIDAE) EM RIACHOS DE BAIXA ORDEM NA AMAZÔNIA BRASILEIRA

PALMAS (TO)
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Tese apresentada ao Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede Bionorte na Universidade Federal do Tocantins como requisito parcial à obtenção do grau de Doutor em Biodiversidade e Biotecnologia.

Orientadora: Prof.ª Dr.ª Paula Benevides de Morais
Co-orientadora: Prof.ª Dr.ª Sheyla Regina Marques Couceiro

PALMAS (TO)
2018

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema de Bibliotecas da Universidade Federal do Tocantins

S237f Santos, Taides Tavares dos.

FUNGOS ASSOCIADOS AO TRATO DIGESTÓRIO DE Phylloicus spp. (TRICHOPTERA: CALAMOCERATIDAE) EM RIACHOS DE BAIXA ORDEM NA AMAZÔNIA BRASILEIRA. / Taides Tavares dos Santos. – Palmas, TO, 2018.

138 f.

Tese (Doutorado) - Universidade Federal do Tocantins – Câmpus Universitário de Palmas - Curso de Pós-Graduação (Doutorado) em Biodiversidade e Biotecnologia, 2018.

Orientadora : Paula Benevides de Morais

Coorientadora : Sheyla Regina Marques Couceiro

1. Celulase. 2. Diversidade fúngica. 3. Interação fungo-inseto. 4. Macroinvertebrados aquáticos. I. Título

CDD 660.6

Elaborado pelo sistema de geração automática de ficha catalográfica da UFT com os dados fornecidos pelo(a) autor(a).

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Tese apresentada ao Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede Bionorte, foi avaliada para a obtenção do título de doutor em Biodiversidade e Biotecnologia e aprovada em sua forma final pelo Orientador e pela Banca examinadora.

Data de aprovação: 14/12/2018.

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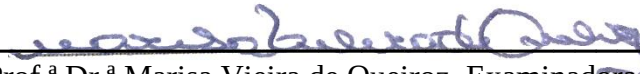

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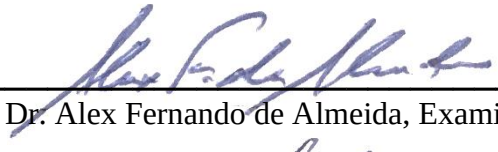

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AGRADECIMENTOS

A Deus, por ter me sustentado, guiado, dado forças, sabedoria e capacidade para a realização deste trabalho. À fiel intercessora e exemplo de todas as virtudes, Nossa Senhora Aparecida, que sempre esteve comigo ao longo de todo o Doutorado.

À minha família, de maneira especial à minha mãe, Maria José, e ao meu pai, Edson, e aos meus irmãos, Elson, Elcia e Taisa, pelo apoio incondicional, pela paciência e compreensão que me dedicaram, que foram cruciais para que eu pudesse vencer mais essa etapa da vida. Aos meus cunhados, Lucas Milani e Robson Borges, que também nunca mediram esforços para me auxiliar nos momentos de lutas e dificuldades.

À minha orientadora, Profª. Dr.ª Paula Benevides de Morais, por ter aceitado me orientar nesse curso de doutorado e me ensinado a ser cientista. Muito obrigado por toda a paciência e empenho dedicado, que foram muito importantes para a realização deste trabalho.

À Universidade Federal do Tocantins e ao Programa de Pós-graduação em Biodiversidade e Biotecnologia da Rede Bionorte (PPG-Bionorte), pela oportunidade de qualificação a mim ofertada.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo suporte financeiro para a realização dessa pesquisa.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo suporte financeiro para a realização dessa pesquisa e pela bolsa de estudo concedida no primeiro ano de atividades do doutorado.

Aos componentes da banca examinadora, por terem aceitado o convite para participar da defesa dessa tese e por todas as contribuições para a melhoria deste trabalho.

À minha coorientadora, Profª. Dr.ª Sheyla Regina Marques Couceiro, por todo o apoio nas atividades de campo, pelo incentivo e cooperação.

Às profissionais técnicas do Laboratório de Microbiologia Ambiental e Biotecnologia (LAMBIO), Cristiane Coelho e Márcia Regina Marson, que sempre estiveram disponíveis, nos bastidores, contribuindo para a que as pesquisas desenvolvidas nesse laboratório continuem sempre indo adiante.

A todos os integrantes da Rede IFFA – Insetos Fragmentadores e Fungos da Amazônia, pela parceria e troca de experiência durante os workshops e encontros da rede. Gostaria de agradecer de forma muito especial à Dr.ª Ana Maria Oliveira Pes, pelo auxílio nas atividades

de campo, pela colaboração na identificação dos insetos e pela concessão da imagem de *Phylloicus* utilizada em um dos artigos oriundos dessa tese.

À Drª. Deyla Paula de Oliveira, por todo o apoio, incentivo e colaboração. De forma especial, agradeço pelo auxílio nas análises de sequências de DNA e análises filogenéticas.

Aos professores do programa de pós-graduação, por todos os ensinamentos nas disciplinas, pelo incentivo e apoio. Ao Prof. Dr. Raphael Pimenta, que, desde o início do curso, me incentivou a persistir na luta construção da ciência de qualidade.

Aos colegas do Programa de Pós-graduação pela parceria e incentivo durante esse período de convivência. De forma especial, à Áurea Welter pelo auxílio nas atividades enzimáticas. À Eskálath Morganna pelo auxílio em análises filogenéticas.

Ao Prof. Dr. Emerson Guarda, por viabilizar a realização das análises espectofotométricas no Laboratório de Pesquisas em Química Ambiental e de Biocombustíveis da UFT.

À Prof.ª Dr.ª Juliana Fonseca e ao Prof. Dr. Alex Fernando, por todo apoio e incentivo durante todo o curso.

A todos os colegas do Laboratório Microbiologia Ambiental e Biotecnologia (LAMBIO), que foram pessoas importantes para que eu pudesse chegar até aqui. De forma especialíssima ao Alef Ricardo, pelo auxílio na confecção de mapas.

Enfim, a todos que contribuíram direta ou indiretamente para que esse trabalho fosse realizado, meus sinceros agradecimentos.

RESUMO

O estudo do trato digestório (TD) de insetos como um habitat para organismos fúngicos representa uma oportunidade para a compreensão das relações simbióticas entre esses organismos. Além disso, representa um *hotspot* para a obtenção de novas linhagens fúngicas com diferentes potenciais de aplicação biotecnológica. Os objetivos do presente trabalho foram avaliar a ocorrência, diversidade e atividade celulolítica de fungos filamentosos e de leveduras associados ao TD de *Phylloicus* (Trichoptera: Calamoceratidae), em riachos da Amazônia Legal, sob diferentes paisagens ecológicas, no Brasil. Larvas de *Phylloicus* foram coletadas em riachos dos estados de Roraima, Pará e Tocantins (n = 137) para o isolamento de fungos filamentosos, e nos estados de Mato Grosso e Pará (n = 113) para o isolamento de leveduras. Tanto os fungos filamentosos associados a três espécies de *Phylloicus* (*P. amazonas, P. elektoros* e *P. fenestratus*), bem como associados a *Phylloicus* spp. foram submetidos à triagem em meio sólido para atividade celulolítica e identificados por meio da combinação de taxonomia clássica e molecular [sequenciamento das regiões espaçadoras internas transcritas (ITS) do rDNA]. Leveduras também foram triadas quanto à produção de celulase e foram identificadas com base na análise de sequências dos domínios D1/D2 da subunidade maior do rDNA. Fungos filamentosos (n = 16) que apresentaram os maiores índices enzimáticos na triagem em meio sólido foram avaliados quanto à produção de celulases [celulase total (FPAse); endoglucanase (CMCase) e celobiohidrolases (avicelase)] por meio de fermentação no estado sólido (FES) utilizando um resíduo agroindustrial (farelo de trigo) como substrato. Entre os resultados desse estudo, verificou-se que fungos filamentosos são muito mais frequentes em associação com *Phylloicus*, ocorrendo em 94,9% dos TDs amostrados, que leveduras, que ocorreram em apenas 31,0% dos TDs amostrados. Um total de 33 táxons de fungos filamentosos foram obtidos. O gênero *Penicillium* foi o mais frequente (18,75%), seguido por *Pestalotiopsis* e *Trichoderma* (10.42%, cada). A ocorrência de táxons de fungos filamentosos entre espécies de inseto hospedeiro variou bastante, sendo que mais da metade dos táxons são exclusivos para uma espécie hospedeira em particular. No que diz respeito às leveduras, foi obtido um total de 20 espécies em associação com *Phylloicus* spp. Os gêneros mais frequentes foram *Candida, Papiliotrema, Rhodotorula* (19,3 %, cada) e *Issatchenkia* (15,8 %). Com relação à atividade celulolítica dos fungos filamentosos, 45,6 % (n = 62) das estirpes testadas mostraram atividade celulolítica. Entre aquelas com maiores índices enzimáticos, seis produziram celulase em um dos três ensaios realizados (FPAse, CMCase e avicelase). Concluiu-se que composição da

micota associada ao TD de *Phylloicus* é variável entre espécies hospedeiras. A atividade celulolítica é uma característica restrita entre as leveduras testadas, enquanto que uma parcela significativa dos fungos filamentosos exibe atividade essa característica, corroborando a possibilidade de ser este um possível papel exercido por esses micro-organismos na interface de interação com seus hospedeiros. A espécie *Cladosporium perangustum* apresenta potencial para aplicação em processos biotecnológicos envolvendo a produção de celulases.

Palavras-chave: celulase; diversidade fúngica; interação fungo-inseto; macroinvertebrados aquáticos; simbiose.

ABSTRACT

The study of the digestive tract (DT) of insects as a habitat for fungal organisms represents an opportunity for understanding the symbiotic relationships between these organisms. In addition, it represents a hotspot for obtaining new fungal lineages with different potentials of biotechnological application. The objectives of the present work were to evaluate the occurrence, diversity and cellulolytic activity of filamentous fungi and of yeasts associated with the DT of *Phylloicus* (Trichoptera: Calamoceratidae), in streams of the Legal Amazon, under different ecological landscapes, in Brazil. *Phylloicus* larvae were collected in the streams of the Roraima, Pará and Tocantins states (n = 137) for the isolation of filamentous fungi, and in the Mato Grosso and Pará states (n = 113) for the isolation of yeasts. The filamentous fungi associated to three species of *Phylloicus* (*P. amazonas*, *P. elektoros* and *P. fenestratus*), as well as associated with *Phylloicus* spp., were subjected to solid-media screening for cellulolytic activity and identified by combining classical taxonomy and molecular [sequencing of internal transcribed spacer (ITS) regions of the rDNA]. Yeasts were also screened for cellulase production and were identified based on sequence analysis of the D1/D2 domains of the large subunit of rDNA. Filamentous fungi (n = 16) that presented the highest enzymatic indices in the solid medium screening were evaluated for cellulase production [total cellulase (FPAse); endoglucanase (CMCase) and cellobiohydrolases (avicellase)] by means of solid-state fermentation (SSF) using an agroindustrial residue (wheat bran) as a substrate. Among the results of this study, it was found that filamentous fungi are much more frequent in association with *Phylloicus*, occurring in 94.9% of the DT sampled, than yeasts, which occurred in only 31.0% of the sampled DTs. A total of 33 filamentous fungi taxa were obtained. The genus *Penicillium* was the most frequent (18.75%), followed by *Pestalotiopsis* and *Trichoderma* (10.42%, each). The occurrence of filamentous fungi taxa among host insect species varied widely, with more than half of the taxa unique to a particular host species. With respect to yeasts, a total of 20 species were obtained in association with *Phylloicus* spp. The most frequent genera were *Candida*, *Papiliotrema*, *Rhodotorula* (19.3% each) and *Issatchenkia* (15.8%). Regarding the cellulolytic activity of filamentous fungi, 45.6% (n = 62) of the strains tested showed cellulolytic activity. Among those with higher enzymatic indexes, six produced cellulase in one of three trials (FPAse, CMCase and avicellase). It was concluded that mycotic composition associated with *Phylloicus* DT is variable among host species. Cellulolytic activity is a restricted feature among the yeasts tested, whereas a significant portion of the filamentous

fungi exhibits this characteristic activity, corroborating the possibility of this being a possible role played by these microorganisms in the interaction interface with their hosts. The species *Cladosporium perangustum* presents potential for application in biotechnological processes involving the production of cellulases.

Keywords: Aquatic macroinvertebrates; cellulase; fungal diversity, fungus-insect interaction; symbiosis.

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% ID	Percentage of similarity between the nucleotide sequences
1 - D	Simpson Index of Diversity
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search
CBS	*Centraalbureau voor Schimmelcultures* Fungal Biodiversity Centre
CFU	Colony-forming Units
CMC	carboxymethylcellulose
CMCase	Endoglucanase
CPOM	Coarse Particulate Organic Matter
d	Index of Margalef
DNA	Ácido Desoxirribonucleico; Deoxyribonucleic Acid
DNS	3.5-dinitrosalicyclic acid
DT	Digestive tract
EI	Enzymatic Indices
Exo-SAP	Exonuclease I and Shrimp Alkaline Phosphatase
FES	Fermentação no Estado Sólido
FNT	Tapajós Nacional Forest
Fo	frequency of occurrence
FPAse	Celulase total
FPOM	Fine Particulate Organic Matter
H’	Index of Shannon
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ITS	Internal transcribed spacer of the rDNA
J	Index of equitability
$MSP.DT^{-1}$	Morphospecies per DT
MT	Mato Grosso state
NCBI	National Center for Biotechnology Information
n_y	Number of isolates
PA	Pará state
PCR	Polymerase Chain Reaction

PDA	Potato Dextrose Agar
PEL	Lajeado State Park
SD	Standard deviation
SISBIO	Sistema de Autorização e Informação em Biodiversidade
SSF	Solid-State Fermentation
STM	Santarém municipality
STQ	Serra do Tepequém
TD	Trato digestório

LISTA DE SÍMBOLOS

ß	Beta
∑	Somatório
™	Trademark

SUMÁRIO

INTRODUÇÃO GERAL

A interação entre fungos e insetos tem sido investigada a partir de diferentes enfoques em anos recentes (León et al., 2016; Ramírez-Camejo et al., 2017; Chen et al., 2018). A maioria dos estudos realizados até o presente momento tem focado, sobretudo, nas interações entre fungos e insetos terrestres, tais como besouros, cupins e moscas, sendo escassos os estudos envolvendo insetos aquáticos (White e Lichtwardt, 2004; Siri e Lastra, 2010), que são organismos de grande variedade taxonômica e de grande importância na decomposição de detritos foliares em ecossistemas aquáticos, que é um processo ecológico-chave para esses ambientes e que envolve a participação de fungos (Chung e Suberkropp, 2009; Cornut et al., 2015).

No presente estudo, focou-se especificamente nas interações harmônicas que ocorrem entre insetos e fungos associados ao trato digestório (TD). Embora já se conheçam alguns aspectos dessa interação, muitos ainda estão obscurecidos. Sabe-se que, em contrapartida ao habitat ofertado por seus hospedeiros, os fungos associados ao TD de insetos podem estar envolvidos em processos que afetam, direta ou indiretamente, o desenvolvimento, a adaptação ou a sobrevivência de seus hospedeiros no ambiente, tais como as contribuições na nutrição dos insetos, quer seja servindo como alimento ou fornecendo suplementos nutricionais a seus hospedeiros (Hongoh e Ishikawa, 2000; León et al., 2016; Stefani et al., 2016).

Do ponto de vista biotecnológico, há evidências de que o TD de insetos pode representar uma fonte de novas linhagens de fungos filamentosos ou leveduras com capacidade de produzirem enzimas envolvidas na degradação de substratos lignocelulósicos, as quais apresentam potencial de aplicação na indústria de biocombustíveis (Schäfer et al., 1996; Gujjari et al., 2011; Suh et al., 2013).

Diante do exposto, evidencia-se a relevância de se realizarem estudos relacionados à diversidade, conservação e aplicação biotecnológica de fungos associados ao TD de insetos, sobretudo em ecossistemas ainda pouco explorados, como os ambientes aquáticos continentais de Cerrado e Floresta Amazônica, que são considerados *hotspots* de biodiversidade. Diante disso, foram realizadas investigações sobre ocorrência, diversidade, potencial celulolítica de fungos filamentosos e leveduriformes associados ao TD de *Phylloicus* (Trichoptera: Calamoceratidae), conforme descrito abaixo. De acordo com Prather (2003), há 55 espécies de *Phylloicus* descritas a partir do sudoeste do Estados Unidos da América, Brasil, Peru e

Venezuela. Esse gênero é especialmente diverso para o Brasil, para o qual são conhecidas 19 espécies, com oito delas sendo endêmicas para esse país (Prather, 2003; Paprocki et al., 2004).

A identificação de *Phylloicus* até o nível taxonômico de espécie requer a coleta de larvas e a manutenção destas em viveiros até a obtenção do indivíduo adulto, que é então comparado com guias taxonômicos (Pes et al., 2005, Hamada e Ferreira-Keppler, 2012). Existem muitos registros de ocorrência de espécies de *Phylloicus* a partir da floresta amazônica brasileira e da mata atlântica (Dumas e Nessimian, 2010; Santos e Nessimian, 2010; Calor, 2011; Quinteiro et al., 2011). Contudo, são escassos os registros de espécies a partir de ecossistemas de Cerrado. Assim, muitas vezes é possível classificar o inseto somente até o nível de gênero.

No **capítulo I** deste documento, intitulado "**Filamentous fungi in the digestive tract of *Phylloicus* larvae (Trichoptera: Calamoceratidae) in streams of the Brazilian Amazon**"[1], é reportada uma avaliação pioneira da ocorrência de fungos filamentosos cultiváveis em associação com o TD de macroinvertebrados do gênero *Phylloicus* em riachos sob diferentes paisagens ecológicas, na Amazônia brasileira.

No **capítulo II**, cujo título é "**The digestive tract of *Phylloicus* (Trichoptera: Calamoceratidae) harbours different yeast taxa in Cerrado streams, Brazil**"[2], é relatada uma investigação sobre a ocorrência, identidade e triagem para atividade celulolítica de leveduras associadas ao TD de insetos aquáticos fragmentadores, do gênero *Phylloicus*, de riachos de Cerrado de duas localidades (Estado do Mato Grosso e do Pará) do Brasil.

No **capítulo III**, intitulado "**A diverse and partially cellulolytic fungal community contributes to the diet of of three species of the aquatic insect *Phylloicus* (Trichoptera: Calamoceratidae) in Amazonian streams**" [3], é descrito o isolamento, identificação molecular e perfil celulolítico de fungos filamentosos associados ao TD de três espécies de *Phylloicus* (*P. amazonas*, *P. elektoros* e *P. fenestratus*), em riachos da floresta amazônica brasileira, e discutido sobre os potenciais papéis dessa comunidade fúngica na dieta de seus hospedeiros.

No **capítulo IV**, intulado "***Cladosporium perangustum* and other cellulolytic fungi from the digestive tract of larval stages of *Phylloicus* (Trichoptera: Calamoceratidae)**"[4], a

[1]Fungos filamentosos no trato digestório de larvas de *Phylloicus* (Trichoptera: Calamoceratidae) em igarapés da Amazônia Brasileira.
[2]O trato digestório de *Phylloicus* (Trichoptera: Calamoceratidae) abriga diferentes taxa de leveduras nos riachos de Cerrado, Brasil.
[3]Uma diversa e parcialmente celulolítica comunidade fúngica contribui para a dieta de três espécies do inseto aquático *Phylloicus* (Trichoptera: Calamoceratidae) em riachos amazônicos.
[4]*Cladosporium perangustum* e outros fungos celulolíticos do trato digestório de estágios larvais de *Phylloicus* spp. (Trichoptera: Calamoceratidae).

produção de celulase por fungos filamentosos associados ao TD de *Phylloicus* é avaliada qualitativamente (triagem em meio sólido contendo carboximeticelulose) e quantitativamente (fermentação no estado sólido). No ensaio quantitativo, foi utilizado um resíduo agroindustrial (farelo de trigo) como substrato.

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CAPÍTULO I:

FILAMENTOUS FUNGI IN THE DIGESTIVE TRACT OF *Phylloicus* LARVAE (TRICHOPTERA: CALAMOCERATIDAE) IN STREAMS OF THE BRAZILIAN AMAZON[5]

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 101), 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 interaction; Symbiosis.

[5]Artigo publicado na revista Boletim do Museu Paraense Emílio Goeldi. Ciências Naturais, v. 13, n. 3, p. 317-325, 2018.

1.1- 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 & 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.

1.2- Material and Methods

1.2.1- 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, Table 1). The sampling of streams in Amazon forest landscapes was carried out in the Tapajós Nacional Forest (FNT), 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 (PEL), 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).

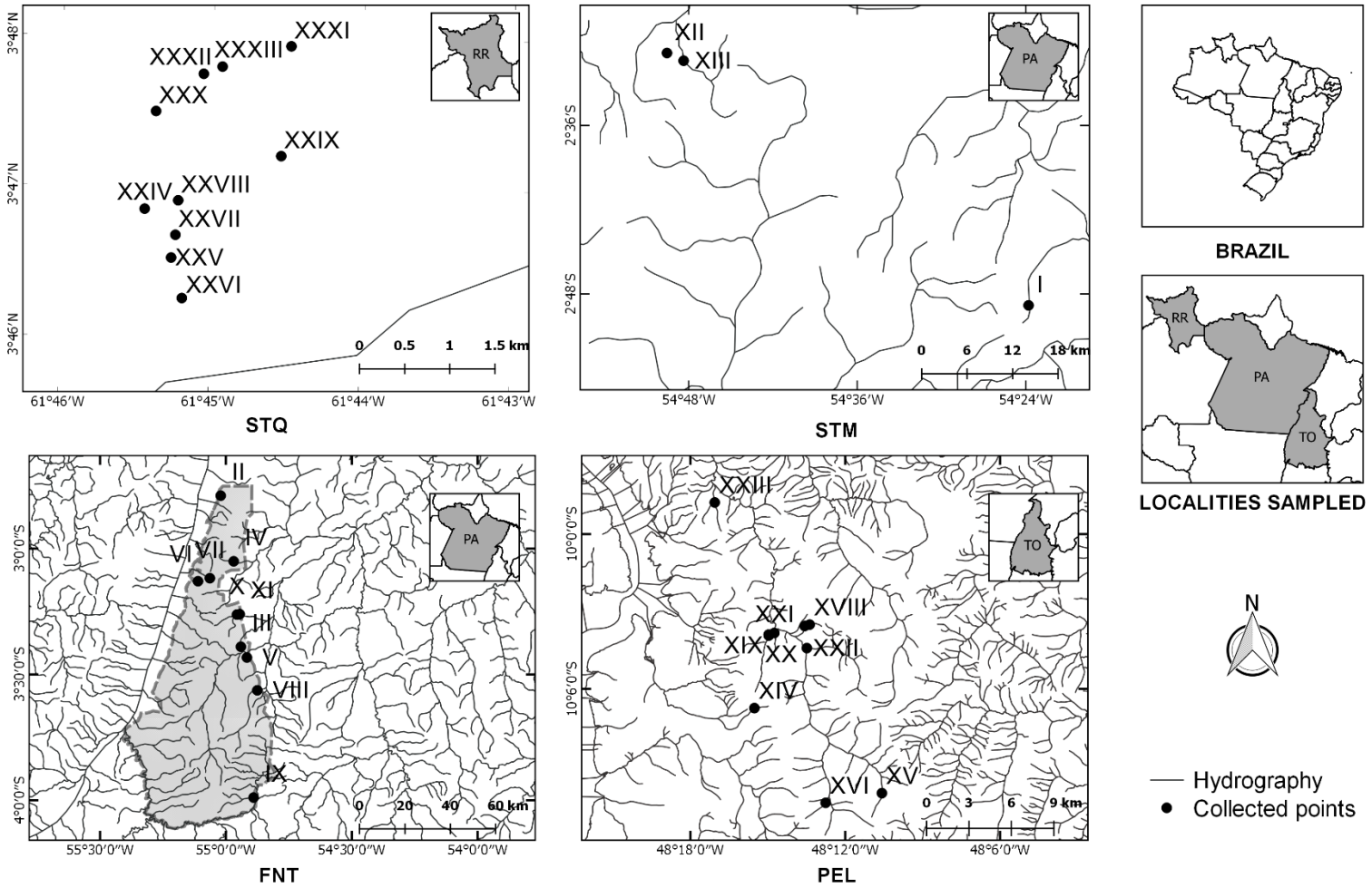

Figure 1. Map of the localities where the study was carried out. Abbreviations: STQ – *Serra do Tepequém*, in the Roraima state; STM – surroundings of the Santarém municipality, in the Pará state; FNT – Tapajós Nacional Forest, in the Pará state; PEL – Lajeado State Park, in the Tocantins state. I to XXXIII indicate the sampled streams.

1.2.2- 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.

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

1.2.3- 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.

1.2.4- 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 *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.).

1.2.5- 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.

1.3- 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 $8.5 \pm 47.0 \times 10^{1}$ $CFU.DT^{-1}$. 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 $5.7 \pm 24.9 \times 10^{1}$ $CFU.DT^{-1}$ (in *cerrado*) to $1.1 \pm 2.2 \times 10^{2}$ $CFU.DT^{-1}$ (in *lavrado* [savanna]) and $1.9 \pm 7.1 \times 10^{2}$ $CFU.DT^{-1}$ (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 ($MSP.DT^{-1} = 6.2 \pm 6.4$) (Table 1). According to the ANOVA, variation in richness of morphospecies among the sampled landscapes was not significant (Figure 4).

Table 1. Occurrence of filamentous fungi in association with 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.

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 morphospecies ± standard deviation
Amazon Forest	STM	I (02º48'49.6"S; 054º23'38.2"W)	n = 03	100.0%	8.0 ± 1.8 x 10^1	7.9 ± 3.1
	FNT	II (02°47'23.0"S; 55°01'14.9"W)	n = 03	100.0%	45.0 ± 9.0	6.8 ± 2.0
	FNT	III (03°23'25.2"S; 54°56'26.3"W)	n = 03	100.0%	7.8 ± 40 x 10^1	5.1 ± 3.1
	FNT	IV (03°03'02.6"S; 54°58'09.3"W)	n = 03	66.6%	3.9 ± 5.2 x 10^1	8.0 ± 8.5
	FNT	V (03°25'59.1"S; 54°54'59.6"W)	n = 03	100.0%	8.3 ± 2.0 x 10^1	8.1 ± 5.3
	FNT	VI (03°07'47.6"S; 55°06'39.0"W)	n = 03	100.0%	13 ± 7.5 x 10^2	7.6 ± 1.5
	FNT	VII (03°07'04.3"S; 55°03'49.5"W)	n = 03	100.0%	10 ± 3.1 x 10^1	4.8 ± 1.7
	FNT	VIII (03°33'48.2"S; 54°52'30.90"W)	n = 03	100.0%	1.8 ± 2.0 x 10^2	6.6 ± 4.2
	FNT	IX (03°59'24.1"S; 54°53'24.6"W)	n = 03	100.0%	2.4 ± 1.9 x 10^2	7.3 ± 4.4
	FNT	X (03°15'44.7"S; 54°57'22.0"W)	n = 10	100.0%	5.6 ± 7.9 x 10^2	7.6 ± 2.1
	FNT	XI (03°15'38.7"S; 54°56'42.8"W)	n = 04	100.0%	1.7 ± 7.4 x 10^2	5.9 ± 4.3
Subtotal 1			**n = 41**	**97.6%**	**1.9 ± 7.1 x 10^2 a**	**6.9 ± 3.2**
Cerrado	STM	XII (02º30'50.8''S; 054º49'33.3''W)	n = 15	86.7%	5.7 ± 4.6 x 10^1	6.0 ± 3.4
	STM	XIII (02º31'23.8''S; 054º48'22.7''W)	n = 48	91.7%	3.5 ± 2.6 x 10^1	5.2 ± 4.7
	PEL	XIV (10°06'44.50"S; 48°15'31.10"W)	n = 00	-	-	-
	PEL	XV (10º10'02.30"S; 48º10'34.70"W)	n = 03	100.0%	4.2 ± 3.6 x 102	8.8 ± 2.0
	PEL	XVI (10º10'24.80"S; 48º12'45.40"W)	n = 03	100.0%	1.7 ± 5.7 x 102	5.5 ± 1.5
	PEL	XVII (10º03'33.60"S; 48º13'34.30"W)	n = 03	100.0%	11 ± 8.9 x 101	3.8 ± 2.5
	PEL	XVIII (10º03'33.40"S; 48º13'49.30"W)	n = 03	100.0%	2.0 ± 1.2 x 102	9.5 ± 3.5
	PEL	XIX (10º03'53.60"S; 48º14'58.00"W)	n = 03	100.0%	11 ± 2.8 x 101	9.2 ± 3.5
	PEL	XX (10°03'55.90"S; 48°14'57.70"W)	n = 03	100.0%	2.1 ± 4.1 x 101	3.9 ± 4.4

Table 1. Occurrence of filamentous fungi in association with 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 (continuation).

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 morphospecies ± standard deviation
Cerrado	PEL	XXI (10º03'49.80"S; 48º14'44.80"W)	n = 03	100.0%	$15 \pm 3.3 \times 10^1$	8.3 ± 1.2
	PEL	XXII (10º04'25.00"S; 48º13'29.10"W)	n = 03	100.0%	$2.1 \pm 7.3 \times 10^2$	3.8 ± 2.5
	PEL	XXIII (09º58'46.30"S; 48º17'03.20"W)	n = 03	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 = 00	-	-	-
	STQ	XXV (03 48'22.50"N; 61 42'32.10"W)	n = 00	-	-	-
	STQ	XXVI (03°46'10.60"N; 61°45'27.00"W)	n = 00	-	-	-
	STQ	XXVII (03°46'43.90"N; 61°45'29.30"W)	n = 00	-	-	-
	STQ	XXVIII (03°46'43.90"N; 61°45'29.10"W)	n = 00	-	-	-
	STQ	XXIX (03°47'00.80"N; 61°44'51.80"W)	n = 00	-	-	-
	STQ	XXX (03°47'16.90"N; 61°45'38.40"W)	n = 03	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 = 00	-	-	-
	STQ	XXXII (03°47'31.60"N; 61°45'17.90"W)	n = 00	-	-	-
	STQ	XXXIII (03°47'32.50"N; 61°45'12.70"W)	n = 03	100.0%	$5.7 \pm 8.9 \times 10^1$	17.9 ± 31.2
Subtotal 3			**n = 06**	**100.0%**	**$1.1 \pm 2.2 \times 10^2$ c**	**15.7 ± 21.3**
TOTAL (Subtotal 1 + Subtotal 2 + Subtotal 3)			**n = 137**	**94.9%(**)**	**$8.5 \pm 47.0 \times 10^1$**	**6.2 ± 6.4**

(*): 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.
Abbreviations: STM: Santarém municipality, Pará state; FNT: Tapajós Nacional Forest, Pará state; PEL: Lajeado State Park, Tocantins state; STQ: *Serra do Tepequém* (STQ), Roraima state.

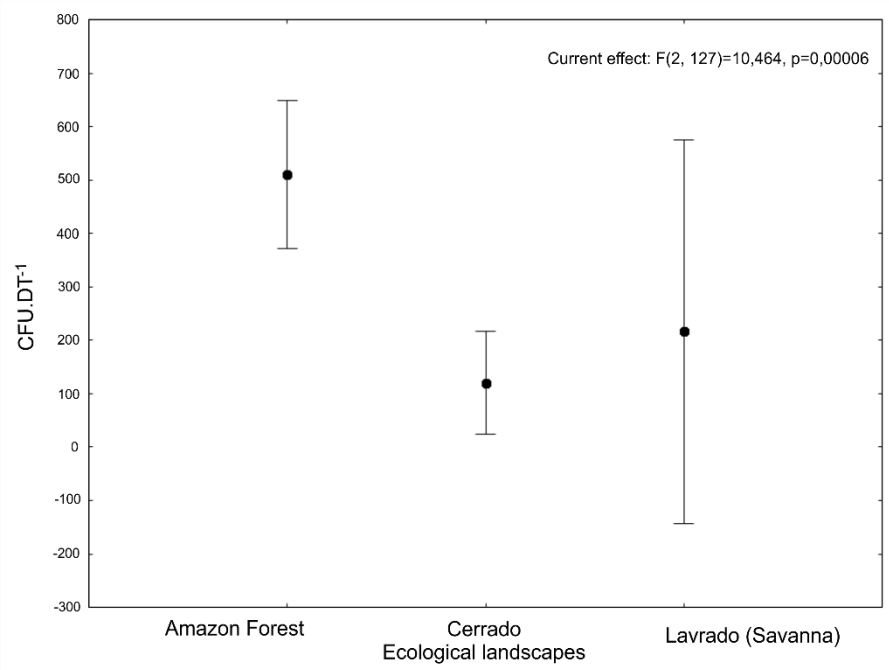

Figure 3. Analysis of variance (ANOVA) of the Colony-forming Units per DT ($CFU.DT^{-1}$) between ecological landscapes (Amazon Forest, Cerrado and Lavrado [Savanna]), at $p < 0.05$, using Statistica v.10 software.

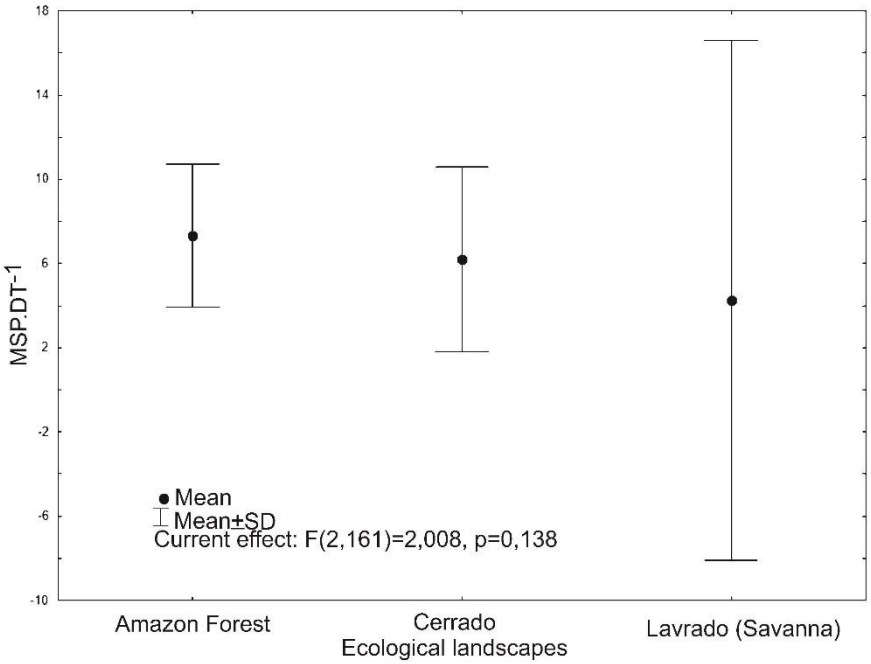

Figure 4. Analysis of variance (ANOVA) of the morphospecies per DT ($MSP.DT^{-1}$) between ecological landscapes (Amazon Forest, Cerrado and Lavrado [Savanna]), at $p < 0.05$, using Statistica v.10 software.

Preliminary identification efforts based on the association between classical and molecular methods (Kern & Blevins, 1999; White *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).

Table 2. Identification of isolates associated with the digestive tract of *Phylloicus* (Trichoptera: Calamoceratidae) larvae collected in streams of Cerrado in Santarém (STM, Pará State) based on the sequencing of the ITS regions of the rDNA.

Isolate code	**Fungal species**	**% ID***	**GenBank acession numbers**
LAG 8.6	*Penicillium simplicissimum*	99	KU059955
PON 3.6	*Paraphaeosphaeria arecacearum*	99	KM873041
PON 9.1	*Paraphaeosphaeria arecacearum*	100	JX496100
PON 15.1	*Penicillium sclerotiorum*	99	KX664361

*Percentage of similarity between the nucleotide sequences obtained in that study with sequences available in the NCBI database.

1.4- 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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CAPÍTULO II:

THE DIGESTIVE TRACT OF *Phylloicus* (TRICHOPTERA: CALAMOCERATIDAE) HARBOURS DIFFERENT YEAST TAXA IN CERRADO STREAMS, BRAZIL[6]

Abstract: The interaction between insects, both larval and adult, and yeasts associated with their digestive tract (DT), has been of interest in recent years, since it can be beneficial for both partners. Studies focusing on this habitat have contributed to the expansion of knowledge about diversity, biogeography and functional characterization of yeasts, especially in ecosystems still poorly exploited, such as the Brazilian Cerrado. We investigated the interaction between larvae of *Phylloicus* spp. (Trichoptera: Calamoceratidae), which is an aquatic insect, and the yeasts isolated from its DT. The larvae were collected from first-order Cerrado streams of two States (Mato Grosso – MT and Pará – PA) in Brazil. Yeasts were cultivated and identified based on sequence analysis of the D1/D2 domains of the large subunit of rRNA genes. A total of 20 yeast species, belonging to six genera of Ascomycota and five Basidiomycota, is harbored in the DT of the larvae. The most frequent genera were *Candida*, *Papiliotrema*, *Rhodotorula* (19.3% each) and *Issatchenkia* (15.8%). *Candida parapsilosis* and *Rhodotorula mucilaginosa* were only yeast species isolated from the DT of larvae in both locations. The most species-rich community was that associated with DT of *Phylloicus* spp. in MT samples (H' = 1.48) as compared to PA samples (H' = 0.67). All species were accidental (frequency < 25%), which is indicative of a loose association of these yeasts with their host. This is the first report of the association of yeasts with the DT of the shredders group of aquatic insects.

Keywords: Aquatic macroinvertebrates; Freshwater; Fungal diversity, Fungus-insect interaction; Symbiosis.

[6]Artigo publicado na revista Symbiosis, v. 77, p. 147-160, 2018. https://doi.org/10.1007/s13199-018-0577-9

2.1- Introduction

In the interface of interaction between insects and yeasts, the body of an insect can offer several sites for yeast colonization, such as the outer surfaces (Rosa et al., 2003; Yamoah et al., 2008), the reproductive system (Ricci et al., 2011) and the digestive tract (DT) (Suh et al., 2003; León et al., 2016). In addition to providing a habitat, the insect hosts also contribute to the dispersal of these microorganisms, acting as vectors among different environments (Lachance et al., 2001; Christiaens et al., 2014). Also, the yeasts associated with the DT of insects are important for the hosts, as they can play roles that contribute to increase health and nutrition of larvae and adults (Noda and Koizumi, 2003; Douglas, 2015).

The association of yeasts with DT from various terrestrial insects, such as beetles (Grünwald et al., 2010; Urbina et al., 2013; Stefani et al., 2016), flies (Morais et al., 2005; Broderick and Lemaitre, 2012) and termites (Schäfer et al., 1996; Handel et al., 2016), has been reported. A wide variety of yeasts has been detected in this habitat, which has been indicated as a hotspot for the discovery of new species of yeasts (Zhang et al., 2003; Suh et al., 2005). Thus, studies focusing on yeasts that occur in the DT from insects represent an opportunity to increase knowledge about the diversity, biogeography and functional characterization of these organisms (Rao et al., 2007; Urbina et al., 2013), especially in ecosystems still poorly exploited, such as the Brazilian Cerrado, which is considered a priority for the study and conservation of biodiversity in the world (Myers, 2000; Klink and Machado, 2005).

Yeasts associated with DT of insects are involved in the nutrition of these organisms, acting as a food resource or providing nutritional supplements (Hongoh and Ishikawa, 2000, León et al., 2016, Stefani et al., 2016). Yeasts associated with longhorned beetles (Coleoptera: Cerambycidae), *Xylopinus saperdioides* (Coleoptera: Tenebrionidae) among other wood-inhabiting that feed on lignocellulosic substrates (Grünwald et al., 2010; Gujjari et al., 2011; Suh et al., 2013) are related to the degradation of these substrates, while endosymbiotic yeast from mosquitoes provide nutritional supplementation (essential amino acids, vitamin B, protein and trace minerals) to their hosts (Urubschurov and Janczyk, 2011). In addition to providing food supplements to their hosts, yeasts may also be part of the insect diet. This is the case of yeasts of the species *Saccharomyces cerevisiae* used as food for larvae of *Aedes aegypti* (Diptera: Culicidae) and other mosquitoes (Asahina, 1964; Sirot et al., 2011).

Despite the importance of aquatic insects and fungi in several ecological processes that occur in aquatic ecosystems, such as the decomposition of plant debris (Graça, 2001; Hieber

and Gessner, 2002), little is known about the interaction between the two biological groups. There are reports related to the occurrence of a large variety of yeasts and filamentous fungi in aquatic environments (Shearer et al., 2007; Brandão et al., 2017). However, the reports related to strains of fungi in association with the DT of aquatic insects are few and generally restricted to fungi of the Trichomycetes class (Zygomycota) (White and Lichtwardt, 2004; Siri and Lastra, 2010).

The aim of this study was to investigate the occurrence of yeasts associated with the DT of aquatic shredder larvae of insects of the genus *Phylloicus* spp. (Trichoptera: Calamoceratidae) from streams in Brazilian Cerrado ecosystems. We describe the successful isolation of yeasts from DT of 113 larvae through classical culture methods and the taxonomic identification of these yeasts based on sequence analysis of the D1/D2 domains of the large subunit of rRNA genes, which is a widely used and consolidated barcode for this purpose. It was found that the DT of *Phylloicus* larvae harbors different yeast taxa (20 species), with biogeographical differences in community composition between the locations sampled. Only two yeast species (*Candida parapsilosis* and *Rhodotorula mucilaginosa*) were isolated from the DT of insects in both locations. Furthermore, all species were accidental (frequency < 25%), which is indicative of a loose association of these yeasts with their host.

2.2- Materials and methods

2.2.1- Characterization of the study areas

The study was conducted in streams situated in Cerrado ecosystems in two Brazilian states (Mato Grosso, MT; Pará, PA) (**Fig. 5**). Cerrado is the second largest biome in Brazil (Ratter et al., 1997) and South America (Klink and Machado, 2005). This vegetation is dominant in Central Brazil, which includes the areas sampled in MT State and occurs as spots in parts of the PA State (Ratter et al., 1997). The selection of streams (MT: *Bacaba* stream - 14º43'06.7"S, 52º21'42.8"W; PA: *Lagoa* stream - 02º30'50.8''S, 054º49'33.3''W and *Ponte Alta* stream - 02º31'23.8''S, 054º48'22.7''W) was based on criteria of conservation and continuity of riparian vegetation.

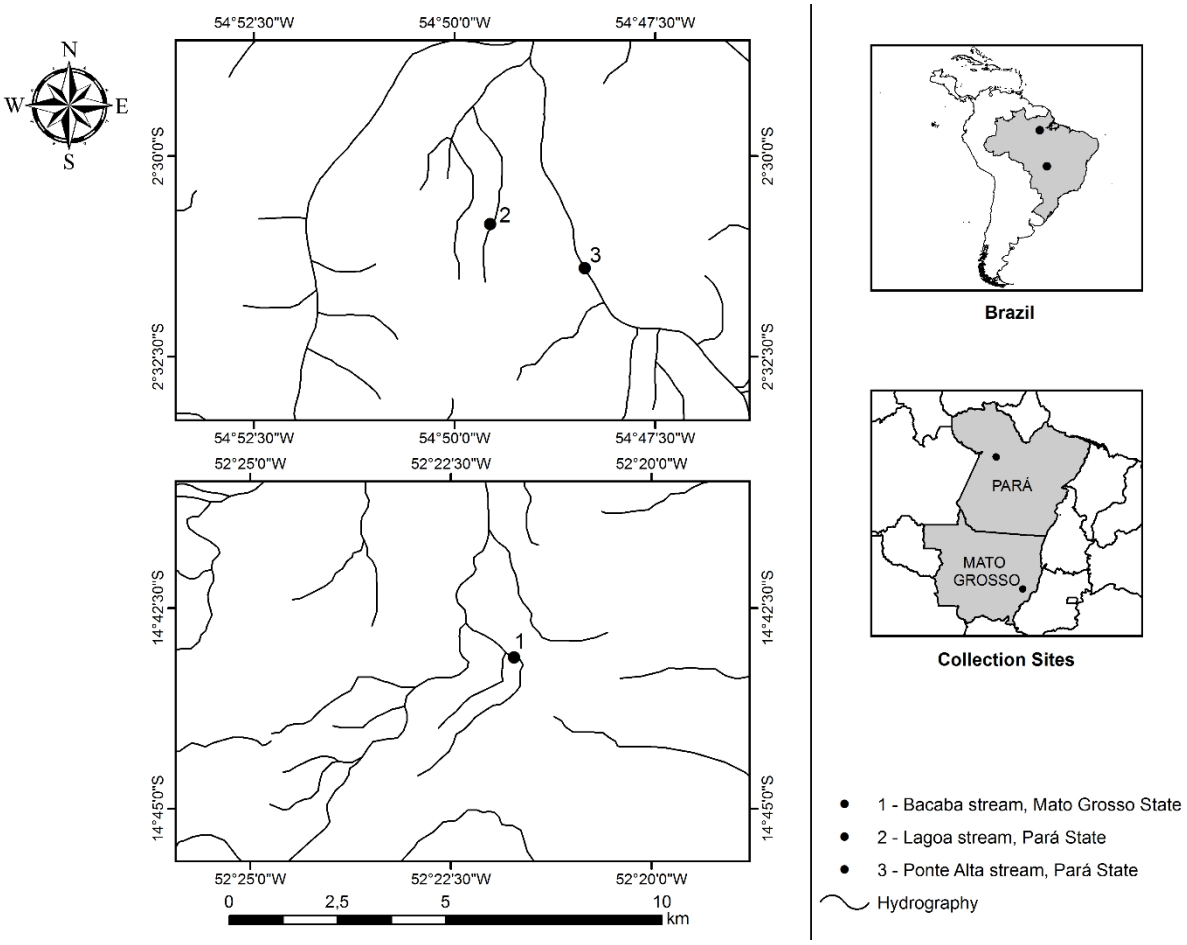

Fig. 5. Map showing *Phylloicus* spp. sampling sites. Locations of the low order streams in Brazilian Pará and Mato Grosso states is indicated.

Collections were done in a stretch of 50 m in each stream, by sampling all available substrates, especially litter banks at every 10 m, with the aid of a D-frame net (mesh of 0.500 mm and an area of 0.465 m^2). At each point, three subsamples were collected, which were screened for shelters of *Phylloicus* spp. in the field. The shelters collected were transferred to sterile tubes containing stream water, and stored for up to two hours in coolers with ice until they were transferred to the laboratory for processing. The larvae were identified based on specific taxonomic keys for the group (Pes et al., 2005; Hamada and Ferreira-Keppler, 2012).

2.2.2- Isolation, purification and conservation of yeast

Phylloicus spp. larvae were carefully removed from shelters and individually subjected to surface disinfection with ethanol 70% for 30 seconds and washed with sterile water. The dissection of larvae was performed with the aid of a stereoscopic microscope, and the DT was transferred to 1.5 mL tubes containing 1.0 mL of sterile distilled water. After homogenizing the content of each tube, 100 µL were inoculated in triplicate Petri dishes (90 mm diameter) containing Potato Dextrose Agar (PDA) culture medium (potato extract: 4.0g; dextrose: 20.0g; agar: 15.0g) supplemented with 0.1µg/mL chloramphenicol. A negative control of the larvae disinfection was performed by inoculating the final water of the disinfection procedure in the same culture media. The choice of medium was made after a trial with three different media currently used for yeast isolation and recommended by Kurtzman et al. (2011a): PDA, YEPD and YMA among which the first one performed better both for population counts and yeast morphotype richness. The plates were incubated at room temperature (25 ± 3 ºC) for 3 to 10 days, being inspected daily, until the growth of colonies with morphological aspects of yeasts (Kurtzman et al., 2011a), that were described based on shape, coloration and size of the colonies, among other relevant morphological characteristics.

Yeast colony forming units (CFU) of each morphotype in each plate were counted for quantitative analysis. One to three representatives of different yeast morphotypes in each plate were purified by repeated streak-inoculation on YM (Yeast-Malt) agar plates (0.3% yeast extract; 0.3% malt extract; 0.5% peptone; 1.0% glucose; 2.0% agar; pH 4) and preserved on GYMP (Glucose-Yeast-Malt-Phosphate: 2.0% glucose; 0.5% yeast extract; 2.0% malt extract; 0.2% $NaH_2PO_4.H_2O$) added with glycerol 20% at -80 ºC in the *Coleção de Culturas Microbianas Carlos Rosa* for later identification. The number of representative colonies was based on the number of CFU of the same morphotype in the plate, noting that the higher the

counts the more colonies were collected from plate for identification purposes. This strategy has the purpose to guarantee that the diversity of yeast species present in the sample will be sampled thoroughly and it is a well-stablished methodological approach in yeast isolation from environmental samples (Rosa et al., 2003; Pimenta et al., 2009; Rivera et al., 2009; Yurkov et al., 2011; Araújo et al., 2012). Morphotyping of CFU growing on plates was used solely for individualization and preliminary characterization of the colonies isolated for identification (Kurtzman et al., 2011a).

2.2.3- DNA extraction, amplification and sequencing

Yeasts were cultivated in YM agar for 24 to 48 hours. Then, DNA extraction was performed according to the methodology proposed by Sambrook et al. (1989), with modifications. After the extractions, the DNA was analyzed in NanoDrop 2000 spectrophotometer (Thermo Scientific, Uniscience, Brazil). Subsequently, the amplification of the D1/D2 variable domains of the large subunit of rRNA genes was carried out as described by Lachance et al. (1999) using the primers NL-1 (5' - GCATATCAATAAGCGGAGGAAAAG - 3') and NL-4 (5'- GGTCCGTGTTTCAAGACGG - 3'). Successful PCR (Polymerase Chain Reaction) amplification was confirmed by electrophoreses on a 1.0 % (w/v) agarose gel (Promega, Madison, WI) stained with GelRed™ (Biotium Inc. California, USA) in 1X TBE buffer (2.0 mmol L^{-1} EDTA; 0.1 mol L^{-1} Tris-HCl; and 0.1 mol L^{-1} boric acid [pH 8,0]) (Sambrook et al., 1989) and visualized under ultraviolet light. PCR products were purified using Exo-SAP (Exonuclease I and Shrimp Alkaline Phosphatase) (USB Corp. Cleveland, USA), according to the manufacturer's recommendations.

Sequencing was performed using BigDye Terminator v3.1 (Life Technologies, Carlsbad, California, USA) on the ABI 3500 xl automatic sequencer (Life Technologies, Carlsbad, California, USA). The sequencing products of both DNA strands were contiguously grouped, aligned and corrected using the Geneious 6.1.8 software (Kearse et al., 2012). A comparative identity search of the nucleotide sequences obtained from the isolates was performed using the BLAST (Basic Local Alignment Search) tool (Altschul et al., 1990) of the NCBI (National Center for Biotechnology Information) Database and in the CBS (*Centraalbureau voor Schimmelcultures* Fungal Biodiversity Centre) Database (http://www.cbs.knaw.nl/Collections/). Identity ≥ 99% were indicative of the same species,

according to Kurtzman et al. (2011b). The sequences were deposited in GenBank under the accession numbers MH636019 to MH636075 (**Table S1**).

2.2.4- Phylogenetic analysis

The sequences of yeasts from this study and additional sequences from ex-type cultures obtained from GenBank were aligned using Clustal W (Thompson et al., 1994). Phylogenetic tree was constructed by the neighbor-joining method using MEGA software version 6.0 (Biodesign Institute, USA). The bootstrap was 2,000 replications to assess the reliable level to the nods of the tree (Tamura et al., 2013). A glomeromycetous sequence of *Glomus mosseae* was used as outgroup as proposed in the phylogenetic analysis of yeasts performed by Lou et al. (2014). Sequences from this study were indicated in the tree by collection code, followed by the abbreviation of the Brazilian state of origin (PA or MT), while GenBank sequences were indicated by accession numbers (**Table S1** and **Fig. 6**).

2.2.5- Cellulolytic activity screening

The strains were previously reactivated on YM agar plates and incubated at 25 ± 3 ºC for 48 h. The production of cellulases was determined according to methodology described by Strauss et al. (2001) and Buzzini and Martini (2002), with modification. After reactivation, the strains were inoculated with Petri dishes containing YP-CMC medium (10.0 g/L yeast extract, 20.0 g/L peptone, 4.0 g/L carboxymethylcellulose and 20.0 g/L of agar) and incubated at 25 ± 3 ºC for 10 days. The hydrolysis halos were revealed according to Maijala et al. (1991), where the plates were flooded with 10 mL of aqueous solution of 0.3 g/L Congo red (30 minutes) and destained with 5 mL of 1.0 mol/L sodium chloride solution (15 minutes). Cellulolytic enzyme-producing strains were identified by the presence of a translucent halo around the colony, in contrast to the more intense red staining of the rest of the medium.

2.2.6- Statistical analysis

Community analysis was based on Krebs (1978) and Ludwig and Reynolds (1988). Data was expressed as the presence/absence of yeasts in insect DT (occurrence). The frequency of occurrence (Fo) was calculated as the percentage of DT in which the yeast species was found

in relation to the total DT in which occurred at least one species of yeast. DT in which yeast growth was not reported were not considered for this calculation. The constancy of any yeast species *y* was based on occurrence (Fo) data and corresponds to the percentage of samples in which the species *y* was present. Yeast species were classified as constant when present in >50% of the samples; accessory when present in 25-50% of the samples and accidental when present in less than 25% of the samples.

The Simpson Index of Diversity (1 - D) was calculated and reflects the probability of two isolates at random in the community belong to different species (Simpson, 1949; McCune and Grace, 2002). It varies from 0 to 1, and higher values indicate higher diversity. It is calculated as $D = 1 - \sum (n / N)^2$, where n refers to the total number of isolates of a particular species and N = the total number of isolates of all species.

The Index of Shannon (H') was applied to measure the degree of uncertainty in identifying a member of the community as belonging to a species or the degree of uncertainty in determining that one yeast isolate belongs to a particular species in a collection of S species and N isolates (Shannon, 1948). The lower Shannon, the lesser uncertainty and thus the lower the diversity. It is calculated as $H' = -\sum_1^S (p_1 \, . \, ln \, p_i)$, where p_i is the frequency of isolation of each species, varying from 1 to S (species richness). Diversity indices were calculated by PAST software (version. 3.19) (Hammer et al., 2001).

Total population counts is expressed as the geometric mean of geometric mean of the number of colony forming units (CFU) of yeasts in all DT sampled. Population counts of each yeast species was calculated as the geometric mean of CFU of the yeast species in DT positive for yeast presence. The CFU in each DT was counted as the mean of colonies of the yeast species-related morphotype in triplicate replica plates. The final count represents the geometric mean of CFU for the number of DT that harbored that yeast species, not the total number of DT sampled.

2.3- Results

Yeasts strains were obtained from 31% (n = 35/113) of the total of digestive tracts analyzed. The population of yeasts ranged from 3.0 to 3.3 x 10^3 CFU/DT, with a geometric mean of yeast isolates per DT of 3.4 x 10^1 CFU/DT (**Table 3**). The geometric mean of yeast isolates per DT in MT samples was 1.6 x 10^2 CFU/DT, while on the PA samples was 9.3 CFU/DT.

A total of 20 yeast species was isolated from the DT of *Phylloicus* spp. (**Table 3**). Twelve species belonged to six genera of the Ascomycota and eight species to five genera of the Basidiomycota. The most species-rich community was that associated with DT of *Phylloicus* spp. from MT (H' = 1.48) as compared to PA samples (H' = 0.67). The Simpson Index of Diversity (1 - D) was also higher for MT (1 - D = 0.74) than for PA samples (1 - D = 0.23).

Table 3. Number of isolates (n_y), population counts and frequency of occurrence (Fo) of yeast species isolated from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) from Mato Grosso and Pará states, Brazil.

Yeast species	% ID (1)	GenBank acession numbers	Mato Grosso state (n = 50)		Pará state (n = 63)				Fo (%) (4)
			Bacaba stream (n = 50)		*Lagoa* stream (n = 15)		*Ponte Alta* stream (n = 48)		
			n_y (2)	Counts(3)	n_y	Counts	n_y	Counts	
Ascomycota									
Aureobasidium thailandense	99%	JQ682650	3	55					5.3
Candida blattae	99%	FJ614695					1	20	1.8
C. boidinii	99%	KY296061	1	7					1.8
C. parapsilosis	99%	EU605804	3	295			1	10	7.0
C. rugosa	99%	EF375701	1	10					1.8
Candida sp. 1	100%	HM461715					2	6	3.5
Candida sp. 2	100%	EU011605					1	3	1.8
Candida sp. 3	100%	LN875214	1	10					1.8
Debaryomyces hansenii	100%	EU131182					1	3	1.8
Issatchenkia siamensis	99%	JQ672607	9	3800					15.8
Lodderomyces elongisporus	99%	KY108338					3	6	5.3
Meyerozyma guilliermondii	99%	EU188617					3	13	5.3

Table 3. Number of isolates (n_y), population counts and frequency of occurrence (Fo) of yeast species isolated from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) from Mato Grosso and Pará states, Brazil (continuation).

Yeast species	% ID [1]	GenBank acession numbers	Mato Grosso state (n = 50)		Pará state (n = 63)				Fo (%) [4]
			Bacaba stream (n = 50)		*Lagoa* stream (n = 15)		*Ponte Alta* stream (n = 48)		
			n_y [2]	Counts[3]	n_y	Counts	n_y	Counts	
Basidiomycota									
Cryptococcus podzolicus	99%	KT895969					1	3	1.8
Hannaella luteola-like	97%	KR136232	1	3000					1.8
Papiliotrema flavescens	99%	MF045447					4	16	7.0
Papiliotrema flavescens-like 1	97%	LT627406			1	1000			1.8
P. flavescens-like 2	98%	MG367282					1	3	1.8
Papiliotrema laurentii	99%	JQ968506					5	30	8.8
Pseudozyma antarctica	99%	JQ650240					3	6	5.3
Rhodotorula mucilaginosa	99%	KY109087	8	1356			3	10	19.3
Total of occurrences			27		1		29		100%
Species richness			8				14		

[1]Percentage of similarity between the nucleotide sequences obtained in that study with sequences available in the NCBI database;

[2]Total number of DT in which the yeast species was found;

[3]Geometric mean of the number of colony forming units (CFU) of yeasts in DT positive for yeast presence;

[4]The frequency of occurrence (Fo) was calculated as the relative occurrence of the yeast species in relation to the total occurrence.

The genera *Candida* (19.3%), *Papiliotrema* (19.3%), *Rhodotorula* (19.3%) and *Issatchenkia* (15.8%) were the most frequent in DT of *Phylloicus*. Regarding the species, *Rhodotorula mucilaginosa* was the most frequent (19.3%), followed by *Issatchenkia siamensis* (15.8%) and *Papiliotrema laurentii* (8.8%).

In the MT samples, the most frequent genera were *Issatchenkia* (33.3%), *Rhodotorula* (29.6%) and *Candida* (22.2%), while the most frequent species were *I. siamensis* (33.3%) and *R. mucilaginosa* (29.6%). On the other hand, in PA samples, the most frequent genus was *Papiliotrema* (36.7%), followed by *Candida* (16.7%). Most frequent species was *P. laurentii* (16.7%), followed by *Papiliotrema flavescens* (13.3%). Basidiomycetous yeasts were frequently isolated from insect DT in PA samples whereas only two species of this phyllum occurred associated with insect DT in the MT samples.

The phylogenetic relationship between yeast species is shown in **Fig. 6**. Species occurrence showed patterns that differed between the sampled locations (MT and PA samples). Only two yeast species (*Candida parapsilosis* and *R. mucilaginosa*) were isolated from the DT of insects in all locations sampled. The yeast species *Candida* sp. 1 (PON 10.13 and PON 11.1) and *Candida* sp. 2 (PON 21.1) presented phylogenetic proximity with *Candida* sp. HM461715.1 isolated from soil in Taiwan. *Candida* sp. 3 (PH44), as indicated in **Table 3** and **Fig. 6**, showed to be sibling to *Candida* sp. LN875214.1 isolated from fruits in French Guiana.

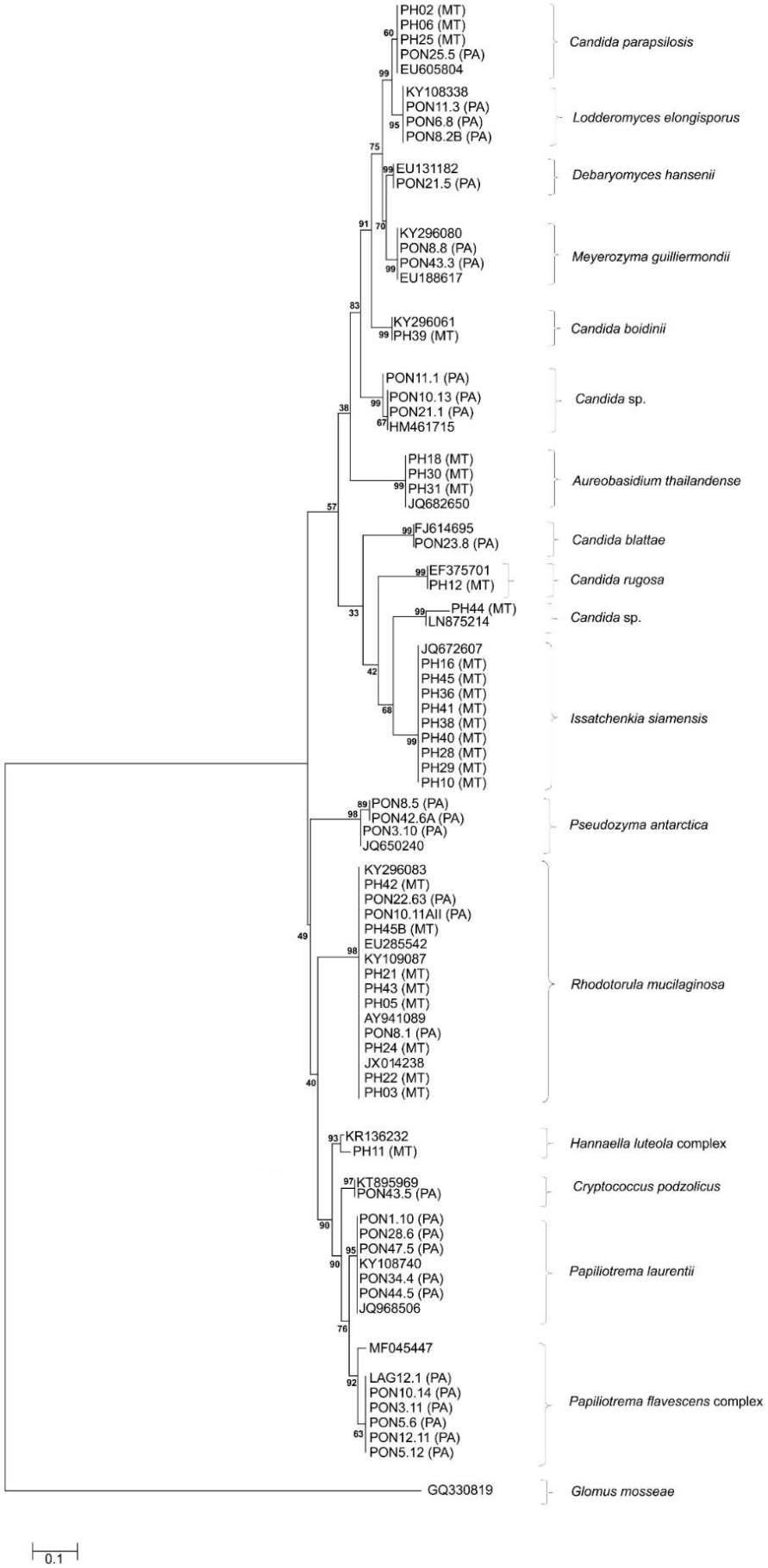

Fig. 6. Phylogenetic relationship between yeast species.

Among isolates which identity could not be confirmed (identity ≤ 99%), the isolates LAG12.1 (named as *Papiliotrema flavescens*-like 1 in **Table 3**) and PON12.11 (named as *Papiliotrema flavescens*-like 2 in **Table 3**) presented phylogenetic proximity with *Papiliotrema flavescens*, from this study, and MF045447, isolated from apple fruit surface in China. The isolate PH11 (named as *Hannaella luteola*-like in **Table 3**) presented phylogenetic proximity with *Hannaella luteola* KR136232, isolated from the necrotic tissue of cacti in Brazil, and proximity to other basidiomycetous yeasts of the clade *Papiliotrema/Cryptococcus*.

Among the yeasts isolated in this study, 31.6% (n = 57) were cellulolytic. The ascomycetous yeasts, *Aureobasidium thailandense* (two isolates), *C. parapsilosis* (two isolates), *C. rugosa* (one isolate), *I. siamensis* (two isolates), *Lodderomyces elongisporus* (three isolates) and *Meyerozyma guilliermondii* (two isolates) showed positive activity for cellulases. *Cryptococcus podzolicus* (one isolate), *P. laurentii* (two isolates) and *R. mucilaginosa* (three isolates) were the basidiomycetous species with positive activity.

2.4- Discussion

The low incidence of DT of *Phylloicus* spp. colonized by yeasts in Cerrado streams of two locations (MT and PA samples) may be related to a possible loose association of yeasts and *Phylloicus* based on the random absorption of yeasts colonizing surrounding environment. There is evidence that existing yeasts in the DT of insects are eaten along with the substrates on which they are growing and form a part of the insect diet (Morais et al., 1994; Stefani et al., 2016). *Phylloicus* is functionally classified as a shredder, that is to say that it feeds on plant leaves fallen in the stream, and partially colonized by decomposers. Among fungi, filamentous species are known as best decomposers while yeasts are known as copiotrophic saprobes in sugar-rich substrates. Thus, it is expected that food items of *Phylloicus* present lower numbers and diversity of yeasts as compared to filamentous fungi. Diet-based acquisition of yeasts might explain both low infection incidence and great differences in yeast communities within and between sampling locations.

It has been demonstrated that insects can benefit from nutritional supplements provided by symbiotic fungi (Sasaki et al., 1996; Noda and Koizumi, 2003). The food source used by aquatic shredders such as *Phylloicus* spp. is a substrate rich in carbohydrates (cellulose, for example) and at the same time, low in nitrogen, similar to the resource used by wood-destroying insects, such as longhorned beetles (Grünwald et al., 2010), in which the presence of yeasts in the DT has already been demonstrated and the active participation of these microorganisms in the nutrition of their host is presumed. In a similar way, it is possible to suppose that the yeasts associated with the DT of *Phylloicus* exerts activities related to the conditioning of the lignocellulosic organic matter consumed by this insect, as presumed for other insects that also feed on similar substrates and present interaction with yeasts in their DT (Gujjari et al., 2011; Suh et al., 2013). Taking into account the low incidence of yeast infection among *Phylloicus* larvae, yeast functions within the host, if any, must be non-essential. The screening for cellulolytic activity, performed in this study, revealed that this characteristic is not generalized for all yeasts obtained, since only a part of the strains was able to degrade carboxymethylcellulose in agar plates. It is known that yeasts are not commonly cellulolytic and few species have this ability (Jiménez et al., 1991; Nakase et al., 1994; Buzzini and Martini, 2002). There are records of strains of cellulolytic yeasts belonging to some of the genera detected in this study, such as *Aureobasidium* (Jiménez et al., 1991), *Candida* (Strauss et al., 2001; Kanti and Sudiana, 2002), *Cryptococcus* (Thongekkaew et al., 2008; Jaiboon et al., 2016),

Meyerozyma (Kuo et al., 2015) and *Rhodotorula* (Kanti and Sudiana, 2002). Regarding the cellulolytic yeasts obtained in this study, only *M. guilliermondii* (Yun et al., 2015) and *R. mucilaginosa* (Hu et al., 2015) were previously described with this capacity.

Methodological culture-based approaches to investigate the occurrence of yeast associated with the DT of insects such as that adopted in this study have been successfully used (Suh et al., 2003; Gujjari et al., 2011; Ricci et al., 2011; Urbina et al., 2013; León et al., 2016), since a high diversity of these microorganisms has been revealed including the discovery of new yeast species (Middelhoven et al., 2004; Suh and Zhou, 2011; Oliveira et al., 2014; Handel et al., 2016). Investigations based exclusively on culture-independent methods or on the combination of both approaches (Gusmão et al., 2010; Grünwald et al., 2010; Lou et al., 2014) have also proved useful as complementary strategies, enabling the detection of fastidious yeasts (Zhang et al., 2003) or confirmed findings by culture-based methods (Molnár et al., 2008).

Here, we demonstrate that the DT of *Phylloicus* might harbour different yeast taxa (twenty-one species). Among the yeast genera found in the DT of *Phylloicus* spp., *Candida*, *Issatchenkia*, *Papiliotrema* and *Rhodotorula* were the most frequent ones. *Candida* is a highly diversified and polyphyletic genus that has been reformulated (Lachance et al., 2011). This genus appears to be ubiquitous in insects and has not been associated with a specific host since it has previously been detected in association with DT from a wide range of insects such as *Odontotaenius disjunctus* (Coleoptera: Passalidae), *Phrenapates bennetti* (Coleoptera: Tenebrionidae) (Nguyen et al., 2006), among others.

Rhodotorula, that presented frequency of occurrence similar to *Candida*, is frequently associated with aquatic habitats (Li et al., 2010; Brandão et al., 2017), that was the probable source of this yeast to *Phylloicus* larvae. Representatives of this genus have already been detected in association with insects (Zacchi and Vaughan-Martini, 2002; León et al., 2016), as well as other substrates, such as plant tissues (Gan et al., 2017; Martins et al., 2018). Taking into account the feeding strategies of *Phylloicus* spp. larvae, the presence of *Rhodotorula* in plant tissues, as well as in aquatic environments, strongly suggest dietary items as their main source of yeast acquisition.

The genus *Issatchenkia* has already been detected in association with the DT of coleopteran insects (Rao et al., 2007) and outer surfaces from stingless bees (Rosa et al., 2003). In addition, Coelho et al. (2010), Silva-Bedoya et al. (2014) and Chang et al. (2016) have reported the occurrence of the genus *Issatchenkia* from aquatic ecosystems, and thus supporting the hypothesis of acquisition of these yeasts by *Phylloicus* spp. from the environment.

The genus *Papiliotrema*, as frequent as the genus *Issatchenkia*, belongs to the family Rhynchogastremataceae, according to recent phylogenetic analysis of the tremellomycetous yeasts (Liu et al., 2015a, b). Two species of this genus (*P. flavescens* and *P. laurentii*) were detected in association with the DT of *Phylloicus*. Yeasts of the genus *Papiliotrema* have also been detected in association with the DT of *Ostrinia nubilalis* (Lepidoptera: Pyralidae) (Molnár et al., 2008) and *Odontotermes obesus* (Isoptera: Termitidae) (Handel et al., 2016).

High frequency of occurrence for a yeast species in samples of a single insect group may indicate a possible symbiotic association between this microorganism and the insect. In this study, none of the yeast species detected in DT of *Phylloicus* larvae could be considered constant (frequency > 50%) or accessory (25-50%). All species were accidental (frequency < 25%) (**Table 3**). Zhang et al. (2003), in investigations related to yeast associated with DT of beetles, verified that, in general, each individual beetle harbors only one species of yeast and in some cases two or three additional species were verified. Similar findings were revealed for yeasts associated with the DT of *Phylloicus*, where most of the digestive tracts sampled contained only one species of yeast or a maximum of three species. As proposed by Zhang et al. (2003) for beetles, these findings may be indicative of specificity between *Phylloicus* species and yeasts.

Most yeast species detected here have already been detected in association with insects (larvae and/or adults), either from external surfaces or from internal organs (**Table 4)**. This is the first report of *A. thailandense*, *Candida boidinni*, *C. rugosa*, *Cr. podzolicus*, *I. siamensis* and *Pseudozyma antarctica* in association with insect DT. The most frequent species from *Phylloicus* spp., *Rhodotorula mucilaginosa*, was also detected in DT of *Dactylopius coccus* and *D. confusus* (León et al., 2016) and from internal parts of *Labidura* sp. (Dermaptera: Labiduridae) (Zacchi and Vaughan-Martini, 2002). We could not find evidence of symbiotic association of *R. mucilaginosa* and *Phylloicus* spp. Future studies of diet preference of these shredders and the expansion of collections in tropical habitats may help to clarify the occasional association found in this study.

Table 4: Reports of occurrence of the yeast species isolated in this study.

Yeast Species	Digestive tract of insects	References	Other natural substrates	References
Aureobasidium thailandense	First report in this study		Leaves and wooden surfaces of *Cerbera odollam* Gaertn; Internal parts of the *Forcipomyia taiwana* (Diptera: Ceratopogonidae) adults; Cashew (*Anacardium occidentale* L.) apple peduncle	Peterson et al., 2013; Chen et al., 2016; Meneses et al., 2017.
Candida blattae	*Corydalus cornutus* (Neuroptera: Corydalidae) and unidentified cockroach adults	Nguyen et al., 2007.	Natural fermentation of Taberna (an alcoholic beverage made from palm sap)	Santiago-Urbina et al., 2016.
C. boidinii	First report in this study		Internal parts of marine invertebrates (crabs and mollusks); External surfaces of *Drosophila* spp. (Diptera: Drosophilidae); Rotting wood from Tropical forest	Araujo et al., 1995; Pimenta et al., 2009; Morais et al., 2013.
C. parapsilosis	Variety of passalid beetles; Black beetles *Pterostichus melanarius* (Coleoptera: Carabidae) adult	Suh et al., 2005; Moubasher et al., 2017.	Adult haemolymph and fourth-instar larvae of *Solenopsis invicta* (Hymenoptera: Formicidae) red imported fire ant; Estuarine waters; Internal parts of the *Culex pipiens* (Diptera: Culicidae) mosquito larvae; Soil microbiome	Ba and Phillips, 1996; Coelho et al., 2010; Steyn et al., 2016; Yurkov et al., 2015.
C. rugosa	First report in this study		Adult haemolymph and fourth-instar larvae of *Solenopsis invicta* (Hymenoptera: Formicidae) red imported fire ant; External surfaces of *Drosophila* spp. (Diptera: Drosophilidae); Phylloplane of sugarcane (*Saccharum officinarum* L.)	Ba and Phillips, 1996; Pimenta et al., 2009; Limtong et al., 2014.

Table 4: Reports of occurrence of the yeast species isolated in this study (continuation).

Yeast Species	Digestive tract of insects	References	Other natural substrates	References
Cryptococcus podzolicus	First report in this study		Rotting wood from Tropical forest; Continental aquatic environments; Soil microbiome	Morais et al., 2013; Silva-Bedoya et al., 2014; Yurkov et al., 2015.
Debaryomyces hansenii	*Odontotermes formosanus* (Isoptera: Termitidae) adults	Mathew et al., 2012.	Adult haemolymph of *Solenopsis invicta* (Hymenoptera: Formicidae) red imported fire ant; Outer surfaces from stingless bees; Continental aquatic environments; External surfaces of *Drosophila* spp. (Diptera: Drosophilidae); Soil microbiome	Ba and Phillips, 1996; Rosa et al., 2003; Medeiros et al., 2008; Pimenta et al., 2009; Yurkov et al., 2015.
Hannaella luteola	*Diabrotica virgifera* (Coleoptera: Chrysomelidae)	Molnár et al., 2008.	Internal parts from *Culex theileri* (Diptera: Culicidae) larvae; Continental aquatic environments	Steyn et al., 2016; Brandão et al., 2017.
Issatchenkia siamensis	First report in this study		Sediments, leaves and fruits from mangrove	Chi et al., 2012.
Lodderomyces elongisporus	*Xylosandrus mutilatus* (Coleoptera: Curculionidae: Scoltylinae)	Suh et al., 2008.	Phylloplane of sugarcane (*Saccharum officinarum* L.); Sea surface microlayer	Limtong et al., 2014; Chang et al., 2016.
Meyerozyma guilliermondii	*Suilla* sp. (Diptera: Heleomyzidae) larvae; *Odontotermes formosanus* (Isoptera: Termitidae) adults	Zacchi and Vaughan-Martini, 2002. Mathew et al., 2012;	External surfaces of *Drosophila* spp. (Diptera: Drosophilidae); Estuarine waters; Internal parts from *Culex theileri* (Diptera: Culicidae) larvae; Soil microbiome	Pimenta et al., 2009; Coelho et al., 2010; Steyn et al., 2016; Yurkov et al., 2015.

Table 4: Reports of occurrence of the yeast species isolated in this study (continuation).

Yeast Species	Digestive tract of insects	References	Other natural substrates	References
Papiliotrema flavescens	*Ostrinia nubilalis* (Lepidoptera: Pyralidae), *Diabrotica virgifera* (Coleoptera: Chrysomelidae) and *Helicoverpa armigera* (Lepidoptera: Noctuidae)	Molnár et al., 2008.	Phylloplane of sugarcane (*Saccharum officinarum* L.); Wild flowers; Continental aquatic environments	Limtong et al., 2014; Han et al., 2015; Brandão et al., 2017.
Pa. laurentii	Passalid beetles	Suh et al., 2005;	Outer surfaces from stingless bees; Wild flowers; Soil microbiome; Continental aquatic environments	Rosa et al., 2003; Han et al., 2015; Yurkov et al., 2015; Brandão et al., 2017.
Pseudozyma antarctica	First report in this study		Outer surfaces from stingless bees; Continental aquatic environments; Outer surfaces from *Drosophila* spp. flies; Sugarcane (*Saccharum officinarum* L.) phyllospheres	Rosa et al., 2003; Medeiros et al., 2008; Pimenta et al., 2009; Nasanit et al., 2015.
Rhodotorula mucilaginosa	*Dactylopius coccus* (Hemiptera: Coccoidea: Dactylopiidae) and *D. confusus*	León et al., 2016.	Inner body content from *Labidura* sp. (Dermaptera: Labiduridae), *Drosophila* sp. (Diptera: Drosophilidae) and *Iridomyrmex humilis* (Hymenoptera: Formicoidea) adults; Internal parts from *Culex theileri* (Diptera: Culicidae) larvae; Continental aquatic environments	Zacchi and Vaughan-Martini, 2002; Steyn et al., 2016; Brandão et al., 2017.

Issatchenkia siamensis and *P. laurentii* were, respectively, the second and third yeast species most frequently isolated in this study. *I. siamensis* has been isolated from sediments, leaves and fruits from mangrove (Chi et al., 2012), but not in association with the DT of an insect. *Papiliotrema laurentii,* yeast species resulting from the sequence-based reclassification of the genus *Cryptococcus* (Liu et al., 2015a, b), is reported as a member of the yeast community of the DT of passalid beetles (Suh et al., 2005) and a variety of natural ecosystems, including soil (Yurkov et al., 2016) and freshwater lakes (Brandão et al., 2017). The frequent isolation of these yeasts in DT of *Phylloicus* reinforces the origin of this community as being plant tissues and aquatic ecosystems.

Candida parapsilosis and *R. mucilaginosa* were the only yeasts isolated from the DT of insects in all locations sampled (MT and PA states). Differences in the yeast micobiota of *Phylloicus* larvae in the two locations may be due to environmental factors such as abiotic factors of the water and the vegetational differences between the two areas. As mentioned before, the probable source of yeasts for *Phylloicus* larvae is the water or the plant materials from riparian vegetation, which has a very variable species richness in tropical and subtropical streams in the world (França et al., 2009; Rezende et al., 2017), but may also be due to taxonomic differentiation of the *Phylloicus* species. Further investigation may clarify variations in the yeast mycobiota in DT of different species of the genus.

Yeast species richness and diversity were lower in the DT of insects found in the PA samples as compared to the MT samples. One possible explanation would be related to environmental quality of the habitat, since the sampling area in MT is a typical Cerrado landscape, whereas the PA area is a fragment of Cerrado inserted in a matrix of Amazon forest. Morais et al. (1992) showed that primary forest environments hold a higher diversity of yeasts associated with *Drosophila* flies as result of more diversified food sources. Park et al. (2018) found differences in diversity indices of fungal genera associated with larvae of *Bradysia agrestis* (Diptera: Sciaridae), a phytopathogen-transmitting insect vector in East Asia by geographically (ecologically) segregated regions.

It may also be possible that the differences are related to variations in the composition of the communities of plants belonging to riparian vegetation of each sampled area and contributing to the diet of *Phylloicus* larvae. Riparian vegetation is the main source of energy for the food web in streams, providing organic matter for a variety of aquatic communities (Gimenes et al., 2010; Tank et al., 2010; Gonçalves and Callisto, 2013). There is a difference in plant species richness present in the riparian vegetation of the different ecosystems and

landscapes in Brazil (Afonso et al., 2000, França et al., 2009, Bambi et al., 2017, Rezende et al., 2017). There is also divergence among fungal communities that colonize decomposing plant species (Krauss et al., 2011; Medina-Villar et al., 2015; Gomes et al., 2016), and in the palatability of these colonized vegetable debris, which influences the food preference of the insect shredders (Graça et al., 2001; Gonçalves et al., 2014).

Geib et al. (2009) showed that host tree species had a marked effect on diversity of the larval gut bacterial community in the Asian longhorned beetle. Shredder insects, such as *Phylloicus* larvae, are selective feeders and their preferences have been related with microbial conditioning among other factors (Graça et al., 2001; Canhoto et al., 2005; Graça and Cressa, 2010). Gonçalves et al. (2016) demonstrated, in a microcosm experiment, that the consumption by the shredder *Schizopelex festiva* (Trichoptera: Sericostomatidae) of leaf discs conditioned by rich fungal assemblages was greater than in those conditioned by single species. Ferreira et al. (2015) assessed the diets of *Phylloicus* larvae in headwater streams of Cerrado and concluded that diets of the same taxon may vary because of basin or regional differences in riparian vegetation density (Ferreira et al., 2015). Although it is currently known that these shredders prefer to consume leaves conditioned by fungi than unconditioned leaves (Graça et al., 1993a, b; Graça and Cressa, 2010), it is possible that *Phylloicus* species are generalist feeders on fungi, and they are randomly feeding on leaves colonized by yeasts in general but not a particular yeast species.

2.5- References

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CAPÍTULO III:

A DIVERSE AND PARTIALLY CELLULOLYTIC FUNGAL COMMUNITY CONTRIBUTES TO THE DIET OF OF THREE SPECIES OF THE AQUATIC INSECT *Phylloicus* (TRICHOPTERA: CALAMOCERATIDAE) IN AMAZONIAN STREAMS[7]

Abstract: We studied fungal taxa associated with the digestive tract (DT) of the aquatic shredder *Phylloicus amazonas, P. elektoros* and *P. fenestratus* larval stages, that occurs in streams of the Brazilian Amazon Forest. Filamentous fungi were isolated, purified and screened for cellulolytic activity. A total of 33 fungal taxa was identified through the combination of classical and molecular [sequencing of the internal transcribed spacer (ITS) regions of the rDNA] taxonomy. The genus *Penicillium* was the most frequent in DT of *Phylloicus* spp. (18.75%), followed by *Pestalotiopsis* and *Trichoderma* (10.42%, each). The occurrence of fungal taxa among hosts was quite variable, with more than half of the associated fungi being exclusive of each host species. Only two fungal species (*Pestalotiopsis microspora* and *Penicillium citrinum*) were found in the three insect species. A significant portion of the fungal community associated with each host presented cellulolytic activity (± 50 % of the strains associated). Although there was no evidence of species-specific symbiotic interaction between filamentous fungi and their hosts, *Phylloicus* spp. consistently has cellulolytic filamentous fungi associated with its DT, corroborating the possible role of these microorganisms in the conditioning of the vegetal debris consumed by the shredder insects in streams.

Keywords: Aquatic macroinvertebrates; Cellulolytic fungi; Fungal diversity, Fungus-insect interaction.

[7]Em preparação para submissão.

3.1- Introduction

The digestive tract (DT) of insects has been revealed as a hotspot for studies of diversity and for understanding the symbiotic relationships between fungi and insects (Suh et al., 2005; Nguyen et al., 2006; Lichtwardt, 2012). New records of occurrence as well as the discovery of new fungal taxa has been possible from the exploitation of this habitat (Suh and Zhou 2011; Misra et al., 2014; Oliveira et al., 2014; Handel et al., 2016). In addition, the functional characterization of the fungal organisms from DT of insects has been contributed to the understanding of the roles played by them in the interaction interface with their hosts (León et al., 2016; Stefani et al., 2016), besides generating insights for potential biotechnological applications, such as the selection of fungal strains producing enzymes of industrial interest (Suh et al., 2013).

Most studies related to the DT of insects as a fungal habitat have focused mainly on terrestrial hosts such as beetles (Gama et al., 2006; Stefani et al., 2016), flies (Broderick and Lemaitre, 2012; Ramírez-Camejo et al., 2017) and termites (Schäfer et al., 1996; Handel et al., 2016). Therefore, the knowledge about the interaction between aquatic insects and fungal organisms is quite limited and restricted to the Trichomycetes class (Zygomycota) associated with a small group of insects (White and Lichtwardt, 2004; Siri and Lastra, 2010; Misra et al., 2014).

Insects and fungi are involved in several ecological processes that occur in aquatic ecosystems, such as the decomposition of plant debris (Graça, 2001; Hieber and Gessner, 2002; Krauss et al., 2011). In these ecosystems, fungal colonization affects the quality of plant debris, by increasing palatability and nutritional value, resulting in differences in performance (growth, survivorship and reproduction) of aquatic insects that feed on this organic matter (Arsuffi and Suberkropp, 1989; Chung and Suberkropp, 2009).

Several works have stressed the importance of fungi in diet and food preference of detritivorous aquatic insects (shredders) (Graça et al., 2001; Canhoto et al., 2005; Graça and Cressa, 2010; Cornut et al., 2015). Among the aquatic shredder insects that occur in Brazil, *Phylloicus* spp. (Trichoptera: Calamoceratidae) is especially diverse (Prather, 2003), with many records of occurrence of *Phylloicus* species for the Brazilian Amazon Forest and Atlantic Forest (Dumas and Nessimian, 2010; Santos and Nessimian, 2010; Calor, 2011; Quinteiro et al., 2011; Gama Neto et al., 2017). Recently, one study reported the frequent occurrence of cultivable filamentous fungi in association with the DT of *Phylloicus* (presence in 94.9% of the DT

analyzed) from streams under different ecological landscapes, in the Brazilian Amazon (Santos et al., in press). However, little is known about the taxonomic identity of the filamentous fungi associated with these shredders, as well as about the existence or not of species-specific interaction between these microorganisms and their hosts.

We performed the isolation and molecular identification of filamentous fungi associated to the DT of three *Phylloicus* species (*P. amazonas* Prather, *P. elektoros* Prather and *P. fenestratus* Flint) from two streams of a protected forest in the Brazilian Amazon aiming to verify a possible species-specific relationship between these two groups. We also tested the spetrum of cellulolytic activity among the fungal community as possible benefit to the insect host by the digestion of plant food resources. We hypothesize that fungi and shredders present a symbiotic relationship in which fungi transform plant detritus in highly palatable and energy-rich food and the insect harbours specific fungal taxa in the variable habitat of low-order streams.

3.2- Material and Methods

3.2.1- Characterization of study area

The sampling was carried out in the Tapajós National Forest, which is a biodiversity conservation unit located in the Pará state, Brazil, with vegetation classified as Dense Ombrophylous Forest (Veloso, 1991), characterized by the dominance of large arboreal individuals and by the abundance of woody lianas, palms and epiphytes. Low-order streams (stream I: 03°15'44.7"S; 54°57'22.0"W; stream II: 03°15'38.7"S; 54°56'42.8"W) (**Fig. 7**) were selected for collection. In each selected stream, a 50 m stretch was used to select the available substrate (especially foliage) at five points 10 m afar, with the aid of a D-frame net (0.500 mm mesh and 0.465 m^2 area). At each point, three subsamples were collected, which were screened in the field for the collection of typical cases of *Phylloicus*. The collections were authorized by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) [*Sistema de Autorização e Informação em Biodiversidade* (SISBIO) license number 55136].

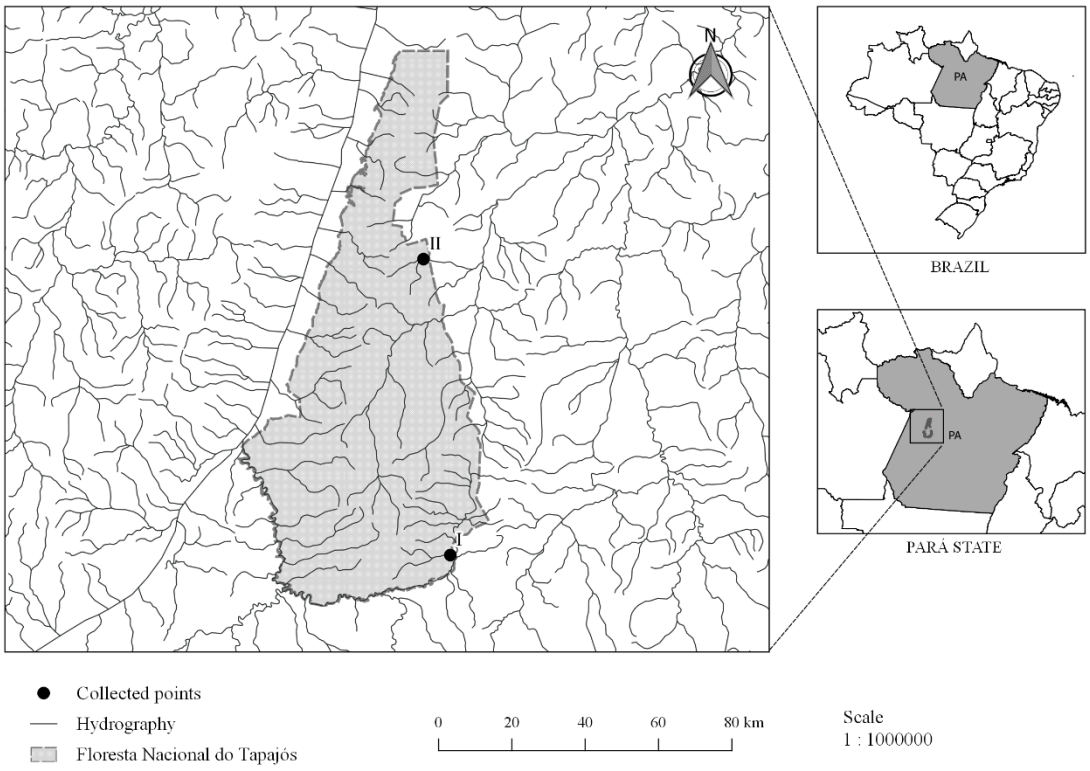

Fig. 7: Map of the sites showing the low order streams and location in the Pará State, Brazil. Abbreviations: PA = Pará State; I and II = streams sampled.

3.2.2- Insect sampling and identification

The larvae of *Phylloicus* spp. were identified through association with adults. Larvae were taken alive and brought to the laboratory, fed with leaves from the streams until the adults were obtained. After the emergence, adults were identified until species using the key of Prather (2003). The larvae sampled for fungal isolation were differentiated through the shape and other characteristics of the cases (dimensions, format and composition and form of plant leaf material). Further on, the carcasses of the larvae were kept in 80 % ethyl alcohol for later identity confirmation throught comparative description of head capsule, spinules, mandibles and labrum and also by larval sclerites within the case by one of the authors (Pes, A. M.).

3.2.3- Isolation, morphological characterization, purification and preservation of filamentous fungi

Under aseptic conditions, the larvae were carefully removed from the cases and individually subjected to surface disinfection (immersion in 70 % ethyl alcohol for 30 seconds, washing with sterile distilled water abundantly). Then, with the aid of a stereoscopic microscope, the DT of each larvae was dissected for dispersion of the contents in 1.0 mL of sterile distilled water, conditioned in 1.5 mL microtubes. After the homogenization of the contents, the inoculation was done in triplicate of 100 μL aliquots in 90 mm diameter Petri dishes containing PDA (Potato, Dextrose and Agar) culture medium (potato extract: 4.0 g, dextrose: 20.0 g, agar: 15.0 g, distilled water: 1000 mL), supplemented with 0.1 $\mu g.mL^{-1}$ chloramphenicol for inhibition of bacterial growth. A negative control of the larvae disinfection was performed by inoculating the final water of the disinfection procedure in the same culture media. Plates were incubated (25 ± 3 °C) for three to ten days, being inspected daily, until the fungal growth.

The fungal Colony-forming Units (CFU), from each DT, were grouped into morphotypes based on macro and micromorphological characterization (Lacap et al., 2003; Ibrahim et al., 2017) and counted for quantitative analysis. One to five representatives of different fungal morphotypes from each DT were purified by successive passages in PDA. The preservation of pure cultures occurred through the Castellani technique (Castellani, 1939) in the *Coleção de Culturas Microbianas Carlos Rosa* for later identification.

3.2.4- DNA extraction, amplification and sequencing

One to five representatives of each morphotype was inoculated in 2.0 % ME broth (malt extract: 20.0 g, distilled water: 1000 mL) and cultured on shaker type oscillatory platform at 150 rpm, 25 ± 3 °C, three to five days. After this period, about 40 mg of mycelium was separated from the liquid medium and used for total DNA extraction using a Wizard® Genomic DNA Purification Kit protocol (Promega Corp., Madison, WI), following a slightly modified protocol from that of Burghoorn et al. (2002). After the extractions, the quantification and quality evaluation of the DNA obtained with the aid of the NanoDrop 2000 spectrophotometer (Thermo Scientific, Uniscience, Brazil) was carried out.

Then, amplification of the internal transcribed spacer (ITS) regions of the rDNA was performed in a thermocycler Mastercycler nexus (Eppendorf, São Paulo, Brazil) using a GoTaq DNA Polymerase kit (Promega Corp., Madison, WI). For this amplification, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) primers were used. Amplification reactions were performed according to Santos et al. (2015). The amplification reaction will be performed to a final volume of 25 µL containing 5 µl of Taq Polymerase buffer (5x); 2.5 µl $MgCl_2$ (25 mM); 1.0 µl of dNTPs (2.5 mM each dNTP); 1.0 µL of the ITS1 primer (5 µM); 1.0 µL of the ITS4 primer (5 µM); 0.25 µL of the enzyme Taq Polymerase (5 U/µL), 5 µL of genomic DNA (10 ng/ µL). Negative control (DNA replaced by water) was used.

The amplified fragments were analyzed by 1% (w/v) agarose gel electrophoresis with GelRed ™ (Biotium, Inc., Fremont, CA) in 1X TBE buffer (2 mM EDTA, 0.1 M Tris-HCl, and boric acid 0 , 1 M [pH 8.0]) (Sambrook et al., 1989) and visualized with UV illumination by a photodocumentation system LPIX EX (Loccus Biotechnology, Cotia, Sao Paulo, Brazil). The 1 Kb DNA Ladder (Promega Corp., Madison, WI) was used as molecular weight marker. Subsequently the PCR products of approx. 300–650 bp were purified using a Kit Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI) and bidirectionally sequenced according to the dideoxy chain-termination method (Sanger et al., 1977) using a BigDye Terminator v 3.1 sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed at Myleus Biotechnology, located in Belo Horizonte, Brazil (http://myleus.com/).

3.2.5- Identification of isolates

The nucleotide sequences generated from each individual were imported into the Geneious 6.1.8 program (Kearse et al., 2012) to be checked, edited and aligned. Sequences of both DNA strands were pooled into contigs, manually corrected and aligned. The alignments were exported in a FASTA extension file (*.fasta/*.fas) for the other analyzes and comparative research of sequence identity using the BLAST (Basic Local Alignment Search) tool (Altschul et al., 1990) of the NCBI (National Center for Biotechnology Information) (GenBank database) and in the CBS (*Centraalbureau voor Schimmelcultures* Fungal Biodiversity Centre) database (http://www.cbs.knaw.nl/Collections/). Identity ≥ 99% were indicative of the same species. The sequences were deposited in GenBank database under the accession numbers MK120544 to MK120591 (**Table S2**).

3.2.6- Phylogenetic analysis

Identical sequences from fungal taxa were treated as duplicates in phylogenetic analyses. The sequences representative of all taxa obtained in this study (36) and additional 36 sequences from GenBank were aligned using Clustal W (Thompson et al., 1994) as implemented in software MEGA version 6.0 (Biodesign Institute, USA) and trimmed. Phylogenetic tree was constructed by the neighbor-joining method. The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree (Tamura et al., 2013). A sequence of *Rhizopus oryzae* (Mucoromycota) from GenBank (AB381938) was used as outgroup as proposed in the phylogenetic analysis of filamentous fungi performed by Xiong et al. (2013). Sequences from this study were indicated in the tree by collection code, while GenBank sequences were indicated by accession numbers (**Figure 8** and **Table S2**).

3.2.7- Evaluation of cellulolytic activity

As proposed by Sunitha et al. (2013), the purified strains were cultured in PDA for seven days. Then, fragments of mycelium (5 mm diameter) were removed from the colonies and transferred to Petri dishes containing CMC medium (carboxymethylcellulose: 5.0 g, glucose: 1.0 g, yeast extract: 0.1 g, peptone: 0.5 g, agar: 16.0 g, distilled water: 1000 mL). After three to five days of incubation at 28 °C, the plates were flooded with 10 mL of 0.2 % aqueous Congo

red solution, which was maintained in contact with the plates for 30 min. This solution was then discarded and the plates were decolorized with 5.0 mL of 1.0 M NaCl solution, which was held in contact with the plates for 15 min and then discarded. The assay was performed in triplicate and the existence of degradation halo was indicative of positive cellulolytic activity, which was indicated the letter "P" in **Table 5**, whereas negative strains were indicated by "N" and fungal taxa that had positive and negative strains were indicated by the letter "V", which means variable cellulolytic activity.

3.2.8- Statistical Analysis

Excel software, version 2016 (Microsoft ™), was used to calculate the geometric mean and standard deviation of the Colony-forming Units per DT (CFU/DT) in relation to the total of DT sampled and in relation to the total of DT from the same *Phylloicus* species. Regarding to community analysis, data was expressed as the presence/absence of fungal taxa in insect DT (occurrence) (named as *ni* in **Table 5**). The same software was used to calculate the frequency of occurrence (*Fo*), which corresponding the percentage of DT in which the fungal taxa was found in relation to the total DT sampled. Each strain represented the presence of the fungal taxa in a DT where these fungal taxa was detected, not the number of filamentous fungi cells present in the DT. *Fo* is calculated as follows: $Fo = (\sum_1^J ni \,/\, N) \times 100$, where *ni* equals the frequency of occurrence of the fungal taxa "*i*" in the DT "*j*"; "*N*" is the total number of DT sampled.

PAST software (version. 3.19) (Hammer et al., 2001) was used to compare the richness (Chao 1) of fungal taxa among hosts (*P. amazonas*, *P. elektoros* and *P. fenestratus*), diversity and equitability (*J*). The diversity was measured through the diversity indexes [Shannon (H') and Margalef (d)]. The Shannon index (H') assigns greater importance to less frequent ("rare") fungal taxa in the sample. It is calculated as $H' = -\sum_1^S (p_1 \,.\, ln\, p_i)$, where "$p_i$" is the frequency of isolation of each fungal taxa, varying from 1 to S (species richness) (Shannon, 1948).

The Margalef (d) index assigns greater importance to different fungal taxa in each sample. It is calculated as $d = (n-1)/\, ln\, N$, where "*n*" equals the number of fungal rates present; "*N*" is the total of individuals found (Margalef, 1958).

Equitability was measured by equability index of Pielou (*J*) (Pielou, 1966), which verifies the distribution of the number of isolates between fungal taxa. The index is based on

H' and is calculated as follows: *J' = H' (observed) / H' max,* where "*H' max"* equals log *S*; "*S*" is total number of fungal taxa.

The pattern of distribution (restricted or shared) of fungal taxa among hosts was visualized in a Venn diagram, built through the web application Venn Diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Excel software, version 2016 (Microsoft ™) was also used to calculate the percentage of negative or positive fungal strains for cellulolytic activity of each host (*P. amazonas*, *P. elektoros* and *P. fenestratus*).

3.3- Results

Fungal strains were obtained from all the digestive tracts analyzed. The fungal CFU/DT ranged from 1.7 x 10^1 to 2.1 x 10^3, with a geometric mean ± standard deviation of 3.9 ± 8.0 x 10^2 CFU/DT.

A total of 33 fungal taxa was isolated from the DT of the three *Phylloicus* spp., from which 22 species, corresponding to 16 genera, belonged to Ascomycota phylum and one species to Mucoromycota (**Table 5**). Eight taxa were identified only up to the genus level (all from the phylum Ascomycota). One Ascomycota (collection code: P9PC5A, **Table S2**) was identified only up to the family level (Bionectriaceae), and two ascomycetous isolates (collection codes: P10PA3 and P10PD3, **Table S2**) were identified only up to the order level (Pleosporales). The phylogenetic relationships among taxa are shown in **Fig. 8**.

Table 5. Identification, frequency of occurrence (*Fo*) and cellulolytic activity of fungal taxa isolated from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) from Amazon Forest, Brazil.

Fungal taxa	**% ID** [a]	**GenBank acession numbers**	**Host**						***Fo*** **(%)** [d]	**Cellulolytic activity** [e]
			P. amazonas **(n = 05)**		***P. elektoros*** **(n = 05)**		***P. fenestratus*** **(n = 04)**			
			n_i [b]	**Counts** [c]	**n_i**	**Counts**	**n_i**	**Counts**		
Arthopyrenia sp.	99%	LT796887	1	3					2.08	P
Arthrinium phaeospermum	99%	MH345868	1	3					2.08	N
Aspergillus aculeatus	99%	JX501412			1	3			2.08	N
As. oryzae	99%	HQ285542	1	7			1	130	4.16	N
Bionectriaceae sp.	98%	MH267845	1	10					2.08	P
Chaetomium cupreum	99%	KU204551			1	33	1	903	4.16	P
Cladosporium perangustum	100%	MG669149	1	10					2.08	P
Clonostachys rosea	99%	KY810806					1	13	2.08	P
Cordyceps spegazzinii	100%	KP133191					2	13	4.16	N
Diaporthe sp.	98%	KU523580					1	3	2.08	N
Epicoccum nigrum	100%	MG602595					1	877	2.08	P
Gliomastix polychroma	99%	MH859647					1	20	2.08	N
Letendraea helminthicola	99%	KJ774053	1	3					2.08	P
Neooccultibambusa pandanicola	99%	MG298941	1	3					2.08	N
Paraconiothyrium sp.	100%	KF746099					2	14	4.16	V
Penicillium citrinum	100%	MG948252	1	3	1	3	1	3	6.25	V
Pe. paxilli	99%	MH856391	1	3	1	3			4.16	P
Pe. sclerotiorum	100%	EF488396	1	7					2.08	P
Penicillium sp.	100%	KM458825			1	7	1	3	4.16	P
Penicillium sumatraense	100%	MH864546					1	7	2.08	P
Pestalotiopsis microspora	99%	MH094237	2	5	1	17	2	12	10.42	N

Table 5. Identification, frequency of occurrence (*Fo*) and cellulolytic activity of fungal taxa isolated from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) from Amazon Forest, Brazil (continuation).

Fungal taxa	**% ID** [(a)]	**GenBank acession numbers**	**Host**						***Fo*** **(%)** [(d)]	**Cellulolytic activity** [(e)]
			P. amazonas **(n = 05)**		***P. elektoros*** **(n = 05)**		***P. fenestratus*** **(n = 04)**			
			n_i [(b)]	**Counts** [(c)]	**n_i**	**Counts**	**n_i**	**Counts**		
Phialemoniopsis cornearis	99%	MH865903					1	13	2.08	P
Pleosporales sp.	99%	MH268068					2	5	4.16	P
Pyrenochaetopsis microspora	99%	LT623227			1	3			2.08	P
Ramichloridium sp. 1	99%	KU204638			1	7			2.08	N
Ramichloridium sp. 2	97%	KU204638					1	30	2.08	P
Tolypocladium album	99%	HQ608068			1	17	1	17	4.16	V
Tolypocladium endophyticum	100%	KF747262			1	3			2.08	N
Trichoderma sp. 1	99%	MK010822			1	13			2.08	N
Trichoderma sp. 2	99%	MH284652	1	3	1	237			4.16	N
Trichoderma spirale	99%	MH512952	1	10					2.08	N
Tr. strigosum	100%	EU718081			1	3			2.08	N
Umbelopsis isabellina	99%	MH863098					1	13	2.08	N
Total of occurrences			14		13		21		100%	

[(a)]Percentage of similarity between the nucleotide sequences obtained in that study with sequences available in the NCBI database;

[(b)]Total number of DT in which the fungal taxa was found;

[(c)]Geometric mean of the number of colony forming units (CFU) of fungal taxa in DT positive for fungal taxa presence;

[(d)] Frequency of occurrence (*Fo*), which was calculated as the relative occurrence of the fungal taxa in relation to the total occurrence;

[(e)]Abbreviations: "P" indicates positive cellulolytic activity; "N" indicates negative (no) cellulolytic activity; "V" indicates variable cellulolytic activity (some strains respond as negative and other as positive).

Fig. 8. Phylogenetic relationship between fungal taxa.

Regarding CFU/DT counts, *Chaetonium cupreum* was the fungal taxon that presented the highest count (903 UFC), followed by *Epicoccum nigrum* (877 CFU), *Trichoderma* sp. 2 (237 CFU) and *Aspergillus oryzae* (130 CFU).

The genus *Penicillium* (18.75%) was the most frequent in DT of the three *Phylloicus* spp., followed by *Pestalotiopsis* and *Trichoderma* (10.42%, each). *Pestalotiopsis microspora* was the most frequent fungal species (10.42%), followed by *Penicillium citrinum* (6.25%). These two species were the only taxa shared among the all species of *Phylloicus* studied here (**Fig. 9**) and all other species were isolated once or two times only. In addition to these two fungal taxa, *Phylloicus amazonas* and *P. elektoros* shared *Penicillium paxilli* and *Trichoderma* sp. 2. *Chaetomium cupreum, Penicillium* sp. and *Tolypocladium album* were shared among DT of *P. elektoros* and *P. fenestratus*. *Phylloicus amazonas* e *P. fenestratus* shared *Aspergillus oryzae* in addition to the two previously mentioned.

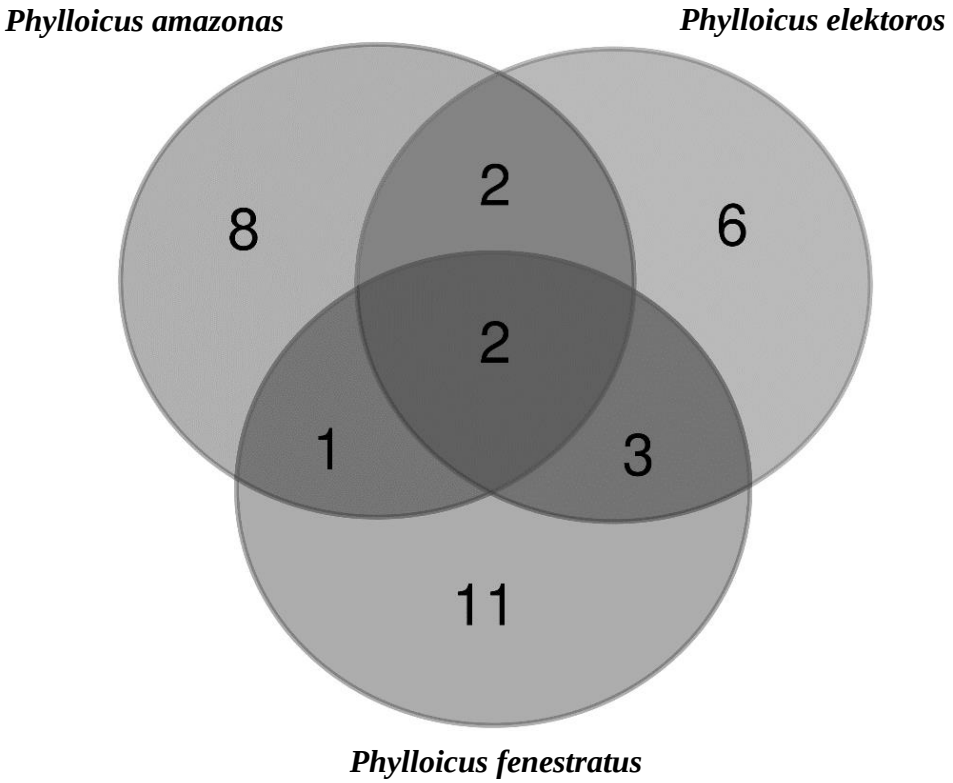

Fig 9. Venn diagram with the number of fungal taxa restricted and shared among *Phylloicus* species.

Diversity and equitability indexes are described in **Table 6**. The diversity index applied indicate higher diversity as well as equitability (*J*) associated with DT of *P. amazonas*.

Table 6. Richness, diversity indexes and equitability of fungal taxa associated with *Phylloicus* spp. (Trichoptera: Calamoceratidae).

Index	**Host**		
	P. amazonas	***P. elektoros***	***P. fenestratus***
Richness (Chao 1)	13	13	17
Shannon (*H'*)	2.44	1.32	1.37
Margalef (d)	2.80	2.05	2.09
Equitability (*J*)	0.95	0.51	0.48

In relation to cellulolytic activity, the behavior of the strains is shown in **Table 5**. Almost half of the all strains associated with the *Phylloicus* spp. (47.9%, n = 23/48) showed cellulolytic activity. Considering each host individually, it was observed that the percentage of positive strains was similar, with 57.1% (n = 8/14) in *P. amazonas*, 53.9% (n = 7/13) in *P. elektoros*, and 47.6% (n = 10/21) in *P. fenestratus*.

3.4- Discussion

The DT of the three *Phylloicus* spp. harbours a diverse community of fungi (33 fungal taxa) in the Amazon forest ecosystem. With the exception of *Umbelopsis isabellina*, which belongs to the phylum Mucoromycota, all taxa associated with *Phylloicus* DT belong to the phylum Ascomycota. Similarly, the abundance of ascomycetous fungi was also higher than that of other phyla in the DT of rove beetles (Coleoptera: Staphylinidae) (Stefani et al., 2016). On the other hand, Ascomycota was not prevalent in the DT of *Dactylopius* (Hemiptera: Coccoidea: Dactylopiidae) (León et al., 2016). A much larger variety of insects had their DT investigated for the presence of yeasts, among which the phylum Ascomycota is predominant (Blackwell and Jones, 1997; Suh et al., 2005). Sung et al. (2008) described *Paleoophiocordyceps coccophagus*, a fungal parasite of a scale insect from the Early Cretaceous (Upper Albian) that provides the oldest fossil evidence of animal parasitism by fungi and also supports both a Jurassic origin of fungal–animal symbioses within Hypocreales (Sordariomycetes, Pezizomycotina, Ascomycota) during the Cretaceous, concurrent with the diversification of insects and angiosperms that may suggest a prevalence of Ascomycota as insect symbionts. Futher investigation of fungal communities associated with DT from other insects may clarify whether or not Ascomycetous fungi prevail as symbionts with insects.

Ascomycota are known to dominate the early succession in decomposing leaves in streams. A work by Vorísková and Baldrian (2013) showed that sequences assigned to the Ascomycota showed highest relative abundances in live leaves and during the early stages of decomposition in streams. Fungi from the Ascomycota phylum also prevailed in the live and senescent leaves on the trees (88.5% and 99.5% of amplicons, respectively). These data are in accordance with previous culture-based studies on various trees (Osono, 2002; Santamaría and Bayman, 2005) and the pyrosequencing analyses of live *Quercus macrocarpa* leaves (Jumpponen and Jones, 2009a, 2009b). Endophytes in plant leaves are prevalently Ascomycota (Rodriguez et al., 2009). As *Phylloicus* is a shredder feeding on coarse particulate organic matter (CPOM) composed primarily of leaves and wood debris, it is expected that Ascomycota fungi will prevail in its diet.

Penicillium, *Pestalotiopsis* and *Trichoderma* were the most frequent isolated genera. The genus *Penicillium* have been detected from the DT of a wide variety of hosts, such as *Rhodnius prolixus* (Hemiptera: Reduviidae) (Moraes et al., 2004), *Dactylopius coccus* (León et al., 2016) and *Drosophila melanogaster* (Diptera: Drosophilidae) (Ramírez-Camejo et al.,

2017). In addition, it has also been verified from a wide variety of environments and substrates, such as plant hosts (Larran et al., 2007; Hanada et al., 2010) and continental aquatic ecosystems (Krauss et al., 2011; Sandberg et al., 2014), which correspond respectively to the food source and habitat of *Phylloicus* larvae.

Fungal taxa from the genus *Pestalotiopsis* and *Trichoderma* have been detected in association with a smaller scope of insects compared to the genus *Penicillium*. The genus *Pestalotiopsis* was detect in the DT of *Diaphania pyloalis* (Lepidoptera: Pyralididae) (Chen et al., 2018) and exoskeletons of *Cydia ulicetana* (Lepidoptera: Tortricidae) (Yamoah et al., 2008), while *Trichoderma* was detect from DT of triatomines (Hemiptera: Reduviidae) (Moraes et al., 2004) and exoskeletons of *Cydia ulicetana* (Lepidoptera: Tortricidae) (Yamoah et al., 2008). As well as *Penicillium*, representatives of both fungal taxa have detected in association with plant hosts and from aquatic ecosystems (Orole and Adejumo, 2011; Rocha et al., 2011; Rönsberg et al., 2013; Liu et al., 2016), indicating that these fungal taxa may be acquired by *Phylloicus* from food items and/or surrounding environment.

The two species most frequently associated with the three *Phylloicus* species were *Pestalotiopsis microspora* (*Fo* = 10.42 %) and *Penicillium citrinum* (6.25 %). The fungus *P. citrinum* was isolated from internal parts of the body of aquatic mosquito larvae (Diptera: Culicidae) (Pereira et al., 2009), while no previous reports of association with insects were found for *P. microspora*. The low frequency of isolation (*n* in **Table 5**) and low counts in individual DT does not support a close association of those fungi and the larvae of *Phylloicus.* In a similar approach, we could not find evidence of close association between *Phylloicus* larvae and yeasts isolated from their DT in savanna streams of Northern Brazil (Santos et al., 2018).

Although the combination of classical and molecular taxonomic approaches has been used, it was not possible to identify all the fungal taxa up to the species level. However, the sequencing of the rDNA ITS regions, which is consolidated as a barcode sequence for the identification of filamentous fungi (Nilsson et al., 2008; Gazis et al., 2011; Schoch et al., 2012), allowed the accurate identification, at the species or genus level, of the majority of isolates from this study. In addition, the phylogenetic analysis performed corroborates the taxonomic associations presented in **Table 5**. The Bionectriaceae isolate of this study (collection code: P9PC5A, **Table S2**), which present 98% similarity with a Bionectriaceae sp. MH267845 isolated from inner bark of *Micrandra spruceana* (Baill.) R. Schult. in Peru, groups robustly in the same clade of the species *Gliomastix polychroma*, which also belongs to the family Bionectriaceae (**Fig. 8**). Similarly, the Pleosporales isolates of this study (collection codes:

P10PA3 and P10PD3, **Table S2**), named as Pleosporales sp. in **Table 5**, which present 99% similarity with Pleosporales sp. MH268068, isolated from inner bark of *Hevea guianensis* Aubl. in Peru, are grouped with other taxa belonging to the order Pleosporales (*Arthopyrenia* sp., *Epicoccum nigrum*, *Letendraea helminthicola*, *Neooccultibambusa pandanicola*, *Paraconiothyrium* sp. and *Pyrenochaetopsis microspora*) (**Fig. 8**).

Regarding the isolates identified up to the taxonomic level of genera in this study, only two (collection codes: P9PH7 and P10PD1, **Table S2**) presented similarity less than 99% with sequences from GenBank and/or CBS database. The isolate P10PD1 (named as *Diaporthe* sp. in **Table 5**) presented phylogenetic proximity with *Diaporthe* sp. KU523580 (**Fig. 8**), isolated from soil in Brazil. On the other hand, the isolate P9PH7 (named as *Ramichloridium* sp. 2 in **Table 5**) presented phylogenetic proximity with *Ramichloridium* sp. 1, from this study, and KU204638 (**Fig. 8**), isolated from inner tissues of *Hirtella racemosa* Lam., in Costa Rica.

The amplification and sequencing of additional genomic regions, such as partial sequences of translation elongation factor 1-α, calmodulin, β-tubulin genes, has been proposed to contribute to the taxonomic elucidation of the standard barcode sequence (rDNA ITS regions) is not sufficient (Udayanga et al., 2012; Santos et al., 2015). This strategy may be used in future efforts of identification of the three isolates (collection code: P9PC5A, P10PA3 and P10PD3, **Table S2**) of this study with incomplete identification (only order or family level, as previously mentioned).

All species of *Phylloicus* larvae from this study come from streams of the same ecological landscape (Amazon Forest), from a geographical region of Brazil (Pará state). Although number of *Phylloicus* specimens collected was not high, the expected richness was similar to actual richness indicating that samplig effort was sufficient (**Table 6**). There was variation in fungal richness, uniformity and diversity among hosts. The species richness (Chao 1) was higher for *P. fenestratus* in comparison with the other host species. However, the species of *Phylloicus* with greater diversity was *P. amazonas* (d = 2.80; H'= 2.44) compared to *P. fenestratus* (d = 2.09; H' = 1.37) and *P. elektoros* (d = 2.05; H'= 1.32), as indicated by the Margalef (d) and Shannon (H') indexes, whose values are sensitive to the specific richness of each host.

The occurrence of fungi in DT of *Phylloicus* has not yet been reported for these Amazonian species. Ceneviva-Bastos et al. (2017) have reported the main food items in guts of the tophic guilds of Ephemeroptera, Plecoptera and Trichoptera in three basins of Brazilian Savanna and fungi were considered important items for the Ephemeroptera *Leptohyphes*,

Miroculis and the Trichoptera *Grumichella* but not for the coarse detritivore *Phylloicus* that presented the most flexible trophic guild, and was classified as omnivore. It is usually accepted that stream macroinvertebrates exhibit plasticity in their feeding habitats, being considered generalists in many cases (Friberg and Jacobsen, 1994; Mihuc and Minshall, 1995; Carvalho and Graça, 2007; Moretti et al., 2009). In general, *Phylloicus* larvae are usually considered typical shredders, and one expects to find a predominance of CPOM, which is defined as leaf fragments and wood debris, but also including fungal cells on accordance with Cummins and Klug (1979), in the DT of the larvae. However, Ferreira et al. (2015) found that fine particulate organic matter (FPOM) [defined as particles from 0.5 m to 1.0 mm among which fungal cells and spores could be included as pointed by Cummins and Klug (1979)] predominated in all instars and they suggest that *Phylloicus* larvae exhibited plasticity in their dietary behavior. FPOM was the most important food resource for the leaf litter-associated insect community in the studies of Palmer et al. (1993), Tomanova et al. (2006), Chará-Serna et al. (2012), Callisto and Graça (2013) and studies reviewed by these authors. FPOM is primarily generated from the decomposition of CPOM by shredders, microorganisms and physical abrasion (Allan, 1995) and constitutes a mostly continuous resource in the streams, and its ubiquity in the guts of leaf litter-associated invertebrates may be explained by its high availability in the habitat. On the other hand, Carvalho and Uieda (2009) showed that *Phylloicus* sp. consumed mostly CPOM and can be classified as the unique specialist shredder in a stream of Southeast Brazil. What emerges from those works is the great variability in feeding behavior of *Phylloicus* and the rarity of data on the presence of fungi associated with their diets.

One could argue that the larvae are probably feeding on fungal cells among particles ingested at ramdom and the fungi found in their DT are the most abundant in the environment. Nevertheless the fungi presenting the highest counts were not the most frequently isolated. *Chaetonium cupreum* presented 903 CFU in the one specimen it was isolated; *Epicoccum nigrum* also presented a high count of 877 CFU in the one DT it was isolated whereas *Pestalotiopsis microspora* counts were 5 to 17 CFU per DT in the five hosts it was found. A work by Santos et al. (in press) shows the fungal counts varied from $5.7 \pm 24.9 \times 10^1$ CFU/DT (in the Cerrado) to $1.1 \pm 2.2 \times 10^2$ CFU/DT (in the *Lavrado* [Savanna]) and $1.9 \pm 7.1 \times 10^2$ CFU/DT (in the Amazon forest). This indicates that for those two particular fungal species *C. cupreum* and *E. nigrum* and also for *Trichoderma* sp. 2 (237 CFU/DT) and *A. oryzae* (130 CFU/DT), counts are exceedingly higher than for other fungal species and even for whole

counts in the study of Santos et al. (in press). Since, we hypothesize that a degree of choice can be found in ingestion of fungi by *Phylloicus* larvae in Amazonian streams.

The composition of the fungal community was different among the host species. The number of fungal taxa with occurrence restricted to one host species was much higher than the total of species shared between two or more hosts. These findings lead to the hypothesis that *Phylloicus* larvae of the three species do have different food preferences and may choose leaves from different plant species that are colonized by those particular fungi found in their DT and a possible feeding preference for the leaf species and not the fungal species. Works showing different fungal communities in leaves of different plants species in streams (Vorísková and Baldrian, 2013; Medina-Villar et al., 2015). Riparian vegetation is composed of a variety of plant species (Afonso et al., 2000; França et al., 2009; Bambi et al., 2016; Rezende et al., 2017), which results in the imput of leaves of different species simultaneously in streams. The fungal colonization of these leaves can be influenced by several factors, including chemical properties and physical structure of the leaf surface (Dang et al., 2007; Ardón and Pringle, 2008; Lecerf and Chauvet, 2008; Ferreira et al., 2012), efficient attachment of conidia to a suitable substrate (specifically in the case of aquatic hyphomycetes) (Dang et al., 2007), replacement of native riparian vegetation by exotic vegetation (Medina-Villar et al. 2015; Gomes et al. 2016), among others.

Another possible explanation is related to different choice of fungal patches in leaves or other substrates in the streams, since the specimens of *Phylloicus* of different species were obtained from the same streams. Usually, shredders feed more readily upon leaves with less lignin, higher nutritional quality, greater conditioning by microorganisms and fewer toxic secondary compounds (Graça and Cressa, 2010; Jabiol et al., 2013). Studies show that *Phylloicus* spp. preferred plant leaves conditioned (colonized) by fungi as compared to non-conditioned ones (Graça et al., 2001; Graça and Cressa, 2010). In addition, it was demonstrated that fungal biomass associated with leaf fragments contributes significantly to the growth of a shredder, *Pycnopsyche gentilis* (Trichoptera: Limnephilidae), through the incorporation of fungal carbon in the body of the insect (Chung and Suberkropp, 2009).

The diet of shredders may vary also with life stage (Malas and Wallace, 1977; Casas, 1996). The strategies for coexistence in three species of caddisflies (Trichoptera) in second-order streams was studied by Malas and Wallace (1977) and observed a greater proportion of fine particles in the early instars of two species (*Dolophilodes* sp. and *Diplectrona* sp.). One possible explanation for our findings of one single or two occurrences of the fungal species in

DT of the sample could be that the larvae were from diffferent stages of development, a condition we have not taken into consideration in the present work and may be further investigated.

This is a first report to occurrence of *Aspergillus oryzae*, *Chaetomium cupreum*, *Penicillium paxilli* and *Tolypocladium album* in DT of an insect. The fungus *Aspergillus oryzae* was detected as an entomopathogenic fungus of the *Locusta migratoria* (Orthoptera: Acrididae) (Zhang et al., 2015). This fungus and *Chaetomium cupreum*, *Penicillium paxilli* and *Tolypocladium album* have already been detected in associations with plant hosts and other sources (Phongpaichit et al., 2006; Verma et al., 2007; Mao et al., 2010, Gazis et al., 2014), with strains of *P. paxilli* and *C. cupreum* producing antimicrobial compounds (Phongpaichit et al., 2006; Mao et al., 2010). This suggests that the potential relationships between *Phylloicus* spp. and DT fungal community can go well beyond the presumed roles of metabolic symbiosis in which the fungi provide cellulolytic and xylanolytic enzymes for digestion of plant substrates by the insect, and may relate to immunity, protection of its hosts against pathogens and parasites, action on the detoxification of substances ingested by insects, among others (Dowd, 1992; Douglas, 2015), which requires further research to clarify.

Insects require several exogenous dietary compounds, such as amino acids, vitamins, specific fatty acids and sterols (Vega and Dowd, 2005; Douglas, 2009). Insect-associated fungi provide these food supplements to their hosts, such as B vitamins provided by fungi associated with beetles, (Gusteleva, 1975; Nardon & Grenier 1989), sterols yeasts for beetles, planthoppers and fire ant larvae (Ba et al., 1995; Noda and Koizumi 2003), as well as by-products and/or enzymes for the degradation of recalcitrant carbon sources such as cellulose or lignin (Hongoh and Ishikawa, 2000, Douglas, 2009; Urubschurov and Janczyk; 2011; León et al., 2016). There are numerous records of fungi producing digestive enzymes to aid in insect host nutrition and detoxification of complex substrates (Shen and Dowd, 1992; Schäfer et al., 1996; Gujjari et al., 2011; Suh et al., 2013), which includes cellulolytic enzymes. In fungi, cellulose breakdown usually occurs outside the host, as in the case of bark beetles with their fungal associates *Ceratocytis* spp. and *Ophiostoma* spp. (both Ascomycota: Pezizomycotina: Sordariomycetes: Ophiostomales) (Harrington, 2005), or *Xiphydria* woodwasps and their *Daldinia decipiens* and *Entonaema cinnabarina* (both Ascomycota: Pezizomycotina) fungi (Srutka et al., 2007). In this study, it was verified that a significant part (± 50 %) of the fungal community sheltered by the three *Phylloicus* species has cellulolytic behavior. Thus, it is plausible to assume that filamentous fungi associated with aquatic insect DT degrade cellulolytic substrates in the

interface of interaction with their hosts, as previously verified for other xylophagous insects that have fungi associated with their DT (Gujjari et al., 2011; Suh et al., 2013).

There are records of cellulolytic strains belonging to some of the genera detected in this study, such as *Chaetomium* (Ankudimova et al., 1999; Soni and Soni, 2010; Al-Kharousi et al., 2015), *Cladosporium* (Andersen et al., 2016) and *Penicillium* (Al-Kharousi et al., 2015; Cunha et al., 2016; Bomtempo et al., 2017) but only the species *Penicillium citrinum* (Dutta et al., 2008) was previously described as cellulolytic.

Although there was a difference in frequency of occurrence among fungal taxa, this frequency was generally low (less than 25%), which is considered as an accidental occurrence in the analysis of community statistics (Santos et al., 2018) therefore, is not indicative of symbiotic interaction. In addition, since the amount of exclusive fungal taxa was much higher than that of shared taxa among hosts, there is no indication of a core microbiome (common and shared microbiome) among all of them. Therefore, the fungal community associated with *Phylloicus* spp. larvae consists mainly of fungal taxa from food items, which come from riparian vegetation (whose plant species are variable) or through water, which is the habitat of these larvae.

3.5- References

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CAPÍTULO IV:

Cladosporium perangustum AND OTHER CELLULOLYTIC FUNGI FROM THE DIGESTIVE TRACT OF LARVAL STAGES OF *Phylloicus* (TRICHOPTERA: CALAMOCERATIDAE) [8]

Abstract: Cellulases are enzymes involved in industrial and biotechnological processes, which result in high-value products and by-products. The aim of this study was to evaluate the production of cellulases by filamentous fungi from the digestive tract (DT) of *Phylloicus* spp. (Trichoptera: Calamoceratidae), which is an aquatic insect from Amazon Forest. A total of 136 strains was screnned for cellulolytic activity in agar plate containing carboxymethylcellulose (CMC medium). Presence of degradation halo was indicative of positive cellulolytic activity and the relation between the total diameter (degradation halo + colony) and the colony diameter was used as an Enzymatic Indices (EI). The strains with the highest EI were selected for the cellulase production assays by solid-state fermentation using agroindustrial substrate (wheat bran). Total cellulase (FPAse), endoglucanase (CMCase) and cellobiohydrolases (avicelase) were determined. Of the total strains tested, 45,6 % (n = 62) showed cellulolytic activity. Among the strains with the highest EI, six presented cellulase production by one of the three applied measurements. *Cladosporium perangustum* was revealed with a good producer of cellulase, since it produced much higher amounts than the other fungi tested (FPAse = 14.60 U/g; CMCase = 12.04 U/g; avicelase = 11.46 U/g). Future studies aiming to determine the ideal production conditions, as well as activity of the cellulolytic enzymes of this strain may enable the use of the same in biotechnological processes, such as the production of biofuels from lignocellulosic biomass.

Keywords: cellulolytic enzymes; solid-state fermentation; wheat bran.

[8]Em preparação para submissão.

4.1- Introduction

Cellulases are enzymes implicated in the degradation of cellulose fibers to soluble sugars such as glucose, cellobiose and other oligomers (Damaso et al., 2012). The enzymatic hydrolysis of cellulose requires a consortium of cellulase enzymes, which act synergistically (Hong et al., 2001; Lynd et al., 2002). Endoglucanases (endo-β-1,4-glucanase, EG, EC 3.2.1.4) randomly attack cellulose chains and release cello-oligosaccharides; cellobiohydrolases (exo-β-1,4-glucanase, CBH, EC 3.2.1.91) cleave cellobiose units from the end of cellulose chains, and β-glucosidase (β-1,4-glucosidase, BG, EC 3.2.1.21) converts the resulting cellobiose to glucose (Bhat et al., 1997).

There is a considerable interest in production of cellulases, since these enzymes are involved in several industrial and biotechnological processes, which result in high-value products and by-products, such as starch processing, animal food production, grain alcohol fermentation, biofuel production, malting and brewing, extraction of fruit and vegetable juices, textile industry, pulp and paper industry (Adsul et al., 2007; Kaur et al., 2007; Isikgor and Becer, 2015).

Several organisms, such as filamentous fungi (Gao et al., 2008; Bomtempo et al., 2017), bacteria (Ariffin et al., 2006; Behera et al., 2014), yeasts (Thongekkaew et al., 2008; Jaiboon et al., 2016) and insects (Sun and Scharf, 2010; Taggar, 2015), produce cellulolytic enzymes in the natural conditions, which are related with the survival and/or adaptation of these organisms in the environment.

Filamentous fungi produce most of the cellulases commercially used (Kirk et al. 2002; Cherry and Fidantsef, 2003). These microorganisms has been isolated from various substrates rich in cellulosic fibers, including live or decomposed plants, agroindustrial residues, soils, internal parts of body of phytophagous insects, and new cellulase producing strains of potential biotechnological interest has been detected (Dutta et al., 2008; Damaso et al., 2012; Suryanarayanan et al., 2012).

Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use, mainly in terms of ethanol production from lignocellulose biomass, wich is a rich and abundant organic resource (Zhang et al., 2006; Damaso et al., 2012). The production of cellulases by filamentous fungi through solid-state fermentation using agroindustrial substrates (wheat bran, corn storver, rice bran, among others), as has been demonstrated by several studies (Gao et al., 2008; Brijwani et al., 2010; Bomtempo et al., 2017),

is among the strategies that have been proposed to reduce the cost of production of these enzymes.

The aim of this study was to evaluate qualitatively (agar plate screening) and quantitatively (solid-state fermentation) the production of cellulases by filamentous fungi from the digestive tract (DT) of *Phylloicus* spp. (Trichoptera: Calamoceratidae), which is an aquatic insect that occurs in Brazil and other countries, and is related with degradation of submerse plant debris in tropical streams.

4.2- Material and Methods

4.2.1- Fungal strains

The filamentous fungi used in this study were isolated from DT of *Phylloicus* spp. (Trichoptera: Calamoceratidae), from streams of the *Floresta Nacional do Tapajós*, a conservation unit in the Pará state, Brazil. The strains (136) (collection codes in **Table S3**) were preserved by the Castellani method (Castellani, 1939) in the *Coleção de Culturas Microbianas Carlos Rosa*, Tocantins state, Brazil.

4.2.2- Agar plate screening for cellulolytic activity

As proposed by Sunitha et al. (2013), the purified strains were preliminarily cultured in PDA (Potato, Dextrose and Agar) culture media (potato extract: 4.0g, dextrose: 20.0g, agar: 15.0g, distilled water: 1000 mL) for seven days. Then, circular fragments of the colonies (5 mm diameter) were removed and used to inoculate Petri dishes containing CMC media (carboxymethylcellulose: 5.0 g, glucose: 1.0 g, yeast extract: 0.1 g, peptone: 0.5 g, agar: 16.0 g, distilled water: 1000 mL). After 3-5 days of incubation at 28 °C, the plates were flooded with 10 mL of 0.2% aqueous Congo red solution (30 minutes) and destained with 1.0 M NaCl solution (15 minutes). All strains that showed yellow areas around the fungal colony in an otherwise red medium were considered positive for the cellulolytic activity. The assay was performed in triplicate. As a criterion for comparing the strains with each other, the Enzymatic Index (EI = D / d) was used, where "D" is the total diameter (halo + colony) and "d" is the colony diameter. The experimental design was completely randomized. The analysis of variance (ANOVA) and comparison of means of the EI was performed by the Tukey test at 5% of significance using PAST software (version 3.19) (Hammer et al., 2001).

4.2.3- DNA extraction, amplification, sequencing

The strains selected for DNA extraction were inoculated in 2.0 % ME broth (malt extract: 20.0 g, distilled water: 1000 mL) and cultured on shaker type oscillatory platform at 150 rpm, 25 ± 3°C, 3 to 5 days. After this period, about 40.0 mg of mycelium was separated

from the liquid medium and used for total DNA extraction using a Wizard™ Genomic DNA Purification Kit protocol (Promega Corp., Madison, WI), following a slightly modified protocol from that of Burghoorn et al. (2002).

The following primers were used to amplify the internal transcribed spacer (ITS) regions of the rDNA (approx. 600 pb): ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The amplification was performed in a thermocycler Mastercycler™ nexus (Eppendorf, São Paulo, Brazil) using a GoTaq™ DNA Polymerase kit (Promega Corp., Madison, WI) in a final reaction volume of 25 µL containing 9.375 µL of ultrapure water; 2.5 µL of 25 mM MgCl2; 1.0 µL of 10 mM dNTPs; 5.0 µL of Taq Polymerase buffer (5X); 1.0 µL of the primer ITS1 (2 mM); 1.0 µL of the primer ITS4; 0.125 µL of the enzyme Taq Polymerase (5 U/µL), 5.0 µL of DNA (10 ng/µL). Negative control (DNA replaced by water) was performed. The amplification conditions were as follows: initial denaturation at 95°C for 2 min, followed by 39 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min.

Successful PCR (Polymerase Chain Reaction) amplification was confirmed by electrophoreses on a 1.0 % (w/v) agarose gel (Promega, Madison, WI) stained with GelRed™ (Biotium Inc. California, USA) in 1X TBE buffer (2.0 mmol L^{-1} EDTA; 0.1 mol L^{-1} Tris-HCl; and 0.1 mol L^{-1} boric acid [pH 8,0]) (Sambrook et al. 1989) and visualized under ultraviolet light in a LPIX-EX™ imaging system (Loccus Biotecnology, Cotia, Brazil). The 1 Kb DNA Ladder (Promega Corp., Madison, WI) was used as molecular weight marker. Subsequently the PCR products were purified using a Kit Wizard™ SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI) and bidirectionally sequenced according to the dideoxy chain-termination method (Sanger et al. 1977) using a BigDye Terminator v 3.1 sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was performed at Myleus Biotechnology, located in Belo Horizonte, Brazil (http://myleus.com/).

4.2.4- Identification of isolates

The nucleotide sequences generated from each individual were imported into the Geneious 6.1.8 program (Kearse et al., 2012) to be checked, edited and aligned. Sequences of both DNA strands were pooled into contigs, manually corrected and aligned. The alignments were exported in a FASTA extension file (*.fasta/*.fas) for the other analyzes and comparative research of sequence identity using the BLAST (Basic Local Alignment Search) tool (Altschul

et al., 1990) of the NCBI (National Center for Biotechnology Information) (GenBank Database) and in the CBS (*Centraalbureau voor Schimmelcultures* Fungal Biodiversity Centre) Database (http://www.cbs.knaw.nl/Collections/). Identity ≥ 99% were indicative of the same species. The sequences were deposited in GenBank under the accession numbers XX000000 to XX000000.

4.2.5- Enzymes production

All enzyme production was conducted by solid-state fermentation (SSF) according to Gao et al. (2008) and Basso et al. (2010), with modifications. The agroindustrial substrate used was wheat bran, obtained dry in the local market. To ensure complete absence of water, it was kept in a drying oven at 60 °C until constant weight. Subsequently, 5.0 g of this dried substrate was added in Erlenmeyer flasks (125 mL) along with mineral saline described by Mandels and Weber (1969) to 60.0 % humidity, at pH 5.0 (0.05 M acetate buffer). The flasks were autoclaved for 15 min, cooled and inoculated with spore suspension (10^6 spores/mL) and incubated under static conditions at 28 °C for 96 h. After this period, 80.0 mL of sodium phosphate solution (0.05 M, pH 7.0) was added to the fermented, which was homogenized with a glass stick and agitated (170 rpm) for 2 h. The contents of each Erlenmeyer flask were then filtered and stored at -20 °C for further analysis.

4.2.6- Cellulolytic enzyme assays

Total cellulase (FPAse), endoglucanase (CMCase) and cellobiohydrolases (avicelase) were determined according to the methodology proposed by Ghose (1987), with modifications. Reducing sugars were estimated using the DNS (3.5-dinitrosalicyclic acid) method described by Miller (1959). The absorbance values at 540 nm were converted to equivalent amounts of glucose by standard curve. For conversion of the amount of glucose to values of enzymatic activity, a unit (U) of enzymatic activity was considered as the amount of enzyme necessary to release 1.0 μmol of glucose per minute under the conditions defined for the reaction. The assays were performed in triplicate and the activities were expressed in U/g of dry substrate used. For all enzymatic activities, colorimetric reaction controls were performed to discard the contributions of the substrate (reaction blank) and the enzyme extract (white enzyme), separately, from the absorbance values obtained.

4.2.6.1- Determination of FPAse activity

For the assay, Whatman n. 1 filter paper in the form of 1 x 6cm strips (approximately 50.0 mg) was used as the substrate. To the 25.0 mL tubes were added, in addition to the filter paper, 1.0 mL of 0.05 M citrate-phosphate buffer, pH 4.8 and 0.5 mL of the enzyme extract, and incubated for 30 min at 50 °C. The reaction was stopped by immersing the tubes in an ice bath and then 2.0 ml of DNS was added and the mixture boiled for 5 min. Then, 10 mL of homogenized distilled water and the absorbances measured at room temperature were added in a spectrophotometer at 540 nm (Miller, 1959).

4.2.6.2- Endoglucanase activity (CMCase)

In tubes with 25.0 mL capacity, 0.5 mL of the enzyme extract was added to 0.5 mL of a 2.0 % solution of carboxymethylcellulose (CMC) in 0.05 M citrate-phosphate buffer, pH 4.8, and incubated for 30 min at 50 °C. The reaction was interrupted and analyzed as described in item 4.2.6.1.

4.2.6.3- Cellobiohydrolase activity (Avicelase)

Enzyme extract (0.5 mL) and 1.0 mL of the 1.0 % microcrystalline cellulose solution (Avicel) was added in tubes (25.0 mL capacity) along with 0.05 M citrate-phosphate buffer, pH 4.8, and incubated for 30 min at 50 °C. The reaction was interrupted and analyzed as described in item 4.2.6.1.

4.3- Results

A total of 45.6 % (62/136) of the strains tested was positive for cellulolytic activity in solid media (**Table S3**). The EI ranged from 1.03 to 3.14 (mean ± standard deviation = 1.30 ± 0.37). The 16 strains with the highest EI, presented in the **Table 7**, were selected for the cellulase production assays by solid-state fermentation using an agroindustrial substrate (wheat bran) as the carbon source.

Table 7. Filamentous fungi strains with the highest enzymatic indices (EI) from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae).

Collection code	Mean Øc*	Mean Øh**	EI	SD***
P5PA5	7.00	22.00	3.14 a	0.00
P6PB5	10.00	25.00	2.50 b	0.00
P6PB1 (III)	9.67	21.00	2.17 c	0.05
P6PB1 (I)	17.00	33.67	1.98 d	0.07
P1PB1	20.33	36.33	1.79 e	0.03
P6PB1 (IV)	12.67	22.00	1.74 e	0.07
P2PB8	18.33	29.00	1.59 f	0.06
P9PB1	7.00	10.67	1.52 f	0.07
P5PC7	6.00	9.00	1.50 f	0.00
P9PE2 (II)	6.00	9.00	1.50 f	0.00
P9PE7	17.00	24.67	1.48 f	0.15
P1PC4	19.33	27.67	1.44 fg	0.07
P9PC5b	19.00	26.33	1.41 g	0.19
P6PA2	20.00	28.00	1.40 g	0.06
P10PD2	23.00	32.00	1.39 g	0.05
P9PC10	12.67	17.33	1.37 g	0.05

(*) Mean diameter of the colony; (**) Mean diameter of the halo; (***) Standard deviation of the EI. Averages followed by the same letter are not statistically significantly different according to Tukey's test, at $p < 0.05$.

All strains tested in this study were previously characterized through macro and micromorphology, as mentioned in the material and methods section. However, those that were selected for the cellulase production assays were identified by molecular taxonomy (**Table 8**), based on the amplification and sequencing of the rDNA ITS regions. All isolates selected are from the phylum Ascomycota. The isolates P1PB1 and P6PA2 could be identified only up to class taxonomic level (Dothideomycetes).

Table 8. Identification of strains with higher enzymatic indices (EI).

Collection code	Fungal taxa	% ID *	GenBank Accession numbers
P5PA5	*Beltrania pseudorhombica*	99%	KR093912
P6PB5	*Cladosporium exasperatum*	100%	MH863865
P6PB1 (III)	*Paraconiothyrium* sp.	99%	KF746099
P6PB1 (I)	*Paraconiothyrium* sp.	99%	KF746099
P1PB1	Dothideomycetes sp. 1	97%	JQ905826
P6PB1 (IV)	*Paraconiothyrium* sp.	99%	KF746099
P2PB8	*Penicillium mallochii*	100%	KY019235
P9PB1	*Pe. sclerotiorum*	100%	EF488396
P5PC7	*Pe. cairnsense*	100%	MH863991
P9PE2 (II)	*Pe. paxilli*	99%	MH856391
P9PE7	*Cladosporium perangustum*	100%	MG669149
P1PC4	*Readeriella guyanensis*	99%	EU707900
P9PC5b	*Pe. citrinum*	100%	MG948252
P6PA2	Dothideomycetes sp. 2	99%	KM266001
P10PD2	*Pe. citrinum*	100%	MG948252
P9PC10	*Letendraea helminthicola*	99%	KJ774053

*Percentage of similarity between the nucleotide sequences obtained in that study with sequences available in the NCBI database

In the **Table 9** is presented the cellulolytic enzyme assays (Fpase, endoglucanase and avicelase) of strains with higher EI in the agar plate screening.

Table 9. Cellulolytic enzyme assays of strains isolated from DT of *Phylloicus* spp. (Trichoptera: Calamoceratidae).

Collection code	**Fungal taxa**	**FPAse (U/g)**	**CMCase (U/g)**	**Avicelase (U/g)**
P5PA5	*Beltrania pseudorhombica*	-	-	-
P6PB5	*Cladosporium exasperatum*	-	-	-
P6PB1 (III)	*Paraconiothyrium* sp.	-	-	-
P6PB1 (I)	*Paraconiothyrium* sp.	-	-	-
P1PB1	Dothideomycetes sp.	2.52	2.35	0.10
P6PB1 (IV)	*Paraconiothyrium* sp.	-	-	-
P2PB8	*Penicillium mallochii*	0.88	-	-
P9PB1	*Pe. sclerotiorum*	0.61	-	-
P5PC7	*Pe. cairnsense*	0.56	-	-
P9PE2 (II)	*Pe. paxilli*	-	-	-
P9PE7	*Cladosporium perangustum*	14.60	12.04	11.46
P1PC4	*Readeriella guyanensis*	0.86	0.21	-
P9PC5b	*Pe. citrinum*	-	-	-
P6PA2	Dothideomycetes sp.	-	-	-
P10PD2	*Pe. citrinum*	-	-	-
P9PC10	*Letendraea helminthicola*	-	-	-

4.4- Discussion

A significant portion (45.6 %, 62 strains) of the filamentous fungi from the DT of *Phylloicus* spp. is positive for the cellulolytic activity, according the agar plate screening performed here, which permits the rapid screening of large populations of fungi for the presence or absence of cellulase. Filamentous fungi potentially cellulolytic can be found in various environments and in very varied proportions. Ruegger and Tauk-Tornisielo (2004) used similar screening methodology with soil filamentous fungi, which allowed us to observe that 45 % (36 strains) of the filamentous fungi tested were positive for cellulase. Sunitha et al. (2013), applying the same methodology of the present study in endophytic fungi of medicinal plants, found that only 32 % (36 strains) of the fungi tested were cellulolytic.

There is evidence of a positive correlation between the results verified in strategies of enzymatic screening with the production of the enzymes themselves (Carder, 1986). Florencio et al. (2012) compared the EI from of cellulolytic enzymatic screening of strains of *Trichoderma* sp. with the production of cellulase by SSF and observed a positive correlation between the two methods. However, this correlation should not be taken into account when strains belonging to different fungal taxa are being analyzed simultaneously, as was done in this study, where 16 strains belonging to 12 different fungal taxa were screened for cellulase production on agar plates and evaluated for to the production of cellulase by means of SSF. The EI has been proposed as a strategy to compare strains positive for cellulase in the screening methods for biotechnology purposes (Nogueira and Cavalcanti, 1996; Ruegger and Tauk-Tornisielo, 2004). In this study, the strains with EI higher were chosen to quantitative tests of cellulase production and not to validate the correlation between the methods for the qualitative and quantitative detection of cellulases.

The genus *Aspergillus*, *Penicillium* and *Trichoderma*, are already well known to have cellulolytic strains (Sukumaran *et al*., 2005; Kim *et al*., 2014) and, the most of the commercial cellulases have been produced from the these genera (Kirk et al. 2002; Cherry and Fidantsef 2003). Among the filamentous fungi with higher EI, the genus *Penicillium* was the most frequent. No cellulolytic strains of *Aspergillus* or *Trichoderma* were detected, although the presence of representatives of this genus was confirmed by means of a classical taxonomy among the fungi tested (data not shown).

It is a well-established fact that culture conditions affect significantly the production of cellulases (Juhász et al., 2005). Several factors affect the synthesis of microbial enzymes in a

solid-state culture system, such as substrate selection and concentration, humidity, temperature, incubation time, pH, additional nutrients, among others (Sohail et al., 2009; Saxena and Singh, 2011). Here, a single culture condition was standardized for all strains evaluated and only six strains presented cellulase production by one of the three applied measurements. Of these, two (*Cladosporium perangustum* and a Dothideomycetes strain) had cellulose production quantified in all assays.

The establishment of the culture conditions imposed here was based on the specialized literature, especially with regard to the employment of SSF using wheat bran as substrate, which has been successful for production of cellulases by filamentous fungi (Camassola and Dillon, 2007; Cunha et al., 2016). However, when subjecting different fungal strains, members of different taxonomic groups, to a single cultivation condition, a great variation in cellulase production was expected between the strains tested, including the occurrence of non-producers, as verified in that study.

Nevetherless, even without adoption an experimental strategy to optimize cultivation conditions, *Cladosporium perangustum* was revealed with a good producer of cellulase, since it produced much higher amounts than the other fungi tested. Strains from the genus *Cladosporium* have already been reported as cellulase producers (Andersen et al., 2016). Although, to the best of our knowledge, this the first account of cellulolytic strain of *C. perangustum.* Future studies aiming to determine the ideal production conditions, as well as activity, of the cellulolytic enzymes of this strain may enable the use of the same in biotechnological processes that require the use of cellulases, such as the production of biofuels from lignocellulosic biomass.

4.5- References

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CONSIDERAÇÕES FINAIS

Este trabalho mostrou que a interação entre fungos e estágios larvais de insetos aquáticos é complexa, e os mecanismos possivelmente vão além do simples valor nutricional dos fungos, podendo estar ligada à facilitação da digestão e produção de metabólitos.

O papel dos fungos e dos insetos aquáticos já está muito bem estabelecido na decomposição de detritos vegetais em riachos. A interação entre esses dois grupos biológicos é ainda bastante obscura e requer o empenho e cooperação de atores de várias áreas da ciência, tais como micólogos, entomólogos, botânicos, ecológicos, climatolologistas, etc., para juntos, canalizarem esforços e estabelecerem prioridades de investigação para buscar a solução para várias questões ainda abertas sobre essa temática. Também é válido mencionar que os habitats aquáticos compostos por riachos de baixa ordem, como os envolvidos nesse estudo, estão, de maneira geral, localizados em áreas geográficas continuamente ameaçadas pelo avanço da fronteira agrícola e pela urbanização, o que reforça a necessidade de composição de uma equipe multi e interdisciplinar verdadeiramente comprometida com a causa da preservação da biodiversidade e da valorização ambiental para a garantia das necessidades das gerações presente e futura.

A ocorrência prevalecente de fungos filamentosos no trato digestório (TD) de *Phylloicus* é um forte indicativo de que esses organismos exercem papéis de interesse para seus hospedeiros, os quais provavelmente vão além da degradação de substratos celulósicos. Com base no que foi aqui investigado, é possível atribuir, aos fungos filamentosos e leveduriformes associados ao TD de *Phylloicus*, papéis como "fornecedores de suplementos nutricionais importantes e não produzidos por seus hospedeiros", "amplificadores da qualidade do recurso alimentar utilizado pelos insetos" ou, ainda, "atuação como item alimentar para o hospedeiro". Contudo, resumir o papel desses organismos fúngicos a apenas isso é uma simplificação, pois a literatura comprova que fungos filamentosos e leveduras são dotados de *pools* gênicos bastante variados, e podem produzir uma vasta gama de substâncias que, por sua vez, podem ter uma infinidade de papéis e serventias nos contextos em que estão inseridos. Como exemplo, pode-se hipotetizar a produção de substâncias químicas úteis para a defesa do hospedeiro contra o ataque de patógenos ou parasitas.

Comprovou-se que a micota associada ao TD de *Phylloicus* é composta por estirpes celulolíticas, incluindo linhagens com potencial emprego biotecnológico, tal como *Cladosporium perangustum*. Os registros prévios de ocorrência e de potencial biotecnológico

dos grupos taxonômicos de leveduras e fungos filamentosos detectados nesse estudo, que há linhagens das mesmas espécies, oriundas de habitats e substratos distintos, comprovadamente capazes de produzirem compostos bioativos de interesse para a área farmacêutica e de cosméticos, de corantes, de pigmentos, de enzimas para aplicação na indústria têxtil, de biocombustível, de alimentos ou de nutrição animal. Isto reforça a hipótese acerca da amplitude de papéis que os fungos podem exercer na natureza e servirem de *insights* para a biotecnologia.

Em futuros esforços, a coleção de microrganismos aqui obtida e taxonomicamente caracterizada poderá servir para a prospecção não só de linhagens produtoras de outras enzimas de interesse biotecnológico, tais como xinalases, amilases e lipases, mas também para a prospecção de ativos biológicos que potencialmente podem ser produzidos por esses fungos na interface de interação com seus hospedeiros (tais como antimicrobianos) e que podem ser úteis para humanidade. A literatura científica respalda esse pressuposto, que perpassa por áreas afins e relacionadas à Biotecnologia, como a Enzimologia, Química de Produtos Naturais e a Engenharia Genética e Bioquímica, o que reforça o cárater inter e multidisplinar do fazer científico e do desenvolvimento de produtos e processos biotecnológicos.

ANEXOS

Table S1. Yeast sequences used in this study for phylogenetic analysis.

Collection code	Identity	Host	Geographical origin	Collector	GenBank accession numbers
PH18	*Aureobasidium thailandense*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636031
PH30	*Aureobasidium thailandense*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636037
PH31	*Aureobasidium thailandense*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636025
PON23.8	*Candida blattae*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636068
PH39	*Candida boidinii*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636040
PH02	*Candida parapsilosis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636026
PH06	*Candida parapsilosis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636029
PH25	*Candida parapsilosis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636034
PON25.5	*Candida parapsilosis*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636069
PH12	*Candida rugosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636020
PON10.13	*Candida* sp. 1	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636062
PON11.1	*Candida* sp. 1	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636063
PON21.1	*Candida* sp. 2	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636065
PH44	*Candida* sp. 3	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636023
PON21.5	*Debaryomyces hansenii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636066
PH10	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636030
PH16	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636021
PH28	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636035
PH29	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636036
PH36	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636038
PH38	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636039
PH40	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636041
PH41	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636042
PH45	*Issatchenkia siamensis*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636045
PON6.8	*Lodderomyces elongisporus*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636056

Table S1. Yeast sequences used in this study for phylogenetic analysis (continuation).

Collection code	Identity	Host	Geographical origin	Collector	GenBank accession numbers
PON8.2B	*Lodderomyces elongisporus*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636058
PON11.3	*Lodderomyces elongisporus*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636064
PON3.4 (*)	*Meyerozyma guilliermondii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636051
PON8.8	*Meyerozyma guilliermondii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636060
PON43.3	*Meyerozyma guilliermondii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636073
PON43.5	*Cryptococcus podzolicus*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636074
LAG12.1	*Cryptococcus* sp. 1	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636046
PON12.11	*Cryptococcus* sp. 2	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636049
PH11	*Hannaella luteola* - like	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636019
PON3.11	*Papiliotrema flavescens*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636053
PON5.6	*Papiliotrema flavescens*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636054
PON5.12	*Papiliotrema flavescens*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636055
PON10.14	*Papiliotrema flavescens*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636048
PON1.10	*Papiliotrema laurentii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636050
PON28.6	*Papiliotrema laurentii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636070
PON34.4	*Papiliotrema laurentii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636071
PON44.5	*Papiliotrema laurentii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636047
PON47.5	*Papiliotrema laurentii*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636075
PON3.10	*Pseudozyma antarctica*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636052
PON42.6A	*Pseudozyma antarctica*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636072
PON8.5	*Pseudozyma antarctica*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636059
PH03	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636027
PH05	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636028
PH21	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636032
PH22	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636022

Table S1. Yeast sequences used in this study for phylogenetic analysis (continuation).

Collection code	Identity	Host	Geographical origin	Collector	GenBank accession numbers
PH24	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636033
PH42	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636043
PH43	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636044
PH45B	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	MT state, Brazil	Morais PB	MH636024
PON8.1	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636057
PON10.11AII	*Rhodotorula.mucilaginosa*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636061
PON22.63	*Rhodotorula mucilaginosa*	Digestive tract of *Phylloicus* spp.	PA state, Brazil	Santos TT	MH636067

(*)This strain is absent from Fig. 6.

Table S2. Fungal sequences used in this study for phylogenetic analysis.

Collection code	Identity	Host	Collector	GenBank accession numbers
P9PC7	*Arthopyrenia* sp.	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120544
P9PA3	*Arthrinium phaeospermum*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120545
P9PJ5	*Aspergillus aculeatus*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120546
P10PC1	*Aspergillus oryzae*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120547
P9PE2 (I)	*Aspergillus oryzae*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120548
P9PJ8	*Chaetomium cupreum*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120549
P10PB3	*Chaetomium cupreum*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120550
P9PE7	*Cladosporium perangustum*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120551
P10PA7	*Clonostachys rosea*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120552
P10PA1 (I)	*Cordyceps spegazzinii*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120553
P10PC2A	*Cordyceps spegazzinii*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120554
P10PD1	*Diaporthe* sp.	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120555
P10PB4	*Epicoccum nigrum*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120556
P10PC5	*Gliomastix polychroma*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120557
P9PC10	*Letendraea helminthicola*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120558
P9PC6	*Neooccultibambusa pandanicola*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120559
P10PA3 (I)	*Paraconiothyrium* sp.	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120560
P10PC6	*Paraconiothyrium* sp.	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120561
P9PC2	*Penicillium citrinum*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120562
P9PI1	*Penicillium citrinum*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120563
P10PD2	*Penicillium citrinum*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120564
P9PE2 (II)	*Penicillium paxilli*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120565
P9PI8	*Penicillium paxilli*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120566
P9PB1	*Penicillium sclerotiorum*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120567

Table S2. Fungal sequences used in this study for phylogenetic analysis (continuation).

Collection code	Identity	Host	Collector	GenBank accession numbers
P10PA10	*Penicillium* sp.	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120568
P9PI7	*Penicillium* sp.	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120569
P10PA11	*Penicillium sumatraense*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120570
P9PB2	*Pestalotiopsis microspora*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120571
P9PC8	*Pestalotiopsis microspora*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120572
P9PF5	*Pestalotiopsis microspora*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120573
P10PA1 (II)	*Pestalotiopsis microspora*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120574
P10PC2B	*Pestalotiopsis microspora*	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120575
P10PA5	*Phialemoniopsis cornearis*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120576
P9PI2	*Pyrenochaetopsis microspora*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120577
P9PH7	*Ramichloridium* sp.1	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120578
P10PB1A	*Ramichloridium* sp.2	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120579
P10PB2	*Tolypocladium album*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120580
P9PJ6	*Tolypocladium album*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120581
P9PI5	*Tolypocladium endophyticum*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120582
P9PH5	*Trichoderma* sp.1	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120583
P9PB4	*Trichoderma* sp.2	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120584
P9PF2	*Trichoderma* sp.2	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120585
P9PE1	*Trichoderma spirale*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120586
P9PG1	*Trichoderma strigosum*	Digestive tract of *Phylloicus elektoros*	Santos TT & Morais PB	MK120587
P10PA9	*Umbelopsis isabellina*	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120588
P9PC5A	Bionectriaceae sp.	Digestive tract of *Phylloicus amazonas*	Santos TT & Morais PB	MK120589
P10PC3	Pleosporales sp.	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120590
P10PD3	Pleosporales sp.	Digestive tract of *Phylloicus fenestratus*	Santos TT & Morais PB	MK120591

Table S3. Filamentous fungi from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) used in this study. Øc = Mean diameter of the colony; Øh = Mean diameter of the halo; EI = enzymatic indices; SD = Standard deviation of the EI; (-) = without halo production.

Collection code	Mean Øc*	Mean Øh**	EI	SD***
P1PB1	20.33	36.33	1.79	0.03
P1PC4	19.33	27.67	1.44	0.07
P2PB8	18.33	29.00	1.59	0.06
P2PC1A	18.00	19.00	1.06	0.00
P2PC1B	17.00	18.00	1.06	0.00
P2PC1C	16.00	17.00	1.06	0.00
P2PC2	31.00	32.00	1.03	0.00
P3PA1	24.33	25.33	1.04	0.00
P3PA3 (I)	29.67	30.67	1.03	0.00
P3PA3 (II)	30.67	31.67	1.03	0.00
P4PC4	17.00	18.00	1.06	0.00
P5PA3	15.00	16.00	1.07	0.00
P5PA5	7.00	22.00	3.14	0.00
P5PC7	6.00	9.00	1.50	0.00
P6PA2	20.00	28.00	1.40	0.06
P6PA3	23.00	25.00	1.09	0.00
P6PB1 (I)	17.00	33.67	1.98	0.07
P6PB1 (III)	9.67	21.00	2.17	0.05
P6PB1 (IV)	12.67	22.00	1.74	0.07
P6PB5	10.00	25.00	2.50	0.00
P7PB2	38.00	45.33	1.19	0.02
P8PA4b	17.00	18.00	1.06	0.00
P8PB2b	20.33	24.67	1.23	0.04
P8PB3 (I)	37.67	39.67	1.05	0.00
P8PB3 (II)	35.67	37.67	1.06	0.00
P8PB4 (II)	17.33	18.33	1.06	0.01
P8PB5	9.67	10.67	1.10	0.01
P8PB6	23.67	30.33	1.29	0.05
P8PC3	14.33	18.67	1.30	0.04
P8PC7	14.33	16.67	1.17	0.06
P9PB1	7.00	10.67	1.52	0.07
P9PC10	12.67	17.33	1.37	0.05
P9PC5b	19.00	26.33	1.41	0.19
P9PC5C	11.67	15.67	1.34	0.01
P9PC7	14.67	15.67	1.07	0.00
P9PE2 (I)	5.67	7.00	1.24	0.11
P9PE2 (II)	6.00	9.00	1.50	0.00
P9PE2 (III)	5.00	6.67	1.33	0.09
P9PE7	17.00	24.67	1.48	0.15
P9PH7	11.33	13.33	1.18	0.02
P9PI1	14.00	19.00	1.37	0.14
P9PI2	13.67	14.67	1.07	0.00
P9PI7	28.00	35.00	1.25	0.05

Table S3. Filamentous fungi from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) used in this study. Øc = Mean diameter of the colony; Øh = Mean diameter of the halo; EI = enzymatic indices; SD = Standard deviation of the EI; (-) = without halo production (continuation).

Collection code	Mean Øc*	Mean Øh**	EI	SD***
P9PI8	7.67	9.67	1.32	0.12
P9PJ8	18.33	19.67	1.07	0.03
P10PA10	14.00	17.00	1.21	0.00
P10PA11	22.67	26.33	1.16	0.02
P10PA13	25.33	26.67	1.05	0.02
P10PA3 (I)	11.33	12.33	1.09	0.00
P10PA3 (II)	21.67	23.00	1.06	0.03
P10PA3 (III)	11.00	12.00	1.09	0.01
P10PA3 (IV)	12.00	13.00	1.08	0.00
P10PA3 (V)	10.67	12.33	1.16	0.04
P10PA5	21.00	23.67	1.13	0.02
P10PA7	38.33	46.67	1.22	0.01
P10PB1 (A)	9.00	11.00	1.22	0.01
P10PB2	18.33	19.33	1.06	0.01
P10PB3	9.67	11.67	1.21	0.01
P10PB4	14.67	18.00	1.23	0.04
P10PC3	10.67	12.67	1.19	0.01
P10PD2	23.00	32.00	1.39	0.05
P10PD3	20.00	24.33	1.22	0.02
P1PB5	14.00	-	-	-
P1PC1	11.33	-	-	-
P3PA10	22.70	-	-	-
P3PA13	25.30	-	-	-
P3PA2	11.33	-	-	-
P3PA5	21.70	-	-	-
P3PA6 (II)	11.00	-	-	-
P3PA8	12.33	-	-	-
P3PA9	10.70	-	-	-
P5PA1	21.33	-	-	-
P5PB1	14.67	-	-	-
P5PB2	9.67	-	-	-
P5PB6	18.00	-	-	-
P5PB8	12.33	-	-	-
P5PB9 (I)	12.67	-	-	-
P5PB9 (II)	21.00	-	-	-
P5PC1	22.33	-	-	-
P5PC2	9.00	-	-	-
P5PC8	11.67	-	-	-
P6PA1a (I)	12.33	-	-	-
P6PA1a (IV)	10.70	-	-	-
P6PA1B	21.00	-	-	-
P6PC3	32.33	-	-	-
P7PA1	9.00	-	-	-

Table S3. Filamentous fungi from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) used in this study. Øc = Mean diameter of the colony; Øh = Mean diameter of the halo; EI = enzymatic indices; SD = Standard deviation of the EI; (-) = without halo production (continuation).

Collection code	Mean Øc*	Mean Øh**	EI	SD***
P7PA9	13.33	-	-	-
P7PB1	12.33	-	-	-
P7PB3	10.50	-	-	-
P7PB5	21.00	-	-	-
P7PC1	9.00	-	-	-
P7PC2	9.67	-	-	-
P7PC4	11.00	-	-	-
P8PA4a	12.00	-	-	-
P8PB1	9.33	-	-	-
P8PB7 (I)	21.33	-	-	-
P8PB7 (II)	25.00	-	-	-
P8PC1 (I)	9.00	-	-	-
P8PC1 (II)	10.00	-	-	-
P8PC11 (II)	14.00	-	-	-
P8PC5	10.33	-	-	-
P8PC9	21.00	-	-	-
P9PA3	32.00	-	-	-
P9PB2	9.00	-	-	-
P9PC2	12.67	-	-	-
P9PC6	25.33	-	-	-
P9PC8	9.33	-	-	-
P9PC9	11.00	-	-	-
P9PE6	12.00	-	-	-
P9PF2	7.67	-	-	-
P9PF5	21.00	-	-	-
P9PH3	38.30	-	-	-
P9PI5	8.00	-	-	-
P9PJ5	11.00	-	-	-
P9PJ6	12.00	-	-	-
P6PA1A (II)	12.00	-	-	-
P6PC2	11.00	-	-	-
P9PE1 (II)	12.67	-	-	-
P9PB4	11.50	-	-	-
P9PE1 (I)	21.00	-	-	-
P9PE2 (I)	11.50	-	-	-
P9PH5	9.33	-	-	-
P9PG1	11.50	-	-	-
P10PB1B (I)	12.00	-	-	-
P10PB1B (II)	10.60	-	-	-
P10PA1 (I)	21.00	-	-	-
P10PA1 (II)	21.67	-	-	-
P10PA6 (I)	10.00	-	-	-
P10PA6 (II)	9.67	-	-	-

Table S3. Filamentous fungi from the digestive tract of *Phylloicus* spp. (Trichoptera: Calamoceratidae) used in this study. Øc = Mean diameter of the colony; Øh = Mean diameter of the halo; EI = enzymatic indices; SD = Standard deviation of the EI; (-) = without halo production (continuation).

Collection code	Mean Øc*	Mean Øh**	EI	SD***
P10PA9	10.00	-	-	-
P10PC1 (II)	12.33	-	-	-
P10PC2A	10.70	-	-	-
P10PC2B	21.00	-	-	-
P10PC5	8.33	-	-	-
P10PC6A	7.67	-	-	-
P10PD1	6.33	-	-	-