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MICROBIOLOGY

A diverse and partially cellulolytic fungal community contributes to the diet of three species of the aquatic insect *Phylloicus* (Trichoptera: Calamoceratidae) in Amazonian streams

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Abstract: Investigations on the fungal community associated with the digestive tract (DT) of insects have provided insights into the diversity of associated microorganisms and their potential roles in the interaction with their hosts. However, most studies have focused on terrestrial insects, with few studies focusing on aquatic insects in Neotropical regions. We studied fungal taxa associated with the DT of larval stages of the aquatic shredders *Phylloicus amazonas, P. elektoros* and *P. fenestratus* in 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 taxonomy. The genus *Penicillium* was the most frequent in DT of *Phylloicus* spp. (18.75%). The occurrence of fungal taxa among hosts was quite variable, with more than half of the associated fungi being exclusive of each host species. A significant portion of the fungal community associated with each host presented cellulolytic activity (± 50%). It was concluded that the fungal community associated with *Phylloicus* spp. larvae consist mainly of fungal taxa from food items, which come from riparian vegetation (whose plant species are variable) or are indigenous of the aquatic ecosystems, which is the habitat of these larvae.

Key words: Aquatic macroinvertebrates, cellulolytic fungi, digestive tract, fungal diversity, fungus-insect interaction.

INTRODUCTION

The digestive tract (DT) of insects has been revealed as a hotspot for diversity studies 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, have been possible from the exploitation of this habitat (Suh & 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 contributed to the understanding of their roles in the interaction with their hosts (León et al. 2016, Stefani et al. 2016). It also generated insights for potential biotechnological applications, such as selecting fungal strains producing enzymes of industrial interest (Suh et al. 2013).

The insect microbiome can influence nutrition, physiology, immunity and behavior of insects (Douglas 2015, Chen et al. 2016). Studies involving the microbiome of these organisms have revealed that the hosts' diet can influence the microbial composition of their DTs (Majumder et al. 2019, Przemieniecki et al. 2020). Insects that feed on woody substrates or leaf debris (e.g., termites, wood roaches, scarab

beetle larvae) generally have some type of relationship with cellulolytic microorganisms, which contribute to the degradation of the cell wall of the plant material consumed (Douglas 2015). However, most studies on such cellulolytic microorganisms in the insect microbiome have been related to bacteria and terrestrial insects (Hatefi et al. 2017, Shelomi et al. 2019, Callegari et al. 2020, Wang et al. 2020).

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 & 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 fungi is limited and restricted to the *Trichomycetes* class (*Mucoromycota*) associated with a small group of insects (White & Lichtwardt 2004, Siri & Lastra 2010, Misra et al. 2014). Insects and fungi are involved in several ecological processes in aquatic ecosystems, such as the decomposition of plant debris (Graça 2001, Hieber & 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 & Suberkropp 1989, Chung & Suberkropp 2009). Various studies point for the importance of fungi in the diet and food preference of detritivorous aquatic insects, especially shredders (Graça et al. 2001, Canhoto et al. 2005, Graça & Cressa 2010, Cornut et al. 2015). Among the aquatic shredder insects that occur in Brazil, *Phylloicus* spp. (Trichoptera: Calamoceratidae) is exceptionally diverse (Prather 2003), with many records of occurrence of *Phylloicus* species for the Brazilian Amazon and Atlantic Forests (Dumas & Nessimian 2010, Santos & Nessimian 2010, Quinteiro et al. 2011, Gama Neto et al. 2017, Souza-Holanda et al. 2020). One study recently reported the frequent occurrence of cultivable filamentous fungi in association with the DT of *Phylloicus* (presence in 94.9% of the DTs analyzed) from streams under different ecological landscapes in the Brazilian Amazon (Santos et al. 2018). However, little is known about the taxonomic identity of the filamentous fungi associated with these shredders and the possible existence of species-specific interaction between these microorganisms and their hosts.

We performed the isolation and molecular identification of filamentous fungi associated with 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 detect a species-specific relationship between these two groups. Collections were limited to these two streams in one location in order to avoid the effect of site/ecosystem in the distribution of fungi and insect species. We also tested the spectrum of cellulolytic activity of the fungal community as a possible benefit to the insect host by the digestion of plant food resources. We hypothesize that aquatic fungi and shredders have a symbiotic relationship in which fungi transform plant detritus in highly palatable and energy-rich food and the *Phylloicus* genus harbours specific fungal taxa that it selects from the fungal community of the highly diverse habitat of low order streams.

MATERIALS AND METHODS

Characterization of the 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 et al. 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. 1) were selected for collection. In each stream, a 50 m stretch was used to select the available substrate (especially foliage) at five points 10 m apart, 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 to collect typical cases of *Phylloicus* spp. 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].

Insect sampling and identification

The larvae of *Phylloicus* spp. were identified by associating their appearance and cases with descriptions of type specimens of each species occurring in the site. For this purpose, larvae were taken alive and brought to laboratory in flasks containing stream water and transferred to polystirene boxes for their maturation until adulthood. During the period, larvae were fed with leaves until adults emerged. These adults were identified to species level 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, composition and form of plant leaf material). Further on, the larvae’s carcasses were stored in 80% ethyl alcohol for

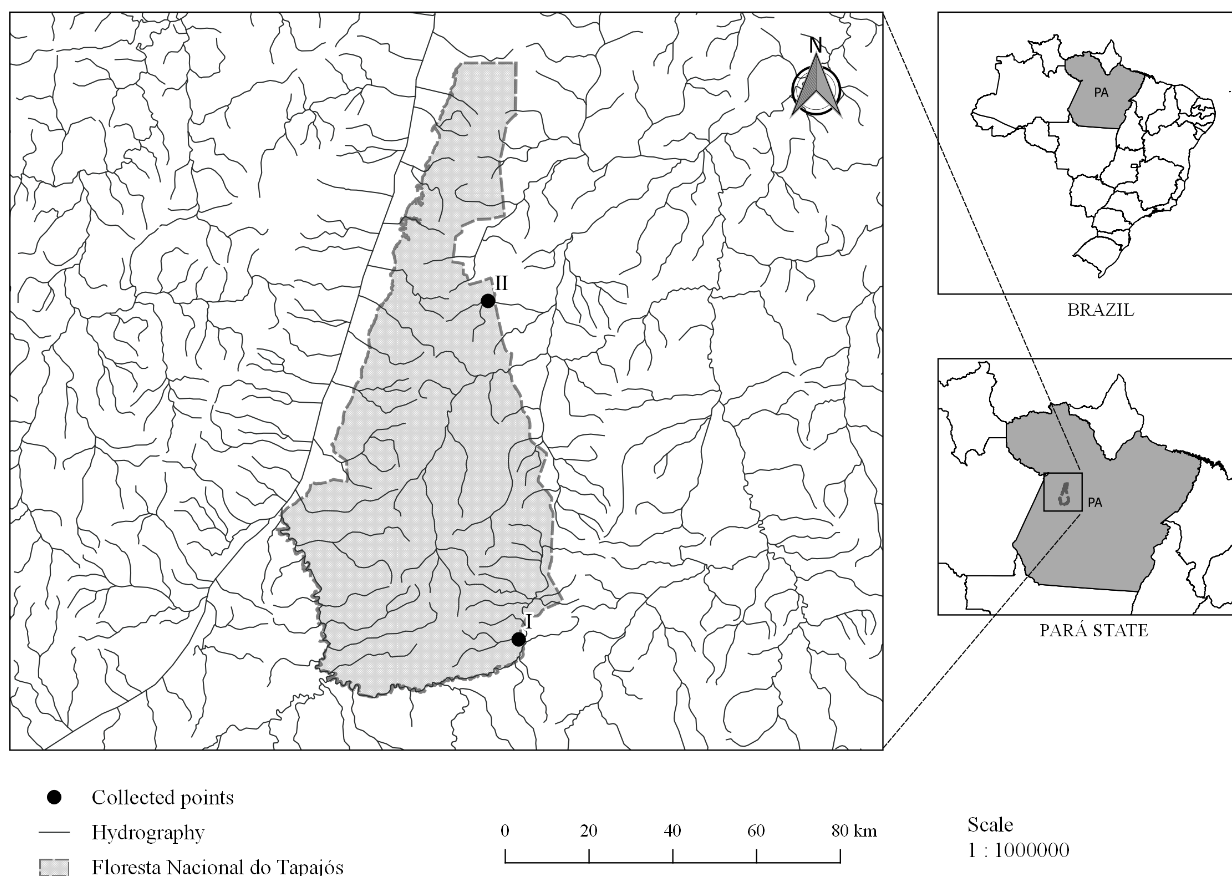

Figure 1. Map of the sites showing the low order streams and location in the Pará State, Brazil. Abbreviations: PA = Pará State; I and II = streams sampled.

identity confirmation through a comparative description of head capsule, spinules, mandibles and labrum and also by larval sclerites within the case by one of the authors (A.M. Pes).

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 in 1.5 mL microtubes. After the homogenization of the contents, the inoculation of the DT content was done in triplicate of 100 μL aliquots in 90 mm diameter Petri dishes containing PDA (Potato Dextrose 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. 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 fungal growth was detected, described and counted.

The fungal Colony-forming Units (CFU), from each DT, were grouped into different morphogroups (named morphotypes) according to their morphological characteristics (colony appearance, colour and type of mycelium) (Fröhlich et al. 2000, Lacap et al. 2003, Ibrahim et al. 2017). The microculture technique was used to identify microscopic structures, following Kern & Blevins (1999). Formation of conidia was observed microscopically with lactophenol cotton blue staining (Seifert et al. 2011).

The CFU of each morphotype in each DT was counted for quantitative analysis. One to five representatives of different fungal morphotypes from each DT were purified by successive inoculation in PDA Ptri plates. The Castellani technique was used for preservation of pure cultures (Castellani 1939) in the *Coleção de Culturas Microbianas Carlos Rosa/UFT* for identification.

DNA extraction, amplification and sequencing

One to five representatives of each morphotype 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, for 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 was assessed with the NanoDrop 2000 spectrophotometer (Thermo Scientific, Uniscience, Brazil).

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. (2016). The amplification reaction was performed to a final volume of 25 μL containing 5.0 μl of Taq Polymerase buffer (5x); 2.5 μL $MgCl_2$ (25 mmol); 1.0 μL of dNTPs (2.5 mmol each dNTP); 1.0 μL of the ITS1 primer (5 μmol); 1.0 μL of the ITS4 primer (5 μmol); 0.25 μL of the enzyme Taq Polymerase (5 U/μL), 5 μL of genomic DNA

(10 ng.µL^{-1}). Negative control (DNA replaced by ultrapure water) was used.

Amplified fragments were analyzed by 1% (w/v) agarose gel electrophoresis with GelRed™ (Biotium, Inc., Fremont, CA) in 1X TBE buffer (2 mmol EDTA, 0.1 mol Tris-HCl, and boric acid 0.1 mol [pH 8.0]) (Sambrook & Russell 2001) and visualized with UV illumination by a photo documentation system LPIX EX (Loccus Biotechnology & Cotia, São Paulo, Brazil). The 1 Kb DNA Ladder (Promega Corp., Madison, WI) was used as a molecular weight marker. Subsequently, the PCR products of approx. 300–650 bp were purified using a Wizard™ SV Gel and PCR Clean-Up System kit (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/).

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 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 identical species. The sequences were deposited in the GenBank database under the accession numbers MK120544 to MK120591 Supplementary Material (Table SI).

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. A phylogenetic tree was constructed by the neighbor-joining method. The bootstrap was 1,000 replications to assess the reliable level to the nodes 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 (Fig. 2 and Table SI).

Evaluation of cellulolytic activity

As proposed by Sunitha et al. (2013), the pure 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 mol. L^{-1} 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 in the letter "P" in Table I, whereas negative strains were indicated by "N" and fungal taxa

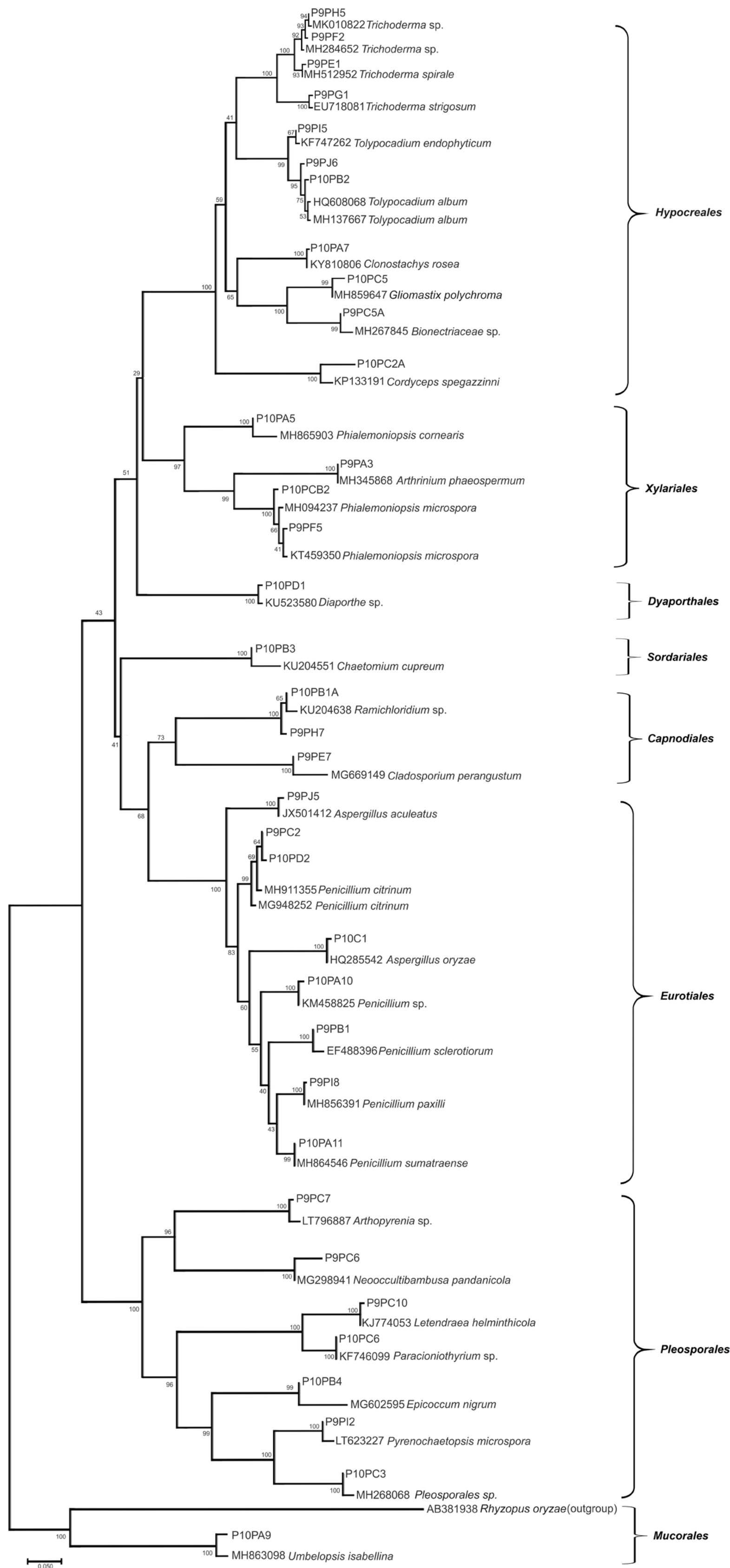

Figure 2. Phylogenetic relationship between fungal taxa.

that had positive and negative strains were indicated by the letter "V", which means variable cellulolytic activity.

Statistical analysis

For community analysis, data were expressed as the presence/absence of fungal taxa in insect DT (occurrence) (named as *ni* in Table I). Each strain isolated from a particular DT and identified as described above was counted as a representative of its fungal taxon in each DT where that fungal taxon was detected, not the counts of that taxon's cells present in the DT. Excel software, version 2016 (Microsoft™), was used to calculate the frequency of occurrence (*Fo*), which corresponded to the percentage of DT in which the fungal taxon was found. *Fo* was calculated as follows: $Fo = (\sum_1^J ni / N) \times 100$, where *ni* equals the number of occurrences of the fungal taxa "*i*" in the DT "*j*"; "*N*" is the total number of DT sampled.

The same Excel software was used to calculate the geometric mean and standard deviation of the Colony-forming Units per DT ($CFU.DT^{-1}$) in order to analyse fungal populations in *Phylloicus* DT.

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 taxon, 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 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 the total number of fungal taxa.

The distribution pattern (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*).

RESULTS

Fungal strains were obtained from all the shredders digestive tracts analyzed. The fungal $CFU.DT^{-1}$ ranged from 1.7×10^1 to 2.1×10^3, with a geometric mean ± standard deviation of $3.9 \pm 8.0 \times 10^2$ $CFU.DT^{-1}$. A total of 33 fungal taxa was isolated from the DT of the three *Phylloicus* species, from which 22 species, corresponding to 16 genera, belonged to the phylum *Ascomycota* and one species to *Mucoromycota* (Table I). Eight taxa were identified only up to the genus level (all from the phylum *Ascomycota*). One *Ascomycota* (collection code: P9PC5A, Table SI) was identified only up to the family level (*Bionectriaceae*), and two ascomycetous isolates (collection codes: P10PC3 and P10PD3, Table SI) were identified only up to the order level (*Pleosporales*). The phylogenetic relationships among taxa are shown in Fig. 2.

Chaetonium cupreum was the fungal taxon that presented the highest count (903 UFC), followed by *Epicoccum nigrum* (877 CFU),

Table I. 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	% Query cover	bp	% 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. (LT796887)	98	573	99	MK120544	1	3					2.08	P
Arthrinium phaeospermum (MH345868)	99	635	99	MK120545	1	3					2.08	N
Aspergillus aculeatus (JX501412)	100	568	99	MK120546			1	3			2.08	N
As. oryzae (HQ285542)	100	587	99	MK120548	1	7			1	130	4.16	N
Bionectriaceae sp. (MH267845)	87	591	98	MK120589	1	10					2.08	P
Chaetomium cupreum (KU204551)	100	335	99	MK120549			1	33	1	903	4.16	P
Cladosporium perangustum (MG256463)	99	396	100	MK120551	1	10					2.08	P
Clonostachys rosea (KY810806)	99	571	99	MK120552					1	13	2.08	P
Cordyceps spegazzinii (KP133191)	99	490	100	MK120554					2	13	4.16	N
Diaporthe sp. (KU523580)	100	579	98	MK120555					1	3	2.08	N
Epicoccum nigrum (MG602595)	100	423	100	MK120556					1	877	2.08	P
Gliomastix polychroma (MH859647)	99	536	99	MK120557					1	20	2.08	N
Letendraea helminthicola (KJ774053)	99	636	99	MK120558	1	3					2.08	P
Neooccultibambusa pandanicola (MG298941)	96	487	99	MK120559	1	3					2.08	N
Paraconiothyrium sp. (KF746099)	99	575	100	MK120560					2	14	4.16	V
Penicillium citrinum (MG948252)	100	534	100	MK120562	1	3	1	3	1	3	6.25	V
Pe. paxilli (MH856391)	99	560	99	MK120565	1	3	1	3			4.16	P

Table I. Continuation.

Pe. sclerotiorum (MH856384)	99	558	100	MK120567	1	7					2.08	P
Penicillium sp. (KM458825)	98	601	100	MK120568			1	7	1	3	4.16	P
Penicillium sumatraense (AY213677)	98	587	100	MK120570					1	7	2.08	P
Pestalotiopsis microspora (MT367357)	100	452	99	MK120571	2	5	1	17	2	12	10.42	N
Phialemoniopsis cornearis (MH865903)	100	422	99	MK120576					1	13	2.08	P
Pleosporales sp. (MH268068)	97	536	99	MK120590					2	5	4.16	P
Pyrenochaetopsis microspora (MK508814)	100	534	99	MK120577			1	3			2.08	P
Ramichloridium sp. 1 (KU204638)	93	481	97	MK120578			1	7			2.08	N
Ramichloridium sp. 2 (KU204638)	95	521	99	MK120579					1	30	2.08	P
Tolypocladium album (HQ608068)	99	554	99	MK120580			1	17	1	17	4.16	V
Tolypocladium endophyticum (KF747262)	96	427	100	MK120582			1	3			2.08	N
Trichoderma sp. 1 (MK870785)	100	593	99	MK120583			1	13			2.08	N
Trichoderma sp. 2 (KR154938)	100	618	99	MK120584	1	3	1	237			4.16	N
Trichoderma spirale (MH512952)	100	543	99	MK120586	1	10					2.08	N
Tr. strigosum (EU718081)	99	605	100	MK120587			1	3			2.08	N
Umbelopsis isabellina (MH863098)	97	621	99%	MK120588					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;
[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).

Trichoderma sp. 2 (237 CFU) and *Aspergillus oryzae* (130 CFU). The genus *Penicillium* was the most frequent (18.75%) 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 all species of *Phylloicus* studied here (Fig. 3). Other fungal species were isolated only once or two times during the present study. 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* and *P. fenestratus* shared *Aspergillus oryzae* in addition to the two previously mentioned.

Diversity and equitability indexes are described in Table II. The diversity index was applied to indicate higher diversity as well as equitability (*J*) associated with DT of *P. amazonas*. The cellulolytic activity of the strains is shown in Table I. Almost half of all strains associated with the three *Phylloicus* species. (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*.

DISCUSSION

The DT of the three *Phylloicus* spp. harbours a diverse community of fungi (33 fungal taxa) in the Amazon forest ecosystem. Fungal strains were obtained from all the digestive tracts analyzed in this study, contrasting with the low occurrence of yeasts (31%) verified by Santos et al. (2019) in association with the DT of *Phylloicus* spp. in Cerrado streams, in Brazil. Except for *Umbelopsis isabellina*, which belongs to the phylum *Mucoromycota*, all taxa associated with *Phylloicus* DT belong to *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

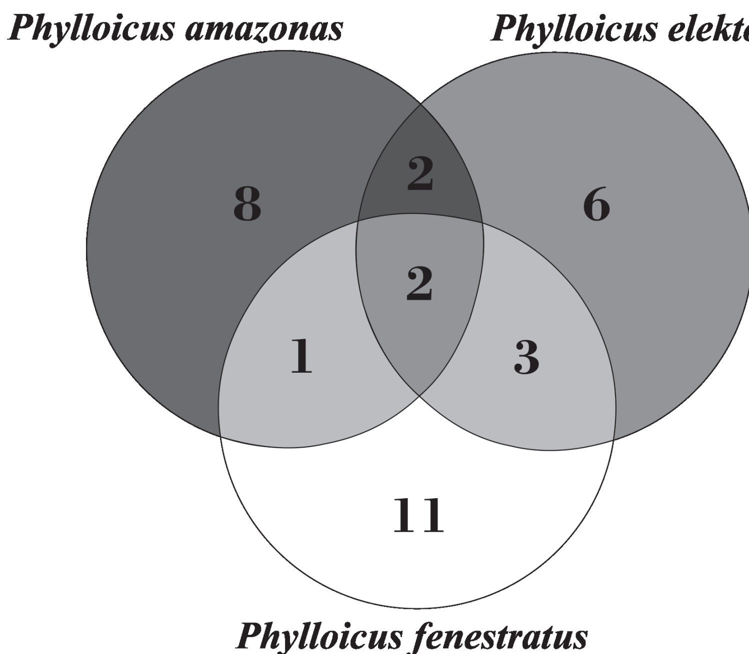

Figure 3. Venn diagram with the number of fungal taxa restricted to and shared by the three *Phylloicus* species sampled in Amazon Forest site Floresta Nacional do Tapajós, Pará-Brazil.

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

Index	Host		
	Phylloicus amazonas	***Phylloicus elektoros***	***Phylloicus 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

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* was predominant (Blackwell & 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. This finding supports a Jurassic origin of fungal–animal symbioses within *Hypocreales* (*Sordariomycetes, Pezizomycotina, Ascomycota*) during the Cretaceous, that occurred at the same time as insects and angiosperms diversified, raising a possible prevalence of *Ascomycota* as insect symbionts. Further investigation of fungal communities associated with DT from other insects may clarify whether or not Ascomycetous fungi prevail as symbionts with insects.

Ascomycota is known to dominate the early succession in decomposing leaves in streams. A work by Vorísková & Baldrian (2013) showed that sequences assigned to the *Ascomycota* showed the highest relative abundances in green leaves fallen to stream and during the early stages of decomposition in streams. Fungi from the *Ascomycota* phylum also prevailed in the non-senescent (green) and senescent (red) 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 & Bayman 2005) and the pyrosequencing analyses of live *Quercus macrocarpa* leaves (Jumpponen & Jones 2009, 2010). 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. It is possible to raise the hypothesis that *Phyloicus* prefers to feed on leaves recently fallen in the streams due to the prevalence of Ascomycetes.

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 group of insects than

the genus *Penicillium*. The genus *Pestalotiopsis* was detected 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 detected from DT of triatomines (Hemiptera: Reduviidae) (Moraes et al. 2004) and exoskeletons of *Cydia ulicetana* (Lepidoptera: Tortricidae) (Yamoah et al. 2008). Along with *Penicillium*, representatives of both fungal taxa have been detected in association with plant hosts and from aquatic ecosystems (Orole & 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 fungi 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 I) and low counts in individual DT does not support a close association between these two fungal species and the larvae of *Phylloicus*. In a similar approach, Santos et al. (2019) could not find evidence of a close association between *Phylloicus* larvae and yeasts isolated from their DT in savanna streams of Northern Brazil.

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 I. The *Bionectriaceae* isolate of this study (collection code: P9PC5A, Table SI) present 98% similarity with a *Bionectriaceae* sp. MH267845 isolated from the inner bark of *Micrandra spruceana* (Baill.) R. Schult. in Peru. It groups robustly in the same clade of the species *Gliomastix polychroma*, which also belongs to the family *Bionectriaceae* (Fig. 2). Similarly, the *Pleosporales* isolates of this study (collection codes: P10PC3 and P10PD3, Table SI), named *Pleosporales* sp. in Table I, present a 99% similarity with *Pleosporales* sp. MH268068, isolated from the inner bark of *Hevea guianensis* Aubl. in Peru. They are grouped with other taxa belonging to the order *Pleosporales* (*Arthopyrenia* sp., *Epicoccum nigrum*, *Letendraea helminthicola*, *Neooccultibambusa pandanicola*, *Paraconiothyrium* sp. and *Pyrenochaetopsis microspora*) (Fig. 2).

Regarding the isolates identified up to the taxonomic level of genera in this study, only two (collection codes: P9PH7 and P10PD1, Table SI) presented less than 99% similarity with sequences from GenBank and/or CBS database. The isolate P10PD1 (named as *Diaporthe* sp. in Table I) showed phylogenetic proximity with *Diaporthe* sp. KU523580 (Fig. 2), isolated from soil in Brazil. On the other hand, the isolate P9PH7 (named as *Ramichloridium* sp. 2 in Table I) presented phylogenetic proximity with *Ramichloridium* sp. 1, from this study, and KU204638 (Fig. 2), 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 in case the standard barcode sequence (rDNA ITS regions) is not sufficient (Udayanga et al. 2012,

Santos et al. 2016). This strategy may be used in future efforts of identification of the three isolates (collection code: P9PC5A, P10PC3 and P10PD3, Table SI) with incomplete identification (only order or family level, as previously mentioned).

All larvae of *Phylloicus* species from this study came from streams of the same ecological landscape (Amazon Forest), from a single geographical region of Brazil (Pará state). Although the number of *Phylloicus* specimens collected was not high, the expected richness was similar to the actual richness, indicating that the sampling effort was sufficient (Table II). There was variation in fungal richness, uniformity and diversity among hosts. The species richness (Chao 1) was higher for *P. fenestratus* than 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 fungal richness calculated for 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 trophic guilds of Ephemeroptera, Plecoptera and Trichoptera in three basins of Brazilian Savanna. Fungi were considered important food 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 an omnivore (Ceneviva-Bastos et al. 2017). It is usually accepted that stream macroinvertebrates exhibit plasticity in their feeding habitats, being considered generalists in many cases (Friberg & Jacobsen 1994, Mihuc & Minshall 1995, Carvalho & 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 includes fungal cells in accordance with Cummins & Klug (1979), in the DT of the larvae. Ferreira et al. (2015) found that fine particulate organic matter (FPOM) [defined as particles from 0.5 m to 1.0 mm] predominated in all instars of *Phylloicus*. They suggest that *Phylloicus* larvae exhibited plasticity in their dietary behavior. Cummins & Klug (1979) included fungal cells and spores in FPOM. In the studies of Palmer et al. (1993), Tomanova et al. (2006), Chará-Serna et al. (2012), Callisto & Graça (2013) and studies reviewed by these authors, FPOM was the most important food resource for the leaf litter-associated insect community. 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 high availability may explain its ubiquity in the guts of leaf litter-associated invertebrates in the habitat. In a study in Southeastern Brazil, Carvalho & Uieda (2009) showed that *Phylloicus* sp. consumed mostly CPOM and can be classified as the unique specialist shredder in that ecosystem. What emerges from those works is the great variability in the 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 random 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 larval specimen it was isolated; *Epicoccum nigrum* also presented a high count of 877 CFU in the one DT it was isolated, whereas the counts of frequent *Pestalotiopsis microspora* were 5

to 17 CFU per DT in the five hosts it was found. Santos et al. (2018) showed that the fungal counts varied from 5.7 ± 24.9 x 10^1 CFU.DT^{-1} (in the Cerrado [Savanna in Central Brazil]) to 1.1 ± 2.2 x 10^2 CFU.DT^{-1} (in the *Lavrado* [Savanna in Northern Brazil]) and 1.9 ± 7.1 x 10^2 CFU.DT^{-1} (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^{-1}) and *A. oryzae* (130 CFU.DT^{-1}), counts are exceedingly higher than for other fungal species and even for whole counts in the study of Santos et al. (2018). 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 have different food preferences and may choose leaves from different plant species colonized by those particular fungi found in their DT and a possible feeding preference for the leaf species and not for the fungal species. Several works show different fungal communities in leaves of different plant species in streams (Vorísková & Baldrian 2013, Medina-Villar et al. 2015). Riparian vegetation is composed of various plant species (Afonso et al. 2000, França et al. 2009, Bambi et al. 2017, Rezende et al. 2017), which results in the simultaneous input of leaves of different species to the 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 & Pringle 2008, Lecerf & 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. This would lead to different fungal communities in leaves fallen in the same stream, and the diet of the three species of *Phyloicus* could reflect this diversity.

The diet of shredders may also vary with the life stage (Malas & Wallace 1977, Casas 1996). The strategies for coexistence in three species of caddisflies (Trichoptera) in second-order streams were studied by Malas & 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 different stages of development, a condition we have not taken into consideration in the present work and it may be further investigated if *Phylloicus* spp. from the same habitat do coexist by partitioning of feeding niche, by selecting fungus species or leaf species in the stream.

This is a first report of the 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 & 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 & Koizumi 2003), as well as by-products and/or enzymes for the degradation of recalcitrant carbon sources such as cellulose or lignin (Hongoh & Ishikawa 2000, Douglas 2009, Urubschurov & 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 & 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). This study 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* (Al-Kharousi et al. 2015, Hu et al. 2018), *Cladosporium* (Andersen et al. 2016) and *Penicillium* (Al-Kharousi et al. 2015, Bomtempo et al. 2017, Li et al. 2020). However, only the species *Penicillium citrinum* (Dutta et al. 2008, Ng et al. 2010) 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 an accidental occurrence in analyzing community statistics (Santos et al. 2019); therefore, it 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 consist 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.

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SUPPLEMENTARY MATERIAL

Table SI.

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