Cultivable cellulolytic fungi isolated from the gut of Amazonian aquatic insects

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ABSTRACT

Filamentous fungi have been targeted by bioprospecting studies because they are effective producers of extracellular enzymes that can potentially be used by the bioindustry. In this study, we isolated filamentous fungi from the guts of Amazonian aquatic insect larvae to evaluate their cellulolytic activity. We collected 69 larvae of shredder insects of three genera: Phylloicus (Trichoptera: Calamoceratidae), Triplectides (Trichoptera: Leptoceridae) and Stenochironomus (Diptera: Chironomidae) in ten streams from a protected area in the central Brazilian Amazon. Production of mycelia was elaborated in PDA (Potato Dextrose Agar) medium. The isolates were transferred to a synthetic medium with carboxymethyl cellulose, and Congo red was used to determine the enzymatic index. The hydrolysis halo, indicating the production of cellulases, was observed in 175 fungal isolates (70% of the total), of which 25 had an enzymatic index ≥ 2.0 and belonged to seven fungal genera. The fungal taxa Cladosporium, Gliocephalotrichum, Penicillium, Pestalotiopsis, Talaromyces, Trichoderma and Umbelopsis were isolated from guts of Phylloicus, Triplectides and Stenochironomus, which are traditionally used in biotechnological applications. Our results indicate the cellulolytic potential of fungi associated with the guts of aquatic Amazonian insects.

KEYWORDS: cellulase, enzymatic hydrolysis, Phylloicus, shredders, Amazonia

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INTRODUCTION

Cellulases produced by fungi are widely used in industry, and the demand for efficient microorganisms has increased over time. Cellulases are currently employed in the production of food, animal feed, second generation ethanol, fruit and vegetable juices, paper, wine and textiles as well as in pulp extraction, starch processing, breweries, laundry and agriculture (Bhat 2000; Choi et al. 2015; Singh et al. 2016). Fungal enzymes are particularly advantageous when compared to animal or plant equivalents, as their production is less costly since the enzymes are secreted as complexes that work in synergy (Dashbhan et al. 2009). Moreover, fungal enzymes have different physicochemical characteristics (e.g., thermostable enzymes of thermophilic organisms), and they are easier to produce on a large scale (Dalmaso et al. 2015).

Many insects that digest wood, foliage and debris (e.g., Isoptera, Coleoptera, Orthoptera, Hymenoptera) (Martin 1983) use lignocellulose as their main food source and are highly efficient in obtaining glucose from cellulose degradation (Sun and Zhou 2011). Naturally, wood-boring insects have been common subjects of studies aiming at the prospect of lignocellulolytic enzymes (Geib et al. 2010). In addition, aquatic leaf-mining Diptera larvae (Koroiva 2013) and other larvae of shredder insects (Rogers and Dora-Peterson 2010) have been considered as potential sources of lignocellulolytic enzymes for the second-generation biofuel industry (Cook and Dora-Peterson 2010; Huang et al. 2010). Although insects can produce endogenous cellulases (Watanabe and Tokuda 2010; Shelomi et al. 2014; Pothula et al. 2019), the polysaccharide hydrolysis is mainly performed by extracellular enzymes produced by microorganisms that colonize the digestive tracts of the insects, especially by fungi like Aspergillus, Penicillium and Fusarium (Haller, Link and Hernández 2015). Several filamentous fungi, such as Cladosporium, Penicillium, and Trichoderma, have been found in the guts of Coleoptera and Diptera such as Silvanidae (Coleoptera) (David et al. 1974), Tenebrionidae (Coleoptera) (Prabha et al. 2011), Platypodidae (Coleoptera) (Henriques et al. 2009) and Culicidae (Diptera) (Fonseca et al. 2008; Pereira et al. 2009; Santos et al. 2018a; Santos et al. 2018b). The study of the gut mycobiota of aquatic insects can lead to the discovery of metabolic processes and interactions with potential biotechnological applications. Thus, the aim of this study was to investigate the cellulase activity of filamentous fungi isolated from the gut of aquatic shredder insects, expecting to identify new cellulolytic enzymes in poorly studied species of Amazonian insects.

MATERIAL AND METHODS

Collecting insects

Larvae of aquatic shredder insects belonging to the genus Phylloicus Müller (Trichoptera: Calamoceratidae), Triplectides Kolenati (Trichoptera; Leptoceridae) and Stenochironomus Kieffer (Diptera; Chironomidae) were collected in ten forest streams located in the Adolfo Ducke Forest Reserve, municipality of Manaus, Amazonas state (Brazil) (02°56'21"N, 59°57'43"W) (Figure 1). The stream beds are composed of sand and leaves, and the streams are shaded by riparian vegetation (e.g., Mendonça et al. 2005). The values of pH ranged from 4.4 to 5.4. The electrical conductivity of the water ranged from 7 to 23 μS cm⁻¹, water velocity from 0.04 to 0.17 cm s⁻¹, dissolved oxygen concentration from 5.8 to 6.8 mg L⁻¹, and the water temperature ranged from 24.8 to 26.8 °C.

Insect larvae were collected randomly in August 2016 with the aid of an aquatic entomological net and identified to the genus level in the field according to Hamada et al. (2014). In total, we captured 69 larvae: 24 Phylloicus spp., 20 Triplectides spp., and 25 Stenochironomus spp. Each larva was removed from its shelter (leaf or stick), sterilized for 30 seconds in 70% ethanol, and stored in a microtube with sterile distilled water. Larvae were processed shortly after collection to avoid the decomposition or excretion of the contents of their digestive tracts.
Isolation, purification and morphological characterization of filamentous fungi

Larvae were dissected under a stereomicroscope using sterilized pins and scissors in the Laboratory of Citotaxonomy and Aquatic Insects of Instituto Nacional de Pesquisas da Amazônia - INPA. Each larva had its guts removed and transferred to a microtube containing 1 mL of sterile distilled water. After homogenizing, a 100 µL aliquot of each sample was inoculated in a Petri dish containing Potato Dextrose Agar (PDA) medium supplemented with 0.1 mg L⁻¹ chloramphenicol and incubated at 27 °C for seven days. For purification, the filamentous fungi that showed no contamination were transferred separately to new Petri dishes containing PDA. After obtaining pure fungal cultures, preservation was carried out by the Castellani method (Castellani 1939). Contaminated strains that were not recovered after the purification step were not considered in data analysis and they are not represented in the results. The fungal isolates were identified based on macromorphological characteristics such as color, shape, size, and border, among other relevant characteristics (Lacap et al. 2003; Ibrahim et al. 2017). To observe the micromorphological characteristics, the fungal isolates were microcultured, stained with lactophenol cotton blue and examined under a Leica Microsystems optical microscope with an attached Leica DFC295 camera and the Leica Application Suite (LAS, 4.2.0) software.

Cellulolytic activity screening

The fungal isolates were evaluated for cellulase production in carboxymethyl cellulose (CMC) medium (NaNO₃: 3.0 g L⁻¹, KH₂PO₄: 1.0 g L⁻¹, MgSO₄: 0.5 g L⁻¹, KCl: 0.5 g L⁻¹, FeSO₄·7H₂O: 10.0 mg L⁻¹, CMC: 10.0 g L⁻¹, agar: 20.0 g L⁻¹) (Ruegger and Täub 2004). The plates were incubated at 27 °C for four days and then subjected to thermal shock for 16 h at 50 °C. The degradation halo was observed using 10 mL of Congo red solution (2.0 g L⁻¹) for 30 min and 5 mL of 0.5 M NaCl solution in 0.1 M Tris-HCl buffer, pH 8.0 (Nogueira and Cavalcante 1996). These assays were performed in triplicate. We calculated the enzymatic index (E.I.) of all isolates that produced a degradation halo. The E.I. was calculated as the ratio between the mean halo diameter and the mean colony diameter among the three replicates (Hankin and Anagnostakis 1975). The fungal isolates that had high enzymatic activity, here defined as E.I. ≥ 2.0 (Lealem and Gashe 1994), were further identified by molecular analysis.

Molecular analyses

The fungal isolates were cultivated in natural potato broth for five days in a rotary shaker (150 rpm) at 27 °C. DNA extraction was performed using a Fungi/Yeast Genomic DNA Isolation kit (Norgen Biotek, ON, CAN) following the manufacturer’s instructions. The fungal ITS1-5.8S-ITS2 regions were amplified by PCR using the forward primer ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and the reverse primer ITS4 5′-TCCTCCGCTTATGATATGC-3′ (White et al. 1990). Amplification was accomplished in a 50 µL reaction mixture containing 2 µL of genomic DNA, 0.3 µL of Taq DNA polymerase - Platinum® 5U/µL, 1.25 µL of reaction buffer (10×), 2.5 µL of dNTPs (2.5 mM each), 2.5 µL of MgCl (50 mM), 0.5 µL of each primer (20 µM), and 15.95 µL of sterile Milli-Q water with the following reaction program: 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. Amplification products were sequenced with an ABI 3500 sequencer (Thermo Fisher Scientific, MA, USA). Analyses were carried out in...
the Molecular Biology Laboratory of Universidade Federal do Amazonas - UFAM.

**Taxonomy**

The consensus sequence of both DNA strands was obtained using Geneious’ 9.0.5 (Kearse et al. 2012). The alignment of the consensus sequences was performed against the database of fungal genomes deposited in GenBank using the Basic Local Alignment Searching Tool (BLAST) (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI). In addition, the UNITE database was also used (Nilsson et al. 2018; https://unite.ut.ee/).

**RESULTS**

The intestinal content of 46 of the 69 aquatic insect larvae collected resulted in fungal growth (Table 1). A total of 248 fungi were isolated from the guts of *Phylloicus*, *Triplectides* and *Stenochironomus* larvae. Among these fungal isolates, 175 produced cellulases: 84 from guts of *Phylloicus* larvae, 55 from *Triplectides* larvae, and 36 from *Stenochironomus* larvae (Table 1). Out of the 175 isolates that produced cellulases, 25 had an E.I. ≥ 2.0 (22.4% of all fungi isolated from guts of *Stenochironomus* larvae, 8% of *Triplectides* and 6.2% of *Phylloicus*) (Table 1).

Most of the identified fungal isolates were Eurotiumycetes, of which 15 were identified as *Penicillium* (Eurotiiales: Aspergillaceae), with E.I. from 2.0 to 4.0. (A1TB1), which had been previously identified as *Penicillium* sp. based on morphology, but was assigned to *Talaromyces purpureogenus* (Stoll) Samson, Yilmaz, Frisvad and Seifert (Eurotiales: Phylloicaceae) based on molecular analysis (Table 2; Figure 2).

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The molecular taxonomic identification confirmed most of our morphology-based identifications (Table 2). Two isolates could be identified only to the genus level: *Gliocephalotrichum* Ellis and Heseltine (Hypocreales: Nectriaceae) and *Umbelopsis* Amos and Bennett (Mucorales: Umbelopsidaceae). Five isolates could be further identified to the species level: *Trichoderma harzianum* Rifai, *Talaromyces purpureogenus*, *Penicillium admetzii* Zalessky, and *P. citrinum* Thom.

**DISCUSSION**

Overall, we found that the guts of *Phylloicus*, *Triplectides* and *Stenochironomus* larvae analyzed in this study are colonized by *Cladosporium*, *Gliocephalotrichum*, *Penicillium*, *Pestalotiopsis*, *Talaromyces* Benjamí, *Trichoderma* and *Umbelopsis* fungi that have species that are traditionally used in biotechnological applications (Jalim et al. 2012; Copete-Pertuz et al. 2019; Geetha et al. 2019; Liuzzi et al. 2019; Salazar-Cerezo et al. 2019; Ge et al. 2020; Slaný et al. 2020).

Interestingly, among the isolates with E.I. ≥ 2.0, fungi of the genus *Penicillium* were more common and more efficient producers of cellulase in solid medium than *Trichoderma*, which is the most well-studied fungal group for cellulase, comprising very powerful decomposers of crystalline cellulose (Galliano et al. 1998; Ahamed and Vermette 2008; Gusakov 2011). *Penicillium* strains can be as good as *Trichoderma* strains in the production of cellulases, based on indicators such as production level and cellulase hydrolytic performance per unit of activity or per milligram of protein (Gusakov 2011; Syed et al. 2013; Carvalho et al. 2014). More recently, Santos et al. (2018b) investigated the filamentous fungi isolated from the guts of *Phylloicus* larvae using both classical and molecular methods and found 21 isolates belonging to the genus *Penicillium*. Our study further corroborates the association between filamentous fungi and aquatic insect larvae.

Other fungal genera with known cellulolytic activity were isolated in this study. *Gliocephalotrichum* is commonly regarded as a saprophytic fungus (Lombard et al. 2014) and this is the first record of its association with insect guts. *Umbelopsis*, which here produced cellulases in a solid medium with carboxymethyl cellulose, colonizes substrates rich in simple carbohydrates and has been found associated with Curculionidae insects (Silva et al. 2015). *Talaromyces* is also known to be associated with insects such as Cynipidae (Hymenoptera) (Seifert et al. 2004) and has been recorded in the midguts of *Aedes aegypti* (Diptera) (Angleró-Rodríguez et al. 2017). Some species of *Talaromyces* (T. emersonii CBS 814.70 and *T. cellulolyticus* Fujii) are able to produce cellulolytic enzymes (Inoue et al. 2014). *Talaromyces purpureogenus*, which is important in bioindustry due to its ability to produce cellulolytic enzymes (Belancic et al. 1995), was recently placed in the genus *Penicillium* (Samson et al. 2011), and *Talaromyces* received all species of the *Penicillium*

**Table 1.** Total number of fungal isolates and number of isolates positive for cellulolytic activity in aquatic shredder insects belonging to three genera sampled in Ducke Reserve, in the central Brazilian Amazon. E.I. = enzymatic index.

| Aquatic insect          | Number of insects | Number of fungal isolates | E.I. ≥ 2.0 |
|-------------------------|-------------------|---------------------------|------------|
|                         | Total  | N with fungal growth | Total | Positive |                      |
| *Phylloicus* spp.       | 24     | 19                        | 112    | 84       | 7                      |
| *Triplectides* spp.     | 20     | 19                        | 87     | 55       | 7                      |
| *Stenochironomus* spp.  | 25     | 8                         | 49     | 36       | 11                     |
| **Total**               | **69** | **46**                    | **248**| **175**  | **25**                 |
subgenus Biverticillium (Samson et al. 2011), since these two taxa form a monophyletic group (Yilmaz et al. 2014).

Our results expand the knowledge on the diversity and functions of fungi associated with the guts of aquatic insects, since few similar studies have been carried out with Amazonian species. Santos et al. (2018b) identified fungi isolated from the gut of Phylloicus larvae in from the Brazilian states of Pará and Tocantins, in the Amazonian and Cerrado savanna biomes, respectively, thus suggesting the occurrence of distinct associated fungi communities across ecosystems. While there are studies on fungi associated with insects, studies on the gut microbiome are lacking (e.g., Fonseca et al. 2008; Pereira et al. 2009). Moreover, studies on the gut mycobiota of aquatic insects in Amazonia have been concentrated on the class Trichomycetes (e.g., Rios-Velásquez et al. 2002; Alencar et al. 2003).

Our results suggest that the mycobiota of aquatic shredder insect guts isolated in this study can effectively degrade cellulose. The presence of cellulolytic fungi is probably related to the conditioning of ingested leaf material through the decomposition of structural compounds by fungal enzymes, which softens the material and increases the palatability of the leaves (Graça et al. 2001; Aßmann et al. 2011; Casotti et al. 2015; Biasi et al. 2017; Reis et al. 2018). Graça et al. (2001) demonstrated experimentally that insects preferred conditioned leaves over unconditioned leaves. Likewise, Reis et al. (2018) showed that high concentrations of tannins in unconditioned leaves are inhibitory and not consumed by

### Table 2. Cellulolytic filamentous fungi isolated from the guts of Phylloicus (Trichoptera), Triplectides (Trichoptera) and Stenochironomus (Diptera) shredder aquatic insects, in the central Amazon region of Brazil. The table shows the morphological and molecular identification of the isolates that had an enzymatic index ≥ 2.0 after growing on a synthetic carboxymethylcellulose medium. M = morphological identification; G = molecular genotyping; E.I. = enzymatic index (mean index ± SD, n = 3); (*) = percentage of similarity between our sequences and those available in the NCBI and UNITE databases.

| Sample # | Host insect | Fungal isolate | Species | Type of identification | E.I. | Identity* | Accession number |
|----------|-------------|----------------|---------|------------------------|------|-----------|-----------------|
| 1        | Phylloicus  | A1PA3          | Cladosporium sp. | M         | 2.3 ± 0.3 |          |                 |
| 2        | Phylloicus  | A1PB5          | Pestalotiopsis sp. | G       | 3.1 ± 0.8 | 97%       | KF887030.1      |
| 4        | Phylloicus  | A2PB4          | Penicillium sp.  | G         | 3.9 ± 0.2 | 99%       | JQ896996.1      |
| 5        | Phylloicus  | A2PC2          | Cladosporium sp. | G         | 2.8 ± 0.5 | 87%       | MH655007.1      |
| 11       | Phylloicus  | A5PA3          | Gloeophalotrichium sp. | G     | 2.9 ± 0.4 | 94%       | MH397480.1      |
| 17       | Phylloicus  | A8PB5          | Penicillium sp.  | M         | 2.1 ± 0.2 |          |                 |
| 20       | Phylloicus  | A9PB4          | Trichoderma harzianum | G     | 2.3 ± 0.1 | 100%      | MN262498.1      |
| 2        | Triplectides| A1TB1          | Talaromyces purpurogenus | G      | 3.0 ± 0.0 | 99%       | MK108916.1      |
| 2        | Triplectides| A1TB3          | Penicillium sp.  | G         | 2.0 ± 0.4 | 97%       | MK775828.1      |
| 8        | Triplectides| A4TA1          | Penicillium sp.  | M         | 2.8 ± 0.3 |          |                 |
| 17       | Triplectides| A4TA3          | Umbelopsp. sp.   | G         | 3.7 ± 0.5 | 98%       | MF101390.1      |
| 17       | Triplectides| A8TA5          | Penicillium admetzii | G      | 4.0 ± 0.6 | 99%       | JN714932.1      |
| 17       | Triplectides| A8TA7          | Penicillium sp.  | G         | 3.9 ± 0.4 | 94%       | KF848945.1      |
| 17       | Triplectides| A8TA9          | Penicillium sp.  | G         | 2.5 ± 0.0 | 93%       | MH268036.1      |
| 3        | Stenochironomus| A1SC3        | Penicillium sp.  | G         | 2.5 ± 0.1 | 85%       | KC181929.1      |
| 4        | Stenochironomus| A2SA2        | Penicillium sp.  | M         | 2.1 ± 0.8 |          |                 |
| 4        | Stenochironomus| A2SA4        | Penicillium sp.  | M         | 2.8 ± 0.8 |          |                 |
| 4        | Stenochironomus| A2SA5        | Penicillium sp.  | M         | 2.2 ± 0.1 |          |                 |
| 5        | Stenochironomus| A2SB2        | Cladosporium sp. | M         | 2.0 ± 0.5 |          |                 |
| 5        | Stenochironomus| A2SB3        | Penicillium admetzii | G      | 2.0 ± 0.3 | 99%       | JN714932.1      |
| 7        | Stenochironomus| A3SA1        | Cladosporium sp. | M         | 3.1 ± 0.5 |          |                 |
| 8        | Stenochironomus| A4SA1        | Penicillium sp.  | G         | 2.3 ± 0.1 | 97%       | MN238763.1      |
| 10       | Stenochironomus| A4SC2        | Penicillium citrinum | G      | 2.1 ± 0.4 | 99%       | KM278038.1      |
| 12       | Stenochironomus| A5SB3        | Cladosporium sp. | M         | 2.5 ± 0.1 |          |                 |
| 13       | Stenochironomus| A5SC1        | Cladosporium sp. | M         | 2.9 ± 0.4 |          |                 |
insect larvae. Gonçalves-Júnior et al. (2017) compared the characteristics of five common leaves found in streams in the Adolpho Ducke Forest Reserve and noted that shredder insects were more frequent on the leaves of *Mabea speciosa* Müll.Arg (Euphorbiaceae), which had a higher polyphenol content, lower amount of cellulose and higher concentration of fungal biomass than the leaves of the other plants. These results indicate that aquatic shredder insect larvae feed on leaves conditioned by fungi, which initiate the decomposition process through extracellular enzymes, facilitating digestion by the larvae (Graça et al. 2001; Aßmann et al. 2011; Casotti et al. 2015; Biasi et al. 2017; Reis et al. 2018).

**CONCLUSIONS**

This study contributes to the knowledge of the mycobiota associated with the guts of aquatic insect larvae, however it remains elusive to what extent the fungal microbiota...
influences its host life. Overall, we expect that future research on shredder aquatic insects microbiota will contribute to our understanding of interactions fungi–host. The presence of cellulolytic fungi in the guts of aquatic insects reinforces the biotechnological potential for cellulolytic enzyme prospection. Our study also brings new information on the identities and functional traits of the symbiotic fungi of aquatic-insects in the Amazon that have not been previously studied. As available data about symbionts increase, so does the understanding of the ecological relationships between these fungi and their insect hosts. More research is essential to better understand the processes that occur in the fungus-insect system, in addition to the biotechnological potential of these processes.

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