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Diversidad de hongos asociados a los intestinos de escarabajos (Passalidae) que habitan en el Parque Nacional Yasuní y evaluación de degradación de celulosa *in vitro*

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SOFÍA ALEJANDRA LÓPEZ CHÁVEZ

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Certifico que la disertación de Licenciatura en Ciencias Biológicas de la candidata Srta. Sofía Alejandra López Chávez ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

M.Sc. Alexandra Narváez Trujillo

Directora de la Disertación

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Con todo mi corazón

a mi hermana Catalina

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LISTA DE ABREVIATURAS

Abreviatura	Significado
ANOSIM	Analysis of similarities
BLAST	Basic Local Alignment Search Tool
CEQCA	Colección de Endófitos Quito Católica
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
g	Gram
h	Hour
ITS	Internal transcribed spacer
M	Molar mass
mg	Miligram
min	Minute
ml	Mililiter
mM	Milimolar mass
Muscle	Multiple sequence alignment by log-expectation
NMDS	Non-metric multidimensional scaling
no.	Number
º C	Celsius degrees
PAST	Paleontological statistics
PDA	Potato dextrose agar

Abreviatura	Significado
RAxML	Randomized Axelerated Maximum Likelihood
rDNA	Ribosomal Deoxyribonucleic Acid
rpm	Revolutions per minute
sec	Second
SIMPER	Similarity percentage
YM	Yeast mold
µl	Microliter
µM	Micromolar mass
µmol	Micromole

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1. RESUMEN

La descomposición de la materia orgánica del suelo en el bosque es esencial para el ciclo de los nutrientes en los ecosistemas edáficos. En estos procesos no sólo están involucrados componentes abióticos, sino también bióticos, tales como los microorganismos y los invertebrados de suelo. Passalidae es una familia del orden Coleoptera que posee hábitos saprófitos y requiere microbios intestinales, los cuales contribuyen a la degradación y digestión de la madera. Los adultos de tres especies de pasálidos se colectaron en el Parque Nacional Yasuní de Ecuador. Hongos filamentosos fueron aislados del tracto intestinal de los escarabajos adultos, los cuales fueron identificados al secuenciar el espaciador transcrito interno (ITS por sus siglas en inglés) y analizados en base a estudios filogenéticos. Los aislados fúngicos fueron asignados a dos filos, cinco órdenes, ocho familias, once géneros y diecinueve especies. *Trichoderma* fue el género más abundante en todas las especies detectadas de pasálidos y en todos los sitios de colecta. La capacidad enzimática para digerir la celulosa fue positiva para los extractos de intestino de escarabajo y para todos los hongos ensayados. Puntuaciones más altas en estos ensayos se lograron con los aislados fúngicos *Clonostachys rossmanie* y *Epicoccum nigrum*. Los análisis de diversidad y de la composición de las especies fúngicas demostraron que los pasálidos albergan una importante diversidad no sólo en el número de especies de hongos, sino en la composición filogenética, y en la compleja dinámica de hongos. La estructura de las comunidades de hongos depende de la especie de hospedero Passalidae y del medio ambiente que lo rodea. La presente investigación proporciona nuevas evidencias de posibles relaciones endosimbióticas que varios tipos de microorganismos son capaces de establecer con escarabajos que se alimentan de madera y que pueden desempeñar un papel

importante en la alimentación de los escarabajos durante la digestión y la fermentación de la celulosa.

Palabras clave: Passalidae, endosimbionte, *Trichoderma,* celulosa

2. ABSTRACT

The decomposition of soil organic matter (SOM) in the forest is essential for the nutrient cycling in edaphic ecosystems. In this processes are not only involved abiotic components, but also biotic such as microorganisms and soil invertebrates. Passalidae is a family of the order Coleoptera that posses saprophytic habits and requires gut microbes to contribute wood's degradation and digestion. Adults of three Passalidae species were collected in Yasuni National Park of Ecuador. Filamentous fungi were isolated from intestinal tracts of adult beetles and identified sequencing the internal transcribed spacer (ITS) region and analyzed with phylogenetic tree construction. The fungal isolates were assigned to 2 phyla, 5 orders, 8 families and 11 genera and 19 species. *Trichoderma* was the most abundant genus detected in all Passalidae species and at all sites sampled. The enzymatic capacity to digest cellulose was positive for extracts of beetle guts and for all fungi tested. Highest scores in these assays were achieved by *Clonostachys rossmaniae* and *Epicoccum nigrum* fungal isolates. Diversity and composition analyses of fungal species demonstrated that Passalidae beetles harbor an important diversity not only of fungal number species but phylogenetic composition, and a complex fungal dynamic. Fungal communities structure depends on Passalidae species host and surrounding environment. The current research provides new evidence of possible endosymbiotic relationships that several types of microbes are able to establish with wood-feeding beetles, which may play an important role to beetles nourishment during cellulose digestion and fermentation.

3. MANUSCRITO PARA PUBLICACIÓN

REVISTA

Applied and Environmental Microbiology

TÍTULO

Diversity of fungi associated with guts of Bess beetles (Passalidae) that inhabit the Yasuni National Park and *in vitro* evaluation of cellulose degradation

AUTORES

Sofía López-Chávez [1#], Alexandra Narváez-Trujillo[1*]

[1]Laboratory of Plant Biotechnology, School of Biological Sciences, Pontificia Universidad Catolica del Ecuador, Quito, Ecuador[1]

#Present address: Address correspondence to Sofía López-Chávez, Laboratory of Plant Biotechnology, School of Biological Sciences, Pontificia Universidad Catolica del Ecuador

salopez@outlook.com

*Present address: Address correspondence to Alexandra Narváez-Trujillo, Laboratory of Plant Biotechnology, School of Biological Sciences, Pontificia Universidad Catolica del Ecuador

anarvaez@puce.edu.ec

Tel. +593 2 2991700 ext. 1810

Fax. +593 2 2991687

INTRODUCTION

Soil organic matter (SOM) in forest ecosystem represents the main source of energy, especially plant litter materials. The decomposition of SOM depends on environmental factors such as pH, temperature, precipitation and oxygen supply, as well as on the action of decomposer organisms, soil fauna and microorganisms (1).

Microbial communities in soil represent a great diversity of biotic interactions (2). During organic decomposition of biomass there is a change in community dynamics, a succession of fungal species occurs and the availability of niches are altered when associations of fungal species are replaced (3). Soil fungal species such as *Phoma*, *Cylindrocarpon*, *Clodosporium*, *Phomopsis, Trichoderma* and *Fusarium* take part in litter decay because of their capacity to break down cellulose bonds (4, 5). As a consequence of these microbiotic interactions in the SOM decomposing process, new resources and microhabitats are also created for soil fauna, such as Collembola, Acari (3) and Passalidae (6) which also play an important role in speeding up the decomposing process.

Soil-inhabiting invertebrates are key components of the decomposer web and nutrient cycling pathways in the edaphic ecosystems. In a wide diversity of ecosystems, soil invertebrates mediate about 15% of the C and 30% of the N cycling (7), and influence physical-chemical properties of soils (8, 9). Therefore, micro-, meso- and macroinvertebrates contribution is enormous in the stabilization and destabilization of SOM (10).

Organic matter commonly has a low nutritional value. Accordingly, saprophytes have to ingest large amounts of organic material to compensate this deficit. Saprophytes consume about 20–30% of the annual input of organic matter (10). During the ingestion of dead organic matter, soil invertebrates inevitably also acquire microorganisms, which have

colonized the decaying litter. Bacteria and fungi have developed different abilities to establish symbiotic relationships especially with its host's digestive tract (11). This gut microbiota plays an essential role in insect growth, development and nutrition, especially with the acquisition of nutrients by fermentation and lignocellulose degradation, nitrogen fixation, amino acid biosynthesis and uric acid degradation (12, 13).

Plant litter is composed mainly by cellulose, hemicellulose and lignin (14). Cellulose is a difficult molecule to breakdown since it consists of linear polymers of β-D-glucopiranose units (glucose) linked by β-1-4 glycosidic bonds (15). Endoglucanases, exoglucanases and β-glucosidases (12) are enzymes required to give way to primary products such as glucose, cellobiose and cello-oligosaccharides (16). Studies have demonstrated that cellulose digestion occurs in many types of insects such as termites, ants, wood roaches, wasps, aphids and a wide range of beetles (17, 18). Some of these, like termites and longhorn beetles have the capacity to produce their own cellulase enzymes in the gut, while other insects complement their cellulose digestion with the cellulase enzymatic capacity of endosymbiont microorganisms (14, 19, 20).

Knowledge regarding the diversity of the microbiota associated to insect's guts is still largely unknown, nonetheless some investigations have identified and characterized them in particular insect groups (17, 21, 22). If we focus only on Coleoptera endosymbiotic interactions, it is possible to visualize the complexity and diversity of relations that exist. Research on beetle guts have resulted mainly in the characterization of isolated yeasts such as *Candida*, *Pichia, Scheffersomyces*, and *Rhodotorula* (23) from beetles like Chrysomelidae (24), Cerambycidae (19, 25, 26) and Passalidae (27–29). In some cases, yeast have been tested for cellulosic ethanol production (30). Likewise, coevolutionary discoveries have been made in ambrosia beetles (Curculionidae) and their obligate mutualisms with plant pathogenic fungi with cellulose-degrading capacity (31–35).

This study focused on a group of passalid beetles (Passalidae: Coleoptera). Six hundred eighty species have been described within this family, distributed mainly in tropical and humid-temperate regions of the world, such as the Neotropical region (36). Their biological cycle mostly occurs in tunnels or galleries built in rotten logs preferably of dicotyledonous angiosperms (37), where they can obtain refuge, nourishment and more stable microenvironmental conditions (38). These insects are cataloged as a highly subsocial group due to their living form. Adults (parents), juveniles, larvae in different developmental stages, and eggs live together in the same tunnel system (38). Parental care creates dependence of juveniles and larvae by feeding a mixture of digested wood, salivary secretions and inoculated feces that contain microbiota with cellulase activity (13). Diet is the key to their fitness, passalids eating only rotting wood in the absence of endosymbiotic microbiota, start losing weight and die much sooner (39).

To take part in the decaying process of fallen trees, Passalidae beetles have developed interactions with other soil decomposing organisms. Harrel (1967) (40) reported a varied assemblage of parasitic and commensalistic organisms that included mites, nematodes and protozoa associated to the passalid beetle *Odontotaenius disjunctus*. However, it was Lichtardt (1968) (40) that reported on the numerous fungi associated with this beetle species and its mites, specifically, Trichomycetes (Zygomycota) found in the hindgut and Laboulbeniales (Ascomycota) common found externally on both the passalids and their mites. Various studies on the microbiota of passalids have revealed a vast diversity of xylose and cellulose-degrading yeast species (11, 13, 27, 29, 41); few studies have reported on other components of this microbiota.

The purpose of this study was to assess the diversity of fungal isolates from the guts of passalids in the Ecuadorian tropical rainforest, as an approximation to their ecological performance and survival. The study also entailed an initial evaluation of the cellulase

activity of gut fungal isolates to verify if fungal enzymes contribute to cellulose hydrolysis and are therefore important components of the community of microorganisms involved in degradation and conversion of biomass in the tropical rainforest. This study also pinpoints the gut microbial communities of this species of Coleoptera as biological reservoirs of new enzymes to be investigated and exploited for biotechnological uses or industrial processes.

MATERIALS AND METHODS

Sample collection, medium, and culture conditions

Passalid adults were collected from three rotting logs in the Yasuní Research Station in the Amazon Basin in Ecuador. Each decaying log was considered a sampling site. Tree no. 1 (00.67543 S 76.39896 W) and tree no. 2 (00.67542 S 76.39896 W) were approximately three meters away from each other. Tree no. 3 (00.67508 S 76.39938 W) was 100 m away. Only tree no. 1 could be identified as *Inga spectabilis* (Fabaceae). All beetles found inside the logs were collected in 50 ml conical centrifuge tubes and kept alive to be processed in the Laboratory of Plant Biotechnology of the Pontificia Universidad Catolica del Ecuador. Beetles were grouped according to their sizes and the tree from which they were collected.

Fungi isolates were obtained from passalid guts. Dissections were performed following the method described in Berkov, *et al* (2007) (19), Nguyen, *et al* (2006) (41), Nguyen, *et al* (2007) (42), and Suh & Blackwell (2004) (43). Insects were isolated in individual Petri dishes. Before dissection, insects were euthanized at -4 ºC for 15 min. Surface sterilization was accomplished by submersion in 75% ethanol for 2-3 min and washed with 0.7% saline solution. This solution was striated on petri dishes containing either potato dextrose agar (PDA) (1x) or 2% malt agar to serve as negative controls. Dissections were performed removing guts aseptically on an ice bath. Six dissected guts from tree no. 3 were taken and preserved in Eppendorf tubes at -16 ºC for posterior enzymatic gut assay. After dissection passalid exoskeletons were preserved in 75% alcohol for morphological identification.

All guts were cut open, emptied and carefully washed in saline solution (Fig. 1), the content of all guts were pooled for fungal isolation. Dissected guts were not divided into regions. Considering that different culture media and different sources can influence number of isolates obtained: 1) whole guts, placed on PDA (1/10x) and 2% malt agar

cultures; 2) macerated guts, striated on PDA (1/10x) and 2% malt agar cultures; and 3) pooled gut contents, striated onto PDA (1/10x), 2% malt agar and yeast mold agar (YM) at pH 3.5 (adjusted with HCl). All culture media were incubated at 25 ºC for a week; daily petri dishes were examined to isolate all possible hyphae found. Morphologically different fungi were isolated, purified twice and maintained in PDA (1x) culture with streptomycin and penicillin antibiotics to eliminate any bacterial contamination. They were placed into permanent stocks and deposited in the Endophyte Collection Quito Católica (CEQCA) at -80 ºC. In order to preserve activity, agar plugs from PDA (1X) fungi chosen for enzymatic assays were stocked in cryovial tubes with autoclaved miliQ water and preserved at 4 ºC until their use. Isolated fungi were divided into 13 groups according to the different beetles species from which they were isolated and from the tree from which each beetle species was collected (Appendix 1).

Fungi DNA extraction and PCR amplification

DNA was obtained from the mycelial growth of a seven day PDA (1X) culture using 5% Chelex following Bahl (2007), Bucheli, *et al*. (2002) (44) and Camacho, *et al*. (1997). The fungal isolates were identified by sequencing the internal transcribed spacer (ITS) region of 5.8S rDNA (45). Primers used were ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 10 µl of Green GoTaq Flexi Buffer 1x, 5 µl of MgCl2 (25 mM), 1 µl of dNTPs (10 mM), 2.5 µl of each primer (ITS1 and IT4) (30 µM), 5 ηg/µl of the template, 0.5 µl of Taq polymerase (5units/µl) and 23.5 µl of miliQ autoclaved water. PCR amplifications were performed using the following protocol: 1 min initial denaturation at 95 ºC; 30 cycles of 1 min denaturation at 95 ºC, 30 sec primer annealing at 55 ºC and 1 min extension at 72 ºC; 5 min extension at 72 ºC and a final

holding at 4 ºC. Paired-end sequencing was performed by Macrogen (Macrogen Inc., Seoul, South Korea) using the Sanger method.

Phylogenetic analyses

Each consensus sequence was compared to ITS annotated DNA sequences of the GenBank database using the Basic Local Alignment Search Tool (BLASTn). BLASTn search resulted in consensus sequences with >99% homologies for several possible species, in these cases phylogenetic trees were constructed to confirm species. For final taxonomical identification, selected sequences from the BLAST analysis were aligned with the consensus sequences using the Muscle (Multiple sequence alignment by log-expectation) software (46). Maximum likehood trees were constructed using the RAxML (Randomized Axelerated Maximum Likelihood) software (47), applying the GTR ɣ model of nucleotide substitution and 100 boot-strapped replicates (48). Given the abundance of *Trichoderma* species, a separate tree was constructed using ITS annotated and referenced DNA sequences from GenBank, *Hypomyces subiculosus* (Appendix 2) was selected as the outgroup, as the possible sister group of *Trichoderma* (49). The remaining monogeneric trees were executed without outgroups. Final phylogenetic analysis was performed with 145 sequences and 200 boot-strapped replicates to give support to each node. The yeast *Kluyveromyces waltii* (Appendix 2) was chosen as the outgroup for the tree (50).

Assays for cellulose degrading activity

Fungi enzymatic extraction

Twelve isolates identified as *Campylocarpon pseudofasciculare, Chaunopycnis alba, Clonostachys rossmaniae, Epicoccum nigrum, Trichoderma atroviride, T. asperellum,*

T. hamatum, T. harzianum, T. virens, Trichoderma sp.1*, T. spirale* and *Scytalidium* sp. were selected for enzymatic assays based on previous reports of endo-β-1,4-glucanase activity (51–53). PDA (1X) media was inoculated with one 3 mm fungal plug preserved at 4 ºC in water. After ten days, the initial PDA (1X) plug inoculum was removed; the remaining culture was chopped and extracted in 25 ml of 0.05 M citrate buffer at pH 5.0. This mixture was maintained on an ice bath for 2 h to obtain a crude mixture of extracellular proteins. Enzyme extracts were obtained by centrifugation in 2 ml Eppendorf tubes at 13,000 rpm for 5 min (18). The resultant supernatants were collected and frozen in aliquots at -20 ºC.

Insect gut enzyme extraction

For gut enzymatic assays, each frozen gut was homogenized with 500 µl of citrate buffer 0.05 M at pH 5.0. Samples were centrifuged at 10,000 rpm for 10 min (12) to obtain total enzyme extract that would contain the pool of cellulose degrading enzymes. The supernatant was collected and frozen at -20 ºC.

Enzymatic assays

Cellulase activity was determined using carboxymethyl cellulose (CMC) as a substrate and the 3,5-dinitrosalicylic acid (DNS) reagent to quantify glycoside hydrolase activity. Cellulose hydrolysis assays were preformed following Ghosh (1987) (54). The reaction mixture contained 50 µl of 2% CMC in 0.05 M citrate buffer at pH 5.0 and 50 µl of the total enzyme extract. Data for the fungi enzymatic assay was collected at 2-hour intervals during an 8-hour incubation period at 37 ºC. In the insect gut enzymatic assay only one data point was used after 12 hours of incubation at 37 ºC.

After each incubation time, 300 µl of DNS reagent was added. Tubes were boiled for 5 min in a vigorously boiling water bath. After boiling, tubes were transferred to a cold-water bath and 2 ml of miliQ water was added. The absorbance was measured at 540 nm using Pharmacia Biotech Ultrospec 2000 spectrophotometer. The negative control contained 50 µl CMC 2% with 50 µl of citrate buffer boiled with DNS reagent. Enzyme blanks were 50 µl of CMC 2% with 50 µl enzyme extractions boiled with DNS reagent. A glucose standard curve was made using CMC 2% at different glucose dilutions 1:1 (1.0 mg/0.5 ml), 1:1.5 (0.67 mg/0.5 ml), 1:2 (0.5 mg/0.5 ml) and 1:4 (0.25 mg/0.5 ml) and boiled with the DNS reagent. Linear regressions of fungi enzymatic and glucose curves were made to calculate coefficients for the cellulase activity. One unit (U/ml) of enzymatic activity is defined as the amount of enzyme necessary to produce 1 µmol of glucose per min at 37 ºC. All enzyme activities represent averages from triplicate measurements of three independent replicates.

Diversity and community composition analysis

Diversity indices and species richness estimator were calculated using fungal sequence data. Shannon's diversity index and Fisher's α values were used to describe fungal species richness. Additionally, individual rarefaction analysis of fungal species accumulation curve was carried out (55). To better understand the myco-ecological dynamics associated to trees and beetles, analyses of fungal species composition were performed using the 13 fungal groups established during fungi isolation (Appendix 1). Non-metric multidimensional scaling (NMDS) was made to analyze fungal community structure (56) and an analysis of similarities (ANOSIM) was also implemented to evaluate differences among fungal community composition. The Sörensen distance measure was used for NMDS and ANOSIM. Similarity Percentage (SIMPER) analysis allowed evaluation of the

contribution of different fungal species to the disimilarity. All analyses were executed in Paleontological statistics (PAST) ver. 2.17c software (57).

RESULTS

Insect identification

A total of 30 Passalidae beetles were collected from the galleries of three different decomposing trees (Appendix 1). Passalid beetles species identification was accomplished using morphological characters and taxonomic keys (Fig. 1) (36, 58–61). All passalid beetles collected belong to the Passalini tribe (58). Four specimens were identified as *Passalus interstitialis* and three as *Passalus variiphyllus*; the remaining individuals were classified as a single species that could not be identified and is therefore reported as *Passalus* sp. (Table 1).

Phylogenetic analyses

We obtained a total of one hundred sixty two fungal isolates from the guts of 30 adult passalid beetles. All guts dissected contained fungal isolates. Ninety-eight fungal isolates were sequenced representing 19 fungal species retrieved from the pasalid beetle guts (Appendix 3). One fungus identified as *Gliocladium* sp., isolated from the control solution, was excluded from all posterior analysis.

The phylogenetic analysis comprised 145 taxa, including queried GenBank sequences (Appendix 2). The majority of the fungal isolates are assigned to the Ascomycota phylum (97%) (Fig. 2), representing the dominant lineage. The most abundant class is Sordariomycetes with 92% of sequences. The order that accounted for the most sequences is Hypocreales, which represent 82% of the sequences (Fig. 3). Within the Hypocreales, the Hypocreaceae family is highly represented by the genus *Trichoderma* that accounted for over 81% of the sequences. *Trichoderma harzianum* is the most abundant species with 41% of all sequences, followed by *Trichoderma spirale* with 16% and *Trichoderma virens*

with 7%. Nectriaceae, another family of the Hypocreales order, represents over 7% of the sequences analyzed. No other single family accounts for more than 2% of isolates. Only three isolates could not be identified up to the species level *Trichoderma* sp. 1, *Trichoderma* sp. 2 and *Scytalidium* sp. The first two had >99% of homology, however *Scytalidium* sp. had 92% of homology according to BLAST, which could indicate a possible new species.

Cellulase enzymatic activity

The fungal cultures *Campylocarpon pseudofasciculare, Chaunopycnis alba, Clonostachys rossmaniae*, *Epicoccum nigrum*, *Scytalidium* sp., *Trichoderma* sp. 1, *T. asperellum, T. atroviride, T. hamatum*, *T. harzianum, T. spirale,* and *T. virens* isolated from the guts of Passalidae beetles were screened for their cellulase activity. All fungal species evaluated show varying levels of CMC hydrolysis (Table 1). The majority of fungal species selected for this assay belong to the *Trichoderma* genus which is known for its cellulase activity (62–65). Among the twelve fungi extracts, *Clonostachys rossmaniae* and *Epicoccum nigrum* exhibit the highest enzymatic activity followed by *Trichoderma* sp. 1, *T. hamatum*, *T. spirale, Chaunopycnis alba* and *T. asperellum* which show similar enzymatic activity. Finally, *Campylocarpon pseudofasciculare, T. virens*, *Scytalidium* sp., *T. harzianum* and *T. atroviride* present the lowest activity (Table 1).

Regarding the insect gut, four out of six beetle guts tested exhibited cellulase activity at 12 hours (Table 2). Although extensive analyses are still needed, the observed enzyme activity from the beetle guts could possibly be an endogenous production in the gut tissue.

Diversity and community composition analyses

Fisher's α for all host fungal species identified was 7.03 and the Shannon's diversity index was 2.13 (Table 3)**.** Rarefaction species-accumulation curve for fungal isolation show that the asymptote indicating saturation of sampling is not observed yet (Fig. 4). Because the number of passalid beetles analyzed for each passalid species obtained from three trees was different, terminal values of the curves cannot be directly compared. Instead, the shape of the curves was compared, with higher initial slopes of the curves representing greater richness. Diversity indexes and the rarefaction curve produced for fungal species in relation to decaying trees showed no differences of fungal diversity among decaying trees (Fig. 5; Table 3). For the fungal species among the three passalid species, diversity indexes show that the hosts *Passalus* sp. and *Passalus variiphyllus* have similar levels of diversity, regardless of their specimen number. *Passalus interstitialis* exhibits a considerably lower diversity of fungal species (Fig. 6; Table 4). Rarefaction analysis, based on the curve shape, was little higher for *Passalus variiphyllus* than *Passalus* sp. *Passalus variiphyllus* shows the lowest fungal species-accumulation curve.

Fungal species composition analyses associated to the three decaying trees was made. In the NMDS, two distinct groups were established, corresponding to tree no. 1 and tree no. 3 (0.131 stress) (Fig. 7). ANOSIM results of fungal species in decaying trees reveal compositional differences based on fungi groups ($R = 0.52$, $P = 0.006$) (Table 5). Important fungi composition differences are found comparing tree no. 3 with tree no. 1 ($P=0.01$) and with tree no 2. ($P=0.04$). SIMPER distinguished an important contribution of two species for differences observed between passalid species and between decaying trees in separate analysis; these two species are *Trichoderma harzianum* and *Trichoderma spirale* contributing together 51% in the tree decaying analysis and 54% in the passalid analysis (Table 7, Table 9). Other species such as *Trichoderma virens, Trichoderma atroviride,*

Trichoderma hamatum and *Fusarium solani* also contributed to dissimilarities (>7%), all other fungal species were below this threshold. In the comparison for decaying trees, there is a significant dissimilarity between tree no. 3 and tree no. 1 (85.73%) and between tree no. 3 and tree no. 2 (64.35%) (Table 8). The fungal species that most contribute to the dissimilarity are *Trichoderma spirale, Trichoderma harzianum, Trichoderma virens* and *Trichoderma atroviride.*

Fungal species composition analyses associated to the three Passalidae species were also carried out. For fungal species composition related to passalid species, the NMDS analysis shows the majority of fungal samples isolated from *Passalus* sp. and *Passalus interstitialis* grouped into one single group (0.134 stress) (Fig. 8). ANOSIM of fungal species showed no significant difference (R= 0.28, *P*= 0.097) between passalid species (Table 6). Meanwhile, SIMPER results correlating passalid species evidence a higher dissimilarity between *P. variiphyllus* and *Passalus* sp. (81.96%), and between *P. variiphyllus* and *Passalus intertitialis* (69.68%) (Table 10). Fungal species such as *Trichoderma spirale, Trichoderma harzianum, Trichoderma virens* and *Epicoccum nigrum* were the main fungal contributors to dissimilarity. Only fungal species showing more than 2% of contribution to dissimilarity were considered for similarity percentage analyses.

DISCUSSION

After performing phylogenetic analyses, testing the cellulase capacity of Passalid beetle's guts and gut-inhabiting fungi, and analyzing fungal ecological diversity contributions, our results suggest that Passalid beetle guts works as a microecosystem with a complexity of phylogenetic composition, species richness and community configuration with enzymatic capabilities. We report on the fungal association to the wood-feeding Passalid (order Coleoptera) in the Yasuní Rainforest and the identification of cellulase activity that can contribute importantly to the degradation of organic matter.

A total of 19 fungal species were isolated from Passalidae adult guts, principally from Hypocreales order which includes saprotrophic and symbiotic species associated to insects (66). As mentioned, studies regarding fungi associated to wood-decomposing insects are scarce and recent. Rojas-Jiménez *et al* (2015) and Vargas-Asensio *et al* (2014) published the results of the unique studies on the association of microbial diversity and Coleoptera families in Costa Rican tropical forests. Interestingly, Hypocreales was the most abundant fungi order isolated. These results are surprisingly similar to the results found in this present study, in which the order Hypocreales accounts for more than 88 of the 98 fungal isolates sequenced.

The genus *Trichoderma* was the most abundant in this study and correlates to the findings by Rojas-Jiménez *et al* (2015) and Vargas-Asensio *et al* (2014), which report this genus as the most abundant. As other fungi with saprophytic habits, the cosmopolitan genus *Trichoderma* uses a wide range of compounds as carbon and nitrogen sources (67) because of their endochitinases (68), exo- and endo-β-1,4-glucanases (4) capabilities. Their enzymatic versatility to degrade various organic substrates in soil, their faster growth rate, their prolific sporulating capacity (5) and their resistance to microbial inhibitors (51) result

in the ability to survive in many ecological niches. The remaining orders isolated showed a lower abundance, being represented by a single fungal species. Other fungal genera found in common to the findings by Rojas-Jiménez *et al* (2015) are *Scytalidium* (Helotiales), *Fusarium* (Hypocreales) and *Mucor* (Mucorales). *Chaunopycnis alba*, isolated in this study is phylogenetically closely related to the genus *Cordyceps,* which is recognized as an insect parasite reported in beetle's gut by Rojas-Jiménez *et al* (2015).

The fungal species isolated from passalid guts in this research, in most of the cases, take part in the decomposition process of soil and litter, because of their capabilities to degrade cello-oligosaccharides (51, 52, 66, 69). *Mucor* and *Epicoccum* belong to the initial phases as early colonizers (3, 70), while versatile fungal degraders such as *Trichoderma* and *Fusarium* (4) which have cellulase, xylase and lipase enzymes, join in this process around one or two years later, when decaying wood is almost completely decomposed. Given that fungal species of soil and litter succession were discovered in this research we suggest that different fungal species found in the gut of passalid beetles perform synergistically to complete several functions during degradation and fermentation of lignocellulosic materials (71); and contribute to satisfying the beetle nutritional needs. Fungi may create affinities for substrates, so members of the phylum Basidiomycota could degrade larger polymeric molecules, Ascomycota fungi can degrade diverse lignocellulosic constituents, while the bacteria degrade and ferment the smaller monomeric and dimeric hexoses and pentoses (71, 72).

It is important to relate ecosystem structure and function to species, and functional diversity. Enzyme activities can be measured and used as an index of microbiological functional diversity (73). Thus, in this occasion glycolysis catabolism conversion from cellulose into glucose was quantified. Determining enzymatic activity depends on enzymatic preparation as well as on the specific substrate used (74). The type of carbon

source for growth in culture conditions plays an important role in the production of lignocellulolitic enzymes (75). In this assay, cellulose activity was not induced using a pre-media culture with cellulose as some articles suggest (76), we used a general media for fungal growth, PDA (1X), so as to evaluate baseline production of cellulose degrading enzymes. A variety of substrates can be used for detecting and quantifying cellulose enzymatic activity (74), we used CMC as a substrate to quantify exclusively endo-β-1,4-glucanase activity considering that fungal cellulose degrading activity is based on principally endo- and exocellulases (17).

Endogenous cellulose activity has been reported in various insects that have wood habitats, as is the case of termites (14, 77). In beetles, the guts of Cerambycidae larvae and adults have been reported to have endogenous cellulase enzymes (76, 78–83), as is the case of the Cerambycidae larvae of *Anoplophora glabripennis*, which exhibit endogenous endo-β-1,4-glucanase activity (15). Up to now there are no reports of endogenous cellulases in passalid guts. The sugar reducing DNS method used in this study revelead that 67% beetle intestines had CMC degrading ability at the one time point evaluated; this could suggest the digestion of this carbon compound by endogenous enzymes of the beetle gut. However, it is evident that additional research is necessary to understand spatial digestion in the beetle intestine and determine the level of collaboration of fungal enzymes to the digestive process of the Passalidae beetle, as well as the contribution of other microorganisms to this complex process and possible endogenous production of cellulose degrading enzymes.

Insects which feed on wood materials which are hard to fragment and have low levels of nitrogenous and carbonaceous nutrients available have developed obligate symbiosis with cellulose-digesting microbes (84). Of the 19 fungal species isolated in this study from Passalidae guts, we tested 12 fungal extracts for cellulase activity. Fungal enzymatic

activity was also assayed by Rojas-Jiménez *et al* (2015) resulting in positive hydrolysis of all lignocellulase sources by *Trichoderma*. Interestingly, similar results were obtained in this study showing a stable cellulase activity during the eight-hour period of enzymatic assays using the extracts of the *Trichoderma* species and all other fungal extracts tested. The cellulose activity was comparably high within fungal extracts in *Clonostachys rossmaniae* strains cultivated under optimized conditions; the fungal isolate of this species also presented positive bioactivity in the degradation of polyurethane plastic *in vitro* (com. pers. Alexandra Narvaez-Trujillo). According to studies by Nilsson (1974), the fungal species *Xygolone* sp. and *Scytalidium* sp. had low enzymatic activity (69), which is also the case in this study for *Scytalidium* sp. that was one of the fungal extracts with the lowest CMC hydrolysis. The use of CMC for evaluation of cellulose degrading enzymes enable determining that there are endo-β-1,4-glucanase enzymes present both in the fungal species as the passalid guts; however additional assays are needed to determine other bioactive cellulose degrading enzymes in the fungal and gut extracts used.

Species richness was plotted as a function of the accumulated number of total fungal isolates. In general, the diversity and species richness of the beetle gut microbiota was not significantly different between decaying trees and between passalid species, except for *Passalus intertitialis,* which showed lower index rates. Although the number of specimens of *Passalus variiphyllus* was low (N=3), this species showed high diversity indexes comparable to *Passalus* sp., which had a higher specimen number (N=24). The great fungal diversity found in the small size sample of *Passalus variiphyllus*, also revealed significant dissimilarity to the other two Passalidae species in the SIMPER analysis. Additionally, in the comparison of diversity indexes for trees and for passalid species, tree no. 3 and *Passalus* sp. indexes were higher, respectively, and closer to asymptote. These results suggest that sampling size may influence the species richness. It is known that for

hyperdiverse groups, such as fungi, it is not possible to carry out a complete survey of the fungal diversity at any investigation site (85). Sampling of the passalid beetles at each decaying tree uncovered a limited number of individual adults; all available individuals were taken. We consider this an exhaustive sampling of each site, despite not having an equitable number of individuals among the different passalid species identified. Additionally, the high number of fungal isolates obtained, by using different culture media, permits a robust estimate of fungal species richness associated to the passalid guts and enables differentiation among sites as well as among passalid species (71).

Our statistical analyses (NMDS, ANOSIM and SIMPER) suggest that each decaying tree could work as a microhabitat in itself, determined by spatial localization, soil composition, and species of felled tree. Tree no. 3, is the most differentiated. Tree no. 3 had the highest number of passalid specimens, so initially this could account for this differentiation, however we must also consider that this tree is spatially more distant to the other two trees sampled. Therefore, we suggest that the difference in fungal composition determined could also be accounted by different environmental conditions, chemical composition of soil and litter, time of tree decomposition and even spatial distances (67, 86). Studies on bacterial community composition in insect guts have demonstrated that host diet and taxonomy influence considerably the bacterial composition in the insect gut (12, 87, 88). Geib *et al* (2009) demonstrated that Cerambycidae beetles show preferences between tree species, which seem to be related to bacterial diversity (12). Environment conditions and chemical composition of soil and litter should be studied thoroughly to determine if these characteristics along with the distance affect fungal composition.

Furtherefore, the diversity and abundance of the *Trichoderma* genus points to it being a necessary gut inhabitant of the passalid beetle and given its known activity as a cellulose degrader (5, 67) most possibly contributes in the degradation of the lignocellulase material

on which this beetle feeds on. Additionally, the fungal community composition analyses revealed that there's a significant difference of fungal composition among Passalidae species, indicating possible host specificity as observed in the study of cellobiose- and xylose-fermenting yeasts of Guatemalan Passalidae beetles' guts carried out by Urbina *et al* (2013).

Our results point to an important contribution to the digestion process in Passalidae beetles and a contribution to biomass conversion in the tropical rainforest, nevertheless we cannot determine conclusively on if the fungal species found in Passalidae guts of this research are real endosymbionts or are transitory organisms inhabitants associated with decomposing trees from which Passalidae nourish. Furthermore, some of these microorganisms could perform as commensals, parasites, or facultative endosymbionts (89). They might even be using the insect as a dispersal mechanism. However, the similarity in results found in this study and the results presented by Rojas-Jiménez *et al* (2015) and Vargas-Asensio *et al* (2014) indicate that fungal species consistently isolated may have a close relationship with their insect hosts as for example the genus *Trichoderma*.

Although, symbiotic relationships evidencing interaction between hosts and fungi are poorly understood, this study provides preliminary results about myco-ecological dynamics in beetle guts, which live in decaying trees. Our results corroborate the presence of certain fungal species associated with the guts of Passalidae beetles. The highly fungal diversity in terms of the number of species obtained, the capacities to degrade cellulose, the communities composition in host gut and decaying trees demonstrate the considerable input that these microbiota bring in to soil ecology. The present work creates new possible lines of research either biochemical characterization of enzymes responsible of cellulose hydrolysis, as well as for ecological contribution not only for soil decomposing processes

but for beetles fitness and evolutionary symbiotic relationships with insects. Additionally, in terms of biotechnology development, the fungal species recovered in this study have the potential to be investigated for industrial application such as the generation of biofuels based on biotic wastes.

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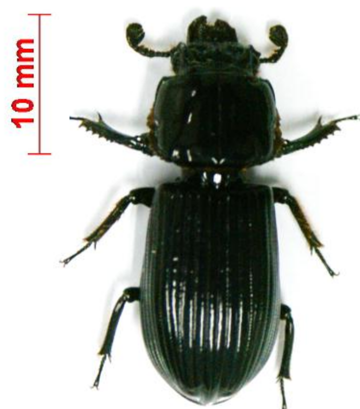
4. FIGURES

A



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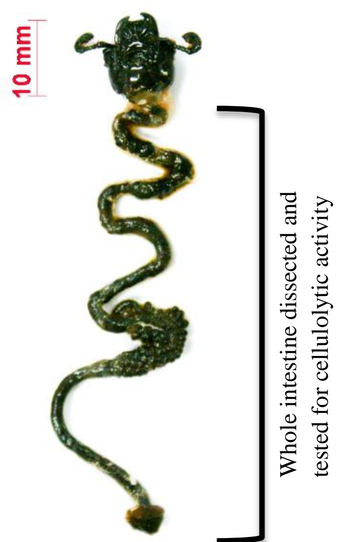

Figure 1. A. Adult passalid beetle, *Passalus intertitialis.* **B.** *Passalus intertitialis* dissected with its entire gut.

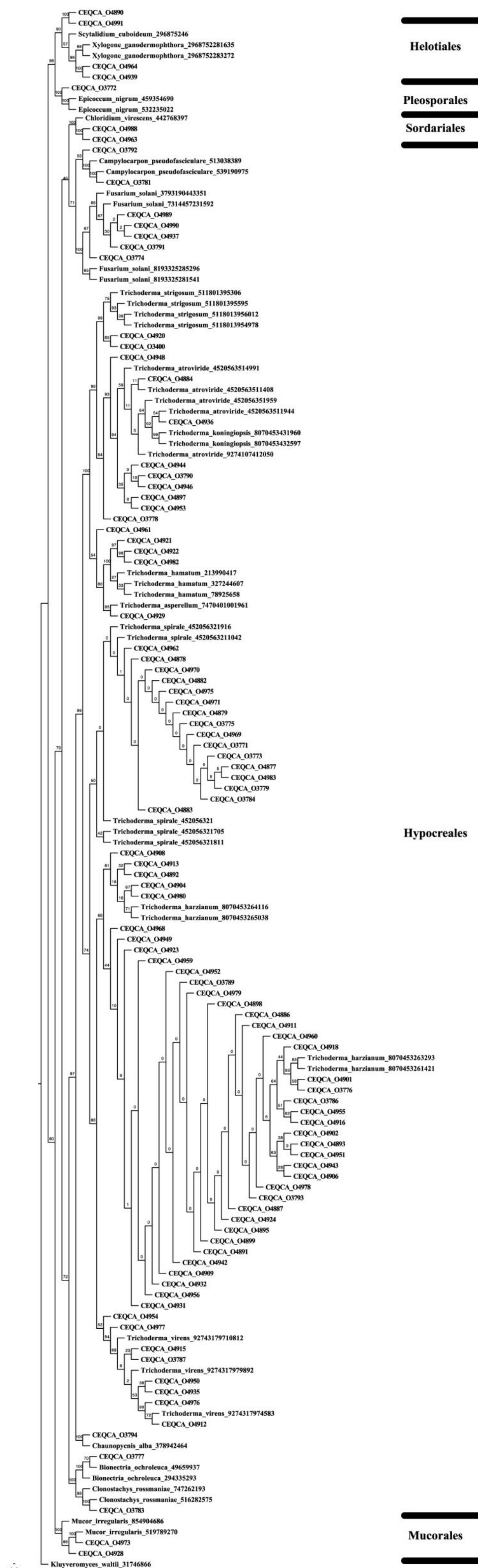

Figure 2. Maximum likehood phylogenetic tree analysis of fungi ITS sequences with emphasis on the Hypocreales order where most of the sequences were classified. Outgroup was *Kluyveromyces waltii*. Strains isolated in this study are shown with the CEQCA code. Numerical values indicate bootstrap percentiles from 200 replicates.

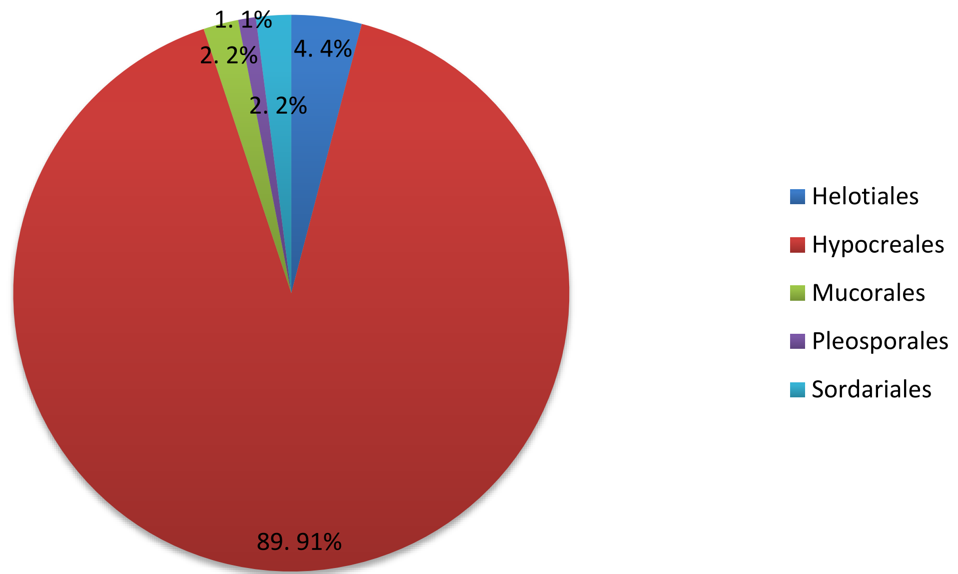

Figure 3. Orders of fungi isolated from passalid guts. Percentage of isolates classified in each order (N=98).

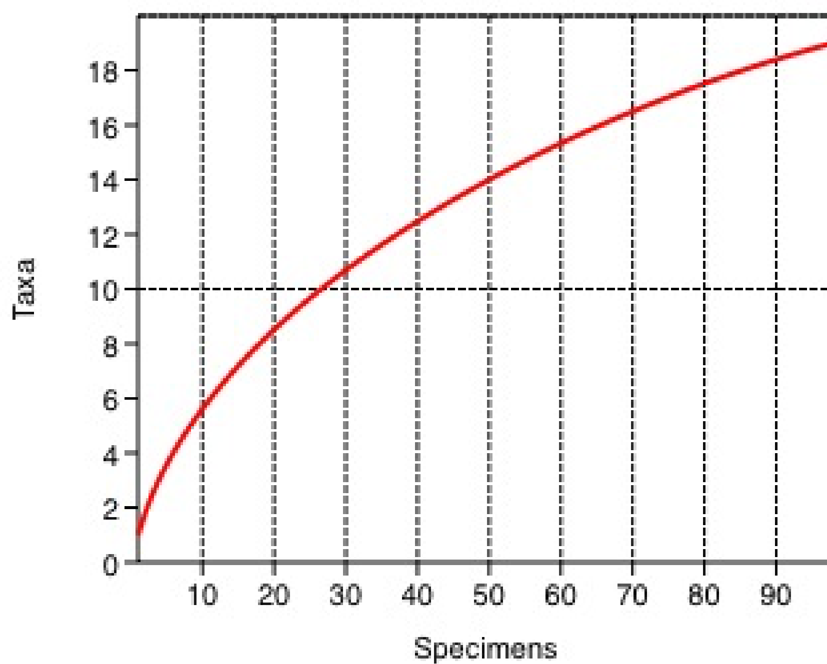

Figure 4. Species-accumulation curve of all host fungal species, isolated from the guts of three Passalidae species (N=98).

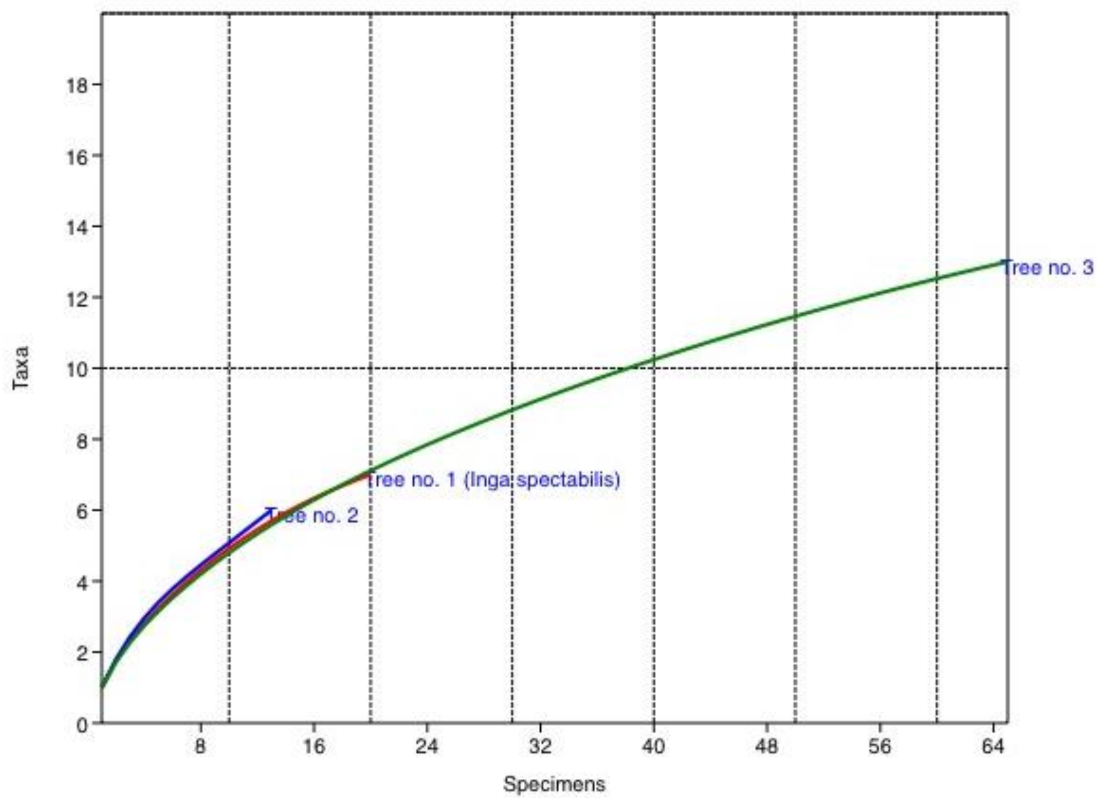

Figure 5. Species-accumulation curve of fungal species isolated from the guts of Passalidae beetles from three decaying trees (tree no. 1 in red line (N=20), tree no. 2 in blue line (N=13) and tree no. 3 in green line (N=65)).

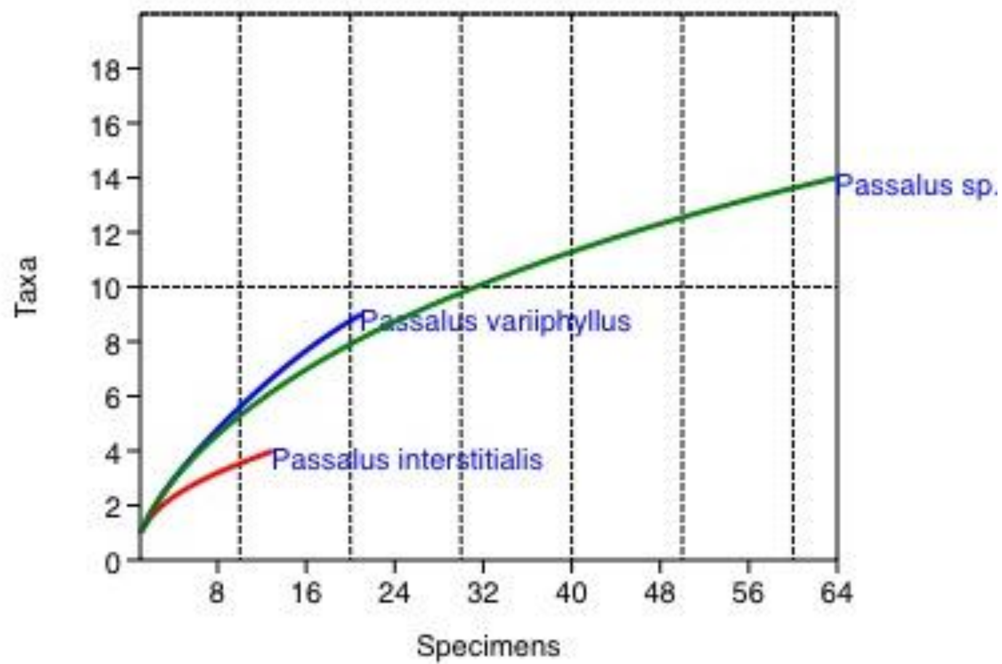

Figure 6. Species-accumulation curve of fungal species isolated from the guts of three passalid species (*Passalus interstitialis* in red line (N=13), *Passalus variiphyllus* in blue line (N=24) and *Passalus* sp. in green line (N=64)).

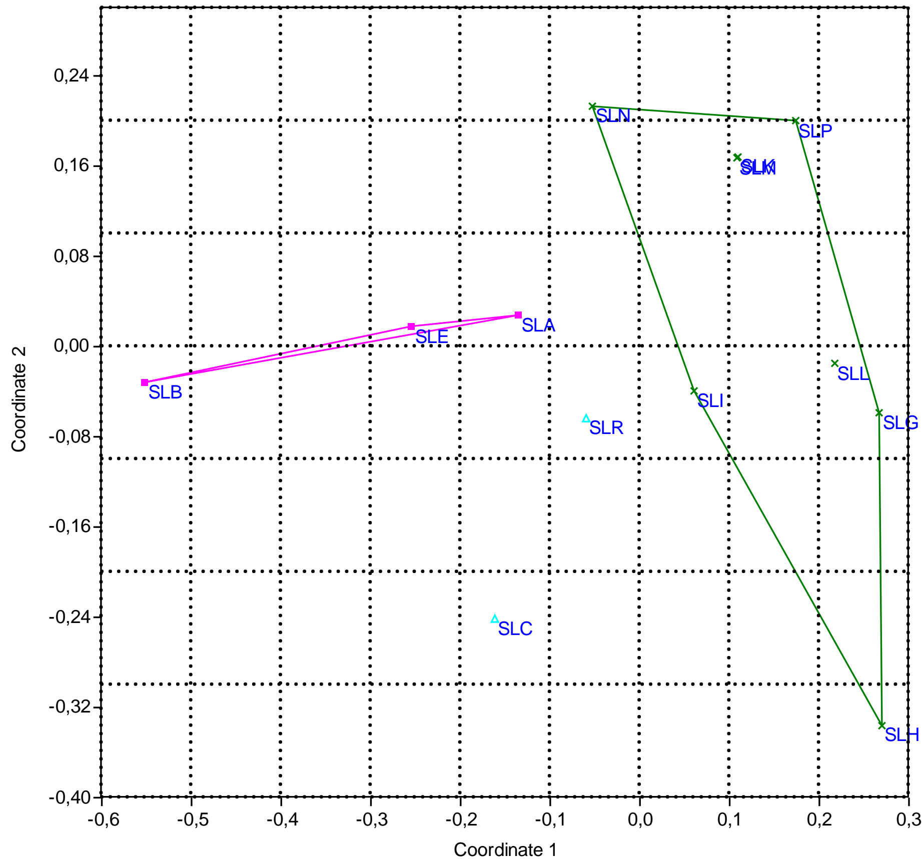

Figure 7. Non-metric multidimensional scaling (NMDS) ordination of fungal isolates from passalid beetles inhabiting three decaying trees en Ecuadorian Amazonia. Tree no. 1 (SLA, SLB and SLE), tree no. 2 (SLC and SLR) and tree no. 3 (SLG, SLH, SLI, SLK, SLL, SLM, SLN and SLP). See Appendix 1 for details regarding abbreviations.

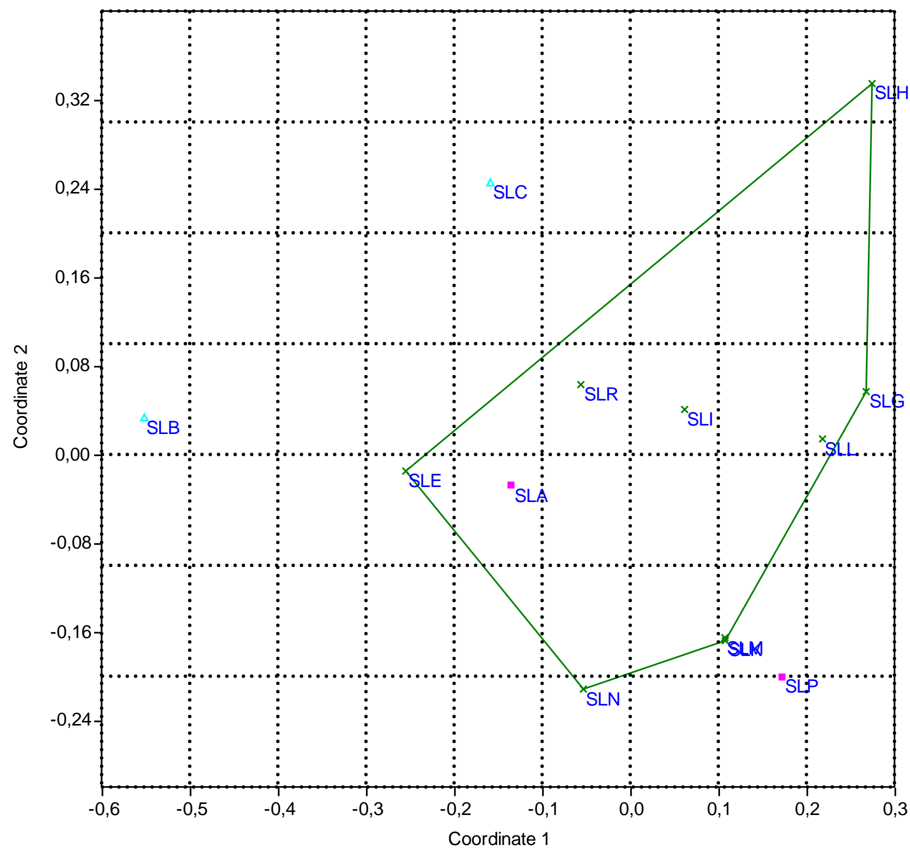

Figure 8. Non-metric multidimensional scaling (NMDS) ordination of fungi isolated from the guts of wood-inhabiting Passalidae beetle species *Passalus interstitialis* (SLA and SLP), *Passalus variiphyllus* (SLB and SLC) and *Passalus* sp. (SLE, SLG, SLH, SLI, SLK, SLL, SLM, SLN and SLR). See Appendix 1 for details regarding abbreviations.

5. TABLES

Table 1. Cellulase activities of extracts of fungi isolated from the guts of three Passalidae species beetles based on reducing sugar methodology.

Collection code	**Fungal species tested**	**Cellulase activity (umol/min)**	**Standard deviation**
CEQCA-O3783	*Clonostachys rossmaniae*	0.08	0.040
CEQCA-O3772	*Epicoccum nigrum*	0.07	0.01
CEQCA-O3778	*Trichoderma* sp.	0.06	0.00
CEQCA-O4922	*Trichoderma hamatum*	0.06	0.01
CEQCA-O4879	*Trichoderma spirale*	0.06	0.02
CEQCA-O3794	*Chaunopycnis alba*	0.04	0.01
CEQCA-O4929	*Trichoderma asperellum*	0.04	0.01
CEQCA-O3792	*Campylocarpon pseudofasciculare*	0.03	0.00
CEQCA-O4950	*Trichoderma virens*	0.03	0.00
CEQCA-O4991	*Scytalidium* sp.	0.03	0.00
CEQCA-O4898	*Trichoderma harzianum*	0.02	0.00
CEQCA-O4944	*Trichoderma atroviride*	0.02	0.00

Table 2. Cellulase activity of the gut of Passalidae beetles. Positive (+) cellulase activity and negative (-) cellulase activity

Passalidae field codes	**Passalidae species**	**Cellulase activity**
LV4AB	*Passalus* sp.	-
LV4BA	*Passalus* sp.	+
LV4BE	*Passalus* sp.	+
LV4CD	*Passalus* sp.	-
LV4FA	*Passalus interstitialis*	+
LV4HC	*Passalus* sp.	+

Table 3. Diversity indexes of fungi isolated and fungal diversity indexes according to each decaying tree.

	Fungal diversity indexes	**Tree no. 1 (*Inga spectabilis*)**	**Tree no. 2**	**Tree no. 3**
Taxa S	19	7	6	13
Individuals	98	20	13	65
Shannon	2.13	1.54	1.52	1.74
Fisher alpha	7.03	3.83	4.32	4.89

Table 4. Fungal diversity indexes according to each Passalidae species.

	Passsalus variiphyllus	***Passalus* sp.**	***Passalus interstitialis***
Taxa S	9	14	4
Individuals	21	64	13
Shannon	1.75	1.89	1.03
Fisher alpha	5.97	5.53	1.97

Table 5. Analysis of similarities (ANOSIM) of fungal composition according to decaying trees (R= 0.5177, P= 0.0058).

	Tree no. 1	**Tree no. 2**	**Tree no. 3**
Tree no. 1	-	0.40	0.01
Tree no. 2	0.40	-	0.04
Tree no. 3	0.01	0.04	-

Table 6. Analysis of similarities (ANOSIM) of fungi composition according to passalid species (R= 0.2816, P= 0.0977).

	Passalus interstitialis	*Passalus variiphyllus*	*Passalus* sp.
Passalus interstitialis	-	1.00	0.56
Passalus variiphyllus	1.00	-	0.06
Passalus sp.	0.56	0.06	-

Table 7. Similarity percentage (SIMPER) analysis shows fungal species that contributed the most (as a percentage) to the total dissimilarity within all three decaying trees.

Comparing decaying trees	**Dissimilarity %**	**Fungal species**	**Av. dissim**	**Contrib. %**	**Cumulative %**	**Mean abund. 1**	**Mean abund. 2**	**Mean abund. 3**
Tree no. 1, tree no. 2, tree no. 3	74.87	*Trichoderma harzianum*	19.20	25.65	25.65	0.67	2.00	4.13
		Trichoderma spirale	19.10	25.51	51.16	3.33	2.50	0.13
		Trichoderma virens	6.49	8.67	59.83	0.00	0.00	1.00
		Trichoderma atroviride	5.63	7.52	67.35	0.00	0.00	1.00
		Trichoderma hamatum	4.33	5.78	73.12	1.00	0.00	0.00
		Fusarium solani	3.84	5.13	78.25	0.00	0.50	0.50
		Trichoderma strigosum	2.28	3.05	81.29	0.67	0.00	0.00
		Clonostachys rossmaniae	2.05	2.73	84.02	0.33	0.00	0.00
		Mucor irregularis	1.80	2.41	86.43	0.00	0.50	0.13
		Epicoccum nigrum	1.51	2.02	88.45	0.00	0.50	0.00
		Trichoderma asperellum	1.51	2.02	90.47	0.00	0.50	0.00
		Campylocarpon pseudofasciculare	1.45	1.93	92.40	0.33	0.00	0.13
		Trichoderma sp. 2	1.14	1.52	93.92	0.33	0.00	0.00
		Chloridium virescens	1.13	1.51	95.43	0.00	0.00	0.25
		Xylogone ganodermophthora	0.90	1.20	96.63	0.00	0.00	0.25
		Scytalidium sp.	0.90	1.20	97.83	0.00	0.00	0.25
		Chaunopycnis alba	0.73	0.97	98.80	0.00	0.00	0.13
		Trichoderma sp. 1	0.45	0.60	99.40	0.00	0.00	0.13
		Bionectria ochroleuca	0.45	0.60	100.00	0.00	0.00	0.13

Table 8. Similarity percentage (SIMPER) analysis shows fungal species that contributed the most (as a percentage) to the total dissimilarity comparing within three decaying trees. Only fungal species showing more than 2% of contribution to dissimilarity were considered

Comparing decaying trees	**Dissimilarity %**	**Fungal species**	**Av. dissim**	**Contrib. %**	**Cumulative %**	**Mean abund. 1**	**Mean abund. 2**
Tree no. 1, tree no. 2	59.52	*Trichoderma spirale*	16.09	27.04	27.04	3.33	2.50
		Trichoderma harzianum	10.40	17.48	44.51	0.67	2.00
		Trichoderma hamatum	7.04	11.82	56.33	1.00	0.00
		Fusarium solani	5.21	8.75	65.09	0.00	0.50
		Trichoderma strigosum	3.67	6.17	71.25	0.67	0.00
		Clonostachys rossmaniae	3.37	5.66	76.91	0.33	0.00
		Epicoccum nigrum	3.36	5.64	82.55	0.00	0.50
		Trichoderma asperellum	3.36	5.64	88.19	0.00	0.50
		Mucor irregularis	3.36	5.64	93.83	0.00	0.50
		Campylocarpon pseudofasciculare	1.84	3.08	96.92	0.33	0.00
		Trichoderma sp. 2	1.84	3.08	100.00	0.33	0.00
Tree no. 1, tree no. 3	85.73	*Trichoderma harzianum*	24.47	28.55	28.55	0.67	4.13
		Trichoderma spirale	21.83	25.46	54.01	3.33	0.13
		Trichoderma virens	7.52	8.77	62.78	0.00	1.00
		Trichoderma hamatum	6.53	7.62	70.39	1.00	0.00
		Trichoderma atroviride	6.51	7.59	77.99	0.00	1.00
		Trichoderma strigosum	3.45	4.03	82.01	0.67	0.00
		Clonostachys rossmaniae	3.08	3.59	85.60	0.33	0.00
		Fusarium solani	2.89	3.37	88.97	0.00	0.50
		Campylocarpon pseudofasciculare	1.97	2.29	91.26	0.33	0.13
		Trichoderma sp. 2	1.73	2.01	93.28	0.33	0.00

Table 8. Continued

Comparing decaying trees	**Dissimilarity %**	**Fungal species**	**Av. dissim**	**Contrib. %**	**Cumulative %**	**Mean abund. 1**	**Mean abund. 2**
Tree no. 2, tree no. 3	*64.35*	*Trichoderma spirale*	*16.15*	*25.09*	*25.09*	*2.50*	*0.13*
		Trichoderma harzianum	*14.60*	*22.68*	*47.78*	*2.00*	*4.13*
		Trichoderma virens	*7.38*	*11.47*	*59.25*	*0.00*	*1.00*
		Trichoderma atroviride	*6.42*	*9.98*	*69.22*	*0.00*	*1.00*
		Fusarium solani	*4.75*	*7.39*	*76.61*	*0.50*	*0.50*
		Mucor irregularis	*3.14*	*4.88*	*81.49*	*0.50*	*0.13*
		Epicoccum nigrum	*3.09*	*4.80*	*86.30*	*0.50*	*0.00*
		Trichoderma asperellum	*3.09*	*4.80*	*91.10*	*0.50*	*0.00*
		Chloridium virescens	*1.29*	*2.01*	*93.10*	*0.00*	*0.25*

Table 9. Similarity percentage (SIMPER) analysis shows fungal species that contributed the most (as a percentage) to the total dissimilarity within all three Passalidae species.

Comparing Passalidae species	Dissimilarity %	Fungal species	Av. dissim	Contrib. %	Cumulative %	Mean abund. 1	Mean abund. 2	Mean abund. 3
P. interstitialis, P. variiphyllus, Passalus sp.	72.12	*Trichoderma spirale*	20.82	28.87	28.87	1.50	5.00	0.33
		Trichoderma harzianum	17.86	24.76	53.63	4.00	1.00	3.22
		Trichoderma virens	4.73	6.56	60.19	0.50	0.00	0.78
		Trichoderma atroviride	4.57	6.34	66.53	0.00	0.00	0.89
		Trichoderma hamatum	3.55	4.92	71.45	0.00	1.00	0.11
		Trichoderma strigosum	3.01	4.17	75.63	0.00	1.00	0.00
		Fusarium solani	2.90	4.01	79.64	0.00	0.00	0.56
		Mucor irregularis	2.01	2.79	82.43	0.00	0.50	0.11
		Epicoccum nigrum	1.81	2.51	84.94	0.00	0.50	0.00
		Trichoderma asperellum	1.81	2.51	87.45	0.00	0.50	0.00
		Chaunopycnis alba	1.74	2.41	89.87	0.50	0.00	0.00
		Campylocarpon pseudofasciculare	1.72	2.39	92.25	0.00	0.50	0.11
		Trichoderma sp. 2	1.51	2.09	94.34	0.00	0.50	0.00
		Chloridium virescens	0.94	1.30	95.64	0.00	0.00	0.22
		Clonostachys rossmaniae	0.85	1.18	96.82	0.00	0.00	0.11
		Xylogone ganodermophthora	0.76	1.06	97.88	0.00	0.00	0.22
		Scytalidium sp.	0.76	1.06	98.94	0.00	0.00	0.22
		Trichoderma sp. 1	0.38	0.53	99.47	0.00	0.00	0.11
		Bionectria ochroleuca	0.38	0.53	100.00	0.00	0.00	0.11

Table 10. Similarity percentage (SIMPER) analysis shows fungal species that contributed the most (as a percentage) to the total dissimilarity comparing within three Passalidae species. Only fungal species showing more than 2% of contribution to dissimilarity were considered.

Comparing Passalidae species	Dissimilarity %	Fungal species	Av. dissim	Contrib. %	Cumulative %	Mean abund. 1	Mean abund. 2
P. interstitialis, P. variiphyllus	69.68	*Trichoderma spirale*	19.31	27.71	27.71	1.50	5.00
		Trichoderma harzianum	18.76	26.93	54.64	4.00	1.00
		Trichoderma strigosum	5.51	7.90	62.54	0.00	1.00
		Trichoderma hamatum	5.51	7.90	70.44	0.00	1.00
		Epicoccum nigrum	3.31	4.75	75.19	0.00	0.50
		Trichoderma asperellum	3.31	4.75	79.94	0.00	0.50
		Mucor irregularis	3.31	4.75	84.70	0.00	0.50
		Campylocarpon pseudofasciculare	2.75	3.95	88.65	0.00	0.50
		Trichoderma sp. 2	2.75	3.95	92.60	0.00	0.50
		Chaunopycnis alba	2.58	3.70	96.30	0.50	0.00
		Trichoderma virens	2.58	3.70	100.00	0.50	0.00
P. interstitialis, Passalus sp.	62.82	*Trichoderma harzianum*	22.99	36.60	36.60	4.00	3.22
		Trichoderma spirale	14.54	23.15	59.75	1.50	0.33
		Trichoderma atroviride	5.71	9.08	68.83	0.00	0.89
		Trichoderma virens	5.58	8.88	77.71	0.50	0.78
		Fusarium solani	3.65	5.82	83.53	0.00	0.56
		Chaunopycnis alba	3.29	5.24	88.77	0.50	0.00

Table 10. Continued

Comparing Passalidae species	**Dissimilarity %**	**Fungal species**	**Av. dissim**	**Contrib. %**	**Cumulative %**	**Mean abund. 1**	**Mean abund. 2**
P. variiphyllus, *Passalus* sp.	81.96	*Trichoderma spirale*	27.44	33.48	33.48	5.00	0.33
		Trichoderma harzianum	12.52	15.28	48.75	1.00	3.22
		Trichoderma hamatum	5.54	6.76	55.51	1.00	0.11
		Trichoderma strigosum	5.46	6.67	62.18	1.00	0.00
		Trichoderma atroviride	4.45	5.43	67.61	0.00	0.89
		Trichoderma virens	4.37	5.33	72.94	0.00	0.78
		Epicoccum nigrum	3.29	4.02	76.96	0.50	0.00
		Trichoderma asperellum	3.29	4.02	80.98	0.50	0.00
		Mucor irregularis	3.27	3.99	84.97	0.50	0.11
		Fusarium solani	2.78	3.39	88.36	0.00	0.56
		Campylocarpon pseudofasciculare	2.75	3.36	91.72	0.50	0.11
		Trichoderma sp. 2	2.73	3.33	95.05	0.50	0.00

6. APPENDIXES

Appendix 1. Fungal groups according the Passalidae species from each decaying tree. Ninety-eight fungal species were identified.

Fungal group no.	Fungal group code	Passalidae field code	Passalidae species	Decaying tree type	CEQCA code	Fungi species isolated
1	SLA	LV1EA	*Passalus interstitialis*	Tree no. 1	CEQCA-O4877	*Trichoderma spirale*
				(*Inga spectabilis*)	CEQCA-O4924	*Trichoderma harzianum*
					CEQCA-O4878	*Trichoderma spirale*
					CEQCA-O4879	*Trichoderma spirale*
2	SLB	LV1DA, LV1DB	*Passalus variiphyllus*	Tree no. 1	CEQCA-O4982	*Trichoderma hamatum*
				(Inga spectabilis)	CEQCA-O3779	*Trichoderma spirale*
					CEQCA-O3771	*Trichoderma spirale*
					CEQCA-O4969	*Trichoderma spirale*
					CEQCA-O4970	*Trichoderma spirale*
					CEQCA-O3792	*Campylocarpon pseudofasciculare*
					CEQCA-O4961	*Trichoderma* sp.2
					CEQCA-O3400	*Trichoderma strigosum*
					CEQCA-O4983	*Trichoderma spirale*
					CEQCA-O4920	*Trichoderma strigosum*
					CEQCA-O4921	*Trichoderma hamatum*
					CEQCA-O3784	*Trichoderma spirale*
3	SLC	LV3AA	*Passalus variiphyllus*	Tree no. 2	CEQCA-O3772	*Epicoccum nigrum*
					CEQCA-O4882	*Trichoderma spirale*
					CEQCA-O4883	*Trichoderma spirale*

Appendix 1. Continued

Fungal group no	Fungal group code	Passalidae field code	Passalidae species	Decaying tree type	CEQCA code	Fungi species isolated
					CEQCA-O3775	*Trichoderma spirale*
					CEQCA-O3776	*Trichoderma harzianum*
					CEQCA-O4918	*Trichoderma harzianum*
					CEQCA-O4928	*Mucor irregularis*
					CEQCA-O4929	*Trichoderma asperellum*
					CEQCA-O4971	*Trichoderma spirale*
4	SLE	LV1DC	*Passalus* sp.	Tree no. 1	CEQCA-O4922	*Trichoderma hamatum*
				(*Inga spectabilis*)	CEQCA-O3783	*Clonostachys rossmaniae*
					CEQCA-O4931	*Trichoderma harzianum*
					CEQCA-O4962	*Trichoderma spirale*
5	SLG	LV4AC, LV4AD,	*Passalus* sp.	Tree no. 3	CEQCA-O4884	*Trichoderma atroviride*
		LV4AE, LV4CA,			CEQCA-O3789	*Trichoderma harzianum*
		LV4CC, LV4HB,			CEQCA-O4923	*Trichoderma harzianum*
		LV4IA			CEQCA-O4932	*Trichoderma harzianum*
					CEQCA-O3790	*Trichoderma atroviride*
					CEQCA-O4988	*Chloridium virens*
					CEQCA-O4935	*Trichoderma virens*
					CEQCA-O3787	*Trichoderma virens*
					CEQCA-O4886	*Trichoderma harzianum*
					CEQCA-O3791	*Fusarium solani*

Appendix 1. Continued

Fungal group no	Fungal group code	Passalidae field code	Passalidae species	Decaying tree type	CEQCA code	Fungi species isolated
6	SLH	LV4BC, LV4BD, LV4CB, LV4HA, LV4HD, LV4IB, LV4IC	*Passalus* sp.	Tree no. 3	CEQCA-O4887	*Trichoderma harzianum*
					CEQCA-O3777	*Bionectria ochroleuca*
					CEQCA-O4890	*Scytalidium* sp.
					CEQCA-O4991	*Scytalidium* sp.
					CEQCA-O4973	*Mucor irregularis*
					CEQCA-O3778	*Trichoderma* sp.1
					CEQCA-O4891	*Trichoderma harzianum*
					CEQCA-O4936	*Trichoderma atroviride*
					CEQCA-O4892	*Trichoderma harzianum*
					CEQCA-O4937	*Fusarium solani*
					CEQCA-O4963	*Chloridium virens*
					CEQCA-O4939	*Xylogone ganodermophthora*
					CEQCA-O4964	*Xylogone ganodermophthora*
					CEQCA-O3774	*Fusarium solani*
					CEQCA-O4968	*Trichoderma harzianum*
					CEQCA-O4893	*Trichoderma harzianum*
					CEQCA-O3781	*Campylocarpon pseudofasciculare*
					CEQCA-O4895	*Trichoderma harzianum*

Appendix 1. Continued

Fungal group no	Fungal group code	Passalidae field code	Passalidae species	Decaying tree type	CEQCA code	Fungi species isolated
7	SLI	LV4AB, LV4BA	*Passalus* sp.	Tree no. 3	CEQCA-O4975	*Trichoderma spirale*
					CEQCA-O4942	*Trichoderma harzianum*
					CEQCA-O4943	*Trichoderma harzianum*
					CEQCA-O4944	*Trichoderma atroviride*
					CEQCA-O4976	*Trichoderma virens*
					CEQCA-O4946	*Trichoderma atroviride*
					CEQCA-O4977	*Trichoderma virens*
					CEQCA-O4948	*Trichoderma atroviride*
					CEQCA-O4897	*Trichoderma atroviride*
8	SLK	LV4CD	*Passalus* sp.	Tree no. 3	CEQCA-O4898	*Trichoderma harzianum*
					CEQCA-O4899	*Trichoderma harzianum*
					CEQCA-O4912	*Trichoderma virens*
					CEQCA-O4978	*Trichoderma harzianum*
					CEQCA-O4949	*Trichoderma harzianum*
					CEQCA-O4979	*Trichoderma harzianum*
9	SLL	LV4BE, LV4IE	*Passalus* sp.	Tree no. 3	CEQCA-O4950	*Trichoderma virens*
					CEQCA-O4951	*Trichoderma harzianum*
					CEQCA-O4952	*Trichoderma harzianum*
					CEQCA-O4953	*Trichoderma atroviride*
					CEQCA-O4901	*Trichoderma harzianum*

Appendix 1. Continued

Fungal group no	Fungal group code	Passalidae field code	Passalidae species	Decaying tree type	CEQCA code	Fungi species isolated
					CEQCA-O4989	*Fusarium solani*
10	SLM	LV4HC, LV4ID	*Passalus* sp.	Tree no. 3	CEQCA-O4902	*Trichoderma harzianum*
					CEQCA-O4913	*Trichoderma harzianum*
					CEQCA-O3786	*Trichoderma harzianum*
					CEQCA-O4954	*Trichoderma virens*
11	SLN	LV4BB	*Passalus* sp.	Tree no. 3	CEQCA-O4955	*Trichoderma harzianum*
					CEQCA-O4904	*Trichoderma harzianum*
					CEQCA-O4980	*Trichoderma harzianum*
12	SLP	LV4DA, LV4FA, LV4GA	*Passalus interstitialis*	Tree no. 3	CEQCA-O4956	*Trichoderma harzianum*
					CEQCA-O4916	*Trichoderma harzianum*
					CEQCA-O4906	*Trichoderma harzianum*
					CEQCA-O3794	*Chaunopycnis alba*
					CEQCA-O4915	*Trichoderma virens*
					CEQCA-O4908	*Trichoderma harzianum*
					CEQCA-O3793	*Trichoderma harzianum*
					CEQCA-O4959	*Trichoderma harzianum*
					CEQCA-O4909	*Trichoderma harzianum*
13	SLR	LV3C	*Passalus* sp.	Tree no. 2	CEQCA-O3773	*Trichoderma spirale*
					CEQCA-O4960	*Trichoderma harzianum*
					CEQCA-O4911	*Trichoderma harzianum*
					CEQCA-O4990	*Fusarium solani*

Appendix 2. Internal transcribed spacer (ITS) annotated and referenced DNA sequences of fungal species used for phylogenetic analysis.

Fungal species	Phylum	GenBank accession No.
Campylocarpon pseudofasciculare	Ascomycota	JX521869
Campylocarpon pseudofasciculare	Ascomycota	KF447567
Chaunopycnis alba	Ascomycota	JN903552
Chloridium virescens	Ascomycota	JX684009
Clonostachys rossmaniae	Ascomycota	KM357316
Clonostachys rossmaniae	Ascomycota	KC806299
Epicoccum nigrum	Ascomycota	KF512824
Epicoccum nigrum	Ascomycota	KC311470
Fusarium solani	Ascomycota	KP326582
Fusarium solani	Ascomycota	KF880406
Fusarium solani	Ascomycota	JN983014
Fusarium solani	Ascomycota	KP132233
Hypomyces subiculosus	Ascomycota	FN859452
Kluyveromyces waltii	Ascomycota	AY046208
Scytalidium cuboideum	Ascomycota	GQ272630
Trichoderma asperellum	Ascomycota	LC123613
Trichoderma atroviride	Ascomycota	KT278863
Trichoderma atroviride	Ascomycota	KJ010952
Trichoderma atroviride	Ascomycota	KR909150
Trichoderma atroviride	Ascomycota	KJ783312
Trichoderma atroviride	Ascomycota	KU319048
Trichoderma hamatum	Ascomycota	JF303861
Trichoderma hamatum	Ascomycota	DQ248967
Trichoderma hamatum	Ascomycota	FJ461581
Trichoderma harzianuum	Ascomycota	KT852833
Trichoderma harzianuum	Ascomycota	AY154949
Trichoderma harzianuum	Ascomycota	KT852833
Trichoderma harzianuum	Ascomycota	KT852806
Trichoderma spirale	Ascomycota	KT278908
Trichoderma spirale	Ascomycota	KC602342
Trichoderma spirale	Ascomycota	KT278897
Trichoderma spirale	Ascomycota	NR077177
Trichoderma spirale	Ascomycota	KT278897
Trichoderma strigosum	Ascomycota	NR103571
Trichoderma strigosum	Ascomycota	DQ083027
Trichoderma strigosum	Ascomycota	EU718074
Trichoderma strigosum	Ascomycota	EU718073
Trichoderma virens	Ascomycota	KT336516
Trichoderma virens	Ascomycota	KP009289

Appendix 2. Continued

Fungal species	Phylum	GenBank accession No.
Trichoderma virens	Ascomycota	KT278905
Xylogone ganodermophthora	Ascomycota	GQ272612
Xylogone ganodermophthora	Ascomycota	KF925449
Mucor irregularis	Zygomycota	JX976247

Appendix 3. Taxonomic identification of passalid's gut fungi. Phylogeny based on maximum likehood

Phylum	Class	Order	Family	Species	CEQCA code	Number of isolates
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Epicoccum nigrum*	CEQCA-O3772	1
Ascomycota	Leotiomycetes	Helotiales	Leotiomycetidae	*Scytalidium* sp.	CEQCA-O4890, CEQCA-O4991	2
Ascomycota	Leotiomycetes	Helotiales		*Xylogone ganodermophthora*	CEQCA-O4939, CEQCA-O4964	2
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Clonostachys rossmaniae*	CEQCA-O3783	1
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma asperellum*	CEQCA-O4929	1
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma hamatum*	CEQCA-O4982,CEQCA-O4921, CEQCA-O4922	3

Appendix 3. Continued

Phylum	Class	Order	Family	Species	CEQCA code	Number of isolates
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma harzianum*	CEQCA-O4924, CEQCA-O4931, CEQCA-O3776, CEQCA-O4918, CEQCA-O4960, CEQCA-O4911, CEQCA-O3789, CEQCA-O4923, CEQCA-O4932, CEQCA-O4886, CEQCA-O4887, CEQCA-O4891, CEQCA-O4892, CEQCA-O4968, CEQCA-O4893, CEQCA-O4895, CEQCA-O4942, CEQCA-O4943, CEQCA-O4898, CEQCA-O4899, CEQCA-O4978, CEQCA-O4949, CEQCA-O4979, CEQCA-O4951, CEQCA-O4952, CEQCA-O4901, CEQCA-O4902, CEQCA-O4913, CEQCA-O3786, CEQCA-O4955, CEQCA-O4904, CEQCA-O4980, CEQCA-O4956, CEQCA-O4916, CEQCA-O4906, CEQCA-O4908, CEQCA-O3793, CEQCA-O4959, CEQCA-O4909	39

Appendix 3. Continued

Phylum	Class	Order	Family	Species	CEQCA code	Number of isolates
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma atroviride*	CEQCA-O4884, CEQCA-O3790, CEQCA-O4936, CEQCA-O4944, CEQCA-O4946, CEQCA-O4948, CEQCA-O4897, CEQCA-O4953	8
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma* sp. 1	CEQCA-O3778	1
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma* sp. 2	CEQCA-O4961	1
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma spirale*	CEQCA-O4877, CEQCA-O4878, CEQCA-O4879, CEQCA-O3779, CEQCA-O3771, CEQCA-O4969, CEQCA-O4970, CEQCA-O4983, CEQCA-O3784, CEQCA-O4962, CEQCA-O4882, CEQCA-O4883, CEQCA-O3775, CEQCA-O4971, CEQCA-O3773, CEQCA-O4975	16
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma strigosum*	CEQCA-O3400, CEQCA-O4920	3

Appendix 3. Continued

Phylum	**Class**	**Order**	**Family**	**Species**	**CEQCA code**	**Number of isolates**
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma virens*	CEQCA-O4935, CEQCA-O3787, CEQCA-O4976, CEQCA-O4977, CEQCA-O4912, CEQCA-O4950, CEQCA-O4915	8
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Campylocarpon pseudofasciculare*	CEQCA-O3792, CEQCA-O3781	2
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium solani*	CEQCA-O4990, CEQCA-O3791, CEQCA-O4937, CEQCA-O3774, CEQCA-O4989	5
Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	*Chaunopycnis alba*	CEQCA-O3794	1
Ascomycota	Sordariomycetes	Sordariales	Chaetosphaeriaceae	*Chloridium virescens*	CEQCA-O4988, CEQCA-O4963	2
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	*Bionectria ochroleuca*	CEQCA-O3777	1
Zygomycota	Mucoromycotina	Mucorales	Mucoraceae	*Mucor irregularis*	CEQCA-O4928, CEQCA-O4973	2

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