Université catholique de Louvain

Faculté des bioingénieurs

Earth and Life Institute

Pole of Applied Microbiology

Laboratory of Mycology

Diversity of saprophytic fungi in the rhizoplane of plants communities naturally recolonizing oil ponds in the Amazonian areas

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Louvain-La-Neuve, Janvier 2018

Acknowledgement

I would like to express my gratitude to my co-promotor Dr. Cony Decock who gave me this opportunity and guided me during my studies. I would not have been able to complete my research and achieve each one of my goals without his help. Thanks you for your supervision, direction, useful comments and engagement through the process to accomplish this PhD.

I would like to sincerely thank to my promotor Prof. Stéphane Declerck, for his good advices and encouragements, for having me in his lab, where I had the opportunity to work with very qualified personnel, and to improve my knowledge.

My special thanks go to Mrt. Veronica Luna, Dra. Josefina Egas and Dr. Grégoire Hennerbert for having gotten me started in mycology and for give me the opportunity to work in their projects. Thank you so much for your support, your confidence in me and your continuous encouragements.

I also gratefully acknowledge the financial support received from the CIUF/CUD (currently ARES, Académie de Recherche et d'Enseignement Supérieur Wallonie-Bruxelles, Commission de la Coopération pour le Dévelopment) through the PIC Project. Thanks for providing the financial means and laboratory facilities to complete this research. Thanks are also addressed to the people involved in the projects PUCE with the financial support received from PUCE when working in Ecuador.

I sincerely thank the thesis jury members for their wise advises to improve this document.

I would like to express my gratitude to the people of Laboratory of Mycology at UCL and to the people in the Carrera de Microbiología at the PUCE, who have willingly shared their precious time and knowledge with me during the research process.

Finally, I would like to thank my family, particularly to my husband, for his non-stop encouragement and their strong support. My friends who helped me along the course of my doctoral studies by giving encouragement and providing the moral and emotional support I needed to complete my thesis. I am very grateful to all everyone that in a way or another were constantly around me during the last five years.

Table of content

List of abbreviations

28S	28S ribosomal RNA is the structural ribosomal RNA (rRNA) for the large component, or large subunit (LSU)
Act	Actin
ANOVA	ANalysis Of VAriance
ARES	Académie de Recherche et d'Enseignement Supérieur Wallonie-Bruxelles
BC	Consensus tree
BCCM/MUCL	Belgian Co-ordinated Collections of Microorganisms/ Mycothèque de l'Université Catholique de Louvain
BH	Bushnell-Haas broth
BI	Bayesian Inference
BLA	Banana Leaf Agar
BLAST	Basic Local Alignment Search Tool
BPP	Posterior probability
BS	Bootstrap support
BSP	Parsimony Bootstrap support
CSC	Consolidated Species Concept
CBS	Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
CESAQ	Centro de Servicios Ambientales y Químicos
CFU	Colony Forming Unit
CHS	Chitin synthase
CIUF	Commission de la Coopération pour le Dévelopment
Cmd	Calmodulin
D1/D2	Variable domain of the 28S rRNA gene
DSF	Dark Septate Fungi
DCPIP	Redox indicator 2, 6-dichlorophenol indophenol
DIC	Differential interference contrast
DNA	Deoxyribose Nucleic Acid
dNTPs	Deoxyribonucleotide triphosphate
DW	Dry Weight
ELIM	Earth and Life Institute
et al.	et alii, Latin expression meaning "and others"
Fig.	Figure

FRFC	Fonds de la Recherche Fondamentale Collective
g	Gram(s)
GAMMA	Gamma distribution
GC	Gas Chromatography Gas
GCPSR	Genealogical Concordance Phylogenetic Species Recognition
gen.	Genus
GTR	General time reversible model
h3	Histone
HTUs	Hypothetical taxonomic units
i.e.	id est, Latin expression meaning "that is" or “namely”
INIFAT	Instituto de Investigaciones Fundamentales en Agricultura Tropical “Alejandro de Humboldt”
ITS	Internal Transcribed Spacer
LSU	The large subunit
MAE	Ministerio Ambiente del Ecuador
MAFFT	Multiple Alignment using Fast Fourier Transform
MCMC	Markov chain Monte Carlo
MEA	Malt Extract Agar
min	Minutes
ml	Mililiter(s)
ML	Maximum likelihood
MLBS	Maximum likelihood bootstrap
MLST	Multilocus sequence typing
mm	Milimeter(s)
MP	Maximum Parsimony
MPT	Most parsimonious trees
MYPD	0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1% dextrose, 20 g. agar, Difco
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
OA	Oatmeal Agar
ºC	Celsius degrees
OTUs	Operational Taxonomic Units
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PEPDA	Proyecto de eliminación de pasivos en el Distrito

	Amazónico (project for the elimination of liabilities in the amazon district)
PhyDE-1	Phylogenetic Data Editor
PP	Posterior probabilities
PRIPA	Plan de restauración integral de Pasivos Ambientales (Environmental Restoration Plan)
PUCE	Pontificia Universidad Católica del Ecuador
RAPD	Random Amplified Polymorphic DNA
RAxML	Randomized Axelerated Maximum Likelihood
RFLPs	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RPB2	Fragment of the second-largest subunit of polymerase II gene
rRNA	Ribosomal Ribonucleic Acid
SNA	Synthetic Nutrient-Poor agar
sp. nov.	species nova, Latin expression meaning "new species"
sp., spp.	Specie, Species
SSU	Small subunit
T	Type strain
TEF1α	Elongation factor 1-alpha
TN93	Tamura-Nei
TPHs	Total Petroleum Hydrocarbons
tub	ß-tubulin
µL	Microliter
µm	Micrometer

Summary

Oil industry is of outmost importance for the Ecuadorian economy and the environment. It generates important incomes but also major aquatic and terrestrial pollution generated in biologically rich ecosystems, affecting the local biological diversity. Pollution takes many forms of which crude oil "pools" is the most directly visible. Numerous oil ponds are registered and mapped in the eastern Amazonian region. Most of these ponds already dated back from the 70^{ies}, and with time, natural regeneration has taken place with the development of native plants communities.

Therefore, in order to fully understand these events of re-colonization of heavily oil-polluted environments, it is important to study more deeply the root system, which develops into highly contaminated organic material. This includes also the fungal component of the rhizosphere, that comprise both symbiotic, saprophytes and pathogenic species (from the external rhizoplane or endophytic). The rhizosphere represents an important niche / ecological system centred on the very near root environment. A large number of fungal strains remain to be explored in terrestrial ecosystem. Hence, focusing on the diversity of fungi in hydrocarbon-polluted soils may represent a straightforward approach to isolate potential novel species with possible degrading capacities.

We aimed to evaluate the diversity of the root endophytic and saprotrophic rhizoplane fungi associated to various

herbaceous plants growing in these Amazonian oil ponds. To achieve this objective, a culture dependent approach of the fungal diversity was applied to the rhizoplane and the root internal tissue (endophytes). For a more in depth taxonomic studies the Consolidated Species Concept (CSC), combining phylogenetic, morphological, and ecological species concepts (Quaedvlieg *et al.*, 2014; Bakhshi *et al.*, 2015) was applied to selected genera.

To assess *in-vitro* the ability of the isolated fungal strains to grow in presence of and to degrade oil molecule (hydrocarbons) was a second objective. To achieve this objective, *in-vitro* screening procedures were carried out on solid and liquid medium in order to test the grow of a selection of representative fungal strains. On the basis of the results of the first screening, a subset of strains (i.e. the best growing) was selected to test in liquid medium their ability to degrade jet fuel.

1077 strains have been obtained in pure culture from the rhizoplanes and internal root tissues of 208 herbaceous plants recolonizing two oil ponds. 779 strains were isolated from the rhizoplane and 298 from the internal tissue of roots.

In order to apply the Consolidate Species Concept, three generic complexes within the Hypocreales were selected for cases studies: *Myrothecium*, *Cylindrocarpon* and *Gliocladiopsis* / *Aquanectria* (sub-section1.2.1–1.2.3). As result of this study, several undescribed species were addressed*: Inaequalispora longiseta* sp. nov., *I. cylindrospora* sp. nov. and *Parvothecium amazonense* sp. nov. were described and illustrated. *Digitiseta* gen. nov., was recognized, typified by *D. setiramosa* comb. nov. *Digitiseta dimorpha* comb. nov. was also proposed, and the new

species *D. parvodigitata* sp. nov. and *D. multidigitata* sp. nov. were described (Gordillo & Decock, 2017). *Dactylonectria amazonica* sp. nov., *D. ecuadoriense* sp. nov., *D. polyphaga* sp. nov., *D. palmicola* sp. nov. and *Campylocarpon amazonense* sp. nov. also were described and illustrated.

Four new species of *Aquanectria* and three new species of *Gliocladiopsis* were described as *Gliocladiopsis ecuadoriensis, G. hennebertii* and *G. singaporiensis*. Finally, some *Aquanectria* species were proposed as new *A. filiformis*, *A. devians*, *A. tenuispora* and *A. tenuissima.*

Our screening procedure looked *in-vitro* at the ability of our strains to grow in presence of and to degrade oil molecule (hydrocarbons). Our results showed that fifteen genera shown some level of tolerance, in solid medium, and also presented the ability to use hydrocarbon in liquid medium. In this study the removal ratio of TPHs in liquid medium achieve by *Trichoderma atroviride* MUCL 54742, *Dichobotrys abundans* MUCL 54589, and *Mucor irregularis* MUCL 54578 reflected significantly higher degradation activity relative to the control. *Trichoderma atroviride* MUCL 54742 exhibited the highest removal ratio.

Author's contribution

The work presented here was strictly realized during the time course of my PhD.

The **Introduction, state of the art, General Discussion, Conclusions** and **Perspectives** were written by myself and not published elsewhere.

Chapter 1: My contribution to this chapter was estimated 70%. The field sampling in Ecuador and identification of the strains in the collection were performed with the help of PhD. Cony Decock (PIC, project partner from BCCM/MUCL), Mtr. Veronica Luna (PIC, project partner from PUCE), Mónica Garces (PhD student UCL), Lic. Gabriela Vargas (Technician from PUCE), María Fernanda Davila, Nicole Sotomayor (undergraduate in PUCE).

The molecular work with the strains and the morphological analysis was performed by myself. The data analysis and writing of the three papers were done by myself.

Chapter 2: My contribution to this chapter was estimated 80%. The experiments as well as data collection were conducted with the help of Lic. Cesar Yaguana (Technician from PUCE), Msc. Elisa Jara (Chemical Technician from PUCE). The data analysis with SPSS 24 was performed with the help of Aurelie Bertrand ("Support en Méthodologie et Calcul Statistique", UCL). The writing of this chapter was done by myself.

I. INTRODUCTION

About 120000 species of fungi have been described to date (Hawksworth, 2001; Hawksworth & Lücking, 2017). However, this is very likely a very low figure. In the last 10 years, several studies have tried to estimate the "actual" number of fungal species on Earth. The very first study dates back from Hawksworth (2001), who estimated about 1.5 million species. This resulted from a worldwide extrapolation of an estimated ratio of the number of plant / number of Fungi of 1 / 6 calculated in the United Kingdoms (Hawksworth, 2001). The most recent estimate is of 3.8 million fungal species (Hawksworth & Lücking 2017); it results from the same extrapolation based on an updated plant / fungus ratio of 1 / 9.8. Other figures, based on the ITS-DNA sequence diversity of the soil fungal and the vascular plant richness, range from 3.5 to 5.1 million (Brien *et al.*, 2005; Blackwell, 2011). Hence, we only know likely less than 5 % of the estimated fungal taxonomic diversity (Hawksworth & Rossman, 1997). The Fungi or the Mycota represents one of the least-explored kingdoms of our planet (Webster & Weber, 2007).

In the last 20 years, sequencing of individual genes first (the Sanger sequencing) and more recently the next-generation sequencing (NGS) or mass sequencing also known as metagenomics (Samson *et al.*, 2010) allowed developing alternative approaches to assess the fungal diversity (Taylor & Berbee, 2014). These new molecular-based approaches yielded many so-called Operational Taxonomic Units (OTUs) that, in most cases, represent distinct species that remains unidentified in the Linnaean system because of the lack of accurately identified reference sequences.

This issue was illustrated recently with the discovery of *Bifiguratus adelaidae* new early member in the lineage of Mucoromycotina (Torres-Cruz *et al.*, 2017). *Bifiguratus adelaidae* was indeed as one of these OTUs inhabiting soil and abundantly shown by metagenomic approach (James & Seifert, 2017). It was once listed as one of the 50 “most wanted fungiThis list emphases the fact that many Fungal OTUs repeatedly shown by metagenomic approaches in environmental DNA are totally unknown as far as their morphology, reproduction and, perhaps more importantly, physiology are concerned, and ahead, their role in ecosystem processes (Nilsson *et al.*, 2016). *Bifiguratus adelaidae* was one of these fungi for which nothing was know in term of morphology, reproduction, and physiology. Its discovery, allowing a better characterization, was made possible thanks to new high-throughput sampling strategies (Torres-Cruz *et al.*, 2017).

For mycologists, documenting undescribed species still is an important task to understand fungal communities and ecosystem functioning. The recognition of species and the Linnaean scheme provide the key knowledge on the biology, distribution, ecology, host range, and control of fungal pathogens or others, associated to industrial process (Crous *et al.*, 2015; James & Seifert, 2017).

The species is the basic unit of biological classification. The species is a hypothesis, which is to be tested when new data are available (Shenoy *et al.*, 2007). The species concept has been regarded from different points of view. Currently, four concepts are commonly addressed: *Morphological*, *Ecological*, *Biological* and *Phylogenetic* (Shenoy *et al.*, 2007).

Morphological Species Concepts is based “on morphological

characteristic" (Shenoy *et al.*, 2007), which, in the case of Fungi, are mostly reduced to the reproductive structure, asexual or sexual. Quantitative / Qualitative morphological characters still are central to descriptions of morphological species. Morphological differences may be often subtle; if fungal speciation is determined by physiological adaptation, there may be little or no selection pressure for qualitative or quantitative changes in morphology.

An accurate morphological description needs the background of a qualified taxonomist because of the complexity of differentiating numerous species with poorly differentiated morphology. Furthermore, for many species, and this are more obvious for the belowground, soil-borne fungi, the observation of the reproductive structures often requires an *in-vitro* cultivation step. However, still, this is not a guarantee of identification because many species do not produce reproductive structures in *in-vitro* cultures (sterile mycelia) (Wang *et al.*, 2009; Seifert *et al.*, 2011).

Biological Species Concepts, "are reproducing populations that are reproductively isolated from other populations" (Shenoy *et al.*, 2007). This approached is not so commonly used in mycology, because less than 15% of fungi can be cultivated and it is also difficult to find opposite mating type allowing testing the biological species concept (Taylor *et al.*, 2006).

In the *Phylogenetic Species Concepts*, "species should represent a monophyletic group of individuals that share at least one uniquely derived character that descends from a common ancestor" (Shenoy *et al.*, 2007). *Phylogenetic* Species recognition has been widely used, even for uncultivable species (Taylor *et*

al., 2006).

Phylogenetic inferences analysis of one, appropriately polymorphic, loci has been used to avoid the inherent problems above mentioned. Good genetic markers should present enough variability allowing species identification with low level of intraspecific variation. It is important to have reference datasets for comparison of the new, unidentified sequences.

The most widely used DNA regions is the ribosomal DNA (rDNA) operon (White *et al.*, 1990; O'Donnell *et al.*, 2009). The ribosomal DNA operon is divided into several translated regions, *viz*. 18S, 5.8S and 28S. The internal transcribed spacer regions (ITS), comprises two segments, ITS1 and ITS2, which join the 18S with 5.8S and the 5.8 S with 28S.

The small subunit 18S (SSU) ribosomal operons of fungi have been studied extensively. It is highly conserved and is not suitable for species boundaries delineations and diagnostics in fungi. The large subunit 28S (LSU) is used in numerous studies, particularly in yeast. It is also rather conserved but for a variable region known as the D1-D3 domain. 18S and 28S evolve relatively slowly, and could be used in studies of higher-order relationships (families and orders).

The ITS gene region has, comparatively to the translated regions, a higher evolutionary rate and constitute a pertinent tool to delimit species. This region has been proposed as the prime tool for the fungal barcode. Nonetheless, the resolution could be still insufficient for species identification in species rich complex, as, for example, the Aspergilli, penicilli, fusari, or the trichodermes. Such genera require the use of other sequences, generally from house-keeping, protein coding genes (Crous *et al.*,

2009; Samson *et al.*, 2010). Proteins coding genes often contains introns, a non-coding, not translated region uniting exons. Introns are more variable that the coding, translated exons, which make them a good target for species identifications.

The commonly used proteins coding genes included elongation factor 1-alpha (*TEF1*α), calmodulin (*Cmd*), actin (*Act*), histone *h3*, ß-tubulin (*tub*), chitin synthase (*CHS*), the second-largest and subunit of polymerase II (*RPB2*), etc. These genes, especially in their introns, have a higher inter-species variability than the ITS regions. These are more useful for elucidate the taxonomy of complex genera (O'Donnell *et al.*, 2009; Lombard *et al.*, 2010a, 2015, 2016; Bakhshi *et al.*, 2015).

For phylogenetic reconstruction, traditional approaches such as neighbor-joining algorithm (distance based) and parsimony (based on preserved character) were used for long time. Nowadays, model-based methods such as Maximum Likelihood and Bayesian inferences are widely used thanks to the improvement of computer power and software implementations (Yang & Rannala, 2012; Raja *et al.*, 2017).

Maximum Parsimony (MP) philosophy is that the most likely explanation for the evolution is the simplest one. When a reasonable number of topologies have been generated, the tree that needs the minimum number of changes is selected as the maximum parsimony tree. For a given set of data, more than one "optimal" or "most parsimonious tree" can be obtained (Lemey *et al.*, 2009).

The Maximum likelihood (ML) method used a specific

evolutionary model and a statistical criterion because; it contemplates the probability that a tree gave rise to the observed data. The tree with the highest probability is the most likely tree (Lemey *et al.*, 2009). Felsenstein (1981) was one of the first to use this approach for phylogenetic estimation via DNA sequence data (Raja *et al.*, 2017). The advantage for using this method over distance or parsimony methods is to understand the process of sequence evolution. However, this type of analysis request strong computational capacities (Raja *et al.*, 2017).

In order to determine the robustness of the trees obtained from MP and ML statistical tests, bootstrap analysis is used. The results of the bootstrap analysis are expressed as percentage that provides support for the branches of the tree. When the value is greater than or equal to 70%, the branch is statistically supported (Alfaro *et al.*, 2003).

Bayesian inference relies on Bayes's theorem, which states that the posterior probability is proportional to the prior information plus the data information. Bayesian inference seeks the tree that maximizes the probability of the tree given the data and model of sequence evolution (Raja *et al.*, 2017). Bayesian inference has increased its popularity thanks to advances in computational methods, especially Markov Chain Monte Carlo algorithms (MCMC) which explore regions of the tree "space" in proportion of their posterior probabilities (Lemey *et al.*, 2009; Yang & Rannala, 2012).

In spite of the different species recognition criteria, sequence-based methods in fungal taxonomy are now the gold standard for consolidated a taxonomic entity. The criterion first

was proposed by Taylor (2000), who introduced the Genealogical Concordance Phylogenetic Species Recognition (GCPSR), “a multigene phylogenetic approach for recognizing fungal species on the basis of genealogical concordance”. The use of this concordance between unlinked genes indicates the absence of genetic exchange and an evolutionary independence between the lineages (Crous *et al.*, 2015). This is the base of the Phylogenetic Species Concept.

Ecological Species Concepts “is based on species that occupies an adaptive zone that evolved separately from all lineages outside its range” (Shenoy *et al.*, 2007). Ecology (substrate, habitat), in the context of defining taxonomic entities, is little considered. Nonetheless, the host relationships could help for taxonomical studies. Today, molecular tools allow evidencing more and more so-called cryptic specie, which, *a posteriori*, provided us some evidences of species specific ecology, such as host, niche or habitat.

Ecological niche plays an important role in the species recognitions; the adaptations to particular substrates, temperature conditions, and competition with other microbes probably help to the speciation and cohesion of the species (natural selection). Ecological adaptations are important clues to the process of speciation, this argument is used to define a species (Jayasiri *et al.*, 2015).

Quaedvlieg *et al.* (2014) introduced the Consolidated Species Concept (CSC) that defines species through a polyphasic approach combining morphological, ecological, and phylogenetic data. This approach considers characteristics with a variable weight in order to reach a conclusion on the proposition

that an entity represents a separate species (Quaedvlieg *et al.*, 2014).

So, combining different data to resolve taxonomic problems provides a more holistic approach towards a classification system.

For reaching a conclusion in the CSC scheme, it is necessary to take into accounts that different conclusions can be drawn about speciation, when applying these species concepts individually. The molecular similarity between different taxa in a robust multi-locus DNA dataset represents high weighted information in any CSC analysis. Although, the differences in morphology and ecology are given less weight, they also are important for the integration of the criterion to reach a conclusion. This approach has become generally accepted during the last decade as a functional species concept within the mycological community (Quaedvlieg *et al.*, 2014; Bakhshi *et al.*, 2015; Leavitt *et al.*, 2015; Lombard *et al.*, 2015; Hawksworth & Lücking, 2017).

This concept has been progressively applied to genera of phytopathogenic fungi (Crous *et al.*, 2015; Liu *et al.*, 2016), such as *e.g. Cercospora* spp. (Bakhshi *et al.*, 2015), *Neonectria* and *Cylindrocarpon* (Chaverri *et al.*, 2011) or the *Teratosphaeriaceae* (Quaedvlieg *et al.*, 2014).

The number of species being recognized so far has been increasing by the use of the consolidated species concepts. Multi-locus sequence approaches to phylogenetic inference gave strong species boundaries to describe a new species. However, several processes could cause discordances between gene trees and species trees; misleading a true evolutionary relationships among closely related taxa may overestimate species clades

(Stewart *et al.*, 2014; Liu *et al.*, 2016).

Therefore, coalescent-based species delimitation methods, used mostly in plants and animal taxa, also could represent a pertinent approached in mycology. The coalescent-based methodology can make quantitative predictions about probabilities of gene trees, and serve as a baseline for investigating causes of gene tree discordance e.g. incomplete lineage sorting, horizontal gene transfer, gene duplication and loss, hybridization, and recombination (Stewart *et al.*, 2014; Liu *et al.*, 2016). Giving more support to a polyphasic analysis.

An other crucial factor to establish species boundaries is the need of a large sampling size, one or only a few individuals could fail in represent the species as a whole (Stewart *et al.*, 2014; Liu *et al.*, 2016). It is a fact that we need to develop a more complete inventory, especially in more diverse ecological niches; this will help us to eventually have a more accurate view of the number of fungal species (Hyde *et al.*, 2007). It is also fundamental to preserve them in a representative collection for formal description or to perform experiments on their physiology (James & Seifert, 2017).

Ecuador harbors a rich biodiversity, thanks to the different topological and climatic conditions, which created diverse environments. However, the local biodiversity has been very unequally explored and studied. Plants and animals are well known and continuously surveyed. But other taxonomic groups, including the Mycota, remain very critically understudied. Recent studies report about 3000 fungi in Ecuador (Læssøe & Petersen 2008) but this could be considered a low figure compared to other tropical regions and to the diversity of plants. Furthermore,

among the Mycota, those developing belowground, in the rhizosphere compartment, mycorrhizal and non-mycorrhizal, are even less well studied.

Biodiversity erosion in Ecuadorian as a result from both direct and indirect human activities also is a major concern for the country. The pollution generated by oil or mining activities in biodiversity rich ecosystems is one of the principal threats to this biodiversity. The petroleum industry generates high amounts of organic residues (Lemos *et al.*, 2002). Its extraction and transportation carry inevitable risks to the environment. Both human and mechanical errors are the main cause of terrestrial environmental pollution. Accidental oil spills into the environment become one of the major problems of pollution of soils, rivers and seas. Around the world, oil spills into the environment resulted in major disturbances of the local ecosystems, *e.g.* Alaska, the Gulf of Mexico, or the Niger Delta region in Nigeria (Adams *et al.*, 2015; Asghar *et al.*, 2016)

Oil represents a major concern for the economy of Ecuador. Since the seventies and the exploitation of the Lago Agrio camp by Texaco Gulf, Ecuador became one of the major exporters of oil, the third in Latin America. Per day, around 500,000 barrels of crude oil are produced (San Sebastián & Hurtig, 2004; Maddela *et al.*, 2015a). The Ecuadorean Amazon basin, known as *el Oriente*, located on the western edge of the Amazon rainforest (Finer *et al.*, 2008; Butler, 2012) harbor the most promising oil and gas resources of Ecuador and perhaps of the world. *El Oriente* includes the provinces of Sucumbíos, Orellana, Napo, Pastaza, Morona Santiago, and Zamora-Chinchipe. This

ecoregion is considered as one of the most biodiverse places on Earth with the highest species diversity per unit area. The Ecuadorian Amazon includes Protected areas such as the Cuyabeno Wildlife Reserve, the Limoncocha biological reserve and the Yasuní National Park (area of 9820 km^2), one of the world's most ecologically complex and fragile places. However, up to 60 % of the Ecuadorian Amazon is occupied by oil companies and 80% of the oil wells are located in the areas of Lago Agrio, Shushufinfi, Orellana and Joya de los Sachas (Bustamante & Jarrín, 2005; Ron, 2012). Oil blocks leased to companies largely overlap with these protected areas and also ancestral indigenous territories.

The economic development of the country favored by the Oil economy has brought not only a vast network of roads, pipelines, and oil facilities in the Amazon region but also millions of gallons of untreated wastes and crude oil discharged without treatment into the environment (San Sebastián & Hurtig, 2004).

Currently, according to Empresa Publica (EP) PETROECUADOR and the actions carried out within the Proyecto de *eliminación de pasivos en el Distrito Amazónico* (PEPDA, aiming at clearing the liabilities in the amazon district) and the *Plan de restauración integral de Pasivos Ambientales* (PRIPA) (Environmental Restoration Plan), 2550 sources of contamination have been documented up to the year 2013, with 357 pits, 993 spills and 1200 oil pools, registered in the province of Sucumbíos and in the province of Orellana (MAE Ministerio del Ambiente Ecuador, 2016).

Decontamination of crude oil-polluted soil is considered as one of the priorities by the Ecuadorian government. A project for the elimination of pollution sources was established for, as much as possible, restoration of ecosystems. The objective of this project was to clean up oil pools and spills (MAE Ministerio del Ambiente Ecuador, 2016). International cooperation initiatives also are working together with municipality's authorities in the Sucumbíos region of Ecuador. CoRenewal, a nonprofit organization is developing new strategies to clean up the abandoned oil pools and water. Their work is mainly focused on the use of IOS-500 Bacteria to degrade petroleum waste. Nonetheless, they also are working on the Amazon MycoRenewal Project, which focuses in testing fungal enzymes of native fungi (white rot fungi) with potential for mycoremediation. Biological treatments are promising alternative to reduce the environmental impact caused by oil spills (Lemos *et al.*, 2002; Saraswathy & Hallberg, 2002; Chávez-Gómez *et al.*, 2003; Zacatenco & Madero, 2010).

The oil ponds used in this study as place of sampling are located in Charapa camp (bloque 50), in the Province of Sucumbíos, approximately 15 km NE of Campo Lago Agrio. Those oil ponds were left abandoned for above 30 years. With time, these ponds were progressively covered by leaf litter and woody debris falling from the neighboring Amazonian forest. These leaf litter and branches in various states of decomposition slowly accumulated, *in fine*, forming a superficial layer of organic, compost-like matter of variable thickness, reaching 30 cm. This organic matter and the resulting loam layer have favored plant establishments, and some of these ponds are now largely,

superficially, re-colonized by plant communities. The local plants, mostly herbaceous species, are rooted in the organic layer but their deepest roots plunge into the crude oil. These plant communities may offer an interesting model of study of re-colonization.

The rhizosphere – the intimate interface between soil and roots – is a highly active micro-environment in which several biogeochemical reactions take place. The central role of microorganism in this micro-ecosystem includes biogeochemical cycling of nutrients and biodegradation. Among the rhizosphere microbiota, fungi are known for their importance in maintenance of nutrient cycling, the soil formation, fertility and over all soil improvement (Borah *et al.*, 2015). The structure of the microbial population is affected by the environmental conditions and anthropogenic activities such as the oil pollution. Therefore, in order to understand globally these plant re-colonization events, it is of importance to study more deeply their root system, which develops into highly contaminated organic material. This includes the fungal component of the rhizosphere, that comprise both symbiotic and saprophytes species (from the external rhizoplane or endophytic).

In the fungal kingdom, there are a large number of species that remain to be explored as far as their physiology, and specifically, their degrading enzymes, is concerned. Hence, focusing on the diversity of fungi in hydrocarbon-polluted soils may represent a straightforward approach to isolate potential novel species with abilities to degrade complex hydrocarbon mixt. Nowadays, molecular tools targeting species or communities

(e.g. next generation sequencing) are fundamental to study fungal genera with complex taxonomy and to picture them in the more extended microbial community. This thesis aims at picturing / characterizing the fungal communities, associated with the rhizosphere (rhizoplane and root internal tissues) of the various plants growing in these peculiar hydrocarbon-polluted environments, mainly from a taxonomic and secondarily from a functional point of view.

II. RESEARCH OBJECTIVES AND OUTLINE OF THE THESIS

Within this context, this thesis aims at picturing / characterizing the fungal communities associated with the rhizoplane and root internal tissues of the various plants growing in these peculiar oil polluted environments, mainly from a taxonomic and secondarily from a functional point of view.

The first objective was to evaluate the diversity of the root endophytic and saprotrophic rhizoplane fungi associated to the various herbaceous plants growing in these Amazonian oil ponds. To achieve this objective, a methodology based on the cultivation of filamentous Fungi was developed. This culture dependent assessment of the fungal diversity was applied to the rhizoplane and the root internal tissue (endophytes). The characterizations of these fungal strains were based on preliminary morphological approach, allowing identification to the generic level. Subsequently, the Consolidated Species Concept (CSC), combining phylogenetic, morphological, and ecological species concepts (Quaedvlieg *et al.*, 2014; Bakhshi *et al.*, 2015) was applied to several genera selected as models. Criteria for the selection of the model genera were their abundance and *a priori* diversity within the isolates the availability of a modern, multi-approach taxonomic scheme allowing optimal description. Also, their potential ecological roles in the micro-ecosystem studied with, ahead, the possibility to extend their studies to other natural (rain forest) or anthropic (agriculture, horticulture, nursery) ecosystems.

The second objective of the thesis was to assess *in-vitro* the ability of the isolated fungal strains to grow in presence of and to degrade oil molecules (hydrocarbons). To achieve this objective, *in-vitro* screening procedures were developed. The first screening was carried out on solid and liquid medium in order to test the grow of a selection of representative strains. On the basis of the results of the first screening, a subset of strains (i.e. the best growing) was selected to test in liquid medium their ability to degrade jet fuel (Fig.1).

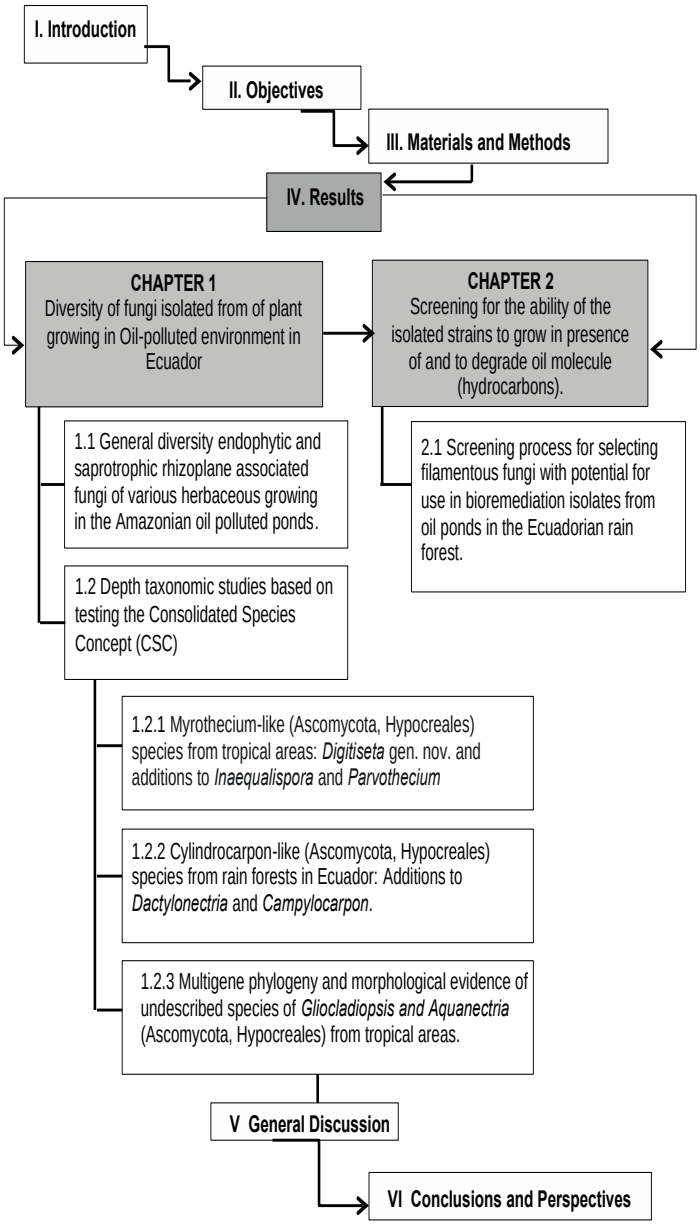


Fig 1. Outline of the thesis

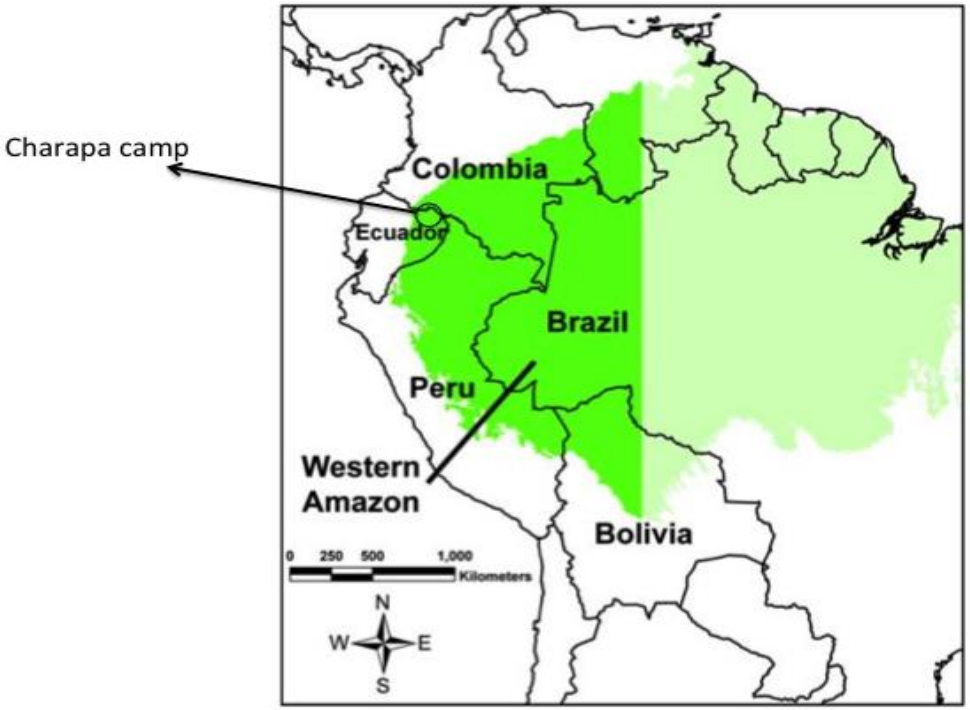
III. MATERIALS AND METHODS

1. Origin of the isolates and methods for sampling and isolation

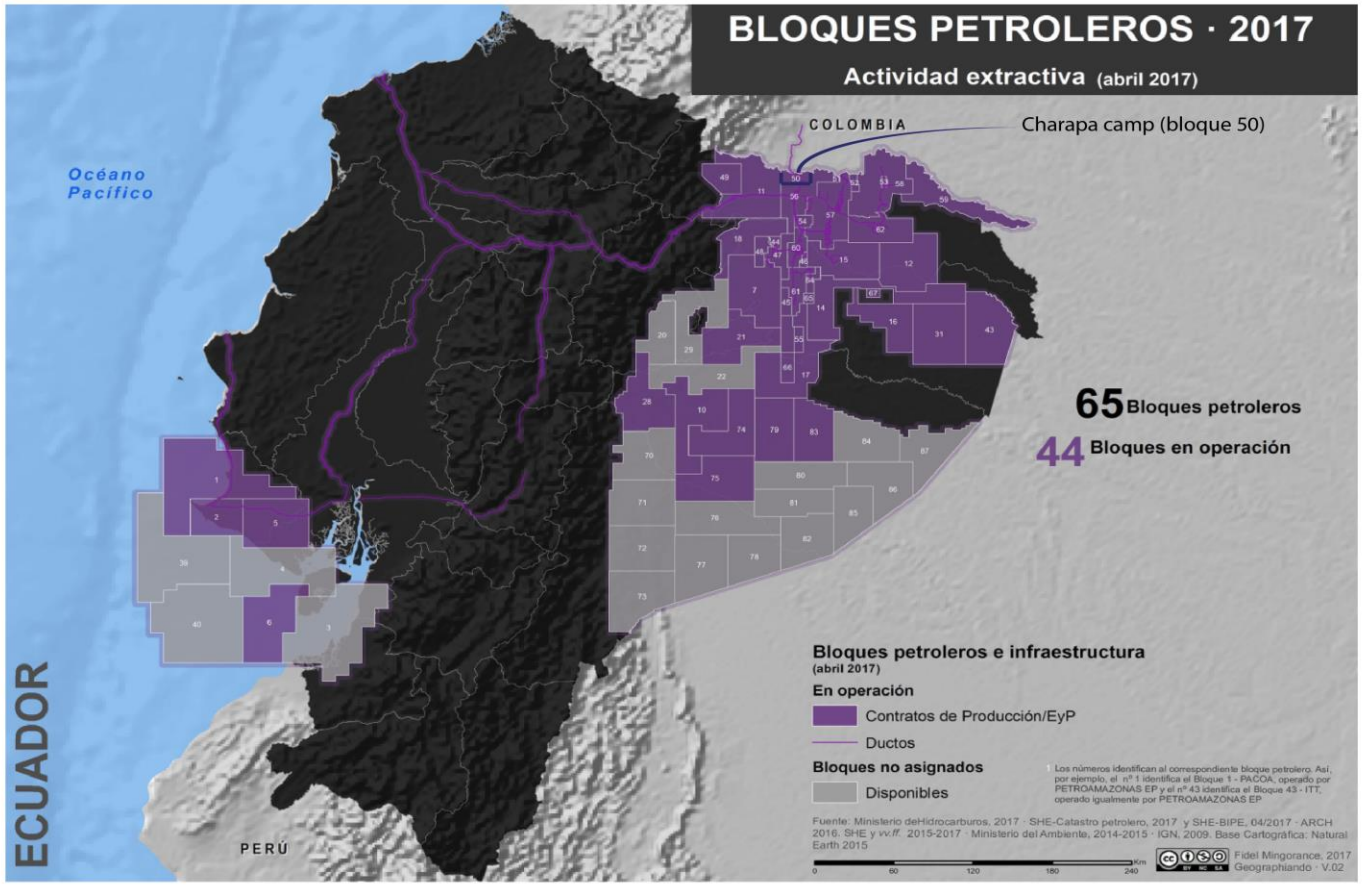
The strains studied in this thesis were obtained from natural substrata, mainly rhizoplane and roots of plant that are natural recolonizing oil ponds in Ecuador and from BCCM/MUCL fungal collection (Louvain-la-neuve, Belgium). The strains use for taxonomical analysis generated in this studied as well as the strains from BCCM/MUCL, and type or reference strains used for comparison are shown in (Table 1 of each sub-section).

1.1. Site and sample procedure

The Ecuadorean Amazon basin, known as *el Oriente*, located on the western edge of the Amazon rainforest (III. Fig. 1) (Finer *et al.*, 2008; Butler, 2012). The Charapa camp (bloque 50) is located in the Province of Sucumbíos, approximately 15 km NE of Campo Lago Agrio, and is bordered to the North by the Republic of Colombia, with an area of 24,358 hectares (approx.) (III. Fig. 2). This area is currently controlled by PetroAmazonas EP Ecuadorian public oil company who collaborated with the development of this thesis within the framework of the PIC Project “Reinforcement of the fungal expertise in Ecuador via case studies of fungal plants interactions in selected ecosystems and the development of biotechnology-oriented fungal resource centres”.



III. Fig. 1. Western Amazon oil basin. Adapted from Finer *et al.*, (2008)



III. Fig. 2. Ecuadorian Oil blocks 2017, Extractive Activity (April 2017)

III. Fig. 2. Ecuadorian Oil blocks 2017, Extractive Activity (April 2017), 65 oil blocks, 44 blocks in operation, Production contracts / EyP, Ducts. Unallocated blocks: Available. Source: Ministry of Hydrocarbons of the Republic of Ecuador, 2017- SHE-oil tanker and 2017 SHE BIPE, 04/2017–ARCH 2016. SHE y vv.ff. 2015-2017 Ministry of the environment 2014-2015. Cartographic base: Natural Earth 2015.[1] The number in white points the oil blocks. (Adapted From: https://geographiando.net/gallery/radiografia-minero-energetica/).

The strains were isolated from the rhizoplane and roots of several Angiosperms (Monocotyledons) and Pteridophytes growing in a layer of organic debris forming a mat floating over oil ponds, in Amazonian forest. Ponds are located at Charapa camp, Sucumbíos Prov., approx. W 76° 48' 57" – S 00° 11' 49" (pond 1 of 330 m^2) and W 76°48'54" – S 00°11'46" (pond 2 of 450 m^2), approx. elev 300 masl (Perez, 2014). The native plant community in which the study area is located corresponds to the Lowland Evergreen Forest of Napo-Curaray according to Guevara *et al.,* (2013) (III. Fig. 3).

The oil pond vegetation was characterized by the botanist A. Pérez (2014) (Annexe 1). This vegetation is dominated by *Carludovica palmata* Ruiz & Pav. (1798) (Cyclanthaceae), *Dimerocostus strobilaceus* Kuntze (1891) (Costaceae), *Heliconia* cf. *chartacea* Lane ex Barreiros (Heliconiaceae), and several species of Araceae. The local neighboring vegetation is a secondary rain forest dominated by species of *Ficus sp.* (Moraceae), *Croton lechleri* Müll. Arg.

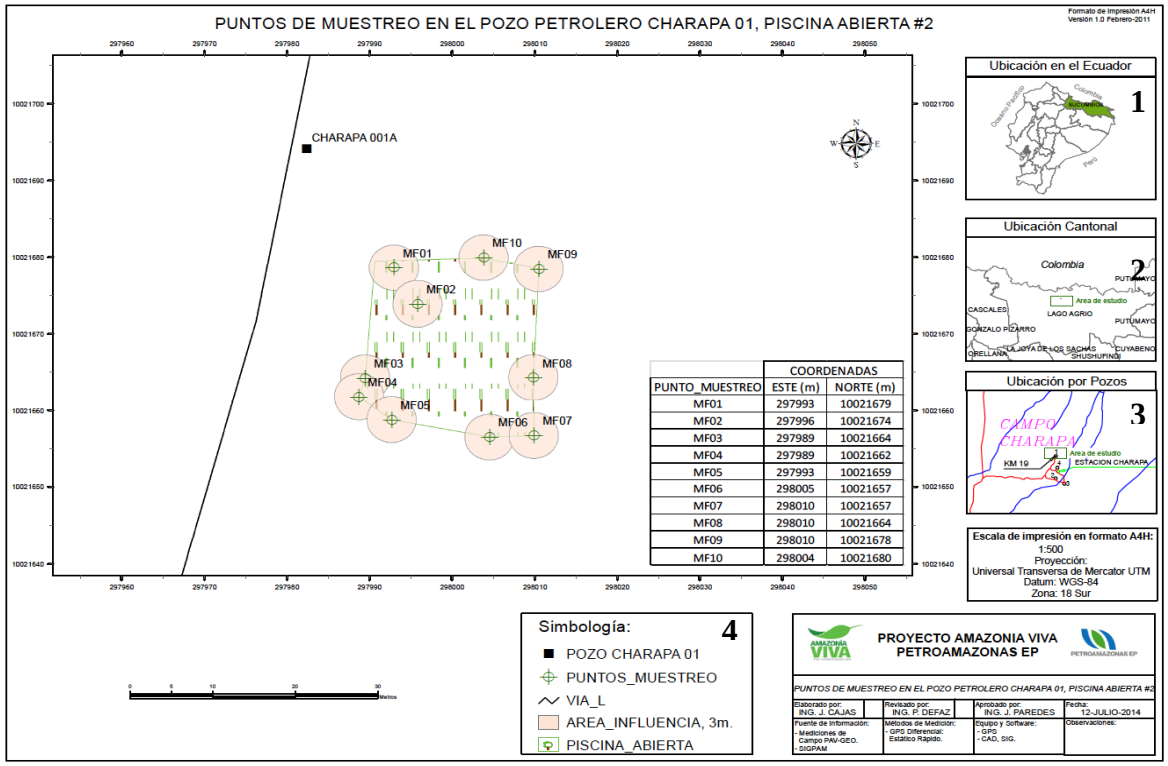
1866 (Euphorbiaceae) and *Sapium glandulosum* Morong (Euphorbiaceae) (III. Fig. 3). Weather conditions are characterized by warm temperatures and high humidity typically found in rain forest climates.

A chemical and physical analyses of the “soil” formed by the organic debris of the pond 2. The level and composition of the oil contamination were determined by the Centro de Servicios Ambientales (CESAQ-PUCE) laboratory; samples of soil from the pond show a high Total petroleum hydrocarbon (TPH) concentration inside the ponds and surrounding soil (i.e. above 5000 mg Kg^{-1} pond and ~1200 mg Kg^{-1} in the surrounding soil).

III. Fig. 3. a. External view of Pond 2 showing the emergent vegetation, Herbaceous layer (2.5 m) dominated; b. Monocotyledonous plant species growing in a superficial layer; c-d. Sample plant collected.

A total of 208 plants were collected, at four sampling times in October 2012, December 2012 and 2013, and June 2014. The

plants were selected randomly inside the pond and the neighboring area. For the first and second sampling, we randomly sampled plants from inside and outside the two ponds. For the third and fourth sampling, we sampled in an estimated 3 meters area of influence in the transect line at the border of the pond (III. Fig. 4), collecting the same plant species from 3 m inside of the pond and 3 m outside of the pond. We also collected a few plant specimens from the center of the pond. Plants with roots and adjacent soils were collected and placed in polyethylene bags, kept stored at 4–7 ºC until processed.

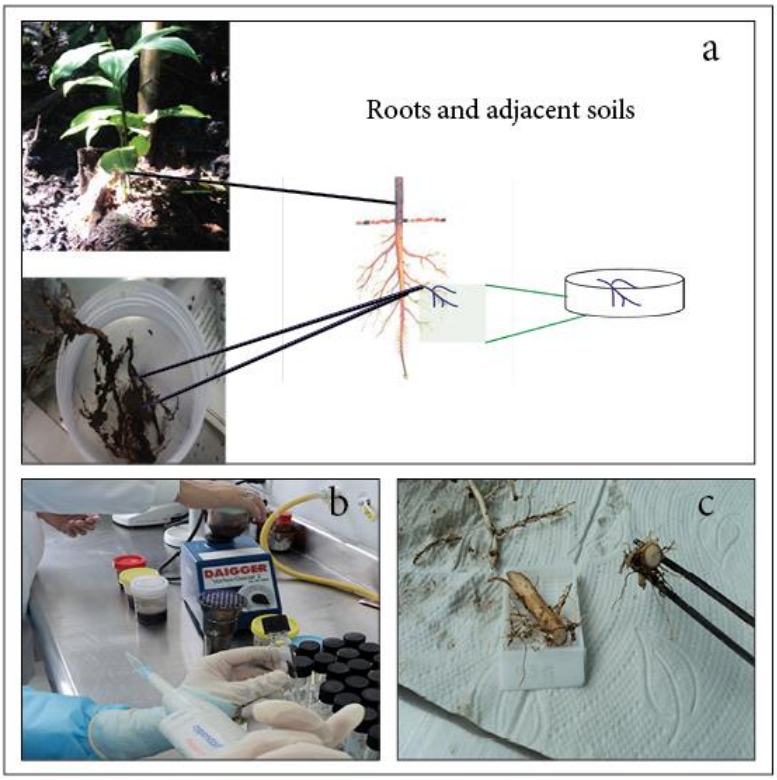


III. Fig. 4. Pond 2 sampling points in Charapa camp. (1. Location in Ecuador; 2. Cantonal location; 3. Location of the wells; 4. Sampling point, Charapa well, Area of influence, Open pond) (June 2014). Adapted from Department of Geomatics of PetroAmazonas.

1.2. Isolation of fungal strains

To isolate rhizoplane saprophytes, roots were gently cleaned from the rough soil particles. Cleaned roots were then rinsed with a sterile solution of 3% (v/v) Tween 80/ water, and the resulting solution diluted 1:10 in sterilized 0,1% (v/v) Tween 80/ water (III. Fig. 3). The dilutions were poured on malt extract agar (MEA, 20 g malt extract Difco and 20 g Agar Difco per L, supplemented with 0.05 g of L-chloramphenicol Himedia) using a Digralsky spreader, in duplicate. The plates were incubated in the dark at 25 ± 1 ºC and were examined every day for 10 days. Pure cultures were obtained from germinating spores or growing hyphae and transferred to Petri dishes with MEA

For endophytes isolation, each root was cleaned to remove soil particles, cut into six 2–5 cm-long pieces, and surface-sterilized using the following sequence of immersions: 1 min 99 % (v/v) ethanol, 5 min 35% (v/v) hydrogen peroxide, 1 min 99 %(v/v) ethanol, then rinsed in sterile, distilled water for a few minutes. Immediately, after rinsing, segments of 2–3 mm length were aseptically excised from the middle of each root piece, placed on MEA, and incubated at 25 ± 1 ºC in the dark (Ahlich & Sieber, 1996). Pure cultures were obtained from growing hyphae and transferred to Petri dishes with MEA (III. Fig. 5).



III. Fig. 5. a. Roots and adjacent soils were collected; b. Isolation of rhizoplane saprophytes ; c. Isolation of endophytes

1.3. Phenotypic studies

The phenotypic studies of the fungal strains were included in this thesis and consisted in the evaluation of macroscopic and microscopic features on different culture media following the criteria established for each genus.

1.3.1. Morphological characterization

In order to reach optimal growth and sporulation, we used different culture media. Cultures were grown on Malt Extract Agar (MEA), Synthetic Nutrient-Poor agar (SNA, Nirenburg 1981) and

Banana Leaf Agar (BLA) (Untereiner *et al.*, 1998). Cultures were incubated at 25 ± 1 ºC with a 12/12 hr in near UV ligtht / dark cycle. Culture characteristics were determined at 7 d after inoculation. Colors of the colonies were described according to Kornerup and Wanscher (1978). The micromorphology of isolates was studied from slides of reproductive structure obtained on BLA medium and SNA medium after 7 d at 25 ± 1 ºC. Fungal structures were mounted in lactic acid, and studied using a phase contrast microscope (Nikon, Japan). The size of each microscopic structure (i.e. condiophores, conidiogenous cells and conidia) was obtained by measuring a minimum of 15 structures of each type, and through the use of an Olympus light BX 53. Photographs of fungal colonies were obtained using a Canon EOS 60D digital camera. Photomicrographs were obtained with Olympus BX 50 with Differential Interference Contrast (DIC) microscopy, also known as Nomarski Interference Contrast (NIC).

1.4. Conservation of the strains

All the strains were conserved on agar slant under liquid paraffin and water, and several were lyophilized. In the first procedure, fungi were cultured on MEA in slant tubes, and covered with liquid paraffin or water. The tubes were stored at 4 ºC temperature. Several strains were kept as second storage in BCCM/MUCL and were freeze-dried and cryopreserved.

2. DNA isolation, amplification and analyses

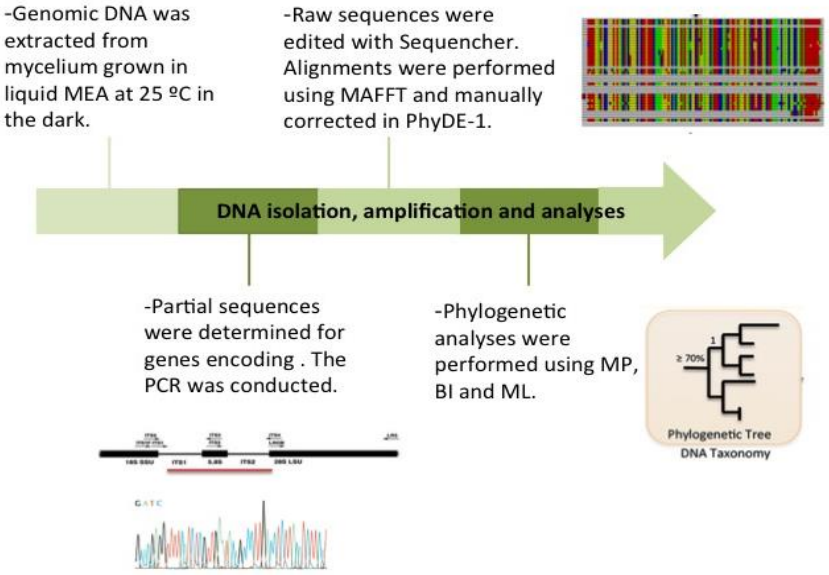
Genomic DNA was extracted from mycelium grown in liquid MEA at 25 ± 1 ºC in the dark. Extractions were carried out using innuPREP Plant DNA kit (Analytik Jena, Germany) following the manufacturer's recommendations.

Partial sequences were determined for genes encoding β-tubulin (*tub2*, region between exon 1 and exon 4) with primer pair T1/Bt-2b also β-tubulin (tub2, region between exons 3 & 4) with primer pair Bt2a/Bt-2b, RNA polymerase II second largest subunit (rpb2, domains 6 & 7 region between exons 3 & 5) with primer pair RPB2-5F/ fRPB2-7cR, translation elongation factor 1-α (*tef1a*, region between exon 1 and exon 4) using the primers ef1/ ef2 also (tef1a, part of the largest exon with EF1-983F /EF1-2218R and histone H3 (*his3*, region between exon 1 and exon 3) gene with H_3-1a/H_3-1b, the nuc rDNA ITS1-5.8S-ITS2 region (ITS) performed with the primer pair ITS5/ITS4 and nuc 28S (28S, region comprising the D1-D3 domains) was amplified with primer pair LR0R/LR6 (Table 1).

The PCR was conducted using a total volume of 25 µl of reaction mixture with these final concentrations: 5 ng DNA, 1 PCR buffer (20mM Tris/HCl pH 8.4, 50 mM KCl), 1µM each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 unit of GoTaq® DNA Polymerase (Promega Corporation, USA). The resulting PCR products presenting a electrophoresis mobility according the gene dimension were stored at -4 ºC until sequencing. Amplicons were sequenced in both directions by Macrogen Inc. (Korea) using the same primers as used for amplification. Raw sequences were edited with

Sequencher®software version 5.1 (Gene Codes Corporation Ann Arbor n.d.). Nucleotide sequence alignments were performed using MAFFT v. 7.213 (Katoh & Standley, 2013) and manually corrected in PhyDE-1 (Müller *et al.*, 2006) when necessary. BLAST searches (Altschul *et al.*, 1990) were performed to search for preliminary affinities of our strains. Reference sequences for phylogenetic analysis were downloaded from Genbank.

Phylogenetic analysis of the strains and sequences used in this study are listed in each sub-section (1.2.1-1.2.3). Newly sequenced strains are included in the data set for phylogenetic analysis. Data sets were set up to conduct the phylogenetic inferences (III.Fig. 6).



III. Fig. 6. Flow chart of DNA isolation, amplification and phylogenetic analyses

Table 1. Primers used in PCR and sequencing for different fungi.

Locus	Product size (bp)	Primer forward (5' →)	Primer reverse (5' →)	T(ºC)	Reference
LSU	~1000	LR0 ACCCGCTGAACTTAAGC	LR6 CGCCAGTTCTGCTTACC	50	Vilgalys & Hester 1990
ITS	~385-545	ITS 5 GGAAGTAAAAGTCGTAACAAGG	ITS 4 TCCTCCGCTTATTGATATGC	55	White et al. 1990
tef1a	~600	ef1 ATGGGTAAGGA(A/G)GACAAGAC	ef2 GGA(G/A)GTACCAGT(G/C)ATCATGTT	60-56	O'Donnell et al. 1998
	~609	EF1-983F GCYCCYGGHCAYCGTGAYTTYAT	EF1-2218R ATGACACCRACRGCRACRGTYTG	47	Rehner and Buckley 2005
RPB2	~793	5F2 GAYGAYMGWGATCAYTTYGG	7cR CCCATWGCYTGCTTMCCCAT	60-52-54	Liu et al., 1999
his3	~447	H_3-1a ACTAAGCAGACCGCCCGCAGG	H_3-1b GCGGGCGAGCTGGATGTCCTT	60	Glass and Donaldson 1995
tub2	~500	T1 AACATGCGTGAGATTGTAAGT	Bt-2b ACCCTCAGTGTAGTGACCCTTGGC	56	O'Donnell and Cigelnik 1997 Glass and Donaldson 1995
	~360	Bt2a GGTAACCAAATCGGTGCTGCTTTC	Bt-2b ACCCTCAGTGTAGTGACCCTTGGC	56	Glass and Donaldson 1995

Phylogenetic analyses were performed using Maximum Parsimony (MP) as implemented in PAUP* 4.0b10 (Swofford, 2003), Bayesian Inference (BI) as implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) and Maximum Likelihood (ML) using RAxML 7.0.4 (Stamatakis, 2006). The best-fit likelihood model of evolution for the different data sets, for BI and ML, was estimated using PartitionFinder (Lanfear, 2012).

For MP analyses, gaps were treated as missing data or fifth base according the analysis. The most parsimonious trees were identified using heuristic searches with 100 random addition sequences, further evaluated by bootstrap analysis, retaining clades compatible with the 50% majority-rule in the bootstrap consensus tree. Analysis conditions were tree bisection addition branch swapping, starting tree obtained via stepwise addition, steepest descent not in effect, MulTrees effective. Clades with bootstrap support value (BS) above 90% were considered strongly supported by the data.

Bayesian analyses were implemented with two independent runs, each with four MCMC simultaneous independent chains, totaling the number required of generations for the data set, starting from random trees, and keeping one tree every 1000th generation. All trees sampled after convergence [average standard deviation of split frequencies < 0.01, confirmed using Tracer v1.4 (Rambaut & Drummond, 2007), were used to reconstruct a 50% majority-rule consensus tree and to estimate posterior probabilities. Clades with BPP above 0.95 were considered strongly supported by the data.

Maximum likelihood (ML) searches were conducted with RAxML involved 1000 replicates under the best-fit model. In

addition 1000 bootstrap (ML BS) replicates were run with the same model. Nodes with maximum likelihood bootstrap values of 70% or greater were considered to be significantly supported.

The newly generated sequences and the alignments used in the cladistics analyses were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and Tree base (https://treebase.org/treebase-web/home.html). GenBank Accession numbers are indicated in the tables of the different publications included in the Results Section.

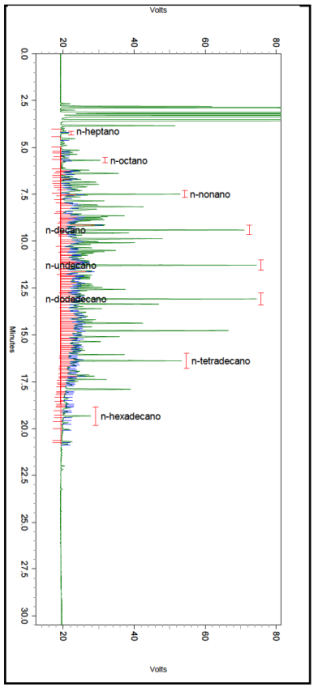
3. Determination of the ability of filamentous fungi to grow on solid and in liquid medium containing jet fuel.

3.1.1. Biological material

Thirty-two strains belonging to 26 genera were selected from strains in Chapter I. The strains were screened for their ability to grow on solid and liquid media containing jet fuel as the unique carbon source.

3.1.2. Hydrocarbon used in the assay

Jet fuel, which is a petroleum derivate, also known as kerosene, was used in the study. It contains hydrocarbons from C8 to C17 distributed between saturated hydrocarbons (80% to 90%) and aromatic (10% to 20%), and is usually free of olefins (Weisman, 1998). Chemical analysis of jet fuel was made by Gas Chromatography (GC) (III. Fig. 7).



Front Signal Results

Time	Name	Area	Amount
4.130	n-heptano	42083	34.759
5.686	n-octano	180063	108.360
7.492	n-nonano	494445	248.627
9.409	n-decano	920922	446.051
11.290	n-undecano	961338	455.443
13.084	n-dodedecano	1047359	482.190
16.386	n-tetradecano	585870	278.051
19.339	n-hexadecano	178286	103.232
Totals		4410366	2156.713

III. Fig. 7. Chromatography profile from jet fuel (Jet A) used in this assay performed by at the chemistry laboratory, Pontifical Catholic University of Ecuador

3.2. Preliminary screening of isolates on solid and in liquid media

3.2.1. Determination of mycelial growth on solid culture medium

The selected fungal strains were screened for their ability to utilize jet fuel as a source of carbon, by measurement of the fungal growth on jet fuel. Fungal growth was determined in Petri dishes by measuring growth diameter. Growth was assessed by comparing fungal culture diameter in presence/absence of jet

fuel.

The fungal strains were grown on MYPD medium (0.3% Malt extract, 0.3% Yeast extract, 0.5% Peptone, 1% Dextrose, 20 g. agar, Difco) (Boonchan *et al.* 2000). The media were prepared by adding 0.1% Tween 80 and 1%, 2% and 3% (v/v) of jet fuel, previously sterilized by filtration through a 0.22 µm filter. The control media was prepared using the same compositions excluding the jet fuel. Both media were inoculated with a 10 mm agar plug with mycelium, taken from actively growing colony. The cultures were incubated at 25 ± 1 ºC in the dark for a period of 7 days. All the tests were conducted in triplicates. The colony diameter was subsequently recorded (Husaini *et al.*, 2008). The relationship between the diameters of growth in the medium was calculated by [% of growth decrease = ((colony diameter without jet fuel – colony diameter with jet fuel) / colony diameter without jet fuel)*100] to determine the difference in growth.

3.2.2. Rapid assessment of biodegradation of hydrocarbons by colorimetric technique in liquid culture.

The screening procedure for testing the degradation capacity of the selected fungi was carried out using the Hanson *et al.* (1993) method modified by Bidoia *et al.* (2012). This method is based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP), which is an enzyme-catalyzed redox electron acceptor that can be used as an indicator of microbial metabolism (Montagnolli *et al.*, 2015; Marchand *et al.*, 2017). DCPIP redox indicator presents a rapid, simple and low cost tool for evaluating the ability of different microorganisms to use a hydrocarbon

substrate by simply observing the discoloration of the media from blue (oxidized) to colorless (reduced) (Montagnolli *et al.*, 2015).

Three parameters were considered to assess the efficacy of fungi in biodegradation: The first one is a measure of discoloration of the culture medium from blue to colorless, measured in absorbance. The second is the dissipation of hydrocarbon (jet fuel) from the liquid medium. The third is related to the biomass production of the fungus in the culture liquid medium (Al-Nasrawi *et al.,* 2012).

Seven-day old fungal cultures were used as inoculum. One plug (10 mm size) was picked from the periphery of the culture grown on malt agar extract (MAE) and transferred carefully into tubes containing 7.5 ml of Bushnell-Haas broth (BH DIFCO™; 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$, $CaCl_2$ pH7); 0.1% Tween 80, 400 µL DCPIP (0.025 g/25 ml of redox indicator) and 50µL jet fuel were added (Bidoia *et al.*, 2012). Abiotic control experiments were performed by incubating the medium without inoculums. The experiments were conducted in triplicates. The tubes were kept under constant agitation (i.e. 100 rpm) at 25 ± 1 ºC. After one week of incubation, the level of discoloration, between dark blue to colorless, indicating jet fuel degradation, was measured by spectrophotometry (absorbance at 600 nm) (Hanson *et al.*, 1993; Al-Nasrawi, 2012; Bidoia *et al.*, 2012; Hanafy *et al.*, 2015; Montagnolli *et al.*, 2015; Marchand *et al.*, 2017). For each tube, we have evaluated the mycelium grow of the strains in liquid medium containing jet fuel by our qualitative scale: for the largest growth (3), for a medium growth (2) and for lower growth (1) and

no growth (0); this further examination helped to select the best strains.

3.3. Assay for test the ability to degrade jet fuel

Degradation experiments were performed separately in 0 ml Erlenmeyer flasks, containing 90ml of BH broth, with 0.1% Tween-80 and 1% (v/v) of sterile jet fuel as the unique carbon source. Prior to adding the jet fuel, the growth medium was sterilized (121 °C for 15 minutes). The fungal inoculum consisted of 10 ml of a suspension of 5-day old spores (using Tween-80 0.01%) isolated from 3 Petri dishes. The experiments were done in sevenfold for each fungal strain. Controls were done for triplicate consisted in jet fuel added to the BH broth without inoculum (abiotic control). All the flasks were incubated for 15 days at room temperature under constant agitation of 100 rpm (Barnstead lab-line max q2000) and a photoperiod of 12 h. (Al-Nasrawi, 2012; Maddela *et al.*, 2015b).

3.3.1. Hydrocarbon extraction and analysis of jet fuel

Ten ml of solution from each flask, including the control, were harvested at the beginning of the experiment (time 0) and after 15 days of incubation. Five ml were used for chemical analysis such as TPHs quantification by Gas Chromatography (GC), which is the more widely used technique in the petroleum hydrocarbon degradation studies (Mittal & Singh 2009). In addition, 1 ml of the solution was used to test the viability and purity of the different strains. The purity was conducted to ascertain the absence of unwanted fungal or other contaminants. The viability consisted in evaluating the growth of the fungus by

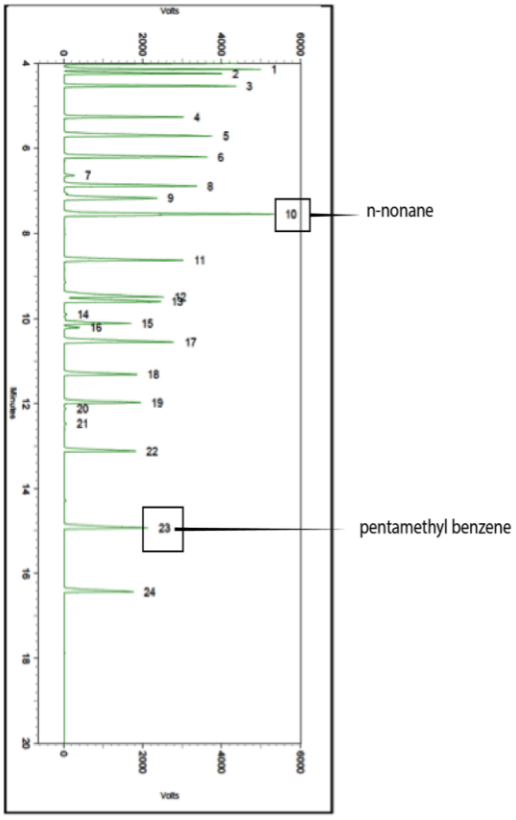
serial dilution method. Samples were serially diluted with sterilized distilled water up to 10^{-6}. The last three higher dilutions were spread on MEA agar medium in Petri dishes and incubated at 25 ± 1 ºC for 7 days in the dark. The remaining 4 ml were stored -4 °C as a backup of the sample, until the end of the assay, in case of additional tests.

The TPH extraction was carried out by adding 6 ml of dichloromethane (DCM) (Sigma-Aldrich) to 5 ml from each sample. The tubes were sonicated in an ultrasonic bath (Branson 3800) twice for 5 minutes each. The mixture was transferred into a 50 ml separating funnel and sequentially extracted three times with equal volumes of the solvent mixtures. The organic fractions from the respective tube were pooled and dried over anhydrous Na_2SO_4, then the residual content was filtered through Whatman No 1 filter. The solvent fractions were evaporated with a rotary evaporator (Rotavapor water bath BM-200) to obtain a final volume of 1 ml in order to proceed to the GC analysis of TPH (Boonchan *et al.*, 2000; Husaini *et al.*, 2008).

The extracts were analyzed within a single batch by GC with flame ionization detector (FID), using a GC-FID Agilent 7890 gas chromatograph (Agilent Technologies, www.agilent.com), equipped with a FID detector and automatic injector. The separation column was an Agilent DB-TPH 30 m x 0.32 mm x 0.25 µm with an injection volume of 1 µL and injection temperature of 300°C. The GC oven program started at 40°C, was held for 1 min, then at 8°C 1 min, then increased to 220°C for 1 min. This enabled a complete run within 24.5 min.

The characterization and quantification of jet fuel components by GC was determined according to the calibration curve of standard ‘O-PONA System Validation Mixture’ Oxygenates & Paraffin, Olefin, Naphthene, Aromatics (O-PONA) by GC to determine TPHs (III. Fig. 8)

Finally, the efficiency of biodegradation was expressed in terms of removal ratios (RRs) as described by The percentage of TPH loss (%D) was given by the formula for removal rate (RR): %D = 100 [(MI - MT) / MI], where MT was the concentration of TPHs in each treatment and MI was the initial TPH concentration present in the medium (Maddela *et al.,* 2015a,b, 2016).



Compound	Retention time (min)
1. iso octane	4.142
2. n–heptane	4.238
3.methyl cyclohexane	4.535
4. toluene	5.263
5. n–octane	5.705
6. ethylbenzene	6.198
7. unknown,	
8.1,2,4–trimethylcyclohexane	6.878
9. o–xylene	7.166
10. n–nonane	7.541
11. 1,2,4–trimethylbenzene	8.629
12. n–decane	9.484
13. 1,2,3 trimethylbenzene	10.109
14. unknown	
15.trans–decahydro naphthalene	10.112
16. unknown	
17.1,2,4,5–tetramethyl benzene	10.546
18. n–undecane	11.314
19,20,21 unknown	
22. n–dodecane	13.114
23. pentamethyl benzene	14.930
24. n–tetradecane	16.426

III. Fig. 8 The TPHs analyzed in GC-FID Agilent 7890 (mg/L) were retention times (min) according to the standard O-PONA System Validation Mixture (ASTM Methods, 2015).

4. Statistical analyses

A linear mixed model was used, this type of statistical analyses is very flexible and capable of fitting a large variety of datasets. The objective of a statistical model is to have a mathematical formula that describes the relationship in the data.

It is based on a straight line which has the formula $\hat{Y} = b_0 + b_1 X_1$ (where b_0 is the intercept and b_1 is the gradient), the linear mixed model can be formulated in a very similar way (equation 1-2) (Analysis, 2014). This model permits us to account for the correlation within the "subjects", and to consider the subjects, as a random sample from a common population distribution, which may be more realistic in many applications (Jensen, 2006).

This assumes that each observation is independent; however they may well be some inter-dependence in the responses in relation to some factor. To deal with this we add a random coefficient into the model, which allows us to assume a different baseline response value for each factor. A mixed model contains the usual fixed coefficient as seen in linear regression, and one or more random coefficient, essentially giving some structure to the error term characterizing variation due to some factor level (Jensen, 2006).

The mycelia growth at each concentration x (Treatment=1) was compared with the mycelia growth in the medium without jet fuel (Treatment=0) to determine the effects of the treatment on fungal growth. The model was also used to determine which strains were the more sensitive to jet fuel.

This model allows taking into account the treatment effect, i.e. the systematic differences in growth between jet fuel and control, through the inclusion of the fixed coefficient β_2. The random coefficients β_{1i} and β_{3i} are included in order to take into account the similarities in the growth of the replicates obtained on the same strain. The coefficients β_{1i} represent the systematic

differences in growth between strains, while the β_{3i} allow for differences in the treatment effect between strains.

Equation 1:

$$Growth_{ij} = \beta_0 + \beta_{1i} + \beta_2 I(x)_{ij} + \beta_{3i} I(x)_{ij} + \epsilon_{ij}$$

i = refers to a strain

j = to a replicate

β_{1i} = random coefficient $\beta_1 \sim N(0, \sigma_1^2)$.

β_{3i}= random coefficient $\beta_3 \sim N(0, \sigma_3^2)$.

β_0= fixed coefficient

β_2= fixed treatment effect

$I(x)_{ij}$= Indicator (Treatment = 1 for x JF) (Treatment = 0 for Control negative)

ϵ_{ij} = $\epsilon \sim N(0, \sigma^2)$ random error with the assumption of a normal distribution with mean 0 and constant variance σ^2

For the liquid medium it was the objective to evaluate if there was a difference between the strains, according to the DCPIP absorbance measures. The following linear mixed model analysis (equation 2) was used.

Equation 2:

$$DCPIP_{ij} = \beta_0 + \beta_{1i} + \epsilon_{ij}$$

$$\text{with } \epsilon \sim N(0, \sigma^2) \text{ and } \beta_1 \sim N(0, \sigma_1^2)$$

for the model was also used to evaluate the strains for which the predicted value of β_{1i} (or, equivalently, of DCPIP absorbance measure) was the lowest. The data were analyzed using the software SPSS Statistics 24.

For the study on degradation potential of the strains, we conducted a one-way ANOVA to find statistical differences between the treatments using the SPSS (version 21.0). A Tukey post hoc test was used to identify the significant differences ($P \leq 0.05$).

IV. RESEARCH RESULTS

CHAPTER I

1. Diversity of fungi isolated from rhizoplane of plant growing in oil-polluted environment in Ecuador

1.1. General diversity of endophytic and saprotrophic rhizoplane associated fungi of various herbaceous growing in the Amazonian oil polluted ponds

Introduction

Ecuador is considered a biodiversity hotspot, which find its origin in the different topological and climatic conditions, which have facilitated the emergence of diverse environments and ecosystems. This biodiversity is still very unequally explored and studied. Plants and animals are well known and continuously surveyed, but other taxonomic groups such as the Mycota remain largely understudied. Meanwhile, in Ecuador as in many places of the world, the biodiversity does not escape the world general trend of erosion occurring at an unprecedented rate as a result of human activities. Anthropogenic threats impacting the Ecuadorian biodiversity include deforestation, oil or mining activities.

Fungi (Mycota) is one of the major lineages of life and also a key constituent of all ecosystem in term of diversity and input into various biogeochemical cycles. The soil Fungal communities are particularly critical in many ecosystems, amongst others, for their interaction with plants.

Recent studies reported about 3000 fungi in Ecuador (Læssøe & Petersen, 2008) but this could be considered a low figure considering the numerous ecosystems and compared to

other regions. Knowing that the ratio plants / fungi range from 1 / 6 to 1 / 9.8 (Hawksworth, 2001; Hawksworth & Lücking, 2017), Ecuador would theoretically harbour approximately between 100.000 and 160000 fungal species (for comparison, 16087 plant species are currently reported in Ecuador, Catalogue of the Vascular Plants of Ecuador date). Furthermore, among the Mycota, the belowground, either soil-borne or root associated (termed the rhizosphere) fungal diversity, mycorrhizal and non-mycorrhizal, have been very little studied.

Many of these soil Fungi can be cultivated on artificial media making possible, in addition to fine taxonomic characterization, the study of their physiological treats. Many Fungi are known as producers of enzymes or secondary metabolites displaying various activities (e.g. degradation of polymers, antibacterial, antiparasitic) (Anke & Sterner, 2002), some of which providing potential solutions to problems of environmental and economic concerns (agriculture, biotechnology, pharmaceutical industry, etc.) (OECD). Identifying and cultivating new strains & species from under-surveyed niches will improve our knowledge about biodiversity and ecosystem functioning, important for biodiversity management. It could result also in unveiling enzymes or metabolites for biotechnological.

One of the most important issues in Ecuador affecting the biodiversity is the uncontrolled treatment of the oil industry residues in the Amazonian rainforest. Amongst these, the so-called “oils ponds” represent likely the most emblematic symptom of this lack of careful management of these residues. These “oil ponds” are repository for poor quality oil left over by the oil

companies within the Amazonian rainforest. *Hitherto*, about 1200 oil ponds are mapped in Ecuador. They are impacting the ecosystems down to the local population.

These “oil ponds” were gradually covered by vegetal debris originating from the surrounding rain forest, and which accumulation and decomposition over time resulted in floating organic, compost-like mats. These floating mats were progressively recolonized by native plants that found suitable conditions for growth although their root systems expend into a highly polluted substrate and plunge beneath deep into crude oil. The plants developed locally a peculiar microecosystem, that is worth being studied as a whole, but also in its specific constituents, of which the plants, microbes, their physiological treats and interactions.

The rhizosphere represents an important niche / ecological system centred on the very near root environment. It includes the different abiotic and biotic components and their interactions. It is a highly active micro-environment in which several biogeochemical reactions take place. The biotic component, apart from the plant root itself, is mainly microbial, dominated by bacteria and Fungi. They constitute the main machineries of the soil microbial biomass (Rajapaksha *et al.*, 2004). Among the rhizosphere microbiota, fungi are known for their importance in nutrient cycling, soil formation, fertility and, over all, soil improvement (Borah *et al.*, 2015).

The rhizosphere fungal communities tend to be in equilibrium with the local biotic / abiotic factors. In this context, any changes in the soil biotic or physico-chemical composition, whether of

natural or anthropogenic origin, may lead to changes and adjustment of the microbial communities (Admon *et al.*, 2001; Bento *et al.*, 2005). These responses depend on the original community composition and its ability to adapt to the new conditions (Adekunle & Adebambo, 2007).

The microorganisms associated with the plants in oil ponds, developed in harsh conditions, might display specific properties. The local physico-chemical conditions may have exerted a selection pressure in favour of certain species or strains, which are worth being analysed both *in*- and *ex-situ*. Hence, they need to be isolated, identified, preserved and screened. These species/strains also may have potential for *ex-situ* applications, e.g. through their enzymatic systems and metabolites production, or helping the revegetation. This microecosystem and its microbial diversity may serve as model to boost microbial studies and expertise in Ecuador, and to search for physiological threats of potential issue for biotechnology and a head bio-economy.

In this frame, the aim of this study was to picture the fungal diversity associated to the roots of different plants growing in the oil ponds, either as endophyte or from the rhizoplane using a culture dependent approach.

Materials and Methods

Site and Sample procedure

The strains were isolated from the rhizoplane and roots of several Monocotyledonous and Pteridophytes growing in a layer

of organic debris forming a mat floating over oil ponds, in Amazonian forest. Two ponds were studied: Pond 1 (76°48’57” W- 00° 11’49” S) has a surface of 330 m^2 and Pond 2 (76°48’54” W –00°11’46” S) a surface of 450 m^2 approx., both at an elev of approx. 300 m asl. Weather conditions are characterized by warm temperatures and high humidity typically found in rain forest climates (Perez, 2014).

The plant community of the oil ponds have been studied by Dr. Alvaro Perez, botanist at PUCE (cf. Annexe 1). The local vegetation is composed of herbaceous plants and dominated by monocotyledons, including *Carludovica palmata* Ruiz & Pav. (1798) (Cyclanthaceae), *Dimerocostus strobilaceus* Kuntze (1891) (Costaceae), *Heliconia* cf. *chartacea* Lane ex Barreiros (Heliconiaceae), and several species of Araceae. The plant community in the surrounding areas is a secondary Lowland Evergreen Forest of the Napo-Curaray facies (Guevara et al., 2013), dominated by species of *Ficus* sp. (Moraceae), *Croton lechleri* Müll. Arg. 1866 (Euphorbiaceae) and *Sapium glandulosum* Morong (Euphorbiaceae).

A total of 208 plants were collected for subsequent fungal sampling. They were sampled randomly in the near central area of each pond and along a transect parallel to their border (3 meters was the estimated zone of influence from the transect), to take plant samples from inside outside (surroundings) the pond.

Isolation of fungal strains

Roots and adjacent soils were collected, placed into polyethylene bags and kept in refrigerator at 4–7 ºC until

processed within 24 hours. To isolate rhizoplane Fungi, roots rough soil particles were gently removed. Cleaned roots were then rinsed with sterile a solution of 3% (v/v) Tween 80/ water, and the resulting solution 1 ml was diluted in 9 ml sterilized 0,1% (v/v) Tween 80/ water. The dilutions were poured on malt extract agar (MEA, 20 g malt extract Difco and 20 g Agar Difco per L, supplemented with 0.05 g of L-chloramphenicol Himedia) using a Digralsky spreader, in duplicate. The plates were incubated in the dark at 25 ± 1 ºC and were examined every day.

To isolate endophytes, each root was cleaned to remove soil particles. They were then cut into six, 2–5 cm-long pieces, and surface-sterilized using the following sequence of immersions: 1 min 99 % (v/v) ethanol, 5 min 35% (v/v) hydrogen peroxide, 1 min 99 %(v/v) ethanol, then rinsed in sterile, distilled water for a few minutes. Immediately, after rinsing, segments of 2–3 mm length were aseptically excised from the middle of each root piece, placed on MEA, and incubated at 25 ± 1 ºC in the dark (Ahlich & Sieber, 1996). Pure cultures were obtained from germinating spores or growing hyphae and transferred to Petri dishes with MEA.

Morphological characterization

Cultures were grown on MEA, at 25 ± 1 ºC with a 12 / 12 hr. near UV light / dark cycle (Untereiner *et al.*, 1998). Culture characteristics were determined at 7 d after inoculation. Colours of the colonies were described according to Kornerup and Wanscher (1978). Description of the conidiophores and conidia is, as a rule, based on morphologically informative structures.

Conservation of the strains

All of the strains were conserved as submerged cultures in liquid paraffin and water, Fungi were cultured on MEA in slant tubes, and the growing part of the agar was covered with the liquid paraffin or water at the Mycology laboratory in the Pontificia Universidad Católica del Ecuador (PUCE). The tubes were stored at 4ºC. A subset of the strains is kept as a back-up storage at BCCM/MUCL under freeze-drying (lyophilisation) and cryopreservation (Fig. 1.1).

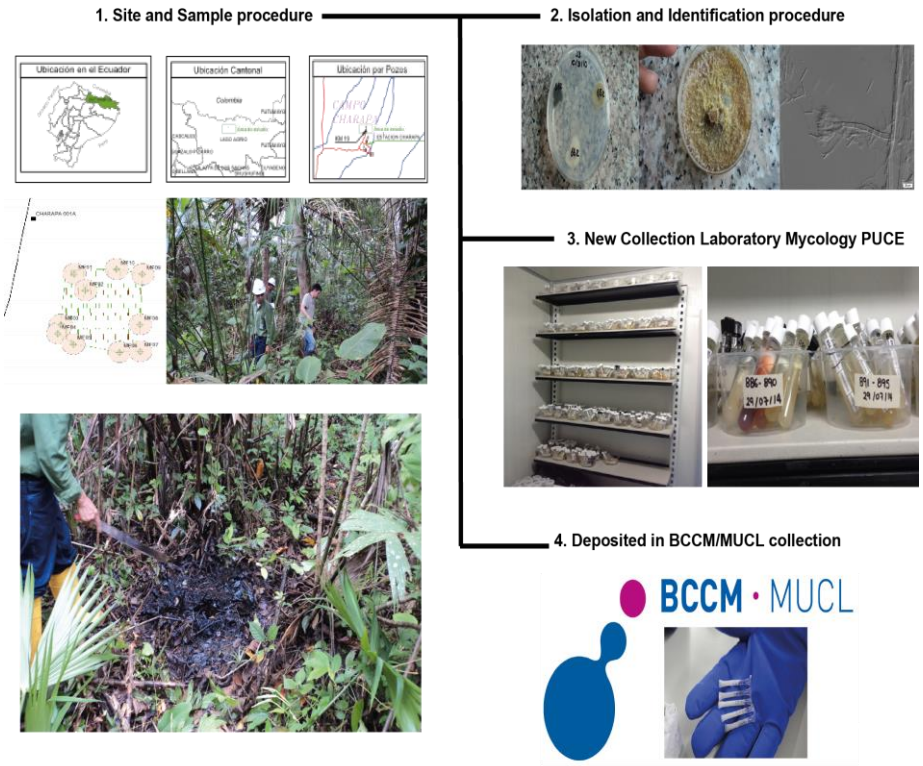


Fig. 1.1. Schematic representation of sampling process for isolation of fungi and deposited in safe collections.

Results

In the present study, 1077 strains were isolated. 779 strains were isolated from the rhizoplane and 298 from the internal tissue of roots. Half of the strains (i.e. 512) could be identified to genus level by morphological methods. The remaining strains remained sterile and could not be identified using morphology-based methods.

The majority of the strains were isolated from roots of Costaceae (i.e. *Costus scaber*, *Costus pulverulentu*, *Costus lima var. scabremarginatus*, etc.) and Araceaes (i.e. *Socratea exorrhiza, Philodendron* sp., *Anthurium* sp., etc) approx. 200 fungal strains from each family. Cyclanthaceae (i.e *Carludovica palmata Ruiz & Pav, Cyclanthus bipartitus*), Marantaceae (i.e. *Monotagma* sp., *Calathea* sp.), Dryopteridaceae (i.e. *Polybotrya* sp.) approx. 90 fungal strains were isolated from each family of plants. Approx. 10 fungal strains were isolated from Fabaceae (i.e. *Inga capitata* Desv.), Melastomataceae (e.i. *Tococa guianensis* Aubl., *Miconia cf. zubenetana*), Malvaceae (i.e. *Theobroma cacao*), Zingiberaceae (*Renealmia cf. thyrsoidea*), Acanthaceae (i.e. *Mendoncia* sp.), Heliconiaceae (i.e. *Heliconia chartacea*), Rubiaceae (i.e. *Psychotria stenostachya*), Poaceae (i.e. *Olyra latifolia*), Piperaceae (i.e. *Piper* sp.) . Finally, less than 10 strains were recovered from Boraginaceae (i.e. *Cordia alliodora*), Urticaceae (i.e. *Urera caracasana* and *Urera baccifera*), Cecropiaceae (i.e. *Pouruma* sp.), Euphorbiaceae (*Acalypha* sp.), Moraceae, Pterophyta (i.e. *Cyathea lasiosora*), Sapindaceae (i.e. *Paullinia* sp.), Leguminoceae (i.e. *Acacia sp*.).

On average, one fungal strain was isolated for each cm of roots analyzed, for plants located within the oil ponds, and 0.5 fungal strain / cm for plants located outside of pond. As far as the rhizoplane is concerned, each cm of root analysed yielded one strain for plants both inside and outside the pond.

Sixty-one genera have been identified. A group of endophytic fungi with melanised hyphae, known as Dark Septate Fungi (DSFs) (Girlanda *et al.*, 2007) and several Basidiomycetes could not be identified due to the lack of sporulation.

The genera *Cladosporium*, *Cylindrocarpon-like*, *Dactylonectria*, *Fusarium,* Gliocladium, *Metarhizium*, *Paecilomyces*, *Penicillium*, *Pestalotia*, *Trichoderma*, *Volutella* and *Xylaria* are the most frequently isolated (> 10 strains per genera, Table 1.2, Fig. 1.2). Other genera isolated at a lower level (< 10 strains) were *Acremonium*, *Alternaria*, *Aspergillus*, *Beauveria*, *Botryodiplodia* spp., *Codinea* spp., *Chloridium*, *Colletotrichum*, *Cunninghamella* spp., *Curvularia*, *Cylindrocladiella*, *Dendrosporium*, *Epicoccum* spp., *Gliocladiopsis* spp., *Mucor*, *Microsphaeropsis*, *Mycoleptodiscus*, *Myrothecium*, *Nectria*, *Parvothecium*, *Pithomyces* like, *Phialophora*, *Phoma* and *Verticillium* spp.

Several genera were represented by singleton: *Aquanectria*, *Coniothyrium*, *Cylindrocladium*, *Cylindrodendrum*, *Dinemasporium*, *Dichobotrys*, *Gelasinospora*, *Geotrichum*, *Hormographiella*, *Helminthosporium*, *Idriella*, *Kendrickiella*, *Leptosphaeria*, *Leptoxyphium*, *Mariannaea*, *Neurospora*, *Peziza*, *Phomopsis*, *Readeriella*, *Spegazzinia*, *Speiropsis*, *Sporothrix*, *Stachybotrys*, *Stilbella* and *Torula-like*. These genera have

cosmopolitan distribution growing in a wide range of substrates, mainly as colonizers of vegetation.

Table 1.2. Genera isolated from root and rhizoplane of plants that grow in crude oil in Charapa camp.

Phylum	Order	Family	Genus
Zygomycota	Mucorales	*Cunninghamellaceae*	*Cunninghamella*
		Mucoraceae	*Mucor*
Ascomycota	Amphisphaeriales	*Amphisphaeriaceae*	*Pestalotia*
	Botryosphaeriales	*Botryosphaeriaceae*	*Botryodiplodia*
	Capnodiales	*Capnodiaceae*	*Leptoxyphium*
		Cladosporiaceae	*Cladosporium*
		Mycosphaerellaceae	*Readeriella*
	Chaetothyriales	*Herpotrichiellaceae*	*Phialophora*
	Chaetosphaeriales	*Chaetosphaeriaceae*	*Chloridium*
			Codinea
			/Chaetosphaeria
			Dinemasporium
	Diaporthales	*Valsaceae*	*Phomopsis*
	Eurotiales	*Aspergillaceae*	*Aspergillus*
		Incertae sedis	*Kendrickiella*
		Trichocomaceae	*Paecilomyces*
			Penicillium
	Glomerellales	*Glomerellaceae*	*Colletotrichum*
		Plectosphaerellaceae	*Verticillium*
	Hypocreales	*Cordycipitaceae*	*Beauveria*
		Clavicipitaceae	*Metarhizium*
		Incertae sedis	*Acremonium*
		Hypocreaceae	*Gliocladium*
			Trichoderma
			Stilbella
		Nectriaceae	*Aquanectria*
			Cylindrocarpon
			Cylindrocladium
			Cylindrodendrum
			Cylindrocladiella
			Dactylonectria
			Fusarium
			Gliocladiopsis
			Mariannaea
			Nectria
			Volutella
		Stachybotryaceae	*Myrothecium*
			Parvothecium
			Stachybotrys
	Magnaporthales	*Magnaporthaceae*	*Mycoleptodiscus*
	Saccharomycetales	*Dipodascaceae*	*Geotrichum*

	Sordariales	*Sordariaceae*	*Gelasinospora*
			Neurospora
	Ophiostomatales	*Ophiostomataceae*	*Sporothrix*
	Pezizales	*Pyronemataceae*	*Dichobotrys*
		Pezizaceae	*Peziza*
	Pleosporales	*Coniothyriaceae*	*Coniothyrium*
		Didymellaceae	*Epicoccum*
			Phoma
		Didymosphaeriaceae	*Spegazzinia*
		Leptosphaeriaceae	*Leptosphaeria*
		Massarineae	*Helminthosporium*
		Pleosporaceae	*Alternaria*
			Curvularia
			Pithomyces -like
		Torulaceae	*Torula*-like
	Xylariales	*Xylariaceae*	*Xylaria*
		Microdochiaceae	*Idriella*
	Incertae sedis	*Dothideomycetes*	*Speiropsis*
	Incertae sedis		*Dendrosporium*
	Incertae sedis	*Pleosporineae*	Microsphaeropsis
	Dark septate Fungi		
Basidiomycota	Agaricales	*Psathyrellaceae*	*Hormographiella*

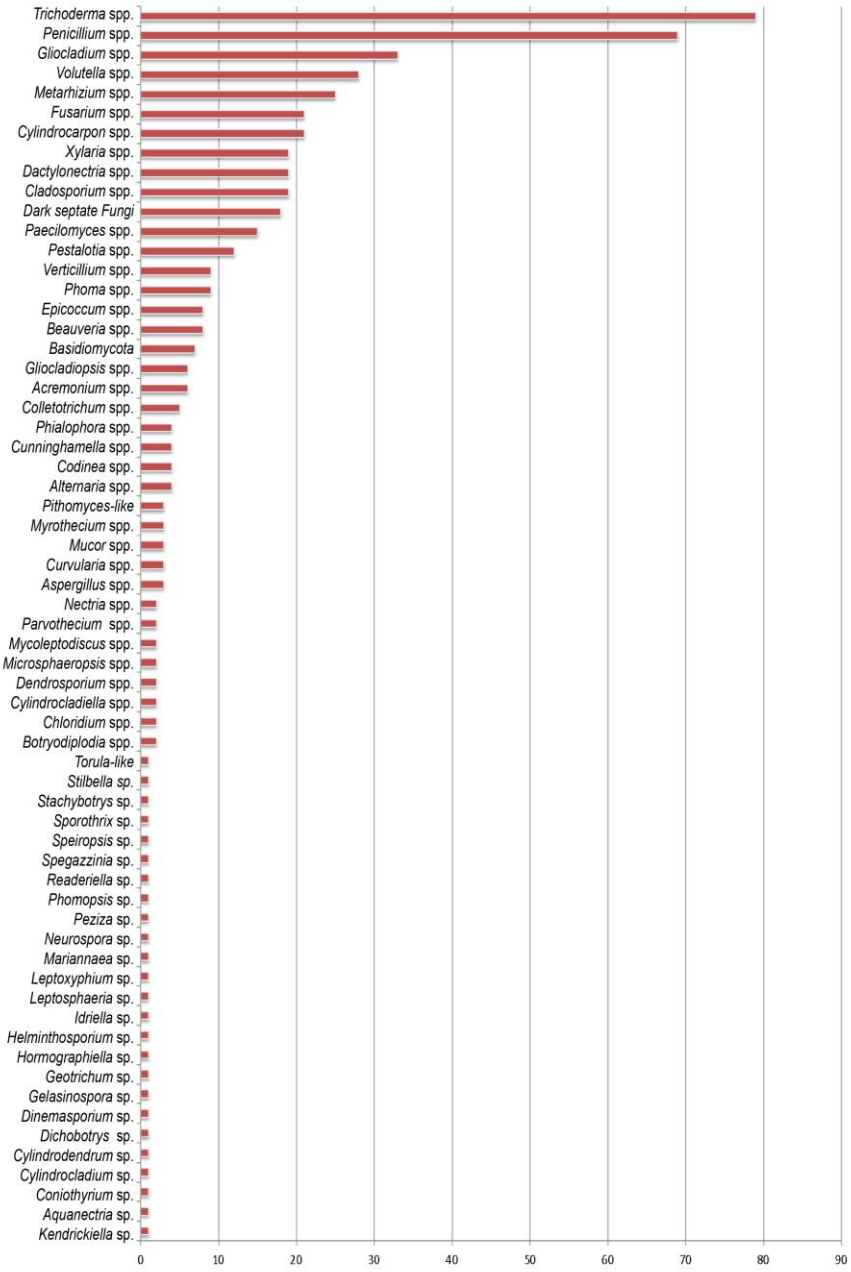


Fig. 1.2. Number of strains isolated from rhizoplane and roots of plants natural recolonized oil ponds.

Andrade & Franken (2013) mentioned that the most common fungal roots endophytes belong Ascomycota, of which mainly Xylariales and Hypocreales and the DSF. According to the number of strains isolated, *Trichoderma* spp., *Dactylonectria* spp. (Hypocreales) and *Xylaria* spp. (Xylariales) were the more frequently isolated in this study (Fig. 1.3).

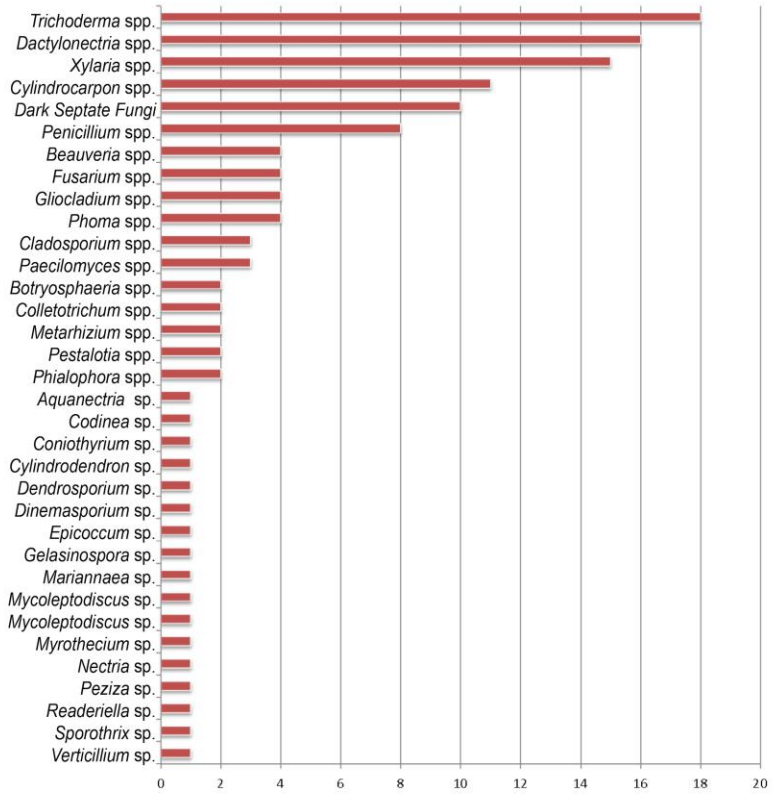


Fig. 1.3. Number of strains isolated from in site the root of plants natural recolonized oil ponds.

DNA sequencing of strains from the PUCE and BCCM/MUCL fungal culture collections revealed numerous taxa for which no name could be found. From these unnamed isolates, we selected three specific groups or complex of genera

for models taxonomic studies, applying the Consolidated Species Concept (Quaedvlieg *et al.*, 2014). They are the *Myrothecium* complex (sub-section 1.2.1), the *Cylindrocarpon* complex (sub-section 1.2.2), and the *Gliocladiopsis* / *Aquanectria* complex (sub-section 1.2.3).

Discussion

For years, mycologists have relied on culture-dependent methods to study the soil fungal diversity and their functional traits. In contrast, the recent development of metagenomics, mass sequencing, culture-independent methodologies allow exploring the soil fungal communities *in situ*, from both taxonomic and functional perspectives (Fierer *et al.*, 2012). These methodologies provide a more complete picture of the species and their functional diversity (Fierer *et al.*, 2012) .

Pernía *et al.* (2012) conducted the first meta-analysis of fungal diversity of crude oil polluted soil. The results showed that the majority of the identified “OTUs” belonged to the Ascomycota (83%), with a low representation of Zygomycota (10%), Glomeromycota (6%), and Basidiomycota (1%). Stefani *et al.* (2015) studied the fungal communities in contaminated soil, using both culture dependant and independent approaches. They also showed that Ascomycota was the dominant phylum, representing 65% of the OTUs. Ascomycota with the orders Saccharomycetales (12%), Hypocreales (10%), Pleosporales (9%), Sordariales (8%), Basidiomycota with Agaricales (13%), and Chytridiomycota with Spizellomycetales (6%) dominated the fungal community (Stefani *et al.*, 2015). Based on the culture dependent approach yielded mostly isolates of Ascomycota with

Hypocreales (40%) and Pleosporales (10%) (Stefani *et al.*, 2015).

In our study the Hypocreales (51%), Eurotiales (17%) and Pleosporales (6%) were the most frequent orders. While some members of the Basidiomycota were recorded in the molecular dataset, none were isolated according to Stefani *et al.* (2015). In our study Basidiomycota (1%) represent at a very low proportion.

Soil microbial diversity is often affected by the presence of hydrocarbon contamination. However, a tolerant and active community could emerge in these conditions (Stefani *et al.*, 2015).

As far as the rhizoplane is concerned, on average, one fungal strain / cm of root was isolated regardless of the level of localisation of sampling, inside or outside the ponds and consequently, the level of contamination. Uzona *et al.* (2015) reported in their study of rhizosphere fungi associated with two tree species (i.e*. Mangifera indica* and *Elaeis guineensis*) growing in oil-contaminated soil, a decrease of the fungal diversity from the unpolluted site through the polluted site. This could be attributed to the contamination, which affects soil properties and microflora (Uzona *et al.,* 2015). In our study both sites are contaminated, and although at different level, this does not seem to affect their fungal communities.

In the case of endophytes for each cm of internal tissue root, one strain was recovered from plant sampled within the pond and 0.5 from plant sampled outside the pond. In a similar research, Bourdel *et al.*, (2016) were able to isolate 41

endophytic fungi from 18 plants roots of two plant species (i.e. *Eleocharis erythropoda* and *Populus balsamifera*) growing in different soils containing high ranges of TPH contamination. According to their cultured-based isolation technique, they were able to isolated one strain per ~ 3 cm of root internal tissue, which is comparable to our results. Moreover, using a molecular approached for evaluation of fungal diversity, Bourdel *et al.*, (2016) revealed that there were no difference in richness between contaminated levels for either plant specie.

Garcés-Ruiz *et al.*, (2017) studied, concomitantly to our study, the arbuscular mycorrhizal species (AMF) associated with three plant species (i.e., *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*) from the same ponds in Ecuador. They showed that the roots had similar AMF diversity indices whatever the site, inside or outside the pond, or plant species selected. The nature of the endophytic fungi, and their localisation within the root growing in the intercellular spaces, protect then from environmental stresses such as oil contamination.

The most common fungi which have been recorded as a biodegrades belongs to following genera *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Pleurotus, Polyporus, Rhizopus, Rhodotolura, Saccharomyces, Talaromyces* and *Torulopsis* (Al-Nasrawi, 2012). Some of the genera isolated in our study correspond to these genera. Our fungal strains were also reported in works dealing with the microbiome from crude oil polluted soil. In the research by Rivera-Cruz *et al.* (2002), 46 oil-degrading fungal species were

isolated. Of these, 39 belonged to the genera *Trichoderma*, *Penicillium*, *Aspergillus*, *Mucor*, and *Paecilomyces*, whereas 7 remained unidentified. These fungi were obtained from the rhizosphere of grasses growing in soils polluted by oil leaks and spills from pipelines and pits. According to Chaillan *et al.* (2004), *Aspergillus* and *Penicillium* are the most commonly isolated genera in oil polluted tropical soils. In our study, *Trichoderma* was the most frequent genus in term of strains, followed by *Penicillium* is the second genus in term of number of strains whereas *Aspergillus* was less frequently isolated. Odriosolla *et al.* (2008), also reported *Aspergillus* sp. from contaminated soil in the Rio Grande harbour area, located in the Southern Brazil.

An important understudied group of endophytes are the so-called Dark septate fungi (DSF) which are a diverse group Ascomycetes colonizing plant roots. DSF have been reported from about 600 plant species (320 genera), in all ecosystems, from the tropics to arctic, or alpine habitats and now in roots of plants recolonizing oil ponds. It forms a group that functionally and ecologically overlaps with soil fungi or saprotrophic rhizosphere-inhabiting fungi (Jumpponen & Trappe, 1998). Some members of this group have mutualistic interactions with plants e.g. members of the form genus *Phialophora* (Jumpponen & Trappe, 1998; Jumpponen, 2001) have been hypothesized to develop mycorrhizal-like associations. Nevertheless, in absence of known nutrient-exchange interfaces, the nature of these interactions is uncertain (Jumpponen & Trappe, 1998).

Because of the potential role of DSF in the nutrition of host plants, it is important to understand the nature and nutritional

strategy of these fungi (Vergara *et al.*, 2017). Although the influence of DSF on its host is still under debate, the identification of DSF with biotechnological potential it could be hypothesized that these fungi would be adapted to accessing organic nutrient pools. Caldwell and Trappe (2000) demonstrated that DSF are capable of producing the necessary extra-cellular enzymes to degrade the major C, N and P polymers.

Rundell *et al.* (2015) reported a high diversity of endophytes in plants stems from different Ecuadorian ecosystems included the Amazonian rain forest. The authors found secondary metabolites produced by these fungi. Endophytes isolated in this thesis are a potential source for new metabolites. Endophytes are well known by their properties as antibacterial, antifungal, antiviral activities (Andrade & Franken, 2013).

Fungi that have been successfully isolated in axenic culture in this study are maintained in the PUCE collection. They could open the possibilities of different new researches, approaches from taxonomy, metabolism, antimicrobial activity, and many other aspects of their biology (Verkley *et al.*, 2015). In chapter 2, some of these fungi were evaluated in the context of biodegradation. It is also important to look forward for a more complete taxonomic description of these strains that will help to for increase the knowledge on mycological diversity in Ecuador.

1.2. In depth taxonomic studies based on testing the Consolidated Species Concept (CSC)

Taxonomic studies were long based on morphological characters. The classification of a fungus into a taxonomic rank was largely based on convergent morphologies. However, morphological characters may be often unclear due to hybridization, cryptic speciation and convergent evolution (Raja *et al.*, 2017). Molecular and DNA-based studies have substantially changed the process for determining fungal classes, sub-classes, orders, families, genera, and species (Jayasiri *et al.*, 2015).

Sordariomycetes (Pezizomycotina, Ascomycota) is one of the largest classes of Ascomycetes. Sordariomycetes are found worldwide, and inhabit terrestrial, freshwater, as well as marine habitats. The most comprehensive study of the class dates back from Kirk *et al*. (2008) whom provided new insight into its structure based on multigene phylogenetic analysis. The Sordariomycetes currently comprised of six subclasses, 32 orders, 105 families, and 1331 genera. (Maharachchikumbura *et al.*, 2016).

The Hypocreales (Sordariomycetes) includes several groups of fungi of major economic importance. For instance, *Trichoderma reesei* produces cellulase used for industrial purposes, *Fusarium venenatum* A3/5 is the source of a low cost mycoprotein (Rossman *et al.*, 1996; Seifert, 2000; Wiebe, 2002). Many species are important plant pathogens, as the Fusari, *Cylindrocarpon* spp., *Colletotrichum* spp. etc. They are found on a vast range of hosts and habitats worldwide. Currently, the order

comprised eight families, 240 genera and approximately 2700 species (Kirk *et al.*, 2008; Crous *et al.*, 2014). The vast majority of Hypocreales reproduce through asexual morphs, a feature which is often crucial for their identification (Lombard *et al.*, 2015). In this study 261 strains were isolated these remain to be more deeply studied from a taxonomic and physiological point of view.

Crous *et al.* (2014) first established the family Stachybotryaceae (Hypocreales) to accommodate the genera *Myrothecium*, *Peethambara*, and *Stachybotrys*, previously classified as *incertae sedis*. These three genera included approximately 210 species. In a comprehensive study of Stachybotryaceae, Lombard *et al.* (2016), identified 33 genera using multi-locus sequence analysis and morphological features. The members of this family are characterized by asexual morphs with mononematous to sporodochial or synnematous conidiomata, usually with phialidic conidiogenous cells that produce 0-1 septate conidia accumulating in hyaline, yellow, orange and, more commonly, various shade of green to black slimy masses[1]. Between our strains 6 belongs to this family (Lombard *et al.*, 2016).

Tulloch (1972) characterized *Myrothecium* (Stachybotryaceae) based on a basic set of morphological characters: sporodochial conidiomata, phialidic conidiogenesis, and conidia of varying shapes accumulating in a green mucoid drop; variable characters included the presence or absence of

[1] A few species produce however dry chain of conidia (e.g. *Memnoniella*).

marginal or tramal sterile “setae” or setoid hyphae and of a conidial, apical, cone-like mucilaginous appendage. However, this circumscription was questioned several times (Samuels & Rossman, 1979; Nag Raj, 1993, 1995a,b; Schroers *et al.*, 1999; Ahrazem *et al.*, 2000; Seifert *et al.*, 2003a; Decock *et al.*, 2008; Chen *et al.*, 2016).

Lombard *et al.* (2016) revised the genus based on morphological and multilocus phylogenetic data. They demonstrated that, *sensu* Tulloch (1972), *Myrothecium* was largely polyphyletic; 13 new Myrothecium-like genera (*Albifimbria*, *Capitofimbria* (monotypic), *Dimorphoseta*, *Gregatothecium*, *Inaequalispora* (monotypic), *Myxospora*, *Neomyrothecium*, *Paramyrothecium*, *Parvothecium* (monotypic), *Smaragdiniseta* (monotypic), *Striaticonidium*, *Tangerinosporium*, and *Xenomyrothecium* (monotypic).

The monotypic genera *Inaequalispora, Parvothecium*, and *Septomyrothecium*, along with *Virgatospora echinofibrosa*, *Peethambara sundara*, and *Albosynnema elegans*, form a distinct, well-supported lineage within the Stachybotryaceae (Lombard *et al.*, 2016).

The Nectriaceae are characterized by uniloculate ascomata that are white, yellow, orange-red or purple. They are associated with phialidic asexual morphs that produce amerosporous to phragmosporous conidia. Lombard *et al.* (2015) have presented the largest studies of nectriaceous fungi based on a very large sampling and a multi-locus phylogenetic analysis. They recognized 47 genera within Nectriaceae. Around 126 strains

within this family were identified by morphological characters though further studies are necessary for a fine characterization.

The phylogenetic relationships have therefore been established among the members of this family, also the phenotypic and ecological characters of genera in the *Nectriaceae* has been established by Lombard *et al.* (2015). The lineages of particular interest to our study, were represented in the tree inferred from the combined 10 genes sequence data set obtained by Lombard *et al.* (2015). From among the eighteen different clades, the Clade I composed by the genera *Penicillifer*, *Corallonectria*, *Dematocladium*, *Aquanectria, Gliocladiopsis*, which are characterised by their penicillate arrangement of fertile branches but do not all share the same ecological niche. Followed by the Clade IV formed by *Thelonectria*, *Cylindrocarpostylus*, *Rugonectria*, *Campylocarpon*, and *Mariannaea* and VI that nest *Neonectria*, *Ilyonectria*, *Cylindrodendrum* and *Dactylonectria*. These two clades include genera, with the exception of *Cylindrocarpostylus* and *Mariannaea*, having soil-borne cylindrocarpon-like asexual morphs. They are associated with basal rot and canker diseases of their plant hosts (Lombard *et al.*, 2015). These findings provide broad phylogenetic support and a foundation for taxonomic research on our study.

Lombard and Crous (2012) provided the most comprehensive review of the genus *Gliocladiopsis*, as a result of this study, two species were accepted as two different taxa: *G. sagariensis* (reinstated as the type species) and *G. tenuis*; another five species *G. curvata*, *G. elghollii* (USA), *G.*

indonesiensis and *G. Mexicana* and *G. pseudotenuis*, were also added to the genus. Liu and Cai (2013) added *G. guangdongensis* based on a single isolate originating from southern China. The latest report was published by Parkinson *et al.* (2017), describing three new species: *G. peggii*, *G. whileyi*, and *G. forsbergii*, isolated from avocado roots, in Australia.

Aquanectria is the closest phylogenetic relative of *Gliocladiopsis* (Lombard *et al*., 2015). *Aquanectria* would differ in its longer, filiform to slightly sinuous conidia (Lombard *et al*., 2015), which may represent an adaptation to aquatic habitats (Baschien *et al.*, 2013); *Aquanectria* thus far includes only two species: *A. penicillioides* (type species) and *A. submersa* (Lombard *et al.,* 2015), which are both aquatic fungi that grow on submerged plant debris (Ingold, 1942; Hudson, 1961; Duarte *et al.*, 2012).

The study continued on the family Nectriaceae clades (IV, VI), and considered some *Cylindrocarpon*-like species. These species are soil-borne cosmopolitan fungi found on various substrates such as saprophyte, some of which cause plant diseases, including tree cankers, black foot, or root rot (Samuels & Brayford, 1994; Halleen *et al.*, 2004, 2006; Hirooka *et al.*, 2005; Castlebury *et al.*, 2006; Chaverri *et al.*, 2011; Cabral *et al.*, 2012b,a; Lombard *et al.*, 2014).

The species were recognized using molecular phylogenetic approaches. *Neonectria* – *Cylindrocarpon* have been repeatedly shown to be polyphyletic. Halleen *et al.* (2004) first segregated *Campylocarpon* into two cylindrocarpon-like species: *C. fasciculare* and *C. pseudofasciculare*. *Campylocarpon* has no

known sexual morph. Both *C. fasciculare* and *C. pseudofasciculare* have thus far been observed only in association with grapevines (Halleen *et al.*, 2004; Abreo *et al.*, 2010; Correia *et al.*, 2013; Dos Santos *et al.*, 2014).

Chaverri *et al.* (2011) provided a comprehensive review of the genus *Cylindrocarpon*, in which they implemented extensive morphological and multi-locus phylogenetic analyses. In addition to *Campylocarpon* and *Neonectria*, they recognized three additional lineages at the genus level: *viz*. *Ilyonectria*, *Rugonectria* and *Thelonectria*. Subsequently, additional studies have revealed that *Ilyonectria sensu* Chaverri *et al.* (2011) is not monophyletic (Cabral *et al.*, 2012b,a; Lombard *et al.*, 2013), and *Dactylonectria* has therefore been introduced to accommodate species which had largely been isolated from diseased grapevines from a variety of regions where they are cultivated (Lombard *et al.*, 2014). Only three *Dactylonectria* species have been isolated from plants other than grapevines thus far: *viz*. *D. anthuriicola* and *D. hordeicola*, which were isolated from *Anthurium* sp. (Araceae) and *Hordeum vulgare* (Poaceae), respectively (Lombard *et al.*, 2014), and *D. pinicola*, which was isolated from *Pinus laricio* (Lombard *et al.*, 2014).

1.2.1. Myrothecium-like (Ascomycota, Hypocreales) species from tropical areas: *Digitiseta* gen. nov. and additions to *Inaequalispora* and *Parvothecium*

Adapted from the research article published in

Mycological Progress (2017)

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Preface

Comprehensive fungal diversity studies associated with plants include the formal recognition of the taxa. The fungal assessment of fungal diversity in oil ponds in the Amazonian rain forest, based mainly on culture-dependent methodologies, yielded many distinct strains which identity have been searched for.

This paper is a revision of some myrothecium-like strains isolated from oil pond in the rain forest (**section 1.1)**, and several isolates held unidentified at the MUCL fungal collection. In previous studies of myrothecium-like species, 13 genera and a bunch of species were recognized (Lombard *et al*., 2016).

This study (**sub-section 1.2.1)** revealed the occurrence of two undescribed species among these new genera accepted by Lombard *et al*. (2016), *viz*. *Inaequalispora* (*I. prestonii*.) and *Parvothecium* (*P. terrestre*). Besides, *Myrothecium setiramosum, M. dimorphum*, and several unidentified strains formed the base of a new lineage, which was worst recognized as a new genus (*Digitiseta* gen. nov.).

Abstract

Inaequalispora and *Parvothecium* are two myrothecium-like, closely related genera of Hypocreales. They are also morphologically similar, sharing sporodochial conidiomata, penicillate conidiophores, fusiform to elipsoidal conidia accumulating in a green slimy drop, and hypha-like setoid extensions emerging through the conidial mass. During a revision of myrothecium-like isolates originating from rainforest areas of South America (Ecuador, Brazil) and Southeast Asia (Singapore), multilocus phylogenetic inferences (based on DNA sequence data of ITS, partial nuc 28S, and partial *tef1a*, *rpb2* and *tub2*) and morphological studies concordantly revealed the occurrence of two undescribed species of *Inaequalispora* (*I. longiseta* sp. nov. and *I. cylindrospora* sp. nov.) and one undescribed species of *Parvothecium* (*P. amazonense* sp. nov.). *Myrothecium setiramosum*, *M. dimorphum*, and two undescribed taxa form the base of a new lineage, sister to the current *Parvothecium* lineage. This lineage is recognized as *Digitiseta* gen. nov., typified by *D. setiramosa* comb. nov. *Digitiseta dimorpha* comb. nov. is also proposed, and the new species *D. parvodigitata* sp. nov. and *D. multidigitata* sp. nov. are described.

Keywords Phylogeny . Systematics . Ecuador . Brazil . Singapore

1. Introduction

Myrothecium, whose interpretation was for a long time based on the monographic revision of Tulloch (1972), was

characterized by a basic set of morphological characters: a sporodochial (to occasionaly synnematous) conidioma, phialidic conidiogenesis and variably shaped conidia accumulating in a green mucoid drop (Tulloch, 1972). Variable characters included the presence or absence of marginal or tramal sterile setae or setoid hyphae and of a conidial, apical, conelike mucilaginous appendage (Tulloch, 1972; Nag Raj, 1993, 1995a,b). Its circumscription was nevertheless questioned several times (Samuels & Rossman, 1979; Schroers *et al.*, 1999; Ahrazem *et al.*, 2000; Seifert *et al.*, 2003a; Decock *et al.*, 2008; Chen *et al.*, 2016), and phylogenetic inferences have shown that this basic set of characters was not apomorphic of a monophyletic *Myrothecium* lineage; on the contrary, this combination of characters is distributed in several lineages representing distinct genera (Decock *et al.*, 2008; Chen *et al.*, 2016; Lombard *et al.*, 2016). Lombard *et al.* (2016) proposed the most comprehensive revisión of the genus, implementing detailed morphological and multi-locus phylogenetic analyses; in addition to *Myrothecium* s.s. (the *M. inundatum* lineage), 13 myrothecium-like genera were recognized (Lombard *et al.*, 2016).

Among these new genera, the monotypic *Inaequalispora* was introduced to accommodate *Myrothecium prestonii* (=*Inaequalispora prestonii*), a little known species originally described from Southeast Asia (Tulloch, 1972). This species was confirmed as a single-species lineage, related to *Septomyrothecium uniseptatum*, as shown previously (Decock *et al.*, 2008; Chen *et al.*, 2016). The monotypic *Parvothecium*, typified by *P. terrestre*, was also introduced in the vicinity of *Inaequalispora* and *Septomyrothecium*.

Inaequalispora, *Parvothecium* and *Septomyrothecium* are morphologically very similar, sharing, in addition to myrothecium-like conidiomata, hypha-like setoid extensions protruding through the conidial mass. These setoid extensions are thick-walled in *Inaequalispora* and *Septomyrothecium* and thin-walled in *Parvothecium* (Lombard *et al.*, 2016). Conidia are aseptate, ~5–10 µm long, in *Inaequalispora* and *Parvothecium* (Lombard *et al.*, 2016) and one-septate, ~15– 20 µm long, in *Septomyrothecium* (Matsushima, 1971; Decock *et al*., 2008).

Inaequalispora, *Parvothecium* and *Septomyrothecium* together with *Virgatospora echinofibrosa*, *Peethambara sundara* and *Albosynnema elegans* form a distinct, well supported lineage within the Stachybotryaceae (Lombard *et al.*, 2016).

Previously, Decock *et al*. (2008) showed that *Myrothecium setiramosum* together with several unnamed myrothecium-like isolates originating from Southeast Asia were related to *I. prestonii* (under *Myrothecium prestonii*) and to several species of *Septomyrothecium* (*S. uniseptatum, S. maraitiense*, and *Septomyrothecium* sp.). These species formed a presumed *Septomyrothecium* clade (Decock *et al.*, 2008). Lombard *et al.* (2016) did not address the taxonomic position and affinities of *M. setiramosum* but, nevertheless, suggested that it could be accomodated in a genus on its own.

In this frame, we re-evaluated the phylogenetic afinities, generic placement and identity of a set of *M. setiramosum* and myrothecium-like strains originating from Southeast Asia (cf. Decock *et al.,* 2008), and additional isolates from South America. As a result, two additional species of *Inaequalispora*, *I.*

cylindrospora and *I. longiseta*, and one species of *Parvothecium*, *P. amazonense*, are described as new. *Digitiseta* gen. nov. is introduced for *M. setiramosum*, with *D. setiramosa* comb. nov., *D. dimorpha* comb. nov., and two additional undescribed taxa, *D. parvidigitata* and *D. multidigitata*.

2. Materials and methods

Site and sample procedure

The strains from Ecuador were isolated from the rhizoplane and roots of plants growing in a superficial layer of organic debris covering oil-ponds in the Amazonian rainforest (Sucumbíos Prov., Charapa camp, approx. 00°11′49″ S, 76°48′57″W and 00°11′46″ S, 76°48′54″ W). The strains from Singapore were isolated from conidia of myrothecium-like colonies emerging from decaying leaves submerged in freshwater (Mac Ritchie Reservoir, approx. 1°20′22″ N, 103°49′31″ E). Several strains from Brazil and Venezuela were received by courtesy of R.F. Castañeda (INIFAT, Cuba).

Morphological characterization

Each inoculum (mycelial plug/germinating conidia) was placed in the center of a 9-cm Petri dish on Potato Dextrose Agar (PDA) and Banana Leaf Agar (BLA) (Nirenburg, 1981; Untereiner *et al.*, 1998). Cultures were incubated at 20 ± 2 ºC with a 12/12-h incident near UV light/dark cycle. Cultural characteristics were determined 7–14 days after inoculation. Colors were coded according to (Kornerup & Wanscher, 1978). The descriptions of the conidiomata are, as a rule, based on those developed on BLA.

DNA isolation, amplification and analyses

DNA was extracted from mycelium grown in liquid malt extract at 20 ± 2 ºC in the dark. Extractions were carried out using innuPREP Plant DNA kit (Analytik Jena, Germany) following the manufacturer's recommendations.

DNA sequences were determined for a fragment of the genes encoding β-tubulin (*tub2*, region between exons 3 and 4), translation elongation factor 1-α (*tef1a*, part of the largest exon), RNA polymerase II second largest subunit (*rpb2*, domains 6 and 7, region between exons 3 and 5), internal transcribed spacer region (ITS1–5.8S–ITS2 region) and nuc 28S ribosomal RNA (28S, region comprising the D1-D3 domains). Reference sequences were selected in Lombard *et al.* (2016) and downloaded from GenBank. Amplifications of the ITS were performed with the primer pair ITS5/ITS4 (White *et al.*, 1990). The *tef1a* gene was amplified using the primer pair EF1-983F/EF1-2218R (Rehner & Buckley, 2005). The fragment of the *tub2* gene was amplified with primer pair Bt2a and Bt-2b (Glass & Donaldson, 1995), the fragment of the *rpb2* gene with primer pair RPB2-5F2 (Sung *et al.*, 2007) and fRPB2-7cR (Liu *et al.*, 1999), and the fragment of the 28S with primer pair LR0R (Rehner & Samuels, 1994) and LR6 (Vilgalys & Hester, 1990). The PCR parameters were as described in Lombard *et al.* (2010c). Sequencing was performed by Macrogen (Seoul, Korea) using the same primers as used for amplification. The amplicons were sequenced in both directions. Raw sequences were edited with Sequencher® software v.5.1 (Gene Codes Corporation, Ann Arbor).

The alignment of the nucleotide sequences was performed with MAFFT v.7.213 (Katoh & Standley, 2013) and manually

corrected in PhyDE-1 (Müller *et al.*, 2006) when necessary.

The final alignment comprises 3682 positions (including gaps) distributed as follows: 28S: 1034 positions; ITS: 582 positions; *tef1a*: 969 positions; *tub2*: 351 positions; *rpb2*: 746 positions. However, several sections of the ITS (26 positions in the ITS2) and *tub2* (21 positions located in the intron 3) were not confidently alignable and were excluded from the analysis. The *tef1a* sequence is missing for *Parvothecium terrestre* CBS 198.89 and CBS 534.88 and *Peethambara sundara* CBS 646.77.

Phylogenetic analyses

The phylogenetic analysis included 26 taxa (Table 1). *Peethambara sundara* was designated as out-group, following Lombard *et al*. (2016). Phylogenetic analyses were performed under both parsimony and probabilistic hypotheses, using maximum parsimony (MP) as implemented in PAUP* 4.0b10 (Swofford, 2003), Bayesian inference (BI) as implemented in MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) and maximum likelihood (ML) using RAxML 7.0.4 (Stamatakis, 2014). The general time-reversible model (GTR), using a distribution of rates at variable sites modeled on a discrete gamma distribution with four rate classes, was estimated as the best-fit likelihood model of evolution for ML.

For the BI, the Kimura (K80) model using the proportion of invariant sites was estimated as the best-fit likelihood model of evolution for nuc rDNA ITS and 28S regions, and the GTR model on a discrete gamma distribution with four rate clases was estimated as the best-fit likelihood model of evolution for *tub2*, *tef1a*, and *rpb2*, using PartitionFinder (Lanfear, 2012).

For MP analyses, gaps were treated as missing data. The most parsimonious trees were identified using heuristic searches with 1000 random addition sequences, further evaluated by bootstrap analysis, retaining clades compatible with the 50% majority rule in the bootstrap consensus tree. Analysis conditions were tree bisection addition branch swapping, the starting tree obtained via stepwise addition, steepest descent not in effect, and MulTrees effective. Clades with bootstrap support values (BS) above 90% were considered strongly supported by the data.

Bayesian analyses were implemented with two independent runs, each with four simultaneous independent chains for 4 million generations, starting from random trees, and keeping one tree every 1000th generation. All trees sampled after convergence (average standard deviation of split frequencies <0.01, confirmed using Tracer v.1.4; (Rambaut & Drummond, 2007), were used to reconstruct a 50% majority-rule consensus tree (BC) and to estimate posterior probabilities. The posterior probability (BPP) of each node was estimated based on the frequency at which the node was resolved among the sampled trees with the consensus option of 50% majority rule (Simmons *et al.*, 2004). Clades with BPP above 0.95 were considered strongly supported by the data.

ML searches conducted with RAxML involved 1000 replicates under the GTRGAMMAI model. In addition, 1000 bootstrap (ML BS) replicates were run with the same model. Clades with maximum likelihood bootstrap values of 85% or greater were considered to be significantly supported.

3. Results

For the Bayesian analysis, the base frequencies for *tub2*,

tef1a, and *rpb2* using the GTR+G model are A = 0.22, C = 0.30, G = 0.26, T = 0.21, and gamma distribution shape parameter is 0.178. For ITS and 28S region, using the K80+I model, the base frequencies are A = 0.24, C = 0.25, G = 0.27, T = 0.22 and the proportion of invariable sites of 0.912. The two Bayesian runs converged to stable likelihood values after 20,100 generations. The first 10% of saved trees were discarded as the burn-in phase. For the MP analysis, 3019 positions were constant, 132 variable but uninformative and 379 parsimony-informative. The heuristic search yielded two most parsimonious trees [length 952 steps, consistency index (CI) 0.64, retention index (RI) 0.82]. In the ML searches with RAxML, the combined dataset aligment had 511 distinct patterns with a proportion of gaps and undetermined characters of 4.65%.

The topology of the trees is highly concordant whichever methology is used (MP, BI, ML). Four well-supported lineages are shown (Figure. 1) in all analyses. *Inaequalispora prestonii*, together with several unnamed isolates (MUCL 48321, MUCL 48282, 48165 and 48121), form a well-supported *Inaequalispora* lineage (Figure. 1; BSP = 97, BSML = 99, PP = 1). This lineage is subdivided into three clades, i.e. the *I. prestonii* branch (Lombard *et al.*, 2016) and two new species clades or phylogenetic species, represented by MUCL 48321 on one side and MUCL 48282, 48165 and 48121 on the other side.

Parvothecium terrestre and several unnamed isolates (i.e. MUCL 48084, 54664 and 54636) together form a second well-supported *Parvothecium lineage* (Figure. 1; BSP = 88, BSML = 100, PP = 1). This lineage is subdivided into two clades, i.e. the *P. terrestre* branch represented by the ex-type strain only

(Lombard *et al.*, 2016), and a new phylogenetic species represented by the MUCL isolates 48084, 54664 and 54636 (Figure. 1; BSP = 100, BSML = 100, PP = 1).

Several isolates (i.e. MUCL 41187, 48180, 48260 and 48271) tentatively identified (Decock *et al.*, 2008) or received as *M. setiramosum*, and which, a priori, belong to a unique morphospecies, together form a third well-supported *M. setiramosum* lineage (Figure. 1; BSP = 100, BSML = 100, PP = 1). The type strain of *M. dimorphum* (Watanabe *et al.*, 2003) and the strain *P. terrestre* CBS 534.88 also nest within this lineage. This lineage is then subdivided into four clades, each equated to a phylogenetic species. One clade corresponds to *M. dimorphum*, which is well characterized phenotypically (Watanabe *et al.*, 2003). A second clade is formed by the strain *P. terrestre* CBS 534.88, indicating that it is not conspecific with *P. terrestre*, contrary to the conclusion of Lombard *et al*. (2016); (the position of this strain is identical, whatever the sequence dataset used, i.e. the MUCL or the Lombard *et al*. 2016 dataset). Two clades host the MUCL *M. setiramosum* strains, i.e. MUCL 41187 on one side and MUCL 48180, 48260 and 48271 on the other side; this indicates that the previous morphospecies concept of *M. setiramosum* encompassed two phylogenetic species.

Septomyrothecium uniseptatum, together with *S. maraitiense* and several unnamed isolates from various geographic origins, form an additional lineage (Figure. 1; BSP = 100, BSML = 100, PP = 1). This *Septomyrothecium* lineage is sister to the *Inaequalispora* lineage, as shown previously (Decock *et al.*, 2008; Chen *et al.*, 2016; Lombard *et al.*, 2016).

Septomyrothecium sp. *1* (MUCL 55084) originating from French Guiana, *Septomyrothecium* sp. *2* (MUCL 51298) originating from Uganda, and *Septomyrothecium* sp. *3* (MUCL 41081) and *Septomyrothecium* sp. *4* (MUCL 41240), both originating from Venezuela, form four distinct long branches, each likely representing a distinct phylogenetic species. These will be discussed elsewhere.

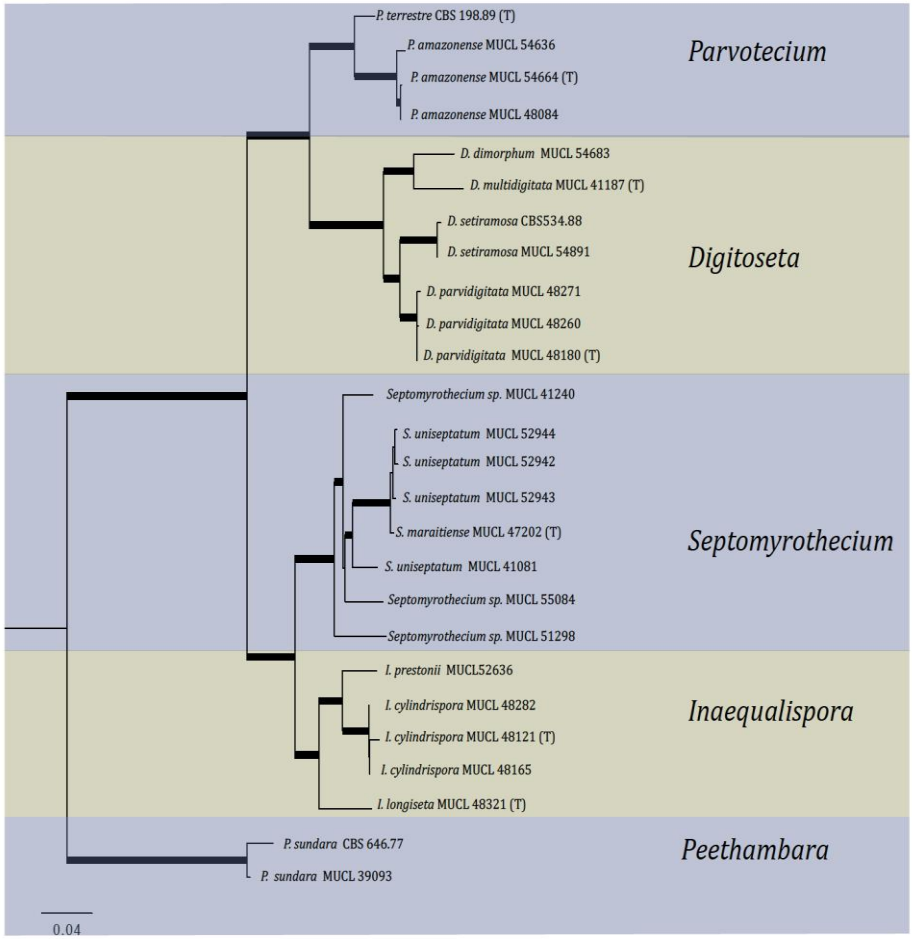


Figure 1. The ML consensus tree inferred from the combined fivegene sequence alignment. Branches with bootstrap support of ≥90%

for Maximum Likelihood (ML) and Maximum Parsimony (MP) and Bayesian posterior probabilities of ≥0.90 (PP) are indicated with bold lines. T type; IT isotype; A authentic

Taxonomic conclusions

Inaequalispora and *Parvothecium* were erected for two myrothecium-like species, I*. prestonii* and *P. terrestre,* forming two distinct clades in multilocus phylogenetic inferences (Lombard *et al.*, 2016). Morphologically, both genera share basic morphological features including sporodochial conidiomata, conidiogenous apparatus, conidiogenesis, conidia accumulating in a green mucoid drop and morphologically (and ontogenetically) related hypha like setoid extensions. They were distinguished by the wall thickness of their setoid extensions, respectively thick- and thin-walled. Both genera are so far monotypic. Inaequalispora and *Parvothecium* are related to *Septomyrothecium*, a myrothecium-like genus distinguished from other myrothecium-like genera in having longer (∼15–20 µm long) 1-septate conidia (Matsushima, 1971).

The present study, using multilocus phylogenetic inferences and a morphological approach, concordantly reveals two additional species of *Inaequalispora* and an additional species of *Parvothecium*. These three phylogenetic species also correspond to three distinct morphotypes, which are differentiated by their conidial (shape and size) and setal (size) features, hence defining concordantly phylogenetic and morphological species. They are described below as *I. longiseta*,

I. cylindrospora and *P. amazonense.*

The case of *M. setiramosum* is more critical and its generic placement could be questioned. The phylogenetic inferences revealed that the current *M. setiramosum* morphospecies concept is polyphyletic. A posteriori, the critical re-examination of the *M. setiramosum* strains and of *P. terrestre* CBS 534.88 revealed some phenotypical singularities, especially as far as the conidial size and the branching of the setoid extensions are concerned, allowing to define concordantly three other phylogenetic and morphological species, in addition to *M. setiramosum* s.s.

These species are obviously not congeneric with *M. inundatum*, as shown previously (Decock *et al.*, 2008; Chen *et al.*, 2016). Lombard *et al*. (2016) suggested that they might belong to a separate genus. The *M. setiramosum* and the current *Parvothecium* lineages are sister clades (Figure. 1), and the latter could host *M. setiramosum* and its relatives. Yet, a priori, the affinity of *P. terrestre* CBS 534.88 with strains of *M. setiramosum* and *M. dimorphum* (Figure. 1) would argue for this option. The resulting, expanded *Parvothecium* would thus include species whose setoid extensions are apically either unbranched and tapering (Lombard *et al.*, 2016) or branched and digitated (Castañeda Ruiz, 1986; Watanabe *et al.*, 2003).

Nonetheless, CBS 534.88 was initially deposited at the CBS by R.F. Castañeda under *M. setiramosum*. This strain, presumably, should have produced sporodochia whose setoid extensions were apically digitated, as originally described in *M. setiramosum* (Castañeda Ruiz, 1986). The re-examination of CBS 534.88 shows that it is degenerated and poorly sporulating;

scarce, aborted sporodochia developed late, only on BLA, and never fully matured, eventually producing few conidia accumulating in small, hyaline mucoid drops. Nevertheless, these aborted sporodochia developed setoid extensions, whose apices are short-branched and digitated (Figure. 2), confirming its original identification. CBS 534.88 is thus an authentic strain of *M. setiramosum*.

Consequently, all the strains (and species) included in the *M. setiramosum* lineage (Figure. 1) have thick-walled, apically digitated, hypha-like setoid extensions (Castañeda Ruiz, 1986; Watanabe *et al.*, 2003). In these features, they would, therefore, differ from the strains and species of the *Parvothecium* lineage, which are characterized by sporodochia with unbranched and thin-walled setoid extensions (Lombard *et al.*, 2016; see Figure. 7, below).

On these phylogenetic and morphological grounds, which show two lineages, each well charaterized phenotypically, we would consider the *M. setiramosum* lineage as the basis of a distinct genus, following the suggestion of Lombard *et al*. (2016). It is described below as *Digitiseta* gen. nov., typified by *Digitiseta setiramosa* comb. nov., and including *D. dimorpha* comb. nov., *D. parvidigitata* sp. nov. and *D. multidigitata* sp. nov.

Taxonomy

Digitiseta Gordillo & Decock, gen. nov.

MycoBank: MB 820512.

Similar to *Parvothecium* and *Inaequalispora*, from which it differs in having setoid hypha-like extensions apically, variably short-branched, digitated.

Type species: Digitiseta setiramosa (R.F. Castañeda) Gordillo & Decock.

Etymology: "*Digitiseta*" (Lat.): referring to the digitate ápices of the setoid hypha-like exensions.

Asexual morph: Conidiomata sporodochial; *sporodochia* delimited by a white margin, topped by a green mucoid mass of conidia, from which emerge setoid, hypha-like extensions; base stromatic, with a textura angulata, from which arise conidiophores and setoid hypha-like extensions; *conidiophores* macronematous, mononematous, in dense clusters, biverticillate, with metulae and phialides; *conidiogenesis* phialidic; *phialides* cylindrical to digitate; *conidia* one-celled, cylindrical, slightly asymmetrical in side view, the base slightly truncate, the apices rounded, hyaline to pale greenish, accumulating in a green mucoid drop; *secondary conidiogenesis* occasionally present at the tip of the digitated branches of the setoid extensions that may transform into a conidiogenous locus; *secondary conidia* small, globose; *setoid hypha like* extensions borne from basal hyphae or, occasionally, laterally on the conidiophore, erect, protruding through and above the conidial mass, hyaline, septate, thick walled, ending variably short-branched, digitated.

Sexual morph: unknown.

Remarks

Digitiseta is morphologically related to *Parvothecium*, which is also, for the time being, its closest relative from a phylogenetic perspective (Figure. 1). It also looks much like *Inaequalispora*. These genera share the sporodochial conidiomata, morphologically and ontogenetically related setoid hypha-like

extensions protruding through the conidial mass, phialidic conidiogenesis and one-celled conidia accumulating in a green mucoid drop. They differ mainly by the termination of their setoid extensions, which have short apical branches in *Digitiseta* and are unbranched in *Inaequalispora* and *Parvothecium*.

A secondary microconidial form was reported in *Digitiseta dimorpha* [2]. In this species, the tips of the digitated branches could transform into a conidiogenous locus, producing small, globose conidia (Watanabe *et al.*, 2003). We have not been able to reproduce this feature in our study of the ex-type strain (MAFF 238296). It was not observed in other species of *Digitiseta* (see below).

Occurrence of secondary microconidia has been described in other genera of asexual ascomycetes. For instance, Becerra-Hernandez *et al.*, (2016) described secondary conidia in *Gyrothrix verticiclada* (Goid.) S. Hughes & Piroz. In this species, secondary conidia are also borne from the very apices of conidiomatal setae that may transform into conidiogenous loci. This phenomenon might, in fact, be environment-dependent.

Digitiseta setiramosa (R.F. Castañeda) Gordillo & Decock, comb. nov. (Figure. 2a–c).

MycoBank: MB 820513.

Basionym: Myrothecium setiramosum R.F. Castañeda, Deuteromycotina de Cuba, Hyphomycetes IV (La Habana): 10 (1986), MycoBank MB130173.≡*Septomyrothecium setiramosum*

[2] *Digitiseta dimorpha* (Ts. Watan.) Decock & Gordillo, comb. nov. (MB820832; basionym: *Myrothecium dimorphum* Ts. Watan.,Mycoscience 44: 284 (2003), MB489474).

(Castañeda) Decock, Cryptogamie, Mycologie 29(4): 328 (2008), MycoBank MB537060.

Culture characteristics: colonies on PDA reaching 12 mm diam. in 7 days, overall orange-white (6A3), remaining sterile; on BLA, mycelium white, effused, and with little developed conidiomata.

Conidiomata sporodochial, few, scarce; *sporodochia* appearing late, little developed, superficial, circular to ellipsoid, up to 1 mm diam. and high, topped by a small, hyaline, mucoid mass of conidia, from which emerge a few setoid, hypha-like extensions; *stroma* with a *textura angularis*, the basal hyphae hyaline, septate; *conidiophores* in clusters, erect, arising from basal hyphae, each composed of a basal stipe and an apical conidiogenous penicillus; *stipe* hyaline, thin-walled, septate; *conidiogenous penicillus* biverticillate with 1 whorl of 3–4 *metulae*, cylindrical to clavate, 8.0–10.5 × 2–3 µm, each giving rise to 1 apical whorl of 3–5 *phialides*, cylindrical, finger-like, straight to slightly incurved inward the penicillus, 7.0–11.5 × 1.5–2 µm; *conidia* cylindrical to slightly asymmetrical, the base slightly truncate, the apices rounded, hyaline to pale greenish (as seen in mass), aseptate, thin- and smooth walled, (6–)7.0–8(−8.5) × 1.5 µm, av. = 7.7 × 1.5 µm, accumulating into a green mucoid mass; *hypha-like setoid* extensions borne from the stromatal hyphae, erect, protruding through the conidial mass, hyaline, thick-walled, septate, overall 60–140 µm long and 1–3 µm wide, the apices short branched, with 3–6 digitated, thick-walled branches.

Sexual morph: unknown.

Substratum and habitat: dead leaf, in leaf litter, forest, known

from *Andira inermis* (W. Wright) DC. (Fabaceae) and *Eugenia glabrata* (Sw.) DC. (Myrtaceae).

Holotype (not seen): CUBA. Matanzas, San Miguel de los Baños, leaf litter of *Eugenia glabrata*, 25 Jun 1985, R.F. Castañeda, INIFAT C85/104.

Authentic strain: CUBA. Santiago de las Vegas, Ciudad de La Habana, leaf litter of *Andira inermis*,9 Jul 1987, collected by R.F. Castañeda, isolated by R.F. Castañeda, INIFAT C87/ 234 = CBS 534.88 = MUCL 54891.

Remarks

The holotype *of D. setiramosa* originates from Cuba (Castañeda Ruiz, 1986). It was not available for study and our description is based on CBS 534.88 (= INIFAT C87/ 234 =MUCL 54891), which is an authentic strain, also originating from Cuba. CBS 534.88 very likely represents *D. setiramosa* s. str. In order to fix the interpretation of the species, this strain might be designated as epitype.

However, given that it is degenerated and poorly sporulating, and thus, although the few sporodochia observed present all the features of the species except for the green mass of conidia, we refrain from designing it as epitype, waiting to have a better sporulating isolate.

Watanabe *et al.* (2003) described *Digitiseta dimorpha* (as *M. dimorphum*) based on a single isolate originating from Japan. The species is also characterized by short-digitated setoid extensions (Watanabe *et al.*, 2003), similar to those found in *D. setiramosa*. The affinities of these two species are confirmed by the present phylogenetic inferences (Figure. 1), hence the new

combination proposed above.

Digitiseta dimorpha differs from *D. setiramosa* and all other species of *Digitiseta* (cf. below) mainly by the shape and size of its conidia, which are ellipsoidal to ovate, 5–8 × 2–2.6 µm (Watanabe *et al.*, 2003). All other species of *Digitiseta* produce cylindrical, longer and narrower conidia, ~7.0–9.0 × 1.5 µm.

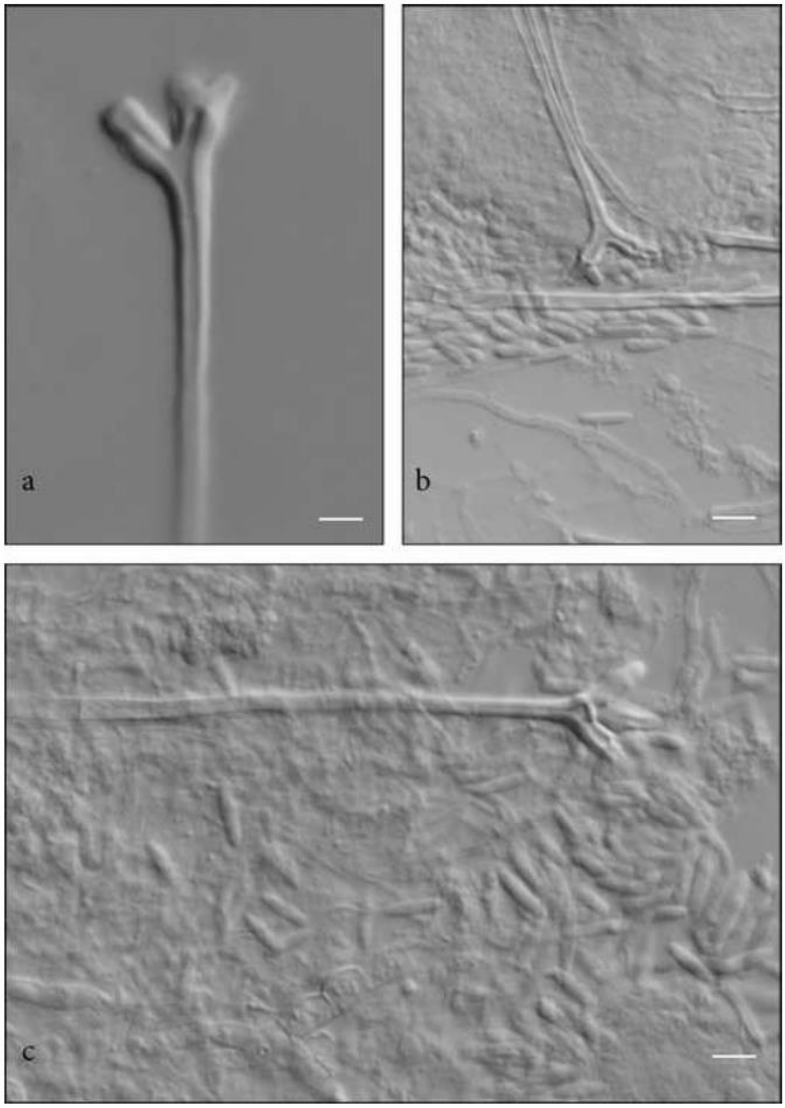


Figure 2. a–c. *Digitoseta setiramosa* (MUCL54891) from the epitype, MUCL54891 a–c. Hyphal-like setoid extensions (scale bars: Figs. a–c = 10 µm). All from cultures on BLA.

Digitiseta multidigitata Decock & Gordillo, sp. nov. (Figure. 3a–c).

MycoBank: MB 820514.

Similar to *D. setiramosa* from which it differs in having larger conidia, mostly 8–9.5 × 1.5 µm, av. = 8.5 × 1.5 µm) and densely, multidigitated setoid hypha-like extensions, with branches of primary and secondary orders.

Holotype: BRAZIL: Mata Atlantica, Joao Pessoa, dead leaf, unidentified angiosperm, collected and isolated by R.F. Castañeda, Sep. 1997, INIFAT C98/76 = MUCL 41187, as dried 2-week-old culture and slides prepared from sporodochia on BLA (living culture ex-holotype MUCL 41187).

Etymology: "multidigitata" (Lat.): referring to the densely branched apices of the setoid extensions.

Culture characteristics: on PDA, colonies reaching 20 mm diam. in 7 days, orange-white (6A3); On BLA, mycelium white, effused

Conidiomata sporodochial; *sporodochia* appearing after 2– 3 weeks, superficial circular to ellipsoid up to 5–12 mm high and 4–10 mm diam., stromatic, superficial, with a white margin surrounding an olivaceous-green (5F3), slimy mass of conidia;

stroma with a *textura angularis,* the basal hyphae hyaline, septate; *conidiophores* in dense clusters, erect, arising from basal hyphae, each composed of a basal stipe and an apical conidiogenous penicillus; *stipe* cylindrical, hyaline, thin-walled, septate; *conidiogenous penicillus* biverticillate, with 1 whorl of 3–4 *metulae*, cylindrical to clavate, or slightly bi-convex, 12–20 × 2 µm, each giving rise to an apical whorl of 3–5 *phialides*, cylindrical, finger-like 10–15 × 1–1.5 µm; conidia aseptate, cylindrical to slightly asymmetrical, base slightly truncate, apex rounded, hyaline thin- and smooth walled, (7.5–)8–9.5(−10.0) × 1.5 µm, av. = 8.5 × 1.5 µm, accumulating into a dark green mucoid mass; *hypha-like setoid extensions* thick-walled, septate, borne from the basal mycelium, hyaline, overall 108–220 µm long and 2 µm wide, apically polytomous with of 6–8 short, digitated, thin- to thick-walled branches, simple or once-branched.

Sexual morph: unknown.

Substratum and habitat: decaying leaf, Neotropical rainforest.

Remarks

Digitiseta multidigitata is closely related to *D. dimorpha* (Figure. 1), from which it differs in having differently shaped, longer and narrower conidia (cf. above). *Digitiseta multidigitata* differs from *D. setiramosa* in having slightly larger conidia (8–9.5 µm long, av. = 8.5 µm vs. 7–8 µm long, av. = 7.7 µm) and from *D. parvidigitata* (see below) in having densely branched apices of its setoid extensions, mostly with 6–8 branches in one or two whorls.

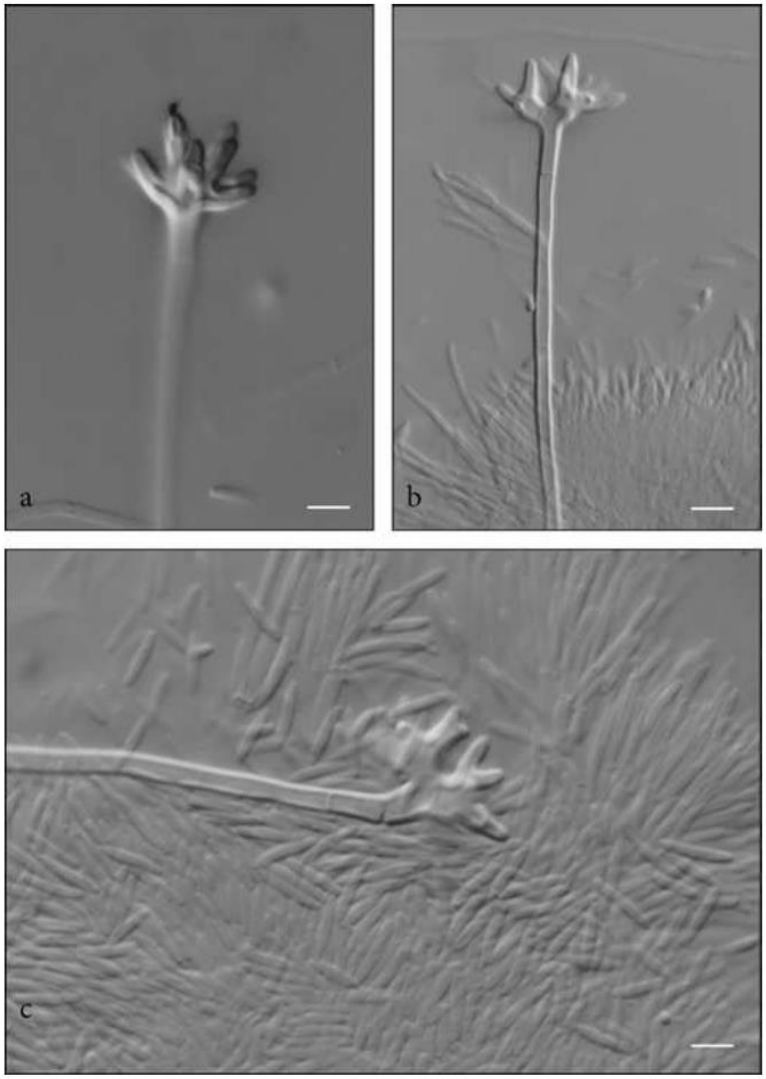


Figure 3. a–c. *Digitoseta multidigitata* (MUCL41187) from the type, MUCL41187 a–c. Hyphal-like setoid extensions (Figs. a–c, scale bars = 10 µm). All from cultures on BLA.

Digitiseta parvidigitata Decock & Gordillo, sp. nov. (Figure. 4 a–c).

MycoBank: MB 820515.

Similar to *D. setiramosa* from which it differs in having larger conidia, 8.0–9.0(10.0) × 1.5 µm, av. = 8.5 × 1.5 µm, and poorly branched setoid hypha-like extensions, commonly with 2–3 digitate processes.

Holotype: SINGAPORE: Mac Ritchie Reservoir, dead leaf, unidentified angiosperm, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock from colonies on natural substrate, dried 2-week-old culture and slides prepared from sporodochia on BLA (living culture ex-holotype MUCL 48180).

Etymology: "*parvidigitata"* refers to the small branched apices of the setoid extensions.

Culture characteristics: colonies on PDA reaching 16 mm diam. in 7 days, overall white at the beginning, then turning pale orange; on BLA, mycelium white, effused.

Conidiomata sporodochial; *sporodochia* appearing after 2– 3 weeks, superficial, circular to ellipsoid, up to 1–2 mm high and 2 mm diam., with a slimy mass of conidia olivaceous green (7F3), from which emerge setoid, hypha-like extensions; *stroma* with a *textura angularis*, the basal hyphae hyaline, septate; *conidiophores* in dense clusters, arising from basal hyphae, erect, each composed of a basal stipe and an apical conidiogenous penicillus; *stipe* hyaline, thin-walled, septate; conidiogenous penicillus biverticillate, with 1 whorl of 3–4 *metulae,* cylindrical to clavate, or slightly bi-convex, 8.0–12.0 × 1.5–2 µm, each giving rise to 1 apical whorl of 3–5 *phialides*, cylindrical, finger-like, 8–11.5 × 1–1.5 µm; *conidia* cylindrical to slightly asymmetrical, the base slightly truncate, the apex

rounded, hyaline thin- and smooth-walled, 8.0–9.0(−10.0) × 1.5 µm, av. = 8.5 × 1.5 µm, accumulating into a dark green mucoid mass; *hypha-like setoid extensions* borne from the basal mycelium and occasionally from a conidiophore, erect, protruding through the conidial mass, thick walled, septate, hyaline, overall 100–160 µm long × 2 µm wide, with terminal polytomous structures composed of 2–3 digitate thin- to slightly thick-walled, branches 3–10 µm long.

Sexual morph: unknown.

Substratum and habitat: dead leaf submerged in freshwater, rainforest.

Additional specimens examined: SINGAPORE: Mac Ritchie Reservoir, dead leaf, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock from colonies on natural substrate, MUCL 48271; ibid., MUCL 48260.

Remarks

Digitiseta parvidigitata is closely related to *D. setiramosa* (Figure. 1), from which it differs in having small branched apices, with only 2–3 digitate processes, rarely once short-dichotomous, against 3–6 in *D. setiramosa*. *Digitiseta multidigitata* differs in having profusely branched ending, with 6–8 digitate processes, in one or commonly two rows.

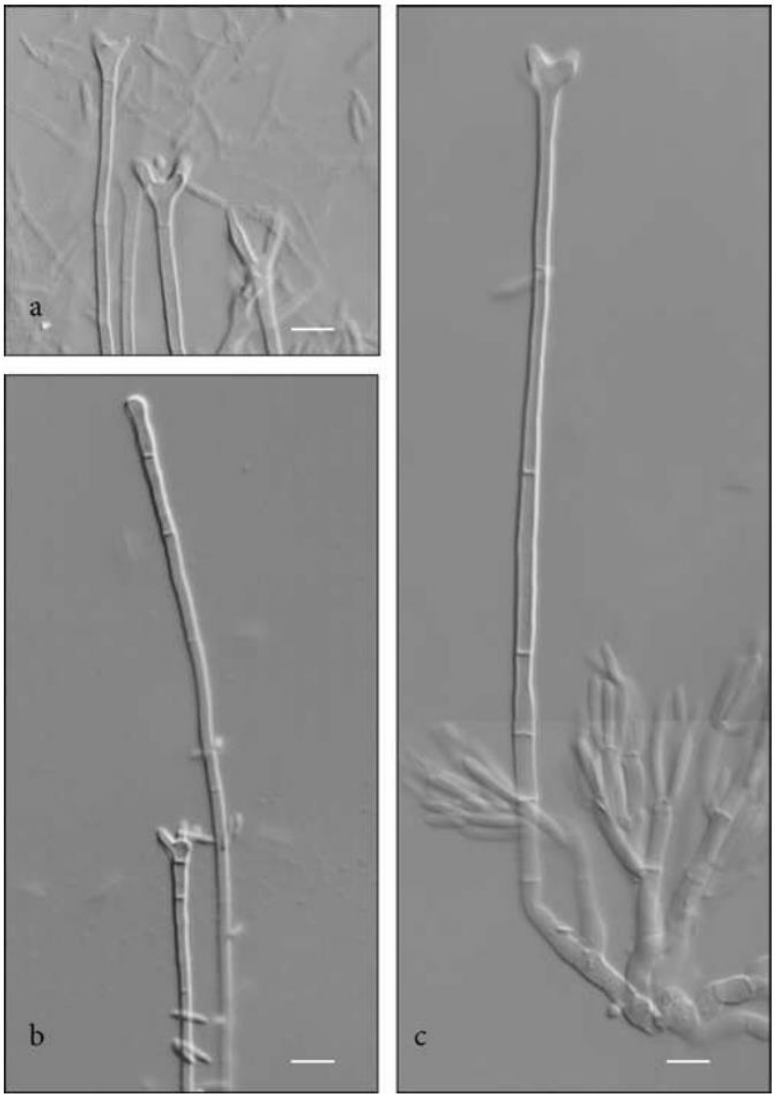


Figure 4. a–c. *Digitoseta parvidigitata* from the type, MUCL48180. a–c. Hyphal-like setoid extensions, conidiophores (Figs. a–e, scale bars = 10 µm). All from cultures on BLA.

Inaequalispora cylindrospora Decock & Gordillo, sp. nov.

(Figure. 5a–d).

MycoBank: MB 820516.

Holotype: SINGAPORE: Mac Ritchie Reservoir, decaying submerged leaf, unidentified angiosperm, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock from conidia from colonies on natural substrate, MUCL 48121 as dried 2-week-old culture and slides prepared from colonies on BLA agar (living culture ex-holotype MUCL 48121).

Etymology: "cylindrospora" (L.), referring to the cylindrical conidia.

Culture characteristics: colonies on PDA reaching 30 mm diam. in 7 days, white first, turning orange-white; on BLA, mycelium white, effused.

Conidiomata sporodochial; sporodochia appearing after 2–3 weeks, superficial, circular to ellipsoid up to 2–4 mm high and 2–3 mm diam., stromatic, with a slimy olivaceous-green mass of conidia (5E3) from which setoid, hypha-like extensions emerge; *stroma* poorly developed, hyaline, with *textura angularis*, the basal hyphae hyaline, septate; conidiophores in dense clusters, arising from basal, short hyphae, erect, each composed of a basal stipe and an apical conidiogenous penicillus; stipe hyaline, thin-walled, septate; conidiogenous penicillus biverticillate apex with a whorl of 3–4 *metulae*, 5–12 × 2–2.5 µm, each giving rise to an apical whorl of 3–4 *phialides*, cylindrical, finger-like, narrowed at the tip, with a collarette, 10–15 × 1.5 µm, av. = 11.9 × 1.5 µm; *conidia* aseptate, cylindrical, base truncate, apex acute and slightly curved, hyaline, thin- and smooth-walled, small apical mucilaginous appendage variably present, (6–)7.0– 8.5(−9) × 1.5

µm, av. = 7.8 × 1.5 µm, accumulating into a dark green mucoid mass; *hypha-like setoid extensions* usually borne from the basal hyphae, occasionally from a conidiophore, erect, protruding through the conidial mass, regularly septate, progressively thicker-walled upwards, hyaline, smooth, straight with rounded ends, 160–360 µm long × 1.5–2 µm wide.

Sexual morph: unknown.

Substratum and habitat: leaf submerged in freshwater, rainforest.

Additional specimens examined: SINGAPORE: Mac Ritchie Reservoir, decaying, submerged plant material, unidentified angiosperm, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock from conidia from colonies on natural substrate, MUCL 48282; ibid. MUCL 48165.

Remarks

Inaequalispora cylindrospora is similar to *I. longiseta* and *I. prestonii*, from which it differs in having longer conidia (7.0–8.5 × 1.5 µm vs. 6.0–7.5 × 1–1.5 µm, and 4–7 × 2.0–2.5 µm, fide Nag Raj 1995b). *Inaequalispora longiseta* also differs in having longer setoid extensions, 160–560 µm long (160–360 µm long in *I. cylindrospora*).

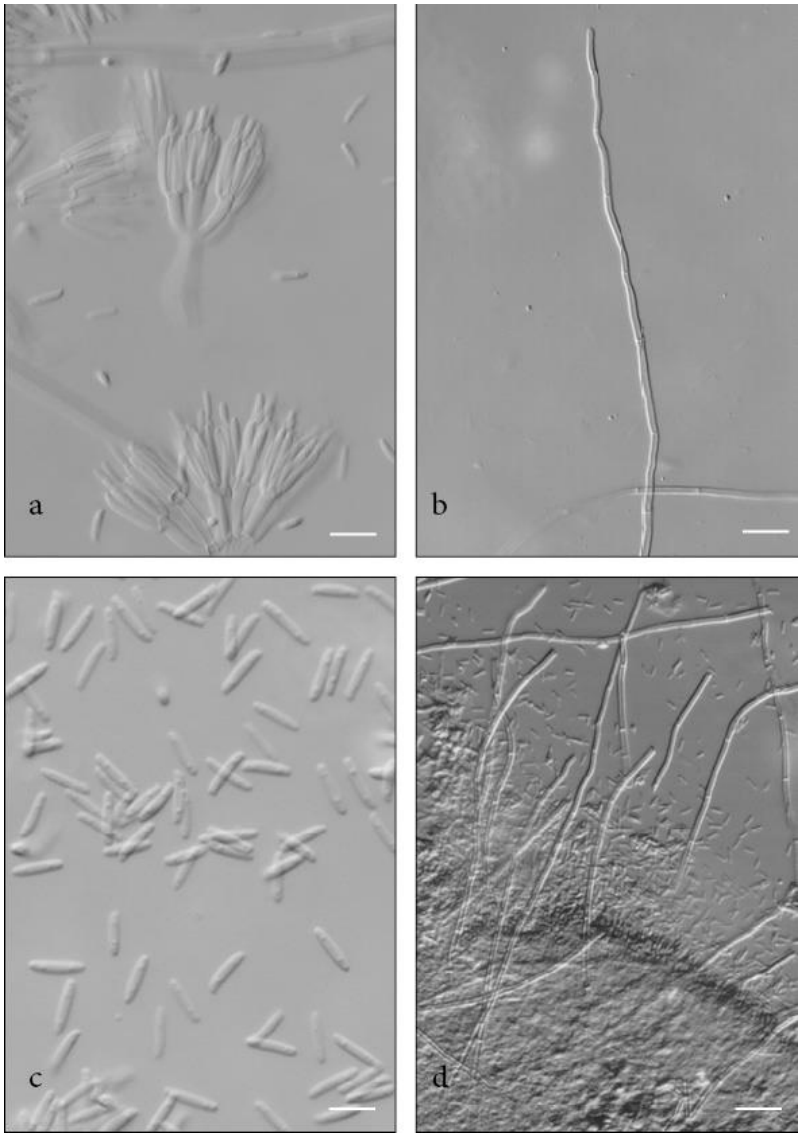


Figure 5. a–d. *Inaequalispora cylindrispora*, from the type, MUCL48121. a. Conidia b-d Hyphal-like setoid extensions; c. Conidiophores; (Figs. a–d, scale bars = 10 µm). All from cultures on BLA

Inaequalispora longiseta Decock & Gordillo, sp. nov. (Figure. 6 a–d).

MycoBank: MB 820517.

Similar to *I. prestonii* from which it differs in having longer setoid hypha-like extensions, 160–560 µm long, and fusiform to narrowly ellipsoidal, conidia.

Holotype: SINGAPORE: Mac Ritchie Reservoir, decaying, submerged plant material, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock from conidia of colonies emerging from the leaf, MUCL 48321, as dried 2-week-old culture and slides of sporodochia on BLA (type culture MUCL 48321).

Etymology: "longiseta" (Lat.), referring to the long hypha like setoid extensions.

Culture characteristics: *colonies* on PDA reaching 35 mm diam. in 7 days, overall white; colonies on BLA mycelium white, effused.

Conidiomata sporodochial; *sporodochia* appearing after 1–3 weeks, superficial, circular to ellipsoid or irregular, up to 3–4 mm high and 2–4 mm diam., with a slimy olivaceous-green mass of conidia (5E3) from which setoid, hypha-like extensions emerge; *stroma* poorly developed, hyaline, with *textura angularis*, the basal hyphae hyaline, septate; conidiophores in dense clusters, arising from basal, short hyphae, erect, each composed of a basal stipe and an apical conidiogenous penicillus; *stipe* hyaline, thin-walled, septate; penicillus biverticillate, bearing a whorl of 3–4 *metulae*, cylindrical to clavate or slightly bi-convex, 10–15 × 2.5 µm, each giving rise to an apical whorl of 3–4 *phialides*,

cylindrical, hyaline, smooth, finger-like, 10–15 × 2.5 µm, straight to very slightly incurved, becoming narrowed at the tip, with a collarette, 10–12.5 × 2 µm; conidia aseptate, fusiform to ellipsoidal, base narrowly truncate, apex acute, slightly curved, hyaline, thin and smooth-walled, (5.0–)6.0–7.5(−8) × (1.0–)1.5 µm, av. = 6.7 × 1.4 µm, accumulating into a dark green mucoid mass; *hypha-like setoid extensions* numerous, borne from the basal hyphae and protruding through the conidial mass, regularly septate, progressively thicker-walled upwards, hyaline, smooth, straight, occasionally bent at the apices, 160– 560 µm long and 1.5–2 µm wide.

Sexual morph: unknown.

Substratum and habitat: dead leaf submerged in freshwater, rainforest.

Remarks

Inaequalispora longiseta is distinguished from *I. prestonii* in having longer setoid hypha-like extensions (respectively 160–560 µm and up to 200 µm fide Lombard *et al.* 2016) and fusiform to narrowly ellipsoidal, longer and narrower conidia (6.0–7.5 × (1–) 1.5 µm, av. = 6.7 × 1.4 µm vs. 4.0–7.0 × 2–2.5 µm, av. = 5.6 × 2.2 µm, fide Nag Raj 1995b). Funnel-shaped mucoid apical appendages were described on conidia of *I. prestonii* (Nag Raj, 1995b). This mucoid appendage is variably present on the conidia of *I. cylindrospora*, and was not observed on conidia of *I. longiseta.*

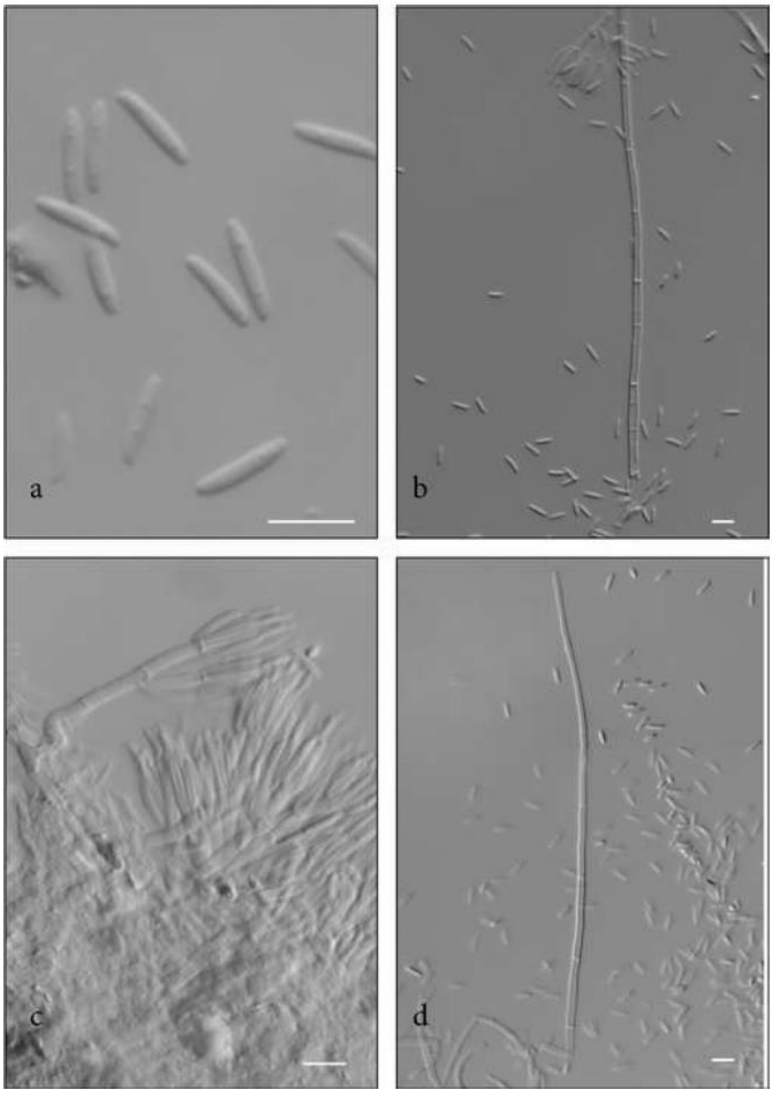


Figure 6. a–d. *Inaequalispora longiseta*, from the type, MUCL48321. a. Conidiophores; b-d Hyphal like setoid extensions; c. Conidia; (Figs. a–d, scale bars = 10 µm). All from cultures on BLA.

Parvothecium amazonense Gordillo & Decock, sp. nov.

(Figure. 7a–c).

MycoBank: MB 820518.

Holotype: ECUADOR: Prov. Sucumbíos, Nueva Loja city, Lago Agrío canton, Charapa camp, secondary rainforest, from the rhizoplane of *Theobroma cacao*, Jan 2013, A. Gordillo & C. Decock, MUCL 54664, as dried 2-week-old culture and slides prepared from colonies on BLA agar (living culture exholotype MUCL 54664, PUCE PHPE 161).

Etymology: "*amazonense"* (Lat.), referring to the type locality, the Amazonian forest.

Culture characteristics: colonies on PDA, reaching 40 mm diam. in 7 days, white with orange; on BLA, mycelium white, effused.

Conidiomata sporodochial; *sporodochia* superficial, circular to ellipsoid or irregular, up to 4–9 high and 4–8 mm diam., with a slimy olivaceous-green mass of conidia (7F3), emerge setoid, hypha-like extensions emerge; *stroma* well developed, hyaline, with *textura angularis*; conidiophores in dense clusters, arising from basal hyphae, erect, each composed of a basal stipe and an apical conidiogenous penicillus; *stipe* hyaline, thin-walled, septate; penicillus biverticillate, with a whorl of 3–4 *metulae*, 6.5–12.5 × 1.5–2.5 µm each giving rise to a apical whorl of 3–5 *phialides,* cylindrical, finger-like, 8.5–14 × 1–1.5 µm; *conidia* aseptate, cylindrical, base slightly truncate, apex rounded, with a small apical, obconical, mucilaginous appendage, thin- and smooth-walled, hyaline to pale greenish, accumulating into a dark green mucoid mass (5.0–)6–7(−7.5) × 1–1.5 µm, av. = 6.5 × 1.4 µm; *hypha-like setoid extensions* long, borne from basal

hyphae, thin-walled and septate, distally rounded, hyaline, smooth, overall 60– 400 µm long and 2 µm wide.

Sexual morph: unknown.

Substratum and habitat: growing in the rhizosphere of *Theobroma cacao*, secondary rainforest.

Additional specimens examined: ECUADOR: Prov. Sucumbíos, Nueva Loja city, Lago Agrío canton, Charapa camp, secondary rainforest, rhizoplane of *Theobroma cacao*, Jan 2013, A. Gordillo & C. Decock, MUCL 54636; SINGAPORE: Mac Ritchie Reservoir, decaying, submerged leaf, unidentified angiosperm, Aug 2006, collected by Olivier Laurence (Mycosphere), isolated by C. Decock, MUCL 48084.

Remarks

Parvothecium amazonense and *P. terrestre* are closely related (Figure. 1) but morphologically distinct. *Parvothecium amazonense* has larger conidia compared to those of *P. terrestre*, i.e. mainly 6–7 × 1–1.5 µm, av. = 6.5 × 1.4 µm, vs. 4–5 × 2–3 µm, av. = 4 × 2 µm (Lombard *et al.,* 2016). These authors also mentioned verrucose metulae and phialides in *P. terrestre*, a feature not observed in *P. amazonense*.

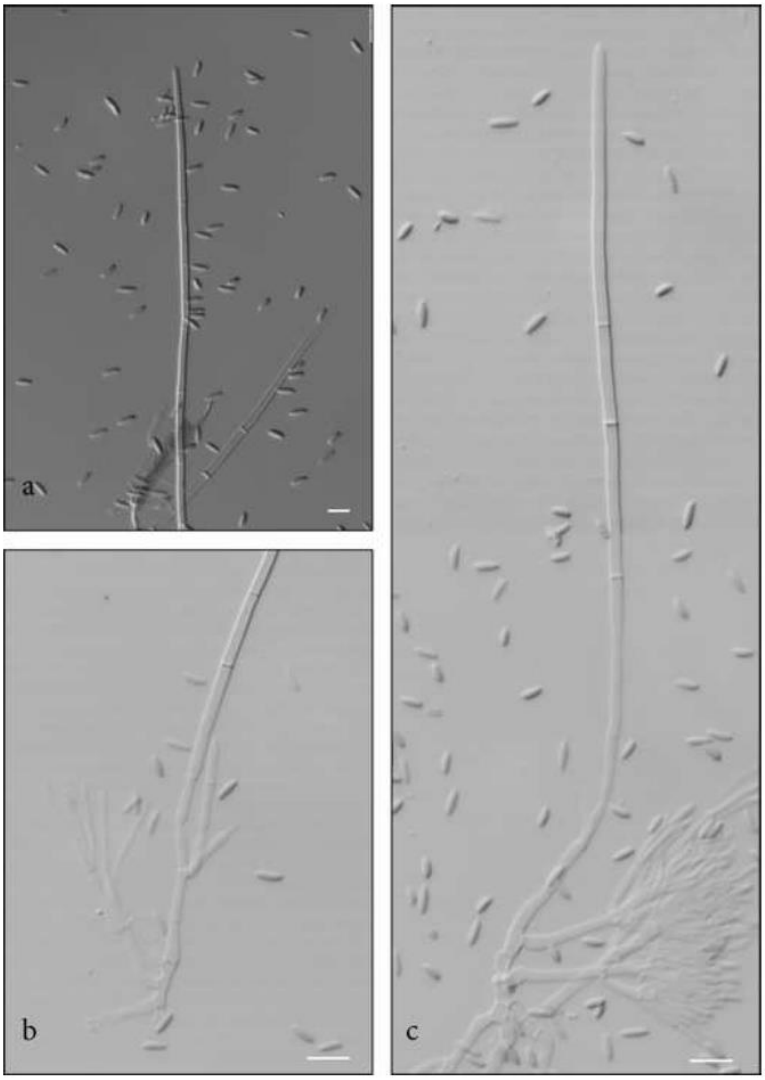


Figure 7. a–c. *Parvothecium amazonense*, from the type, MUCL54664. a–c. Hyphal-like setoid extensions, conidia and conidiophores (Figs. a–c, scale bars 10 µm). All from on BLA cultures.

Table 1. List of species, collections, and sequences used in the phylogenetic analyses

Genus and species names	Substrate	Country	GenBank accession number				
Voucher specimens/cultures reference			**ITS**	**LSU**	***rpb2***	***tef1-α***	***tub2***
Digitiseta Gordillo & Decock							
Digitiseta dimorpha (Ts.Watan.) Decock & Gordillo							
MUCL 54683 (T)		Japan	KY389329	KY389349	KY389367	KY769935	KY366460
Digitisetamultidigitata Decock & Gordillo							
MUCL 41187 (T)	Rotten leaf	Brazil	KY389325	KY389345	KY389363	KY769934	KY366456
Digitiseta parvidigitata Decock & Gordillo							
MUCL 48180 (T)	Dead leaf	Singapore	KY389326	KY389346	KY389364	KY769931	KY366457
MUCL 48271	Dead leaf	Singapore	KY389327	KY389347	KY389365	KY769932	KY366458

MUCL 48260	Dead leaf	Singapore	KY389328	KY389348	KY389366	KY769933	KY366459
Digitiseta setiramosa Gordillo & Decock							
MUCL 54891 = CBS 534.88	Leaf litter	Cuba	KU846472	KU846494	KU846511	KY769930	KU846552
= INIFAT C87/234 (A)							
Inaequalispora L. Lombard & Crous							
Inaequalispora cylindrospora Decock & Gordillo							
MUCL 48165	Submerged leaf	Singapore	KY389319	KY389339	KY389357	KY769925	KY366449
MUCL 48121 (T)	Submerged leaf	Singapore	KY389320	KY389340	KY389358	KY769924	KY366450
MUCL 48282	Submerged leaf	Singapore	KY389321	KY389341	KY389359	KY769923	KY366451
Inaequalispora longiseta Decock & Gordillo							
MUCL 48321 (T)	Submerged leaf	Singapore	KY389318	KY389338	KY389356	KY769926	KY366448
Inaequalispora prestonii (M.C. Tulloch) L. Lombard & Crous							
MUCL 52636 (IT)	Forest soil	Malaysia	KY389317	KY389337	KY389355	KY749867	KY366447

Parvothecium L. Lombard & Crous							
Parvothecium amazonense Gordillo & Decock							
MUCL 54636	Cacao rizosphere	Ecuador	KY389322	KY389342	KY389360	KY769927	KY366452
MUCL 54664 (T)	Cacao rizosphere	Ecuador	KY389323	KY389343	KY389361	KY769928	KY366453
MUCL 48084	Submerged leaf	Singapore	KY389324	KY389344	KY389362	KY769929	KY366454
Parvothecium terrestre L. Lombard & Crous							
CBS198.89 (T)	Soil	Brazil	KU846468	KU846489	KU846506	−	KU846548
Peethambara sundara Subram. & Bhat							
MUCL39093 = CBS 521.96	Dead twig	Nepal	KU846470	KU846491	KU846508	KY779740	KU846550
CBS 646.77	Dead twig	India	KU846471	AF193245	KU846509	−	KU846551
Septomyrothecium Matsush.							
Septomyrothecium maraitiense Decock							
MUCL 47202 (T)	Decaying leaf	French Guiana	KY389330	KU846493	KU846510	KY769915	KY366461

Septomyrothecium sp. 1							
MUCL 55084	Contaminant	French Guiana	KY389334	KY389352	KY389370	KY769919	KY366465
Septomyrothecium sp. 2							
MUCL 51298	Legume pod	Uganda	KY389335	KY389353	KY389371	KY769920	KY366466
Septomyrothecium sp. 3							
MUCL 41081 = CBS 100966	Dead leaf	Venezuela	KU846472	KU846494	KU846511	KY769921	KU846552
Septomyrothecium sp.4							
MUCL 41240	Dead leaf	Venezuela	KY389336	KY389354	KY389372	KY769922	KY366468
Septomyrothecium uniseptatum Matsush.							
MUCL52944 (A)	No data	Japan	KY389331	KU846495	KU846512	KY769916	KY366462
MUCL 52943 (A)	No data	Japan	KY389332	KY389350	KY389368	KY769917	KY366463
MUCL52942 (A)	No data	Japan	KY389333	KY389351	KY389369	KY769918	KY366464

T type; IT isotype; A aut

1.2.2. Cylindrocarpon-like (Ascomycota, Hypocreales) species from the Amazonian rain forests in Ecuador: additions to *Campylocarpon* and *Dactylonectria*

Ana GORDILLO & Cony DECOCK

Adapted from the research article published in

Cryptogamie Mycologie (2017)

Preface

As previously evidenced **sub-section 1.2.1**, the application of a polyphasic approached for the recognition of new species is nowadays the most accurate strategy to describe taxa.

This paper (**sub-section 1.2.2)** results from an in-deep revision of Ecuadorian tropical species of cylindrocarpon-like isolates. From these studies, several new terminal clades emerged, some of them were described in the following chapter.

Four new species in *Dactylonectria* were formally described, also one specie of *Campylocarpon* was addressed. They were isolated from externally sane, asymptomatic roots, either from their internal tissues as endophytes or from their rhizoplanes. They are therefore not associated with specific disease symptoms such as root rots or black foot, contrary to all the other *Dactylonectria* and *Campylocarpon* species.

Abstract – *Dactylonectria* and *Campylocarpon* are two related genera of Hypocreales sharing a cylindrocarpon-like asexual morph, mostly known as soil-borne pathogens. During a study of the fungal communities of roots (endophyte) and rhizoplanes of plants growing in a layer of compost-like vegetal materials covering crude oil ponds in rain forest areas of the Amazonian Ecuador, a set of isolates with a cylindrocarpon-like asexual morph were studied. Multilocus phylogenetic inferences (based on partial DNA sequences from nuclear ribosomal DNA genes (ITS, 28S) and the housekeeping genes β-tubulin, translation elongation factor 1-α and Histone 3) and morphological studies revealed the occurrence of five undescribed species, of which four belong to *Dactylonectria* and one to *Campylocarpon*. They are described as *Dactylonectria amazonica*, *D. ecuadoriense*, *D. polyphaga*, *D. palmicola* and *Campylocarpon amazonense*.

Hypocreales / phylogeny / systematics / South America.

INTRODUCTION

Cylindrocarpon Wollenw.[3] and *Cylindrocarpon*-like species are cosmopolitan fungi. They are mostly known as soil-borne pathogens causing plant diseases such as black foot or root rots, but also tree cankers (Samuels & Brayford, 1994; Halleen *et al.*, 2004, 2006; Hirooka *et al.*, 2005; Kobayashi *et al.*, 2005;

[3] As a rule, authorship of scientific names listed in the table 1 are not repeated in the text.

Castlebury *et al.*, 2006; Chaverri *et al.*, 2011; Cabral *et al.*, 2012b,a; Lombard *et al.*, 2014).

Cylindrocarpon was first introduced by Wollenweber (1913) with *C. cylindroides* Wollenw. as type, a species with an asexual morph characterized by long, sausage-shaped, 3–5-septate macroconidia and smaller, ellipsoid, aseptate microconidia. Later on, Wollenweber (1917) associated *Cylindrocarpon* with the sexual form *Neonectria* Wollenw. (*C. cylindroides* was associated with *N. ramulariae* Wollenw.). Subsequently, more than 140 names have been published in *Cylindrocarpon*, at species or subspecies level (*index fungorum* www.indexfungorum.org).

The first revision of *Neonectria* – *Cylindrocarpon* dates back from Booth (1966), who recognized four informal groups on the basis of morphological features of both the sexual and asexual morph, including the anatomy of the perithecial outer layer and the presence or absence of microconidia and chlamydospores. Chaverri *et al.* (2011) proposed the first comprehensive treatment of *Cylindrocarpon* – *Neonectria* of the molecular era, implementing multi-locus phylogenetic inferences combined to morphological analyses. In addition to *Neonectria* (gathering mostly species of Booth *Cylindrocarpon* group 1) and *Campylocarpon* [previously segregated by Halleen *et al.* (2004)], they acknowledged at generic level three additional lineages, two of which overlapping with Booth informal groups: *Thelonectria* P. Chaverri & C. Salgado, which corresponds to Booth *Cylindrocarpon* group 2, *Ilyonectria* which corresponds to Booth *Cylindrocarpon* group 3, and *Rugonectria* P. Chaverri & Samuels. Further studies then revealed that *Ilyonectria* sensu Chaverri *et al.* (2011) also was polyphyletic (Cabral *et al.*, 2012 b, a; Lombard *et al.*, 2013); *Dactylonectria* was therefore introduced to

accommodate a bunch of species, the majority of which were isolated from diseased grapevines (Lombard *et al.*, 2014). More recently, Aiello *et al.* (2017) introduced *Pleiocarpon* L. Lombard & D. Aiello for a single species, *P. strelitziae* L. Lombard & D. Aiello, associated with basal rot of *Strelitzia reginae* (Strelitziaceae) in Southern Italy.

In this taxonomic frame, we re-evaluated the generic placement, identity and phylogenetic affinities of a set of cylindrocarpon-like strains, isolated from asymptomatic roots of herbaceous plants growing in a layer of compost-like vegetal materials covering weathered crude oil ponds in the Eastern Amazonian Ecuador. A combination of morphological and multilocus DNA-based phylogenetic approaches show these strains to be distributed into five new terminal clades or branches, that represent as much new species. Four species belongs to *Dactylonectria* and one to *Campylocarpon*. These taxa are described and commented.

MATERIAL AND METHODS

Site and Sample procedure

The strains from Ecuador were isolated from internal tissues and rhizoplanes of roots of herbaceous plants growing in a superficial layer of compost-like vegetal debris covering two weathered oil ponds (for detailed methodologies, cf. Gordillo and Decock 2016). Both ponds are located at Charapa camp, Province of Sucumbíos, approx. W 76°48'57" – S 00°11' 49" and W 76°48'54" – S 00°11'46", elevation approx. 300 m asl.

Morphological characterization

Inoculums (mycelial plugs / germinating conidia) were placed in the centre of a 9 cm Petri dish, on Potato Dextrose Agar (PDA) and on a piece of sterile banana leaf on water agar (Banana Leaf Agar, BLA) (Untereiner *et al.*, 1998). Cultures were incubated at 25ºC with a 12/12 hrs incident near UV light / dark cycle. Cultural characteristics were determined at 7 days after inoculation on PDA. Colours of the colonies are described according to Kornerup and Wanscher (1978). As a rule, the reproductive structures (e.g. conidiophores, conidia, perithecia, ascospores) measured are those produced on BLA. Measurements for length and width of conidia and ascospores are given as (Minimum–) Lower Limit of a 95% Confidence Interval – Upper Limit of a 95% Confidence Interval (–Maximum). For the other measurements, only the extreme values are given (Cabral *et al.*, 2012a)

Taxon sampling, DNA isolation, PCR amplification and sequencing

The taxa included in the phylogenetic analysis are listed in Table 1. DNA was extracted from mycelium grown in malt extract broth (2%) at 25ºC in the dark, using the innuPREP Plant DNA kit (Analytik Jena, Germany) following the manufacturer's recommendations.

DNA sequences were determined for parts of the genes encoding the nuclear ribosomal internal transcribed spacers region, including the ITS1, ITS2 and 5.8S subunit (ITS), the nuclear ribosomal large subunit (28S, region comprising the D1–D3 domains), β-tubulin (*tub2*, region between exons 1 and 4), translation elongation factor 1-α (*tef1a*, region between exons 1

and 4) and histone H3 (*his3*, region between exons 1 and 3). Amplification and sequencing of the ITS, 28S, *tef1a*, *tub2* and *his3* were performed, respectively, with the primer pairs ITS5 / ITS4 (White *et al.*, 1990), LR0R (Rehner & Samuels, 1995) / LR6 (Vilgalys & Hester, 1990), ef1 / ef2 (O'Donnell *et al.*, 1998), T1 (O'Donnell & Cigelnik, 1997) / Bt-2b (Glass & Donaldson, 1995) and H3-1a / H3-1b (Glass & Donaldson, 1995). The PCR conditions are as described in Lombard *et al.* (2010c). Sequencing was performed by Macrogen Ltd. (Seoul, Korea) using the same primers as for amplification. The amplicons were sequenced in both directions. Raw sequences were edited with Sequencher®software version 5.1 (Gene Codes Corporation Ann Arbor n.d.).

Phylogenetic analysis

The affinities of our Amazonian cylindrocarpon-like strains were first searched for using the Blast search engine at GenBank (Altschul *et al.*, 1990). Subsequently, based on the blast search results, sequence data sets were set up to conduct phylogenetic inferences. The nucleotide alignments were performed with MAFFT v7.213 (Katoh & Standley, 2013) and manually corrected in PhyDE-1 (Müller *et al.*, 2006) when necessary.

Each data set was partitioned into ITS1, ITS2, 5.8S, 28S and exons / introns for the protein coding genes. The best-fit evolutionary model for each defined partition was estimated using Partition Finder (Lanfear, 2012), following the Akaike information criterion (AIC). Phylogenetic analyses were performed under probabilistic hypothesis, using Bayesian inferences (BI) as implemented in MrBayes v3.1.2 (Ronquist &

Huelsenbeck, 2003) and Maximum likelihood (ML) using RAxML 7.0.4 (Stamatakis, 2006). For the Bayesian inferences, the best-fit models for each partition were implemented as partition-specific model. All the parameters were linked across partitions.

Bayesian analyses were implemented with two independent runs, each with four MCMC simultaneous, independent chains, for ten million generations for the first data set and six million generations for the second data set, starting from random trees and keeping one tree every 1000th generation. All trees sampled after convergence [average standard deviation of split frequencies < 0.01, confirmed using Tracer v1.4 (Rambaut & Drummond, 2007)] were used to reconstruct a 50% majority-rule consensus tree (BC) and to estimate posterior probabilities (PP). Clades with PP above >0.95 were considered significant supported by the data.

Maximum Likelihood trees were obtained using RAxML v.7.2.8 (Stamatakis, 2006). The analysis first involved 1000 ML searches, under a GTRGAMMA model and all other parameters estimated by the software. ML Bootstrap support values (BS) were obtained running 1000 multi-parametric bootstrapping replicates, under the same model. A node was considered to be strongly supported if it showed a BPP ≥ 0.95 and/or ML BS ≥ 80%.

Phylogenetic congruency between the loci was tested using a 70% reciprocal bootstrap criterion in ML analysis of each individual locus (Mason-Gamer *et al.*, 1996; Lombard *et al.*, 2014).

RESULTS

Phylogenetic analyses

The amplicons of the ITS, 28S, *tub2*, *tef1a* and *his3* of our Amazonian strains ranged ~ 500–750 bases each. The BLAST search at GenBank (Altschul *et al.*, 1990) of the ITS, 28S, *tub2*, *tef1a* and *his3* DNA sequences demonstrated each homology mostly with members of *Dactylonectria*. A single strain showed affinities with *Campylocarpon*. Subsequently, a *Dactylonectria* and a *Campylocarpon* sequence data sets were built.

The first data set includes the sequences of the five loci cited above for 40 *Dactylonectria* strains, representing the 10 known species and our related Amazonian strains (Table 1). Several 28S sequences, however, are missing (Table 1). This data set was subdivided into 23 partitions viz. ITS1, 5.8S, ITS2, 28S, *tef1a* Introns 1, 2, 3 and 4, *tef1a* Exons 1, 2, 3 and 4, *tub2* Introns 1, 2 and 3, *tub2* Exons 1, 2, and 3, *his3* Exons 1, 2 and 3, *his3* Introns 1, 2 and 3. The models estimated as the best-fit likelihood model of evolution for each partition, subsequently used for the BI, are summarized in Table 2.

The 70% reciprocal bootstrap tree topologies showed no conflicts between phylogenies resulting from the *tub2*, *tef1a* and *his3* gene regions. These individual phylogenies resolved each the same lineages, of which, of interest for our studies, the *D. vitis*, *D. pauciseptata*, and *D. anthuriicola* lineages. They also resolved each the same terminal clades within these lineages. This was already the case for a closely related cylindrocarpon-like data set used by Lombard *et al.* (2014) or Cabral *et al.* (2012a). The polymorphism of the *tub2*, *tef1a* and *his3* sequences make them suitable for species discrimination within each lineages.

However, the ITS and 28S genes region revealed conflicting as far as the terminal clades are concerned. The ITS and 28S resolved equally the *D. vitis*, *D. pauciseptata*, and *D. anthuriicola* lineages. However, within each of these lineages, the ITS and 28S sequences did not allow differentiating the terminal species clades shown by the house-keeping genes; their phylogenetic signals, at that level, are null or very weak. This was also reported for the related cylindrocarpon-like data set used by Lombard *et al.* (2014) for the 28S, which has very little phylogenetic signals.

Nonetheless, as emphasized by Cunningham (1997) combining incongruent partitions could increase phylogenetic accuracy. This was the case in previous phylogenetic studies of cylindrocarpon-like species (Lombard *et al.*, 2014) but also in other genera of Hypocreales (e.g. Gehesquiere *et al.*, 2016). Therefore, the five gene regions also were combined in the present study.

The concatenated *Dactylonectria* data set resulted in 3077 positions (including gaps). *Dactylonectria hordeicola* CBS 16289 was used as outgroup, following Lombard *et al.* (2014). The two Bayesian runs converged to stable likelihood values after 45000 generations. The first 25% of saved trees were discarded as the "burnin" phase. In the ML the searches with RAxML, the combined data set had 406 distinct patterns with a proportions of gaps and undetermined characters of 8.47%. The best scoring ML tree is shown at Fig. 1 (–lnL –8071.916899).

The topologies obtained for the two data sets were overall highly concordant between Bayesian and Maximum likelihood inferences.

The analysis of this data set (Fig. 1) resolve the lineages corresponding to the known *Dactylonectria* species, confirming previous results (Lombard *et al.*, 2014). Our Amazonian *Dactylonectria* strains are distributed into four terminal clades / branches (Fig. 1, PS1, PS2, PS3 and PS4), distinct from all the other known species clades.

The clade PS1, formed by 6 strains [MUCL 55431, MUCL 55432, MUCL 55424, MUCL 55425, MUCL55205 & MUCL 55226] and PS2, represented by 2 strains [MUCL 55430 & MUCL 55433] are both closely related to the *D. vitis* branch and the unnamed branch represented by the strain Cy228 (Cabral *et al.*, 2012a). The clade PS3, represented by 11 strains [MUCL 54780, MUCL 54802, MUCL 54771, MUCL 55206, MUCL 55208, MUCL 55209, MUCL 55238, MUCL 55427, MUCL 55428, MUCL 55429, MUCL55435] is closely related to the *D. anthuriicola* branch (BSML = 100, PP = 1). The clade PS4, represented by a single strain [MUCL 55426], belongs to the *D. pauciseptata* lineage (Fig. 1).

The second data set includes sequences of three loci (ITS, *tub2* and *his3*) from 26 *Campylocarpon* strains, including the type strains of *C. fasciculare* and *C. pseudofasciculare* (Halleen *et al.*, 2004), two sets of Brazilian strains identified as *C. fasciculare* (Correia *et al.*, 2013) and *C. pseudofasciculare* (Dos Santos *et al.*, 2014), and our related single Amazonian strain (Table 1). The ITS sequences are missing for the strains Cy1UFM and CBS 113560. As well, the *his3* sequences are missing for one set of Brazilian strains (Correia *et al.*, 2013) and some strains from South Africa (Halleen *et al.*, 2004) (Table 1). This data set was subdivided into 14 partitions, viz. ITS1, 5.8S, ITS2, Intron 1 tub, Exon1 tub, Intron 2 tub, Exon 2 tub, Intron 3 tub, Exon 3 tub,

Exon 1 *his3*, Intron 1 *his3*, Exon 2 *his3*, Intron 2 *his3*, Exon 3 *his3*. The models estimated as the best-fit likelihood model of evolution for BI for each partition are summarized in Table 3.

The 70% reciprocal bootstrap tree topologies showed no conflicts between ITS, *tub2* and *his3* gene regions for the Campylocarpon data set. The concatenated Campylocarpon data set comprises 1588 positions (including gaps). Ilyonectria radicicola CBS 264.65 was used as outgroup (Halleen *et al.*, 2004). The two Bayesian runs converged to stable likelihood values after 30000 generations. The first 25% of saved trees were discarded as the "burnin" phase. In the ML searches with RAxML the combined data set alignment had 327 distinct patterns with a proportions of gaps and undetermined characters of 31.45%. The best scoring ML tree is shown at Fig. 2 (–lnL –4375.971595).

The *Campylocarpon* lineage (Lombard *et al.*, 2014) (Fig. 2) is divided into two clades, of which only one is well supported. The well-supported clade (BSML = 100, PP = 0.99) is itself divided into two well-supported subclades. The first subclade (BSML = 96, PP = 1) includes the type strain of *C. fasciculare* (CBS 112613) together with several strains originating from grapevine in South Africa (Fig 2); it represents *C. fasciculare* s.s. The second clade (BSML = 94, PP = 0.66) is composed of several grapevine associated strains, originating from Northeaster Brazil (Correia *et al.*, 2013).

The second clade is not supported (Fig. 2). It includes the type strains of *C. pseudofasciculare*, the strains CBS 112592 and BV7, a set nine strains originating from southern Brazil (Dos Santos *et al.*, 2014). Our Amazonian strain MUCL 55434 (branch

PS5) forms an isolated branch, which relationships with other *Campylocarpon* are unresolved (Fig. 2).

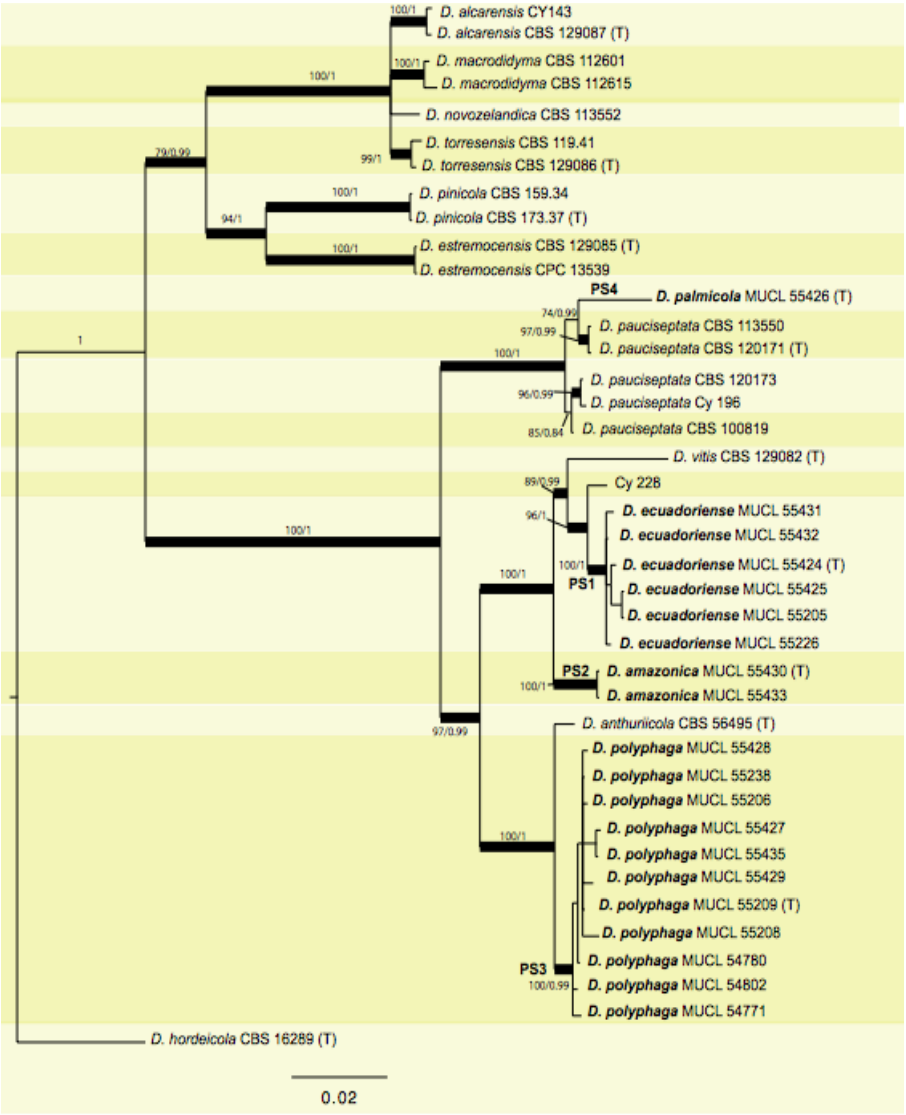


Fig. 1 The ML best tree (–lnL –8071.916899) inferred from the combined five-gene (ITS, 28S, *tub2*, *tef1a* and *his3*) DNA sequence alignment. ML Bootstrap support (BS) values and posterior probability (PP) are indicated and highlighted in bold lines (≥ 80% BS and ≥ 0.95 Bayesian PP.) (T) = Type.

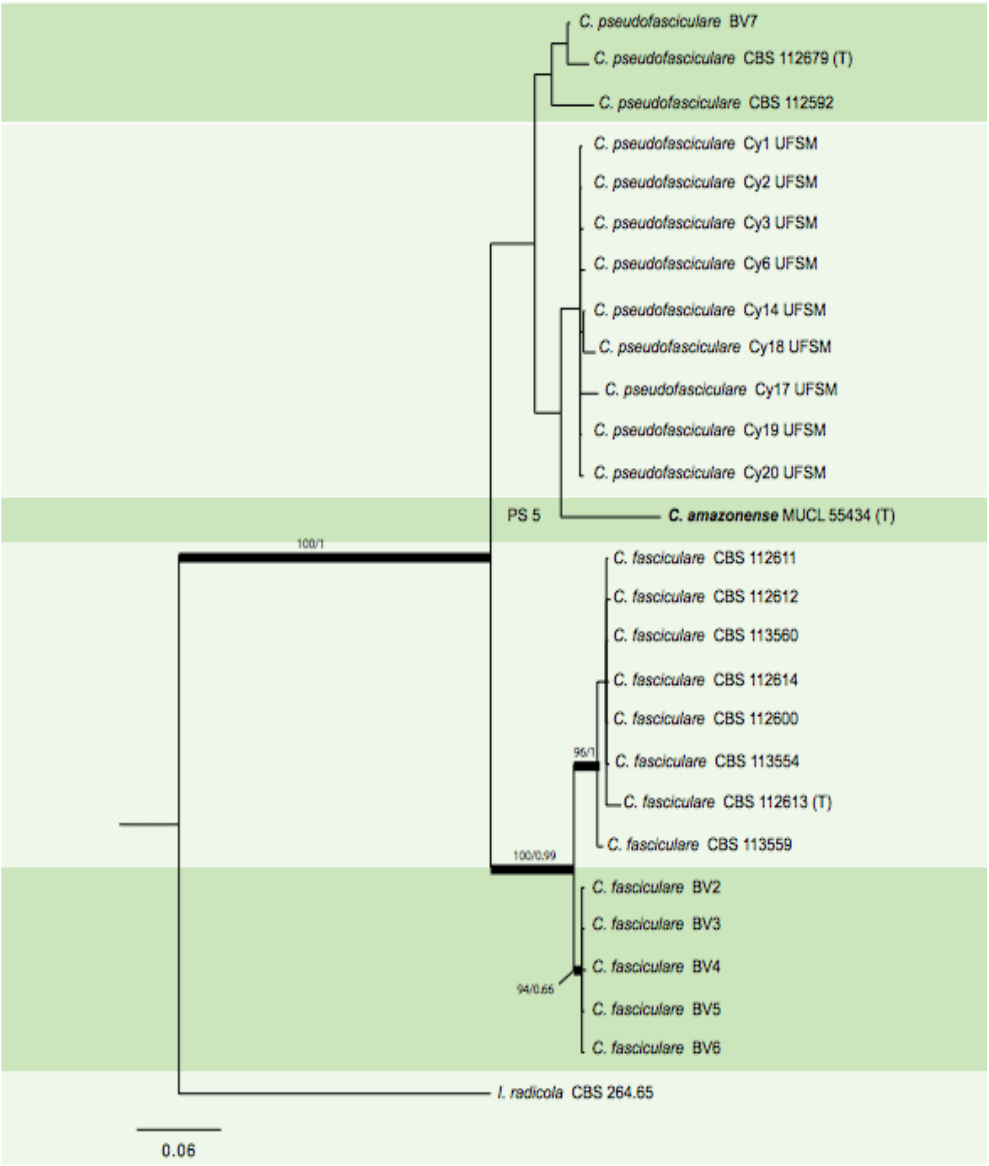


Fig. 2 The MB best tree (–lnL –4375.971595) inferred from the combined tree-gene (ITS, *tub2* and *his3*) sequence alignment. ML Bootstrap support (BS) values and posterior probability (PP) are indicated and highlighted in bold lines (≥ 80% BS and ≥ 0.95 Bayesian PP.) (T) = Type.

Morphological analysis

Morphological studies of our Amazonian isolates reveal that the strains of the new clades / branches PS1–5 also present each phenotypic singularities allowing morphological distinction from each other (Table 4, 5) but also morphological distinction from their closest phylogenetic relatives. The main morphological differences are in the conidial shape and size (Table 4, 5). Amongst our set of strains, those forming PS4 has the longest 3-septate conidia, averaging 42 × 7.3 µm whereas those forming PS3 has the smallest conidia, averaging 34.5× 7.7 µm. The strains forming PS1 and PS2 have relatively similar conidia, differing by their width, respectively 7–8 and 5.5–6.5 µm. Noteworthy, our single isolate MUCL 55426 (PS4) also forms in vitro a sexual form, absent in our other Amazonian strains, and, so far, not reported in other strains of the *D. pauciseptata* lineage. Our *Campylocarpon* strain (PS5) has typical, curved conidia, with up to 5 septa.

Taxonomic conclusions

Phylogenetic studies of a set of Amazonian cylindrocarpon-like isolates revealed the occurrence of five clades or branches, which are equated to as much phylogenetic species. Four species belong to *Dactylonectria* and one to *Campylocarpon*. Each of these phylogenetic species also presents a phenotype allowing distinguishing them (Table 5) but also allowing their differentiation from their closest phylogenetic relatives (Table 4). These species, therefore, are considered as five undescribed

taxa, proposed below as *Campylocarpon amazonense* (PS5), *Dactylonectria amazonica* (PS2), *D. ecuadoriense* (PS1), *D. palmicola* (PS4), and *D. polyphaga* (PS3).

TAXONOMY

Campylocarpon amazonense Gordillo & Decock, sp. nov. **Figs 3 a–c**

MycoBank: MB 822796

Holotype: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, rhizoplane, *Cordia alliodora* (Ruiz & Pav.) Oken (Boraginaceae), Jul. 2014, A. Gordillo & C. Decock, MUCL 55434, as a two-week-old dried culture on BLA (living culture ex-holotype MUCL 55434 = PUCE PHPE4-18-1014).

Etymology. "*amazonense*" (Latin): from the area of origin, the Amazonian rain forest.

Culture characteristics: colonies on PDA reaching 35 mm diam. in 7 days, with abundant aerial mycelium over the whole colony or in sectors, cottony to felty or forming hyphal strands, white to light brown first (6D5) then yellowish brown, sometimes partly covered by off-white slime, the reverse brown (6E8).

Conidiophores first simple, arising laterally from aerial hyphae, consisting of a short cell from which emerge 1–3 phialides, later gathered in fascicles 80 µm high, 100 µm diam., with a basal stipe 7.5–14.5 × 3–4.0 µm, supporting 1–3 branches, 11.5–31 × 3–4.0 µm, each with a single phialide; **phialides** narrowly flask-shaped, the widest point near the middle, (14–) 15–20 (–27) µm long, 2.5–3.5 (–4.0) µm wide at the base, 3.0–4.0 µm at the widest point, and 1.5–2.3 µm near the

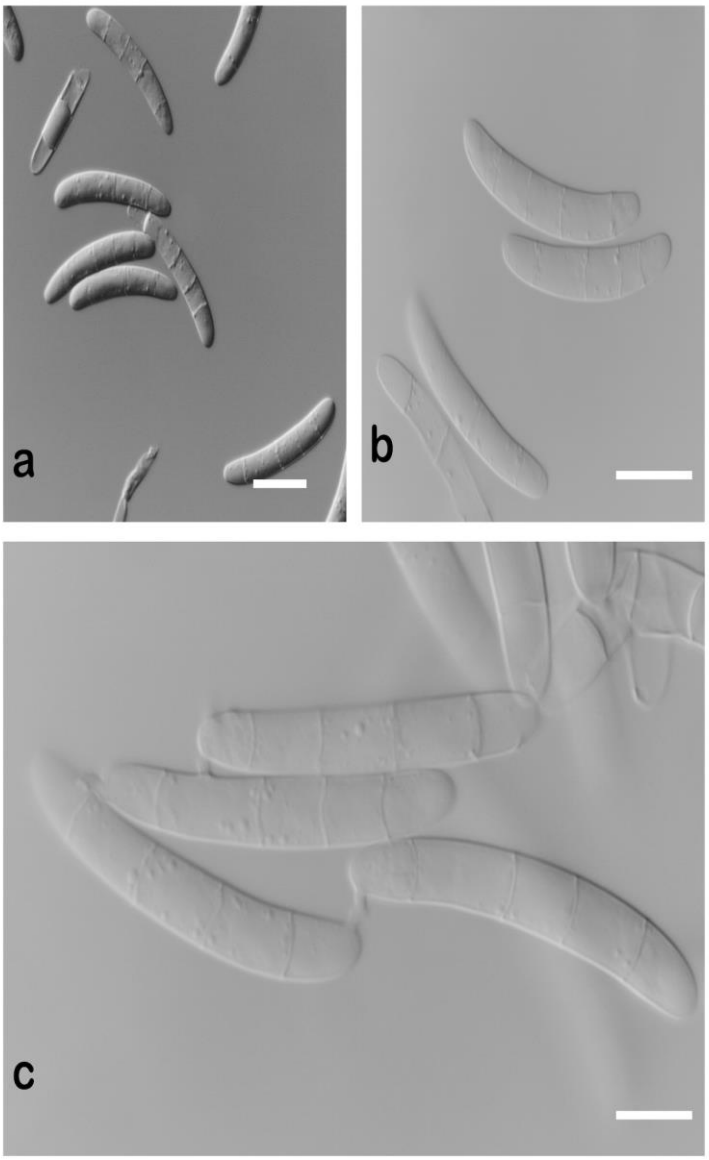
aperture (av. = 17.3 × 3.6); **macroconidia** (2–) 3–5 septate, cylindrical, faintly to moderately curved, with obtuse ends, sometimes tapering at the base; 2-septate conidia (23–) 24–33 (–33.5) × (5.5–) 5.3–6.0 (–6.2) µm (av. = 27.8 × 5.8 µm), 3-septate conidia (28–) 31–46 (–47) × (5.5–) 5.9–7 (–7.8) µm (av. = 39 × 6.5 µm), 4-septate conidia (31–) 32–49 (–49) × (6.2–) 6.0–7.0 (–7.8) µm (av. = 40 × 6.5 µm), 5-septate conidia (39–) 40–54 (–54.6) × (6.2–) 6.7–8 (–7.8) µm (av. = 50 × 7.3 µm); **conidial masses** off-white, first hemispherical, then sliding over the mycelium; **microconidia** not observed; **chlamydospores** not observed.

Sexual morph: not observed.

Substratum, host and habitat: rhizoplane of *Cordia alliodora* (Ruiz & Pav.) Oken (Boraginaceae).

Remarks: *Campylocarpon amazonense* shares with *C. fasciculare* and *C. pseudofasciculare* the 3–5-septate, slightly curved macroconidia. It differs from *C. pseudofasciculare* in having smaller macroconidia and from *C. fasciculare* in having narrower macroconidia (cf. Table 4).

Campylocarpon amazonense is the third species described in the genus, and the first that is not associated with grapevine.



Figs. 3 a–c. *Campylocarpon amazonense*, from MUCL 55434 (type). a-c. Macroconidia (Figs. a, b scale bars = 20 μm; c, scale bars = 10 μm). All from cultures on BLA.

Dactylonectria amazonica Gordillo & Decock, sp. nov. **Figs 4 a–f**

MycoBank: MB 822799

Holotype: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, from the rhizoplane of *Piper* sp. (Piperaceae), Jun. 2014, A. Gordillo & C. Decock, MUCL 55430, as two-week-old dried culture on BLA (living culture ex-holotype MUCL 55430, PUCE PHPE4-34-768).

Etymology. *"amazonica"* (Latin): from the area of origin, the Amazonian rain forest.

Culture characteristics: colonies on PDA 15 mm diam. in 7 days, with sparse aerial mycelium, the margin light brown (6D6), light brown (6D4) towards the centre, the reverse brown (6F8).

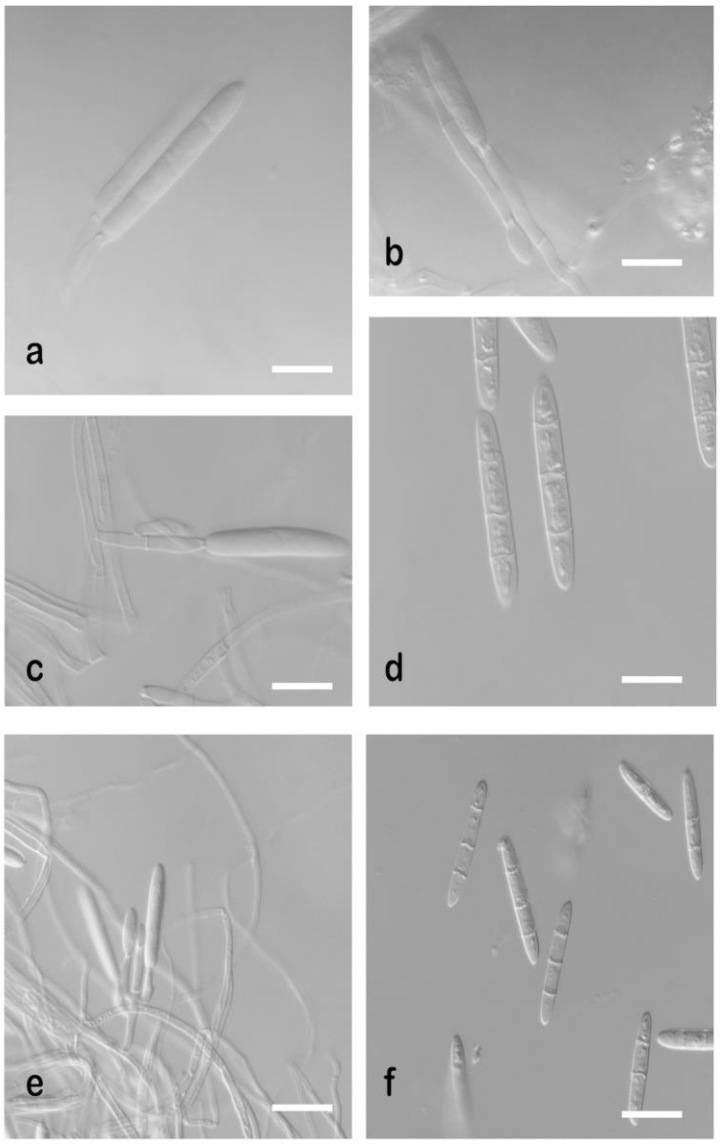
Conidiophores forming macroconidia solitary to loosely aggregated, arising laterally or terminally from aerial mycelium, unbranched or sparsely branched, stipe 30 × 2.5 µm, bearing 1–3 phialides; **phialides** subcylindrical, slightly tapering towards the apical conidiogenous loci, 11.0–15.5 µm long, 2.5–3.0 µm wide at the base, 2.3–4.0 µm at the widest point, approx. in the middle, 1.5–2.3 µm near the aperture; **macroconidia** cylindrical, straight or faintly curved, with both ends rounded, mostly without a visible hilum, predominantly 3-septate, rarely 4-septate; 3-septate conidia (31–) 31–41 (–43) × (5.5–) 5.7–6.5 (–7.0) µm (av. = 37 × 6.2 µm), with a length:width ratio of 5.7–6.1, accumulating in flat, slimy domes; **conidiophores** forming microconidia formed on surface mycelium, mono- or bi-verticillate; **phialides** narrowly flask-shaped, the widest point near the middle, 7–11 × 2.3 µm; **microconidia** formed in heads, aseptate, subglobose to ovoid,

rarely ellipsoid, mostly with a visible, centrally located or slightly laterally displaced hilum, 6–10 × 4 µm (av. = 8.4× 4 µm), with a length:width ratio of 2.1 µm; **chlamydospores** globose to subglobose to ellipsoid, 8–12 × 6–10 µm, smooth, thick-walled, formed intercalary in chains or in clumps, becoming golden-brown.

Sexual morph: not observed.

Substratum, host and habitat: rhizoplane of *Piper* sp. (Piperaceae), Neotropical rain forest.

Additional specimens examined: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, root, *Piper* sp. (Piperaceae), Jun. 2014, A. Gordillo & C. Decock, living culture as MUCL 55433 = PUCE PHPE4-34-950.



Figs. 4 a–f. *Dactylonectria amazonica* from MUCL 55430 (type). a, d, f. 3-septate macroconidia; b, c, e. Conidiophores (Figs. a, c, d scale bars = 20 μm; b, e, f scale bars = 40 μm). All from cultures on BLA.

Dactylonectria ecuadoriense Gordillo & Decock, sp. nov. **Figs 5 a–f**

MycoBank: MB 822801

Holotype: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, rhizoplane, *Piper* sp. (Piperaceae), Nov. 2013, A. Gordillo & C. Decock, MUCL 55424, as a two-week-old dried culture on BLA (living culture ex-holotype MUCL 55424 = PUCE PHPE3-1-621).

Etymology. *"ecuadoriense"* (Latin): from the country of origin, Ecuador.

Culture characteristics: colonies on PDA reaching 30 mm diam. in 7 days, with sparse aerial mycelium, the marginal area light brown (6D6) to brownish orange (5C7), light brown toward the centre (5D6), the reverse brown (6D8).

Conidiophores forming macroconidia solitary to loosely aggregated, arising laterally or terminally from aerial hyphae, unbranched or sparsely branched, stipe 30 × 3.0 µm, bearing 1–3 phialides; **phialides** subcylindrical, slightly tapering towards the apical conidiogenous loci, 8.0–20 µm long, 2–3 µm wide at the base, 2.5–4.0 µm at the widest point, near the middle, 1.5–2 µm near the aperture; **macroconidia** cylindrical, straight to faintly curved, with both ends rounded, without visible hilum, predominantly 3-, rarely 4-septate, (31–) 34–43 (–43) × (7.0–) 7.0–8.0 (–8.5) µm (av. = 38 × 8 µm), length:width ratio = 4.4–5.4, accumulating in slimy drops, forming flat domes; **conidiophores** forming microconidia on aerial mycelium, mono- or biverticillate; **phialides** narrowly flask-shaped, the widest point near the middle, 7.8–12 × 2 µm wide; **microconidia** formed in slimy heads, aseptate, subglobose to ovoid, rarely ellipsoid, 3.0–4.5 ×

3.0–4.5 (–5.5) µm (av. = 3.8 × 3.7 µm), with a length:width ratio of 0.9–1; **chlamydospores** intercalary, formed in short chains or in cluster, individually globose, subglobose to ellipsoid, 7.8–14 × 4–11.7 µm, smooth, thick-walled, becoming golden-brown.

Sexual morph: not observed.

Substratum, host and habitat: rhizoplane of several angiosperms and one pteridophyte, including *Socratea exorrhiza* (Mart.) H Wendl (Arecaceae), *Carludovica palmate* Ruiz & Pav. (Cyclanthaceae), *Piper* sp. (Piperaceae) and *Cyathea lasiosora* (Mett. Ex Kuhn) Domin (Cyatheaceae, Pteridophyta), in Neotropical, Amazonian rain forest.

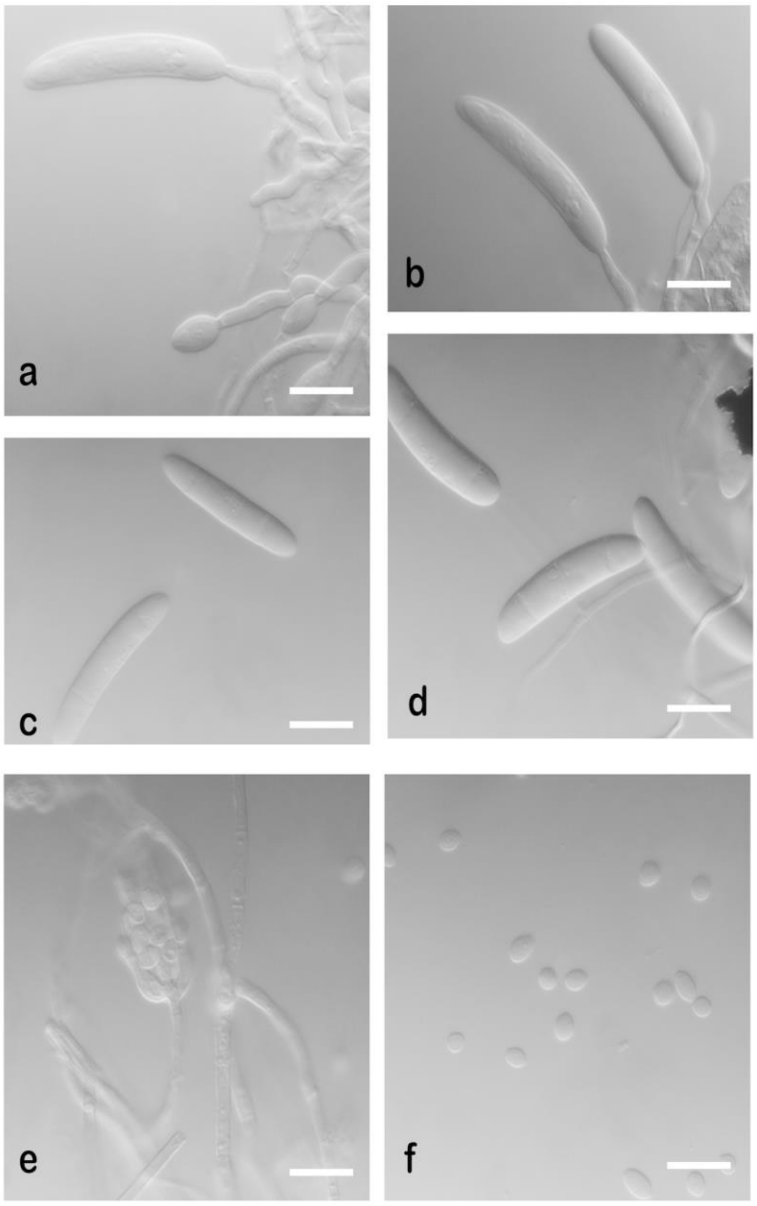
Additional specimens examined: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, rhizoplane, *Carludovica palmate* (Cyclanthaceae), Jun. 2014, A. Gordillo & C. Decock, living culture MUCL 55431 = PUCE PHPE4-23-912; ibid., rhizoplane, *Socratea exorrhiza* (Arecaceae), Jun. 2014, A. Gordillo & C. Decock, living culture MUCL 55432 = PUCE PHPE4-37-925; ibid., root, *Cyathea lasiosora* (Cyatheaceae, Pteridophyta), Nov. 2013, A. Gordillo & C. Decock, living culture MUCL 55226 = PUCE PHPE3-26-671; ibid., rhizoplane, *Piper* sp. (Piperaceae), Nov. 2013, A. Gordillo & C. Decock, living culture MUCL 55205 (= MUCL 55425) = PUCE PHPE3-1-622.

Remarks: *Dactylonetria amazonica* (PS2) and *D. ecuadoriense* (PS1) are morphologically similar and share some ecological parameters; both species share the same microhabitat and, partly, their host ranges. Morphologically (Table 5), they mainly differ in the width of their 3-septate macroconidia, respectively 5.7–6.5 µm (av. = 6.2 µm) and 7.5–8 µm (av. = 7.7

µm). *Dactylonectria palmicola* has longer macroconidia whereas *D. polyphaga* has smaller macroconidia (Table 5).

In a phylogenetic perspective, *D. amazonica* and *D. ecuadoriens*e are related to *D. vitis*, which is known so far only from Vitis vinifera in southern Europe (Cabral *et al.*, 2012a). They differ from *D. vitis* in having smaller 3-septate macroconidia; the conidial size ranges in *D. amazonica* and *D. ecuadoriense* are, respectively, mostly 34–39 × 5.7–6.5 µm (av. = 37 × 6.2 µm) and 34–40 × 7.5–8 µm (av. = 37.9 × 7.7 µm), reaching up to 43 µm in both. The size range is 41.5–43.5 × 7.9–8.2 µm (av. = 42.5 × 8.0 µm), reaching up to 51.5 µm in *D. vitis* (Cabral *et al.*, 2012a).

In addition, *D. amazonica* and *D. ecuadoriense* on one side and *D. vitis* on the other side are known from drastically different environments with very different humidity regime and vegetation, viz. a hyper humid area of the Amazonian rain forest and a spot of the Mediterranean area of Southern Europe.



Figs. 5 a–f. *Dactylonectria ecuadoriense* from MUCL 55424 (type). a–b. Conidiophore; c–d. 3-septate macroconidia; e. Conidiophores microconidia; f. Microconidia (Figs. a–f, scale bars: = 15 μm). All from cultures on BLA.

Dactylonectria palmicola Gordillo & Decock, sp. nov. **Figs 6 a–f**

MycoBank: MB 822804

Holotype: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, rhizoplane, *Euterpe precatoria* (Arecaceae), Nov. 2013, A. Gordillo & C. Decock, MUCL 55426, as a two-week-old dried culture on BLA (living culture ex-holotype MUCL 55426 = PUCE PHPE3-11-641).

Etymology. *"palmicola"* (Latin): refers to the common name of the host plant, "palm".

Culture characteristics: colonies on PDA reaching 41 mm diam. in 7 days, with sparse or no aerial mycelium, margin white to greyish orange (5B3), orange grey (5B2) in sectors towards the centre, the reverse brownish yellow (5C7) to greyish orange (5B5).

Perithecia formed (homothallically) in vitro, developing directly on the agar surface or on sterile pieces of banana leaf, solitarily or aggregated, ovoid to obpyriform, with a flattened apex, smooth to finely warted, dark red, darkening in 3 % KOH, 260–310 µm high × 160–280 µm diam.; **perithecial** wall made of two little distinguishable layers; **outer layer** composed of 1–3 rows of angular or subglobose cells; **inner layer** composed of angular to oval cells in subsurface face view; **asci** narrowly clavate to cylindrical, 72–60 × 6–8 µm, 8-spored; **ascospores** ellipsoid to oblong- ellipsoid, somewhat tapering towards both ends, centrally 1-septate, smooth to finely warted, frequently guttulate, hyaline, (12.5–) 13–16 (–17) × (3.5–) 3.5–4.2 (–4.7) µm (av. = 14.7 × 3.9 µm).

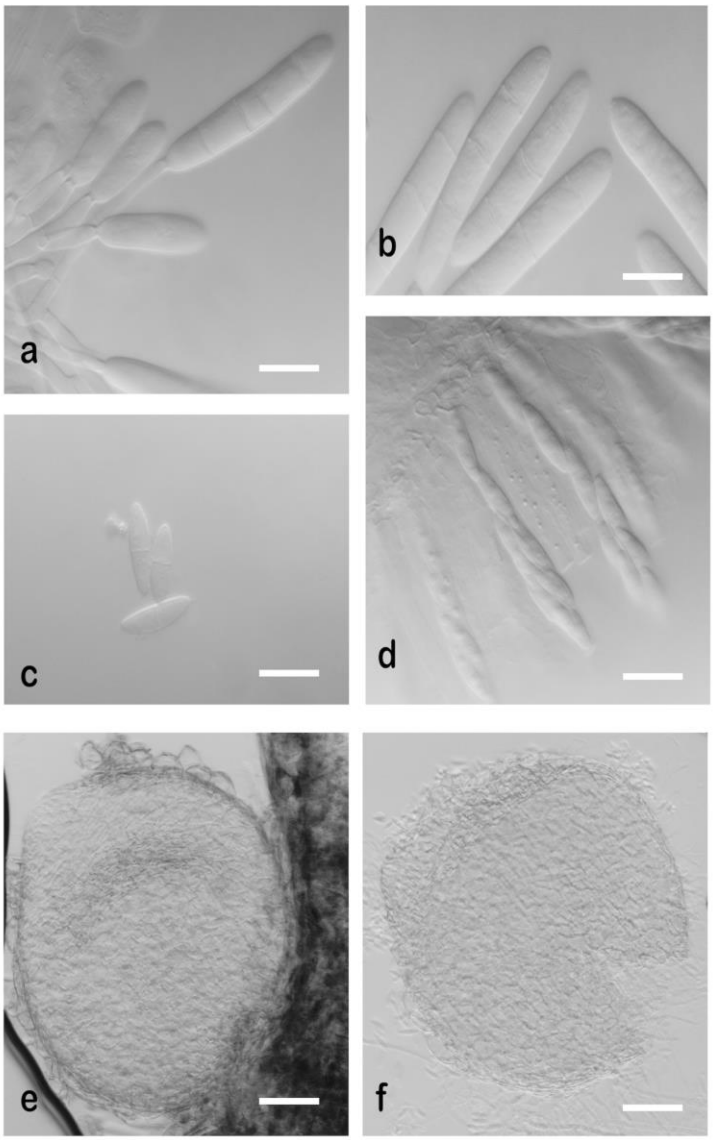
Conidiophores forming macroconidia simple or loosely aggregated, arising laterally or terminally from aerial or surface hyphae, unbranched or sparsely branched, stipe 6.2–23.5 × 2.3–4.0 µm, bearing 1–3 phialides; **phialides** subcylindrical, slightly tapering towards the apical conidiogenous loci, 10–15 × 2.3–4.0 µm wide; **macroconidia** cylindrical, straight or faintly curved, with both ends more or less broadly rounded, mostly without a visible hilum, predominantly 3-septate, (37.5–) 39–47 (–47.5) × (6.0–) 7.0–7.5 (–8.0) µm (av. = 42 × 7.3 µm), with a length:width ratio of 6, accumulating in slimy, flat domes; **conidiophores** forming microconidia formed on hyphae at agar surface, mono- or bi-verticillate; phialides narrowly flask-shaped, the widest point near the middle, 8.0–11 × 2 µm; **microconidia** formed in heads, aseptate, subglobose to ovoid, rarely ellipsoid, mostly with a visible, centrally located or slightly laterally displaced hilum, (4.0–) 4–6 (–6.0) × (3.0–) 2.5–4.2 (–4.0) µm (av. = 5 ×3.5 µm), with a length:width ratio of 0.78–1.6; **chlamydospores** globose to subglobose to ellipsoid, 10–18× 12–20 µm, smooth, thick-walled, formed intercalary in chains or in clumps, becoming golden-brown.

Substratum, host and habitat: rhizoplane of *Euterpe precatoria* (Arecaceae), Neotropical, Amazonian rain forest.

Remarks: *Dactylonectria palmicola* differs from the other Amazonian species in having a sexual morph and the longest macroconidia (Table 5).

This species is phylogenetically related to *D. pauciseptata*, from which it differs in having narrower macroconidia, mostly 7.0–7.5 µm (av. = 7.3 µm) vs 8.5–9.5 (av. = 9 µm, Schroers *et al.* (2008). *Dactylonectria palmicola* also produces a sexual form in

vitro, which is so far unknown in *D. pauciseptata*. These two species also differ by their habitat; *D. pausiceptata* is known from *Vitis* sp., in vineyard in Portugal and Slovenia (Cabral *et al.*, 2012a) whereas *D. palmicola* originates from a hyper humid area of the Amazonian rain forest.



Figs. 6 a–f. *Dactylonectria palmicola* from MUCL 55426 (type). a. Conidiophores and macroconidia; b. 3–septate macroconidia; c. Ascospores; d. Asci; e–f. Perithecia (Figs. a–d. scale bars = 15 μm; e–f. scale bars = 35 μm). All from cultures on BLA.

Dactylonectria polyphaga Gordillo & Decock, sp. nov.

Figs 7 a–f

MycoBank: MB 822805

Holotype: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, root *Costus* sp. (Costaceae), Nov. 2013, A. Gordillo & C. Decock, MUCL 55209, as a two-week-old dried culture on BLA (living culture ex-holotype MUCL 55209 = PUCE PHPE3-4-628).

Etymology. *"poly-"* (Greek): meaning "many", and *phagein* (Greek): meaning "to eat".

Culture characteristics: colonies on PDA reaching 26 mm diam. in 7 days, with sparse aerial mycelium, the margin brownish yellow (5C7), golden brown toward the centre (5D7), the reverse brown (6E7).

Conidiophores forming macroconidia solitary to loosely aggregated, arising laterally or terminally from aerial mycelium, unbranched or sparsely branched, stipe 19 × 3.2 µm, bearing 1–3 phialides; **phialides** subcylindrical, slightly tapering towards the tip, 7.8–22 µm long, 2–3 µm wide at the base, 2.3–4.0 at the widest point, 1.5–2.5 µm near the aperture; **macroconidia** cylindrical, straight or minutely curved, with both ends more or less broadly rounded, mostly without a visible hilum, predominantly 3-septate, rarely 1–2- or 4-septate; 3-septate conidia (31–) 31–37 (–39) × (7.0–) 7.0–8.0 (–7.8) µm (av. = 34.5 × 7.7 µm), with a length:width ratio of 4.4–5, accumulating in slimy, flat domes; **conidiophores** giving rise to microconidia formed on mycelium at agar surface, mono- or bi-verticillate; **phialides** narrowly flask-shaped, typically with widest point near the middle, 7–11 µm long, 2 µm wide; **microconidia** formed in

heads, aseptate, subglobose to ovoid, rarely ellipsoid, mostly with a visible, centrally located or slightly laterally displaced hilum, 4.0–4.7 × 3.0–4.0 µm, with a length:width ratio of 1.2; **chlamydospores** globose to subglobose to ellipsoid, 8–10 × 8–12 µm, smooth, thick-walled, formed intercalary in chains or in clumps, becoming golden-brown.

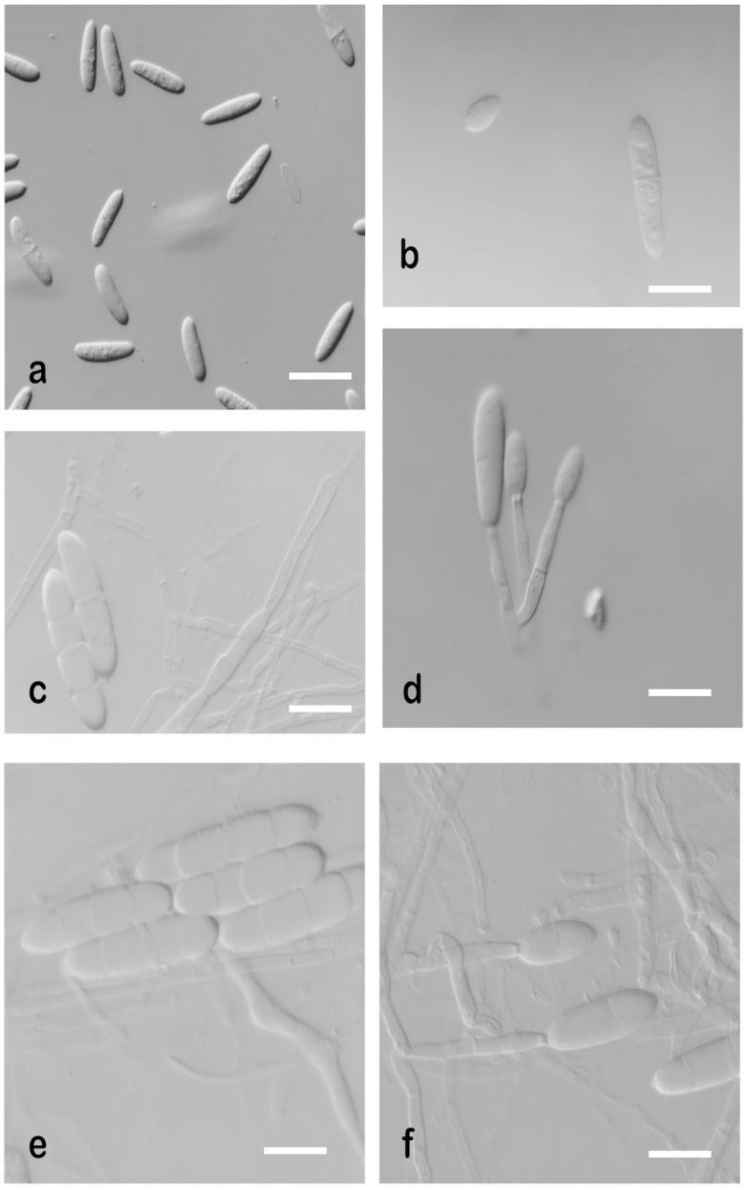
Sexual morph: not observed.

Substratum, host and habitat: rhizoplanes and roots of *Piper* sp. (Piperaceae), *Asplenium* sp. (Aspleniaceae), *Costus* sp., *C. scaber* (Costaceae), *Anthurium* sp. (Araceae), *Miconia* sp. (Melastomataceae), *Euterpe precatoria* (Arecaceae) Neotropical rain forest.

Additional specimens examined: ECUADOR, Prov. Sucumbíos, Nueva Loja, Lago Agrío canton, Charapa camp, secondary rain forest, root, *Costus* sp. (Costaceae), Nov. 2013, A. Gordillo & C. Decock, living culture MUCL 55208; ibid., root, *Asplenium* sp. (Aspleniaceae, Pteridophyta), Jan. 2013, A. Gordillo & C. Decock, living culture MUCL 54802 = PUCE PHPE2-19-240; ibid., root, Costus scaber Ruiz & Pav (Costaceae), living culture MUCL 54771 = PUCE PHPE2-35-299; ibid., root, *Anthurium* (Araceae), living culture MUCL 54780 = PUCE PHPE2-04-399); ibid., root, *Costus* (Costaceae), living culture MUCL 55238 = PUCE PHPE3-4-626; ibid., Nov. 2013, root, *Piper* sp. (Piperaceae), living culture MUCL 55206 = PUCE PHPE3-1-624; ibid., rhizoplane, *Acalypha* (Euphorbiaceae), living culture MUCL 55427 = PUCE PHPE3-20-657; ibid., Jul. 2014, root, *Miconia* sp. (Melastomataceae), MUCL 55435 = PUCE PHPE4-22-1016; ibid., Jun. 2014, root, *Euterpe precatoria* (Arecaceae), living cultures MUCL 55429 = PUCE PHPE4-26-750, MUCL 55428 = PUCE PHPE4-40-745.

Remarks: *Dactylonectria polyphaga* differs from the other Amazonian species in having the smallest macroconidia (Table 5).

This species is phylogenetically related to *D. anthuriicola* (Fig 1); they differ in the size range of their 3-septate macroconidia, respectively 32–36 × 7.3–7.9 (av. = 34.5× 7.7 µm) versus 29.5–32 × 7.5–8.1 µm (av. = 30.8 × 7.8 µm, Cabral *et al.* (2012a).



Figs. 7 a–f. *Dactylonectria polyphaga* from MUCL 55209 (type). a, c, e. 3–septate macroconidia ; b. Microconidia; d, f. Conidiophores (Figs. a, scale bars = 40 μm; b–f scale bars = 20 μm). All from cultures on BLA.

DISCUSSION

Dactylonectria was introduced to accommodate a bunch of species mostly associated with black foot symptoms of grapevine in Australia, Europe, New Zealand, South Africa and USA (Cabral *et al.*, 2012b,a; Lombard *et al.*, 2014). Ten species are currently known. *Dactylonectria alcacerensis*, *D. macrodidyma*, *D. novozelandica* and *D. vitis* are only known from grapevines (Cabral *et al.*, 2012b,a; Lombard *et al.*, 2014). *Dactylonectria estremocensis*, *D. pauciseptata*, *D. pinicola* and *D. torrecensis* are known from grapevines but also from other hosts including conifers (Lombard *et al.*, 2014). So far, only two species are known exclusively from plants other than grapevine, viz. *D. anthuriicola* on *Anthurium* sp. (Araceae) and *D. hordeicola* on *Hordeum vulgare* (Poaceae). Furthermore, as far as we have been able to ascertain, there is no record of *Dactylonectria* from from South America and more specifically from the Amazonian rain forest.

Campylocarpon, originally, also was described in association with grapevine, causing black foot symptoms, in South Africa (Halleen *et al.*, 2004). *Campylocarpon* species, however, were recorded in several areas of South America, also associated to disease symptoms of grapevine: *C. fasciculare* was reported from Brazil (Correia *et al.*, 2013) whereas *C. pseudofasciculare* was reported from Uruguay (Abreo *et al.*, 2010), Peru (Alvarez *et al.*, 2012), North-eastern (Correia *et al.*, 2013) and southern Brazil (Dos Santos *et al.*, 2014).

Four *Dactylonectria* and one *Campylocarpon* species are described here above, originating from the Amazonian rain forest

in Ecuador. These species were all found directly (endophytic) or indirectly (rhizoplane) associated with several herbaceous angiosperms and one pteridophyte (Table 1). They were isolated from externally sane, asymptomatic roots, either from their internal tissues as endophytes or from their rhizoplanes. They are therefore not associated with specific disease symptoms such as root rots or black foot.

Their hosts were growing in a very disturbed, heavily polluted microhabitat in the Amazonian rain forest, which consists of a floating layer of decomposing organic debris, mostly vegetal, forming a compost-like substrate, 10-20 cm thick, and accumulating through the years over crude oil pools. These *Dactylonectria* and *Campylocarpon* species should be searched for in the neighbouring, undisturbed ecosystem to circumscribe their host range and ecology.

The phylogenetic inferences presented above also show some diversities within the *D. pauciseptata* lineage as defined by Lombard *et al.* (2014). In our analyses, the *D. pauciseptata* lineage is divided into four clades or branches. The first clade (Fig. 1, BSML = 100, PP = 1) include the type strain of *D. pauciseptata* (CBS 120171, Slovenia) and the strain CBS 113550 (New Zealand); it corresponds to *D. pauciseptata* s.s. A second clade includes two strains, viz. CBS 120173 (Portugal) and Cy 196 (Slovenia) (Cabral *et al.*, 2012a). *Dactylonectria palmicola* (MUCL 55426, Ecuador) and the strain CBS 100819 (New Zealand) form each an isolated branch. *Dactylonectria pauciseptata* sensu Lombard *et al.* (2014) might be polyphyletic and could encompass four phylogenetic species. In addition to *D. pauciseptata* and *D. palmicola*, two potential species are worth being studied more carefully (Fig. 1).

The South American records of *C. fasciculare* and *C. pseudofasciculare* also are worth to be studied more carefully. The phylogenetic analyses show that the *Campylocarpon* lineage is divided into a well-supported *C. fasciculare* lineage and a poorly supported *C. pseudofasciculare* lineage (Fig. 2). The C. fasciculare lineage (BSML = 100, PP = 0.99) is subdivided into two clades. The first clade (BSML = 96, PP = 1) includes the type strain of *C. fasciculare* (CBS 112613) together with several isolates from South African grapevine (Fig 2); it represents C. fasciculare s.s. The second clade (BSML = 94, PP = 0.66) is composed of several grapevine associated strains originating from Northeaster Brazil (Correia *et al.*, 2013); it may represent also an unnamed phylogenetic species. The *C. pseudofasciculare* strains are distributed into two poorly supported clades and the branch represented by the single *C. amazonense* strain (Fig. 2). The first (although not well supported) clade includes the type strain of *C. pseudofasciculare* (CBS 112679, *C. pseudofasciculare* s.s) and the strains CBS 112592 and BV7, both of uncertain identity. A second (also not well supported) clade is composed of nine isolates, originating in southern Brazil (Dos Santos *et al.*, 2014) that may represent an unnamed phylogenetic species.

Table 1 . List of *Cylindrocarpon-like* isolates used for the various phylogenetic inferences

Genus & species names	**Substrate**	**Country**	**GenBank accession numbers**				
Voucher specimens/cultures reference			**ITS**	**28S**	***his3***	***tub2***	***tef1a***
Campylocarpon Halleen *et al*.							
Campylocarpon amazonense Gordillo & Decock							
MUCL55434 (T)	Rhizoplane*, Cordia alliodora*	Ecuador	MF683709	MF683729	MF683688	MF683646	MF683667
Campylocarpon fasciculare Schroers *et al*.							
CBS 112613 (T)	Trunk, *Vitis vinifera,*	South Africa	AY677301	HM364313	JF735502	AY677221	JF735691
CBS 112611	Rootstock, *Vitis vinifera*	South Africa	AY677299	–	–	AY677225	–
CBS 112612	Root, *Vitis vinifera*	South Africa	AY677300	–	–	AY677216	–
CBS 113560	Root, *Vitis vinifera*	South Africa	AY677304	–	–	AY677217	–
CBS 112614	Trunk, *Vitis vinifera*	South Africa	AY677302	–	–	AY677220	–
CBS 112600	Root, *Vitis vinifera*	South Africa	AY677298	–	–	AY677219	–
CBS 113554	Rootstock, *Vitis vinifera*	South Africa	–	–	–	AY677223	–
CBS 113559	Root, *Vitis vinifera*	South Africa	AY677303	–	–	AY677218	–
BV2	Rootstock, *Vitis vinifera*	Brazil	JX521864	–	–	JX521835	–
BV3	*Vitis vinifera*	Brazil	JX521865	–	–	JX521836	–
BV4	Rootstock, *Vitis vinifera*	Brazil	JX521866	–	–	JX521837	–
BV5	Rootstock, *Vitis vinifera*	Brazil	JX521867	–	–	JX521838	–

BV6	Rootstock, *Vitis vinifera*	Brazil	JX521868	–	–	JX521839	–
Campylocarpon pseudofasciculare Halleen *et al*.							
CBS 112679 (T)	Root, *Vitis vinifera*	South Africa	AY677306	–	JF735503	AY677214	JF735692.1
CBS 112592	Root, *Vitis vinifera,*	South Africa	AY677305	–	–	AY677215	–
BV7	Rootstock, *Vitis vinifera*	Brazil	JX521869	–	–	JX521840	–
Cy1UFSM	*Vitis labrusca*	Brazil	–	–	KF633164	KF633144	–
Cy2UFSM	*Vitis rotundifolia x vinífera*	Brazil	KF447564	–	KF633173	KF633145	–
Cy3UFSM	*Vitis rupestris*	Brazil	KF447565	–	KF633166	KF633146	–
Cy6UFSM	*Vitis labrusca*	Brazil	KF447566	–	KF633169	KF633147	–
Cy14UFSM	*Vitis labrusca*	Brazil	KF447567	–	KF633158	KF633148	–
Cy17UFSM	*Vitis rupestris* x *riparia*	Brazil	KF656730	–	KF633161	KF633149	–
Cy18UFSM	*Vitis berlandieri x rupestris*	Brazil	KF447568	–	KF633162	KF633150	–
Cy19UFSM	*Vitis berlandieri x rupestris*	Brazil	KF447569	–	KF633163	KF633151	–
Cy20UFSM	*Vitis labrusca*	Brazil	KF447570	–	KF633165	KF633152	–
Dactylonectria L. Lombard & Crous							
Dactylonectria alcacerensis (A. Cabral *et al.*) L. Lombard & Crous							
CBS 129087 (T)	*Vitis vinifera*	Portugal	JF735333	KM231629	JF735630	AM419111	JF735819
Cy134	*Vitis vinifera*	Spain	JF735332	–	JF735629	AM419104	JF735818
Dactylonectria anthuriicola (A. Cabral & Crous) L. Lombard & Crous							

CBS 564.95 (T)	Root, *Anthurium* sp.	Netherlands	JF735302	KM515897	JF735579	JF735430	JF735768
Dactylonectria amazonica Gordillo & Decock							
MUCL55430 (T)	Rhizoplane, *Piper* sp.	Ecuador	MF683706	MF683726	MF683685	MF683643	MF683664
MUCL55433	Root, *Piper* sp.	Ecuador	MF683707	MF683727	MF683686	MF683644	MF683665
Dactylonectria ecuadoriense Gordillo & Decock							
MUCL55424 (T)	Rhizosplane, *Piper* sp.	Ecuador	MF683704	MF683724	MF683683	MF683641	MF683662
MUCL55432	Rhizosplane, *Socratea exorrhiza*	Ecuador	MF683702	MF683722	MF683681	MF683639	MF683660
MUCL55226	Root, *Cyathea lasiosora*	Ecuador	MF683703	MF683723	MF683682	MF683640	MF683661
MUCL55431	Rhizosplane, *Carludovica palmata*	Ecuador	MF683701	MF683721	MF683680	MF683638	MF683659
MUCL55425	Rhizoplane, *Piper* sp.	Ecuador	MF683705	MF683725	MF683684	MF683642	MF683663
MUCL55205	Root, *Piper* sp.	Ecuador	MF683700	MF683720	MF683679	MF683637	MF683658
Dactylonectria estremocencis (A. Cabral *et al.*) L. Lombard & Crous							
CBS 129085 (T)	*Vitis vinifera*	Portugal	JF735320	KM231630	JF735617	JF735448	JF735806
CPC 13539	*Picea glauca*	Canada	JF735330	–	JF735627	JF735458	JF735816
Dactylonectria hordeicola L. Lombard & Crous,							
CBS 162.89 (T)	*Hordeum vulgare*	Netherlands	AM419060	KM515898	JF735610	AM419084	JF735799
Dactylonectria macrodidyma (Halleen *et al*.) L. Lombard & Crous							

CBS 112601	Root, *Vitis vinifera*	South Africa	AY677284	KM515899	JF735644	AY677229	JF735833
CBS 112615 (T)	Root, *Vitis vinifera*	South Africa	AY677290	KM515900	JF735647	AY677233	JF735836
Dactylonectria novozelandica (A. Cabral & Crous) L. Lombard & Crous							
CBS 112608	Root, *Vitis vinifera*	South Africa	AY677288	KM515901	JF735632	AY677235	JF735821
Dactylonectria palmicola Gordillo & Decock, sp. nov.							
MUCL55426 (T)	Rhizoplane, *Euterpe precatoria* (Arecaceae)	Ecuador	MF683708	MF683728	MF683687	MF683645	MF683666
Dactylonectria pauciseptata (Schroers & Crous) L. Lombard & Crous							
CBS 120171 (T)	Root, *Vitis* sp.	Slovenia	EF607089	KM515903	JF735587	EF607066	JF735776
CBS 100819, LYN 16202/2	Root, *Erica melanthera*	New Zealand	EF607090	KM515902	JF735582	EF607067	JF735771
CBS 113550	base of trunk, *Vitis* sp.	New Zealand	EF607080	–	JF735583	EF607069	JF735772
CBS 120173, KIS10468	Root, *Vitis* sp.	Slovenia	EF607088	–	JF735589	EF607068	JF735778
CBS Cy196	*Vitis* sp.	Portugal	JF735305	–	JF735590	JF735433	JF735779
Dactylonectria pinicola L. Lombard & Crous,							
CBS 159.34	No data	Germany	JF735318	KM515904	JF735613	JF735446	JF735802
CBS 173.37 (T)	*Pinus laricio*	UK: England	JF735319	KM515905	–	JF735447	JF735803
Dactylonectria polyphaga Gordillo & Decock							
MUCL55209 (T)	Root, *Costus* sp. (Costaceae)	Ecuador	MF683689	MF683710	MF683668	MF683626	MF683647
MUCL55208	Root, *Costus* sp. (Costaceae)	Ecuador	MF683699	MF683719	MF683678	MF683636	MF683657

MUCL55238	Root, Costus sp. (Costaceae)	Ecuador	MF683696	MF683717	MF683675	MF683633	MF683654
MUCL55428	Root, *Euterpe precatoria* (Arecaceae)	Ecuador	MF683692	MF683713	MF683671	MF683629	MF683650
MUCL55429	Root, *Euterpe precatoria* (Arecaceae)	Ecuador	MF683691	MF683712	MF683670	MF683628	MF683649
MUCL55206	Root, *Piper* sp. (Piperaceae)	Ecuador	MF683693	MF683714	MF683672	MF683630	MF683651
MUCL55435	Root, *Miconia* sp. (Melastomataceae)	Ecuador	MF683694	MF683715	MF683673	MF683631	MF683652
MUCL55427	Rhizosphera, *Acalypha (Euphorbiaceae)*	Ecuador	MF683695	MF683716	MF683674	MF683632	MF683653
MUCL54780	Root, *Anthurium* sp. (Araceae)	Ecuador	MF683690	MF683711	MF683669	MF683627	MF683648
MUCL54802	Root, *Asplenium* sp. (Apleniaceae)	Ecuador	MF683698	MF683718	MF683677	MF683635	MF683656
MUCL54771	root, *Costus scaber* (Costaceae)	Ecuador	MF683697	–	MF683676	MF683634	MF683655

Dactylonectria torresensis (A. Cabral *et al*.) L. Lombard & Crous

CBS 119.41	*Fragaria* sp.	Netherlands	JF735349	KM515906	JF735657	JF735478	JF735846
CBS 129086 (T)	*Vitis vinifera*	Portugal	JF735362	KM231631	JF735681	JF735492	JF735870
Dactylonectria vitis (A. Cabral *et al*.) L. Lombard & Crous							
CBS 129082 (T)	*Vitis vinifera*	Portugal	JF735303	KM515907	JF735580	JF735431	JF735769

[T] = Ex-type isolates.

Table 2. Summary of data sets *Dactylonectria* of ITS, 28S, *tef1*, *Btub* and *h3*

	Data set								
Partitions	ITS1, ITS2, *tub* E1, *tub* I1*, tef1a I1*	5.8S, nrLSU, *tef1a* E3	*tub* E3, , *his3* E1, *his3* E2, *his3* E3, *tef1a* E1	*tub* I3, *tef1a* I2, *tef1a* I3	*tef1a* E2, *tef1a* I4	*his3* I1, *tef1a* I4	*tub* I2	*tub* E2	*his3* I2
Model selected	GTR+G	GTR+I	GTR+I+G	HKY+G	JC	GTR+G	SYM+G	SYM+G	GTR+I+G
Freq. A	0.237	0.246	0.169	0.217	0.263	0.226	0.258	0.225	0.392
Freq. C	0.321	0.217	0.381	0.324	0.253	0.367	0.279	0.291	0.387
Freq. G	0.22	0.291	0.243	0.178	0.263	0.164	0.226	0.253	0.094
Freq. T	0.222	0.246	0.207	0.282	0.221	0.243	0.236	0.23	0.126
Proportion of invariable sites		0.916	0.61						0.148
Gamma shape	1.068		0.717	1.007		1.626	1.835	0.448	2.77

I = Intron; E = Exon

Table 3. Summary of data sets *Campylocarpon* of ITS, *Btub* and *h3*

	Datasets					
Parttions	ITS1	5.8S	ITS2	*tub* I1 *tub* I2 *tub* I3	*tub* E1, *tub* E2, *tub* E3, *his3* E1 *his3* E2 *his3* E3	*his3* I1 *his3* I2
Model selected	SYM+G	JC	HKY+I	GTR	GTR+I	HKY+I
			Base frequencies			
Freq. A	0.234	0.306	0.142	0.278	0.187	0.271
Freq. C	0.323	0.224	0.39	0.32	0.367	0.376
Freq. G	0.212	0.216	0.291	0.177	0.245	0.148
Freq. T	0,231	0.254	0.178	0.226	0.2	0.205
Proportion of invariable sites			0.567		0.752	0.193
Gamma shape	1.108					

I = Intron; E = Exon

Table 4. Comparison of the conidial size of *Campylocarpon* species

Species	**Substrate**	**Conidial features**		
		# of septa	**Conidial size (µm)**	**Conidial av size (µm)**
C. amazonense Gordillo & Decock	rhizoplane of *Cordia alliodora*.	2-septate 3-septate 4-septate 5-septate	(23.5–) 24–32 (–33.5) × (5.5–) 5.3–6.0 (–6.2) (28–) 33–44 (–47) × (5.5–) 5.9–7 (–7) (31–) 33–47 (–49) × (6.2–) 6.0–7.0 (–7.8) (39–) 46.2–54.2 (54.6–) × (6.2–) 6.7–8 (–7.8)	28 × 5.8 39 × 6.5 40 × 6.5 50 × 7.3
C. fasciculare Schroers *et al.*	Roots, rootstock and trunk of *Vitis vinifera*, causing	2-septate 3-septate 4-septate 5-septate	(28.5–) 35–43.5 (–47) × (6–) 6.5–7.5 (–9) (29–) 38–44.5 (–53) × (5.5–) 6.5–8 (–9) (39–) 47–51.5 (–58)×(6.5–)	38 × 7 41.5 × 7.5 49 × 8 –

	black foot disease.		7.5–8.5 (–9) 44.5–54 × 7.5–9	
C. pseudofasciculare Halleen *et al.*	Roots of asymptomatic *Vitis vinifera* in nursery	2-septate 3-septate 4-septate 5-septate	24–36 × 6–7 (29–) 37–48 (–68.5) × (6.0) 6.5–7.5 (–9.5) (40.5–) 46.5–53.5 (–62) × (6.5–) 7–8.5 (–9.5) (35.6) 51–59 (–68) × (6.5–) 7.5–8.9 (–10)	– 44 × 7 51 × 8 55 × 8

Table 5. Comparison of the conidial features of the new *Dactylonectria* species from the Ecuadorian Amazonia.

Species	**Substrate**	**# of septa**	**Conidial size (µm)**	**Conidial av. size (µm)**
Dactylonectria amazonica	Rhizoplane	3-septate conidia	(31–) 31–41 (–43) × (5.5–) 5.7–6.5 (–7.0)	37 × 6.2
Dactylonectria ecuadoriense	Root (endophyte) and rhizoplane	3-septate conidia	(31–) 34.0–43.0 (–43.0) × (7–) 7–8 (–8.5)	38 × 8
Dactylonectria palmicola	Rhizoplane	3-septate conidia	(37–) 39–47 (–47.0) × (6–) 7.0–7.5 (–8)	42 × 7.3
Dactylonectria polyphaga	Root (endophyte)	3-septate conidia	(31–) 31–37 (–39) × (7.0–) 7 – 8 (–7.8)	34.5× 7.7

1.2.3. Multigene phylogeny and morphological evidence for undescribed species of *Aquanectria* and *Gliocladiopsis* (Ascomycota, Hypocreales) from tropical areas.

Adapted from the research article summited in

Mycologia (2017)

Ana Gordillo & Cony Decock

Preface

This paper (**sub-section 1.2.3)** is a continuation of our revision of the Ecuadorian strains and some additional isolates from tropical areas. A handful of taxa or phylogenetic species were shown using the principle of multiple gene genealogy concordance.

Several *Gliocladiopsis*-like isolates from the rainforest in Ecuador, Singapore and French Guiana were described as new species and their phylogenetic relationship were discussed (**sub-section 1.2.3)**. Three new *Gliocladiopsis* species were addressed, and four additional *Aquanectria* species were described.

Abstract: *Aquanectria* and *Gliocladiopsis* are two closely related genera of Hypocreales. They are also morphologically similar, forming hyaline, penicillate conidiophores and hyaline, straight to sinuous, 0–1-septate phialoconidia. During a revision of gliocladiopsis-like isolates originating from rain forest areas of South America (Ecuador, French Guyana) and Southeast Asia (Singapore), multilocus phylogenetic inferences revealed the occurrence of seven new phylogenetic species. Based on partial DNA sequences encoding β-tubulin, translation elongation factor 1-α, histone H3, and the nuc rDNA internal transcribed spacer regions. These phylogenetic species revealed unique combinations of phenotype, allowing morphological distinction from their closest phylogenetic relatives. Four new species of *Aquanectria* and three new species of *Gliocladiopsis* are described and illustrated. Three of the four *Aquanectria* species deviate from the other species in the genus having shorter conidia, which are in the size range observed in *Gliocladiopsis* species. They are placed in *Aquanectria* based on the phylogenetic signal, but this also makes the morphological distinction between these genera obsolete.

Key words: *Aquanectria* / *Gliocladiopsis* / Hypocreales /phylogeny /systematics / tropical areas.

INTRODUCTION

Gliocladiopsis (Hypocreales) is characterized by complex, hyaline, penicillate conidiophores, which consist of a simple septate stipe bearing 2 to 4 successive whorls of branches

subtending whorls of phialides. Conidia are hyaline, cylindrical, 0–1-septate, accumulating in whitish to pale yellowish mucoid drops (Lombard & Crous, 2012). So far, a connection to a sexual morph is known only for *G. pseudotenuis* (syn. *Glionectria tenuis* Crous & C.L. Schoch, Schoch *et al.* 2000).

Originally described with a single species, *Gliocladiopsis sagariensis* (Saksena, 1954), the genus was subsequently either reduced to synonymy under *Cylindrocarpon* (Agnihothrudu, 1959) or reduced to synonymy under *Cylindrocladium* (Barron, 1968), with the type species considered as a synonym of *Cylindrocarpon tenue* Bugnic. It was not until 1993 that Crous and Wingfield (1993) reconsidered the status of *Gliocladiopsis*, based on a single species, *G. tenuis* (syn. *G. sagariensis*). Later on, Crous and Peerall (1996) and Crous and Wingfield (1997) added G. *irregularis* and *G. sumatrensis*.

Lombard and Crous (2012) provided the most comprehensive revision of the genus, combining morphological and phylogenetic species concepts. One of the results of this study was that *G. sagariensis* and *G. tenuis* were accepted as two separate taxa (Lombard & Crous, 2012). Additionally, five species were added to the genus, *viz*. *G. curvata, G. elghollii, G. indonesiensis, G. mexicana* and *G. pseudotenuis*, whereas two sterile strains were recognized as two phylogenetic species, left unnamed as *Gliocladiopsis* sp. 1 and *Gliocladiopsis* sp. 2 (Lombard & Crous, 2012). Nonetheless, Liu and Cai (2013) proposed that *Gliocladiopsis* sp. 2 could be equated to *G. irregularis*. Liu and Cai (2013) also added *G. guangdongensis* on

the basis of a single strain originating from Southern China (Liu & Cai, 2013). Parkinson *et al.* (2017) described three new species, *G. peggii*, *G. whileyi* and *G. forsbergii*, isolated from avocados roots, in Australia.

As far as ecology is concerned, limited information is currently available. *Gliocladiopsis* species are primarily soil-borne fungi, isolated as soil-borne pathogens (Booth, 1966; Crous *et al.*, 1997) and from diseased roots (Dann *et al.*, 2012; Parkinson *et al.*, 2017) but also from asymptomatic rhizomes (Li *et al.*, 2008). Their trophic or nutritional relationships, including their potential relevance as soil-borne pathogens (Booth, 1966) or, to the contrary, as growth promotors (Dann *et al.*, 2012) is poorly understood. Nevertheless, the type strain of *G. guangdongensis* was isolated from wood submerged in freshwater (Liu & Cai, 2013) whereas unidentified *Gliocladiopsis* isolates were also isolated as endophytes from the flower of *Rafflesia cantleyi* Solms-Laubach (Rafflesiacae) (Refaei *et al.* 2011, ITS sequence at GenBank HM368445) and from seeds of *Cecropia insignis* (Cecropiaceae) in Panama (U'Ren *et al.*, 2009), suggesting a broader ecological range.

Gliocladiopsis is morphologically reminiscent of *Aquanectria*, which is also its closest phylogenetic relative (Lombard *et al.*, 2015). The conidiophores, conidiogenous cells, and conidiogenesis and, to some extents, the conidia are similar in both genera. *Aquanectria* would differ in having longer, filiform to slightly sinuous conidia (Lombard *et al.*, 2015), what might represent an adaptation to aquatic habitats (Baschien *et al.*,

2013); *Aquanectria* includes *hitherto* only two species, *A. penicillioides* (type species) and *A. submersa* (Lombard *et al.*, 2015), which are both aquatic fungi growing on submerged plant debris (Ingold, 1942; Hudson, 1961; Duarte *et al.*, 2012). In this frame, we re-evaluated the identity and phylogenetic afinities of a set of gliocladiopsis-like strains originating from rain forest areas of South America (Ecuador and French Guiana) and Southeast Asia (Singapore). Using multilocus, DNA-based phylogenetic approaches, these strains were shown to be dispersed into seven new terminal clades, which could be equated to as much new phylogenetic species (Taylor *et al.*, 2000). Each of these phylogenetic species also presents a distinct phenotype, allowing morphological distinction from their closest phylogenetic relatives.

Three species nest within the *Gliocladiopsis* lineage as defined by Lombard and Crous (2012) or Parkinson *et al.* (2017). Their morphology agrees with this placement. One species belongs to the *Aquanectria* lineage as defined by Lombard *et al.* (2015), what also is concordant with its phenotype. The other three phylogenetic species form together a single lineage, related (sister) to the *Aquanectria* lineage. However, this is discordant with their phenotypes, of which the conidial shape and size that would point toward *Gliocladiopsis* rather than *Aquanectria*. *Aquanectria* is considered as their natural placement on the sole basis of the phylogenetic affinities; however, this placement and, ahead, the pertinence of having two genera could be questioned.

METHODS AND MATERIALS

Site and sample procedure.– The strains from Ecuador were isolated from the rhizosphere and roots of several Monocotyledons and one Pteridophyte growing in a layer of organic debris forming a mat floating over oil ponds, in Amazonian forest. The local vegetation is dominated by *Carludovica palmata* Ruiz & Pav. (1798) (Cyclanthaceae), *Dimerocostus strobilaceus* Kuntze (1891) (Costaceae), *Heliconia* cf. *chartacea* Lane ex Barreiros (Heliconiaceae), and several species of Araceae. Weather conditions are characterized by warm temperatures and high humidity (Perez, 2014).

Isolation of fungal strains. – In Ecuador, roots and adjacent soils were collected, placed into polyethylene bags and kept in a refrigerator at 4–7 C until processed within 24 hours. Roots were gently washed to remove the rough soil particles then rinsed with sterile water. This rinsing solution was diluted in 9 mL sterilized water (1 vol solution into 9 vol water). Of each dilution, 1 mL was poured on malt extract agar supplemented with 50 ppm of L-chloramphenicol (MEA50, Untereiner *et al.*, 1998), in duplicate. The plates were incubated in the dark at 25ºC and examined every day for fungal growth. Pure cultures were obtained from germinating spores or growing hyphae and transferred to MEA in 9 cm diam Petri dishes.

To isolate endophytes, roots were washed to remove soil particles, cut into six 5 cm-long segments, and surface-sterilized using successive baths, 1 min in 99 % (v/v) ethanol, 5 min in 35% (v/v) hydrogen peroxide, 1 min in 99 %(v/v) ethanol, then rinsed with sterile, distilled water for a few minutes. Immediately after

rinsing, segments of 2–3 mm length were aseptically excised from the middle of each root piece and placed on MEA. The plates were incubated at 25ºC in the dark (Ahlich & Sieber, 1996) and examined every day for fungal growth. Isolates were obtained by excising hyphal tips emerging from the root' pieces.

The strains obtained were deposited at BCCM/MUCL (Agro-food & Environmental Fungal Collection).

The strains from Singapore were obtained by Cony Decock through single conidia isolation from gliocladiopsis-like colonies emerging from decaying leaves submerged in freshwater. They were deposited at BCCM/MUCL.

The strain from French Guiana was obtained by Cony Decock through single conidia isolation from a gliocladiopsis-like colony emerging from a fragment of a basidiome of a *Phellinus* sp. (Basidiomycota, Hymenochaetaceae), on artifical culture media. It was deposited at BCCM/MUCL.

Morphological characterisation. – Cultures were grown on Malt Extract Agar (MEA), Synthetic Nutrient-Poor agar (SNA) and Banana Leaf Agar (BLA), at 25 C with a 12/12 hr near UV light / dark cycle (Untereiner *et al.*, 1998). Culture characteristics were determined at 7 d after inoculation. As a rule, the descriptions of the conidiophores and conidia are based on morphologically informative structures developed on BLA. For conidial measurements, the 95 % confidence levels were determined, the extremes of the conidial measurements are given inside parenthesis. For the other structures only the extremes are

presented. Colors of the colonies were described according to Kornerup and Wanscher (1978).

DNA isolation, amplification, sequencing and data sets. – DNA was extracted from mycelium grown in malt broth at 25 C in the dark, using innuPREP Plant DNA kit (Analytik Jena, Germany) following the manufacturer's recommendations. Partial sequences were determined for genes encoding β-tubulin (*tub2*, region between exon 1 and exon 4), translation elongation factor 1-α (*tef1a*, region between exon 1 and exon 4) and histone H3 (*his3*, region between exon 1 and exon 3) and the nuc rDNA ITS1-5.8S-ITS2 region (ITS). The *tub2* gene fragment was amplified using primer pair T1 (O'Donnell & Cigelnik, 1997) and Bt-2b (Glass & Donaldson, 1995). The *tef1a* gene fragment was amplified using the primers ef1and ef2 (O'Donnell *et al.*, 1998). The *his3* gene fragment was amplified with primer pair H_3-1a and H_3-1b (Glass & Donaldson, 1995). The ITS region was amplified with the primer pair ITS5 and ITS4 (White *et al.*, 1990). The PCR conditions are as described in (Lombard *et al.*, 2010c). Amplicons were sequenced in both directions by Macrogen Inc. (Korea) using the same primers (cf. above).

Raw sequences were edited with Sequencher®software version 5.1 (Gene Codes Corporation Ann Arbor n.d.). Reference sequences (Lombard & Crous, 2012; Lombard *et al.*, 2015; Parkinson *et al.*, 2017) were downloaded from Genbank. Nucleotide sequence alignments were determined using MAFFT v. 7.213 (Katoh & Standley, 2013) and manually corrected in PhyDE-1 (Müller *et al.*, 2006) when necessary. Three data sets

were set up to conduct three phylogenetic inferences. Species, strains and sequences used in this study are listed in TABLE 1.

The first data set comprises 56 strains of *Gliocladiopsis* (thirteen species), *Aquanectria* (two species), *Dematiocladium* (one species), *Penicillifer* (one species), *Corallonectria* (one species), *Cylindrocladiella* (one species), our unidentified gliocladiopsis-like isolates, and *Calonectria* (two species) selected as outgroup (Lombard *et al.*, 2015). The second dat set comprises the 13 described species of *Gliocladiopsis* and our unidentified gliocladiopsis-like strains that, as a result of the first set analysis, were found to belong to the *Gliocladiopsis* lineage (Lombard & Crous, 2012). *Calonectria brachiatica* and *C. brassicae* also were selected as out-group (Lombard & Crous, 2012). The third data set includes the described species of *Aquanectria* (Lombard *et al.*, 2015) and several of our unidentified gliocladiopsis-like strains that, as a result of the first set analysis, were found to belong to or to be related to *Aquanectria* (Lombard *et al.*, 2015). The concatenated alignments are deposited at TreeBASE (http://www.treebase.org/treebase/index.html) under accession numbers S21752, S21531 and S21757.

Phylogenetic analysis. – Phylogenetic analyses were performed using maximum parsimony (MP) as implemented in PAUP* 4.0b10 (Swofford, 2003), Bayesian inference (BI) as implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) and maximum likelihood (ML) using RAxML 7.0.4 (Stamatakis,

2006). The best-fit likelihood model of evolution for the different data sets was estimated using PartitionFinder (Lanfear, 2012).

For MP analyses, gaps were treated as missing data. The most parsimonious trees were identified using heuristic searches with 1000 random addition sequences, further evaluated by bootstrap analysis, retaining clades compatible with the 50% majority-rule in the bootstrap consensus tree. Analysis conditions were tree bisection addition branch swapping, starting tree obtained via stepwise addition, steepest descent not in effect and MulTrees effective. Clades with bootstrap support value (BS) above 90% were considered strongly supported by the data.

Bayesian analyses were implemented with two independent runs, each with four MCMC simultaneous independent chains, starting from random trees, and keeping one tree every 1000th generation. All trees sampled after convergence [average standard deviation of split frequencies < 0.01, confirmed using Tracer v1.4 (Rambaut, A. & Drummond, 2007), were used to reconstruct a 50% majority-rule consensus tree (BC) and to estimate posterior probabilities. Clades with PP above 0.95 were considered strongly supported by the data.

Maximum likelihood (ML) searches were conducted with RAxML involved 1000 replicates under the GTRGAMMA model. In addition, 1000 bootstrap (BSML) replicates were run with the same model. Nodes with maximum bootstrap values ≥ 85% were considered to be significantly supported.

Phylogenetic congruency between the loci was tested using

a 70% reciprocal bootstrap criterion in ML analysis of each individual locus (Mason-Gamer *et al.*, 1996; Lombard *et al.*, 2014).

RESULTS

Phylogenetic analysis. – The amplification products of the *tub2*, *tef1a*, *his3* and ITS ranged ~ 500–550 bases each. The *tub2* gene fragment is missing for the type strain of *Dematiocladium celtidis*. The *his3* gene fragments could not be amplified for the strains MUCL 48728, 48412 and 54818. It is also missing for the type strain of *Aquanectria penicillioides* and *Dematiocladium celtidis.* The *tef1a* gene fragment is missing for the strain *Aquanectria submersa, Gliocladiopsis forsbergii*, *Gliocladiopsis peggii* and *Gliocladiopsis whileyi*.

The 70% reciprocal bootstrap tree topologies showed no conflicts between phylogenies resulting from the individual DNA regions (ITS, *tub2*, *tef1a* and *his3*) as far as the terminal clades are concerned. These individual phylogenies resolved each the same terminal clades / branches or phylogenetic species; the polymorphism of these DNA markers make them suitable for species discrimination within each of the lineages shown. Concerning the *Gliocladiopsis* (sensu Lombard *et al.,* 2015) and the *Aquanectria* sensu (Lombard *et al.,* 2015) lineages, the results are confused. These individual phylogenies resolved *Aquanectria* (sensu Lombard *et al.,* 2015) as a well-supported clade only in *tef1a* and *tub2*-based phylogenies, whereas *Gliocladiopsis* (sensu Lombard & Crous, 2012 or Parkinson *et al.*,

2017) is resolved as a well-supported clade in none of the individual phylogenies. Furthermore, these DNA region partitions region also revealed conflicting as far as the relationships between *Aquanectria* and *Gliocladiopsis* are concerned. The *tef1a*-based phylogenetic inference only did group the *Gliocladiopsis* and *Aquanectria* clades in well-supported lineage. The phylogenetic inferences based on the other genes remain confused as far as the relationships between these two clades are concerned.

Nonetheless, we have no info on the individual gene phylogenies by Lombard *et al.* (2015). Furthermore, as emphasized by Cunningham (1997), combining incongruent gene partitions could increase phylogenetic accuracy. This was the case in previous phylogenetic studies of cylindrocarpon-like species (Lombard *et al*., 2014) but also in other genera of Hypocreales (e.g. Gehesquiere *et al.*, 2016; Lombard *et al.*, 2017). Therefore, the five gene regions also were combined in the present study.

The first data set comprises 2447 positions, including gaps. Four segments located in the introns 1 and 2 of *his3* (108 positions), in the introns 1 and 3 of *tub2* (50 positions), in the ITS2 (110 positions) and in the introns 1 and 2 of *tef1a* (19 positions) could not be confidently aligned and were excluded from the analysis. The general time reversible model (GTR+I+G) using proportion of invariant sites and distribution of rates at variable sites modelled on a discrete gamma distribution with four rate classes was estimated as the best-fit likelihood model of

evolution for BI and ML. The base frequencies are A = 0.22, C = 0.33, G = 0.22, T = 0.22, the gamma distribution shape parameter = 0.73, and the proportion of invariable sites = 0.41.

For the MP analysis, 1368 positions were constant, 237 variable-uninformative and 612 parsimony-informative. The heuristic search yielded 57 most parsimonious trees (length 977 steps, consistency index (CI) 0.648, retention index (RI) 0.773. The differences in the topologies between the 20 trees results from variable relationships within the main *Gliocladiopsis* lineage, especially within the *G. curvata* clade. The two Bayesian runs converged to stable likelihood values after 330100 generations. In the ML searches, the combined data set had 1024 distinct patterns with proportion of gaps and undetermined characters of 10.60%. The topologies obtained were overall highly concordant in the resolution of the terminal species clades, whatever the methodologies (cladistic or probabilistic).

The second data set comprises 2264 positions, including gaps. This analysis includes the segments that were removed from the first data set (cf. above). The general time reversible model (GTR+I+G), using proportion of invariant sites and distribution of rates at variable sites modeled on a discrete gamma distribution with four rate classes was estimated as the best-fit likelihood model of evolution for BI and ML. The base frequencies are A = 0.22, C = 0.32, G = 0.22, T = 0.22, the gamma distribution shape = 0.68 and the proportion of invariable sites = 0.48.

For the MP analysis, 1606 positions were constant, 77 variable-uninformative and 356 parsimony-informative. The heuristic search yielded 118 most parsimonious trees (length 679 steps, CI = 0.772, RI = 0.88. The differences in the topologies between the 118 trees results from variable relationships within the main *Gliocladiopsis* lineage and within the *G. curvata* clade. The two Bayesian runs converged to stable likelihood values after 650000 generations. In the ML searches, the combined data set alignment had 558 distinct patterns with proportion of gaps and undetermined characters of 8.16%. The topologies obtained were overall highly concordant in the resolution of the terminal species clades, whatever the methodologies.

The third data set comprises 2268 positions, including gaps. The best estimated model was JC+G with unequal base frequencies for ITS (A = 0.23, C = 0.28, G = 0.24, T = 0.23, gamma distribution shape parameter of 0.49) and HKY+G with unequal base frequencies for *tub2, his3* and *tef1a* (A = 0.23, C = 0.33, G = 0.21, T = 0.21, gamma distribution shape parameter of 0.26).

For the MP analysis 1751 positions were constant, 148 variable-uninformative and 157 parsimony-informative. The heuristic search yielded one single parsimonious trees (length 378 steps, CI = 0.93, RI = 0.88. The two Bayesian runs converged to stable likelihood values after 10000 generations. In the ML searches with RAxML the combined data set alignment had 343 distinct patterns with proportion of gaps and

undetermined characters of 13.78%. The topologies obtained were highly concordant in the terminal clades in all cases.

The first data set phylogenetic analyses resolved 28 species clades (FIG. 1). Twenty-two species clades are distributed into three well-supported related lineages (FIG. 1) *viz*. the *Aquanectria* (BS = 100, BSML = 100, PP = 1) and the *Gliocladiopsis* (BS = 100, BSML = 99, PP = 1) lineages and an additional lineage (BS = 100, BSML = 100, PP = 1), sister to the *Aquanectria* clade. *Dematiocladium*, *Penicillifer*, *Corallonectria*, *Cylindrocladiella* are basal to the *Gliocladiopsis – Aquanectria* lineage.

The *Gliocladiopsis* lineage, whether using the first (FIG. 1) or second data set (FIG. 2), is divided into several well-supported sublineages, confirming previous results (Lombard & Crous, 2012; Parkinson *et al.*, 2017).

The first sublineage (FIGS. 1, 2, BS = 95, BSML = 97, PP = 1) corresponds to the *G. sagariensis* - *G. elghollii* clade, also shown by Lombard and Crous (2012). The strain MUCL 54818 nests within this clade, forming a well distinct branch. The second sublineage (BS = 90, BSML = 94, PP = 1) corresponds to the *G. tenuis* - *Gliocladiopsis* sp. 1 clade, also shown by Lombard and Crous (2012). The strain MUCL 54740 nests within this clade, forming a subclade (BS =100, BSML = 99, PP = 1) with *Gliocladiopsis* sp. 1.

The strains MUCL 48412 and MUCL48728 form a distinct clade (FIGS. 1, 2, BS = 87, BSML = 93, PP = 1), representing a

third, distinct phylogenetic species, which is related to *G. curvata*, *G. forsbergii* and *G. whileyi* (FIG. 2).

The *Aquanectria* lineage, as originally defined (Lombard et al., 2015), contains *A. penicillioides* and *A. submersa* (Lombard et al., 2015). MUCL 54681 is closely related to but distinct from these two species, forming a well distinct branch (FIGS. 1, 3), hence it is interpreted as a novel phylogenetic species.

Four strains (MUCL 48016, MUCL 48047, MUCL 48197 and MUCL 53250) form a distinct, well-supported lineage (FIGS. 1, 3, BS = 100, BSML = 100, PP = 1). This lineage is sister to the *Aquanectria* lineage (Lombard *et al.*, 2015). It is further divided into three distinct subclades, corresponding to MUCL 48016 and 48047, MUCL 48197, and MUCL 53250, considered here as three additional phylogenetic species.

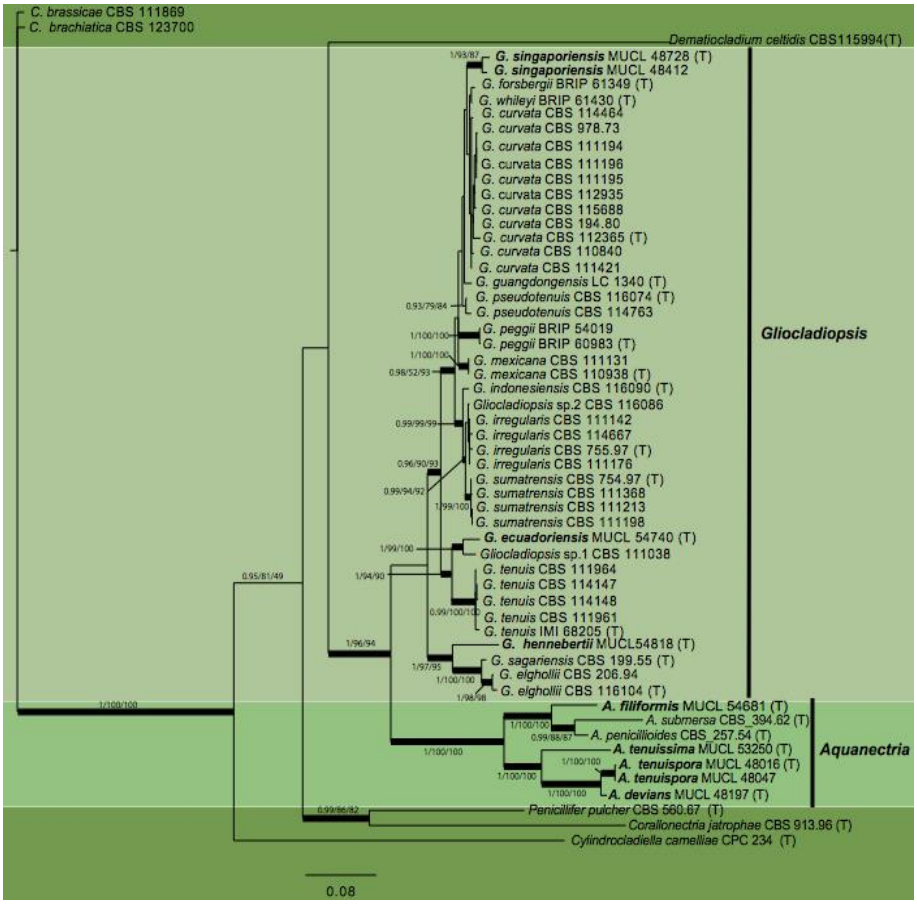


Figure 1. MULTILOCUS PHYLOGENETIC TREE (BAYESIAN INFERENCE) combined four-gene sequence alignment from *Aquanectria* and *Gliocladiopsis* data set. New phylogenetic species are indicated bold. Bootstrap support values and posterior probability are indicated with bold lines when PP (≥ 0.95)/ ML (≥ 85%)/ MP (≥ 90%). Thickened lines represent nodes also present in ML and MP trees. Scale bar shows 10 changes. The tree was rooted to *Calonectria brachiatica* and *Calonectria brassicae*.

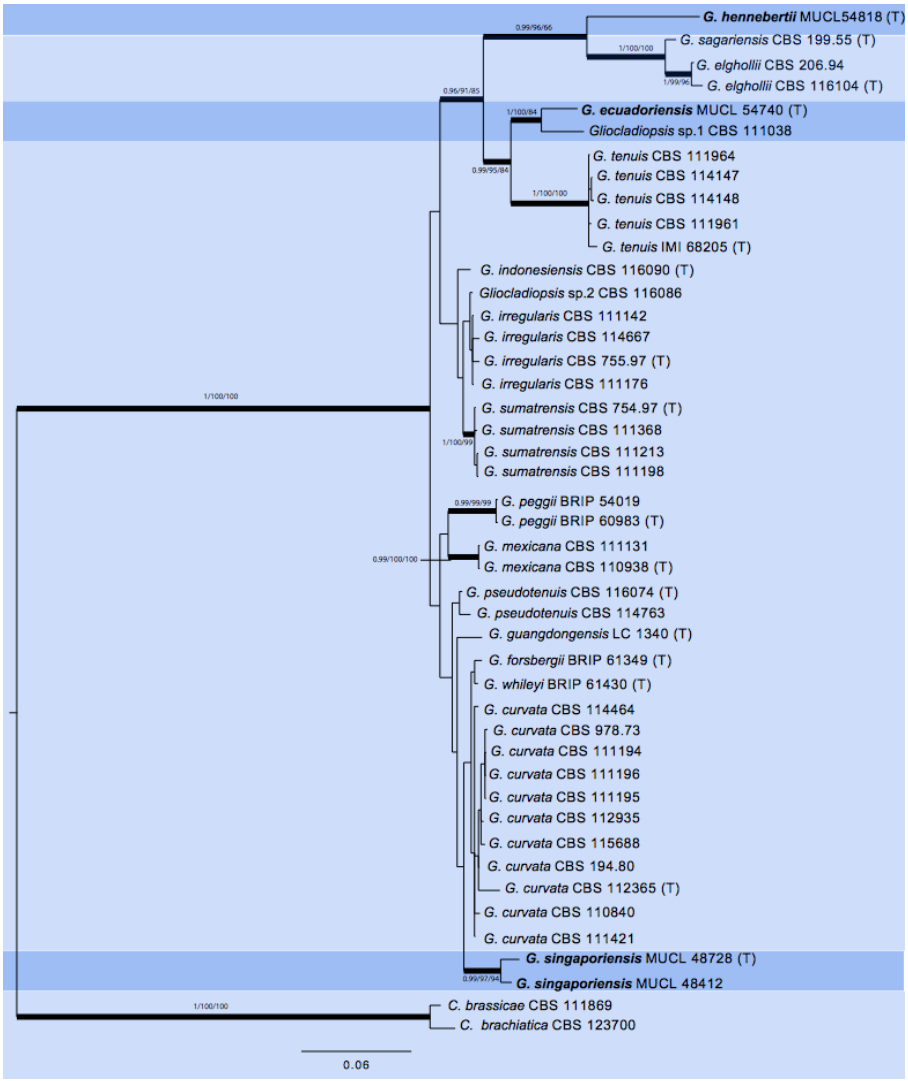


Figure 2. MULTILOCUS PHYLOGENETIC TREE (BAYESIAN INFERENCE) combined four-gene sequence alignment from *Gliocladipsis* data set. New phylogenetic species are indicated bold. Bootstrap support values and posterior probability are indicated with bold lines PP (≥ 0.95)/ ML (≥ 85%)/ MP (≥ 90%). Thickened lines represent nodes also present in ML and MP

trees. Scale bar shows 10 changes. The tree was rooted to *Calonectria brachiatica* and *Calonectria brassicae*.

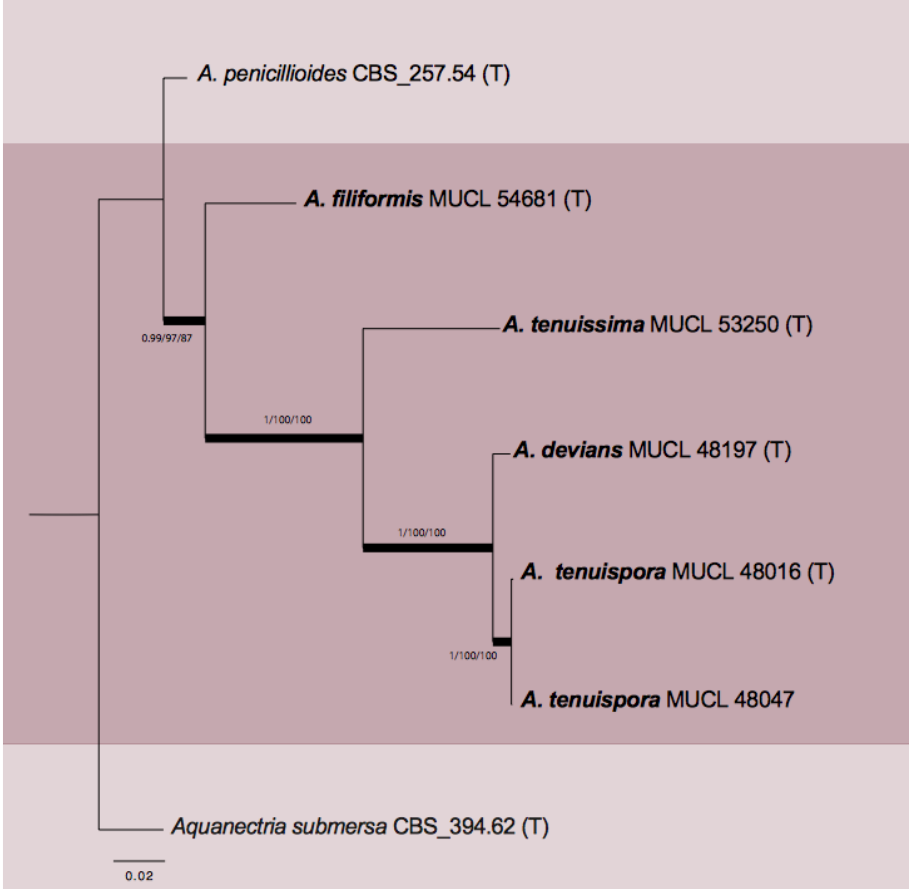


Figure 3. MULTILOCUS PHYLOGENETIC TREE (BAYESIAN INFERENCE) combined four-gene sequence alignment from *Aquanectria* data set. New phylogenetic species are indicated bold. Bootstrap support values and posterior probability are indicated with bold lines PP (≥ 0.95)/ ML (≥ 85%)/ MP (≥ 90%). Thickened lines represent nodes also present in ML and MP

trees. Scale bar shows 10 changes. The tree was rooted to *Aquanectria sumersa.*

Taxonomic conclusions. – Several gliocladiopsis-like strains originating from South America and Southeast Asia are shown to represent seven new lineages (FIGS 1–3), which are interpreted as so many phylogenetic species. Examination of the strains pertaining to each of these PSs revealed singular combinations of phenotype, especially as far as the branching pattern of the conidiophores and the conidial shape and size are concerned, allowing morphological distinction from their closest phylogenetic relatives**.**

Three species nest within the *Gliocladiopsis* lineage (Lombard & Crous, 2012). Their morphology also agrees with the morphological circumscription of the genus. They have the penicillate conidiophores and cylindrical conidia, 0–1-septate conidia, which size range is 12–22 µm long. They are described below as *Gliocladiopsis ecuadoriensis*, *G. hennebertii* and *G. singaporiensis*.

MUCL 54681 nests together with *A. penicillioides* and *A. submersa* (Lombard *et al.*, 2015). The morphology of this strain also agrees with the current morphological circumscription of the genus (Lombard *et al.*, 2015). *Aquanectria* was distinguished from *Gliocladiopsis* by having much longer conidia, straight to variably sinuous. *Aquanectria penicillioides* and *A. submersa* have sinuous conidia, which size range is, respectively, 45–55 × 2–3 µm (Ingold, 1942; Ranzoni, 1956; Lombard *et al.*, 2015) and

26–44 × 1.5–2.5 µm (Hudson, 1961). MUCL 54681 produces straight to only occasionally slightly sinuous conidia, which size range is 25–30 × 2 µm. It is described below as *Aquanectria filiformis* sp. nov.

The three phylogenetic species represented by MUCL 48016 and 48047, MUCL 53250, and MUCL 48197 formed together a well-supported clade (BS = 100, BSML = 100, PP = 1), which is sister to the *Aquanectria* lineage (FIG. 1) (Lombard *et al.*, 2015). However, their phenotypes are, to some extend, deviating from *Aquanectria* as originally defined (Lombard *et al.*, 2015). They have much shorter conidia, which are in the size range observed in the *Gliocladiopsis* species; morphology-based taxonomy would have placed these strains, doubtlessly, in the latter genus. However, placing emphasis on the phylogenetic signals (e.g. Lombard *et al.*, 2016), they are described below as *Aquanectria devians*, *Aquanectria tenuispora* and *Aquanectria tenuissima*, what is commented.

TAXONOMY

Aquanectria devians Gordillo & Decock, sp. nov. FIGS. 4a–b

MycoBank: MB 823328

Typification: SINGAPORE. MacRitchie Reservoir Park, approx. N 1° 20' 41" – E 103° 49' 20", decaying leaf submerged in freshwater, Dec 2001, collected by Olivier Laurence, isolated by Cony Decock from colonies on natural substrate (holotype *MUCL 48197*, as two-week-old dried culture on BLA). Ex-type

culture MUCL 48197. GenBank: *his3*: KX671150; ITS: KX671144; *tef1-α*: KX671136; *tub2*: KX611506.

Etymology: *devians* (Lat.), referring to small size of the conidia, the smallest conidia *hitherto* known in the genus.

Culture characteristics: colonies on SNA effuse, reaching 5 mm diam in 7 days, 40 mm diam in 14 days; aerial mycelium hyaline; *colonies* on MEA reaching 40 mm diam in 7 days, 65 mm diam in 14 days, with a concentric growth pattern; *aerial mycelium* finely floccose, white, the reverse dark brown (KW 6F5).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending the phialidic conidiogenous cells; *stipe* hyaline, thin-walled, 80–160 × 2 µm with 1(–3) levels of hyaline branches; *primary branches* aseptate 15.5–17 × 2 µm; *secondary branches* aseptate 8.0–12.5 × 2 µm; *tertiary branches* absent to rare 8.0–10 × 2 µm; *phialides* cymbiform, 8–12 × 2 µm, in whorls of 2–4; *conidia* cylindrical, straight with rounded ends, hyaline, smooth, 0 or 1 septate, (10–) 11–16 (–17) × 1.6–2 µm, av = 14 × 1.6 µm; *chlamydospores* not observed; *sexual morph* not observed.

Substratum: submerged leaf in fresh water.

Habitat: rain forest

Distribution: known only from Singapore.

Remarks.— *Aquanectria devians* has the smallest conidia within *Aquanectria*, averaging 14 µm long, in which features it differs from all other species of the genus. In that sense, it is the most “deviating” species of *Aquanectria* as originally conceived (Lombard *et al.*, 2015).

Gliocladiopsis irregularis has conidia similar in length, averaging 13 × 2.5 µm (Crous & Peerall, 1996). However, conidia are cylindrical, 2.5–3 µm wide in *G. irregularis* (Crous & Peerall, 1996) and cylindrical but thinner, 1.6–2 µm wide in *A. devians* (FIG. 4a).

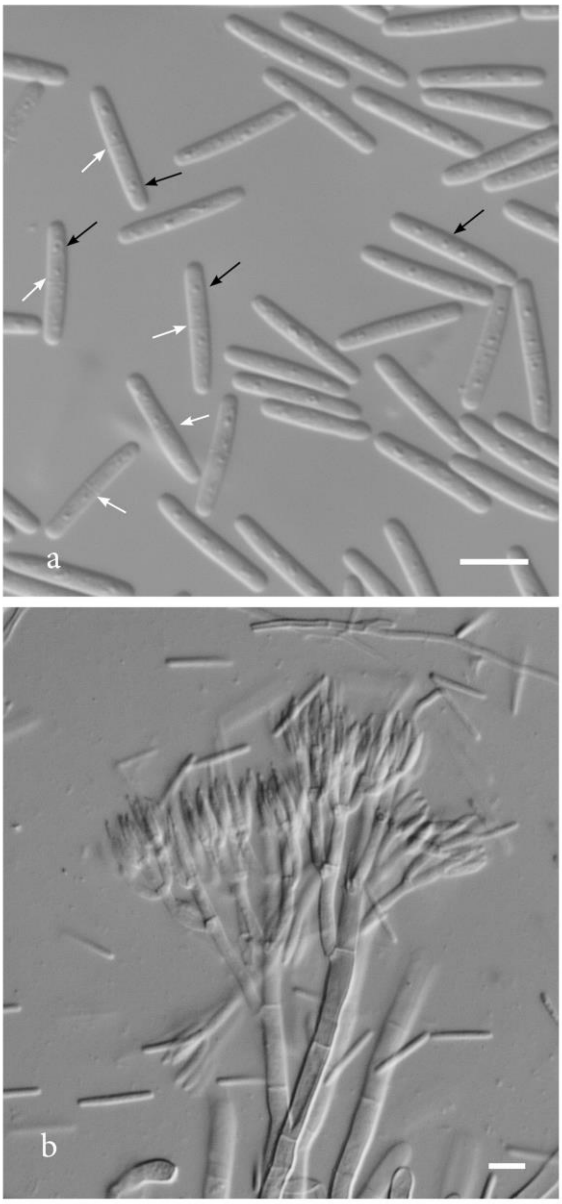


Figure 4. *Aquanectria devians* from the type, MUCL48197. a. Conidia; b. Conidiophores; white arrow: septa, black arrow: guttulates (Figs. a–b, scale bars = 10 μm).

Aquanectria filiformis Gordillo & Decock, sp. nov. FIGS. 5a–b

MycoBank: MB 823329

Typification: ECUADOR. SUCUMBÍOS PROV: Nueva Loja, Lago Agrío canton, Charapa camp, approx. W 76° 48' – S 00° 11', secondary rain forest, from root of *Monotagma* sp. (Marantaceae), Nov 2012, *A. Gordillo & C. Decock*, *PUCE PHPE1-14-54* (holotype MUCL 54681, isotype PUCE PHPE1-14-54, as two-week-old dried culture on BLA). Ex-type culture MUCL 54681, PUCE PHPE1-14-54). GenBank: *his3*: KX671145; ITS: KX671137; *tef1-α*: KX671129; *tub2*: KX611499.

Etymology: filiformis (Lat.), referring to the shape of the conidia.

Culture characteristics: colonies on SNA effuse, reaching 4 mm diam in 7 days, 15 mm diam in 14 days; aerial mycelium hyaline; colonies on MEA reaching 21 mm diam in 7 days, 42 mm diam in 14 days with a concentric growth pattern; *aerial mycelium* floccose, greyish brown (KW 5D3), yellowish brow (KB 5E8) toward the center, pale orange (KW 5A3) toward the margin, the reverse dark brown (KW 5E4).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending the phialidic conidiogenous cells; *stipe* hyaline, thin-walled, up 31–47 × 3 µm, with 1 (–3) series of hyaline branches; *primary branches* aseptate, 16–31 × 2 µm; *secondary branches* aseptate, 9–14 × 2 µm; *tertiary branches* absent to rare, aseptate, 9–12.5 × 1.5 – 2 µm with

terminal branches bearing 2–3 phialides*; phialides* cylindrical to cymbiform, 12–17 × 2 µm; *conidia* filiform, straight to occasionally slightly sigmoid, with rounded ends, hyaline, smooth, guttulate, 0–1 septate, (23–) 24–30 (–31) × 1.5–2 µm, av = 27 × 2 µm; *chlamydospores* sparse, in chains of 2–3 ovoid to cylindrical brownish cells, individually 16–23 × 10–13 µm; *sexual morph* not observed.

Substratum: root of *Monotagma* sp. (Marantaceae).

Habitat: secondary Amazonian rain forest.

Distribution: known *hitherto* only from eastern Ecuador.

Remarks.— *Aquanectria filiformis* is related to *A. penicillioides* and *A. submersa* (FIGS. 1, 3), from which it could be distinguished by its conidial size and shape. *Aquanectria penicillioides* has longer (45–55 × 2–3 µm), curved to sigmoid conidia (Ingold, 1942; Lombard *et al.*, 2015). The conidia of *A. submersa* were originally described as 29–51 (or 26–44) × 1.5–2 µm (Hudson, 1961). The conidia of *A. filiformis* are (23–) 24–30 (–31) × 1.5–2 µm (av = 27 × 2) mostly straight (FIG. 5e), unfrequently slightly sigmoid.

Aquanectria penicillioides and *A. submersa* are described as “aquatic” fungi growing on plant material submerged in freshwater (Ingold, 1942; Ranzoni, 1956; Hudson, 1961; Duarte *et al.*, 2012; Lombard *et al.*, 2015). *Aquanectria filiformis* was isolated from a non-strictly aquatic habitat. Nevertheless, its

microhabitat, a superficial layer of organic debris covering an oil pond, is regularly flooded during the frequent heavy rains.

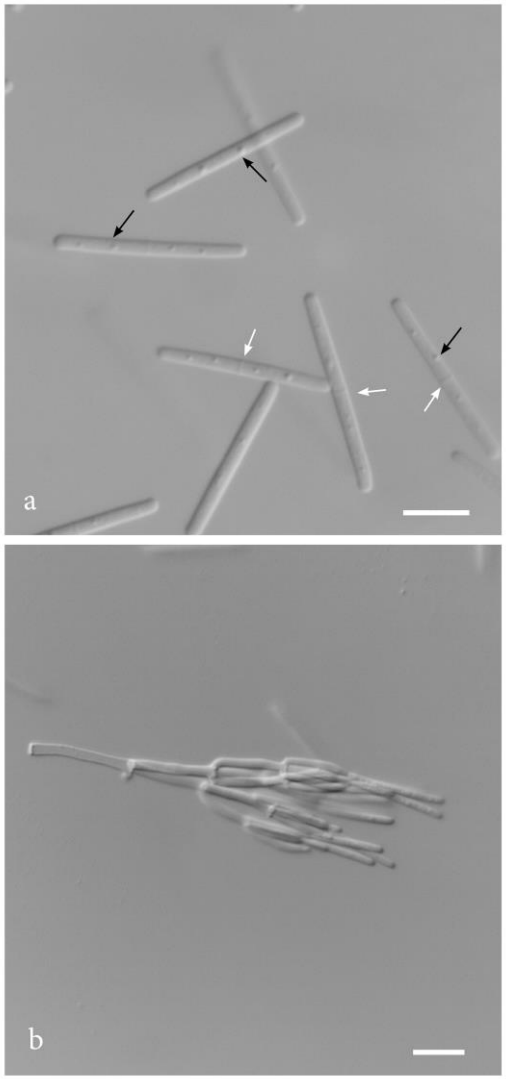


Figure 5. *Aquanectria filiformis*, from the type, MUCL54681. a. Conidia; b. Conidiophores; white arrow: septa, black arrow: guttulates (Figs. a, b. scale bars = 10 μm).

Aquanectria tenuissima Gordillo & Decock, sp. nov. FIGS. 6a–b

MycoBank: MB 823330

Typification: FRENCH GUIANA. Nouragues Nature Reserve, approx. N 4°05' – W 52°41' emerging from a fragment of a basidiomata of a *Phellinus sp*. (voucher specimen FG-10-218) plated on Malt Extract Agar, during field work, Aug. 2010, *C. Decock*, *FG-10-218c* (holotype *MUCL 53250*, as two-week-old dried culture on BLA. Ex-type culture MUCL 53250. GenBank: *his3*: KX671148; ITS: KX671142; *tef1-α*: KX671134; *tub2*: KX611504.

Etymology: *tenuissima* (Lat.), referring to the small conidia.

Culture characteristics: colonies on SNA effuse, reaching 31 mm diam in 14 days; aerial mycelium hyaline; *colonies* on MEA reaching 30 mm diam in 7 days, 55 mm diam in 14 days, with a concentric growth pattern; *aerial mycelium* floccose, brown (KW 6E7), greyish-orange toward the margin (KW 6B5), the reverse yellowish brown.

Conidiophores hyaline, penicillate, with a basal stipe and apical series of branches subtending the phialidic conidiogenous cells; *stipe* hyaline, thin-walled, 25–100 × 2–4 µm with 1(–3) hyaline branches; *primary branches* aseptate 16–31 × 2 µm; *secondary branches* aseptate 9–12 × 2 µm; *tertiary branches* absent to occasional 9–11.5 × 2 µm; *phialides* cymbiform 8–10(–12) × 2 µm, arranged in terminal whorls of 2–4; *conidia*

cylindrical, straight with rounded ends, hyaline, smooth, oil guttule, 0 or 1 septate, 16–23 (–24) × 2 µm, av = 20 × 2 µm; *chlamydospores* in short chains of bright brown cells, individually 6–12 × 12 µm; *sexual form* not observed.

Substratum: unknown, isolated as a culture contaminant.

Habitat: rain forest.

Distribution: known *hitherto* only from French Guiana

Remarks.— The conidia of *A. tenuispora* and *A. tenuissima* are similar. These two species could be differentiated by the length of their stipe, respectively 40–55 µm and 25–100 µm long, and the size of their phialides, respectively, 9–13 × 2 µm (av = 12 × 2 µm) and 8–10 (–12) × 2 µm (av = 9 × 2 µm). *Aquanectria devians* (MUCL 48197) is distinguished from *A. tenuispora* and *A. tenuissima* in having smaller conidia *viz*., respectively, (10–) 11–16 (–17) µm (av = 14 × 1.6 µm), (16–) 17–20 (–21) × 2 µm (av = 19 × 2 µm) and 16–23 (–24) × 2 µm (av = 20 × 2 µm).

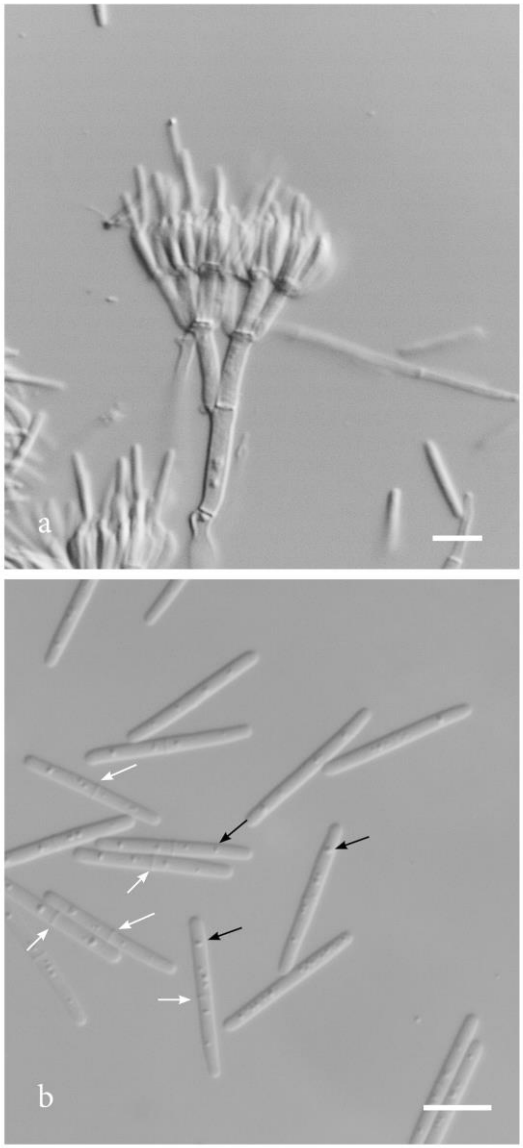


Figure 6. *Aquanectria tenuispora*, from the type, MUCL53250. a. Conidiophores; b. Conidia. Conidia; white arrow: septa, black arrow: guttulates (Figs. a. scale bars = 7μm; b. scale bars = 10 μm).

Aquanectria tenuispora Gordillo & Decock, sp. nov. FIGS. 7a–d

MycoBank: MB 823331

Typification: SINGAPORE. Mac Ritchie Reservoir Park, approx. N 1° 20' 41" – E 103° 49' 20", Nov 2001, decaying leaf submerged in freshwater, unidentified angiosperm, collected by *Olivier Laurence*, isolated by *Cony Decock* from colonies on natural substrate (holotype MUCL 48016, as two-week-old dried culture on BLA). Ex-type culture *MUCL 48016*. GenBank: *his3*: KX671147; ITS: KX671141; *tef1-α*: KX671133; *tub2*: KX611503.

Etymology: tenuispora (Lat.), referring to the small size of the conidia

Culture characteristics, colonies on SNA effuse, reaching 35 mm diam in 7 days, 62 mm diam in 14 days; *aerial mycelium* hyaline; *colonies* on MEA reaching 28 mm diam in 7 days, 50 mm diam in 14 days, with concentric growth pattern; *aerial mycelium* floccose, brown (KW6E6), greyish-brown toward the margin (KW6E4), the reverse dark brown (KW 6F5).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending the phialidic conidiogenous cells; *stipe* hyaline, thin-walled, 40–55 × 2–3 µm, with apical 1(–3) series of hyaline branches; *primary branches* aseptate 14–20 × 2–3 µm; *secondary branches* aseptate 8–16 × 2–3 µm; *tertiary branches* absent to rare aseptate 9–12 × 2 µm; *phialides* in whorl of 2–4, cylindrical to cymbiform 9–13 × 2 µm; *conidia* cylindrical,

straight with rounded ends, hyaline, smooth, oil guttule, 0 (–1) septate, (16–) 17–20 (–21) × 2 µm, av = 19 × 2 µm; *chlamydospores* present in short chains of 3–5 cells, individually 9–17 × 8–12 µm; *sexual morph* not observed.

Substratum: submerged leaf in freshwater reservoir.

Habitat: rain forest litter.

Distribution: known *hitherto* only from Singapore

Other specimens examined: SINGAPORE. MacRitchie Reservoir Park, Nov 2001, decaying, submerged leaf litter in freshwater, unidentified angiosperm, collected by Olivier Laurence, isolated by Cony Decock from colonies on natural substrate (living culture MUCL 48047).

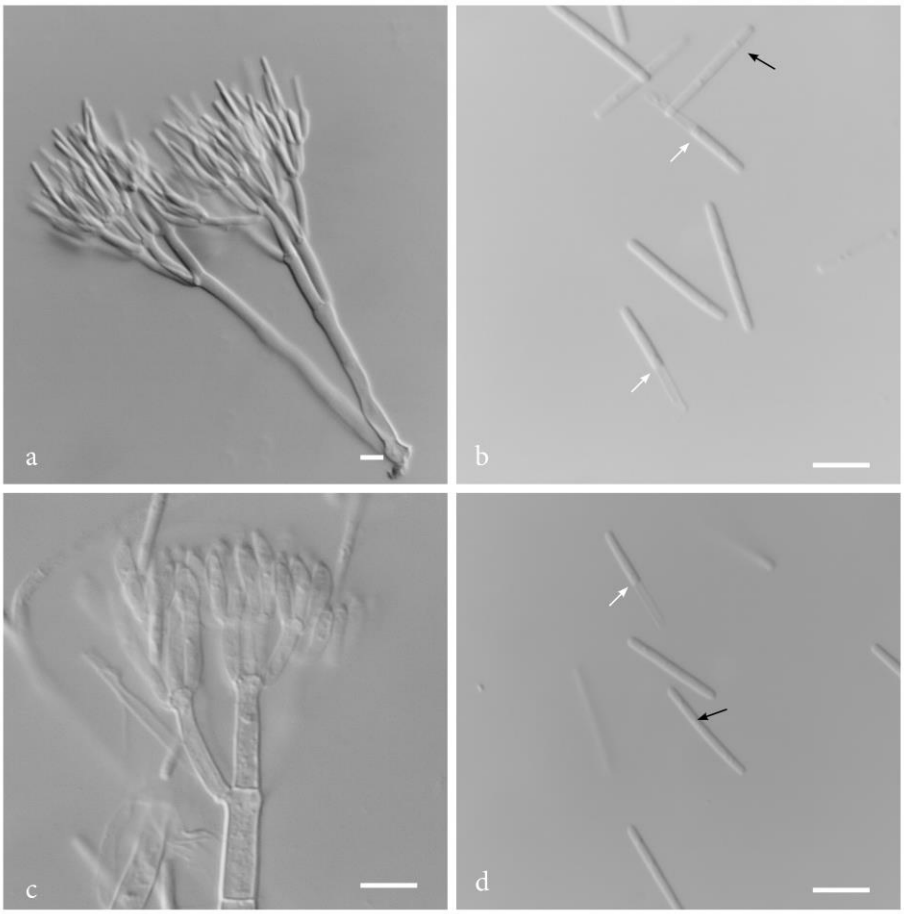


Figure 7. *Aquanectria tenuissima*, from the type MUCL48016. a.b Conidiophores; c,d. Conidia; white arrow: septa, black arrow: guttulates (Figs. a. scale bars = 7μm; b. scale bars = 10 μm).

Gliocladiopsis ecuadoriensis Gordillo & Decock, sp. nov. FIGS. 8a–d

MycoBank: MB 823333

Typification: ECUADOR. SUCUMBÍOS PROV.: Nueva Loja, Lago Agrío Canton, Charapa Camp, approx. W 76° 48' – S 00° 11', secondary rain forest, from the rhizoplane of *Polybotrya sp.* (Dryopteridaceae), Jan 2013, *A. Gordillo & C. Decock*, *PUCE PHPE2-20-368* (holotype MUCL 54740, isotype PUCE PHPE2-20-368, as two-weeks-old dried culture on BLA). Ex-type culture MUCL 54740, PUCE PHPE2-20-368. GenBank: *his3*: KX671146; ITS: KX671139; *tef1-α*: KX671131; *tub2*: KX611501.

Etymology: "*ecuadoriensis*" (Lat.), refers to the country of origin, Ecuador.

Culture characteristics: colonies on SNA effuse, reaching 44 mm diam in 7 days, 75 mm diam in 14 days; *aerial mycelium* mostly white; *colonies* on MEA reaching 30 mm diam in 7 days, 50 mm diam in 14 days; *aerial mycelium* floccose, greyish orange (KW 5B4), darker, yellowish brown toward the center (KB 5E8), brownish orange toward the margin (KW 5C4), the reverse olive brown (KW 4F8).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending phialidic conidiogenous cells; *stipe* hyaline, thin-walled, septate, 20–140 × 2–4 µm bearing 2 (–3) series of hyaline branches; *primary branches* aseptate, 16–21 × 2–3 µm; *secondary branches* aseptate, 12–20 × 2 µm; *tertiary branches* aseptate, 9–20 × 2 µm; *phialides* in whorls of 2–4, cymbiform to cylindrical 9–16 × 2 µm; *conidia* cylindrical, straight with rounded ends, hyaline, smooth, 0 or 1 septate, (8–) 8–16 (–16) × 1.6–2 µm, av = 13 × 2 µm;

c*hlamydospores* in short chains of 3–5 ovoid to spherical, smooth-walled, bright brown cells, individually 14–24 × 8–16 µm; *sexual morph* not observed.

Substratum: rhizoplane of *Polybotrya* sp. (Dryopteridaceae).

Habitat: secondary Amazonian rain forest.

Distribution: known *hitherto* only from Ecuador, western Amazonia.

Remarks.— *Gliocladiopsis ecuadoriensis* (MUCL 54740) is closely related to *Gliocladiopsis* sp. 1 represented by CBS 111038; both strains could be considered as conspecific although slightly genetically divergent (FIG. 2). However, the lack of reproductive structure in the strain CBS 111038 (Lombard & Crous, 2012) hampers confirmation. It could be of interest to grow this strain on BLA, a media which could stimulate sporulation (Gordillo & Decock, 2017). At a lesser degree, *G. ecuadoriensis* also is related to *G. tenuis* (FIG. 2).

Gliocladiopsis ecuadoriensis and *G. tenuis* are distinguished by their conidial size, 8–16 × 1.6–2 (av = 13.2 × 2 µm) and 16.5–20 × 1.5–2 µm (av = 18 × 2 µm) (Crous & Wingfield, 1993; Lombard & Crous, 2012). The number of branches of the conidiogenous apparatus also differentiates these two species; *G. ecuadoriensis* has predominantly primary and secondary branches, rarely tertiary, whereas *G. tenuis* produces regularly tertiary to quaternary branches (Crous & Wingfield, 1993).

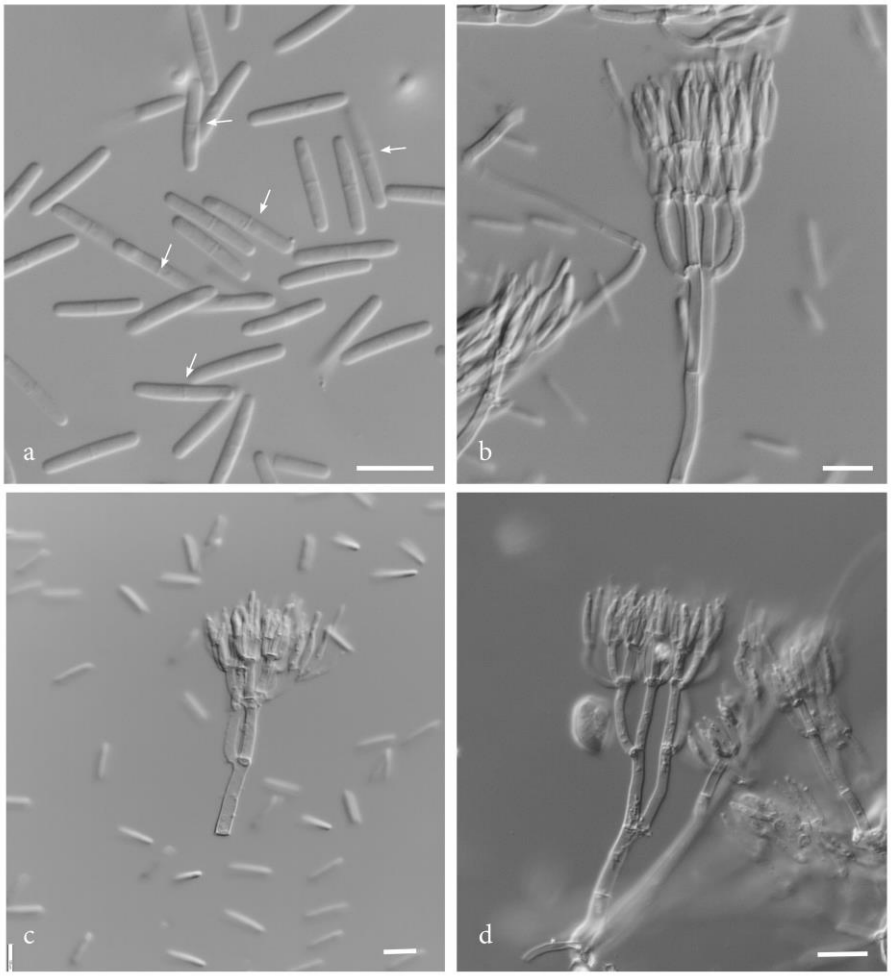


Figure 8. *Gliocladiopsis ecuadoriensis* from the type, MUCL54740 a. Conidia b–d. Conidiophores; white arrow: septa. (Figs. a – d. scale bars = 10 μm). .

Gliocladiopsis hennebertii Gordillo & Decock, sp. nov. FIGS. 9a–b

MycoBank: MB 823334

Typification: ECUADOR. SUCUMBÍOS PROV.: Nueva Loja, Lago Agrío canton, Charapa camp, approx. W 76° 48' – S 00° 11', secondary rain forest, from the rhizoplane of *Costus scaber* (Costaceae), Jan 2013, *A. Gordillo & C. Decock*,*PUCE PHPE2-33-332* (holotype *MUCL 54818*, isotype *PUCE PHPE2-33-332*, as two-week-old dried culture on BLA). Ex-type culture MUCL 54818, PUCE PHPE2-33-332. GenBank: ITS: KX671140; *tef1-α*: KX671132; *tub2*: KX611502.

Etymology: "*hennebertii*" (Lat.), dedicated to Prof G.L. Hennebert, former director of MUCL and initiator of the cooperation with PUCE, Ecuador.

Culture characteristics: *colonies* on SNA effuse, reaching 23 mm diam in 7 days, 55 mm diam in 14 days; *aerial mycelium* hyaline; *colonies* on MEA reaching 23 mm diam in 7 days, 37 mm diam in 14 days; *aerial mycelium* floccose, greyish at center (KW 5D3), brownish grey (KW 5B2) toward the margin, the reverse yellowish brown (KW 5F8).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending the phialidic conidiogenous cells; *stipe*, thin-walled, septate, 27–39 × 2–4, with 2–3 (–4) series of hyaline branches; *primary branches* aseptate, 27–39 × 3–4 µm; *secondary branches* aseptate, 13–25 × 2–3 µm; *tertiary branches* aseptate, 9–17 × 2–3 µm; quaternary branches absent to occasional 8–12 × 2–3 µm, *phialides* in terminal whorls of 2–4 per branch, cylindrical, 12–21(–23) × 2–3 µm; *conidia* cylindrical, hyaline, smooth, guttulate, straight with rounded ends, 0 or 1

septate, (16–) 18–21 (–22) × 1.5–2 µm, av = 20 × 2 µm; *chlamydospores* sparse, in short chains of ovoid to cylindrical cells, individually 16–22 ×8–16 µm; *sexual form* not observed.

Substratum: rhizoplane of *Costus scaber* Ruiz & Pav. (Costaceae)

Habitat: secondary Amazonian rain forest.

Distribution: known *hitherto* only from Ecuador, western Amazonia

Remarks.— *Gliocladiopsis hennebertii* is related to *G. elghollii* and *G. sagariensis*; these three species form a well-supported subclade (FIGS. 1, 2).

Gliocladiopsis hennebertii and *G. sagariensis* could be distinguished by the length of their primary and secondary branches and phialides, respectively 27–39 µm, 13–25 and 12–21(–23) µm long *vs* 14–22 µm, 8–12 µm and 10–15 µm long (Saksena 1954). Their conidia are overall similar although marginally smaller in *G. hennebertii* compared to *G. sagariensis*, *viz*. 16–22 × 1.5–2 µm (av = 20 × 2 µm) and 18–24 × 1.5–2 µm (Saksena 1954), respectively.

The branching pattern of the penicillus in *G. sagariensis* is uncertain. The ex-type isolated held at the CBS is sterile (Lombard & Crous, 2012). Saksena (1954) noted that "*fructifications are usually in three to four stages*" although only primary, secondary branches and phialides are mentioned in the

description (three “stages”). The drawings and pictures also illustrate tertiary branches, what would mean, adding phialides, four “stages”. Quaternary branches should therefore be absent.

Gliocladiopsis hennebertii could be distinguished from *G. elghollii* by the absent to occasional presence of quaternary branches, present in abundance in the latter, and in having narrower conidia, respectively 1.5–2 µm *vs* 2–4 µm wide (Lombard & Crous, 2012).

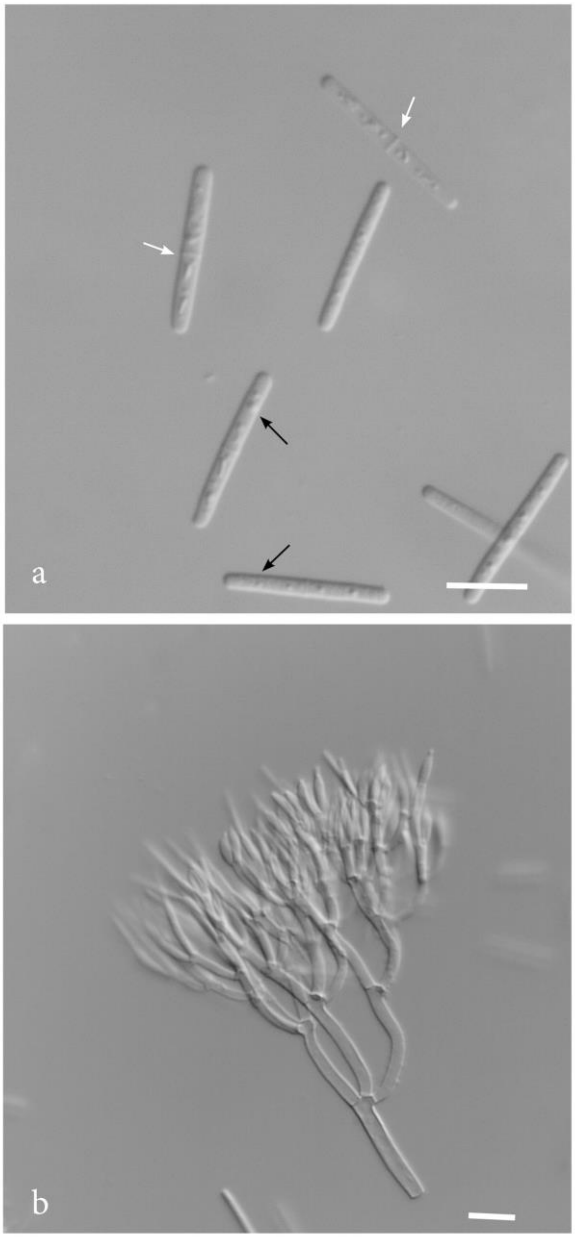


Figure 9. *Gliocladiopsis hennebertii* from the type, MUCL54818. a. Conidia, b. Conidiophores; white arrow: septa, black arrow: guttulates (Figs. a – d. scale bars = 10 μm).

Gliocladiopsis singaporiensis Decock & Gordillo, sp. nov. FIGS. 10a–d

MycoBank: MB 823335

Typification: SINGAPORE. Lower Peirce Reservoir, approx. N 1° 22' 12" – E 103° 49' 13", decaying leaf submerged in freshwater, Apr 2002, collected by *Olivier Laurence* (Mycosphere S.a.r.l.), isolated by *Cony Decock* from conidia picked up from colonies on natural substrate (holotype MUCL 48728, as two-week-old dried culture on BLA. Ex-type culture MUCL 48728. GenBank: ITS: KX671138; *tef1-α*: KX671130; *tub2*: KX611500.

Etymology: "*singaporiensis*" (Lat.) refers to the country of origin, Singapore.

Culture characteristics: *colonies* on SNA effuse, white, reaching 40 mm diam in 7 days, 70 mm diam in 14 days; aerial mycelium hyaline; *colonies* on MEA reaching 35 mm diam in 7 days, 60 mm diam in 14 days, *aerial mycelium* floccose, initially homogeneously white then light brown (KW 5D4), orange grey (KW 5B2) toward the margin, the reverse olive brown (KW 4D5).

Conidiophores hyaline, penicillate, with a basal stipe and an apical series of branches subtending phialidic conidiogenous cells; *stipe* hyaline, thin-walled, septate, 34–140 × 3–4 µm, with 2–3(–4) series of hyaline branches: *primary branches* aseptate, 20–31 × 3–4 µm; *secondary branches* aseptate, 16–22 × 3–4

µm; *tertiary branches* aseptate, 10–20 × 2 µm; *quaternary branches*, 9–16 × 2–3 µm; *phialides* in whorls of 2–4, cymbiform to cylindrical, 8–14 × 2 µm; *conidia* cylindrical, hyaline, smooth, straight with rounded ends, 0 (–1) septate, (13–) 14–19 × 1.5–2 µm, av = 16 × 2 µm; *chlamydospores* sparse, in short chains of ovoid to cylindrical, individually 8–14 × 9–13 µm; *sexual morph* not observed.

Substratum: submerged leaf litter in stream.

Habitat: leaf litter, Southeast Asian rain forest.

Distribution: known *hitherto* only from Singapore.

Other specimens examined: SINGAPORE: Lower Peirce Reservoir, approx. N 1° 22' 12" – E 103° 49' 13", decaying leaf submerged in freshwater, unidentified angiosperm, Apr 2002, collected by Olivier Laurence (Mycosphere S.a.r.l.), isolated by Cony Decock from conidia picked up from colonies on natural substrate, culture MUCL 48412.

Remarks.— *Gliocladiopsis singaporiensis* has an isolated position, closed related to the subclade formed by *G. curvata*, *G. forsbergii* and *G. whileyi* (FIG. 2). *Gliocladiopsis singaporiensis* could be differentiated from these species by the conidial shape and/or size and the number of branches in the penicillus. *Gliocladiopsis singaporiensis* has slightly shorter and especially narrower conidia (13–19 × 2 µm, av = 16 × 2 µm) compared to those of *G. curvata* (17–21 (–23) × 3–5 µm, av = 19 × 3 µm, Lombard & Crous, 2012) and those of *G. whileyi* (17–21 (–22) ×

1.5–3 µm, Parkinson *et al.*, 2017). Conidia are also straight in *G. singaporiensis* and slightly curved in the two latter species (Lombard & Crous, 2012; Parkinson *et al.*, 2017). Quaternary branches are frequent in *G. singaporiensis* and *G. whileyi* (Parkinson *et al.*, 2017) but absent to rare in *G. curvata* (Lombard & Crous, 2012).

Gliocladiopsis forsbergii differs from *G. curvata*, *G. singaporiensis* and *G. whileyi* in having penicilli with up to five levels of branches (Parkinson *et al.*, 2017). *Gliocladiopsis irregularis, G. mexicana*, *G. pseudotenuis* and *G. sumatrensis* all lack quaternary branches (Lombard & Crous, 2012), in which feature they differ from *G. singaporiensis*.

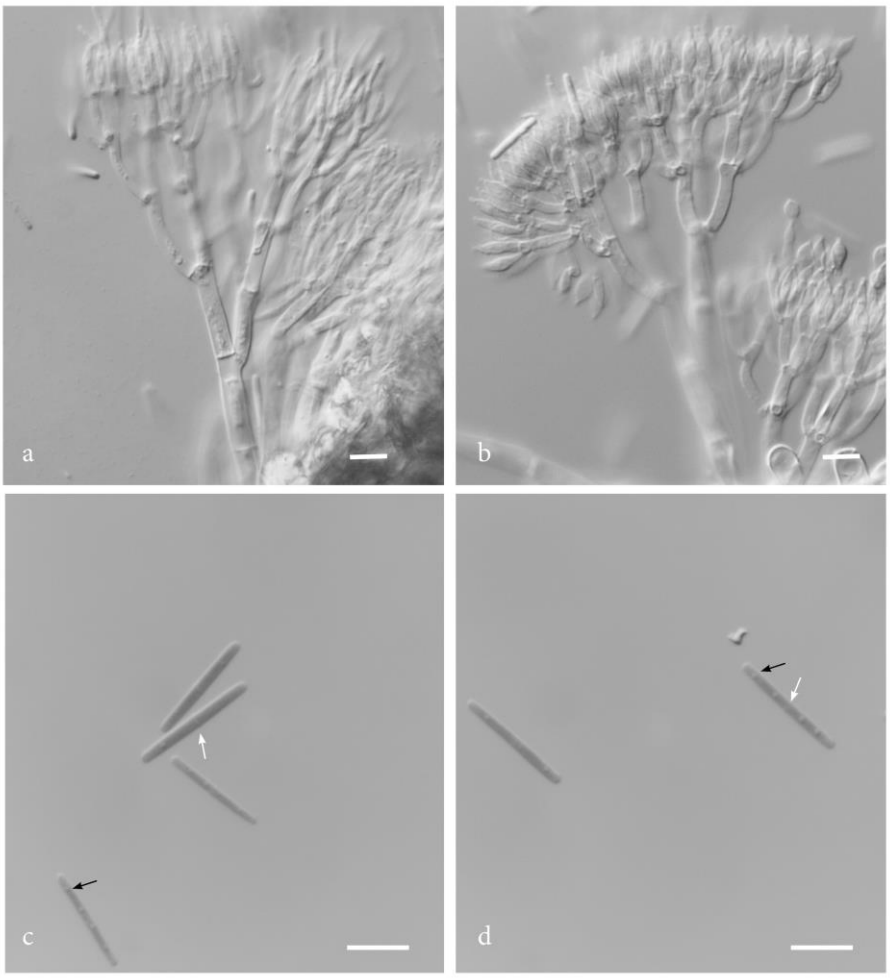


Figure 10. *Gliocladiopsis singaporiensis* from the type, a–b. Conidiophores; c.d. Conidia; white arrow: septa, black arrow: guttulates (Figs. a – d. scale bars = 8 μm).

DISCUSSION

Aquanectria and *Gliocladiopsis* are closely related sister taxa (Lombard *et al.*, 2015) that can hardly be distinguished

morphologically, although they tend to occur in different ecological niches. The finding of *Aquanectria devians*, *A. tenuispora* and *A. tenuissima* raised questions about the delimitation and the pertinence of differentiating *Aquanectria* from *Gliocladiopsis*. Their phenotypes are strongly reminiscent of *Gliocladiopsis*, as evidenced by their conidia, which shape and size overlap with those of *Gliocladiopsis* species (Lombard & Crous, 2012). Their conidial sizes are, on average, ≤ 20 µm long, far smaller than the conidia observed in *Aquanectria sensu* Lombard *et al.* (2015). In the sole morphological perspective, they would have been placed, doubtlessly, in *Gliocladiopsis*. Nonetheless, these species form together a well-supported clade (FIG. 1) that is more closely related (sister) to the *Aquanectria* lineage (*sensu* Lombard *et al.* 2015) than to the *Gliocladiopsis* lineage (*sensu* Lombard and Crous 2012). Their intron 1 of *his3*, for instance, is confidently alignable with that of *A. submersa* and *A. filiformis* (*sensu* Lombard and Crous 2012) but hardly alignable with that of the *Gliocladiopsis* species.

Following the current trend of molecular-based classification (*e.g.* Lombard *et al.* 2016), placing emphasis on the phylogenetic signals of the different markers used, *Aquanectria* is here considered as their “taxonomic” placement. This option reduces, *de facto*, the morphological (conidiophores, conidiogenous cells) and ontogenetic criteria (conidiogenesis) to subordinate elements. This also makes obsolete the *hitherto* sole apparent (or known) morphological distinction between the closely related *Aquanectria* and *Gliocladiopsis* (Lombard and Crous 2012), *viz*. the shape and size of the asexual spores. The sexual morph of

Gliocladiopsis and *Aquanectria* species are still very poorly known, impeding to draw conclusions. It results in a morphologically heterogeneous *Aquanectria*, divided into a “typical” *Aquanectria* and a gliocladiopsis-like morphotype, each corresponding to a well-supported subclade.

Species of this expanded *Aquanectria* lineage would share the autecological feature of inhabiting an aquatic or temporarily flooded habitat; the type strains of *A. devians* and *A. tenuispora* as well as the type strains of *A. penicillioides* and *A. submersa* originate from aquatic or periodically flooded habitat. The habitat of the type strain of *A. tenuissima* is uncertain.

One alternate option would have been to merge *Aquanectria* and *Gliocladiopsis*, emphasizing the key morphological and ontogenetic features of the hyaline, penicillate conidiogenous apparatus, the conidiogenous cells, and the conidiogenesis that are identical in both genera. This expanded *Gliocladiopsis* also would form a well-supported, well-delimited lineage within the *Nectriaceae* (sensu Lombard *et al.* 2015). The resulting *Gliocladiopsis* also would be morphologically more homogeneous but for the then atypical morphotype represented by the monophyletic *A. penicillioides*, *A. submersa* and *A. filiformis*, which conidia are much longer and variably sinuous compared to the typical morphotype. Eventually, whether placing these three latter species in *Gliocladiopsis* or *Aquanectria*, the long and variably sigmoid conidia could represent a derived adaptation to a strictly aquatic habitat (Baschien *et al.*, 2013), apomorphic for the *A. penicillioides*, *A. submersa* and *A. filiformis* subclade, but

of secondary importance and not pertinent to distinguish closely related genera.

TABLE 1. List of species, collections, and sequences used in the phylogenetic analyses.

Genus /Species names			GenBank accession number			
Voucher specimens/cultures reference	**Substrate**	**Country**	***tub2***	***his3***	**ITS**	***tef1-α***
Aquanectria L. Lombard & Crous						
Aquanectria penicillioides (Ingold) L. Lombard & Crous						
CBS 257.54; ATCC 16261	Acer sp.	USA	KM232000	–	KM231743	KM231865
Aquanectria submersa (H.J. Huds.) L. Lombard & Crous						
CBS 394.62	Unknown	UK	KM231999	KM231458	HQ897796	–
Aquanectria filiformis Gordillo & Decock						
MUCL 54681	Endophyte-root, *Monotagma sp.*	Ecuador	KX611499	KX671145	KX671137	KX671129
Aquanectria devians						
MUCL 48197	Submerged leaf litter in freshwater	Singapore	KX611506	KX671150	KX671144	KX671136
Aquanectria tenuispora						
MUCL 48047	Submerged leaf litter in freshwater	Singapore	KX611505	KX671149	KX671143	KX671135
MUCL 48016	Submerged leaf litter in freshwater	Singapore	KX611503	KX671147	KX671141	KX671133
Aquanectria tenuissima						
MUCL 53250	Unknown	French Guiana	KX611504	KX671148	KX671142	KX671134
Calonectria de Not.						

Calonectria brachiatica L. Lombard, et al.						
CBS 123700	*Pinus maximinoi*	Colombia	FJ696388	FJ696396	GQ280555	GQ267296
Calonectria brassicae (Panwar & Bohra) L. Lombard et al.						
CBS 111869	–	–	AF232857	DQ190720	GQ280576	FJ918567
Corallonectria C. Herrera & P. Chaverri 2013						
Corallonectria jatrophae (Möller) C. Herrera & P. Chaverri						
CBS 913.96(T); GJS 96-18	Unknown tree	Puerto Rico	KC479787	KM231457	KC479758	KM231863
Cylindrocladiella Boesew						
Cylindrocladiella camelliae (Venkataram. & C.S.V. Ram) Boesew						
CPC 234 (T); PPRI 3990; IMI 346845	*Eucalyptus grandis*	South Africa	AY793471	AY793509	AF220952	JN099087
Dematiocladium Allegr., Aramb., Cazau & Crous						
Dematiocladium celtidis Allegr., Aramb., Cazau & Crous						
CBS 115994 (T)	*Celtis tala*	Argentina	–	–	AY793430	KM231864
Gliocladiopsis S.B. Saksena						
Gliocladiopsis curvata L. Lombard & Crous						
CBS 978.73	Soil	Brazil	JQ666119	JQ666009	JQ666043	JQ666085
CBS 194.80	*Persea americana*	Ecuador	JQ666120	JQ666010	JQ666044	JQ666086
CBS 110840	Greenhouse	Belgium	JQ666121	JQ666011	JQ666045	JQ666087

CBS 111194	Soil	Mauritius	JQ666122	JQ666012	JQ666046	JQ666088
CBS 111195	Soil	Mauritius	JQ666123	JQ666013	JQ666047	JQ666089
CBS 111196	Soil	Mauritius	JQ666124	JQ666014	JQ666048	JQ666090
CBS 111421	Soil	Ecuador	JQ666125	JQ666015	JQ666049	JQ666091
CBS 112365(T)	*Archontophoenix purpurea*	New Zealand	JQ666126	JQ666016	JQ666050	JQ666092
CBS 112935	*Syzygium aromaticum*	Indonesia	JQ666127	JQ666017	JQ666051	JQ666093
CBS 114464	Soil	Ecuador	JQ666128	JQ666018	JQ666052	JQ666094
CBS 115688	–	Japan	JQ666129	JQ666019	JQ666053	JQ666095
Gliocladiopsis ecuadoriensis Gordillo & Decock						
MUCL 54740	Rhizospher*, Polybotrya sp.*	Ecuador	KX611501	KX671146	KX671139	KX671131
Gliocladiopsis elghollii L. Lombard & Crous						
CBS 206.94	*Chamaedorea elegans*	USA	JQ666130	JQ666020	JQ666054	JQ666096
CBS 116104 (T)	*Chamaedorea elegans*	USA	JQ666131	JQ666021	JQ666055	JQ666097
Gliocladiopsis forsbergii L.E. Parkinson, E.K. Dann & R.G. Shivas						
BRIP 61349 (T)	*Persea americana*	Australia	KX274037	KX274054	KX274071	–
Gliocladiopsis guangdongensis F. Liu & L. Cai						
LC 1340	Submerged wood	China	KC776124	KC776120	KC776122	KC776118

Gliocladiopsis hennebertii Gordillo & Decock						
MUCL 54818	Rhizospher, *Costus scaber*	Ecuador	KX611502	–	KX671140	KX671132
Gliocladiopsis indonesiensis L. Lombard & Crous						
CBS 116090 (T)	Soil	Indonesia	JQ666132	JQ666022	JQ666056	JQ666098
Gliocladiopsis irregularis Crous & Peerally						
CBS 755.97(T)	Soil	Indonesia	JQ666133	JQ666023	AF220977	JQ666099
CBS 111142	*Araucaria sp.*	Malaysia	JQ666134	JQ666024	JQ666057	JQ666100
CBS 111176	*Araucaria sp.*	Malaysia	JQ666135	JQ666025	JQ666058	JQ666101
CBS 114667	*Araucaria sp.*	Malaysia	JQ666136	JQ666026	JQ666059	JQ666102
Gliocladiopsis mexicana L. Lombard & Crous						
CBS 110938 (T)	Soil	Mexico	JQ666137	JQ666027	JQ666060	JQ666103
CBS 111131	Soil	Mexico	JQ666138	JQ666028	JQ666061	JQ666104
Gliocladiopsis peggii L.E. Parkinson, E.K. Dann & R.G. Shivas						
BRIP 55019	*Persea americana*	Australia	JN243766	JN243767	JN243765	–
BRIP 60983 (T)	*Persea americana*	Australia	KX274038	KX274065	KX274083	–
Gliocladiopsis sagariensis S.B. Saksena						
CBS 199.55 (T)	Soil	India	JQ666141	JQ666031	JQ666063	JQ666107

Gliocladiopsis singaporiensis Decock & Gordillo						
MUCL 48728	Submerged leaf litter in freshwater	Singapore	KX611500	–	KX671138	KX671130
MUCL 48412	Submerged leaf litter in freshwater	Singapore	–	–	–	–
Gliocladiopsis sumatrensis Crous & M.J. Wingf.						
CBS 754.97 (T)	Soil	Indonesia	JQ666142	JQ666032	JQ666064	JQ666108
CBS 111198	Soil	Indonesia	JQ666143	JQ666033	JQ666065	JQ666109
CBS 111213	Soil	Indonesia	JQ666144	JQ666034	JQ666066	JQ666110
CBS 111368	Soil	Indonesia	JQ666145	JQ666035	AF220978	JQ666111
Gliocladiopsis pseudotenuis L. Lombard & Crous						
CBS 114763	*Vanilla sp.*	Indonesia	JQ666139	JQ666029	JQ666062	JQ666105
CBS 116074 (T)	Soil	China	JQ666140	JQ666030	AF220981	JQ666106
Gliocladiopsis tenuis (Bugnic.) Crous & M.J. Wingf						
CBS 111961	*Coffea sp.*	Vietnam	JQ666146	JQ666036	JQ666067	JQ666112
CBS 111964	*Coffea sp.*	Vietnam	JQ666147	JQ666037	JQ666068	JQ666113
CBS 114147	Soil	Vietnam	JQ666148	JQ666038	JQ666069	JQ666114
CBS 114148	Soil	Vietnam	JQ666149	JQ666039	JQ666070	JQ666115
IMI 68205 (T)	*Indigofera sp*.	Indonesia	JQ666150	JQ666040	AF220979	JQ666116

Gliocladiopsis whileyi L.E. Parkinson, E.K. Dann & R.G. Shivas						
BRIP 61340 (T)	*Persea americana*	Australia	KX274052	KX274069	KX274086	–
Gliocladiopsis sp.1						
CBS 111038	Soil	Colombia	JQ666151	JQ666041	JQ666071	JQ666117
Gliocladiopsis sp.2						
CBS 116086	Soil	Indonesia	JQ666152	JQ666042	JQ666072	JQ666118
Penicillifer Emden						
Penicillifer pulcher Emden						
CBS 560.67(T); ATCC 18931; MUCL 11607						
BRIP 61340	Soil	The Netherlands	KM231998	KM231456	KM231742	KM231862

T= type
The missing numbers (“–“)

CHAPTER II

2.1. Preliminary screening process for selecting filamentous fungi with potential for use in bioremediation

Preface

In **Chapter 1,** we evaluated the diversity of endophytic and saprotrophic fungi isolated from the roots and rhizoplane of various herbaceous plants growing in oil ponds in the Amazon region of Ecuador. Pure cultures were obtained allowing further studies of their physiological properties.

In **Chapter 2**, thirty-two strains of the above-mentioned strains were preliminary tested *in vitro* for their growth potential on solid and liquid medium in presence of a standardized jet fuel. A number of strains were also tested in liquid medium for their capacity to degrade jet fuel.

1. INTRODUCTION

The oil industry may have many deleterious impacts on terrestrial and aquatic ecosystems, when consciously or accidentally released into the environment. The diverse pollutions caused in the north of the Ecuadorian Amazon region by Texaco (1972-1992) are amongst the major environmental issues faced by the country (San Sebastián & Hurtig, 2004; Fontaine, 2005; Buccina *et al.*, 2013). Oil hydrocarbons are composed by some molecules that are toxic to many organisms, including flora and fauna but also the native microbial communities (Lemos *et al.*, 2002; Pernía *et al.*, 2012) and the humans (Adekunle & Adebambo, 2007; Moolgavkar *et al.*, 2014).

Physical and chemical methods are the more common remediation strategies used in oil-polluted areas. They include dispersion, dilution, sorption, volatilization and abiotic transformation of the hydrocarbons. These methods requires heavy machinery and may result in important side effects such as residual air pollution (Bidoia *et al.*, 2012). In the recent years, bioremediation has emerged as a credible alternative; for the low cost and for being an environmental friendly technology (Rosenberg & Ron, 2005; D'Annibale *et al.*, 2006; Gadd, 2007; Bidoia *et al.*, 2012). The potential of oil bioremediation has been reported for a variety of terrestrial and cold marine ecosystems, including arctic, alpine, and Antarctic soils (Margesin & Schinner, 2001).

Petroleum consists of a mixture of hydrocarbons, which exhibited different susceptibilities to biodegradation by oil

degrading microorganisms. Each individual compound require a specific route for activation and degradation (Yanto & Tachibana, 2014). The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera; a diverse group of bacteria and fungi have shown this ability (Atlas, 1981). Fungi are well known for their capacity to degrade or to metabolize a wide variety of materials and compounds, processes known as myco-degradation and myco-deterioration (Singh, 2006).

Numerous groups of non-ligninolytic fungi including Zygomycetes and Ascomycetes are able to transform or degrade hydrocarbons (Atlas, 1981; Salicis *et al.*, 1999; Ravelet *et al.*, 2000; Chaillan *et al.*, 2004; Pernía *et al.*, 2012). Several fungi were reported to metabolize total petroleum hydrocarbons (TPHs) via enzymes that include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450, and epoxide hydrolase (Wu *et al.*, 2008, 2010; Balaji *et al.*, 2014). The resulting products are often much less toxic than the original hydrocarbons. These include trans-dihydrodiols, phenols, quinones, dihydrodiol epoxides, and tetraols, which may be conjugated to form glucuronides, glucosides, xylosides, and sulfates (Cerniglia, 1997; Cerniglia & Sutherland, 2010).

Maddela *et al.* (2015b) were the first to report petroleum hydrocarbon-degrading microorganisms from the Amazonian Ecuador. These authors' isolated bacteria (*Bacillus cereus* and *Bacillus thuringiensis*) and fungi (*Geomyces pannorum* and *Geomyces* sp.) from crude oil contaminated Amazonian rainforest soils, which were able to grow using diesel oil as the

unique carbon source under laboratory and field conditions. This opened the door for extensive studies on degradation/detoxification capacities of fungi isolated from oil-polluted soils.

In the present study, numerous fungal strains were isolated from herbaceous plants growing in a superficial layer of organic material (compost-like) covering crude oil ponds and tested *in vitro* for their ability to grow in presence of and to degrade oil molecule (hydrocarbons). To achieve this objective, *in vitro* screening procedures were developed. The first screening was carried out on solid and liquid medium in order to test the growth of a selection of representative fungal strains. On the basis of these preliminary results, a subset of fungal strains was selected to test their ability to degrade jet fuel in liquid medium.

2. MATERIALS AND METHODS

2.1. Determination of the ability of filamentous fungi to grow on solid and in liquid medium containing jet fuel.

2.1.1. Biological material

Strains of filamentous fungi were isolated from the roots or the rhizoplane of plants recolonizing polluted ponds in Charapa camp, Sucumbíos Prov., pond 1 of 330 m^2 (W 76° 48' 57" – S 00° 11' 49") and pond 2 of 450 m^2 (W 76°48'54" – S 00°11'46"), approx. elev. 300 m asl.

Thirty-two strains belonging to 26 genera were selected (Table 2.1). The strains were screened for their ability to grow on solid and liquid media containing jet fuel as carbon source (Fig. 2.1).

Table 2.1. Filamentous fungal strains selected for determination of their ability to use jet fuel as carbon source.

MUCL	Identification	Origin of plants in relation to oil ponds	
54543	*Trichoderma sp.*	Inside	Rhizoplane
54546	*Trichoderma sp.*	Inside	Root
54552	*Trichoderma sp.*	Outside	Rizhoplane
54565	*Volutella sp.*	Inside	Rhizoplane
54578	*Mucor sp.*	Outside	Rhizoplane
54579	*Stilbella sp.*	Inside	Rhizoplane
54584	*Cylindrocladiella sp.*	Inside	Rhizoplane
54589	*Dichobotrys sp.*	Outside	Rhizoplane
54591	*Cunninghamella sp.*	Inside	Rhizoplane
54631	*Verticillium sp.*	Inside	Rhizoplane
54659	*Botryodiplodia sp.*	Inside	Rhizoplane
54675	*Cylindrocarpon sp.*	Inside	Rhizoplane
54699	*Cylindrocarpon sp.*	Inside	Root
54707	*Paecilomyces sp.*	Inside	Root
54718	*Paecilomyces sp.*	Inside	Root
54735	*Volutella sp.*	Inside	Rhizoplane
54742	*Trichoderma sp.*	Inside	Rhizoplane
54752	*Aspergillus sp.*	Inside	Rhizoplane
54762	*Leptoxyphium sp.*	Inside	Rhizoplane
54768	*Colletotrichum sp.*	Inside	Root
54806	*Mariannaea sp.*	Outside	Root
54808	*Gelasinospora sp.*	Outside	Root
54811	*Acremonium sp.*	Inside	Rhizoplane
54820	*Metarhizium sp.*	Inside	Root
54825	*Mycoleptodiscus sp.*	Inside	Root

54828	*Phialophora sp.*	Outside	Root
54833	*Nectria sp.*	Outside	Root
54843	*Botryosphaeria sp.*	Inside	Rhizoplane
54849	*Pestaliota sp.*	Outside	Rhizoplane
54855	*Microsphaeropsis sp.*	Inside	Rhizoplane
54915	*Gliocladium sp.*	Outside	Rhizoplane
54917	*Beauveria sp.*	Inside	Root

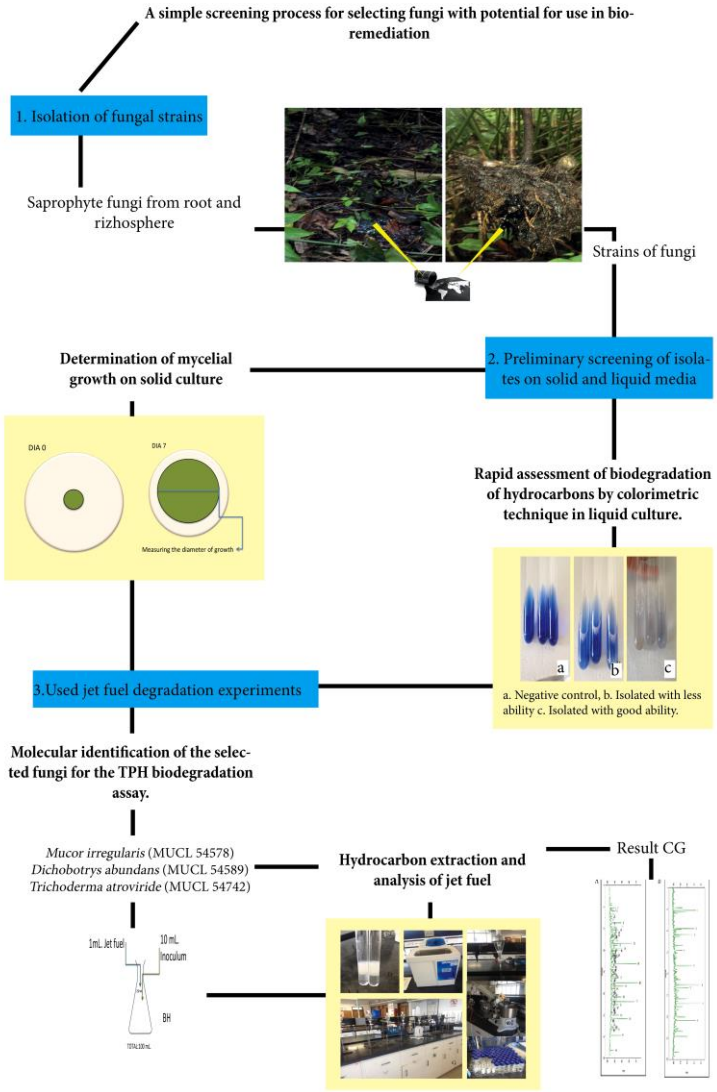
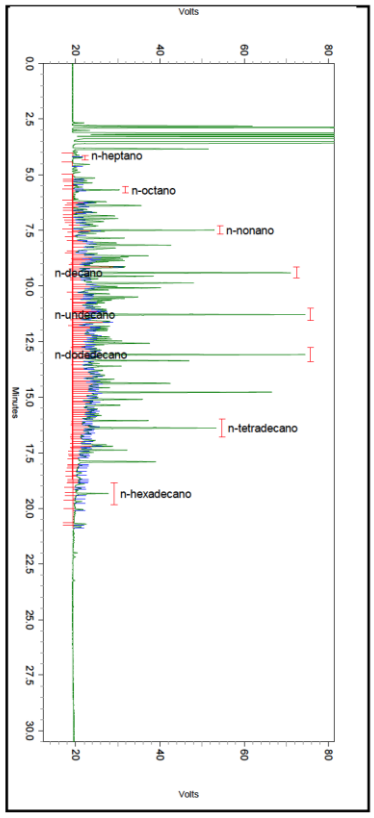


Fig. 2.1. Schematic representation of screening process for selecting fungi* with potential for use in bioremediation (*strains isolated from rhizoplane of plants grown in oil ponds in Ecuador)

2.1.2. Hydrocarbon used in the assay

Jet fuel, which is a petroleum derivate, also known as kerosene, was used in the study. It contains hydrocarbons from C8 to C17 distributed between saturated hydrocarbons (80% to 90%) and aromatic (10% to 20%), and is usually free of olefins (Weisman, 1998). Chemical analysis of jet fuel was made by Gas Chromatography (GC) (Fig. 2.2).



Front Signal Results

Time	Name	Area	Amount
4.130	n-heptano	42083	34.759
5.686	n-octano	180063	108.360
7.492	n-nonano	494445	248.627
9.409	n-decano	920922	446.051
11.290	n-undecano	961338	455.443
13.084	n-dodedecano	1047359	482.190
16.386	n-tetradecano	585870	278.051
19.339	n-hexadecano	178286	103.232
Totals		4410366	2156.713

Fig. 2.2. Chromatography profile from jet fuel (Jet A) used in this assay performed by at the chemistry laboratory, Pontifical Catholic University of Ecuador

2.2. Preliminary screening of fungal strains on solid and in liquid media

2.2.1. Determination of mycelial growth on solid culture medium

The selected strains were screened for their growth on a solid media containing jet fuel as a source of carbon, by measurement of the fungal growth was determined in 9 cm Petri dishes by measuring growth diameter. Growth was assessed by comparing fungal culture diameter in presence / absence of jet fuel.

The fungal strains were grown on MYPD medium (0.3% Malt extract, 0.3% Yeast extract, 0.5% Peptone, 1% Dextrose, 20 g. agar, Difco) (Boonchan *et al.,* 2000). The media were prepared by adding 0.1% Tween 80 and 1%, 2% and 3% (v/v) of jet fuel, previously sterilized by filtration through a 0.22 µm filter. The control media was prepared using the same compositions excluding the jet fuel. Both media were inoculated with a 10 mm agar plug with mycelium, taken from actively growing colony. The cultures were incubated at 25 ± 1 ºC in the dark for a period of 7 days. All the tests were conducted in triplicates. The colony diameter was subsequently recorded (Husaini *et al.*, 2008). The relationship between the diameters of growth in the medium was calculated by [% of growth decrease = ((colony diameter without jet fuel – colony diameter with jet fuel)/ colony diameter without jet fuel)*100] to determine the difference in growth.

2.2.2. Rapid assessment of biodegradation of hydrocarbons by colorimetric technique in liquid culture.

The screening procedure for testing the degradation ability of the selected fungi was carried out using the Hanson *et al.* (1993) method, modified by Bidoia *et al.* (2012). This method is based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP).

Three parameters were considered to assess the efficacy of fungi in biodegradation: The first one is a measure of discoloration of the culture liquid medium from blue to colorless, measured in absorbance. The second is the dissipation of hydrocarbon (jet fuel) from the liquid medium. The third is related to the biomass production of the fungus in the culture liquid medium (Al-Nasrawi *et al.,* 2012).

Seven-day old fungal cultures were used as inoculum. One plug (10 mm size) was picked from the margin of the culture grown on malt agar extract (MAE) and transferred carefully into tubes containing 7.5 ml of Bushnell-Haas broth (BH DIFCO™; 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$, $CaCl_2$ pH7); 0.1% Tween 80, 400 μL DCPIP (0.025 g / 25 ml of redox indicator) and 50μL jet fuel were added. The experiments were conducted in triplicates. The tubes were kept under constant agitation (i.e. 100 rpm) at 25 ± 1 ºC. After one week of incubation, the level of discoloration, between dark blue to colorless, indicating jet fuel degradation, was measured by spectrophotometry (absorbance at 600 nm) (Hanson *et al.,* 1993; Al-Nasrawi *et al.,* 2012; Bidoia *et al.,* 2012;

Hanafy *et al.,* 2015). For each tube, we have evaluated the mycelium grow of the strains in liquid medium containing jet fuel by a semi-quantitative scale: for the largest growth (3), for a medium growth (2), for lower growth (1) and no growth (0); this further examination helped to select the best strains.

2.3. Assay for test the ability to degrade jet fuel

Degradation experiments were performed separately in 250 ml Erlenmeyer flasks, containing 90 ml of BH broth, with 0.1% Tween-80 and 1% (v/v) of filter sterilized jet fuel as the unique carbon source. Prior to adding the jet fuel, the growth medium was sterilized (121 °C for 15 minutes). The fungal inoculum consisted of 10 ml of a suspension of 5-day old spores (using Tween-80 0.01%) isolated from 3 Petri dishes and inoculated into an Erlenmeyer flask. The experiments were done in sevenfold for each of the strains. Control consisted in jet fuel added to the BH broth without inoculum (abiotic control) in triplicate. All the flasks were incubated for 15 days at room temperature under constant agitation of 100 rpm (Barnstead lab-line max q2000) and a photoperiod of 12 h. (Al-Nasrawi, 2012; Maddela *et al.*, 2015b).

2.3.1. Molecular identification of the fungi degrading jet fuel.

The fungi chosen for this experiment were a selection of those having the ability to grow in liquid and or solid medium with jet fuel. Identification was made by classical morphology and by molecular biology using ITS- or *tef-1α*-based identification. The

internal transcribed spacers (ITS) 1 and 2 were amplified and sequenced with the primer pair ITS5/ITS4 (White *et al.,* 1990). The translation Elongation Factor 1-α gene was amplified and sequenced using the primers ef-1/ef-2 (*tef-1α*) to identify *Trichoderma* (O'Donnell *et al*., 1998). DNA was extracted from mycelium grown in liquid malt extract at 20 ± 2 ºC in the dark. Extractions were done using innuPREP Plant DNA kit (Analytik Jena, Germany) following the manufacturer's recommendations. The PCR parameters are as described in Lombard *et al*. (2010).

Amplicons were sequenced in both directions by Macrogen Inc. (Korea) using the same primers as used for amplification. Raw sequences were edited with Sequencher®software version 5.1 (Gene Codes Corporation Ann Arbor n.d.). Indications of the identity of the selected fungi were obtained using the Blast search engine at GenBank (Altschul *et al.*, 1990).

2.3.2. Hydrocarbon extraction and analysis of jet fuel

Ten ml of solution from each flask, including the control, were harvested at the beginning of the experiment (time 0) and after 15 days of incubation. Five ml were used for chemical analysis such as TPHs quantification by Gas Chromatography (GC), which is the more widely used technique in the petroleum hydrocarbon degradation studies (Mittal & Singh 2009). In addition, 1 ml of the solution was used to test the viability and purity of the different strains. The purity was conducted to ascertain the absence of unwanted fungal or other contaminants. The viability consisted in evaluating the growth of the fungus by

serial dilution method. Samples were serially diluted with sterilized distilled water up to 10^{-6}. The last three higher dilutions were spread on MEA agar medium in Petri dishes and incubated at 25 ± 1 ºC for 7 days in the dark. The remaining 4 ml were stored -4 °C as a backup of the sample, until the end of the assay, in case of additional tests.

The TPH extraction was carried out by adding 6 ml of dichloromethane (DCM) (Sigma-Aldrich) to 5 ml from each sample. The tubes were sonicated in an ultrasonic bath (Branson 3800) twice for 5 minutes each. The mixture was transferred into a 50 ml separating funnel and sequentially extracted three times with equal volumes of the solvent mixtures. The organic fractions from the respective tube were pooled and dried over anhydrous Na_2SO_4, then the residual content was filtered through Whatman No 1 filter. The solvent fractions were evaporated with a rotary evaporator (Rotavapor water bath BM-200) to obtain a final volume of 1 ml in order to proceed to the GC analysis of TPH (Boonchan *et al.*, 2000; Husaini *et al.*, 2008).

The extracts were analyzed within a single batch by GC with flame ionization detector (FID), using a GC-FID Agilent 7890 gas chromatograph (Agilent Technologies, www.agilent.com), equipped with a FID detector and automatic injector. The separation column was an Agilent DB-TPH 30 m x 0.32 mm x 0.25 µm with an injection volume of 1 µL and injection temperature of 300°C. The GC oven program started at 40°C, was held for 1 min, then at 8°C 1 min, then increased to 220°C for 1 min. This enabled a complete run within 24.5 min.

The characterization and quantification of jet fuel components by GC was determined according to the calibration curve of the standard “O-PONA System Validation Mixture” Oxygenates & Paraffin, Olefin, Naphthene, Aromatics (O-PONA) by GC to determine TPHs. The TPHs analyzed in GC (mg/L) were: n-
n-octane (retention time 4.142 min), n-heptane (4.237 min), methyl cyclohexane (4.533 min), toluene (5.261 min), n-octane (5.705 min), ethyl benzene (6.198 min) 4-trimethylcyclohexane (6.878 min), o-xylene (7.166 min), n-nonane (7.541 min), 1,2,4-trimethylbenzene (8.629 min), n-decane (9.484 min), 1,2 (10.109 min), 1,2,4,5-tetramethyl benzene (10.546 min), n-undecane (11.314 min), n-dodecane (13.114 min), 3-trimethyl benzene (9.596 min), trans-decahydro naphthalene pentamethyl benzene (14.930 min) and n-tetradecane (16.426 min), according to the standard O-PONA System Validation Mixture (ASTM Methods, 2015).

Finally, the efficiency of biodegradation was expressed in terms of removal ratios (RRs) as described by Joo *et al.* (2008). The percentage of TPH loss (%D) was given by the formula for removal rate (RR): %D = 100[(MI - MT)/MI], where MT was the concentration of TPHs in each treatment and MI was the initial TPH concentration present in the medium (Maddela *et al.* 2015a,b, 2016).

2.4. Statistical analyses

For the identification of the strains having the highest tolerance to the different concentrations (i.e. 1%, 2% and 3%) of jet fuel, the following linear mixed model (equation 1) was used. The mycelia growth at each concentration x (Treatment=1) was compared with the mycelia growth in the medium without jet fuel (Treatment=0) to determine the effects of the treatment on fungal growth. The model was also used to determine which strains were the more sensitive to jet fuel.

This model allows taking into account the treatment effect, i.e. the systematic differences in growth between jet fuel and control, through the inclusion of the fixed coefficient β_2. The random coefficients β_{1i} and β_{3i} are included in order to take into account the similarities in the growth of the replicates obtained from the same strain. The coefficients β_{1i} represents the systematic difference in growth between strains, while the β_{3i} allows for differences in the treatment effect between strains.

Equation 1:

$$Growth_{ij} = \beta_0 + \beta_{1i} + \beta_2 I(x)_{ij} + \beta_{3i} I(x)_{ij} + \epsilon_{ij}$$

i = refers to a strain

j = to a replicate

β_{1i} = random coefficient $\beta_1 \sim N(0, \sigma_1^2)$.

β_{3i}= random coefficient $\beta_3 \sim N(0, \sigma_3^2)$.

β_0= fixed coefficient

β_2= fixed treatment effect

$I(x)_{ij}$= Indicator (Treatment = 1 for x JF) (Treatment = 0 for Control negative)

ϵ_{ij} = $\epsilon \sim N(0, \sigma^2)$ random error with the assumption of a normal distribution with mean 0 and constant variance σ^2

For the test in liquid medium, the objective was to evaluate the difference between the strains, according to the DCPIP absorbance measures. The following linear mixed model analysis (equation 2) was used.

Equation 2:

$$DCPIP_{ij} = \beta_0 + \beta_{1i} + \epsilon_{ij}$$

$$\text{with } \epsilon \sim N(0, \sigma^2) \text{ and } \beta_1 \sim N(0, \sigma_1^2)$$

The model was also used to evaluate the strains for which the predicted value of β_{1i} (or, equivalently, of DCPIP absorbance measure) was the lowest. The data were analyzed using the software SPSS Statistics 24.

For the study on degradation potential of the strains, we conducted a one-way ANOVA to find statistical differences between the treatments using the SPSS (version 21.0). A Tukey post hoc test was used to identify the significant differences ($P \leq 0.05$).

3. RESULTS

3.1. Preliminary screening of fungal strains on solid and liquid media

Thirty-two strains were tested for their ability to grow in presence of jet fuel at 1%, 2% and 3% on solid and in liquid medium. All of them were able to grow on it, but with different efficiency compared to controls. At the end of the experiment, two strains were excluded for contamination problems during the course of experiment (MUCL 54808, MUCL 54849).

Using 1% of jet fuel in solid medium, sixteen strains did not show any decrease in the growth rate. Moreover, some of them had an increase of their growth rate (i.e. *Trichoderma* spp. MUCL 54543, 54546, 54552, 54742, *Mucor* sp. MUCL 54578, *Cylindrocladiella* sp. MUCL 54584, etc.). Ten strains showed a decrease of their growth rate < 30% (i.e. *Volutella* sp. MUCL 54735, *Aspergillus* sp. MUCL 54752, etc.) and three strains showed a decrease of their growth rate of < 50% (i.e. *Cunninghamella* sp. MUCL 54591, *Botryosphaeria* sp. MUCL 54843, *Gliocladium* sp. MUCL 54915) respectively. On the other hand, one strain *Microsphaeropsis* sp. MUCL 54855 was strongly inhibited with a growth rate decrease of 53 % (Table 2.2).

At 2% of jet fuel in solid medium, six strains showed equal growth or increased growth rate (i.e. *Trichoderma* spp. MUCL 54543, 54546, 54552, 54742, *Dichobotrys* sp. MUCL 54589, etc.). Ten strains showed a decrease of their growth rate < 30% (i.e. *Stilbella* sp. MUCL 54579, *Verticillium* sp. MUCL 54631, etc.) and nine strains showed a decrease of their growth rate < 50%

(i.e. *Cunninghamella* sp. MUCL 54591, *Botryodiplodia* sp. MUCL 54659, etc.). Finally, four strains had and inhibition of more than > 50% of mycelium growth (i.e. *Metarhizium* sp. MUCL 54820, *Mycoleptodiscus* sp. MUCL 54825, etc) (Table 2.2).

With 3% of jet fuel in solid medium, only five strains had equal growth rate (i.e. *Trichoderma* spp. MUCL 54543, 54546, 54552, 54742, *Dichobotrys* sp. MUCL 54589, *Mucor* sp. MUCL 54578). Ten strains showed a decrease of their growth rate < 30% (i.e. *Stilbella* sp. MUCL 54579, *Verticillium* sp. MUCL 54631) and seven strains showed a decrease of their growth rate < 50% (i.e. *Botryodiplodia* sp., *Aspergillus* sp., etc.) On the other hand, eight strains display an inhibition of growth > 50% (*Beauveria* sp. MUCL 54917, *Microsphaeropsis* sp. MUCL 54855, etc.) (Table 2.2).

Table 2.2. Percentage of Growth decrease in different concentration of jet fuel

MUCL CODE	STRAIN	% Growth decrease *		
		1% jet fuel	2% jet fuel	3% jet fuel
54543	*Trichoderma sp.*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
54546	*Trichoderma sp.*	0.0 ± 0.0	0.0 ± 0.0	23.7 ± 5.5
54552	*Trichoderma sp.*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
54565	*Volutella sp.*	21.7 ± 15.5	-12.7 ± 12.5	0.0 ± 13.0
54578	*Mucor sp.*	0.0 ± 0.0	21.3 ± 18.7	0.0 ± 0.0
54579	*Stilbella sp.*	20.2 ± 4.3	18.0 ± 4.6	11.3 ± 10.3
54584	*Cylindrocladiella sp.*	-33.1 ± 39.7	20.0 ± 11.3	38.7 ± 5.1
54589	*Dichobotrys sp.*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
54591	*Cunninghamella sp.*	35.3 ± 5.9	39.0 ± 3.5	80.3 ± 5.1
54631	*Verticillium sp.*	10.9 ± 5.9	22.7 ± 4.0	25.3 ± 12.5
54659	*Botryodiplodia sp.*	15.7 ± 3.4	33.0 ± 6.9	39.3 ± 2.9
54675	*Cylindrocarpon sp.*	-12.9 ± 17.9	13.3 ± 5.8	20.3 ± 2.5
54699	*Cylindrocarpon sp.*	2.8 ± 4.8	17.0 ± 0.0	26.0 ± 1.7
54707	*Paecilomyces sp.*	-23.2 ± 41.1	10.7 ± 17.1	17.3 ± 9.9
54718	*Paecilomyces sp.*	-32.9 ± 50.4	42.0 ± 1.7	30.7 ± 11.6
54735	*Volutella sp.*	-33.0 ± 44.7	43.7 ± 7.6	62.3 ± 6.7
54742	*Trichoderma sp.*	0.0 ± 0.0	0.0 ± 0.0	35.3 ± 17.5
54752	*Aspergillus sp.*	19.9 ± 18.9	44.3 ± 11.5	33.0 ± 11.0
54762	*Leptoxyphium sp.*	26.7 ± 23.1	20.3 ± 4.6	20.3 ± 4.6
54768	*Colletotrichum sp.*	11.4 ± 10.3	16.7 ± 14.4	29.0 ± 15.7
54806	*Mariannaea sp.*	-3.6 ± 40.6	53.7 ± 8.1	65.3 ± 8.7
54808	*Gelasinospora sp.*	39.2 ± 3.4	0.0 ± 0.0	72.3 ± 1.5
54811	*Acremonium sp.*	14.5 ± 6.1	23.0 ± 0.0	34.7 ± 6.4
54820	*Metarhizium sp.*	26.6 ± 12.1	52.3 ± 6.8	63.3 ± 3.5
54825	*Mycoleptodiscus sp.*	-8.3 ± 46.4	55.0 ± 9.2	57.0 ± 6.9
54828	*Phialophora sp.*	26.7 ± 23.1	32.3 ± 22.5	39.0 ± 11.5

54833	*Nectria sp.*	2.8	±	4.8	19.3	±	10.4	20.0	±	9.0
54843	*Botryosphaeria sp.*	48.4	±	2.5	45.7	±	1.2	55.0	±	7.0
54849	*Pestaliota sp.*	-90.0	±	79.4	-6.3	±	29.2	28.0	±	13.2
54855	*Microsphaeropsis sp.*	52.8	±	6.3	51.3	±	4.6	69.7	±	2.1
54915	*Gliocladium sp.*	33.8	±	7.9	48.3	±	7.0	49.0	±	11.3
54917	*Beauveria sp.*	0.0	±	0.0	40.0	±	8.9	53.3	±	11.9

* If the number is negative, then it represents an increase and if is 0, there is no effect. Values represent Means ± SD of N replicates.

The difference between mycelium growth in medium with 1%, 2%, and 3% jet fuel and without jet fuel (control) was significantly dependent on the strain ($p<0.004$, <0.00 and <0.00 for 1%, 2%, and 3% jet fuel, respectively). The strains in which the effect of the jet fuel on the growth is the strongest was shown by the statistical linear mixed model (equation 1), which allowed predicting the mean value of the response of the mycelial growth (mm) for each concentration of jet fuel. Several strains were able to grow in presence of the different concentrations of jet fuel, showing a tolerance to the hydrocarbons, but with different efficiency compared to controls (Figs. 2.3, 2.4, 2.5).

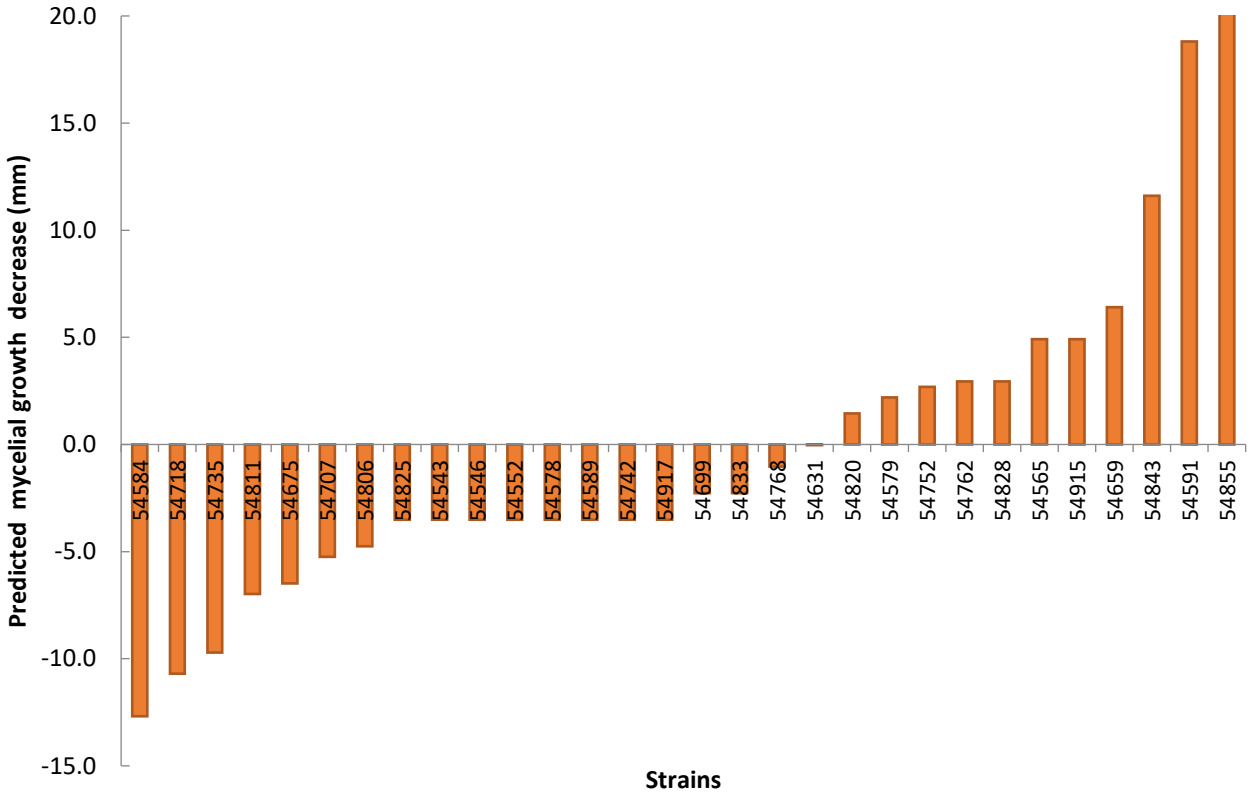


Fig. 2.3. Predicted mean values from mycelium growth decrease in 1%, of jet fuel.

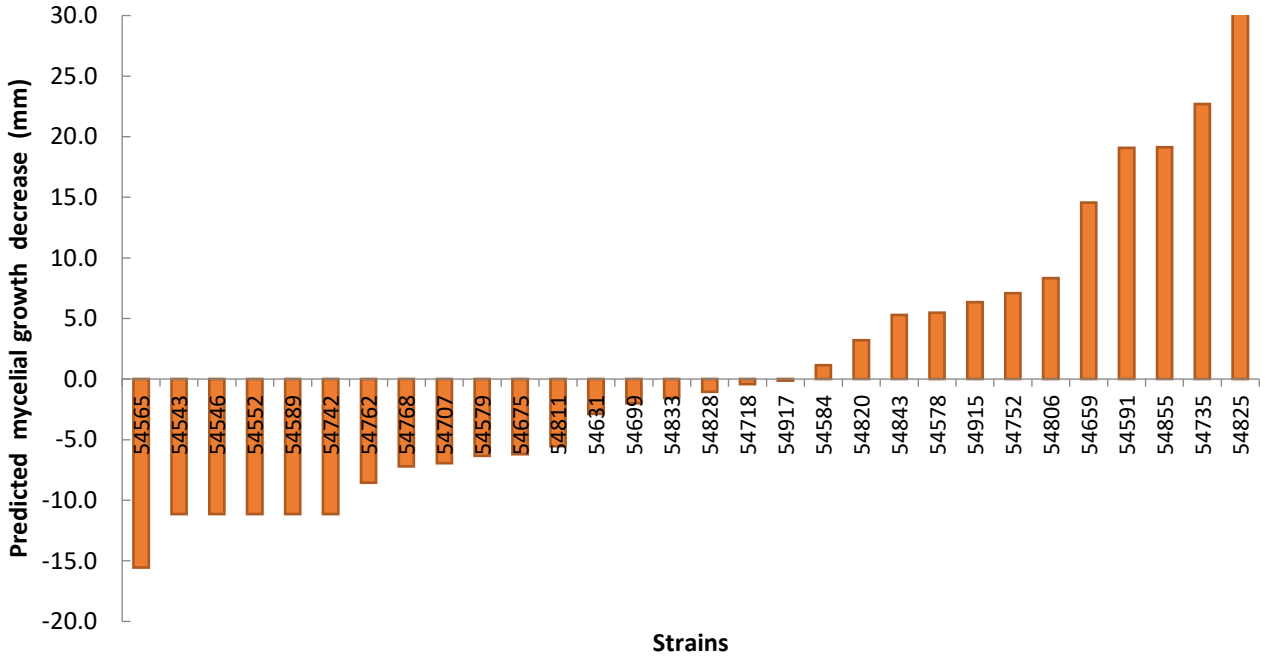


Fig. 2.4. Predicted mean values from mycelium growth decrease in 2%, of jet fuel.

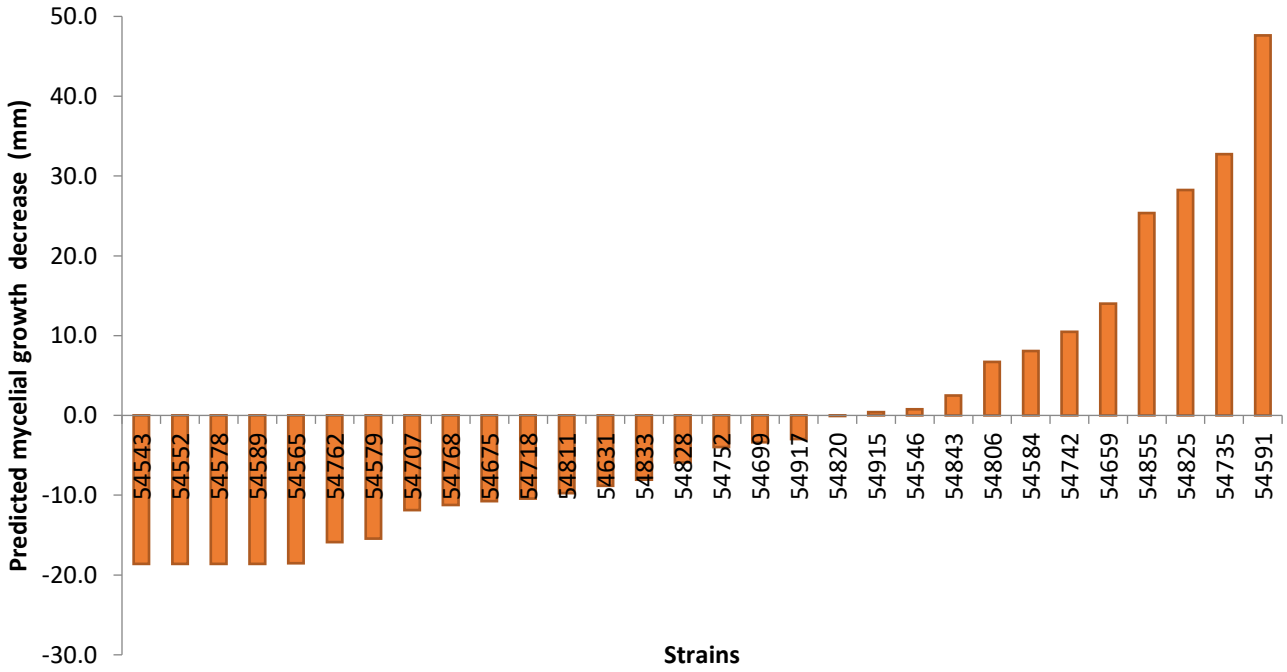


Fig. 2.5. Predicted mean values from mycelium growth decrease in 3%, of jet fuel.

The experiment using DCPIP as a redox indicator, measuring absorbance, showed significant differences between strains in their ability to use jet fuel as a source of C ($p<0.00$). Almost all the strains showed activity (discoloration) and growth in liquid medium with jet fuel. The average absorbance obtained by the control with out fungi was 1,6 (blue). Nine strains (i.e. *Trichoderma* spp. MUCL 54742, *Verticillium* sp. MUCL 54631, *Aspergillus* sp. MUCL 54752, *Colletotrichum* sp. MUCL 54768, *Metarhizium* sp. MUCL 54820, *Mycoleptodiscus* sp. MUCL 54825, *Phialophora* sp. MUCL 54828, *Nectria* sp. MUCL 54833 and *Gliocladium* sp. MUCL 54915) showed a mean predicted absorbance up to 0.3, ten strains (i.e. *Volutella* sp. MUCL 54565, *Mucor* sp. MUCL 54578, *Stilbella* sp. MUCL 54579, *Cylindrocladiella* sp. MUCL 54584, *Dichobotrys* sp. MUCL 54589, *Cunninghamella* sp. MUCL 54591, *Cylindrocarpon* sp. MUCL 54675, Acremonium sp. MUCL 54811, *Botryosphaeria* sp. MUCL 54843 and *Microsphaeropsis* sp. MUCL 54855) showed a mean predicted absorbance up to 0.50, and the remaining ten strains (i.e. *Trichoderma* sp. MUCL 54543, *Trichoderma* sp. MUCL 54552, *Botryodiplodia* sp. MUCL 54659, *Cylindrocarpon* sp. MUCL 54699, *Paecilomyces* sp. MUCL 54707, *Paecilomyces* sp. MUCL 54718, *Volutella* sp. MUCL 54735, *Leptoxyphium* sp. MUCL 54762 and *Mariannaea* sp. MUCL 54806) presented a predicted value of absorbance up to 0.75. *Beauveria* sp. MUCL 54917 was the only strain with a high a predicted value of absorbance of 1,19 (Fig. 2.6). The fungal biomass produced was

assessed and scored semi quantitatively in a four level scale: 3 higher growth, 2 medium growth, 1 little growth, 0 non-growth). Eleven strains displayed (3) higher growth (i.e. MUCL 54565, MUCL 54631, MUCL 54735, MUCL 54742, MUCL 54752, MUCL 54768, MUCL 54825, MUCL 54828, MUCL 54843, MUCL 54855 and MUCL 54820), others twelve presented (2) medium growth (i.e. MUCL 54543, MUCL 54546, MUCL 54552, MUCL 54578, MUCL 54579, MUCL 54584, MUCL 54589, MUCL 54591, MUCL 54675, MUCL 54707, MUCL 54718, MUCL 54811), finally seven-displayed (1) little development (i.e. MUCL 54659, MUCL 54699, MUCL 54762, MUCL 54806, MUCL 54833, MUCL 54915 and MUCL 54917) (Fig. 2.6).

Three strains, for which the growth was distinctively better on solid and liquid medium, were selected for further study of oil degradation using analytical methods.

MUCL CODE	STRAIN	Absorbance	Predicted absorbance*	Observation biomass
54543	*Trichoderma* sp.	0.79	0.75	2
54546	*Trichoderma* sp.	0.50	0.52	2
54552	*Trichoderma* sp.	0.61	0.60	2
54742	*Trichoderma* sp.	0.20	0.28	3
54565	*Volutella* sp.	0.49	0.50	3
54578	*Mucor* sp.	0.32	0.37	2
54579	*Stilbella* sp.	0.40	0.43	2
54584	*Cylindrocladiella* sp.	0.27	0.33	2
54589	*Dichobotrys* sp.	0.47	0.49	2
54591	*Cunninghamella* sp.	0.40	0.43	2
54631	*Verticillium* sp.	0.15	0.23	3
54659	*Botryodiplodia* sp.	0.78	0.74	1
54675	*Cylindrocarpon* sp.	0.47	0.49	2
54699	*Cylindrocarpon* sp.	0.61	0.60	1
54707	*Paecilomyces* sp.	0.64	0.63	2
54718	*Paecilomyces* sp.	0.53	0.54	2
54735	*Volutella* sp.	0.62	0.61	3
54752	*Aspergillus* sp.	0.11	0.20	3
54762	Leptoxyphium sp.	0.55	0.55	1
54768	*Colletotrichum* sp.	0.18	0.26	3
54806	*Mariannaea* sp.	0.51	0.52	1
54811	Acremonium sp.	0.33	0.38	2
54820	*Metarhizium* sp.	0.12	0.21	3
54825	*Mycoleptodiscus* sp.	0.18	0.26	3
54828	*Phialophora* sp.	0.15	0.24	3
54833	*Nectria* sp.	0.23	0.30	1
54843	*Botryosphaeria* sp.	0.47	0.49	3
54855	*Microsphaeropsis* sp.	0.25	0.31	3

54915	*Gliocladium* sp.	0.18	0.26	1
54917	*Beauveria* sp.	1.42	1.19	1

*Predicted mean resulted of the linear mixed model analysis (equation 2).

3.2. Impact of fungi on the degradation of jet fuel

Three strains were selected from the previous experiments and tested for their ability to degrade hydrocarbon in liquid cultures: *Mucor irregularis* MUCL 54578, *Dichobotrys abundans* MUCL 54589 and *Trichoderma atroviride* MUCL 54742. O

GC measured residual jet fuel resulting from the biodegradations by the fungi. In presence of the fungi, many “peaks” on the chromatograph, representing hydrocarbons compounds, disappeared as compared to the original (Fig. 2.5 – 2.6). The highest percentages of loss of hydrocarbons in presence of fungi were obtained with *Trichoderma atroviride* MUCL 54742 and *Dichobotrys abundans* MUCL 54589 after 15 days of incubation (Table 2.2). These figures 2.5 – 2.6 showed that many peaks (compounds) disappeared or were reduced, including those monitored thanks to the standard O-PONA (red dots), but also others that we were not able to characterize.

Purity and viability of the strains were checked at each sampling time. No contaminations were detected and fungi remained alive during the time course of experiment.

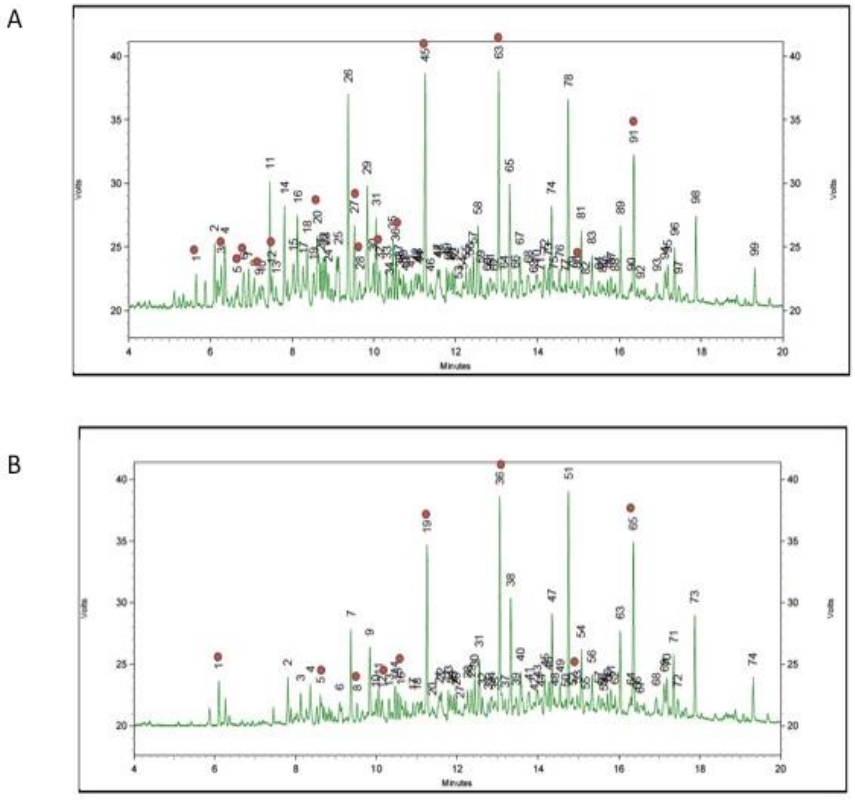


Fig. 2.5. Gas chromatography profiles of jet fuel (TPHs) degradation by control negative (A =0 days, B=15 days).

Red dots are the Hydrocarbons compounds analyzed based to the O-PONA standard mix in GC-FID (mg/L).

COMPOUND	RED DOTS	
	A	B*
n-octane	1	np

ethyl benzene	3	1
1, 2, 4-trimethylbenzene	6	5
o-xylene	9	np
n-nonane	12	np
1, 2, 4-trimethylbenzene	20	np
n-decane	27	8
1, 2, 3-trimethylbenzene	28	np
trans-decahydro naphthalene	32	12
1, 2, 4, 5-tetramethyl benzene	36	15
n-undecane	45	19
n-dodecane	63	36
pentamethyl benzene	80	53
n-tetradecane	91	65

*np= no longer present in figure B, what might be due to (evaporation) volatilization.

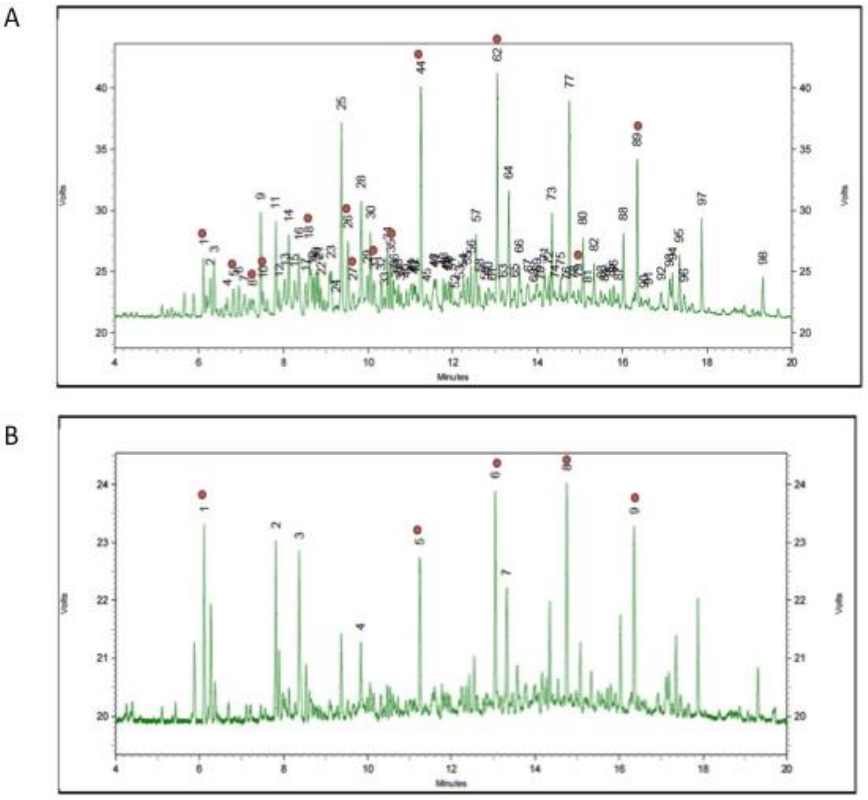


Fig. 2.6. GC chromatograms of jet fuel (TPHs) degradation by *Trichoderma atroviride* MUCL 54742. (A =0 days, B=15 days).

Red dots are the Hydrocarbons compounds analyzed based to the O-PONA standard mix in GC-FID (mg/L). In the figure B compounds are present but in less quantity probably partially degraded.

COMPOUND	RED DOTS	
	A	B*
ethyl benzene	2	1
1, 2, 4-trimethylbenzene	5	np
o-xylene	8	np
n-nonane	10	np
1, 2, 4-trimethylbenzene	18	np
n-decane	26	np
1, 2, 3-trimethylbenzene	27	np
trans-decahydro naphthalene	31	np
1, 2, 4, 5-tetramethyl benzene	35	np
n-undecane	44	5
n-dodecane	62	6
pentamethyl benzene	79	8
n-tetradecane	89	9

*np= Figure B, are no longer present, likely evaporated or degraded.

The data obtained on the total TPHs (mg/L) reported in the GC analysis was transformed as removal rate (RR, cf. table 2.2). Under the given conditions, a significant difference was observed between the treatments with *Mucor irregularis* MUCL 54578,

Dichobotrys abundans MUCL 54589 and *Trichoderma atroviride* MUCL 54742, compared to the control without fungi (p=0.00) (Table 2). The RR for the control (abiotic treatment) were 41.81%, while in the treatment with fungi, the RR were 71.17%, 90.40% and 93.36% for, respectively, *Mucor irregularis* MUCL 54578, *Dichobotrys abundans* MUCL 54589 and *Trichoderma atroviride* MUCL 54742 (Table 2).

Table 2.2. Activity by fungal strains from petroleum contaminated soil

Treatment	N	Total mg/L Day 1	Total mg/L Day 15	Percentage of Removal Rate (%RR)
Control neg.	2	721.90 ± 32.99	424.25 ± 110.7	41.81 ± 7.516[a]
Mucor irregularis MUCL 54578	5	797.88 ± 30.7	232.60 ± 45.3	71.17 ± 5.424[b]
Dichobotrys abundans MUCL 54589	4	794.08 ± 106.1	78.61 ± 19.2	90.40 ± 1.500[c]
Thichoderma atroviride MUCL 54742	5	741.50 ± 89.3	53.06 ± 22.2	93.36 ± 2.577[c]

Values represent Means ± SE of N replicates. Values in the same column followed by identical letter did not differ significantly (P<0.05, Tukey's test). Removal ratio (RR) = (IC - RC/IC) *100 %, where IC and RC are initial and residual concentrations of hydrocarbons, respectively affected.

4. DISCUSSION

Bioremediation with plants and/or microorganisms is nowadays considered as a promising method for cleaning oil contaminated sites (Lemos *et al.*, 2002; Saraswathy & Hallberg,

2002; Chávez-Gómez *et al.*, 2003; Zacatenco & Madero, 2010). Many bacteria and fungi are able to degrade hydrocarbon molecules and are termed as oil-degrading microorganisms (Maddela *et al.*, 2016). Crude oil (but also, e.g. used motor oils) discharges into soil spread horizontally and vertically through the soil porosity and adsorb to the surfaces of soil particles (Husaini *et al.*, 2008). Oil-hydrocarbons have distinct susceptibilities to biodegradation by microorganisms, each requiring a specific route for activation and degradation (Yanto & Tachibana, 2014). Hydrocarbons sensitive to microbial attack rank in decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Singh, 2006). For the fungi tested in the biodegradation assay, the general tendency was observed for the low molecular weight as the aliphatic compounds (i.e. n-alkane, cyclohexane).

Oil-degrading bacteria have been studied extensively for their capacity of degrading petroleum hydrocarbons. Fungal remediation or myco-remediation is now more and more considered. Fungi are known to produce many enzymes (i.e. peroxidase, manganese dependent peroxidase, and laccase lignin), with potential for degradation and removal of hydrocarbons. New findings have shown that litter-decomposing fungi and non-ligninolytic fungi isolates have some potential to transform hydrocarbons significantly (Steffen *et al.*, 2002; Potin *et al.*, 2004; D'Annibale *et al.*, 2006). An advantages of fungi over bacteria is the production of an extensive mycelium ramifying through the contaminated substratum and spreading beyond the top layer of the soil (Husaini *et al.*, 2008).

Hydrocarbon-tolerant fungal strains are poorly known. Most of the identified hydrocarbon-degrading fungi are restricted to the Ascomycota and Basidiomycota (Harms *et al.*, 2011; Bell *et al.*, 2014).

The present study evaluated the ability of 30 strains to grow with jet fuel as a sole carbon source, in order to find out the most promising strains for bioremediation. The strains were isolated from roots of plants growing in oil-ponds in the tropical forest. It is interesting to note that some of the fungi were “stimulated” by the presence of jet fuel and displayed a vigorous growth with respect to the controls; others expressed tolerance growing regardless of the jet fuel concentrations. Stress-tolerant species of different genera, able to grow under conditions of water and nutrient limitations, or high temperature, and in the absence of oxygen i.e. *Mucor, Fusarium, Gliocladium, Penicillium*, and *Trichoderma* had been targeted in myco-remediation (Singh, 2006).

Trichoderma is abundantly present in all soils. They are fast growing fungi. Most species are saprophytic, but some also were isolated as plant endophytes. *Trichoderma* spp. MUCL 54543, 54546, 54552 and 54742 were evaluated in solid and liquid medium and displayed a good growth in both cases, with a positive reaction with DCPIP, especially the strain MUCL 54742. Rivera-Cruz *et. al.* (2002) also isolated *Trichoderma* sp. which exhibited high growth rates, showing tolerance and use of carbon from benzo(a)pyrene. Husaini *et al.* (2008), in preliminary screening assay, reported that *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg exhibited hydrocarbon biodegradation

abilities. Balaji *et al.* (2014) reported strains of *Trichoderma* able of secreting extracellular enzymes degrading hydrocarbons present in oil-contaminated soil.

Chaillan *et al.* (2004) reported *Paecilomyces variotii* Bainier as a oil-degrading fungi. *Paecilomyces* sp. also was reported showing tolerance to and using carbon from benzo(a)pyrene (Rivera-Cruz *et al.*, 2002; Pernía *et al.*, 2012). *Paecilomyces lilacinus* (Thom) Samson was mentioned by Cerniglia & Sutherland (2010) degrading PAHs with three or more rings. In our study, *Paecilomyces* spp. MUCL 54718, 54707 were growing in solid medium regardless of the concentration; at the concentration of 1%, both strains were showed an increase of the growth rate, whereas in high concentration, the growth rate slightly decreased. In liquid medium, we observe a medium growth (2) with a positive reaction of DCPIP.

Mucor was found to be an effective genus for degradation (Gadd, 2007; Pernía *et al.*, 2012; Fernández-Luqueno *et al.*, 2017). Ravelet *et al.* (2000) isolated and tested several *Mucor spp.* in screening processes. A strain of *Mucor racemosus* was one of the most effective biodegrading pyrene (PAH). Other research by Balaji *et al.* (2014) shown that a strain of *Mucor* sp. is able of secreting extracellular enzymes degrading hydrocarbons. Our strain *Mucor* sp. MUCL 54578 show tolerance to the concentration of jet fuel in solid medium, with a moderated growth in liquid medium and reaction positive of DCPIP.

Cylindrocarpon spp. MUCL 54699 and 54675 grew well in solid media with lower inhibition. However, in liquid medium, MUCL 54699 displayed fewer biomass, even though both were positive to the reaction of DCPIP at different levels. Cerniglia & Sutherland (2010) showed that strains of *Cylindrocarpon destructans* were able to degraded PAHs with three or more rings, but our *Cylindrocarpon* strains were not considered because of their potential role as plant pathogens (Seifert *et al.*, 2003b; Petit & Gubler, 2005; Hamid *et al.*, 2009; Aiello *et al.*, 2017).

According to some authors, *Cunninghamella* appeared to be efficient fungi to metabolize PAHs (Chaillan *et al.*, 2004; Singh, 2006; Pernía *et al.*, 2012). Some species of *Cunninghamella* had been more deeply studied i.e. *Cunninghamella elegans*, which metabolizes PAHs of from two to five aromatic rings to detoxified products (Salicis *et al.*, 1999; Rosenberg & Ron, 2005). Researches on fluoranthene and naphthalene had demonstrated that *Cunninghamella bainieri* and *C. elegans* also could metabolized this compounds (Cerniglia & Sutherland, 2010).

In our study, *Cunninghamella* sp. MUCL 54591 did not grow well in solid medium at the different concentrations of jet fuel, but exhibited a moderated growth and DCPIP positive reaction in liquid medium.

Cylindrocaldiella spp. have not been reported among the fungi identified as hydrocarbon degrading fungi, as far as we have been able to ascertain. *Cylindrocladiella* sp. MUCL 54584

exhibited an important development in solid medium at low jet fuel concentration, but was inhibited at concentration 2% and 3% of jet fuel. This strains also presents the ability to utilize the substrate, but with a moderated growth in liquid medium.

Acremonium sp. MUCL 54811 was able to growth in presence of jet fuel in solid and liquid medium showing tolerance to the different concentration of jet fuel, with a positive reaction of DCPIP. *Acremonium spp.* have been reported to degrade crude oil hydrocarbons in several studies (April *et al.*, 2000; Singh, 2006; Pernía *et al.*, 2012; Sardrood & Goltapeh, 2015). *Acremonium murorum* (Corda) W. Gams and *Acremonium kiliense* Grütz are reported as able to degraded mixture of PAHs (Cerniglia & Sutherland, 2010; Al-jawhari, 2015).

In our study, *Colletotrichum* sp. MUCL 54768 and *Phialophora* sp. MUCL 54828 displayed growth in solid and liquid medium with a positive response of DCPIP. *Colletotrichum* spp. have been reported to degrade hydrocarbons (Husaini *et al.*, 2008; Cerniglia & Sutherland, 2010). *Phialophora* spp. were mentioned by Ravelet *et al.* (2000) in an study on biodegradation. Cerniglia & Sutherland (2010) reported that *Phialophora alba* J.F.H. Beyma was able to degraded PAHs.

Dichobotrys sp. MUCL 54589 exhibited tolerance in solid medium and had a moderate growth in liquid medium, with a positive reaction of DCPIP. In the literature, this genus has not been reported in degradation studies. It is noteworthy that *Trichophaea abundans* (syn. *Dichobotrys abundans*) was isolated

from different soils in extreme condition of pH, from acidic (pH 3.9) to weakly alkaline (pH 7.4). This could be interpreted as a stress tolerant fungi (Šimonovičová *et al.*, 2014).

Leptoxyphium sp. MUCL 54762, and *Nectria* sp. MUCL 54833 has not been reported from oil-polluted environments. These two strains grew in solid medium with relative low inhibition but, in liquid medium, they did not show a good growth even though the reaction with DCPIP was positive in both cases.

Other genera unreported in oil contaminated soils are *Stilbella* and *Volutella*. In our studied *Stilbella* sp. MUCL 54579 and *Volutella* sp. MUCL 54565 responded well when hydrocarbon was the unique source of carbon, as measured by DCPIP and in solid medium. However, for the strain *Volutella* sp. MUCL 54735, the mycelial growth in solid medium was more inhibited. In liquid medium display biomass growth but the reaction DCPIP was low.

Verticillium, of which *Verticillium lecanii* is known to degrade PAHs (Salicis *et al.*, 1999; Singh, 2006, Cerniglia & Sutherland 2010). *Verticillium* sp. MUCL 54631, in our study, had a positive development in solid and liquid medium.

Moreover, strain such as *Aspergillus* sp. MUCL 54752 exhibited a good development in liquid medium with biomass and DCPIP positive reaction, but in solid medium presented inhibition. In general, the Aspergilli are rich in species assimilating hydrocarbons (April *et al.*, 2000; Ravelet *et al.*, 2000; Singh, 2006). Husaini *et al*. (2008) reported an *Aspergillus* sp. exhibiting

hydrocarbon biodegradation abilities in preliminary screening assay evaluating the average growth rate of fungal isolates on minimal media containing 1% (v/v) of used motor oil. Balaji *et al.* (2014) reported several Aspergilli able of secreting extracellular enzymes degrading hydrocarbons. April *et al.* (2000) mentioned a particular rapid grow of Aspergilli on oil. It will be necessary to continue our studies for a more accurate conclusion of the potential of our strains.

In the present studied *Botryodiplodia* sp. MUCL 54659, *Mariannaea* sp. MUCL 54806 and *Gliocladium* sp. MUCL 54915 has week development in presence of jet fuel in solid and liquid medium. A low response of DCPIP was shown except for *Gliocladium* sp. MUCL 54915. *Botryodiplodia theobromae* has been reported as oil-degrading i.e. (Pernía *et al.*, 2012; Sardrood & Goltapeh, 2015). *Gliocladium roseum* also was shown to degrade PAHs (Ravelet *et al.*, 2000; Cerniglia & Sutherland, 2010). Also, our result showed that *Beauveria* sp. MUCL 54917 displayed mycelium growth in solid medium but his response in liquid medium was week. Some species of *Beauveria*, such as *Beauveria alba* and *Beauveria bassiana* have been mentioned as able to degrade PAHs *i.e.* Singh (2006) or Cerniglia & Sutherland (2010).

Microsphaeropsis sp. MUCL 54855, *Mycoleptodiscus* sp. MUCL 54825 and *Metarhizium* sp. MUCL 54820 have a week growth in solid medium but exhibited a good development in liquid medium and positive reaction of DCPIP. To the best of our knowledge, these genera have been not reported as oil

degrading fungi. *Botryosphaeria* sp. MUCL 54843 did not exhibit a good mycelial growth in solid medium, but produced an important biomass in liquid medium, with activity of degrading jet fuel. *Botryosphaeria* is a genus with a cosmopolitan distribution, with saprobic, parasitic and endophytic species (Crous *et al.*, 2006).

The different behavior of strains belonging to the same genus highlighted a marked variability between the strains. For instance, *Vollutela* sp. MUCL 54565 has a good growth in solid medium with different concentration of jet fuel and *Vollutela* sp. MUCL 54735 was clearly inhibited in the higher concentrations. As mentioned by Bovio *et al.* (2017), in the case of different strains of the same species, *Penicillium citrinum* showed two insensitive and two stimulated strains by the presence of crude oil or in the case of *Syncephalastrum racemosum*, 9 strains presented inhibition, whereas one was insensitive and one stimulated by crude oil.

A correlation between level of discoloration and biomass yields could not be drawn; i.e. *Nectria* sp. MUCL 54833 or *Gliocladium* sp. MUCL 54833 grew less than *Trichoderma* sp. MUCL 54742 but they obtained similar discoloration. This was already mentioned by Bovio *et al.* (2017); in their study, *Penicillium citreonigrum* MUT 267 grew less than *Trichoderma harzianum* MUT 290, but both produced a comparable discoloration. Furthermore, in our study *Vollutella* sp. MUCL 54565, despite a vigorous growth in liquid medium, showed a moderate DCPIP reaction.

Some differences between fungal growth in solid and liquid medium were observed depending on the strain. Several factors are recognized to affect the growth of filamentous fungi as the inoculum concentration, agitation intensity and composition of the medium. It is not excluded that some fungi used in the present study had better abilities to grow on solid rather than on liquid medium (Singh, 2006). Besides, some fungi form pellets in liquid medium, what might lead to a reduction in the production of metabolites caused by nutrient transfer limitations in the cells of pellets. This could be a reason for the different response for the ability to use jet fuel even though the strains grew in solid medium. Further analysis should be addressed with each potential degrading strain (Singh, 2006).

In a similar study in Ecuador, Maddela *et al.* (2015b) found that the RRs for the fungus *Geomyces pannorum* were 77.3% and 43.4% in the case of diesel fuel and crude oil, respectively. In the case of the *Geomyces sp.* isolates, these ratios were 11.4% and 44.9%. The biodegradation rate for diesel fuel was significantly higher than the rate for crude oil. This finding indicates the complexity of the crude oil relative to diesel fuel in the degradation process. In our study with jet fuel, the RR for *Mucor irregularis* MUCL 54578, *Dichobotrys abundans* MUCL 54589, *Trichoderma atroviride* MUCL 54742 was 71.17%, 90.40% and 93.36%, respectively. These values are comparable to those achieved in diesel fuel, as both are petroleum derivates.

As a general point of view, fungi strains exhibited removal abilities of petroleum hydrocarbons. Further analyses will be

necessary before any conclusions can be made. Better characterization of their ability to degrade the different fractions of hydrocarbons should be performed. Similarly conclusion were made for Bovio *et al*. (2017) when their results found that *P. citreonigrum* MUT 267 and *A. terreus* MUT 271, in preliminary crude oil degradation experiments, clearly show their high potential for bioremediation.

The genus *Trichoderma* had been several times mentioned as oil degrading fungi (Ravelet *et al.*, 2000; Rivera-Cruz *et al.*, 2002; Husaini *et al.*, 2008; Balaji *et al.*, 2014). The strains in the current study grew successfully in presence of jet fuel. *Trichoderma atroviride* MUCL 54742 in particular exhibited the highest RR among the TPHs quantified in jet fuel. Bovio *et al.*, (2017) found contradictory results with *Trichoderma harzianum* MUT 290 in the crude oil degradation tests. Despite of the high DCPIP reaction and vigorous growth, *Trichoderma harzianum* MUT 290 showed a reduction of about 14% of the hydrocarbons analyzed.

A strategy for the exploitation of fungus could involve the use of plants. The plants may stimulate the growth of hydrocarbon degrading bacterial and fungal component in their rhizosphere; their extensive root system could develop a reliable joint environment in order to protect them from the toxic effects of pollution. Chibuike (2013) supported the idea that the ability of some fungi to biodegrade pollutants could be faster and more efficient when these fungi are used in combination with mycorrhizal fungi. Alarcón *et al.* (2008) used both

Cunninghamella echinulata and *Sphingomonas paucimobilis* in combination with arbuscular mycorrhizal fungi and found that the combined inoculant exhibited a higher amount of degradation than the treatment in which the strains were used separately.

Many strains were found to be able to growth with jet fuel as their unique source of carbon *in vitro* in both solid and liquid media; hence, they are possible candidates for bioremediation tools. However, future studies are needed to determine their response to crude oil, and these experiments need to be applied at the field level so that methods for effective bioremediation can be established for crude-oil-contaminated sites in Ecuador.

V. GENERAL DISCUSSION

The Amazonian rain forest in eastern Ecuador is one of the richest and most complex hotspot of biodiversity in South America. Recent studies have reported that the area has the highest number of tree species ever recorded for a 1 ha of *terra firme*, tropical rain forest. At least 473 species representing 187 genera and 54 families were found (Valencia *et al.*, 1994). The microbial diversity, including fungi, is directly positively correlated to the botanical diversity. The ratio Plant / Fungi is estimated between 1 per 6 or 1 / 9.8 (Hawksworth, 2001; Hawksworth & Lücking, 2017); hence the fungal diversity also should reach astonishing numbers. For instance, in a Colombian Amazonian forests, neighboring Ecuador, López-Quintero and colleagues collected 632 specimens of macromycetes, of which 328 could not be identified to the species level, and a significant proportion likely were undescribed species (Hawksworth & Lücking, 2017).

The long-term stability of these environments, however, is affected by the expansion of human settlements and both agricultural and industrials activities. The region has tremendous economic value for Ecuador due to the large amount of minerals, oil, timbers and potential agricultural lands (Varea *et al.*, 1995).

Oil contamination of soils in the Ecuadorean Amazon forest is a major threat for the biodiversity but also a challenge for remediation, including bio-remediation, and natural or human-facilitated re-colonization by plants.

Natural recolonization of heavily oil-polluted areas in the Ecuadorian rain forest offers a unique, extreme, and little explored environmental context that is worth to be studied in term

of biological diversity, dynamic of re-colonization and potential for remediation. Naturally recolonized oil ponds are inhabited by a diversity of plants, which in turn offer habitats for a diversity of organisms, including symbiotic and saprotrophic Fungi. These Fungi, very likely, find their origins in the neighboring non-polluted soils. Their selection by the environmental edaphic conditions, their role in the germination, growth, maintenance and nutrition of plants (Schulz *et al.*, 2006) or the role of saprotrophic rhizosphere fungi in depollution or bioremediation processes (Caldwell & Trappe, 2000) also is worth to be analyzed.

Approaching the fungal diversity

The first assessment of fungal diversity in naturally recolonized oil ponds in the Amazonian rain forest was based mainly on culture-dependent methodologies (Chapter 1).

For years, mycologists and more globally microbiologists have relied on culture-dependent methods to approach the soil microbiome. In the recent years, new culturing techniques have been developed, yielding larger numbers of isolates, allowing to picture better the soil fungal diversity (Ferrari *et al.* 2011, James & Seifert 2017). Culturing improvement methodologies include e.g. the use of high and low nutrient media (Ferrari *et al.*, 2011; Stefani *et al.*, 2015). Cultivation allows, in addition to the very necessary taxonomic works (Raja *et al.*, 2017), subsequent accurate studies of their physiology, metabolism and biochemistry, in controlled conditions (James & Seifert, 2017),

which is useful for understanding the detailed metabolism and functions of these organisms (Pham & Kim, 2012). Archiving living fungal cultures in collections provide the essential basis for fungal systematics and biodiversity research. It is important also for documenting research in ecology, genetics, and plant pathology (Verkley *et al.*, 2015; Raja *et al.*, 2017). Given that the fungal isolates also may display functions or produce metabolite of biotechnological interest, it is worth investing in a better species recovery (Ferrari *et al.*, 2011; Raja *et al.*, 2017)

However, in a strictly diversity perspective, culture-dependent approaches, whatever their level of optimization, still underestimate the diversity (Porras-Alfaro & Bayman, 2011; Johnston *et al.*, 2017). One simple reason for that is the existence of (still) unculturable taxa.

The development of molecular methodologies, of which the development of DNA sequencing methodologies, nowadays provide additional powerful tools to picture the soil microbial, including the fungal, communities, in term of “species” diversity but also functional diversity. The current tendency to approach the global biodiversity is to use massive DNA sequencing through the so-called “New Generation Sequencing” (NGS). Actually, this represents the future for the whole soil fungal communities’ characterization, though still with some bottlenecks.

NGS allows evidencing so-called OTUs (Operational Taxonomic Units). These OTUs, at best, could be equated to the basic unit of the taxonomic classification, the “Linnaean” species. NGS allow evidencing a much larger number of OTUs than any other methods. However, in many cases, and this is especially

obvious for poorly surveyed and species rich environment, these OTUs can hardly be assigned to any “Linnaean” species. The major reason for this is the lack of reference DNA-based taxonomic works that contribute to describe these “Linnaean” species (Hibbett & Taylor, 2013; Hawksworth & Lücking, 2017; James & Seifert, 2017; Johnston *et al.*, 2017; Raja *et al.*, 2017).

To maximize the characterization of the telluric fungi diversity, a combination of both culture-dependent and culture-independent approaches is highly recommended (Porras-Alfaro & Bayman, 2011; Singh *et al.*, 2012; Stefani *et al.*, 2015; Hawksworth & Lücking, 2017). These approaches are complementary and result in a more comprehensive and accurate picture of the fungal communities than each methods used alone (Singh *et al.*, 2012; Stefani *et al.*, 2015; Johnston *et al.*, 2017).

In our study, nonetheless, we used culture-dependant methods to assess the fungal diversity of the roots and rhizoplanes of several plants growing in a layer or organic material over oil ponds. This choice was guided by the main global objective of the CUD-PIC project which was to set up the fundation of an *ex-situ* culture collection in Ecuador. The objective was then to isolate as much as possible pure strains for a two-fold purposes: on one side, fine, state of the art, taxonomical characterization of this collection, and ahead, to describe the local fungal diversity. On the other side, to dispose of a set of pure cultures for later screening of physiological properties, of which the ability to degrade complex hydrocarbon molecules, and ahead the development of biotechnology for a

future bioeconomy as emphasized in the Ecuadorian “Well living National Plan” 2013-2017 (National Secretariat of Planning and Development (Senplades), 2013).

Within our study, 1077 strains were isolated in pure culture from the rhizoplane and internal root tissues of 208 herbaceous plants recolonizing two oil ponds. About half of the strains (512) were identified to generic level or complex of genera based on their morphology; the remaining strains could not be identified based on morphological features, due to absence of sporulation *in vitro*, which is a frequent limitation (Raja *et al.*, 2017).

Two hundred and ninety-eight of these fungal strains were obtained from root internal tissues (endophytes). One hundred and twenty-seven were identified, representing 61 genera. A group of endophytic fungi with melanized hyphae known as Dark Septate Fungi (DSFs) and several Basidiomycetes could not be identified morphologically. The taxonomy and identification of these DSF from roots are difficult (Porras-Alfaro & Bayman, 2011).

Every plant species has a community of endophytes. Recent estimation of their diversity show that up to 50 different genera could be associated with the roots of a single plant species (Porras-Alfaro & Bayman 2011).

Trichoderma, *Dactylonectria* (Hypocreales) and *Xylaria* (Xylariales) were the most frequently fungal strain genera in our study. Dark Septate Fungi also were commonly found.

In order to test the so-called Consolidate Species Concept (Quaedvlieg *et al.*, 2014), and hopefully, to name our strains in

the “Linnaean” scheme, a set of 44 strains from three complexes of genera within the Hypocreales were selected for cases studies: *Myrothecium*, *Cylindrocarpon* and *Gliocladiopsis* / *Aquanectria* (sub-section1.2.1 - 1.2.3). Morphological, Phylogenetic and the Ecological (substrate, habitat) Species Concepts were critically compared to address the identification of these strains resulting in a CSC concept.

The choice of these complexes was guided by the existence of recent state of the art taxonomic revisions allowing to infer the identity and affinities of our own strains. The interest in these groups also resulted from their importance as: etiological agents of diseases of economically important crops, such as stem cankers and root diseases (e.g. *Cylindrocarpon* black foot disease of grapevines affecting both nurseries and young vineyards, Halleen *et al.*, 2004; Chaverri *et al.*, 2011; Cabral *et al.*, 2012a; Dos Santos *et al.*, 2014; Lombard *et al.*, 2014); as potential bio-stimulator (*Gliocladiopsis*, Dann *et al.* (2012) e.g. avocado plants inoculated with *Gliocladiopsis sp*. improved the plant growth); as potential biotechnological applications e.g. several *Myrothecium* species has cellulolytic activity, resulting helpful in biodegradation of waste paper. Several *Myrothecium* also produce several secondary antifungal and antibiotic metabolites (Lombard *et al.*, 2016).

Consolidated Species Concepts

Myrothecium was first revised by Tulloch (1972), and characterized by a single morphological feature, *viz*. sporodochia producing phialoconidia gathering in a variably green, mucilaginous mass. This feature could be considered by that time as the hypothetic uniting character or a taxonomic hypothesis. This hypothesis was several times discussed, based on non-morphological data (of which the presence/absence of metabolites (Ahrazem *et al.*, 2000) but without any consequence, as far as taxonomy was concerned. Now, thanks to molecular techniques such as independent, DNA, genomic data, this hypothesis could have been tested.

The most recent revision of *Myrothecium* was performed by Lombard *et al.* (2016) who implemented detailed morphological, ecological, and multi-locus phylogenetic studies. These approaches revealed that the hypothesis of Tulloch does not stand; the generic concept of Tulloch is polyphyletic, and the green mucilaginous mass of phialoconidia is not apomorphic for a single *Myrothecium* lineage. Lombard *et al.* (2016) evidenced a complex of genera and recognized formally thirteen of them. Many of these new genera were, however, monotypic.

Our studies brought new insight in two of these monotypic genera, by adding species, confirming or refining the generic status and circumscription.

Two species were added to the previously monotypic *Inaequalispora* (Lombard *et al.*, 2016), *viz*. *I. longiseta* and *I. cylindrospora*. *Inaequalispora* was described to accommodate the single species *Myrothecium prestonii* (= *Inaequalispora*

prestonii). The addition of two new species confirmed the monophyly of this genus and the morphological circumscription.

One species also was added to the previously monotypic *Parvothecium* (Lombard *et al.*, 2016), *P. amazonense. Parvothecium* is typified by *P. terrestre*, which was the single known species to date. The morphological circumscription of *P. terrestre* and consequently of *Parvothecium*, was based on two isolates. However, our studies have shown that these two isolates first were not conspecific, and second also were not congeneric. The addition of our unnamed “Myrothecium-like” isolates and of *Myrothecium setiramosum* and *M. dimorphum* (which were not considered by Lombard *et al.* (2016)) showed that the *Parvothecium* lineage *sensu* Lombard *et al.* (2016) could be subdivided into two sublineages. These sublineages also were each characterized by an apomorphic feature, *viz.* simple (sublineage 1) or apically digitated (sublineage 2) setoid hyphae. This raised the question of the generic concepts. Two options were considered: an expanded *Parvothecium* or a *Parvothecium sensu stricto* and a newly defined entity. The second option was chosen following Lombard *et al.* (2016) (sub-section 1.2.1). Both genera are thus supported by morphological and phylogenetic data.

The first comprehensive treatment of *Cylindrocarpon* – *Neonectria* was proposed by Chaverri *et al.* (2011), implementing multi-locus phylogenetic inferences combined to morphological studies. Several new genera were considered and delimited in this complex. *Neonectria*, *Campylocarpon*, *Thelonectria*, *Rugonectria*, and *Ilyonectria*. *Ilyonectria*, later on, was shown to

be also polyphyletic (Lombard *et al.* 2013). Lombard *et al.* (2013) introduced *Dactylonectria* and more recently Aiello *et al.* (2017) also introduced *Pleiocarpon. Dactylonectria* and *Campylocarpon* were introduced to accommodate a bunch of species mostly associated with black foot symptoms of grapevine. *Hitherto*, only *Campylocarpon* was recorded from the Neotropics (Correia *et al.*, 2013; Dos Santos *et al.*, 2014).

In this study, we have identified for the first time species of *Dactylonectria* in the Neotropics. These species were shown to be closely related to *D. vitis*, *D. anthuriicola* and *D. pauciseptata* but representing new species: *D. amazonica*, *D. ecuadoriense*, *D. polyphaga* and *D. palmicola.* These species are overall similar, sharing predominantly 3-septate macroconidia (Cabral *et al.*, 2012a). We also have demonstrated that an additional, new *Campylocarpon* species, inhabiting root' tissue of an Amazonian plant, occurs in the rain forest of Ecuador, *Campylocarpon amazonense*.

The phylogenetic inferences in this study also revealed a larger than expected genetic diversities within the *D. pauciseptata* lineage as defined by Lombard *et al.* (2014). It raised the question of the species concept within the *D. pauciseptata* lineage, which could be polyphyletic.

Lombard *et al.* (2014) showed that the *Campylocarpon* lineage was divided into two clades, representing *C. fasciculare* and *C. pseudofasciculare*. In this study, these two lineages also were shown to be more complex than previously described. The addition of several strains from Brazil in the phylogenetic analyses allowed evidencing new clades that could represent

new phylogenetic species. These phylogenetic species are worth to be studied more carefully, in a taxonomic but also phytopathological perspective. These Brazilian isolates were isolated from diseased grapevines (Correia *et al.*, 2013; Dos Santos *et al.*, 2014) (sub-section1.2.2).

Saksena (1954) described *Gliocladiopsis* with a single species, *Gliocladiopsis sagariensis*. Lombard and Crous (2012) presented the most recent and more comprehensive revision of the genus combining morphological and phylogenetic species concepts. *Aquanectria*, described by Lombard *et al.* (2015), is the closest relative of *Gliocladiopsis* and both genera also are morphologically very similar.

In this study, three new species were shown in the *Gliocladiopsis* lineage (Lombard & Crous, 2012). One new species also was shown to belong to the *Aquanectria* lineage as defined by Lombard *et al.* (2015). In both cases their morphology agrees with this placement (concordant morphological and phylogenetic signals).

However, three phylogenetic species forming together a single lineage were found to be related (sister) to the *Aquanectria* lineage but their phenotype was discordant with this placement. Indeed, their conidial shape and size would rather point toward *Gliocladiopsis* than *Aquanectria*. This, again, raised the question of their generic placement or the circumscription of these genera.

Following the current trend of molecular-based classification (e.g. Lombard *et al.,* 2016), placing emphasis on the phylogenetic signals of several markers, *Aquanectria* could be considered as their “natural” placement. Nevertheless, this option, *de facto*, would reduce the morphological (conidiophores, conidiogenous

cells) and ontogenetic criteria (conidiogenesis) to subordinate elements. Following this interpretation, the resulting *Aquanectria* also would be morphologically heterogeneous, divided into a "typical" *Aquanectria* and a gliocladiopsis-like morphotype, each corresponding to a well-supported subclade. This also makes obsolete the *hitherto* sole apparent (or known) morphological distinction between the closely related *Aquanectria* and *Gliocladiopsis* (Lombard & Crous, 2012), viz. the shape and size of the asexual spores.

An alternate option would have been to merge *Aquanectria* and *Gliocladiopsis*, emphasizing the key morphological and ontogenetic features of the hyaline, penicillate conidiogenous apparatus, the conidiogenous cells, and the conidiogenesis that are identical in both genera. Spores-producing structures and spores could and their underlying genetic machinery (genes / gene cluster) could have more weight that the several markers used.

This expanded *Gliocladiopsis* also would form a well-supported, well-delimited lineage within the Nectriaceae (Lombard *et al.*, 2015), and would be morphologically more homogeneous but for the atypical morphotype represented by the monophyletic *A. penicillioides*, *A. submersa* and *A. filiformis*, which conidia are much longer and sinuous compared to the typical morphotype. Eventually, whether placing these three latter species in *Gliocladiopsis* or *Aquanectria*, the long and sigmoid conidia could represent a derived adaptation to a strictly aquatic habitat (Baschien *et al.*, 2013).

These studies (sub-section 1.2.3) raised question as to the interpretation of the data in the frame of the CSC (Quaedvlieg *et al.*, 2014).

As far as ecology is concerned, limited information is currently available; species of *Gliocladiopsis*, *Dactylonectria* and *Campylocarpon* are primarily soil-borne fungi (Dann *et al.*, 2012; Correia *et al.*, 2013; Lombard *et al.*, 2014; Parkinson *et al.*, 2017). *Dactylonectria* and *Campylocarpon* are often recorded as soil-borne pathogens isolated from diseased roots (Correia *et al.*, 2013; Lombard *et al.*, 2014).

Our species of *Dactylonectria* and *Campylocarpon* also are soil-borne fungi. However, they were also isolated from internal tissues of asymptomatic, externally sane roots. Our species were therefore not associated with specific disease such as root rots or black foot (Li *et al.*, 2008) (sub-section 1.2.2, 1.2.3).

The role of endophyte is, in most cases, poorly understood. In addition to a mutualistic and commensal relationships with their host plants, endophytes also could include latent pathogens or latent saprotrophs (Porras-Alfaro & Bayman, 2011). In other term, endophytism could represent only a segment of the life cycle of the fungal species, living in apparent “harmony” within the host tissue, but that could evolve either toward pathogenicity if the balance is modified, or, once the host tissue is senescent, toward saprophytism.

Biodegradation potential of tropical native hydrocarbon-degrading fungi

A diversity of Fungi was found associated with roots or rhizoplanes of the different plants colonizing the oil ponds. It could be hypothesized that the physico-chemical conditions of the polluted environment have an impact on the soil fungal diversity. Therefore, the fungal communities of these highly oil polluted soils could represent only a fraction of the soil fungal communities of the natural neighboring areas. The new environment could exert a selection pressure on the fungal communities toward “resistant” or “adapted” species / strains.

Their survival in harsh conditions suggests adaptive abilities, which could be related to their diverse enzymatic potential. Caldwell *et al.* (2000) showed that saprotrophic or root endophyte fungi are able to produce extra-cellular enzymes degrading the major C, N and P polymers. These extracellular enzymes could have a significant role in bioremediation. Some new findings of litter-decomposing fungi and indigenous non-lignolytic fungi, reported that their enzymes can transform significantly polycyclic aromatic hydrocarbons (PAHs) (Steffen *et al.*, 2002; Potin *et al.*, 2004; D’Annibale *et al.*, 2006)

Our screening procedure looked *in vitro* at the ability of our strains to grow in presence of and to degrade oil molecules (hydrocarbons). Some of the characteristics that are desirables for the use of fungi in bioremediation include rapid growth, high tolerance and potential for using these compounds.

Fifteen genera (i.e. *Acremonium, Colletotrichum*, *Cylindrocarpon, Cylindrocladiella Cunninghamella*, *Dichobotrys*, *Leptoxyphium Mucor*, *Nectria*, *Paecilomyces*, *Phialophora, Stilbella, Trichoderma, Verticillium, and Volutella sp.* 1 (MUCL 54565)), showed some level of tolerance in solid medium. They also showed abilities to use hydrocarbons, evidenced by the change of 2,6-Dichlorophenolindophenol (DCPIP) from blue (oxidized) to colorless (reduced) in liquid medium (Al-Nasrawi, 2012; Bidoia *et al.*, 2012; Hanafy *et al.*, 2015; Marchand *et al.*, 2017). Six genera (i.e. *Aspergillus, Botryosphaeria*, *Metarhizium, Microsphaeropsis*, *Mycoleptodiscus,* and *Volutella*) did not grow well on solid medium; the inhibition of the different concentrations was evident, even thought in liquid media they displayed a good growth and presented a positive response to DCPIP. Four genera (i.e. *Gliocladium, Mariannaea*, *Beauveria* and *Botryodiplodia*) presented fewer growth in solid and liquid medium.

Among these fungal taxa, we found different responses in relation to their tolerance and ability to mineralize hydrocarbons. The majority of the isolates showed some level of tolerance to the presence of hydrocarbon.

From the nine genera reported by Balaji *et al.* (2014), three are recorded in our studies*, Aspergillus, Mucor* and *Trichoderma*. The others genera reported by Balaji *et al.* (2014) were not tested in the present screening. *Curvularia, Penicillium, Drechslera, Fusarium, Lasiodiplodia* and *Rhizopus* were reported as capable of secreting extracellular enzymes with potential PAH-degrading capacities (Balaji *et al.*, 2014).

Oil degrading fungi belonging to the genera *Trichoderma*, *Aspergillus*, *Mucor*, *Paecilomyces* and *Penicillium* were isolated and selected for their hydrocarbon biodegradation potential in several studies, e.g. Ravelet *et al.* (2000), Rivera-Cruz *et al.* (2002), Husaini *et al.* (2008), or Marchand *et al.* (2017).

Trichoderma atroviride (MUCL 54742), *Dichobotrys abundans* (MUCL 54589)*, and Mucor irregularis* (MUCL 54578) showed a higher removal ratio (RR) of THPs in liquid than the control negative. However, further studies should be addressed for the evaluation of these strains. For instance, an enzymatic approaches could be developed. Microcosms assays using oil-soil polluted also could be tested. Recent studies pointed out the use of an integrative approaches in bioremediation. The use of plants and oil degrading microorganism, with their different strategies against the pollutant, could enhance the degradation response (Chibuike, 2013; Bell *et al.*, 2014)

VI. CONCLUSIONS & PERSPECTIVES

This study has explored the fungal diversity of the rhizoplanes and internal roots tissues of various plants growing in an oil-contaminated environment in the Ecuadorian Amazon rainforest. The main purpose of this study was to characterize the fungal communities using a culture-dependent approach. Our results support the viability of this approach; 1077 strains were isolated. More specifically, 779 strains were isolated from rhizoplanes, and 298 strains were isolated from the internal tissue of roots. Approximately half of the strains (512) developed reproductive structures *in vitro*, which allowed for their identification at the genus level (section 1.1). Sixty-one genera were identified, including various Dark Septate Fungi (DSF) and several isolates of Basidiomycetes. A total of 127 endophytes strains were identified, *Trichoderma*, *Dactylonectria* (Hypocreales), and *Xylaria* (Xylariales) being the most frequently isolated root endophytes (section 1.1).

Pure culture isolated is a critical component for the identification of fungal species, which is fundamental for the basic (ecology, taxonomy) or applied (genomics, bioprospecting scientific) research (Stefani *et al.*, 2015; Raja *et al.*, 2017).

This study employed the Consolidated Species Concept (CSC) to describe the species. This integrative approach enabled the recognition and description of one new genus (*Digitiseta* gen. nov.) and two new combinations (*D. setiramosa* comb. nov. and *Digitiseta dimorpha* comb. nov.); in addition, seventeen new species were described (*Inaequalispora longiseta* sp. nov., *I.*

cylindrospora sp. nov., *Parvothecium amazonense* sp. nov., *Dactylonectria parvodigitata* sp. nov., *D. multidigitata* sp. nov., *D. amazonica* sp. nov., *D. ecuadoriense* sp. nov., *D. polyphaga* sp. nov., *D. palmicola* sp. nov., *Campylocarpon amazonense* sp. nov. *Gliocladiopsis ecuadoriensis* sp. nov., *G. hennebertii* sp. nov., and *G. singaporiensis* sp. nov.. Finally, four new *Aquanectria* species have been proposed: *A. filiformis* sp. nov., *A. devians* sp. nov., *A. tenuispora* sp. nov., and *A. tenuissima* sp. nov.) (sub-section 1.2.1- 1.2.3).

This diversity of new taxa could have been described thanks to the existence of comprehensive taxonomic and phylogenetic studies. This approached is the model to follow with the remaining isolates obtained during this study. Taxonomic identification is a critical step to ensure reproducibility of studies that utilize fungi as a source material in industrial or agrochemical products and scientific names are crucial in communicating information about fungi (Raja *et al.*, 2017).

Some fungi, including root endophytes, especially DSFs, do not always sporulate in culture media, remaining sterile. Dark Septate Fungi constitute a polyphyletic form-group of fungi representing several orders of the Pezizomycotina (Knapp *et al.*, 2015). To date, only 40 DSF species have been described (Vergara *et al.*, 2017). Several reports had described their potential relevance as beneficial fungi (Knapp *et al.*, 2015; Vergara *et al.*, 2017), even though other studies (Jumpponen *et al.*, 1998; Grünig *et al.*, 2002) reveal neutral or pathogenic activities. Their trophic or nutritional relationships are poorly understood. Our knowledge on the diversity and distribution of

these fungi is still limited and further taxonomic and functional studies need to be addressed; our Amazonian strains should contribute to their diversity. (Grünig *et al.*, 2002; Knapp *et al.*, 2015).

Other genera recovered in this study also would be worth analyzing in much details, such as e.g. *Colletotrichum*, which was recently elected the eighth most important group of plant pathogenic fungi in the world (Cannon *et al.*, 2012). *Colletotrichum* contains important plant pathogens, both in the field and in post-harvest. *Colletotrichum* also are frequent isolated as endophytes. Many species spend a part of their life cycle as symptomless endophytes within living plant tissues (Cannon *et al.*, 2012). *Colletotrichum* spp. from non-cultivated plants in natural and semi-natural habitats are much less commonly studied than those associated with cultivated plant hosts. Our rain forest isolates could harbor a higher than expected diversity (Cannon *et al.*, 2012).

Mycologists worldwide are interested in searching for the undescribed biodiversity, particularly among tropical plants, which are often parts of unexplored environments.

Further analyses with a combined approached between culture dependent and culture independent methods are still necessary for a deeper survey of this microbiome. These two methods are complementary and provide two views of the microbial communities (Stefani *et al.*, 2015; Johnston *et al.*, 2017). Modern technologies, such us next-generation sequencing (NGS, e.g. Ion Torrent, Illumina)) are becoming more affordable. Their advantages is the generation of a larger number

of “reads” of DNA sequences that have could be used to identify OTUs, even though their short lengths could be a problem at species level (Johnston *et al.*, 2017). Better screening programs such as transcriptomics and proteomics should be employed to obtain more detailed information regarding ecology and physiology of the soil this fungi (Porras-Alfaro & Bayman, 2011; Raja *et al.*, 2017).

Studies focusing on the use of culture collection with biotechnological applications in cases of biocontrol must be performed. Future researches should consider the problem of the intensive use of toxic agrochemicals on crops, a challenge which needs to be adressed for the development of sustainable agriculture practices. Biological products also represent an area that requires more investigation and scientific efforts. Our culture collection has several fungal strains that should be tested as part of phytopathogen control efforts. Several strains of *Trichoderma* spp. (numerous strains) have been described as biocontrol agents, and some also exhibit some properties that stimulate plant growth

Endophytes are routinely used for isolating secondary metabolites. Besides, their potential roles in the plant community should be studied in more details. Some studies mentioned root endophyte as promoting plant growth, which can be beneficial to agriculture, even though in many cases, their functions overlap with those of mycorrhizal fungi (Porras-Alfaro & Bayman, 2011; Vergara *et al.*, 2017).

One possible use of these strains was considered in Chapter 2 as a foundation for deeper research. Environmental clean-up is

a significant challenge and the development of new low-cost strategies for bioremediation of polluted areas is a priority.

In order for bioremediation projects to be carried out, the structure and function of the microbial communities must be known. Degrading fungi have been isolated and characterized herein (section 1.1) and this study evaluated the *in-vitro* the ability of the isolated strains to grow in the presence of and degrade oil molecules (hydrocarbons). *In-vitro* screening procedures were also performed. The first screening was performed on solid and liquid media in order to test the growth of a selection of representative strains. These processes proved to be promising methods that can be explored further in future studies.

The first screening helped in the selection of a subset of strains. The THP removal ratios achieved in liquid medium by *Trichoderma atroviride* MUCL 54742, *Dichobotrys abundans* MUCL 54589 and *Mucor irregularis* MUCL 54578 reflected significantly higher degradation activity relative to the control. *Trichoderma atroviride* MUCL 54742 exhibited the highest removal ratio.

Further analyses are still needed to continue this study. Our understanding of this topic is still limited. Additional *in vitro* and *in situ* bioaugmentation and biostimulation assays need to be performed. Inoculants for bioaugmentation in bioremediation processes should be developed. Efficient crude oil bioremediation should be evaluated and the effects of pollution and bioremediation processes need to be analyzed. Future approaches, however, should be part of an integrated study of

the interaction between plants, fungi, and bacteria, which are all unique and active players in bioremediation due to the different strategies involved.

VII. OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

1. Scientific publications

1.1. Research Articles Published

- Gordillo A & Decock C (2017). *Myrothecium*-like (Ascomycota, Hypocreales) species from tropical areas: *Digitiseta* gen. nov. and additions to *Inaequalispora* and *Parvothecium*. Mycol Progress. DOI 10.1007/s11557-017-1302-4
- Gordillo A & Decock C (2017). *Cylindrocarpon*-like (Ascomycota, Hypocreales) species from rain forests in Ecuador: Additions to *Dactylonectria* and *Campylocarpon*. Cryptogamie Mycologie. DOI /10.7872/crym/v38.iss4.2017.1

1.2. Research Articles submitted

- Gordillo A & Decock C (2017). Multigene phylogeny and morphological evidence for undescribed species of *Gliocladiopsis* and *Aquanectria* (Ascomycota, Hypocreales) from tropical areas. In revision (minor) for Mycologia

2. Conference Participation

- Gordillo A, Decock C. and Luna V. (2016). Presentation. Diversidad de hongos saprófitos asociados a comunidades de plantas que de forma natural recolonizan piscinas de petróleo en áreas amazónicas de Ecuador. Conference at XL Jornadas Nacionales de Biología, Centro de Investigaciones Biotecnológicas del Ecuador – Escuela

Politécnica del litoral (CIBE-ESPOL. Guayaquil-Ecuador 16-18th Nov.

- Gordillo A, (2016) Presentation. Hongos saprófitos asociados a comunidades de plantas que de forma natural recolonizan piscinas de petróleo en áreas amazónicas de Ecuador. Simposio Diversidad fúngica en ecosistemas naturales y contaminados y colecciones de sultivos de microorganismos. Pontificia Universidad Católica del Ecuador (PUCE) Quito-Ecuador 28 Sep.
- Gordillo A, (2016) Presentation. Refuerzo de la experticia de hongos en el Ecuador a través del studio de casos de las interacciones plantas-hongos en ecosistemas seleccionados y el desarrollo biotecnológico orientados a recursos de hongos. Sociedad Ecuatoriana de Microbiología (SEM) filial de la Asociación latinoamericana de microbiología (ALAM). PUCE Quito-Ecuador 29 Jun.
- Gordillo A, Cevallos S (2015) Presentation. Contribución al estudio de la diversidad fúngica en el Ecuador. JORNADA MICOLÓGICA UTPL. Loja-Ecuador 25-27 th March.
- Gordillo A, Garcés M, Cevallos S, Luna V, Decock C (2015) Poster. Reinforcement of the fungal expertise in Ecuador via case studies of fungal plants interactions in selected ecosystems and the development of biotechnology-oriented fungal resource centres. Biodiversity and Development, a global heritage. Royal Belgian Institute of Natural Sciences. Brussels-Belgium 26 Nov.

VIII. REFERENCES

Abreo E, Martinez S, Bettucci L, Lupo S. 2010. Morphological and molecular characterisation of *Campylocarpon* and *Cylindrocarpon* spp. associated with black foot disease of grapevines in Uruguay. *Australasian Plant Pathology* **39**: 446–452.

Adams GO, Fufeyin PT, Okoro SE, Ehinomen I. **2015**. Bioremediation, Biostimulation and Bioaugmention: A Review. *International Journal of Environmental Bioremediation & Biodegradation* **3**: 28–39.

Adekunle A, Adebambo O. **2007**. Petroleum hydrocarbon utilization by fungi isolated from *Detarium senegalense* (J. F Gmelin) seeds. *Journal of American Science* **3**: 69–76.

Admon S, Green M, Avnimelech Y. **2001**. Biodegradation Kinetics of Hydrocarbons in Soil during Land Treatment of Oily Sludge. *Bioremediation journal* **5**: 193–209.

Agnihothrudu V. **1959**. Notes on fungi from north-east India. *Transactions of the British Mycological Society* **42**: 458–462.

Ahlich K, Sieber TN. **1996**. The profusion of dark septate endophytic fungi in non-ectomycorrhizal fine roots of forest trees and shrubs. *New Phytologist* **132**: 259–270.

Ahrazem O, Begoña Gómez M, Prieto A, Bernabé M, Leal JA. **2000**. Heterogeneity of the genus *Myrothecium* as revealed by cell wall polysaccharides. *Archives of Microbilogy* **173**: 296–302.

Aiello D, Polizzi G, Crous PW, Lombard L. **2017**. *Pleiocarpon* gen . nov . and a new species of *Ilyonectria* causing basal rot of *Strelitzia reginae* in Italy. *IMA Fungus* **8**: 65–76.

Al-jawhari IFH. **2015**. Ability of some fungi isolated from a sediment of Suq-Al Shuyukh marshes on biodegradation of crude oil. *International Journal of Current Microbiology and Applied Science* **4**: 19–32

Al-Nasrawi H. **2012**. Biodegradation of Crude Oil by Fungi Isolated from Gulf of Mexico. *Journal of Bioremediation and Biodegradation* **3**: 147.

Alarcón A, Davies FT, Autenrieth RL, Zuberer D a. **2008**. Arbuscular mycorrhiza and petroleum-degrading microorganisms enhance phytoremediation of petroleum-contaminated soil. *International journal of phytoremediation* **10**: 251–63.

Alfaro ME, Zoller S, Lutzoni F. **2003**. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**: 255–266

Altschul S, Gish W, Miller W, Myers E, Lipman D. **1990**. Basic

Local Alignment Search Tool. *Journal of Molecular Biology* **215**: 403–410.
Alvarez LA, Tamayo D, Castillo C, Munive C, Agustí-Brisach C, Gramaje D, Armengol J. **2012**. Ocurrence of grapevine trunk pathogens in nurseries and vineyards in the northern and southern coast of Peru. *Phytopathologia Mediterranea* **51**: 425.
Analysis R. **2014**. Module 3 - Multiple Linear Regression. : 1–68 https://www.unifr.ch/appecon/assets/files/.../multivariate-lineare-regression.pdf%0A. Accessed, 27-12-2017
Andrade D, Franken P. **2013**. Fungal Endophytes in plant roots: taxonomy, colonization patterns and functions. In: Aroca R, ed. Symbiotic Endophytes.
Anke H, Sterner O. **2002**. Insecticidal and Nematicidal Metabolites from Fungi. In: Osiewacz HD, ed. Industrial Applications. The Mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research). Berlin: Springer, 109–127.
April TM, Foght JM, Currah RS. **2000**. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. *Canadian Journal of Microbiology* **46**: 38–49.
Asghar H, Rafique H, Zahir ZA, Khan M, Akhtar M, Naveed M, Saleem M. **2016**. Petroleum Hydrocarbons-Contaminated Soils: Remediation Approches. In: Hakeem KR, Akhtar J, Sabir M, eds. Soil Science: Agricultural and Environmental Prospectives. Switzerland, 105–123.
Atlas RM. **1981**. Microbial Degradation of Petroleum Hydrocarbons : an Environmental Perspective. *Microbiological Reviews* **45**: 180–209.
Bakhshi M, Arzanlou M, Babai-Ahari A, Groenewald JZ, Braun U, Crous PW. **2015**. Application of the consolidated species concept to *Cercospora* spp. from Iran. *Persoonia: Molecular Phylogeny and Evolution of Fungi* **34**: 65–86.
Balaji V, Arulazhagan P, Ebenezer P. **2014**. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. *Journal of Environmental Biology* **35**: 521–529.
Barron GL. **1968**. *The genera of Hyphomycetes from soil*. Baltimore, USA: The Williams & Wilkins Company.
Baschien C, Tsui CKM, Gulis V, Szewzyk U, Marvanová L. **2013**. The molecular phylogeny of aquatic hyphomycetes with affinity to the *Leotiomycetes*. *Fungal Biology* **117**: 660–672.
Becerra-Hernandez C, Gonzales D, De Luna E, Mena-Portales J. **2016**. First report of pleoanamorphy in *Gyrothix verticlada* with and *Idriella*-like synanamorph. *Cryptogamie, Mycologie* **37**: 241–252.
Bell TH, El-Din Hassan S, Lauron-Moreau A, Al-Otaibi F, Hijri M,

Yergeau E, St-Arnaud M. **2014**. Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-contaminated soils is related to plant phylogeny. *The ISME journal* **8**: 331–43.
Bento FM, Camargo F AO, Okeke BC, Frankenberger WT. **2005**. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. *Bioresource Technology* **96**: 1049–1055.
Bidoia ED, Montagnolli RN, Lopes PRM. **2012**. Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique : a case study. *Applied Microbiology and Microbial Biotechnology* **3**: 1277–1288.
Blackwell M. **2011**. The fungi: 1, 2, 3 ... 5.1 million species? *American Journal of Botany* **98**: 426–438.
Boonchan S, Britz ML, Stanley G a. 2000. Degradation and Mineralization of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Defined Fungal-Bacterial Cocultures. *Applied and Environmental Microbiology* **66**: 1007–1019.
Booth C. **1966**. The genus *Cylindrocarpon*. *Mycological Papers* **104**: 1–56.
Borah G, Bhattacharyya LH, Vipin P, Bhattacharyya PN. **2015**. Rhizospheric Fungal Diversity Associated with Meyna spinosa Roxb. A Threatened and Ethno- Medicinally Important Plant of North-East India. *International Journal of Current Microbiology and Applied Sciences* **4**: 295–303.
Bourdel G, Roy-bolduc A, St-arnaud M, Hijri M. **2016**. Concentration of Contamination Shapes Fungal Endophytic Community Structure in Plant Roots. *Frontiers in Microbiology* **7**: 1–11.
Bovio E, Gnavi G, Prigione V, Spina F, Denaro R, Yakimov M, Calogero R, Crisafi F, Varese GC. **2017**. The culturable mycobiota of a Mediterranean marine site after an oil spill: Isolation, identification and potential application in bioremediation. *Science of the Total Environment* **576**: 310–318.
Brien HEO, Parrent JL, Jackson JA, Moncalvo J, Vilgalys R. **2005**. Fungal Community Analysis by Large-Scale Sequencing of Environmental Samples †. *Appl. Envir. Microbiol.* **71**: 5544–5550.
Buccina S, Chene D, Gramlich J. **2013**. Accounting for the environmental impacts of Texaco's operations in Ecuador: Chevron's contingent environmental liability disclosures. *Accounting Forum* **37**: 110–123.
Bustamante T, Jarrín MC. **2005**. Impactos sociales de la actividad petrolera en Ecuador: un análisis de los indicadores. *ICONOS. Revista de Ciencias Sociales*: 19–34.

Butler R. **2012**. Oil Extraction: the Impact Oil Production in the Rainforest. https://rainforests.mongabay.com/0806.htm Accessed, 27-10-2017

Cabral A, Groenewald JZ, Rego C, Oliveira H, Crous PW. **2012a**. *Cylindrocarpon* root rot: multi-gene analysis reveals novel species within the *Ilyonectria radicicola* species complex. *Mycological Progress* **11**: 655–688.

Cabral A, Rego C, Nascimento T, Oliveira H, Groenewald JZ, Crous PW. **2012b**. Multi-gene analysis and morphology reveal novel *Ilyonectria* species associated with black foot disease of grapevines. *Fungal biology* **116**: 62–80.

Caldwell BA, Trappe JM. **2000**. Utilization of major detrital substrates by dark-septate, root endophytes. *Mycologia* **92**: 230–232.

Cannon PF, Damm U, Johnston PR, Weir BS. **2012**. *Colletotrichum* - current status and future directions. *Studies in Mycology* **73**: 181–213.

Castañeda Ruiz RF. **1986**. Deuteromycotina de Cuba. *Serie Taxonomica del INIFAT* **IV**: 1–37.

Castlebury L, Rossman AY, Hyten AS. **2006**. Phylogenetic relationships of *Neonectria/Cylindrocarpon* on Fagus in North AmericaMention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by . *Canadian Journal of Botany* **84**: 1417–1433.

Cerniglia CE. **1997**. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *Journal of industrial microbiology & biotechnology* **19**: 324–33.

Cerniglia CE, Sutherland JB. **2010**. Degradation of Polyciclic Aromatic Hydrocarbons by fungi. In: Timmis K, McGenity T, van del Meer JR, de Lorenzo V, eds. Handbook of Hydrocarbon and Lipid Microbiology. Berlin: Springer-Verlag Berlin Heidelberg, 2080–2101.

Chaillan F, Le Flèche A, Bury E, Phantavong YH, Grimont P, Saliot A, Oudot J. **2004**. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in Microbiology* **155**: 587–595.

Chaverri P, Salgado C, Hirooka Y, Rossman a Y, Samuels GJ. **2011**. Delimitation of *Neonectria* and *Cylindrocarpon* (Nectriaceae, Hypocreales, Ascomycota) and related genera with *Cylindrocarpon*-like anamorphs. *Studies in mycology* **68**: 57–78.

Chávez-Gómez B, Quintero R, Esparza-García F, Mesta-Howard a. M, Zavala Díaz De La Serna FJ, Hernández-Rodríguez CH, Gillén T, Poggi-Varaldo HM, Barrera-Cortés J, Rodríguez-Vázquez R. **2003**. Removal of phenanthrene from soil by co-cultures

of bacteria and fungi pregrown on sugarcane bagasse pith. *Bioresource Technology* **89**: 177–183.
Chen Y, Ran SF, Dai DQ, Wang Y, Hyde KD, Wu YM, & Jiang Y-. 2016. Mycosphere Essays 2. *Myrothecium*. *Mycospheremycosphere* 759437: 64–80.
Chibuike G. 2013. Use of mycorrhiza in soil remediation: A review. *Scientific Research and Essays* **8**: 1679–1687.
Correia KC, Câmara MPS, Barbosa MAG, Sales R, Agustí-Brisach C, Gramaje D, León M, García-Jiménez J, Abad-Campos P, Armengol J, *et al.* 2013. Fungal trunk pathogens associated with table grape decline in Northeastern Brazil. *Phytopathologia Mediterranea* **52**: 380–387.
Crous PW, Hawksworth DL, Wingfield MJ. **2015**. Identifying and Naming Plant-Pathogenic Fungi: Past, Present, and Future. *Annual Review of Phytopathology* **53**: 247–267.
Crous PW, Kendrick WB, Alfenas AC. **1997**. New species of hyphomycetes associated with Eucalyptus. *South African Journal of Botany* **63**: 286–290.
Crous P. W. & Peerall A. 1996. *Gliocladiopsis irregularis* sp. nov. and notes on cylindrocladium spathiphylli. *Mycotaxon*.
Crous PW, Shivas RG, Quaedvlieg W, Bank M Van Der, Zhang Y, Summerell BA, Guarro J, Wingfield MJ, Wood AR, Alfenas AC, *et al.* 2014. Fungal Planet description sheets : 214 – 280. : 184–306.
Crous PW, Slippers B, Wingfield MJ, Rheeder J, Marasas WFO, Philips AJL, Alves A, Burgess T, Barber P, Groenewald JZ. **2006**. Phylogenetic lineages in the Botryosphaeriaceae. *Studies in Mycology* **55**: 235–253.
Crous PW, Verkley GJM, Groenewald JZ, Samson RA. **2009**. *Fungal Biodiversity*. Utrecht, The Netherlands, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
Crous PW, Wingfield MJ. **1993**. A re-evaluation morphologically of *Cylindrocladiella*, similar genera and a comparison with. *Mycolical Research* **97**: 433–448.
Cunningham CW. **1997**. Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* **14**: 733–740.
D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M. **2006**. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. *Applied and Environmental Microbiology* **72**: 28–36.
Dann EK, Cooke AW, Forsberg LI, Pegg KG, Tan YP, Shivas RG. **2012**. Pathogenicity studies in avocado with three nectriaceous fungi, *Calonectria ilicicola*, *Gliocladiopsis* sp. and *Ilyonectria liriodendri*.

Plant Pathology **61**: 896–902.
Decock C, Huret S, Bivort C. **2008**. Anamorphic fungi from French Guyana. *Septomyrothecium maraitiense* sp. nov. and *S setiramosum* comb. nov. (anamorphic Hypocreales, Ascomycota). *Cryptogamie, Mycologie* **29**: 321–331.
Duarte S, Seena S, Bärlocher F, Cássio F, Pascoal C. **2012**. Preliminary insights into the phylogeography of six aquatic hyphomycete species. *PloS one* **7**: e45289.
Fernández-Luqueno F, López-Valdez F, Sarabia-Castillo C, García-Mayagoitia S, Pérez-Ríos S. **2017**. Bioremediation of Polycyclic Aromatic Hydrocarbons-Polluted Soil at Laboratory and Field Scale: A Review of the Literature on Plants and Microorganisms. In: Anjum NA, Singh S, Narendra G, eds. Enhancing Cleanup of Environmental Pollutants. Springer International Publishing AG 2017, 1–317.
Ferrari BC, Zhang C, van Dorst J. **2011**. Recovering greater fungal diversity from pristine and diesel fuel contaminated sub-antarctic soil through cultivation using both a high and a low nutrient media. *Frontiers in Microbiology* **2**: 1–14.
Fierer N, Leff JW, Adams BJ, Nielsen UN, Bates ST, Lauber CL, Owens S, Gilbert JA, Wall DH, Caporaso JG. **2012**. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences* **109**: 21390–21395.
Finer M, Jenkins CN, Pimm SL, Keane B, Ross C. **2008**. Oil and gas projects in the Western Amazon: Threats to wilderness, biodiversity, and indigenous peoples. *PLoS ONE* **3**.
Fontaine G. **2005**. Microconflictos ambientales y crisis de gobernabilidad en la Amazonía ecuatoriana. *Iconos. Revista de Ciencias Sociales.* **3**: 1–15.
Flayyih I, Jawhari HA-. **2014**. Ability of Some Soil Fungi in Biodegradation of Petroleum Hydrocarbon. *Journal of Applied & Enviromental Microbiology* **2**: 46–52.
Gadd GM. **2007**. Fungi and Industrial Pollutants. In: Kubicek CP, Druzhinina IS, eds. Environmental and Microbial Relationships. Berlin, 69–84.
Garcés-Ruiz M, Senés-Guerrero C, Declerck S, Pirttilä AM, Pagano MC. **2017**. Arbuscular Mycorrhizal Fungal Community Composition in *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria* from Weathered Oil Ponds in the Ecuadorian Amazon. *Frontiers in Microbiology* **8**: 1–13.
Gehesquiere B, Crouch JA, Marra RE, Van Poucke K, Rys F, Maes M, Gobin B, Hofte M, Heungens K. **2016**. Characterization

and taxonomic reassessment of the box blight pathogen *Calonectria pseudonaviculata*, introducing *Calonectria henticotiae* sp. nov. *Plant Pathology* **65**: 37–52.
Gene Codes Corporation, Ann Arbor MU. Sequencher® version 5.4 sequence analysis software.
Girlanda M, Ghignone S, Luppi AM. **2007**. Diversity of sterile root-associatedfungi of two Mediterranean plants. *New Phytologist* **155**: 481–498.
Glass L, Donaldson GC. **1995**. Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. *Appl. Envir. Microbiol.* **61**: 1323–1330.
Gordillo A, Decock C. **2017**. *Myrothecium*-like (Ascomycota, Hypocreales) species from tropical areas: *Digitiseta* gen. nov. and additions to *Inaequalispora* and *Parvothecium*. *Mycological Progress*: 1–12.
Grünig CR, Sieber TN, Rogers SO, Holdenrieder O. **2002**. Spatial distribution of dark septate endophytes in a confined forest plot. *Mycological Research* **106**: 832–840.
Guevara J, Pitman N, Mogollon H, Ceron C, Palacios W. **2013**. Sistema de clasificación de los ecosistemas del Ecuador continental. Subsecretaría de Patrimonio Natural (a). *Ministerio de Ambiente del Ecuador*: 143.
Halleen F, Ourie P, Rous PEWC, Halleen F, Fourie PAH, Crous PWC. **2006**. A review of black foot disease of grapevine. *Phytopathologia Mediterranea* **45**: S55–S67.
Halleen F, Schroers HJ, Groenewald JZ, Crous PW. **2004**. Novel species of *Cylindrocarpon* (Neonectria) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (Vitis spp.). In: Studies in Mycology. 431–455.
Hamid M, Sandra A, Zia B, Josep A. **2009**. Characterization of *Cylindrocarpon liriodendri* Associated with Black Foot Disease of Grapevine in Iran. **645**: 642–645
Hanafy AA El, Anwar Y, Mohamed SA, Al-garni SMS, Sabir JSM, Osama A, Zinadah HA, Ahmed MM, Sampling A. **2015**. Isolation and Molecular Identification of Two Fungal Strains Capable of Degrading Hydrocarbon Contaminants on Saudi Arabian Environment. *International journal of Biological, Biomolecular, Agricultural, Food and Biotechnological enginee* **9**: 1210–1213.
Hanson HG, Desai JD, Desai AJ. **1993**. A rapid and simple screening technique for potential crude oil degrading microorganisms. *Biotechnology Techniques* **7**: 745–748.
Harms H, Schlosser D, Wick LY. **2011**. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nature*

Reviews Microbiology **9**: 177–192.
Hawksworth DL. **2001**. The magnitude of fungal diversity : the 1.5 million species estimate revisited. *Mycological Research* **105**: 1422–1432.
Hawksworth DL, Lücking R. **2017**. Fungal Diversity Revisited : 2 . 2 to 3 . 8 Million Species. *Microbiology Spectrum* **5**: 1–17.
Hawksworth DL, Rossman AY. **1997**. Where are all the undescribed fungi? *Phytopathology* **87**: 888–891.
Hibbett DS, Taylor JW. **2013**. Fungal systematics: is a new age of enlightenment at hand? *Nature reviews. Microbiology* **11**: 129–33.
Hirooka Y, Kobayashi T, Natsuaki KT. **2005**. *Neonectria castaneicola* and *Neo. rugulosa* in Japan. *Mycologia* **97**: 1058–1066.
Hudson HJ. **1961**. *Heliscus submersus* sp. nov., an aquatic hyphomycete from Jamaica. *Transactions of the British Mycological Society* **44**: 91–IN8.
Husaini A, Roslan H, Hii KSY, Ang CH. **2008**. Biodegradation of aliphatic hydrocarbon by indigenous fungi isolated from used motor oil contaminated sites. *World Journal of Microbiology and Biotechnology* **24**: 2789–2797.
Hyde KD, Bussaban B, Paulus B, Crous PW, Lee S, Mckenzie EHC, Photita W, Lumyong S. **2007**. Diversity of saprobic microfungi. *Biodiversity and Conservation* **16**: 7–35.
Ingold CT. **1942**. Aquatic hyphomycetes of decaying alder leaves. *Transactions of the British Mycological Society* **25**: 339–IN6.
James TY, Seifert KA. **2017**. Description of *Bifiguratus adelaidae* : The hunt ends for one of the 'Top 50 Most Wanted Fungi'. *Mycologia* **109**: 361–362.
Jayasiri SC, Hyde KD, Ariyawansa HA, Bhat J, Buyck B, Cai L, Dai YC, Abd-Elsalam KA, Ertz D, Hidayat I, *et al.* 2015. The Faces of Fungi database: fungal names linked with morphology, phylogeny and human impacts. *Fungal Diversity* **74**: 3–18.
Jensen WA . **2006**. Chapter 4 Linear Mixed Models. : 48–69. https://theses.lib.vt.edu/theses/available/etd-04202006-134123/.../Chapter4.pdf. Accessed, 27-12-2017
Johnston P, Park D, Smissen R. **2017**. Comparing diversity of fungal from living leaves using culturing and high-troughput enviromental sequencing. *Mycologia* (accepted manuscript).
Joo H, Ndegwa PM, Shoda M, Phae C, Cm C. 2008. Bioremediation of oil-contaminated soil using *Candida catenulata* and food waste. Environmental Pollution 156: 891–896.
Jumpponen A. **2001**. Dark septate endophytes - Are they mycorrhizal? *Mycorrhiza* **11**: 207–211.
Jumpponen A, Trappe JM. **1998**. Dark septate endophytes: A

review of facultative biotrophic root-colonizing fungi. *New Phytologist* **140**: 295–310.
Katoh K, Standley DM. **2013**. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.
Kirk P, Cannon P, Minter D, Stalpers J. **2008**. *Ainsworth & Bisby`s Dictionary of the Fungi* (Wallingford: CAB International, Ed.).
Kobayashi T, Hirooka Y, Natsuaki KT, Kawashima Y, Ushiyama K. **2005**. New canker diseases of *Abies veitchii* and *Acer crataegifolium* caused by *Neonectria castaneicola*. *Journal of General Plant Pathology* **71**: 124–126.
Knapp DG, Kovács GM, Zajta E, Groenewald JZ, Crous PW. **2015**. Dark septate endophytic pleosporalean genera from semiarid areas. *Persoonia* **35**: 87–100.
Kornerup A, Wanscher JH. **1978**. *Methuen handbook of colours* (E Methuen, Ed.). London.
Læssøe T, Petersen JH. **2008**. Equatorial fungi – mycological biodiversity in Ecuador Svampe 58 2008. : 1–53. (https://www.mycokey.com/Ecuador/LaessoPetersen2008.pdf)
Lanfear R. **2012**. PartitionFinder v1.1.0 and PartitionFinderProtein v1.1.0. **29**: 1695–1701.
Leavitt SD, Moreau CS, Lumbsch HT. **2015**. The Dynamic Discipline of Species Delimitation: Progress Toward Effectively Recognizing Species Boundaries in Natural Populations. In: Upreti D, Divakar PK, Shukla V, Bajpai R, eds. Reecent Advances in Lichenology. Springer India, 11–44.
Lemey P, Salemi M, Vandamme A. **2009**. *The Phylogenetic Handbook: A practical approch to phylogenetic analysis and hypothesis testing*. Cambridge: Cambridge University Press.
Lemos JLS, Rizzo AC, Millioli VS, Soriano AU, de Moura Sarquis MI, Santos R. **2002**. Petroleum degradation by filamentous fungi. In: 9th International Petroleum Environmental Conference. Nuevo México, 21–25.
Li J, Zhao J, Xu L, Zhou L, Li X, Wang J. **2008**. Endophytic fungi from rhizomes of *Paris polyphylla* var. *yunnanensis*. *World Journal of Microbiology and Biotechnology* **24**: 733–737.
Liu, Cai. **2013**. A Novel Species of *Gliocladiopsis* from Freshwater Habitat in China. *Cryptogamie, Mycologie* **34**: 233–241.
Liu F, Wang M, Damm U, Crous PW, Cai L. **2016**. Species boundaries in plant pathogenic fungi: a *Colletotrichum* case study. *BMC Evolutionary Biology* **16**: 81.
Liu YJ, Whelen S, Hall BD. **1999**. Phylogenetic Relationships Among Ascomycetes : Evidence from an RNA Polymerse II Subunit.

Molecular Biology and Evolution **16**: 1799–1808.
Lombard L, Bezuidenhout CM, Crous PW. **2013**. *Ilyonectria* black foot rot associated with Proteaceae. *Australasian Plant Pathology* **42**: 337–349.
Lombard L, Cheewangkoon R, Crous P. **2017**. New *Cylindrocladiella* spp. from Thailand soils. *Mycosphere* **8**: 1088–1104
Lombard L, Crous PW. **2012**. Phylogeny and taxonomy of the genus *Gliocladiopsis*. *Persoonia* **28**: 25–33.
Lombard L, Crous PW, Wingfield BD, Wingfield MJ. **2010a**. Multigene phylogeny and mating tests reveal three cryptic species related to *Calonectria pauciramosa. Studies in mycology* **66**: 15–30.
Lombard L, Crous PW, Wingfield BD, Wingfield MJ. **2010b**. Phylogeny and systematics of the genus *Calonectria*. *Studies in mycology* **66**: 31–69.
Lombard L, Zhou XD, Crous PW, Wingfield BD, Wingfield MJ. **2010c**. *Calonectria* species associated with cutting rot of Eucalyptus. *Persoonia* **24**: 1–11.
Lombard L, Houbraken J, Decock C, Samson RAA, Meijer M, Réblová M, Groenewald JZZ, Crous PWW. **2016**. Generic hyper-diversity in Stachybotriaceae. *Persoonia - Molecular Phylogeny and Evolution of Fungi* **36**: 156–246.
Lombard L, van der Merwe N a., Groenewald JZ, Crous PW. **2015**. Generic concepts in Nectriaceae. *Studies in Mycology* **80**: 189–245.
Lombard L, Van Der Merwe NA, Groenewald JZ, Crous PW. **2014**. Lineages in Nectriaceae: re-evaluating the generic status of *Ilyonectria* and allied genera. *Phytopathologia Mediterranea* **53**: 515–532.
Maddela NR, Burgos R, Kadiyala V, Carrion AR, Bangeppagari M. **2016**. Removal of petroleum hydrocarbons from crude oil in solid and slurry phase by mixed soil microorganisms isolated from Ecuadorian oil fields. *International Biodeterioration and Biodegradation* **108**: 85–90.
Maddela NR, Masabanda M, Leiva-Mora M. **2015a**. Novel diesel-oil-degrading bacteria and fungi from the Ecuadorian Amazon rainforest. *Water Science and Technology* **71**: 1554–1561.
Maddela NR, Scalvenzi L, Pérez M, Montero C, Gooty JM. **2015b**. Efficiency of Indigenous Filamentous Fungi for Biodegradation of Petroleum Hydrocarbons in Medium and Soil: Laboratory Study from Ecuador. *Bulletin of Environmental Contamination and Toxicology* **95**: 385–394.
MAE Ministerio del Ambiente Ecuador. **2016**. *Pasivos ambientales y reparación integral: experiencias de gestión en el Ecuador*. Quito-Ecuador.

Maharachchikumbura SSN, Hyde KD, Jones EBG, Mckenzie EHC, Bhat JD, Dayarathne MC, Huang S. **2016**. Families of Sordariomycetes. *Fungal Diversity* **79**: 1–317.
Marchand C, St-arnaud M, Hogland W, Bell TH. **2017**. International Biodeterioration & Biodegradation Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil. *International Biodeterioration & Biodegradation* **116**: 48–57.
Margesin R, Schinner F. **2001**. Bioremediation (Natural Attenuation and Biostimulation) of Diesel-Oil-Contaminated Soil in an Alpine Glacier Skiing Area. *Applied and Environmental Microbiology* **67**: 3127–3133.
Mason-Gamer RJ, Kellog E. 1996. Testing for phylogenetic conflict among molecular data sets in the tribe Triticeae (Gramineae). *Systematic Biology* **45**: 524–545.
Matsushima T. **1971**. Some interesting fungi imperfecti. *Bulletin Natural Sicence Museum* **14**: 460–480.
Mittal A, Singh P. **2009**. Isolation of hydrocarbon degrading bacteria from soils contaminated with crude oil spills. *Indian Journal of Experimental Biology* **47**: 760–765.
Montagnolli RN, Lopes PRM, Bidoia ED. **2015**. Screening the Toxicity and Biodegradability of Petroleum Hydrocarbons by a Rapid Colorimetric Method. *Archives of Environmental Contamination and Toxicology* **68**: 342–353.
Moolgavkar SH, Chang ET, Watson H, Lau EC. **2014**. Cancer mortality and quantitative oil production in the Amazon region of Ecuador, 1990-2010. *Cancer Causes and Control* **25**: 59–72.
Müller J, Müller KF, Neinhuis C, Quandt D. **2006**. PhyDE - Phylogenetic Data Editor. *Www.Phyde.De*.
Nag Raj TR. **1993**. *Coelomycetous* anamorph with appendage-bearing conidia. *Mycologue Publications*, Waterloo.
Nag Raj TR. **1995a**. Lomachashaka Revisited. *Mycotaxon* **LIII**: 311–324.
Nag Raj TR. **1995b**. What is *Myrothecium prestonii*? *Mycotaxon* **LIII**: 295–310.
National Secretariat of Planning and Development (Senplades). **2013**. National Development Plan/ Good living National Plan 2013-2017. : 1–130.
Nilsson RH, Wurzbacher C, Bahram M, R. M. Coimbra V, Larsson E, Tedersoo L, Eriksson J, Duarte C, Svantesson S, Sánchez-García M, *et al.* 2016. Top 50 most wanted fungi. *MycoKeys* **12**: 29–40.
Nirenburg HI. **1981**. A simplified method for identifying *Fusarium* spp. occurring on wheato Title. *Canadian Journal of Botany* **59**:

1599–1609.
O'Donnell K, Cigelnik E. **1997**. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**: 103–116.
O'Donnell K, Gueidan C, Sink S, Johnston PR, Crous PW, Glenn A, Riley R, Zitomer NC, Colyer P, Waalwijk C, *et al.* 2009. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genetics and Biology* **46**: 936–948.
O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC. **1998**. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 2044–2049.
Odriosolla E, Centeno de la Rosa F, Tavares C, Virgínia A, Sanzo L, Fernandes J, Burkert DM, Kalil J, André C, Burkert V. **2008**. Pre-screening of filamentous fungi isolated from a contaminated site in Southern Brazil for bioaugmentation purposes. *Journal of Biotechnology* **7**: 1314–1317.
OECD Organisation for Economic Co-operation and Development. 2001 Biological Resource Centres: underpinning the future of life sciences and biotechnology. http://dx.doi.org/10.1787/9789264193550-en
Parkinson LE, Shivas RG, Dann EK. **2017**. Novel species of *Gliocladiopsis* (Nectriaceae, Hypocreales, Ascomycota) from avocado roots (*Persea americana*) in Australia. *Mycoscience* **58**: 95–102.
Perez A. **2014**. Diversity , composition and floristic structure of Charapa 1 well pad , Sucumbios-Ecuador.
Pernía B, Demey JR, Inojosa Y, Naranjo-Briceño. **2012**. Biodiversidad y potencial hidrocarbonoclástico de hongos aislados de crudo y sus derivados : Un meta-análisis Biodiversity and hydrocarbonoclastic potencial of fungi isolated from crude and petroleum derivates : a meta-analysis Abstract. Revista Latinoamericana de Biotecnología Ambiental y Algal **3**: 1–39.
Petit E, Gubler WD. **2005**. Characterization of *Cylindrocarpon* Species , the Cause of Black Foot Disease of Grapevine in California. *Plant Disease* **89**: 1051–1059.
Pham VHT, Kim J. **2012**. Cultivation of unculturable soil bacteria. *Trends in Biotechnology* **30**: 475–484.
Porras-Alfaro A, Bayman P. **2011**. Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. *Annual Review of Phytopathology* **49**: 291–315.
Potin O, Rafin C, Veignie E. **2004**. Bioremediation of an aged

polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. *International Biodeterioration and Biodegradation* **54**: 45–52.
Quaedvlieg W, Binder M, Groenewald JZ, Summerell BA, Carnegie AJ, Burgess TI, Crous PW. **2014**. Introducing the consolidated species concept to resolve species in the teratosphaeriaceae. *Persoonia: Molecular Phylogeny and Evolution of Fungi* **33**: 1–40.
Raja HA, Miller AN, Pearce CJ, Oberlies NH. **2017**. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *Journal of Natural Products* **80**: 756–770.
Rajapaksha RMCP, Bååth E, Ba E. **2004**. Metal Toxicity Affects Fungal and Bacterial Activities in Soil Differently. *Applied and environmental microbiology* **70**: 2966–2973.
Rambaut, A. & Drummond A. **2007**. Tracer 1.4.
Ranzoni F V. 1956. The Perfect Stage of *Flagellospora penicillioides*. *American Journal of Botany* **43**: 13.
Ravelet C, Krivobok S, Sage L, Steiman R. **2000**. Biodegradation of pyrene by sediment fungi. *Chemosphere* **40**: 557–563.
Refaei J, Jones E, Sakayaroj J, Santhanam J. **2011**. Endophytic fungi from *Rafflesia cantleyi* : species diversity and antimicrobial activity. *Mycosphere* **2**: 429–447.
Rehner S, Buckley E. **2005**. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- sequences : evidence for cryptic diversification and links to *Cordyceps* teleomorphs Abstract : *Mycologia* **97**: 84–98.
Rehner S a., Samuels GJ. **1994**. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycological Research* **98**: 625–634.
Rehner SA, Samuels GJ. **1995**. Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Canadian Journal of Botany* **73**: 816–823.
Rivera-Cruz M, Ferrera-Cerrato R, Volke-Haller V, Fernandez-Linares L, Rodriguez-Vasquez R. **2002**. Adaptación y selección microbiana autóctona en medios de cultivos enriquecidos con benzo(a)pireno. *Agrociencia* **36**: 503–514.
Ron K. **2012**. Determinación de residuos totales de petróleo (fracción diesel) en aguas de la Reserva de Producción faunística Cuyabeno, mediante la técnica de cromatografía de gases detector de ionización de llama.
Ronquist F, Huelsenbeck JP. **2003**. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Rosenberg E, Ron E. **2005**. Bioremediation of petroleun contamination. In: Crawford R, Crawford D, eds. Biotechnology Research. Idaho, USA: Cambridge University Press, 416.
Rossman AY, Mycologia S, Feb NJ, Taylor P, Rossman AY. **1996**. Morphological and Molecular Perspectives on Systematics of the Hypocreales. *Mycologia* **88**: 1–19.
Rundell S, Spakowicz D, Narváez-Trujillo A, Strobel S. **2015**. The Biological Diversity and Production of Volatile Organic Compounds by Stem-Inhabiting Endophytic Fungi of Ecuador. *Journal of Fungi* **1**: 384–396.
Saksena SB. **1954**. A new genus of *Moniliaceae*. *Mycologia* **46**: 660–666.
Salicis F, Krivobok S, Jack M, Benoit-Guyod JL. **1999**. Biodegradation of fluoranthene by soil fungi. *Chemosphere* **38**: 3031–3039.
Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B. **2010**. *Food and Indoor Fungi*. Utrecht, The Netherlands, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
Samuels G, Brayford D. **1994**. Species of *Nectria* (sensu lato) with red perithecia and striate ascospores. *Sydowia* **46**: 75–161.
Samuels GJ, Rossman AY. **1979**. Conidia and classification of the nectrioid fungi. *Whole fungus ; the sexual-asexual synthesis.* **1**: 167–179.
San Sebastián M, Hurtig A-K. **2004**. Oil exploitation in the Amazon basin of Ecuador: a public health emergency. *Revista panamericana de salud publica = Pan American journal of public health* **15**: 205–211.
Dos Santos R, Blume E, Muniz M, Harakawa R, Garrido R, Rego C. **2014**. Characterization of *Campylorarpon pseudofascicuare* associated with black foot of grapevine in southern brazil. *Phytopathologia Mediterranea* **53**: 406–415.
Saraswathy A, Hallberg R. **2002**. Degradation of pyrene by indigenous fungi from a former gasworks site. *FEMS Microbiology Letters* **210**: 227–232.
Sardrood BP, Goltapeh EM. **2015**. An Introduction to Bioremediation. In: Goltapeh EM, ed. Fungi as Bioremediators, Soil Biology. Springer-Verlag Berlin Heidelberg, 3–29.
Schoch C, Crous P, Wingfield M, Wingfield B. **2000**. Phylogeny of *Calonectria* and selected hypocrealean genera with cylindrical macroconidia. *Studies in Mycology* **45**: 45–62.
Schroers H, Samuels GJ, Gams W. **1999**. *Stephanonectria* , a new genus of the Hypocreales (Bionectriaceae), and its sporodochial anamorph. *Sydowia* **51**: 114–126.

Schroers H-J, Zerjav M, Munda A, Halleen F, Crous PW. **2008**. *Cylindrocarpon pauciseptatum* sp. nov., with notes on *Cylindrocarpon* species with wide, predominantly 3-septate macroconidia. *Mycological research* **112**: 82–92.
Schulz B, Boyle C, Sieber T. **2006**. *Microbial root endophytes. Springer*. Berlin Heidelberg New York.
Seifert K a. 2000. How should we look at anamorphs.pdf. *Studies in Mycology* **45**: 5–18.
Seifert K, Louis-Seize G, Sampson G. **2003a**. *Myrothecium acadiense*, a new hyphomycete isolated from the weed Tussilago farfara. *Mycotaxon* **LXXXVII**: 317–327.
Seifert K a, McMullen CR, Yee D, Reeleder RD, Dobinson KF. **2003b**. Molecular Differentiation and Detection of Ginseng-Adapted Isolates of the Root Rot Fungus *Cylindrocarpon destructans*. *Phytopathology* **93**: 1533–1542.
Seifert K, Morgan-Jones G, Gams W, Kendrick B. **2011**. *The Genera of Hyphomycetes*. Utrecht, The Netherlands, The Netherlands: CBS-KNAW Fungal Biodiversity Centre.
Shenoy BD, Jeewon R, Hyde KD. **2007**. Impact of DNA sequence-data on the taxonomy of anamorphic fungi. *Fungal Diversity*: 1–54.
Simmons MP, Pickett KM, Miya M. **2004**. How Meaningful Are Bayesian Support Values? *Molecular Biology and Evolution* **21**: 188–199.
Šimonovičová A, Nováková A, Pangallo D, Hnátová V, Hubka V. **2014**. The occurrence of heat-resistant species of *Trichophaea abundans* in different types of soil in Slovakia and Czech Republic. *Biologia* **69**: 168–172.
Singh H. **2006**. *Mycoremediation: Fungal Bioremediation*. New Jersey: Wiley-Interscience.
Singh P, Raghukumar C, Murti R, Verma P, Shouche Y. **2012**. Fungal diversity in deep-sea sediments revealed by culture-dependent and culture-independent approaches. *Fungal Ecology* **5**: 543–553.
Stamatakis A. **2006**. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
Stamatakis A. **2014**. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312–1313.
Stefani FOP, Bell TH, Marchand C, De La Providencia IE, El Yassimi A, St-Arnaud M, Hijri M. **2015**. Culture-dependent and -independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. *PLoS ONE* **10**: 1–16.

Steffen KT, Hatakka A, Hofrichter M. **2002**. Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. *Applied Microbiology Biotechnology* **60**: 212–217.
Stewart JE, Timmer LW, Lawrence CB, Pryor BM, Peever TL. **2014**. Discord between morphological and phylogenetic species boundaries : incomplete lineage sorting and recombination results in fuzzy species boundaries in an asexual fungal pathogen. *BMC Evolutionary Biology* **14**: 1–14.
Sung GH, Sung JM, Hywel-Jones NL, Spatafora JW. **2007**. A multi-gene phylogeny of *Clavicipitaceae* (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. *Molecular Phylogenetics and Evolution* **44**: 1204–1223.
Swofford DL. **2003**. PAUP*. Phylogenetic analysis using parsimony (*and other methods), Computer programme.
Taylor JW, Berbee M. **2014**. Fungi from PCR to Genomics: The Spreading Revolution in Evolutionary Biology. In: Esser K, McLaughlin DJ, Spatafora JW, eds. The Mycota VII Systematics and Evolution Part A. Heidelberg: Springer, 1–20.
Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC. **2000**. Phylogenetic species recognition and species concepts in fungi. *Fungal genetics and biology : FG & B* **31**: 21–32.
Taylor JW, Turner E, Townsend JP, Dettman JR, Jacobson D. **2006**. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **361**: 1947–1963.
Torres-Cruz TJ, Billingsley Tobias TL, Almatruk M, Hesse CN, Kuske CR, Desirò A, Benucci GMN, Bonito G, Stajich JE, Dunlap C, *et al.* 2017. *Bifiguratus adelaidae* gen. et sp. nov., a new member of Mucoromycotina in endophytic and soil-dwelling habitats. *Mycologia* **109**: null-null.
Tulloch M. **1972**. The genus *Myrothecium* Tode ex Fr. *Mycological Papers* **130**: 1–42.
U'Ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE. **2009**. Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analysing fungal environmental samples. *Mycological Research* **113**: 432–449.
Untereiner WA, Bonjean B, Decock C, Evrard P, De Frahan MH, Jamin N, Massart L, Nélissen L, Robert V, Bosschaerts M, *et al.*

1998. *MUCL catalogue of strains (Fungi-Yeast)*. Brussels, Belgium: Belgian Office for Scientific, Technical and Cultural Affairs.
Uzona C, Godwin O, Ogechi N. **2015**. Effect of crude oil pollution on the rhizosphere microbial communities of *Mangifera indica L* and *Elaeis guineensis* Jacq in Rivers State, Nigeria. *Journal of biodiversity and Environmental Sciences* **6**: 152–165.
Valencia R, Balslev H, Paz Y Miño C G. **1994**. High tree alpha-diversity in Amazonian Ecuador. *Biodiversity and Conservation* **3**: 21–28.
Varea A, Ortiz P, Martínez E, Bustamante T, Navarro M, Garzón P, Villamil H, Garcés A. **1995**. *Marea negra en la Amazonía Conflictos socioambientales vinculados a la actividad petrolera en el Ecuador*. Quito-Ecuador: Ediciones Abya-Yala.
Vergara C, Campos K, Soares L, de Souza S, Santos L, Claudete S-C, da Silva K, Duarte G, Xavier G, Zilli J. **2017**. Contribution of dark septate fungi to the nutrient uptake and growth of rice plants. *Brazilian Journal of Microbiology*: 1–12.
Verkley GJM, Rossman AY, Crouch JA. **2015**. The role of herbaria and culture collections. In: McLaughlin DJ, Spatafora JW, eds. Systematics and Evolution, The Mycota Vll, Part B. Berlin: Springer-Verlag Berlin Heidelberg 2015, 205–225.
Vilgalys R, Hester M. **1990**. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.
Wang W, McGhee D, Gibas CFC, Tsuneda a., Currah RS. **2009**. *Phialocephala urceolata* sp. nov., from a commercial, water-soluble heparin solution. *Mycologia* **101**: 136–141.
Watanabe T, Watanabe Y, Nakamura K. **2003**. *Myrothecium dimorphum* sp. nov. a soil fungus from beach sand in the Bonin (Ogasawara) Islands, Japan. *Mycoscience* **44**: 283–286.
Webster J, Weber R. **2007**. *Introduction to fungi. ,* (CU Press, Ed.). New York.
White TJ, Bruns TD, Lee SB, Taylor W. **1990**. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis MA, Gelfard H, Sninsky JS, White TJ, eds. PCR-protocols and applications. New York: Academic Press, 315–322.
Wiebe M. **2002**. Myco-protein from *Fusarium venenatum*: A well-established product for human consumption. *Applied Microbiology and Biotechnology* **58**: 421–427.
Wollenweber HW. **1913**. *Ramularia*, *Mycosphaerella, Nectria, Calonectria*. Eine morphologisch pathologische Studie zur Abgren-zung von Pilzgruppen mit cylindrischen und sichelfo ¨rmigen Konidienformen. *Phytopathology* **3**: 197–242.

Wollenweber H. **1917**. Fusaria autographice delineata. *Published by the aythor*.
Wu Y, Luo Y, Zou D, Ni J, Liu W. **2008**. Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with *Monilinia* sp. degradation and microbial community analysis. *Biodegradation* **19**: 247–257.
Yang Z, Rannala B. **2012**. Molecular phylogenetics: principles and practice. *Nature Reviews Genetics* **13**: 303–314.
Yanto DHY, Tachibana S. **2014**. Potential of fungal co-culturing for accelerated biodegradation of petroleum hydrocarbons in soil. *Journal of Hazardous Materials* **278**: 454–463.
Zacatenco P, Madero DG a. **2010**. Filamentous fungi remove weathered hydrocarbons from polluted soil of tropical México. *Environmental Protection* **26**: 193–199.

IX. ANNEXE

Botanist report Charapa ponds, Sucumbíos- Ecuador

M.Sc. Alvaro Peréz

Diversity, composition and floristic structure of Charapa 1 well pad, Sucumbios-Ecuador

To determine and assess the diversity, composition and floristic structure of Charapa 1 well pad a quantitative and qualitative inventory was conducted (Table 1). Field work was conducted on Friday, August 8, 2014.

Table 1. Location of Charapa 1 well pad.

Site	Coordinates		Altitude (masl)	Habitat
	West	South		
Pool 1	76° 48' 57"	00° 11' 49"	309	Secondary forest
Pool 2	76° 48' 54"	00° 11' 46"	309	Secondary forest

Ecological parameters as: basal area, species richness and abundance, relative density, relative dominance, importance value index to species and family and aboveground biomass estimation were calculated using data from a quantitative inventory. Diversity and forest composition were assessed using a qualitative inventory. Vertical forest structure was performed based on the height of all individuals recorded in the quantitative and qualitative inventory.

1. Study area

The plant community in which the study area is located corresponds to Lowland Evergreen Forest (Palacios *et al.* 1999.) or Lowland Evergreen Forest of Napo-Curaray (Guevara *et al.* 2013.); which originally housed a high diverse vegetation with over 200 species ≥ 10 cm DBH in one hectare (Valencia *et al.*, 2004). Currently, much of the natural vegetation has been cleared for field crops, cattle, oil extraction activities, among others, leaving few remnants of natural vegetation.

The vegetation type that grows in the study area is a secondary forest, dominated by grasses, such as *Dimerocostus strobilaceus* (caña agria), *Carludovica palmata* (toquilla), *Heliconia* cf. *chartacea* (platanillo) and several species of Araceae; while the tree layer consists of several species of *Ficus* (higuerón), *Croton lechleri* (sangre de drago) and *Sapium glandulosum* (lechero).

2. Methodology

2.1 Field work

Quantitative inventory

At pool 1 a quadrant of 30 x 11 m (330 m^2) was established, while at pool 2 a quadrant of 25 × 18 m (450 m^2) was established. Each quadrant cover the total area of a pool. In each quadrant all individuals with a diameter equal to or greater than 2 cm DBH (diameter at breast height = 130 cm) were recorded. Taxonomic identification (family, genus, species, common name), diameter (by using a diameter tape) and the estimated height were recorded for each individual (Figure 1).

Figure 1. Tree census at Charapa 1 well pad, DBH measurement

Qualitative inventory

For the floristic inventory plant collections (herbs, shrubs, trees) were performed. Plant collections were made on walks in and around the study area, in order to obtain a representative sample of the species composition and to collect physiognomic, structural and environmental information of the forest.

Those individuals who were not identified to species level directly in the field were collected and pressed for transportation to QCA Herbarium facilities in Quito.

2.2 Laboratory analysis

All individuals collected in the field were identified at the QCA Herbarium facilities by comparison with previously determined specimens and using taxonomic keys; also digitized samples and/or photos available on virtual herbaria of institutions like the Field Museum of Chicago (http://fm1.fieldmuseum.org/vrrc/), Missouri Botanical Garden (http://www.tropicos.org/),New York Botanical Garden (http://sciweb.nybg.org/science2/vii2.asp) and Royal Botanic Gardens Kew (http://apps.kew.org/herbcat/navigator.do) were reviewed.

The scientific name of all species was reviewed in the Catalogue of Vascular Plants of Ecuador (Jørgensen & León-Yanez 1999) and using Tropicos data base of the Missouri Botanical Garden (http://www.tropicos.org/).

2.3 Data Analysis

To analyze data from the quantitative inventory, formulas from Campbell *et al.* 1986 were used.

2.3.1 Basal area (ba) in cm^2

"Basal area" of an individual tree is defined as the DBH area on cross section of the stem or trunk. The basal area of any species recorded at any quadrant is the sum of all their basal areas with DBH ≥ 2 cm.

$$AB = \left(\frac{\pi D^2}{4} \right)$$

where:

D = diameter at breast height; π = constant 3.1416

2.3.2 Relative density (rd)

The "relative density" of a given species is proportional to the number of individuals of that species, related to the total number of individuals in the quadrant.

$$RD = \frac{\text{No. of individuals of any species}}{\text{Total No. of individuals at the quadrant}} \text{ x } 100$$

2.3.3 Relative Dominance (rdm)

The "Relative dominance" of a given species is the proportion of their basal area, with respect to the basal area of all individuals in the quadrant.

$$RDM = \frac{\text{Basal area of a species}}{\text{Basal area from all species}} \text{ X } 100$$

2.3.4 Importance Value Index (IVI)

Two parameters (Relative Density and Relative Dominance) were summed to obtain the "Importance Value". The sum of "Importance Value" for all species in the quadrant is always equal to 20. Therefore, a species that exceed a value of 20 in quadrant (10% of total value) is "important" and a common component of the sampled forest.

$$IVI = RD + RDM$$

2.3.5 Richness and species abundance

The term "Richness" refers to the species abundance per individuals; namely, the total species number divided by all sampled individuals. This data allows a direct comparison in terms of species richness, even if the number of individuals is variable between samples.

The data is always a value between 0 and 1, if all individuals in a sample were different species, it would have a value of 1; a value of 0.5 means a high diversity of species.

2.3.6 Simpson diversity index

This index measures the probability that two randomly selected individuals from a population of *N* individuals come from the same species. If a given *i* (*i* = 1,2, ..., *S*) species is represented in the community by *Pi* (Proportion of individuals), the probability of picking two random individuals from the same species, is the joint probability [(*Pi*) (*Pi*), or Pi^2].

$$\lambda = \sum pi^2$$

where:

Σ = Sum *pi* = the number of individuals of species *i* divided by the total number of individuals in the sample.

It is heavily influenced by the most dominant species (Magurran 1988). As the Simpson index (λ) reflects the degree of dominance in a community, the diversity of it can be calculated as:

$$D = \frac{1}{\lambda}$$

2.3.7 Aboveground biomass

To determine aboveground biomass from all sampled individuals the diameter (D), high (H) and wood density (ρ) were used. In general, aboveground biomass is determined by the equation:

$$\text{AGB} = F \times \rho \times \left(\frac{\pi D^2}{4}\right) \times H$$

The average of four different equations obtained from the literature, and currently used to estimate the aboveground biomass of tropical forests, was used. The average was used because two of them overestimate biomass (Chavé 2005, 2008) and two of them underestimate (Brown 1989; Nelson 1999).

The equations used in this study were:

Chavé 2005:

$$AGBest = \rho \times \text{EXP}\left(-1.499 + 2{,}148\ln(D) + 0.207(\ln(D))^2 - 0.0281(\ln(D))^3\right)$$

Brown 1989:

$$AGBest = \text{EXP}\left(-2.409 + 0{,}9522(\ln(D))^2 - \text{HT}\right)$$

Nelson 1999:

$$AGBest = \text{EXP}(-1.997 + 2{,}413\ln(D))$$

Chavé 2008:

$$AGBest = 1{,}066\ \text{EXP}(-1.864 + 2{,}608\ln(D) + \ln\rho)$$

3. Results

3.1 Quantitative Characterization

3.1.1 Pool 1

It corresponds to a terra firme area with a flat relief. The quadrant was 30 × 11 m (330 m^2) that covered the total area of the abandoned pool plus about 1 m more at each side. The vegetation is dominated by pioneer species of trees and herbs. Among the most representative tree species are *Ficus insipida* (higuerón), *Ficus* cf. *americana* (higuerón), *Hieronyma alchorneoides* (mascarey) and *Croton lechleri* (sangre de drago); while the herbaceous species are *Dimerocostus strobilaceus* (caña agria), *Carludovica palmata* (toquilla), *Heliconia* cf. *chartacea* (platanillo) and several species of Araceae.

The values of the ecological parameters calculated for families and species found in pool 1 are shown below (Table 2).

Table 2. Ecological parameters calculated for plant species found at Pool 1

FAMILY	SPECIES	Fr	BA (cm^2)	RDn	RDm	IVI
Moraceae	*Ficus insipida*	3	4786.34	7.32	37.66	44.98
Moraceae	*Ficus* cf. *americana*	6	356.99	14.63	2.81	17.44
Phyllanthaceae	*Hieronyma alchorneoides*	2	1089.27	4.88	8.57	13.45
Euphorbiaceae	*Croton lechleri*	2	954.29	4.88	7.51	12.39
Fabaceae	*Schizolobium parahyba*	1	907.46	2.44	7.14	9.58
Malvaceae	*Ceiba samauma*	1	875.71	2.44	6.89	9.33
Lauraceae	*Nectandra* cf. *membranacea*	1	854.87	2.44	6.73	9.17
Melastomataceae	*Miconia zubenetana*	2	427.71	4.88	3.37	8.24
Urticaceae	*Cecropia* cf. *ficifolia*	1	555.43	2.44	4.37	6.81
Annonaceae	*Rollinia pittieri*	1	506.45	2.44	3.98	6.42
Anacardiaceae	*Spondias mombin*	1	440.93	2.44	3.47	5.91
Euphorbiaceae	*Tetrorchidium macrophyllum*	2	105.63	4.88	0.83	5.71
Arecaceae	*Euterpe precatoria*	2	98.55	4.88	0.78	5.65
Solanaceae	*Solanum sessile*	2	86.30	4.88	0.68	5.56
Urticaceae	*Urera caracasana*	2	80.45	4.88	0.63	5.51
Sapindaceae	*Allophylus floribundus*	1	149.50	2.44	1.18	3.62
Euphorbiaceae	*Sapium glandulosum*	1	124.63	2.44	0.98	3.42
Fabaceae	*Inga* sp. 1	1	107.46	2.44	0.85	3.28
Boraginaceae	*Cordia alliodora*	1	63.59	2.44	0.50	2.94
Piperaceae	*Piper* sp. 1	1	52.78	2.44	0.42	2.85
Fabaceae	*Inga* cf. *ruiziana*	1	33.17	2.44	0.26	2.70
Rubiaceae	*Coffea arabica*	1	11.34	2.44	0.09	2.53
Hypericaceae	*Vismia* sp.	1	9.62	2.44	0.08	2.51
Rubiaceae	*Pentagonia* cf. *williamsii*	1	9.62	2.44	0.08	2.51
Achariaceae	*Neosprucea grandiflora*	1	9.07	2.44	0.07	2.51
Piperaceae	*Piper aequale*	1	8.55	2.44	0.07	2.51
Annonaceae	*Guatteria* sp.	1	3.80	2.44	0.03	2.47
TOTAL: 41 individuals, 18 families, 27 species. Basal area=12709.48 cm^2						
Simbology: Fr: Frecuency; BA: basal area; RDn: Relative density; RDm: Relative dominance; IVI: Importance Value Index						

3.1.1.1 Richness and species abundance

A total of 41 individuals of trees and shrubs with DBH ≥ 2 cm were recorded; they were classified into 18 families, 24 genera and 27 species. This result suggests a high species richness (0.65). The total basal area was 12709.48 cm^2 (127.09 m^2), due to the presence of large diameter trees, as is the case of *Ficus insipida* with 69 cm DBH.

3.1.1.2 Relative Density (RDn)

The most abundant species were *Ficus* cf. *americana* and *Ficus insipida* with 6 and 3 individuals respectively (22.5%); the remaining 77.5% of the species were represented by two and one individual (Figure 2,Table 2).

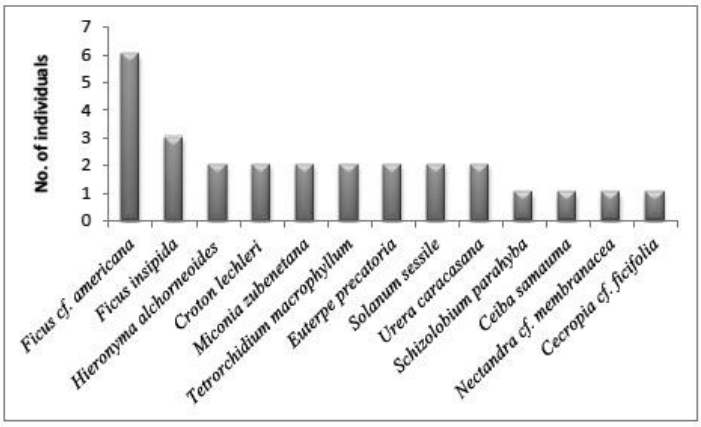


Figure 2. Relative density of species recorded at Pool 1

3.1.1.3 Relative Dominance (RDM)

Ficus insipida (Moraceae) is the most dominant species (37.66 RDM) despite its represented by three individuals, this is because one individual had a 69 cm DBH. Moreover, *Ficus* cf. *americana* with 6 individuals recorded a value of 2.81 RDM. The second dominant species was *Hieronyma alchorneoides* with 8.57 RDM (Figure 3, Table 2).

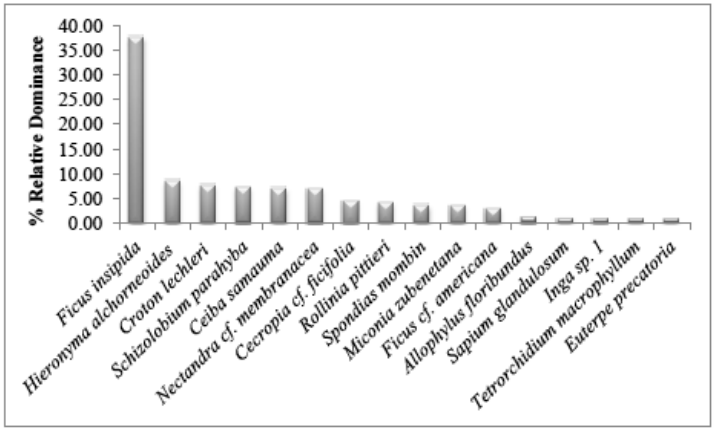


Figure 3. Relative dominance of species recorded at Pool 1

3.1.1.4 Importance Value Index (IVI)

Although *Ficus insipida* was represented by three individuals, it is the species with the highest IVI (44.98), because one of its individuals recorded 69 cm DBH. The second species was *Ficus* cf. *americana* (IVI = 17.44) followed by *Hieronyma alchorneoides* (IVI = 13.45) (Figure 4, Table 2).

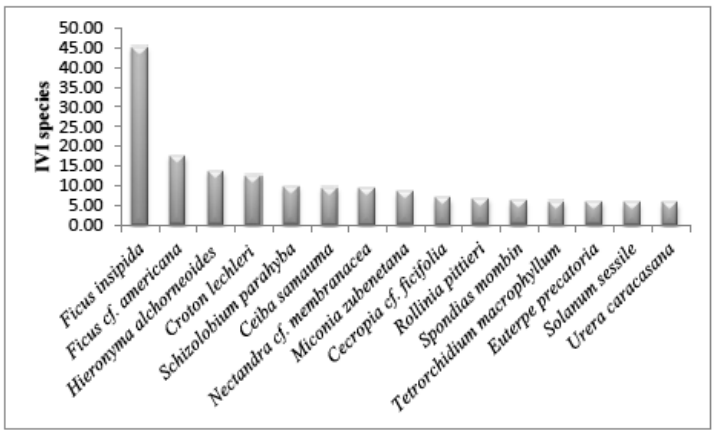


Figure 4. Importance Value Index (IVI) for species recorded at Pool 1

A total of 18 families were recorded at pool 1; from these, Moraceae with 9 individuals and 2 species (21.95% of all individuals at pool 1) was the most important (IVI = 62.42); followed by Euphorbiaceae with 5 individuals and 3 species (12.5% of all individuals at pool 1) recorded an IVI = 21.52. Fabaceae with 3 individuals and 3 species (7.31% of all individuals at pool 1) recorded an IVI = 15.56 (Figure 5).

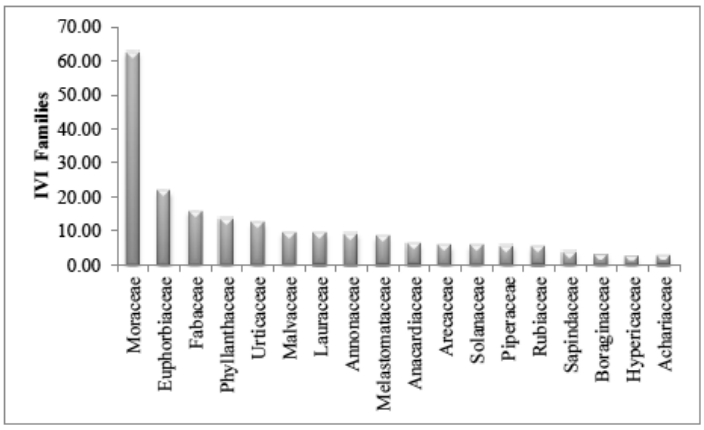


Figure 5. Importance Value Index (IVI) for families recorded at Pool 1

3.1.1.5 Simpson Diversity Index

This quadrant recorded a Simpson diversity index of 0.054; which is interpreted as a high diversity, this was because from 41 individuals registered in this area, 27 were different species.

3.1.2 Pool 2

It corresponds to a terra firme area with a flat relief. The quadrant was 25 × 18 m (450 m2) that covered the total area of the abandoned pool plus about 1 m more at each side. The vegetation is dominated by pioneer species of trees and herbs. Among the most representative tree species are *Ficus insipida* (higuerón), *Ficus* cf. *americana* (higuerón), and several species of *Miconia*; while in the herbaceous was dominated by *Dimerocostus strobilaceus* (caña agria) and several species of *Costus* (caña agria), *Carludovica palmata* (toquilla), *Heliconia* cf. *chartacea* (platanillo) and several species of Marantaceae.

The values of the ecological parameters calculated for families and species found in pool 2 are shown below (Table 3).

Table 3. Ecological parameters calculated for plant species at Pool 2

FAMILY	SPECIES	Fr	BA (cm^2)	RDn	RDm	IVI
Moraceae	*Ficus insipida*	5	8241,85	5,81	42,85	48,66
Melastomataceae	*Miconia prasina*	10	671,61	11,63	3,49	15,12
Moraceae	*Ficus* cf. *americana*	9	405,02	10,47	2,11	12,57
Euphorbiaceae	*Croton lechleri*	3	1149,01	3,49	5,97	9,46
Moraceae	*Ficus piresiana*	2	1096,06	2,33	5,70	8,02
Araliaceae	*Schefflera morototoni*	3	840,50	3,49	4,37	7,86
Fabaceae	*Inga* sp. 2	1	1237,23	1,16	6,43	7,59
Burseraceae	*Protium* cf. *nodulosum*	5	187,95	5,81	0,98	6,79
Melastomataceae	*Miconia* sp.	3	500,62	3,49	2,60	6,09
Melastomataceae	*Miconia* cf. *elata*	3	322,83	3,49	1,68	5,17
Solanaceae	*Solanum sessile*	4	84,04	4,65	0,44	5,09
Rutaceae	*Zanthoxylum* sp.	2	444,37	2,33	2,31	4,64
Arecaceae	*Euterpe precatoria*	2	443,91	2,33	2,31	4,63
Boraginaceae	*Cordia alliodora*	2	423,16	2,33	2,20	4,53
Euphorbiaceae	*Sapium glandulosum*	2	387,20	2,33	2,01	4,34
Rubiaceae	*Palicourea guianensis*	3	134,73	3,49	0,70	4,19
Verbenaceae	*Aegiphila integrifolia*	2	309,80	2,33	1,61	3,94
Malvaceae	*Ochroma pyramidale*	1	433,52	1,16	2,25	3,42
Fabaceae	*Acacia* sp.	1	397,41	1,16	2,07	3,23
Araliaceae	*Dendropanax* sp.	2	162,67	2,33	0,85	3,17
Anacardiaceae	*Tapirira* cf. *guianensis*	2	144,39	2,33	0,75	3,08
Fabaceae	*Inga edulis*	1	298,50	1,16	1,55	2,71
Euphorbiaceae	*Acalypha diversifolia*	2	47,30	2,33	0,25	2,57
Piperaceae	*Piper hispidum*	2	42,52	2,33	0,22	2,55
Bignoniaceae	*Jacaranda copaia*	1	226,87	1,16	1,18	2,34
Melastomataceae	*Miconia zubenetana*	1	200,96	1,16	1,04	2,21
Meliaceae	*Trichilia septentrionalis*	1	96,72	1,16	0,50	1,67

Moraceae	*Ficus gomelleira*	1	65,01	1,16	0,34	1,50
Myrtaceae	*Psidium guajava*	1	59,42	1,16	0,31	1,47
Fabaceae	*Senna macrophylla*	1	42,99	1,16	0,22	1,39
Euphorbiaceae	*Tetrorchidium macrophyllum*	1	40,69	1,16	0,21	1,37
Piperaceae	*Piper* sp. 1	1	26,41	1,16	0,14	1,30
Moraceae	*Clarisia biflora*	1	22,05	1,16	0,11	1,28
Annonaceae	*Guatteria* sp.	1	19,63	1,16	0,10	1,26
Phyllanthaceae	*Hieronyma alchorneoides*	1	12,56	1,16	0,07	1,23
Rubiaceae	*Psychotria stenostachya*	1	8,04	1,16	0,04	1,20
Myristicaceae	*Virola pavonis*	1	4,52	1,16	0,02	1,19
Siparunaceae	*Siparuna thecaphora*	1	3,80	1,16	0,02	1,18
TOTAL: 86 individuals, 22 families, 38 species. Basal area=19235.84 cm²						
Simbology: Fr: Frecuency; BA: Basal area; RDn: Relative Density; RDm: Relative Dominance; IVI: Importan Value Index						

3.1.2.1 Richness and species abundance

A total of 86 individuals of trees and shrubs with DBH ≥ 2 cm were recorded, they were classified into 22 families, 30 genera and 38 species. This results suggests a high species richness (0.44). The total basal area was 19235.84 cm^2 (192.35 m^2), due to the presence of large diameter trees, as is the case of *Ficus insipida* with 69.8 cm DBH.

3.1.2.2 Relative Density (RDn)

The most abundant species were *Ficus* cf. *americana* and *Miconia prasina* with 10 and 9 individuals respectively (22%); followed by *Ficus insipida* and *Protium* cf. *nodulosum* with 5 individuals; the remaining 31% of the species were represented by two one individual (Figure 6, Table 3).

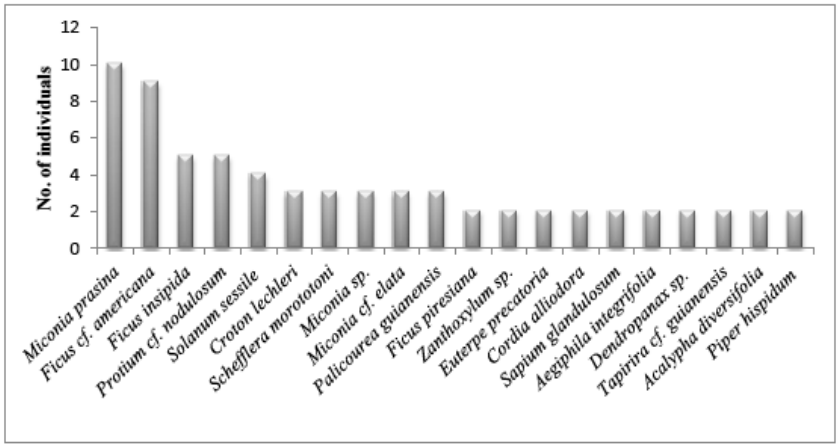


Figure 6. Relative density of species recorded at Pool 2

3.1.2.3 Relative Dominance (RDM)

Ficus insipida (Moraceae) is the most dominant species (42.85 DMR) despite its represented by five individuals, this is because two of its individuals recorded a DBH greater than 60 cm. The second dominant species was *Inga* sp. 2 with 6.43 RDM. *Croton lechleri* and *Ficus piresiana* registered a RDM of 5.97 and 5.70 respectively (Figure 7, Table 3).

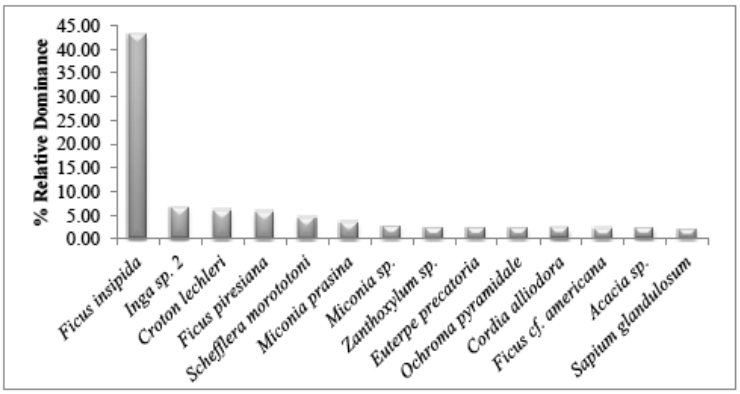


Figure 7. Relative Dominance of species recorded at Pool 2

3.1.2.4 Importance Value Index (IVI)

Although *Ficus insipida* was represented by five individuals, it is the species with the highest IVI (48.66), because two of its individuals recorded a DBH greater than 60 cm. Followed by *Miconia prasina* with an IVI = 15.12 and *Ficus* cf. *americana* with an IVI = 12.57. The remaining species were represented by an IVI less than 10 (Figure 8, Table 3).

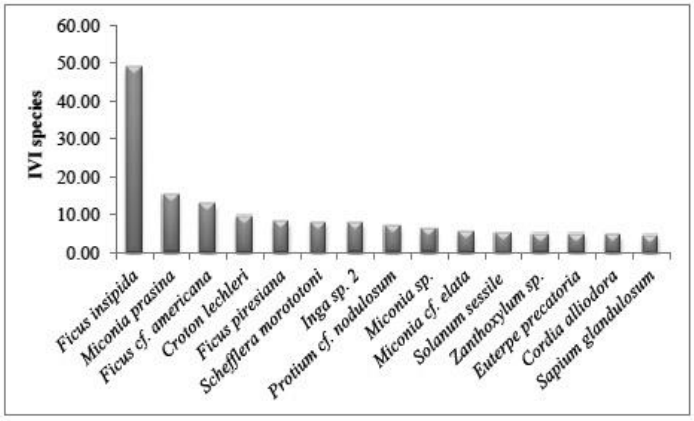


Figure 8. Importance Value Index (IVI) for species recorded at Pool 2

A total of 22 families were recorded at pool 2; from these, Moraceae with 17 individuals and 5 species (19.76% of all individuals at pool 2) was the most important (IVI = 72.03); followed by Melastomataceae with 17 individuals and 4 species (19.76% of all individuals at pool 2) recorded an IVI = 28.58. Euphorbiaceae with 8 individuals and 4 species (9.3% of all individuals at pool 2) recorded an IVI = 17.75 (Figure 9).

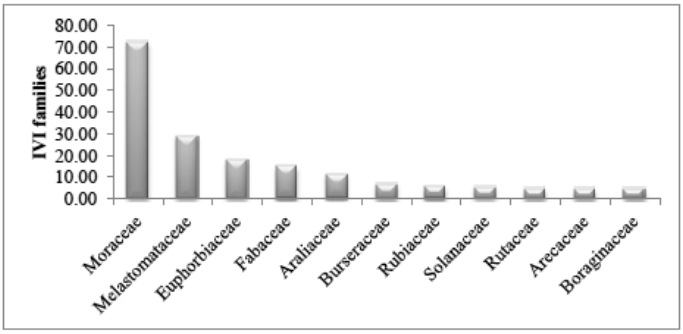


Figure 9. Importance Value Index (IVI) for families recorded at Pool 2

3.1.2.5 Simpson Diversity Index

This quadrant recorded a Simpson diversity index of 0.047; which is interpreted as a high diversity, this was because from 86 individuals registered in this area, 38 were different species.

3.1.3 Aboveground biomass

Changes in patterns of biomass storage have a significant involvement in forest ecology. Approximately, 50% of forest biomass increasing represents the carbon fixed; this percentage varies between species, and within each individual depending on the height and structure.

Total aboveground biomass for the study area was 22.64 tons. Pool 2 had greater aboveground biomass because it recorded more than 45 individuals than pool 1 (Figure 10).

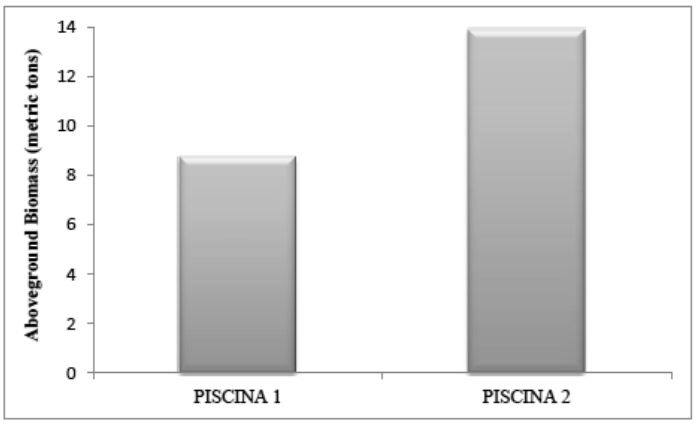


Figure 10. Total aboveground biomass for Charapa 1 well pad was 22 Tm

3.2 Qualitative Characterization

The floristic inventory at the study area recorded a total of 81 species, they were classified into 36 families and 62 genera (Appendix 1 and 2). The Fabaceae family was the most diverse with 8 species, followed by Arecaceae, Melastomataceae, Moraceae and Rubiaceae with 5 species each. Araceae, Costaceae, Piperaceae and Euphorbiaceae recorded 4 species each. At generic level, Arecaceae has 5 genera, followed by Euphorbiaceae, Fabaceae and Rubiaceae with 4 genera each, and Malvaceae and Araceae with 3 genera each(Figure 11).

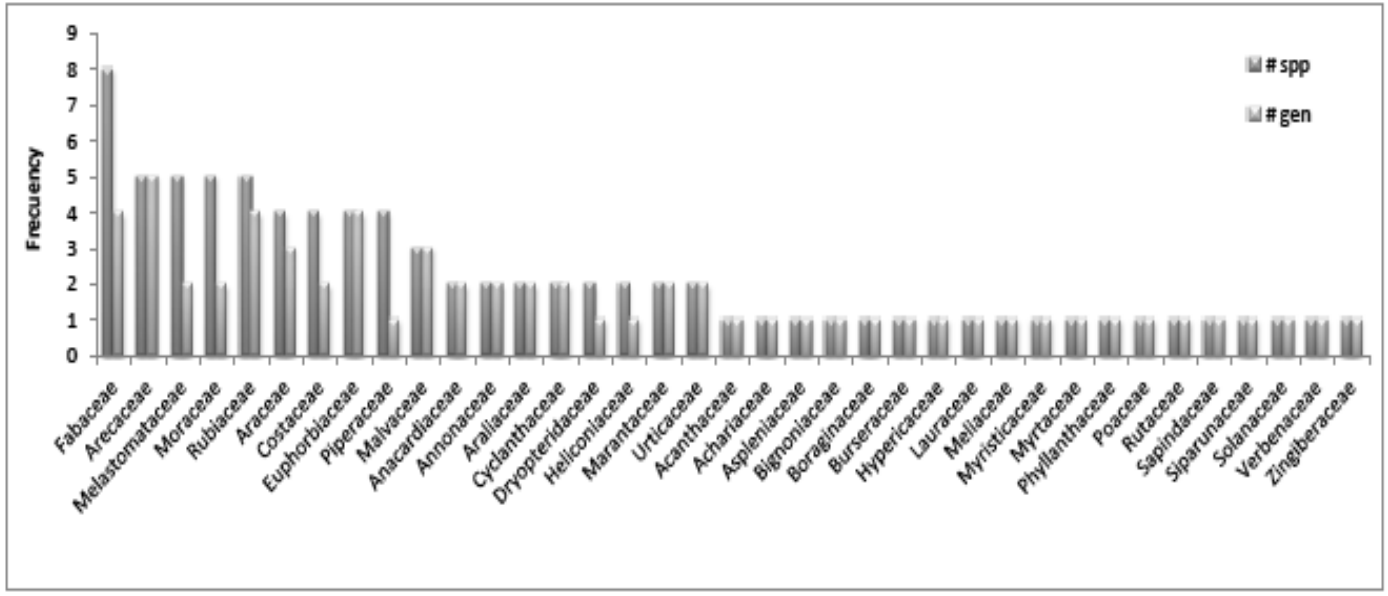


Figure 11. Species and genera diversity by families recorded by the qualitative inventory

3.2.1 Forest strata characterization and vegetation profiles

Composition and forest structure, recorded through the qualitative and quantitative inventories, found at the study area correspond to a secondary forest. The vertical stratification registered at Charapa 1 well pad consisted of three layers: the "herbaceous" layer which reaches 2.5 meters high and covered about 95% of the area, the most abundant species were *Dimerocostus strobilaceus*, *Costus lima* var. *scabremarginatus*, *Costus scaber* and *Costus pulverulentus*, commonly known as caña agria, *Carludovica palmata* (toquilla), *Heliconia* cf. *chartacea* (platanillo), *Renealmia* cf. *thyrsoidea* and several species of Araceae and Marantaceae; the "subarboreous" layer reaches up to 12 m high and covered about 70%of the area, the most frequent species are *Ficus* cf. *americana* (higuerón), *Palicourea guianensis* (amarilla), *Psychotria stenostachya*, *Euterpe precatoria* (palma) and *Urera caracasana* (ortiga);the tree layer ranges from 15 to 30 m high and covered about 80% of the area, the most frequent species were *Ficus insipida* (higuerón), *Nectandra* cf. *membranacea* (canelo), *Croton lechleri* (sangre de drago) and *Hieronyma alchorneoides* (mascarey). Around the study area the land is used for cattle, pasture and cultivation of traditional species (yuca, banano, cacao) (Figure 12 and 13).

Figure 12. Herbaceous layer (2.5 m) dominated by *Carludovica palmata* (toquilla)

Figure 13. External view of Pool 2 showing the emergent trees (*Ficus insipida* and *Ficus piresiana*)

4. Conclusion and Discussion

The study area was dominated by secondary forest, and its vertical structure was composed by three vertical layers (herbaceous, subarboreus and trees). Light-tolerant species and fast growing species were the dominant species.

Alpha diversity of Charapa 1 well pad was relatively high (Pool 1 = 0.054, Pool 2 = 0.047), this is because more than 60% of the species were represented by one or two individuals; however, its necessary remember that the vegetation at the study area is dominated by secondary forest.

Comparison between ecological parameter from pool 1 and 2 is inadequate because they have different sized areas. However, it is evident that the herbaceous layer dominate the study area, and that *Ficus insipida*, despite growing up in a highly polluted environment, recorded higher DBH and height (698 cm and 35 m respectively), similar to those recorded at matured forests.

It's important to identify the species that grow at the study area, because they can be used in bioremediation, reforestation and restoration plans for polluted areas.

Literature cited

- Campbell, D., Daly, D., Prance, G. and U. Maciel. 1986. Quantitative Ecological Inventory of Terra firme and Varzea Tropical Forest on the Rio Xingu, Brazilian Amazon. *Brittonia* 38(4) 369-393.
- Brown, S., Gillespie, A.J.R. y A.E. Lugo. 1989. Biomass Estimation methods for tropical forest with applications to forest inventory data. *Forest Scince* 35 (4) pp: 881-902.
- Chave, J., Andalo, C., Brown, S., Cirus, M.A., Chambers, J.Q., Eamus, D., Folster, H., Fromard, F., Higuchi, N., Kira, T., Lescure, J.P., Nelson, B.W., Ogawa, H., Puig, H., Riera B. y T. Yamakura. 2005. Tree allometry and improved estimation of carbón stocks and balance in tropical forests. *Oecologia* 145: 87–99.
- Chave, J., Condit, R., Muller-Landau, H.C., Thomas, S.C., Ashton, P.S., Bunyavejchewin, S., Co, L.L., Dattaraja, H.S., Davies, S.J., Esufali, S., Ewango, C.E.N., Feeley, K.J., Foster, R.B., Gunatilleke, N., Gunatilleke, S., Hall, P., Hart, T.B., Hernández, C., Hubbell, S.P., Itoh, A., Kiratiprayoon, S., LaFrankie, J.V., Lao. S.L., Makana, J., Noor, N.S., Kassim, A.R., Samper, C., Sukumar, R., Suresh, H.S., Tan, S., Thompson, J., Tongco, M.D.C., Valencia, R., Vallejo, M., Villa, G., Yamakura, T., Zimmerman, J.K. y E.C. Losos. 2008. Assessing Evidence for a Pervasive Alteration in Tropical Tree Communities. *PLoS Biology* 6 (3): 455 – 462.
- Guevara, J., Pitman, N., Mogollón, H., Cerón, C., Palacios, W. 2013. pg: 178-180 en: Ministerio del Ambiente del Ecuador 2012. Sistema de Clasificación de los Ecosistemas del Ecuador Continental. Ministerio del Ambiente del Ecuador. Quito.
- Jorgensen, P.M. & S. León-Yanez. 1999. Catálogo de Plantas Vasculares del Ecuador. Missouri Botanical Garden. San Louis.
- Magurran A. 1988. Diversidad Ecológica y su Medición. Ediciones Vedra S.A.

- The New York Botanical Garden. 2007. Disponible en: http://sciweb.nybg.org/science2/vii2.asp.
- Nelson, B.W., Mesquita, R., Pereira, J.L.G, Aquino de Souzac, S.G., Teixeira Batistad, G. y L. Bovino Coutoe. 1999. Allometric regressions for improved estimate of secondary forest biomass in the central Amazon. *Forest Ecology and Management,* 117(1–3): pp 149–167.
- Palacios, W., Cerón, C., Valencia, R. y R. Sierra. 1999. Las Formaciones Naturales de la Amazonía del Ecuador. En: Sierra, R. (Ed.). 1999. Propuesta Preliminar de un Sistema de Clasificación de Vegetación para el Ecuador Continental. Proyecto INEFAN/GEF-BIRF y EcoCiencia. Quito, Ecuador. Pp. 114-116.
- Royal Botanic Gardens, Kew. 2012. Disponible en: http://apps.kew.org/herbcat/navigator.do.
- Tropicos.org. Missouri Botanical Garden. 14 Nov 2012, Disponible en: http://www.tropicos.org.
- Valencia, R., Foster, R. Villa, G., Condit, R., Svenning, C. J., Hernández, C., Romoleroux, K., Lossos, E., Margaard, E. & Balslev, H. 2004. Tree species distribution and local habitat variation in the Amazon: a large forest plot in eastern Ecuador. *Journal of Ecology* 92:214-229.

Appendix 1. Species list recorded at Charapa 1 well pad

FAMILY	SPECIES	COMMON NAME
Acanthaceae	*Mendoncia* sp.	
Achariaceae	*Neosprucea grandiflora* (Spruce ex Benth.) Sleumer	
Anacardiaceae	*Spondias mombin* L.	hobo
	Tapirira cf. *guianensis* Aubl.	
Annonaceae	*Rollinia pittieri* Saff.	chirimoyo
	Guatteria sp.	
Aspleniaceae	*Asplenium* sp. 1	helecho
Araceae	*Anthurium* sp. 1	
	Philodendron sp. 1	
	Philodendron sp. 2	
Araliaceae	*Xanthosoma* sp. 1	camacho
	Dendropanax sp.	
	Schefflera morototoni (Aubl.) Maguire, Steyerm. & Frodin	palo de fósforo
Arecaceae	*Astrocaryum urostachys* Burret	palma
	Euterpe precatoria Mart.	palma
	Geonoma cf. *deversa* (Poit.) Kunth	palma
	Iriartea deltoidea Ruiz & Pav.	palma
	Socratea exorrhiza (Mart.) H. Wendl.	bombon
Bignoniaceae	*Jacaranda copaia* (Aubl.) D. Don	arabisco
Boraginaceae	*Cordia alliodora* (Ruiz & Pav.) Oken	laurel
Burseraceae	*Protium* cf. *nodulosum* Swart	copal
Costaceae	*Costus lima* var. *scabremarginatus* Maas	caña agria morada
	Costus pulverulentus C. Presl	caña agria
	Costus scaber Ruiz & Pav.	caña agria
	Dimerocostus strobilaceus Kuntze	caña agria
Cyclanthaceae	*Carludovica palmata* Ruiz & Pav	paja toquilla
	Cyclanthus bipartitus Poit. ex A. Rich.	oreja de conejo
Dryopteridaceae	*Polybotrya* sp. 1	helecho
	Polybotrya sp. 2	helecho

Euphorbiaceae	*Acalypha diversifolia* Jacq.	pata de venado
	Croton lechleri Müll. Arg.	sangre de drago
	Sapium glandulosum (L.) Morong	lechero
	Tetrorchidium macrophyllum Müll. Arg.	
Fabaceae	*Acacia* sp.	guarango blanco
	Inga capitata Desv.	guabo
	Inga cf. *ruiziana* G. Don	guabilla
	Inga edulis Mart.	guaba de bejuco
	Inga sp. 1	guabilla
	Inga sp. 2	guabo porotillo
	Schizolobium parahyba (Vell.) S.F. Blake	guarango
	Senna macrophylla (Kunth) H.S. Irwin & Barneby	
Heliconiaceae	*Heliconia* cf. *chartacea* Lane ex Barreiros	platanillo
	Heliconia sp. 1	platanillo
Hypericaceae	*Vismia* sp.	
Lauraceae	*Nectandra* cf. *membranacea* (Sw.) Griseb.	canelo
Malvaceae	*Ceiba samauma* (Mart.) K. Schum.	ceibo
	Ochroma pyramidale (Cav. ex Lam.) Urb.	boya
	Theobroma cacao L.	cacao
Marantaceae	*Calathea marantina* (Willd. ex Körn.) K. Koch	bijao
	Monotagma sp. 1	bijao
Melastomataceae	*Miconia* cf. *elata* (Sw.) DC.	
	Miconia prasina (Sw.) DC.	
	Miconia sp.	
	Miconia zubenetana J.F. Macbr.	
	Tococa guianensis Aubl.	
Meliaceae	*Trichilia septentrionalis* C. DC.	
Moraceae	*Clarisia biflora* Ruiz & Pav.	moral
	Ficus cf. *americana* Aubl.	lechero
	Ficus gomelleira Kunth & C.D. Bouché	matapalo
	Ficus insipida Willd.	higuerón
	Ficus piresiana Vázq. Avila & C.C. Berg	higuerón

Myristicaceae	*Virola pavonis* (A. DC.) A.C. Sm.	coco
Myrtaceae	*Psidium guajava* L.	guayaba
Phyllanthaceae	*Hieronyma alchorneoides* Allemão	mascarey
Piperaceae	*Piper aequale* Vahl	
	Piper hispidum Sw.	
	Piper sp. 1	
	Piper sp. 2	
Poaceae	*Olyra latifolia* L.	bambu
Rubiaceae	*Coffea arabica* L.	café
	Palicourea guianensis Aubl.	amarilla
	Pentagonia cf. *williamsii* Standl.	
	Psychotria stenostachya Standl.	
	Psychotria caerulea Ruiz & Pav.	
Rutaceae	*Zanthoxylum* sp.	
Sapindaceae	*Allophylus floribundus* (Poepp.) Radlk.	
Siparunaceae	*Siparuna thecaphora* (Poepp. & Endl.) A. DC.	guayusa de monte
Solanaceae	*Solanum sessile* Ruiz & Pav.	palo de agua
Urticaceae	*Cecropia* cf. *ficifolia* Warb. ex Snethl.	guarumo
	Urera caracasana (Jacq.) Gaudich. ex Griseb.	ortiga
Verbenaceae	*Aegiphila integrifolia* (Jacq.) B.D. Jacks.	
Zingiberaceae	*Renealmia* cf. *thyrsoidea* (Ruiz & Pav.) Poepp. & Endl.	platanillo

Appendix 2. Common species registered at Charapa 1 well pad

Costaceae - *Dimerocostus strobilaceus* Kuntze - caña agria

Costaceae - *Costus lima* var. *scabremarginatus* Maas -caña agria morada

Marantaceae - *Calathea marantina* (Willd. ex Körn.) K. Koch - bijao

Arecaceae - *Euterpe precatoria* Mart. - palma (plántulas)