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Total Petroleum Hydrocarbon Degradation by Endophytic Fungi from the Ecuadorian Amazon

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Abstract

The capacity of 133 fungal endophyte isolates for degrading petroleum hydrocarbons was evaluated. The endophytes were isolated from leaf and stem tissues from 23 plants collected in a natural habitat contaminated with crude oil in southwestern Ecuador. Their capacity for hydrocarbon biodegradation was tested by an *in vitro* colorimetric qualitative test during 10 days, using the Minimal Salt Medium and crude oil as the carbon source. Taxonomic identification of the endophytic fungi that showed bioactivity in the qualitative test was carried out by analysis of the ITS gene of the region 18S of the rDNA. Endophytes showed the best results in the previous qualitative test where selected for a quantitative *in vitro* test for 30 days. Residual hydrocarbons were tracked by infrared spectroscopy (IR) and gas chromatography (GC) with a flame ionization detector. The maximum removal rates of total petroleum hydrocarbons were 99.6% (IR) and 99.8% (GC), corresponding to fungi of the genus *Verticillium* sp. and *Xylaria* sp. 1 respectively. This is the first report of biodegradation of crude oil hydrocarbons by endophytic fungi in a tropical ecosystem. The results suggest these fungal isolates are potential hydrocarbon biodegraders that could be used in bioremediation processes.

Keywords

Fungal Endophyte, Biodegradation, Bioremediation, Hydrocarbon, Infrared Spectroscopy, Gas Chromatography

1. Introduction

Petroleum is a non-renewable resource of economic importance for several countries [1], including Ecuador. However, crude oil exploitation is a highly contaminant industry. Water and soil pollution might occur in any stage of production. In a highly biodiverse country, as Ecuador, the impact on habitats

for local flora and fauna is concerning, in addition to the effects on people's health [2] [3].

Petroleum hydrocarbons (PHs) are categorized into four broad classes of chemical compounds, namely, the aliphatic, aromatic, resin-based, and asphaltene-based hydrocarbons. Each group possesses different physicochemical characteristics and susceptibility to degradation [4] [5].

All hydrocarbon compounds derived from petroleum sources are generally described as total petroleum hydrocarbons (TPHs) and are categorized as aromatic, aliphatic, resin-based and asphaltene-based hydrocarbons. Aliphatic hydrocarbons correspond to linear or cyclic hydrocarbon chains of variable size and structure. It is widely studied for their uses as fuels and solvents. Aromatic hydrocarbons have a variable structure, size and complexity and range from monocyclic structure compounds to compounds formed by long and complex chains, constituted by aromatic rings [6]. Polycyclic aromatic hydrocarbons have been mostly studied, because some of them constitute a wide variety of products of biological, chemical and industrial importance [6]. However, they are also among the pollutants with the greatest biological impact due to their carcinogenic and mutagenic effects [7] [8] [9]. Degradation of these different compounds is highly related to their physical chemical characteristics [10] [11] [12].

Microbial groups, such as bacteria, yeast, and fungi have been identified as principal agents in the degradation of PHs, even though their degradation efficiencies are varying. However, bacteria are the most active and primary degraders of spilled oil in the environment [13]. Remediation techniques have been developed using microorganisms, such as bacteria, yeast and fungi, with varying degradation efficiencies [14]. It is generally suggested that these microorganisms have the metabolic capacity to use hydrocarbons as a carbon source [15]. Also phytoremediation is considered as cost-efficient and eco-friendly and can be implemented after an initial degradation either by bioremediation or physical, chemical and thermal processes.

Bioremediation of petroleum hydrocarbons using native bacterial and fungal isolates from the Ecuadorian soil microbiome has been previously reported [14]. Generally, bioremediation techniques rely on bacteria given their rapid reproduction cycles, their voracity and the facilities for the study of their metabolic pathways [16] [17]. However, recently various studies have reported fungal organisms that exceed bacterial capacities in hydrocarbon biodegradation [18]. Fungi are used due to their particular metabolic capacities, which mediate the transformation of a wide variety of organic compounds to less complex compounds that are later incorporated into their metabolisms [19] [20]; these processes are facilitated due to the characteristics of their complex enzymatic system and their vigorous hyphae growth [15] [19] [21]. Thus, they offer an undeniable potential for their use in bioremediation processes, accordingly, the study of the diversity and identification of species and fungal strains of biotechnological utility should be a priority in an avid field of constant innovation such

as environmental bioremediation.

In recent years, endophytic fungi have generated major attention in the scientific community due to their wide diversity and particular metabolic capacities [22]. These microorganisms colonize plant tissues, without causing damage or apparent symptoms of disease in their host [13] [23] [24]. They are located in the intercellular spaces and the symplast [25]. Plant and fungi coexist, establishing between them a mutualistic interaction, dependent on the virulence of the fungus and the defenses of the plant. Both elements are influenced by environmental factors and the developmental stage of each organism [24] [26] [27].

Plant-endophyte interaction, be it with bacterial or fungal endophytes, generally improve plant adaptation to the environment by increasing tolerance to biotic and abiotic stress, changes in temperature and salinity, development of resistance to diseases, herbivory, insects, nematodes, bacteria and other pathogenic fungi [28]. The development of resistance to normally lethal or highly hazardous pollutants, such as crude oil, occurs when plants are able to metabolize harmful compounds by extracellular enzymes secreted by endophytic fungi, this process could be developed inside the plant or at soil level by enzymatic secretion through the root [29].

In Ecuador, main oil reservoirs are found in the Cretaceous Hollin and Napo geological formations in the Oriente basin. The Oriente basin, which covers about 100,000 km^2, lies between the Andes on the west and the Guyana shield on the east. The basin extends northward into the Putumayo basin in Colombia, and southward into the Maranon basin in Peru [30]. The principal reservoir, the Lower Cretaceous Hollin Formation is characterized by structurally controlled oil accumulations found in the Cretaceous sandstones of this formation. Geochemical analyses indicate that the oil migrated into these structures from Cretaceous source rocks in the eastern Cordillera and southernmost Oriente basin [31]. The oil is trapped in structures of Cretaceous-Oligocene age [32]. On the Hollin-Loreto roadway that connects Ecuador's capital city, Quito, to the Ecuadorian Amazon basin, outcrops can be observed with these oil accumulations. Various plant species have colonized, adapted and proliferated successfully in habitats contaminated with petroleum [33], with this consideration in mind, the vegetation found on the outcrops on the Hollin-Loreto roadway most probably exhibit similar adaptive features.

Therefore, the exploration and identification of the endophytic fungal diversity these plants harbor could lead to the discovery of fungi with metabolic capacities to biodegrade petroleum hydrocarbons by using these as a carbon source, facilitating the adaptation and host plants survival to adverse conditions. These findings could contribute to the innovation and development of new technologies for environmental bioremediation.

This study is primarily aimed to describe plant fungal endophytic diversity of plant communities in a tropical lowland ecosystem exhibiting long term petroleum contamination, as well as to evaluate petroleum hydrocarbon biodegrada-

tion capacities of the endophytic fungi isolated from plants adapted to crude oil contaminated habitats.

2. Methods

2.1. Sampling Site and Plant Collection

The collection site is located at the locality "Las Minas" Km 10.1 roadway Hollín-Loreto, Napo Province, Ecuador (0˚42'39.63"S, 77˚44'33.13"W). The zone is contaminated with crude oil from natural springs. Botanical samples for taxonomic identification were collected from plants growing on oil outcrops. Leaves and stems samples were collected in zip-lock bags from each plant for endophytic fungi isolation.

2.2. Endophytic Fungi Isolation from Plant Tissues

Stem samples were surface sterilized and segments of each stem tissue were removed by longitudinal cuts and incubated in Petri dishes with Water Agar (WA) and Potato Dextrose Agar (PDA 1:10), at room temperature [34]. Leaf samples were sterilized, then cut into small quadrangular fragments (0.5 mm^2) and incubated in Petri dishes with Malt Extract Agar (MEA 1:10) at room temperature [27]. Each fungus isolated from stem and leaf tissues were transferred to Petri dishes with the 1X PDA by the terminal hyphae method [34], where it remained in culture for seven days.

2.3. Qualitative Oil Degradation Essays by Endophytic Fungi

To determine hydrocarbon degradation bioactivity by the endophytic fungal isolates colorimetric tests were carried out on 125 ml Erlenmeyer flasks, with 50 ml Minimum Salts Medium (MSM) enriched with 1% crude oil [18] [35] as a carbon source, Tween®80 as surfactant and Dichlorophenol-indophenol (DCFIF) as metabolic activity indicator [18]. Three agar plugs (1 cm^2) with individual fungi from axenic cultures of seven days growth were added to the culture medium. This assay was incubated at room temperature, with continuous agitation at 180 rpm during the entire experimental time [18]. The reduction of culture medium, hence usage of petroleum hydrocarbons, were evidenced by gradual color change of the medium over a 10 day period, from deep blue to colorless, these results were tabulated estimating the degree of clarification of the culture media [18]. Additionally, a negative control was carried out using a non-petroleum tolerant fungus. All essays were carried out in triplicate.

2.4. Quantitative Degradation Essays and Total Petroleum Hydrocarbon Evaluation by Infrared Spectroscopy and Gas Chromatography

The endophytic fungi that showed the highest levels of clarification in the qualitative essays were selected for these essays. Three endophyte fungus plugs (1 cm^2) from axenic cultures of seven days growth were added to 125 ml Erlen-

meyer flasks with 50 ml of MSM enriched with 1% crude oil and Tween®80 [35]. The assay was incubated at room temperature with continuous agitation at 180 rpm during the entire assay. The amount of persistent hydrocarbon in the medium was evaluated after 30 days incubation [35]. Additionally, a negative control without endophyte was carried out. All the essays were carried out in triplicate. To determine hydrocarbon removal rate, residual petroleum oil were extracted from the culture medium and subjected to a Total Petroleum Hydrocarbon (TPH) analysis using infrared (IR) spectroscopy at PUCE's Center for Environmental and Chemical Services (CESAQ-PUCE).

Five endophytes with the best results by IR quantification were selected for evaluation of hydrocarbon degradation capacity by gas chromatography (GC). This new hydrocarbon degradation test was carried out in the same conditions as described [35]. Once the experiment concluded, the mycelium was removed from the liquid phase by filtration. A liquid-liquid ultrasound assisted extraction was performed with the liquid phase, with 6 ml of dichloromethane (DCM) and 5 ml of culture medium, sonicated with a Branson®3800 water bath for three periods of 5 min each. The organic phase was recovered after the mixture decantation with two additional portions of DCM [36]. The recovered phase was dried with anhydrous sodium sulfate, concentrated with a rotary evaporator and the resulting volume was adjusted to 2 ml with DCM. The final extracts were analyzed by gas chromatography (GC) (Agilent 7890A gas chromatograph), with flame ionization detector (FID), Agilent DB-TPH capillary column (30 m × 320 μm × 0.25 μm). The injector and detector temperatures were 250˚C and 320˚C, respectively. The programmed parameters were 40˚C for 1 min, a ramp from 40˚C to 220˚C with increments of 8˚C/min. The final temperature of 220˚C was maintained for 1 min. The control sample was prepared with 2 ml of DCM.

2.5. Taxonomic Identification of Endophytic Fungi

The taxonomic identity of the endophytes classified as degraders in the qualitative assays was determined by sequence analysis of the internal transcribed spacer (ITS) region of the 5.8S gene of ribosomal DNA (rDNA). Thus, DNA extractions were performed with Chelex® 100 in accordance with previously reported procedures [37] [38]. Subsequently, the extracted DNA was amplified using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [39]. The Polymerase chain reaction (PCR) was performed in a 50 μl mixture containing 10 μl of Green GoTaq® Flexi Buffer 1X, 5 μl of $MgCl_2$ (25 mM), 1 μl of dNTPs (10 mM), 2.5 μl of each primer (ITS1 and ITS4) (30 μM), 5 ηg/μl of the template, 0.5 μl of Taq polymerase (5 units/μl) and 23.5 μl of Milli-Q® autoclaved water. PCR amplifications were performed using the following protocol: 1 min initial denaturation at 95˚C; 30 cycles of 1 min denaturation at 95˚C, 30 sec primer annealing at 55˚C and 1 min extension at 72˚C; 5 min extension at 72˚C and a final holding at 4˚C [40], the resulting amplicons were sequenced at Macrogen® (Seoul, South Korea).

For phylogenetic identification of endophytic fungi, the consensus sequences were analyzed by comparing them with other ITS sequences from the National Center for Biotechnology Information (NCBI GenBank®) database using the Basic Local Alignment Sequence Tool fornucleotide (BLASTn) search. For taxonomic identification, the highest homology sequences obtained by the BLASTn search were used in alignment with the consensus sequences using the bioinformatic software Muscle (Multiple sequence alignment by log-expectation) [41]. Finally, a phylogenetic tree of maximum likelihood was constructed with 130 sequences from the isolated fungi, using the bioinformatic program RAxML (Randomized Axelerated Maximum Likelihood) [42] applying the nucleotide substitution model GTR ɣ (General Time Reversible) and 1000 bootstrap replicates [43]; *Spizellomyces punctatus* (Chytridiomycota) was used as external group.

2.6. Statistical Analysis

A natural-logarithm transformation was used to normalize the TPH (ppm) quantification data distribution, corroborated with a Shapiro-Wilk test ($p < 0.05$). Subsequently, an analysis of variance (ANOVA) was performed with the IR TPH quantification data and a Tukey multiple comparison test was performed ($p < 0.05$). The data analyses were conducted using the Statistical Package for the Social Sciences software (SPSS, version 22).

3. Results

3.1. Plant Sample Identification

A total of 23 plants were collected at "Las Minas" sit developing on rocky substrates exhibiting crude oil or on mud (mixture of crude oil, earth and water). The 23 plants were classified in 12 angiosperm families, 1 gymnosperm and 1 bryophyte (**Table 1**). The botanical samples were taxonomically identified by the Pontificia Universidad Católica del Ecuador Herbarium (HQCA) staff. Among the angiosperms, the families Melastomataceae, Boraginaceae and Poaceae are predominant in the area, reaching together 70% of the vegetation present, while the rest of vegetation is represented by the families Rubiaceae, Gunneraceae and Urticaceae, among others (**Table 1**). Two botanical samples were not identified due to the limited material collected; in both cases the plants were unique individuals and exhibited reduced size.

3.2. Endophytic Fungi Isolation

A total of 156 endophytic fungi were isolated from stem and leaf samples obtained from the 23 plant specimens collected; these 156 isolates were grouped into 133 morphotypes.

3.3. Qualitative Oil Degradation Essays by Endophytic Fungi

The qualitative tests showed several endophytic isolates with crude oil degrading

Table 1. Taxonomic identification of the collected plants and number of endophytes isolated per plant.

Plant ID	Family	Genus	Species	Endophytes isolated
FM001	MELASTOMATACEAE	-	-	7
FM002	GLEICHENIACEAE	*Gleichenia*	sp.	5
FM003	MARATTIACEAE	*Marattia*	sp.	12
FM004	POLYPODIACEAE	*Polipodium*	sp.	9
FM005	ORCHIDACEAE	-	-	2
FM006	Unidentified	-	-	10
FM007	LYCOPODIACEAE	*Lycopodium*	sp.	5
FM008	BORAGINACEAE	-	-	9
FM009	MELASTOMATACEAE	*Conostegia*	sp.	5
FM010	CYPERACEAE	*Carex*	sp.	2
FM011	POACEAE	-	-	6
FM012	POACEAE	-	-	2
FM013	BRYOPHYTA	-	-	4
FM014	BORAGINACEAE	-	-	4
FM015	MELASTOMATACEAE	*Miconia*	*crassa*	4
FM016	GUNNERACEAE	-	-	8
FM017	Unidentified	-	-	3
FM018	EUPHORBIACEAE	*Croton*	sp.	9
FM019	URTICACEAE	*Cecropia*	*maxima*	4
FM020	POACEAE	-	-	6
FM021	BIGNONIACEAE	*Jacaranda*	sp.	5
FM022	RUBIACEAE	-	-	8
FM023	POACEAE	-	-	4

capacities (40% of the isolates) while the remaining fungi none showed any growth in the culture media (60% of the isolates). The parameter evaluated was the culture medium clearance degree, according to visual observation (Figure 1). In these essays, 10 fungi completely cleared the medium before the 10 days established to conclude the test. The remaining fungi showed partial or null changes (Table 2).

3.4. Quantitative Essays and Total Petroleum Hydrocarbon Determination

The fungi with the best qualitative assay performance (10 endophytes) were subjected to a test that allows quantification of petroleum hydrocarbons in the culture medium at the beginning and at the end of the essay. Among the fungi with better performance are *Clonostachys* sp. 2, *Aspergillus* sp., *Verticillium* sp.,

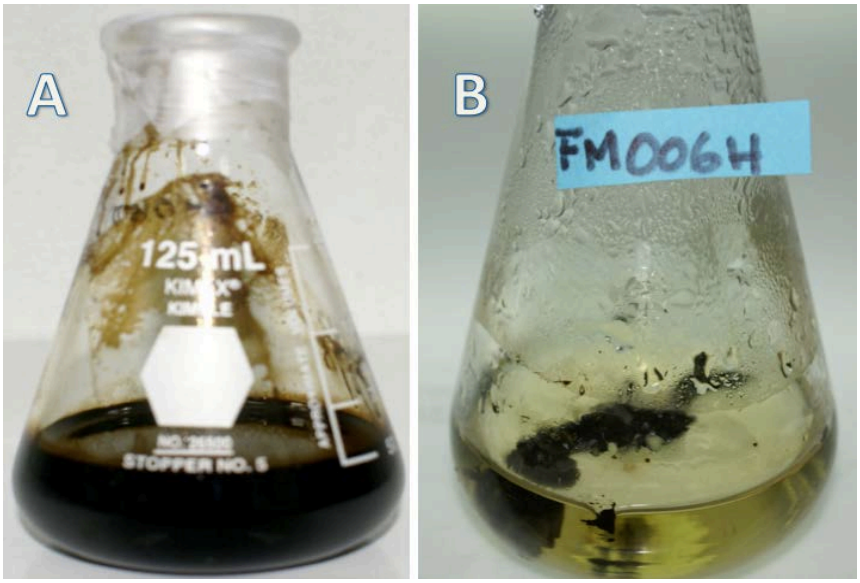


Figure 1. Qualitative test of hydrocarbon biodegradation. (A) Negative control: where the initial appearance of the culture medium product of crude oil is observed (B) Positive result: appearance of the culture medium upon the biodegradation of hydrocarbons by an endophyte fungus, in this case the resulting degradation with *Xylaria* sp. 8 is observed, observing a total clarification of the culture medium after 10 days of testing.

Table 2. Positive results of endophytic fungi for hydrocarbon qualitative biodegradation essays. Clarification level: mild (+), medium (++), total (+++).

No.	CEQCA code	Result	Clarification level	Time
1	CEQCA-N4992	Positive partial	+	10 days
2	CEQCA-N5028	Positive partial	++	10 days
3	CEQCA-N5097	Positive partial	++	10 days
4	CEQCA-N5096	Positive	+++	7 days
5	CEQCA-N5011	Positive	+++	5 days
6	CEQCA-N5012	Positive	+++	8 days
7	CEQCA-N5071	Positive	+++	5 days
8	CEQCA-N5005	Positive partial	+	10 days
9	CEQCA-N5090	Positive partial	+	10 days
10	CEQCA-N5091	Positive partial	++	10 days
11	CEQCA-N5006	Positive	+++	4 days
12	CEQCA-N5007	Positive	+++	7 days
13	CEQCA-N5058	Positive partial	++	10 days
14	CEQCA-N5107	Positive	+++	4 days
15	CEQCA-N5122	Positive partial	+	10 days
16	CEQCA-N5034	Positive partial	++	10 days
17	CEQCA-N5035	Positive partial	+	10 days
18	CEQCA-N5036	Positive partial	+	10 days

Continued

19	CEQCA-N5037	Positive partial	++	10 days
20	CEQCA-N5103	Positive partial	++	10 days
21	CEQCA-N5074	Positive partial	++	10 days
22	CEQCA-N5033	Positive partial	+	10 days
23	CEQCA-N5102	Positive	+++	6 days
24	CEQCA-N5087	Positive partial	+	10 days
25	CEQCA-N5088	Positive partial	++	10 days
26	CEQCA-N5050	Positive partial	+	10 days
27	CEQCA-N5051	Positive partial	+	10 days
28	CEQCA-N5060	Positive	+++	8 days
29	CEQCA-N5057	Positive partial	++	10 days
30	CEQCA-N5101	Positive partial	+	10 days
31	CEQCA-N5043	Positive	+++	5 days
32	CEQCA-N5044	Positive partial	+	10 days
33	CEQCA-N5045	Positive partial	+	10 days
34	CEQCA-N5081	Positive partial	++	10 days
35	CEQCA-N5076	Positive partial	++	10 days
36	CEQCA-N5077	Positive partial	+	10 days
37	CEQCA-N5063	Positive partial	+	10 days
38	CEQCA-N5105	Positive partial	++	10 days
39	CEQCA-N5120	Positive partial	+	10 days
40	CEQCA-N5117	Positive partial	+	10 days
41	CEQCA-N5098	Positive	+++	8 days
42	CEQCA-N5042	Positive partial	+	10 days
43	CEQCA-N5049	Positive partial	+	10 days
44	CEQCA-N5093	Positive partial	++	10 days
45	CEQCA-N5094	Positive partial	++	10 days
46	CEQCA-N5040	Positive partial	+	10 days
47	CEQCA-N5082	Positive partial	++	10 days
48	CEQCA-N5027	Positive partial	+	10 days
49	CEQCA-N5031	Positive partial	++	10 days
50	CEQCA-N4995	Positive partial	+	10 days
51	CEQCA-N5021	Positive partial	++	10 days
52	CEQCA-N5115	Positive partial	++	10 days
53	CEQCA-N5118	Positive partial	+	10 days

Colletotrichum sp. 1, *Phomopsis* sp. 1 and *Xylaria* sp. 1, all of them with a hydrocarbon removal rate average higher than 90% at the end of the essay. How-

ever, individuals with lower degrading capacities such as *Clonostachys* sp. 1, *Colletotrichum* sp. 2, *Colletotrichum* sp. 3 and *Saccharicola* sp. 1, with degradation levels higher than 31% (Table 3). The TPH results obtained previously by IR quantification were corroborated by a new TPH analysis quantified by GC and carried out only with the fungi that showed the best results (5 endophytes) (Table 3).

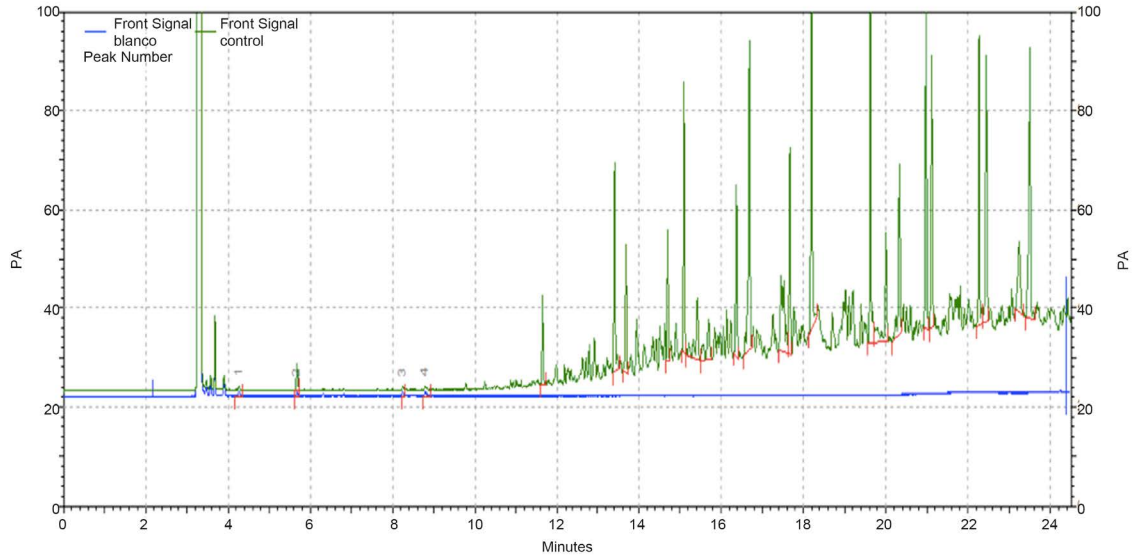
The TPH evaluation by GC allowed the identification of aliphatic compounds (octane, nonane, undecane, dodecane and tetradecane) and aromatics (1,2,3-trimethylbenzene, trans-decahydro naphthalene, 1,2,4,5-tetramethylbenzene and pentamethylbenzene) in the control samples. While the culture media where the endophytic fungi were incubated showed that most of the compounds detected in the controls were completely degraded by the fungi *Aspergillus* sp. and *Xylaria* sp. 1. The fungi *Clonostachys* sp. 2, *Verticillium* sp. and *Colletotrichum* sp. 1 showed total degradation of the compounds identified in the controls with the exception of n-nonane that showed partial degradation to the controls (14%, 15% and 19% respectively) (Table 4 & Figure 2).

3.5. Taxonomic Identification of Endophytic Fungi

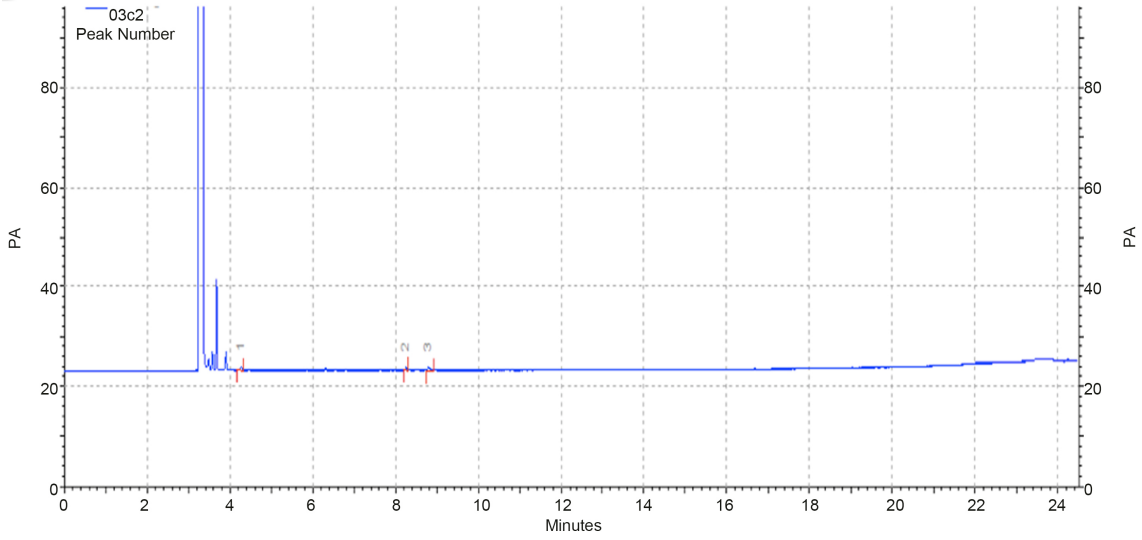
A maximum likelihood phylogenetic tree was generated with 53 endophytic fungi that showed hydrocarbon-degrading bioactivity in the qualitative essays, (Figure 3). The phylogenetic analysis showed a total diversity of three phyla, subdivided in 5 classes, 12 orders, 17 families and 14 genera. The majority of fungal isolates belong to the Ascomycota phylum (94.3%), which represents the dominant lineage. The most abundant class is Sordariomycetes, with 81% of the isolates. The greatest number of fungal isolates are classified within the Diaporthales and Xylariales orders, 26% and 22% of the isolates respectively. Within the Diaporthales

Table 3. Hydrocarbon removal rates quantified by infrared spectroscopy (IR) and gas chromatography (GC).

Endophyte	IR		CG	
	Removal rate%	Standard deviation	Removal rate%	Standard deviation
Verticillium sp.	99.64	0.21	92.41	4.55
Xylaria sp. 1	98.78	0.98	99.82	0.02
Aspergillus sp.	97.65	0.61	99.82	0.03
Colletotrichum sp. 1	97.46	0.02	99.75	0.01
Clonostachys sp. 2	97.41	0.94	99.69	0.01
Phomopsis sp. 1	90.67	1.76	-	-
Colletotrichum sp. 3	86.53	0.12	-	-
Saccharicola sp. 1	78.94	0.26	-	-
Colletotrichum sp. 2	70.37	2.31	-	-
Clonostachys sp. 1	31.46	0.01	-	-



(A)



(B)

Figure 2. Chromatograms resulting from the quantification of HTP by gas chromatography (GC) of the quantitative tests. (A) Results of the control sample (green), together with the white DCM sample (blue), in red the peaks with the identified compounds are observed. (B) Results obtained with the endophytic fungus *Clonostachys* sp. 2.

Table 4. Quantification of persistent petroleum hydrocarbons in the culture medium by gas chromatography (GC) after 30 days of incubation with endophytic fungi.

Compound	Control		*Verticillium* sp.	*Clonostachys* sp. 2	*Xylaria* sp.1	*Colletotrichum* sp. 1	*Aspergillus* sp.
	Retention time	Area	Area	Area	Area	Area	Area
n-octane	5.675	93,622	-	-	-	-	-
n-nonane	8.241	13,390	11,391	11,560	-	10,450	-
1,2,3-trimetil bencene	10.242	34,895	-	-	-	-	-
Trans-decahidro naftalene	10.465	16,317	-	-	-	-	-

Continued

1,2,4,5-Tetrametil bencene	11.003	28,923	-	-	-	-	-
n-undecane	11.517	45,834	-	-	-	-	-
n-dodecane	13.482	124,133	-	-	-	-	-
Pentametil bencene	15.315	204,225	-	-	-	-	-
n-tetradecane	16.868	241,542	-	-	-	-	-

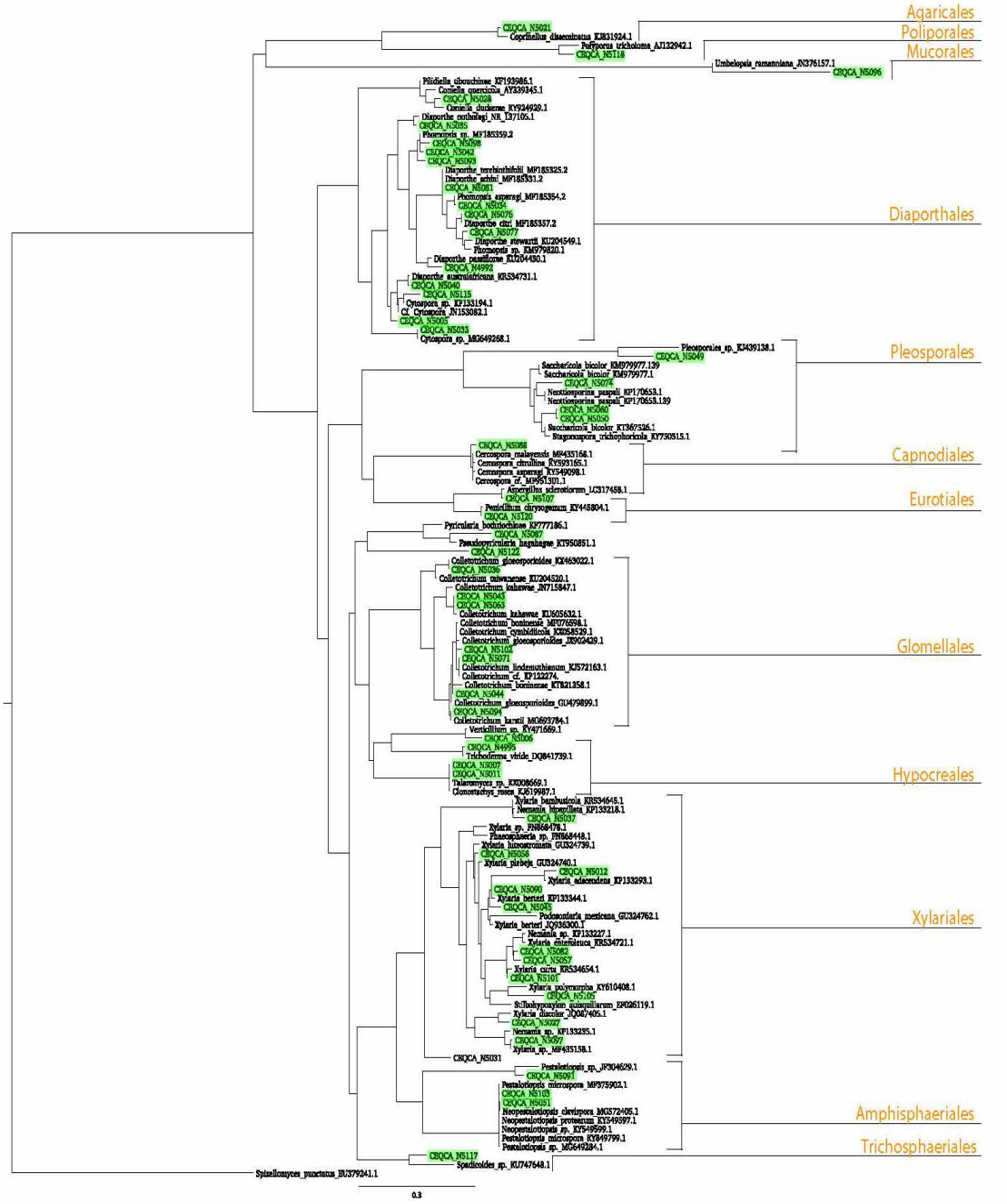


Figure 3. Maximum likelihood phylogenetic tree of the ITS sequences of endophyte hydrocarbon biodegrading fungi. The isolates are shown with the code CEQCA (green), grouped in their respective taxonomic orders (yellow). Spizellomyces punctatus was used as an external group.

order, the family Diaporthaceae is mostly represented by the genus *Diaporthe*, with 4.5% of isolates. Within the Xylariales order, the family Xylariaceae stands out as the only representative, whose members belong to the genus *Xylaria*. *Colletotrichum*, genus belonging to the Glomerellaceae family, highlights for being the most represented with 5.3% of the fungal isolates. Two isolates only could be identified upto order level, one isolate until class level and one at family level. The endophytic fungus Sordariomycetes sp. did not show close homology to any identified specimen at a higher taxonomic level than class. Xylariales sp., Pleosporales sp. and Pyriculariaceae sp. isolates showed a 90%, 94% and 91% BLAST homology level based, respectively (Table 5).

3.6. Statistical Analysis

The Shapiro-Wilk test showed a normal distribution of the transformed data, an analysis of variance (ANOVA) was performed, demonstrating the existence of highly significant differences ($p < 0.01$) between groups (endophytes *versus* control). The Tukey multiple comparison analysis (Table 6) showed differences between endophytes, constituting homogeneous subgroups based on the degree of variability of the means of each treatment. Three subgroups were established; the first subgroup, comprising the five performing endophytes was selected for an additional trial with CG.

Table 5. Taxonomic identification of 53 hydrocarbon-degrading endophytic fungi isolates. Abreviations: Collection of endophytes Quito-Católica (CEQCA), percentaje of identity (ID), query cover percentaje (QC).

No	CEQCA code	ID (%)	QC (%)	Genus and species (BLAST)	Established ID	Family
1	CEQCA-N4992	97	99	*Diaporthe passiflorae*	*Diaporthe* sp. 5	Diaporthaceae
2	CEQCA-N4995	99	100	*Trichoderma viride*	*Trichoderma* sp.	Hypocreaceae
3	CEQCA-N5005	99	100	*Cytospora* sp.	Valsaceae sp. 1	Valsaceae
4	CEQCA-N5006	99	100	*Verticillium* sp.	*Verticillium* sp.	Plectosphaerellaceae
5	CEQCA-N5007	100	100	*Clonostachys rosea*	*Clonostachys* sp. 1	Bionectriaceae
6	CEQCA-N5011	100	100	Clonostachys sp.	*Clonostachys* sp. 2	Bionectriaceae
7	CEQCA-N5012	99	100	*Xylaria adscendens*	*Xylaria* sp.1	Xylariaceae
8	CEQCA-N5021	100	98	*Coprinellus disseminatus*	*Coprinellus* sp.	Psathyrellaceae
9	CEQCA-N5027	99	100	*Xylaria discolor*	*Xylaria* sp.6	Xylariaceae
10	CEQCA-N5028	98	100	*Coniella* sp.	*Coniella* sp.	Schizoparmaceae
11	CEQCA-N5031	90	84	Xylariales sp.	Xylariales sp.	-
12	CEQCA-N5033	100	100	*Cytospora* sp.	Valsaceae sp. 2	Valsaceae
13	CEQCA-N5034	99	100	*Phomopsis asparagi*	*Phomopsis* sp. 4	Diaporthaceae
14	CEQCA-N5035	98	100	*Diaporthe nothofagi*	*Diaporthe* sp. 4	Diaporthaceae
15	CEQCA-N5036	100	100	*Colletotrichum taiwanense*	*Colletotrichum* sp. 7	Glomerellaceae
16	CEQCA-N5037	99	99	*Xylaria* sp.	*Xylaria* sp.9	Xylariaceae
17	CEQCA-N5040	99	100	*Diaporthe australafricana*	*Diaporthe* sp. 2	Diaporthaceae

Continued

18	CEQCA-N5042	99	100	*Phomopsis* sp.	*Phomopsis* sp. 2	Diaporthaceae
19	CEQCA-N5043	100	100	*Colletotrichum kahawae*	*Colletotrichum* sp. 3	Glomerellaceae
20	CEQCA-N5044	100	100	*Colletotrichum* sp.	*Colletotrichum* sp. 6	Glomerellaceae
21	CEQCA-N5045	99	100	*Xylaria* sp.	*Xylaria* sp.10	Xylariaceae
22	CEQCA-N5049	94	100	Pleosporales sp.	Pleosporales sp.	-
23	CEQCA-N5050	97	100	*Saccharicola* sp.	*Saccharicola* sp. 2	Lophiostomataceae
24	CEQCA-N5051	100	99	*Neopestalotiopsis protearum*	*Pestalotiopsis* sp. 2	Pestalotiopsidaceae
25	CEQCA-N5057	99	100	*Nemania* sp.	*Xylaria* sp. 3	Xylariaceae
26	CEQCA-N5058	99	99	*Xylaria plebeja*	*Xylaria* sp. 8	Xylariaceae
27	CEQCA-N5060	97	100	*Saccharicola* sp.	*Saccharicola* sp. 1	Lophiostomataceae
28	CEQCA-N5063	100	100	*Colletotrichum kahawae*	*Colletotrichum* sp. 4	Glomerellaceae
29	CEQCA-N5071	99	100	*Colletotrichum* sp.	*Colletotrichum* sp.1	Glomerellaceae
30	CEQCA-N5074	97	100	*Saccharicola bicolor*	*Saccharicola* sp. 3	Lophiostomataceae
31	CEQCA-N5076	100	100	*Diaporthe citri*	*Diaporthe* sp. 3	Diaporthaceae
32	CEQCA-N5077	99	100	*Diaporthe stewartii*	*Diaporthe* sp. 1	Diaporthaceae
33	CEQCA-N5081	100	100	*Diaporthe* sp.	*Diaporthe* sp. 6	Diaporthaceae
34	CEQCA-N5082	100	99	*Xylaria enteroleuca*	*Xylaria* sp.7	Xylariaceae
35	CEQCA-N5087	91	99	Pyriculariaceae sp.	Pyriculariaceae sp.	Pyriculariaceae
36	CEQCA-N5088	99	100	*Cercospora* sp.	Mycosphaerellacae sp.	Mycosphaerellaceae
37	CEQCA-N5090	99	100	*Xylaria berteri*	*Xylaria* sp.5	Xylariaceae
38	CEQCA-N5091	95	100	*Pestalotiopsis* sp.	*Pestalotiopsis* sp. 3	Pestalotiopsidaceae
39	CEQCA-N5093	99	99	*Phomopsis* sp.	*Phomopsis* sp. 3	Diaporthaceae
40	CEQCA-N5094	100	100	*Colletotrichum Karstii*	*Colletotrichum* sp. 5	Glomerellaceae
41	CEQCA-N5096	99	100	*Umbelopsis ramanniana*	Mucoraceae sp.	Mucoraceae
42	CEQCA-N5097	99	100	*Nemania* sp.	*Xylaria* sp.2	Xylariaceae
43	CEQCA-N5098	100	100	*Phomopsis* sp.	*Phomopsis* sp. 1	Diaporthaceae
44	CEQCA-N5101	100	100	*Nemania* sp.	*Xylaria* sp.4	Xylariaceae
45	CEQCA-N5102	99	99	*Colletotrichum lindemuthianum*	*Colletotrichum* sp. 2	Glomerellaceae
46	CEQCA-N5103	100	99	*Neopestalotiopsis clavispora*	*Pestalotiopsis* sp. 1	Pestalotiopsidaceae
47	CEQCA-N5105	89	100	*Xylaria* sp.	*Xylaria* sp.11	Xylariaceae
48	CEQCA-N5107	100	100	*Aspergillus esclerotiorum*	*Aspergillus* sp.	Trichocomaceae
49	CEQCA-N5115	99	99	*Cytospora* sp.	Valsaceae sp. 3	Valsaceae
50	CEQCA-N5117	92	100	Spadicoides sp.	Helmisthosphaeriaceae sp.	Helminthosphaeriaceae
51	CEQCA-N5118	99	98	*Polyporus tricholoma*	*Polyporus* sp.	Polyporaceae
52	CEQCA-N5120	100	100	*Penicillium chrysogenum*	*Penicillium* sp.	Trichocomaceae
53	CEQCA-N5122	99	100	Sordariomycetes sp.	Sordariomycetes sp.	-

Table 6. Tukey multiple comparisons analysis with the results of the quantification of HTP by infrared spectroscopy.

Endophyte	Subgroup		
	1	2	3
Clonostachys sp. 1	1.2933		
Colletotrichum sp. 2	1.3967		
Saccharicola sp. 1	2.1933	2.1933	
Colletotrichum sp. 3	2.2200	2.2200	
Phomopsis sp. 1	2.2433	2.2433	
Clonostachys sp. 2		2.8033	2.8033
Colletotrichum sp. 1		2.9667	2.9667
Aspergillus sp.		3.1633	3.1633
Xylaria sp. 1		3.3100	3.3100
Verticillium sp.			3.6800
Control			3.8433
Sig.	0.152	0.054	0.088

4. Discussion

Plant adaptation to highly contaminated habitats is restricted to a handful of species that tolerate such conditions [44]. Evidence has shown that endophytes contribute to plant adaptation to stress conditions, stimulating growth, productivity, carbon sequestration and tolerance to contaminants [45].

Deng and Cao [45] suggested that the degree of substrate contamination by hydrocarbons negatively affect plant species diversity and abundance. However, the sampling site of this study exhibits geological oil outcrops with typical Amazonian tropical humid forest vegetation developing *in situ*, turning this area into a point of interest for the diversity study and capabilities of endophytic fungi from plants adapted to this type of habitat.

The endophytic fungi isolates were subjected to *in vitro* tests to evaluate their hydrocarbon biodegradation capacity [18]. The qualitative essays showed the hydrocarbon biodegradation potential of 53 endophytic fungi, as indicated in Table 2. The essays are based on the fact that during microorganisms' oxidation reactions, electrons are transferred to an electron acceptor such as oxygen (O_2), nitrates (NO_3^-) or sulfates (SO_4^{2-}) [46] [47]. By incorporating an electron accepting reagent into the culture medium, such as DCPIP, it is possible to demonstrate the organisms' capacities to use petroleum hydrocarbons as a carbon source through the DCPIP color change, from blue (oxidized) to colorless (reduced) [48]. This redox technique demonstrates that 53 fungal isolates possess the ability to degrade crude oil. Within these 53 endophytes, a subgroup composed of 10 individuals presented outstanding results in the qualitative essays, clarifying completely the culture medium in less time than established for the

essay (<10 days) (**Figure 1**).

With exception of plant families Orchidaceae sp. and Poaceae sp., all the collected plants have fungal organisms with variable capacities to degrade hydrocarbons in this essay. Evidence obtained by [49] and [50] demonstrates the plant endophytic composition could be affected by contaminants, stimulating the proliferation of organisms tolerant to these conditions. Interestingly, the plants with the highest number of endophytic isolates, *Marattia* sp. (Marattiaceae) and FM006 (unidentified), with 12 and 10 isolates respectively, correspond to plants with the highest number of fungi with hydrocarbon biodegradation capacities (**Table 2**), this results support the hypothesis that endophytes contribute to a better adaptation of the host plant to contaminated substrates [28].

Twenty-three plant samples were collected in the site (**Table 1**), including stem and leaves samples. The families Melastomataceae, Boraginaceae and Poaceae stood out for their predominance in the area, representing up to 70% of the species present in the area.

To a lesser degree, individuals from the families Rubiaceae, Gunneraceae and Urticaceae were found. A previous study [33] reported several families in common with this study (Melastomataceae, Boraginaceae, Poaceae, Euphorbiaceae, Bignoniaceae and Rubiaceae) as common species in the tropical Ecuadorian rainforest but evidenced an abnormally higher abundance of them in contaminated secondary forest in contrast to unpolluted secondary forest and pristine forest [33]. In our study the predominant families were Melastomataceae, Rubiaceae, Fabaceae and Euphorbiaceae. The marked predominance of the individuals of the family Melastomataceae could demonstrate a relationship between the family and the colonization of this type of habitat [33]. Likewise, the highest number of fungal endophytes were isolated from plants species of the Melastomataceae, Marattiaceae, Polypodiaceae and Euphorbiaceae which may indicate a strong interaction between the plant and the fungal endophytes, similar to bacteria that are in intimate association with plants which are known to consume alkanes and aromatic hydrocarbon exudates granting them the capacity to in degrade organic contaminants [51].

A total of 133 endophytic fungi were isolated from stem and leaf tissues of 23 plants species (**Table 1**). These figures are congruent with the suggestion that Ecuadorian endophytes are hyperdiverse [52]. The taxonomic positions of 53 fungi were established through a phylogenetic analysis (**Figure 3**). The phylogenetic tree shows the four phyla in the fungi kingdom, Ascomycota, Basidiomycota, Zygomycota and *Spizellomyces punctatus* (phylum Chytridiomycota) as outgroup. The analysis shows that most of the isolated ascomycetes constitute the orders Diaporthales, Xylariales and Glomerellales.

Within the order Diaporthales 14 fungi isolates belonging to three families were identified: Diaporthaceae, Valsaceae and Schizoparmaceae. Among the 14 isolates of the order Diaporthales, the endophyte *Phomopsis* sp. 1 (**Table 3**) showed a hydrocarbon removal rate of 90.7%. Biodegradation of phenanthrene and

4-hydroxybenzoic acid has been reported by fungi from the genus *Phomopsis* [53] [54]; however, this is the first report of crude oil biodegradation for fungi from the genus.

Within the Xylariales order, 12 fungi were identified, 11 belonging to the family Xylariaceae and 1 identified until order level (**Table 5**). Among the 12 individuals, the endophyte *Xylaria* sp. 1 showed a hydrocarbons removal rate of 98.8% (**Table 3**). Pyrene and benzopyrene biodegradation has been reported by *Xylaria regalis* [55] but there are no reports to crude oil biodegradation.

The order Glomerellales groups 8 fungi, all belonging to the family Glomerellaceae and the genus *Colletotrichum* (**Table 5**). Within this order, three individuals with hydrocarbon removal rates ranging from 70.4% to 97.5% (**Table 3**). *Colletotrichum gleosporoides* is known to produce extracellular lipases [56], enzymes that have been widely studied for their ability to degrade hydrocarbons [57] [58].

However, when submitting *Colletotrichum gleosporoides* to hydrocarbon biodegradation essay the lipase production capacity was inhibited, possibly due to the impact of the toxic experimental conditions [58].

The order Hypocreales has three identified isolates; the hydrocarbon removal rate to *Clonostachys* sp. 1 was 31.5% and 97.4% to *Clonostachys* sp. 2 (**Table 3**). There are no previous reports about hydrocarbon biodegradation by individuals from the genus *Clonostachys*. But, metabolic capacities studies with individuals from the genus *Clonostachys* determine zearalenones biodegradation [59].

Aspergillus sp. showed a removal rate of 97.6% (**Table 3**). Previous studies conducted with free-living fungi reported the capacities of *Aspergillus fumigatus* and *Penicillium chrysogenum* for the production of laccases [58], family of enzymes that are actively involved in the hydrocarbons biodegradation processes by oxidizing them [57] [58]. Additionally, there are biodegradation reports of phenanthrene, pyrene and benzopyrene by *Aspergillus niger* [60]. Biodegradation of fluoranthene by *Penicillium* sp., Pyrene by *Penicilliumjanczewskii* and *P. janthinellum* and crude oil by *Penicillium* sp. [61] [62]. These works results were obtained with free-living fungi but, belong to the same genus of the endophytes of our study, thus believe the enzymes involved in hydrocarbon biodegradation processes could be the same ones already reported.

The order Pleosporales is represented by four fungi, three belong to the genus *Saccharicola*, family Lophiostomataceae (**Table 5**). The endophyte *Saccharicola* sp. 1 showeda hydrocarbon removal rate of 78.9% (**Table 3**). This is the first report of hydrocarbon biodegradation by a fungus from the genus *Saccharicola* (97% ID). However, the production of extracellular protease enzymes has been confirmed in individuals of the genus [63], additional studies have identified fungal proteases due to their hydrolytic activity could participate in hydrocarbon degradation processes [57] [58].

Quantification of TPH by IR is based on the measurement of the absorbed energy to produce stretching and bending vibrations of the molecules. The dif-

ferent functional groups and bond types absorb radiation at different frequencies and intensities. The absorption intensity is directly proportional to the bonds number. This is correlated to the hydrocarbons concentration present in the sample [64]. These results were compared with a control sample to establish the hydrocarbon removal rates.

Previous studies with the same hydrocarbon biodegradation methodology using free-living mushrooms, reported removal rates ranges from 14.9% to 43.4% [35]. In this study we obtained variable degradation rates, from 31.5% (minimum), to 99.6% (maximum) of hydrocarbon removal, as shown in **Table 3**.

The analysis also determined a high variation (degradation capacity) among the tested endophytes. The replicates of the trials showed no statistically significant differences.

Additionally, Tukey multiple comparisons analysis grouped the endophytes that showed statistically homogeneous performances among themselves, establishing 3 subgroups, the first subgroup agglutinates the five endophytes with the best performances (*Xylaria* sp. 1, *Clonostachys* sp. 2, *Colletotrichum* sp. 1, *Verticillium* sp. and *Aspergillus* sp.) (**Table 6**). In order to corroborate the results obtained with the best five endophytes, a new analysis was carried out, quantifying the hydrocarbon present in the culture medium by GC. In this TPH quantification method, a mobile phase (inert gas N_2) and a stationary phase (capillary column) are used to separate the hydrocarbon mixture from the samples in their individual components, carried by the mobile phase through the stationary phase. The separation is carried out by a combination of factors including the boiling point, polarity and affinity differences between the different hydrocarbons and the column, making the concentrations estimation had a greater accuracy and allows establish differences between the quantification methods used.

Removal rates may be affected by several factors, including the test temperature. In the present study, the tests were carried out at a temperature of 23˚C in absence of an incubator with agitation, but evidence shows that the optimum temperature for carrying out this type of essays ranges between 28˚C and 31˚C [65] [66], suggesting the results could be improved modifying the experimental temperature.

Another factor was the addition of a surfactant to the culture medium. There are studies where the culture medium was not supplemented with a surfactant to emulsify the mixture between crude oil and water, making the biodegradation process even more complex for the evaluated microorganisms [67]. In this case, Tween®80, the surfactant used in this study, is nontoxic and a stimulant of manganese peroxidase production [68]; the results obtained indicate that it was the appropriate reagent to optimize the enzymatic activity without toxic effects.

The crude concentration evaluated in this study was 1%; further studies could evaluate the performance of these endophytic fungi at higher concentrations, the optimum concentrations for fungi range between 1% and 5% [69]. The crude oil concentration used is determinant to obtain optimal results. Evidence provided

by [70] shows that the degradation percentage of a bacterial consortium culture decreased from an initial 78% to 52% by increasing the crude oil concentration from 1% to 10%.

The results shown in **Table 3** demonstrate the enormous potential of isolated fungi to eventual uses in the bioremediation field. Six fungi (*Xylaria* sp. 1, *Clonostachys* sp. 2, *Colletotrichum* sp. 1, *Verticillium* sp., *Aspergillus* sp. and *Mucoraceae* sp.) showed removal rates higher than 90%, under the tested conditions, are highly encouraging results for a fungal assay and even more in the case of endophytic fungi.

Reference [50] isolated endophytic bacteria from roots of plants that growing in a contaminated with crude oil environment and evaluated the capacities to biodegrade crude by bacterial isolates at 1% concentration; obtaining similar hydrocarbon removal rates to those obtained in this study. The isolated *Streptomyces* sp. HIh9 showed a 97.5% hydrocarbon removal rate after 7 days of incubation, while in our study the endophyte fungus *Verticillium* sp. showed 99.64% after 30 days of incubation, which evidences that plants harbor endophytic contaminant degrading organisms as adaptation mechanism to hostile habitats, due to the plant alone is unable to metabolize these compounds. This study demonstrates the potential for using fungal endophytes in biodegradation of crude oil, either through biotechnological applications or by improving phytoremediation strategies aiding plants to tolerate contaminant-induced stress, especially in countries with economies based on fossil fuel exploitation.

Petroleum hydrocarbon biodegradation is a complex process, depending on the crude oil nature, composition, environmental conditions and soil microbial community. The presence of hydrocarbon-degrading microorganisms is an adaptive response to the environmental contamination conditions [71]. Adaptation mechanisms include selective enrichment and genetic changes that stimulate the complex hydrocarbon biodegradation. The plants forced to develop in this environment stimulate the growth of hydrocarbon-degrading microorganisms to detoxify the plant from substances that normally could not be processed by itself. Fifty-three hydrocarbon-degrading endophytic fungi were isolated from plants growing in crude oil contaminated soils at Ecuadorian Amazon. We identified six endophytic fungi that showed Total Petroleum Hydrocarbon removal rates higher than 90% after 30 days of incubation. The endophyte *Xylaria* sp. 1 showed the best TPH removal rate 98.78% (IR) and 99.8% (CG).

5. Conclusion

Diversity of fungal endophytes determined in this study supports previous reports of hyperdiversity of this group of microorganisms in Ecuador. Our results suggest that bioremediation processes could potentially use hydrocarbon-degrading endophytic fungi isolated from native plant species growing in natural oil outcrops. These may also be used in support of phytoremediation strategies at later stages of remediation of contaminated sites given that the endophytes isolated

from these plant species may present long-term adaptation mechanisms that can improve colonization of contaminated habitats, as well as uptake by plant species.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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