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First report of two *Aspergillus* species isolated from mangrove forest in Ecuador

Primer estudio de dos especies de *Aspergillus* aisladas de bosques de manglar en Ecuador

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Abstract

Mangroves forests are located in tropical and subtropical regions. The adaptation and distribution in coastal regions is influenced by temperature, humidity, tidal and saline fluctuations; therefore, there are exposed to multiple environmental fluctuations. Mangroves are inhabited by wildlife but also is supported by a diverse community of microorganisms, including fungi. Several fungi in mangroves have multiple ecological roles as saprotrophs or as an opportunistic pathogen, many of them are also used in the industry, as the genus *Aspergillus*, that are important in biomedicine, industrial and environmental applications. In this study, we isolated species of fungi from mangrove stems and propagules. They were identified by both morphological and by its molecular characteristics. Here, we report the first isolated of *Aspergillus niger* and *Aspergillus aculeatus* from mangroves in Ecuador. Research such as these highlights the importance to determine the role of fungi in the mangrove ecosystem.

Keywords: Mangrove, *Aspergillus niger*, *Aspergillus aculeatus*, molecular characterization.

Resumen

Los bosques de manglar están distribuidos en las zonas costeras de las regiones tropicales y subtropicales de todo el mundo, siendo especies tolerantes a altas temperaturas, humedad, mareas y las fluctuaciones salinas. Por lo tanto, se ven expuestos a múltiples fluctuaciones y condiciones ambientales extremas. El ecosistema de manglar no solo es hábitat de vida silvestre, sino que también es colonizado por diversas comunidades de microorganismos, como los

hongos. Varios de estos hongos tienen múltiples funciones ecológicas, ya sea saprófitos o patógenos oportunistas. Actualmente el interés de estudiar estos microorganismos radica en su potencial biotecnológico dada su capacidad para tolerar ambientes hostiles. Ejemplo de ello son algunas especies del género *Aspergillus*, las cuales son utilizadas en biomedicina, industrial y la bioremediación. En el presente estudio se aislaron e identificaron de acuerdo con sus características morfológicas y moleculares especies de hongos del género *Aspergillus*. En este estudio se reportan los primeros aislados de *Aspergillus niger* y *Aspergillus aculeatus* de manglares en Ecuador. Investigaciones como ésta resaltan la importancia de determinar el rol de los hongos en el ecosistema de manglar.

Palabras clave: Manglar, *Aspergillus niger*, *Aspergillus aculeatus*, caracterización molecular.

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1 Introduction

Mangrove forests are estuaries located on tropical and subtropical regions; this ecosystem is characterized by its highly salinity-tolerance (Gopal and Chauhan, 2006). The distribution of mangroves is strongly influenced by temperature, humidity, water currents and variations in tide and wind flow. Altogether with the high abundance and variety of microorganisms, make it an important dynamic ecotone between the terrestrial and marine environment (Sridhar et al., 2011). Taking into account microorganisms, fungi are important decomposers of organic matter and they play a fundamental role in productivity and biodiversity of this ecosystem (Friggens et al., 2017). The fungi that inhabit mangrove forest are saprophytes, symbionts or parasites, both in filaments and in yeast (Rodríguez et al., 2013). These fungi can colonize roots, stems and branches submerged in water or can be found in the surface of the water (Li et al., 2016). The diversity of fungi depends on their metabolism, since the strata is associated with daily changes in salinity, intermittent flooding due to tide, exposure to salt fog and substrate availability (Hrudayanath et al., 2013).

Human activities constitute the main problem to mangroves, and among the main human activities are the habitat destruction, pollution and overexploitation of resources (Díaz, 2011). Many cities have settled in the nearby mangroves, which, therefore, are constantly exposed to pollution that is formed by anthropogenic activities, thus exposing them to a variety of chemicals, including heavy metals that are considered a serious problem for the mangrove ecosystem, since they accumulate on the surface of the sediments, hence increasing their concentration in the area (Fernández et al., 2014)

The most common genera of fungi isolated from *Rhizophora* spp. mangrove are *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Curvularia*, *Cylindrocephalum*, *Drechslera*, *Fusarium*, *Myrothecium*, *Nigrospora*, *Penicillium*, *Pestalotia*, *Phyllosticta*, *Trichoderma* and *Verticilium* (Sarma, 2012). However, few studies describe the role of these genera in the mangrove ecosystem. Most of the fungi identified in mangrove ecosystems are endophytes; these fungi are considered of great importance since they have been able to produce bioactive metabolites to modify the defense mechanisms of their host, allowing both to subsist in the environment (Sánchez et al., 2013). Several fungi (e.g., *Aspergillus*, *Absidia*, *Cunninghamella*, *Mucor* and *Rhizopus*) are capable of accumulating heavy metals in their cell compartments, offering an alternative in bioremediation of contaminated areas at low cost compared to tradition decontamination methods (Cardoso et al., 2010).

In many cases, mangrove forests are formed by high density natural monocultures where trees are constantly exposed to pathogens (Ramírez et al., 2006). However, phytopathogenic records in mangrove ecosystems are poorly reported (Pan et al., 2018). The majority of fungi that colonize mangroves trees are related to Ascomycota division, several parasitic species belonging to this group can produce plant death (Pan et al., 2018). Although the relationship between fungal diversity and mangroves is not clear, marine fungi are known to be responsible for the breakdown of mangrove wood in the most common species, including *Rhizophora*, which have been described to be more efficient digestible biomass than bacteria (Steinke and Jones, 1993; Kathiresan et al., 2011). Fungus *Trichoderma* and *Traustreochystrids* genus are efficient saprophytic due to enzymatic activity and potential microorganism to degraded cellulose, starch, lipid, proteins and lignin, whereas *Thichosporon*, *Fusarium* and *Aspergillus* exhibited the maximum cellulase and protease activity in the leaves of mangrove (Kathiresan et al., 2011)

Aspergillus is widely distributed in nature due to its easy dispersal strategy of its conidia and its small size; this strategy allows them to remain in the environment for a long period of time (Abarca, 2000). The fungal genus of *Aspergillus* is highly interesting, containing everything from industrial cell factories, model organisms, and human pathogens, since it has a prolific production of bioactive secondary metabolites (Kjærbølling et al., 2018). These fungi are important in the organic matter breakdown and host defense against highly pathogenic microorganism (Ramírez et al., 2006). The applications of these fungi are required to improve future omics studies (Shu-Lei et al., 2020). In the present study we present the first report of two species of *Aspergillus* isolated from mangroves in Ecuador and identified by molecular and morphologically characteristics.

2 Material and Methods

2.1 Sampling and collection area

The Parque Histórico de Guayaquil - PHG (Guayaquil Historical Park) is a remnant of mangrove forest located in Samborondón city, Ecuador. Lesions with symptoms associated with the presence of fungi in branches were identified, subsequently collected and stored in airtight bags for further analysis.

2.2 Morphological characterization of fungi samples

Morphological characterization was performed on cultures with evident generation of reproductive structure or in strains with more than 20 days of cultivation time. By the use of tape, the mycelium was separated and placed on a slide with a drop of lactophenol and sealed with a coverslip. The visualization of structures was done in a Nikon Eclipse E100 optical microscope with an integrated digital camera. In order to determine taxonomic affiliation, both, the mycobank database (*http://mycobank.org*) and taxonomic keys (de Hoog et al., 2001) were used.

2.3 Isolation and purification of fungi

The plant material was disinfected with 1 % sodium hypochlorite with repeated washings with sterile water. Fragments were subsequently cut with a sterile scalpel and deposited in SDA culture media (Saboraud Dextrose Agar, OXOID), supplemented with 15 μg/mL chloramphenicol for isolation and differentiation of fungi. Culture plates were incubated at room temperature in dark conditions until micellar growth was observed. This process was repeated until pure isolates were obtained. Samples previously isolated were inoculated in 150 mL of PW liquid culture medium (Peptone Water, CRITERION and Nutrient Broth) supplemented with 15 *n*g/mL chloramphenicol and incubated for 15 to 20 days until biomass appeared. Mycelium was filtered using sterile filtration units, biomass was dried at 42 °C for two days and stored at −80 °C until further analysis.

2.4 DNA extraction

Fungal material was incubated with lysozyme (10 μg/mL) at 37 °C for two hours. DNA extraction was performed using the Power Soil DNA Isolation kits (QIAGEN, Carlsbad, USA) with the following modifications: Fungi were previously dried and frozen at −80 °C and transferred to the Powerbead tubes; this material was mixed by vortex for three minutes. Subsequently, 60 μL of C1 buffer was added and a vigorous vortex was performed for three additional minutes, 20 μL of proteinase K was added and mixed with vigorous vortex for 1 minute. The mixture was centrifuged at 8,000 rpm 1 minute, the supernatant was rescued in a new eppendorf tube and 250 μL of C2 buffer was added, then an additional mixed by vortex for 5 seconds was performed and incubated at −20 °C for 5 minutes. Samples were centrifuged at room temperature at 8,000 rpm for 1 minute and the supernatant was transferred to a new tube; subsequently, 200 μL of C3 solution was added, vortexed for 1 minute and incubated at −20 °C for 5 minutes.

For DNA isolation, samples were centrifuged at 8,000 rpm for 1 minute and supernatant was transferred to a new tube with 1mL of C4 solution. The mixture passed through the columns by centrifugation at 8,000 rpm for 1 minute; thereafter, 500 μL of C5 solution was added and centrifuged at 8,000 rpm for 1 minute. For DBA elution, 100 μL ultrapure water was added to the center of the column to elute the genetic material, centrifuged at 13,000 rpm for 1 minute and stored at −20 °C until later use. DNA integrity was assessed by 1 % agarose gel electrophoresis in 1*times* TAE buffer (Tris, Acetate, EDTA) supplemented with SyBR green nucleic (Invitrogen) by comparing the intensity and molecular weight band of DNA with a 100 bp ladder (Tracklit - Invitrogen). Electrophoresis conditions were performed at 100 volts, 35 milliamps.

2.5 Amplification of the ITS-1 and ITS-2 regions by Polymerase Chain Reaction (PCR)

After DNA extraction, fungi were identified either genus or species level from the amplification of the intergenic regions of rDNA using ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 universal primers (TCCTCCGCTTATTGATATGC) (White et al., 1990). PCR conditions were performed in a final volume of 30 μL with final concentration of 0.2 5mM dNTPs, 2.5 mM MgCl2, 2 μM primers, 2 U taq pol and 1× PCR Buffer. The program includes denatu-

ration at 95 °C for 5 minutes, followed by 35 cycles of 94 °C for 59 seconds, 50 °C for seconds and extension at 72 °C for 1 minute, with a final extension of 72 °C for 10 minutes.

2.6 Sequence analysis

Purified amplicons were sequenced in Macrogen (South Korea) and edited in Geneious Prime Software (version 2019.1) in order to obtain a consensus sequence. Taxonomic affiliation was confirmed by BLAST alignment algorithm (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). Phylogenetic analysis was enhanced using Clustal W algorithm and Geneious Prime Software. A tree was generated by Neighbor Joining (Saitou and Nei, 1987) and a distance model of Kimura was used (Kimura, 1983) using MEGA 7 software, with 1,000 iteration bootstrap and eliminating gaps. The results were displayed with iTOL (Letunic and Bork, 2016). ITS sequences related to *Aspergillus* reported on NCBI database used in this research were listed in the supplementary Table 1A. The teleomorph of *Aspergillus* sp. used in this study was *Emiricella nidulans* (HQ026740.1), tree resolution was optimized using is *Aspergillus elegans* (NR077196.1 and MH992144.1), the outgroup was *Saccharomyces cerevisiae* (MG775707.1).

3 Results

Within the substrates collected in the present study, lesions allegedly caused by fungi were analyzed (Figure 1). From this plant, five strains of fungi were isolated and purified from branches and one strain isolated from propagules (Table 1). At the moment of growing in solid culture medium, white mycelia was formed, which later turned either black or grey with submerged mycelium (Figure 2 a-f). When observing the microscopic characteristics, it was determined that these isolates have smooth hyphae, with spherical vesicles covered by filiads and spherical conidia (Figure 2 h-l). Considering macro and microscopic characteristics, classical classification was performed by the use of taxonomic keys (de Hoog et al., 2001) and Mycobank database (*http://mycobank.org*). As a result, these fungi belong to *Aspergillus* genus but by ITS analysis, KCR3.2, KCR4.1sp and KCR15.1 correspond to *Aspergillus niger*, while KCR4.1.1., KCR7.2.1. and KCR14 are related to *Aspergillus aculeatus*.

Of all the 90 nucleotide sequences, 438 positions in the ITS region were used in the phylogenetic analysis. At first glance, all the species analyzed in this study belong to the Nigri section (Gams et al., 1986). The analysis is supported by a Bootstrap with 1000 iterations and by the Neighbor Joining algorithm. The tree shows well supported clades between fungi species in groups of *A. niger* and *A. tubingensis* and between *A. aculeatus* and *A. japonicus* (Figure 3). The dendrogram shows that both *A. niger* and *A. tubingensis* would be the same species, and this is also observed between *A. aculeatus* and *A. japonicus*.

As shown in Table 2, *A. niger* and *A. tubingensis* in the largest alignment with the size 3 626 085 bp are similar in 89.6%, and between *A. aculeatus* with *A. japonicus* with the largest alignment of 3 727 362 bp, the pairwise identity is 90.9%. This shows that they are very close to each other but also differ with members of other clades (i.e. *A. niger* with *A. japonicus* 78.3% similar). At first glance, it can be seen phylogenetically that ITS region does not work well in the resolution of fungi groups. In order to get satisfactory results, it is necessary to complement these analyses with morphological identification and in this case, comparison between genomes.

4 Discussion

In Ecuador, the relevance of this study is related as a baseline of a mycobiome approach in mangrove fungi identification. Two species of *Aspergillus* were identified and purified, *A. niger* and *A. aculeatus*, which were previously reported in other mangroves forest in Malasya, Mexico, China and Indonesia (Sathiya et al., 2009; Lumbreras-Martínez et al., 2018; Deng et al., 2013; Li et al., 2017; Prihanto et al., 2019). Despite fungi are widely distributed in mangrove trees, and because some of them have ecological and physiological functions to increase the tolerance to biotic and abiotic stress conditions (Shu-Lei et al., 2020), the studies about diversity of fungi in mangroves are main focused in cultivable fungi but native uncultured fungus are poorly known.

Figure 1. Lesions associated with the presence of fungi in mangrove stems.

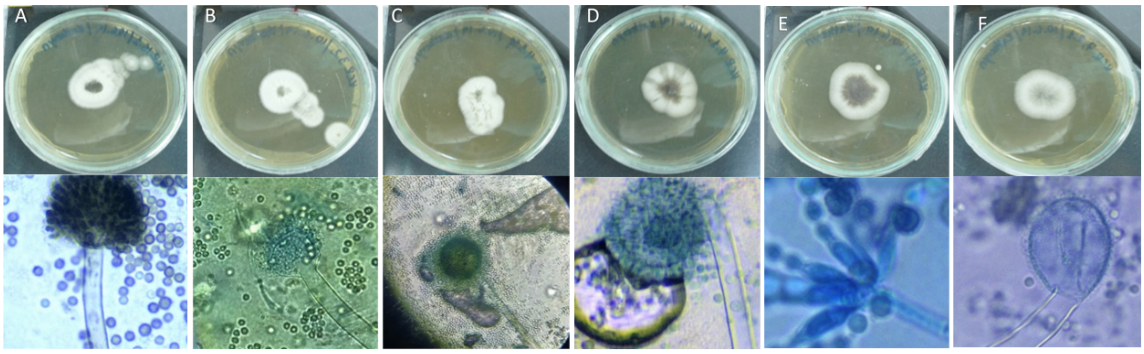

Figure 2. Morphological characteristics of fungal strains isolated from mangroves. (A-F) the top images show micellar growth in solid culture medium. A (isolated KCR 15.1); B (asylum KCR 3.2); C (isolated KCR 4.1 SP); D (isolated KCR 4.1.1); E (isolated KCR 14); F (isolated KCR 7.2.1); at the bottom image show microscopic structures of mangrove fungus isolates.

Table 1. Molecular characterization of fungus isolated from branching and propagules of mangrove

Sample	**Strain**	**Molecular Characterization**					
		Description	Max Score	Total Score	Query Cover (%)	Per. Ident (%)	E-value
Stems	KCR3.2	*Aspergillus niger*	1064	1224	100	99.83	0,0
	KCR4.1sp	*Aspergillus niger*	1048	1208	100	99.82	0,0
	KCR15.1	*Aspergillus niger*	1027	1178	100	99.82	0,0
	KCR4.1.1	*Aspergillus aculeatus*	874	874	100	100	0,0
	KCR14	*Aspergillus aculeatus*	946	946	83	99.81	0,0
Propagule	KCR7.2.1	*Aspergillus aculeatus*	1031	1031	100	100	0,0

Table 2. Genome comparison using LASTZ algorithm: Percentage values is the pairwise identity between two genome sets, numerical value is the largest sequence compare in two genome sets.

	A. niger	*A. tubingensis*	*A. aculeatus*	*A. japonicus*
A. niger	xxx	89.6 %	78.3 %	78.3 %
A. tubingensis	3 626 085	xxx	78.2 %	78.4 %
A. aculeatus	3 727 362	4 803 603	xxx	90.9 %
A. japonicus	1 444 553	4 803 603	3 727 362	xxx

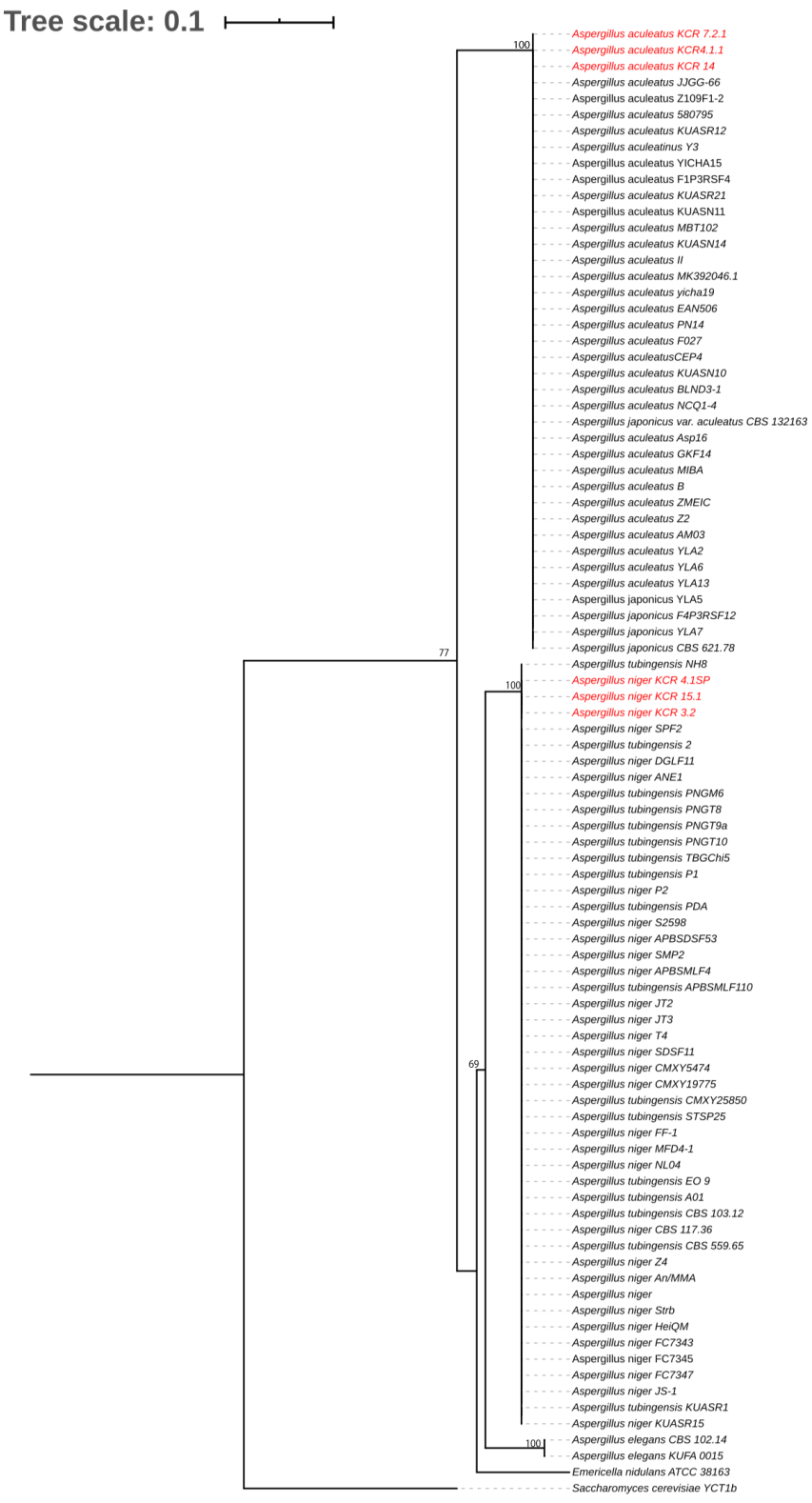

Figure 3. Phylogenetic analysis of fungi of the *Aspergillus* genus isolated from mangrove plant material. The sequences were aligned by Clustal W. The analysis was performed with a support of Bootstrap 1000 iterations and using the Neighbor Joining algorithm with the Kimura 2-parameter method. Teleomorph of *Aspergillus* is *Emiricella nidulans* (HQ026740.1) and outgroup *Saccharomyces cerevisiae* (MG775707.1) and two species of *Aspergillus elegans* (NR 077196.1, MH992144.1).

Traditionally, identification, taxonomy and classification of *Aspergillus* had been based according to morphological features (Patki et al., 2015). Nevertheless, taxonomic classification based on molecular characterization became an indispensable tool, since it allows to distinguish its phylogenetic characteristics (Samson et al., 2014). Considering previous published work about phylogenetic relationship of *Aspergillus*, it is indicated that, although the amplification of ITS regions is widely accepted as a DNA barcode for fungi in general (Conrad et al., 2012), phylogenetic analysis was not resolutive to distinguish uniseriate clades in Nigri section, especially with the aggregation of *A. niger* sequences, which could lead to misidentification of species (Abarca, 2000; Perrone et al., 2008). Our results are consistent with many of the research; the species *A. aculeatus* and *A. japonicus* were phylogenetically closed and similar *A. niger* and *A. tubingensis*. This also explains because there are minor differences between some species belonging to section Nigri, the species *A. niger sensu stricto*, *A. tubingensis*, *A. foetidus*, and *A. brasiliensis* are morphologically identical and altogether have been called *A. niger* aggregate (Munique et al., 2009). This is also consistent with Yokoyama et al. (2001) who described that most of the species of section Nigri are morphologically similar and the molecular identification by mtDNA and rDNA has been confused.

Resolutive molecular techniques as RFLP, AFLP, PFGE or Next Generation Sequencing - NGS would be greatly enhance knowledge and understanding of this fungus (Leong et al., 2006; Perrone et al., 2008; Quainoo et al., 2017). Consistently, genome comparison gives the best resolution to determine percentages of similarity between its sequences (Quainoo et al., 2017). In this study we performed the genetic similarity comparison to determine the genetic diversity between *A. niger*, *A. fumigatus* and between *A. aculeatus* and *A. japonicus*. The results showed high similarity with an average nucleotide identity (ANI) of approximately 90% of the species analyzed (Table 2). These results can be used to explain many different aspects, such as the strain background lineage, showing that this approach is highly useful for new fungal geneticists.

It has been shown that *Aspergillus* sp. is wide distributed in the environment, and it is considered as one of the most frequent phytopathogens by its ability to produce toxins that alter plants metabolisms (Pavón et al., 2012). Some of these fungi colonize roots, facilitating their growth and improving the quality of the grass. Other characteristics are solubilization of phosphorus, halotolerance, attenuation of saline stress to the plants (Li et al., 2017). *Aspergillus* is characterized by producing mycotoxins and secondary metabolites that are important in the degradation of organic matter and as defense mechanisms toward other microorganisms (Pavón et al., 2012).

The importance of this study also relies in the biotechnological capacity that both mangrove ecosystem and its microbial component can offer. One capacity is the physiological characteristic that fungi present with the production of specific protein, for example metabolites to inhibit cell growth of other microorganisms in a high salinity environment (Nicoletti et al., 2018). It has also been proven that some *Aspergillus* sp. help in bioremediation processes of environments contaminated by heavy metals, degradation of extracellular cellulose and hemicellulose (Marrero et al., 2012; Huachi et al., 2014; Araujo et al., 2016).

Regarding *A. niger*, it has been widely used to obtain enzymes for the industry such as: α-Amylase, catalase, cellulase, hemicellulase, lipase and organic acids that could be an alternative to replace petrochemicals (Patki et al., 2015; Ameen et al., 2016; Wang et al., 2016; Morthensen et al., 2017; Wang et al., 2019; Nascimento et al., 2019; Hossain et al., 2016). It is also used for production processes of citric acid that is carried out by solid state, submerged and surface fermentation (López et al., 2006). *A. niger* began being used in the biochemical fermentation industry and industrial biotechnology, since this specie of fungus produces a diverse range of proteins, enzymes and second metabolites (Cairns et al., 2018). The relevance of *A. niger* for the environment relies on its ability to bioaccumulate toxic metals such as lead, cadmium, copper and chromate (Rivera et al., 2015). Several studies have shown that *A. niger* has greater capacity to remove phenolic compounds, oil from contaminated soils and heavy metals, compared to granular activated carbon, used in several biosorption cycles (Araujo et al., 2016; Marzan et al., 2017; Villalba et al., 2018). In their study (Ghyadh et al., 2019) noted that the fungal *Aspergillus niger* showed

the highest efficiency in reducing concentrations of heavy elements by 100%. *Aspergillus aculeatus* showed high resistance to cadmium (CD) toxicity, protected the photosystem II against CD stress and increase the efficiency of photosynthesis process in perennial ryegrass. These results suggest that *A. aculeatus* could be useful to pretreating CD contaminated soils (Han et al., 2018). It has also an important effect in the attenuation of saline stress, since it produces indole-3-acetic acid and siderophores that confer tolerance to saline stress plants (Li et al., 2017). Research regarding the metabolic capacity of *A. niger* and *A. aculeatus* and their interaction with metal ions are limited (Emri et al., 2018). Although there are studies on different species of *Aspergillus* in mangroves, reports of *A. niger* and *A. aculeatus* are scarce or none. Moreover, most of the studies focus on biotechnological and bioremediation potential. *Aspergillus* species reduces high concentrations of heavy metals, but also produces a large number of mycotoxins and secondary metabolism, which may be capable of producing a bulk of bioactive compounds that are used at pharmaceutical industry (Frisvad et al., 2018; Shu-Lei et al., 2020). We need more research about *Aspergillus* species and their applications to produce different bioactives secondary metabolites.

Exploratory studies, as done in this work, allows to generate new concerns about the participation of fungi on the mangrove ecosystems. As well as assessing their possible role as protector for their hosts or determining whether they are pathogenic fungi that cause deterioration and breakdown of wood in mangroves. Briefly, this study was focused on endophytic fungus, turning it a potential candidate to the future applications for more investigations. Therefore, diversity of fungi in mangrove ecosystems are necessary worldwide, starting with the genomes sequencing, since it may represent a useful strategy for finding new metabolic pathways and, subsequently, new bioactive compounds and enzymes.

5 Conclusion

This study we report the molecular and morphological characterization of fungus isolated from mangrove belonging to clade Nigri, *Aspergillus niger* and *Aspergillus aculeatus*. This work is an effort to understanding the distribution of fungus species and highlight the importance to determine the fungus role in the mangrove ecosystem.

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Appendix

Table 1. A: List of GenBank accession number of species used in the present study.

Species	*Aspergillus aculeatus*	*Aspergillus japonicus*	*Aspergillus niger*	*Aspergillus tubingensis*
ITS sequences, accession number	MK644143.1	LC496497.1	KY082744.1	MN589663.1
	MG548756.1	MK035987.1	KY400582.1	MN239975.1
	MK371746.1	LC496498.1	KY566164.1	KY593521.1
	MN187297.1	MH861173.1	MF379661.1	KY593522.1
	MF564097.1		MG575468.1	KY593523.1
	MK271293.1		MG669185.1	KY593524.1
	MK035984.1		MG675233.1	MF143083.1
	MN187365.1		MG733652.1	MF379660.1
	MN187971.1		MG734750.1	MG279093.1
	MK886612.1		MG734751.1	MG733758.1
	MN187974.1		MG833314.1	MG991653.1
	MK911714.1		MG840739.1	MH045586.1
	MK392046.1		MG991588.1	MH398047.1
	MK418753.1		MG991627.1	MH540151.1
	MK518394.1		MH064151.1	MH854604.1
	MK559536.1		MH109325.1	MH858714.1
	MN088378.1		MH181162.1	MN187071.1
	MN173148.1		MH855726.1	
	MN186997.1		MH892847.1	
	MN396714.1		MK028957.1	
	MN509058.1		MK256745.1	
	MH865976.1		MK372989.1	
	MH656795.1		MK577432.1	
	MK713418.1		MK693450.1	
	MK733917.1		MK693453.1	
	MK788185.1		MK949087.1	
	MH892843.1		MN187307.1	
	MH892845.1			
	MK811100.1			
	LC496490.			
	LC496491.1			
	LC496492.1			