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Molecular Identification of Filamentous Fungi Diversity in North Coast Beaches Sands of Puerto Rico

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Abstract:
The Northern region has a great variety of beaches with diverse microbial characteristics. Beach sands receive direct contamination from the garbage generated by people, which serves as nutrient for fungi growth. The objectives of this investigation were to assess the filamentous fungi diversity of four popular beaches; identify the genus and species; and identify the taxonomic relationship between the most abundant fungi. The beaches studied are located in the towns of Vega Baja, Manatí, Barceloneta and Arecibo. One sample of dry sand per month from three equidistant points was acquired every month for a year in each beach. The samples were homogenized according to dry (December-April) and humid (May-November) seasons, for a total of four composite samples per season. The DNA of each sample was isolated and quantified; and, upon sequencing, evaluated by metagenomics analysis with MG-RAST. There were 104 fungi species identified by DNA sequencing analysis. The most abundant were: *Aspergillus penicillioides, Aspergillus terreus, Microascus sp., Arthrographis kalrae, Paramicrosporidium sp., Dokmaia sp*., *Gliomastix polychroma* and *Aspergillus sp*. The taxonomic analysis demonstrated that there is no relationship in the genus of the most abundant species. As significant finding, 66 species of new registries were identified, including *Malassezia restricta*, *Arthrographics eremomycyces*, and *Cephaliophora tropica*. Not only were many of the species pathogenic, several genera of filamentous fungi have been previously isolated from patients in nasal culture, and can cause eye, respiratory and skin disease. The majority of these fungi use direct contact and air transport as transmission vehicle to the host.

Keywords: PCR, DNA, pathogen, beach, sand, filamentous fungi, soil fungi.

INTRODUCTION

Sand, due to the changing environment, encourages the development of fungi that have very special characteristics. These are difficult to detect with the naked eye. The quantitative and qualitative composition of the communities depends on a variety of chemical and biological factors: chemical composition, pH, the surrounding vegetation, the presence of large and small animals, and climatic factors (Abril *et al.,* 1991). Microbiological contamination is greater in sand than in adjacent waters, since sand acts as a passive port for cumulative pollution. The amount of garbage that is observed in the beaches allows to question if this can be a potential source of infection. Diseases of the skin, hair, and nails caused by fungi are common throughout the world and their incidence continues to increase. Filamentous fungi are producers of many skin diseases in the tropics (Gugnani, 2000). These fungi are indicated as potentially dangerous for human health and are found in the sand of the beach. It is important to know the diversity and quantity of these to protect this natural resource that is part of the country's economy, tourist visitors, recreation areas, and public health. In the summer season the number of consultations related to skin infections caused by fungi increases. This is because in the summer there are adequate conditions such as the increase in temperature and humidity that facilitate the proliferation of these microorganisms (Jain *et al.,* 2010). Concern has been shown about real and potential health risks due to exposure to sand on beaches (Néstor *et al.,* 1984; Mendes *et al.,* 1997).

Beaches around the world are very important for the country's economy. Conducting studies of the variety of fungi shows the quality of water and sand. Public health is directly or indirectly associated with human contamination in the sand (Sabino, 2014). There are fungal communities that live in and out of the sea. Aquatic fungi can thrive as mandatory marine species or as optional marine fungi. Terrestrial fungi can also grow in marine environments when conditions are adequate (Alwakeel, 2016). In a sand study of the deserts of Saudi Arabia and Jordan, the analysis was performed through the sequencing of generation of the 18S rRNA genes and by sequencing the internal transcribed spacer region (ITS). The most identified genera were *Fusarium* (*F. redolens, F. solani, F. equiseti), Chaetomium* (*C. madrasense*) *and Albifimbria* (*A. terrestris*). It should be noted that this study demonstrated unexpectedly large fungal biodiversity in the desert sand of the Middle East and its possible role and implications in human health (Murgia, 2018).

Some methods have been identified by phenotypic characterization, where both macroscopic and microscopic characteristics are shown. The presence or absence of hyphae, spore formation and their release mechanisms has been observed. The biological, ecological, biochemical tests and the study of its structure are also observed (Webster, 2009). The evolution of the changes since DNA nucleotide analysis has been a crucial part of the change in the traditional fungal taxonomy (Guarro *et al.,* 1999).

In medical laboratories the use of molecular analysis has proven to be fast and accurate for the identification of the fungus present in people. Many published studies for the identification of pathogenic fungi in humans indicate that the most studied species are *Aspergillus, Fusarium, Scedosporium* and other pathogens (Gilgado *et al.,* 2005; Balajee *et al.,* 2009). DNA is the main source of genetic information of living beings. Therefore, the science of phylogeny establishes the evolutionary relationships between organisms where its objective is the comparison of molecular fingerprints, based on the comparison of sequences of coding and non-coding regions of DNA (Gotelli, 2004; Padial *et al.,* 2010).

The objectives of this investigation were to assess the filamentous fungi diversity of four popular beaches; Identify the genus and species of the filamentous fungi of the dry sand of the beach, estimate the taxonomic relationship between the most abundant fungi in the different beaches, and identify the pathogenic species and the total of species.

MATERIALS AND METHODS

Location and during of study

Samples of dry sands were taken from the beaches of Vega Baja (Marbella), Manatí (Tubos), Barceloneta (Criollas) and Arecibo (Caza Pesca), for one year, once a month. Surface samples of dry sand with sterile spatulas in three points equidistant and parallel to the coastline to obtain representative samples of each beach.

Preparation of sample

Samples from each beach were divided by dry and wet season, homogenized to form a composite sample, placed in a sterile plastic bag for each beach and divided into two seasons for each beach. For a total of eight samples, four of wet season and four dry seasons. The dry season months are December, January, February, March, April. Those of wet season are May, June, July, August, September, October and November (Jury *et al.,* 2007; Torres, 2014; Echevarría, 2019).

Extraction of DNA

DNA was extracted using ultra-Clean soil DNA Isolation Kit (MO BIO lab) kit according to vendor protocol. (Catalog no. 12888-50). https://mobio.com/products/dna-isolation/soil/powersoil-dna-isolation-kit.html

DNA quantification

The quantification and purity of the DNA, it was determined using the QUBIT instrument. The sample measurements and standards can be found in the QUBIT Assays protocol, which can be found at the following web address. https://www.thermofisher.com/pr/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/qubit-assays.html

Polymerase chain reaction (PCR) and DNA sequence analysis

The samples were sent and analyzed in the Molecular Research Laboratory (MRDNA) in US, Texas. In the following link you can find more information http://www.mrdnalab.com/.

The laboratory (MRDNA) performed the shotgun DNA sequence, analyzing genome sequencing steps including the isolation and purification of genomic DNA, fragmentation, ligation to sequencing and purification adapters. After the amplification and denaturing steps, the libraries can be pooled and sequenced. 50 ng of DNA from each sample was used to prepare the DNA libraries using the Nextera sample preparation kit (Illumina). The pooled library was loaded onto a 600 Cycles v3 Reagent (Illumina) cartridge and the sequencing was performed on a Miseq sequencer (Illumina). Diversity analysis bTEFAP® 454 3k ITS1-4 in house assay.

Transcriptome sequence (RNA) Transcriptome sequencing steps include the isolation, purification, chemical fragmentation of mRNA and its conversion into double-stranded cDNA using random hexamer primers. The construction of sequencing libraries was initiated with the generation of blunt-ended cDNA, followed by ligation to the RNA sequencing adapters. After the amplifications and denaturation, the libraries were grouped and sequenced. 2 µg of total RNA (or 250 ng of mRNA) of each sample was used to prepare the libraries using TruSeq RNA sample preparation kits (Illumina). The libraries were quantified and grouped, and the sequencing was performed as previously mentioned.

The cDNA sequencing steps include the purification and generation of blunt-ended cDNA, followed by ligation to the adapters for sequencing, amplification, denaturation and sequencing. 250 ng of double-stranded cDNA from each sample was used to prepare the libraries using TruSeq RNA sample preparation kits (Illumina). The grouped library was sequenced as previously mentioned in a Miseq (Illumina).

Sequencing Analysis

Samples were analyzed using the MG-RAST metagenomic analysis server (http://metagenomics.anl.gov/). The metagenomic

sequences were annotated using the evidence-based annotation method. Sequences were compared against protein databases using BLASTX at an E-value cutoff: 1 x 10^{E-5}. The predicted genes were tabulated and classified into functional categories from lower orders (individual genes) to higher orders (cellular processes). The relative abundance of each gene was calculated by dividing the hits of similarity of genes of an individual by the total hits against any of the database. The final operational taxonomic units (OTUs) were taxonomically classified using BLASTN against a cured database derived from Green Genes, RDP II and NCBI (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi, http: //rdp.cme.msu.edu, www.ncbi.nlm.nih.gov) and compiled at each taxonomic level. The OTUs were grouped at 3% divergence (97% similarity) through the 18S rRNA, which corresponds to the level of species, while 95% similarity (5% divergence) corresponds to the level of gender.

Taxonomic tree

For the construction of the taxonomic tree, NCBI Taxonomy Browser was used to locate the taxonomic identifier of the species or genera to be included in the construction of the taxonomic tree with phylo T version 2015.1.

RESULTS AND DISCUSSION

The presence of fungi on the beaches studied in the dry and wet season was analyzed in terms of species and abundance. A total of 38 species were identified on the Caza Pesca beach in Arecibo, of which one (2.6% of the species) was found in both seasons, seven (18.4% of the species) were found during the wet season only, and thirty (78.9% of the species) were found during the dry season. The most abundant species in the wet season is *Aspergillus pecillioides* (45.35%) of the sequences in the sample, while during the dry season *Aspergillus terrus* predominates (14.26%). The fungus *Aspergillus pecillioides*, is halotolerant and is associated with allergic rhinitis. Its route of exposure is through inhalation (Nazareth *et al.,* 2014; Petrova *et al.,* 2011; Hamilos, 2010), while *Aspergillus terreus* can cause onychomycosis in the nails (St-German *et al.,* 2011).

On the Marbella beach in Vega Baja, a total of 34 species were identified, of which five (14.7% of the species) were found in both seasons, 16 (47.1% of the species) were found during the wet season only, and 13 (38.2% of the species) were found during the dry season. The most abundant species in the wet season is *Microascus sp*. (80.95% of the sequences in the sample), while during the dry season *Arthrographis kalrae* predominates (83.41%). The genus *Aspergillus penicillioides* (45.35%) is found in the soil, mainly in decaying vegetative material. It is considered an opportunistic pathogen of animals, insects and humans (Sandoval *et al.,* 2016). He was identified in Puerto Rico, as it causes onychomycosis, sinusitis. It has been isolated on commercial land and garden soil (Berger, 2015; Sugiura *et al.,* 2010; Vos *et al.,* 2012).

On the beach Criollas in Barceloneta a total of 34 species were identified at the molecular level, of which one (2.9% of the species) was found in both seasons, two (5.9% of the species) were found during the wet season only and 31 (91.2% of the species) were found during the dry season. The most abundant species in the wet season is *Dokmaia sp.* (95.44% of the sequences in the sample), while during the dry season, *Paramicrosporidium* predominant (14.47%). The genus *Dokmaia sp*.is considered an endogenous fungus isolated from plant leaves (Promputtha, 2003). The genus *Paramicrosporidium* is obligate intracellular parasites of various species of animals, which affect both vertebrates and invertebrates (Corsaro *et al.,* 2014).

On the beach, Tubos in Manatí identified a total of 18 species, of which two (11.1% of the species) were found in both seasons, ten (55.6% of the species) were found during the wet season only, and six (33.3% of the species) were found during the dry season. The most abundant species in the wet season is *Aspergillus* (18.38% of the sequences in the sample), while during the dry

season *Gliomastix polychroma* predominant (55.09%). The genus *Aspergillus*, are saprophytic filamentous fungi of the soil, are also found in spoilage foods, leaves and decomposing garbage. Several species are pathogenic to human (Varga *et al.,* 2011). The genus *Gliomastix polychroma* - its natural habitat is in the remains of plants, wood, cellulose, earth and cellulosic material. They can cause opportunistic infections in humans and animals (Alejandra *et al.,* 2012). Some of the diseases they can cause are mycetoma, onychomycosis, hyalohyphomycosis, keratitis, endocarditis and meningitis among others (Finsher *et al.,* 1991; Fernandez *et al.,* 2013). The Tubos beach in Manatí has two species of fungi that are found in both seasons, wet and dry. These are *Aspergillus penicillioides* and *Malassezia restrictedta*. It should be noted that both fungi are pathogenic, with *M. restricta* causing dermatitis and skin conditions (Nazareth *et al.,* 2014; Petrova *et al.,* 2011; Hamilos, 2010; Ambujavalli *et al.,* 2015).

Figure 1 shows most of the fungus species that are unique to the determined beach. Some beaches share species in common, for example, the beach of Caza Pesca and Marbella share one (2.4%) species in common during the wet season (w). Table 1 shows the breakdown of dry season species for each beach. In (Figure 2), the Venn diagram shows most of the fungus species that are unique to the determined beach. Some beaches share species in common, for example, the beach of Caza Pesca and Criollas that share five (6.4%) species in common during the dry season (d); from beach Marbella and Tubos that share two (2.6%) and Criollas beach with Tubos that share one (1.3%) species.

In table 2, show a breakdown of dry season species for each beach (d). The species with the greatest dominance was *Dokmaia sp*, which obtained 95.44% and was found at Criollas beach in the wet season. In (Table 3 shows the percentage of sequence per sample in each beach per season. The species with the greatest dominance was *Dokmaia sp*, which obtained 95.44% and was found at Criollas beach in the wet season. The other predominant species was *Arthrographis kalkarae*, which obtained 83.41%, in the dry season at Marbella beach.

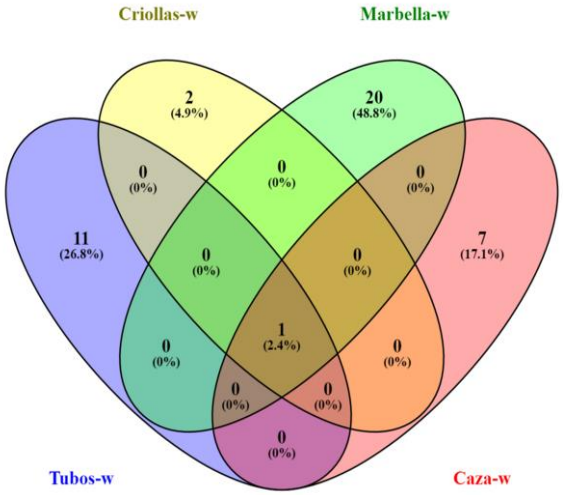

Fig. 1. The Venn diagram shows most of the fungus species that are unique to the beach determined in the wet season (w).

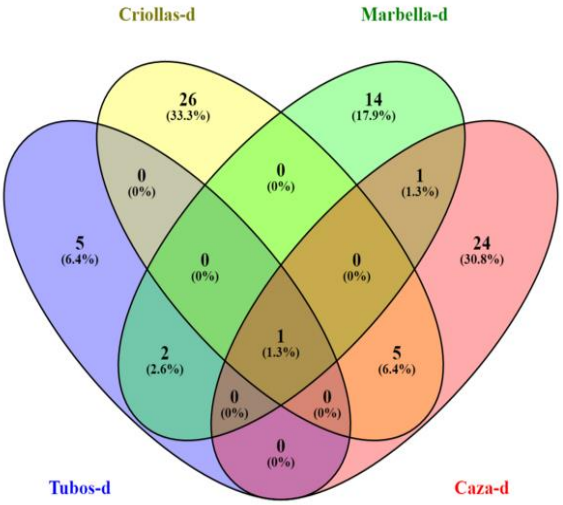

Fig. 2. The Venn diagram shows most of the fungus species that are unique to the beach determined in the dry season (d).

Table 1. Breakdown of wet season species per beach (w).

Caza Pesca -w	Marbella-w	Criollas-w	Tubos-w
Aspergillus penicillioides	*Penicillium citrinum*	*Dokmaia monthadangii*	*Aspergillus penicillioides*
Cladosporium sp.	*Aspergillus nidulans*	*Dokmaia sp.*	*Aspergillus sp.*
Clavulina sp.	*Cladosporium cladosporioides*	*Aspergillus penicillioides*	*Cephalosporium curtipes*
Cordyceps memorabilis	*Aspergillus penicillioides*		*Engyodontium album*
Curvularia lunata	*Lentinula edodes*		*Lecanicillium saksenae*
Euoidium mutisiae	*Gibellulopsis nigrescens*		*Malassezia restricta*
Lecanicillium psalliotae	*Aspergillus niger*		*Metarhizium flavoviride*
Malassezia globosa	*Phaeosphaeria eustoma*		*Mycoleptodiscus sp.*
	Hortaea sp.		*Nigrospora sp.*
	Corollospora gracilis		*Penicillium sp.*
	Aspergillus terreus		*Periconia sp.*
	Corollospora portsaidica		*Pyrenochaeta sp.*
	Corollospora marítima		
	Acremonium sp.		
	Lasiodiplodia pseudotheobromae		
	Scolecobasidium dendroides		
	Sigmoidea parvula		
	Microascus sp.		
	Strumella coryneoidea		
	Aspergillus tamarii		
	Myrothecium atroviride		
8 species	**21 species**	**3 species**	**12 species**

Table 2. Breakdown of dry season species for each beach (d).

Caza pesca -d	Marbella-d	Criollas-d	Tubos-d
Agaricostilbum hyphaenes	*Microdochium sp.*	*Acremonium psammosporum*	*Aspergillus penicillioides*
Aspergillus aculeatus	*Dactylellina lobata*	*Acremonium sp.*	*Cordyceps memorabilis*
Aspergillus penicillioides	*Lulwoana sp.*	*Aspergillus niger*	*Corollospora portsaidica*
Aspergillus sp.	*Rhizocarpon disporum*	*Aspergillus oryzae*	*Geosmithia pallida*
Aspergillus terreus	*Aspergillus penicillioides*	*Aspergillus parasiticus*	*Gliomastix polychromo*
Auricularia polytricha	*Teratosphaeria toledana*	*Aspergillus penicillioides*	*Malassezia restricta*
Chytridium lagenaria	*Malassezia restricta*	*Aspergillus tamarii*	*Sarocladium glaucum*
Cladosporium cladosporioides	*Arthrographis kalrae*	*Aspergillus terreus*	*Wickerhamomyces sp.*
Coprinopsis sp.	*Triparticalcar sp.*	*Boletus floridanus*	
Devriesia lagerstroemiae	*Faurelina indica*	*Calcarisporiella thermophila*	
Galactomyces geotrichum	*Coniosporium sp.*	*Catenaria sp.*	
Graphium dubautiae	*Mycosphaerella sp.*	*Cephaliophora tropica*	

Hygrocybe splendidissima	*Corollospora portsaidica*	*Chaetomium aureum*	
Hypomyces cervinigenus	*Metarhizium anisopliae*	*Chaetomium sp.*	
Microascus trigonosporus	*Corollospora maritima*	*Epichloe festucae*	
Microdochium sp.	*Microascus sp.*	*Laetisaria sp.*	
Mycogone perniciosa	*Strumella coryneoidea*	*Lasiodiplodia pseudotheobromae*	
Oedogoniomyces sp.	*Arthrographis eremomyces langeronii*	*Magnaporthe grisea*	
Paraphaeosphaeria sp.		*Myrothecium roridum*	
Passalora sp.		*Nakataea magnaporthe salvinii*	
Penicillium citrinum		*Paramicrosporidium fungal sp.*	
Penicillium sp.		*Paxillus involutus*	
Phaeosphaeria halima		*Penicillium citrinum*	
Phaeosphaeria typharum		*Pestalotiopsis sinensis*	
Phlyctochytrium sp.		*Phaeosphaeria halima*	
Phoma sp.		*Phaeosphaeria typharum*	
Sarocladium strictum		*Phoma sp.*	
Stachybotrys bisbyi		*Sordaria fimicola*	
Sterigmatomyces elviae		*Stachybotrys sp.*	
Stromatonectria caraganae		*Stachybotrys zeae*	
Teratosphaeria sp.		*Strobilomyces afroboletus luteolus*	
		Verticillium sp.	
31 species	**18 species**	**32 species**	**8 species**

Table 3. Predominant species, season, most abundant fungal species and percentage of sequences of samples.

Beach	Genus and species	NCBI taxID	Sample percentage
Caza Pesca wet season	*Aspergillus penicillioides*	41959	45.35%
Caza Pesca dry season	*Aspergillus terreus*	33178	14.26%
Marbella wet season	*Microascus sp.*	5594	80.95%
Marbella dry season	*Arthrographis kalrae*	241728	83.41%
Criollas dry season	*Paramicrosporidium fungal sp.*	1493516	14.47%
Criollas wet season	*Dokmaia sp.*	397757	95.44%
Tubos dry season	*Gliomastix polychroma*	706482	55.09%
Tubos wet season	*Aspergillus sp.*	5065	18.38%

The most abundant genus (with a count of species greater than 2000) where the species *Arthrographis*, obtained the highest count (11311). The results revealed the presence of pathogenic fungal species in the sand samples of each beach, indicating possible risks to public health. This for people who meet the part of the dry sand on the beach (Berger, 2015). The taxonomic analysis

shows that, except for the species found at Tubos beach in the wet season and Caza Pesca in both seasons, there is no relationship in the genus of the most abundant species of fungi identified (Figure 3). The genus with the highest abundance of species was *Aspergillus*. The most divergent genus was *Paramicrosporidium sp.* The genus with the highest abundance of species is *Aspergillus*. The most divergent genus *Paramicrosporidium*. The most abundant genus with a count of more than 2000, where the species *Arthrographis*, obtained the highest count (11311). The other genues was *Aspergillus* (8852), *Microascus* (7241), *Gliomastix (*3301), *Cordyceps* (2849), *Dokmaia* (2630) and *Lecanicillium* (2246). All were pathogens.

The total of all the species found in the analysis by beach and season is summarized in (Supplementary Table S1). Some species of pathogenic fungi found together with the disease they cause are summarized in (Supplementary Table S2). Given the search of literature in databases and repositories (Cantrell *et al.,* 2006), the following genus and species not reported in Puerto Rico were identified previously. See Supplementary table S3.

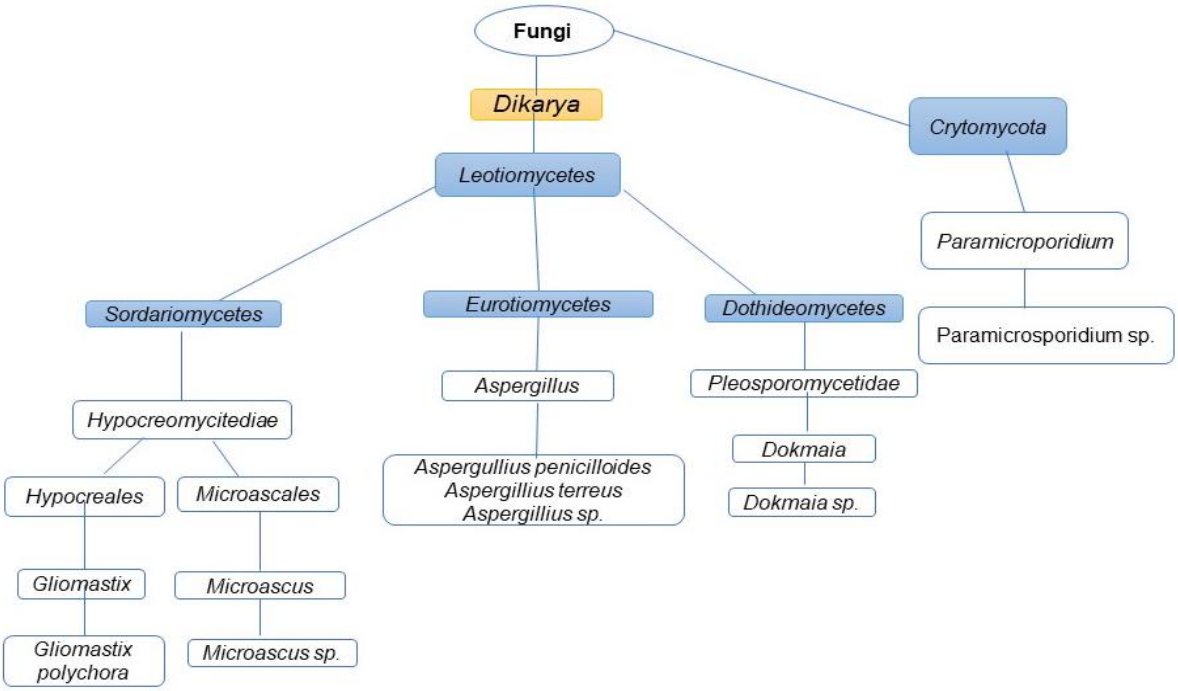

Fig. 3. The taxonomic tree shows that there is no relationship in the genus of the most abundant species of fungi identified by molecular analysis.

CONCLUSION

Identificated 104 species and 76 genus of fungi. The taxonomic analysis showed that, with the exception of the species found in Tubos beach in the wet season and in Caza Pesca in both seasons, there is no relationship in the genus of the most abundant species of the fungi identified by molecular analysis.

The species *Arthrographis* obtained the count of 1131 and this was identified in the

beach of Marbella in Vega Baja in the dry season. At the Criollas beach in Barceloneta, a count of 8852 of the genus *Aspergillus* was obtained. On the beach in Marbella, both wet and dry season, and at the Caza Pesca beach of Arecibo, the count of the genus *Microascus* was 7241. In The Tubos, Manatí, in the dry season, the most abundant genus with a count of 3301 was Gliomastix.

On the beach of Caza Pesca in Arecibo, in wet season and on the beach the Tubos in Manatí, in the dry season, the most abundant genus was *Cordyceps* with a count of 2849. The genus *Dokmaia*, with a count of 2630, was identified in the Criollas beach in Barceloneta in wet season. The genus *Lecanicillium* obtained a count of 2246 and was identified in the Tubos beach in Manatí, in the wet season, and in Caza pesca in Arecibo, in the wet season.

Comparing the four beaches in the wet season, in the samples, it was identified that they share a common species, which means 2.4%. The species in common was *Aspergillus penicilloides*. That same species was detected in common on the four beaches in both seasons. The most abundant were: *Aspergillus penicillioides, Aspergillus terreus, Microascus sp., Arthrographis kalrae, Paramicrosporidium sp., Dokmaia sp., Gliomastix polychroma and Aspergillus sp.*

In the dry season, at Criollas beach, five (6.4%) species were detected in common with the Caza Pesca beach. The beach Marbella has in common one (1.3%) species with The Criollas and the beach the Tubos to buy two (2.6%) species with the beach Marbella. In the dry season they all have the species *Aspergillus penicilloides* in common. In the samples were isolated: *Aspergillus emericella nidulands, Clodosporioides, Rhizocarpo disporum, Aspergillus penicillioides, Aspergillus oryzae, Acremonium psammosporum, Aspergillus niger, Aspergillus sp. Phoma sp. Malassezia restricted, Graphium dubautiae, Arthrographis kalkarae, Aspergillus aculeatus, Curvularia lunata, Aspergillus terreus, Aspergillus parasiticus, Cladosporium sp, Acremonium, Malassezia globosa, Aspergillus tamarii* and *Eremomyces langeonii.*

In the fungi identified in the study, such as *Acremonium, Arthrographics kalkarea*, *Cladosporium, Graphium, Malassezia and Phoma*, its transmission vehicle is endogenous, its host is human and cause severe local or multisystemic infections, especially in suppression immune (Berger, 2015).

As the literature indicates, when visiting the beaches, you should use a towel to sit on the sand, also use glasses to protect the eyes from contact with the spores. This works as a shield of protection for the spores that transports the air and that are in the sand and that also are acquired by direct contact, entering by wounds in the skin and the eyes (OMS, 2003).

This research showed that the filamentous fungi isolated from the samples are mostly pathogenic and with the reference of other studies it indicates that we have to start monitoring and creating controls so that it does not become a public health problem.

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CONFLICT OF INTEREST

All the authors have declared that no conflict of interest exists.

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