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Fungi Associated with Sand and Plants from Marine Coastlines: Potential Relevance for Human Health

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Abstract

The fungal community associated with beach sand and plants located along marine coasts are an under-studied area of research despite its potential relevance to human health. In this study, we isolated and identified the cultivable mycobiota associated with sand and plants collected along the coast of Gran Canaria (Spain) using culture-dependent and -independent methods. Clinically relevant species belonging to *Cryptococcus* spp. and related genera such as *Naganishia* and *Papilotrema* were isolated and identified from shoreline plants. Moreover, *Candida tropicalis* was isolated from beach sand, and *Aspergillus fumigatus* and *Aspergillus terreus* strains were associated with both types of samples (*i.e.*, plants and beach sand). We conclude that beach sand and shoreline plants are potential reservoirs of fungi of high clinical interest. We recommend including beach sand and plants from the environment when assessing the quality of marine coastal systems. Our results open a framework for studying the natural marine environment and its role in the epidemiology of infectious diseases in order to more accurately manage public health.

Keywords

Sand Beach, Shoreline Plants, Reservoir of Pathogenic Fungi, *Cryptococcus* spp., *Aspergillus* spp.

1. Introduction

The importance of understanding which environmental fungi and yeasts could affect public health is growing every year [1] [2] [3] [4]. In this context, the role of environmental mycobiota in disease-causing clinical samples, particularly in immunocompromised individuals, has been regularly reported [5]. Several articles have aimed to fill this knowledge gap regarding the identification of fungal

pathogenic natural reservoirs and the clinical implications of these isolated fungi, such as environmental *Aspergillus* strains [6] [7]; environmental black yeast, including *Aureobasidium* spp. and *Monilliela* spp. [8]; emerging environmental *Candida* spp. strains [9] [10]; and environmental *Cryptococcus neoformans* and *Cryptococcus gattii* strains [11].

However, different environments require more in-depth research, such as sandy beaches, coastal marshes, rural and urban plants, and coastal shrubs and trees. In particular, the fungal flora of coastal beach sand and plants in the immediate vicinity of marine bathing areas is a neglected field of study, despite its potential impact on human health [1] [4]. Therefore, the identification of environmental niches that act as potential fungal pathogenic reservoirs is crucial for the control of mycotic diseases that may be relevant to human health.

The aim of this paper is to isolate and identify the cultivable mycobiota associated with sandy beaches and plants from the marine coastal systems of Gran Canary Island (Canary Archipelago, Spain), through culture-dependent and -independent methods, with particular attention to isolating fungi that are of high clinical importance.

2. Materials and Methods

2.1. Description of the Study Area, Sample Collection, and Preparation

The Canary Islands are located in the southeastern sector of the North Atlantic Ocean, approximately between 27˚ to 29˚ N and 14˚ to 18 ˚W, and are closed to the occidental African coast (**Figure 1(A)**). These islands are a typical example of a "sun, sand, and sea" tourism international destination [12]. Specifically, Gran Canaria is the second largest island in the Canary Islands archipelago (1560 km^2) and supports a large part of this international tourism, as well as local tourism and resident users, as its features make it perfect for recreational sea bathing.

Four sampling zones from Gran Canaria were selected along the east coast of the island (**Figure 1(B)**), according to their exposure to marine currents, anthropogenic activity pressure, periodicity of sand beach cleaning, and presence of plants along the coastline. Las Canteras beach is the northernmost sampling zone (**Figure 1(C)**), which is characterised by intense anthropogenic activity, exposure to ocean currents, and regular sand cleaning by the municipality of Las Palmas de Gran Canaria (the capital of the island). The second is the beach of San Cristóbal, located in the southern part of Las Palmas de Gran Canaria (**Figure 1(D)**). It is characterised as a small and enclosed beach with scarce seawater renewal, as is protected by the breakwater of the port of San Cristóbal. Its sand is cleaned only sporadically and the anthropogenic pressure is high. The third is Hoya del Pozo (**Figure 1(F)**), a beach with high anthropogenic pressure, which is exposed to moderate sea currents and subject to sporadic sand cleaning. Finally, the southernmost samplingzone is Playa del Águila (**Figure 1(E)**), which is located in a shallow-current area. It is characterised by high anthropogenic activity andperiodic sand cleaning. Sampling was carried out prior to the beach sand cleaning

works during the high tourist season in Gran Canaria in 2023 (July-August).

Two samples were harvested from each sampling zone, as shown in **Figure 1**. Sand sampling was carried out through collecting 600 g of marine substrate (top 4 cm) using sterile polyethylene canisters. The canisters were immediately transported in a cool box (4˚C and darkness) to the laboratory for analysis. All samples were processed within a maximum time interval of 24 h. For analysis, 200 g of sand was placed in an orbital shaker at 300 rpm for 30 min with 0.9% NaCl (w/v) solution at a ratio of 1:1. The supernatants were collected and stored individually in sterile polyethylene canisters at 20˚C until use.

Four different plants and shrubs (hereafter referred to as plants) were harvested from the same sampling zones (**Figure 1**). One of them was *Opuntia maxima*, an invasive exotic species (**Figure 1(C)**; **Figure 2(A)**); two others were *Echiumdecaisnei* (**Figure 1(D)**; **Figure 2(B)**) and *Kleinianeriifolia* (**Figure 1(E)**; **Figure 2(D)**), which are endemic shrubs from the Canary Islands; and the last was *Lotus kunkelii*, which is locally endemic to the Gran Canary east coast (**Figure 1(F)**; **Figure 2(C)**).

Figure 1. Location of the sampling zones and sites in Gran Canaria (Spain). (A) Canary archipelago; (B) island of Gran Canaria; (C) Las Canteras beach. Sand sampling site 1 (LC1). Sand sampling site 2 (LC2). *Opuntia maxima* sampling, specimen 1 (O1), specimen 2 (O2), and specimen 3 (O3); (D) San Cristóbalbeach. Sand sampling site 1 (SC1). Sand sampling site 2 (SC2). Echiumdecaisnei sampling, specimen 1 (E1), specimen 2 (E2), and specimen 3 (E3); (E) Playa del Águila area. Sand sampling site 1 (PA1). Sand sampling site 2 (PA2). Kleinianeriifoliasampling, specimen 1 (K1), specimen 2 (K2), and specimen 3 (K3); (F) Hoya del Pozo area. Sand sampling site 1 (HP1). Sand sampling site 2 (HP2); Lotus kunkeliisampling, specimen 1 (L1), specimen 2 (L2), and specimen 3 (L3).White pushpins show sand sampling sites, and green pushpins show plant sampling sites (satellite images obtained from Visor IDECanarias, http://visor.grafcan.es/).

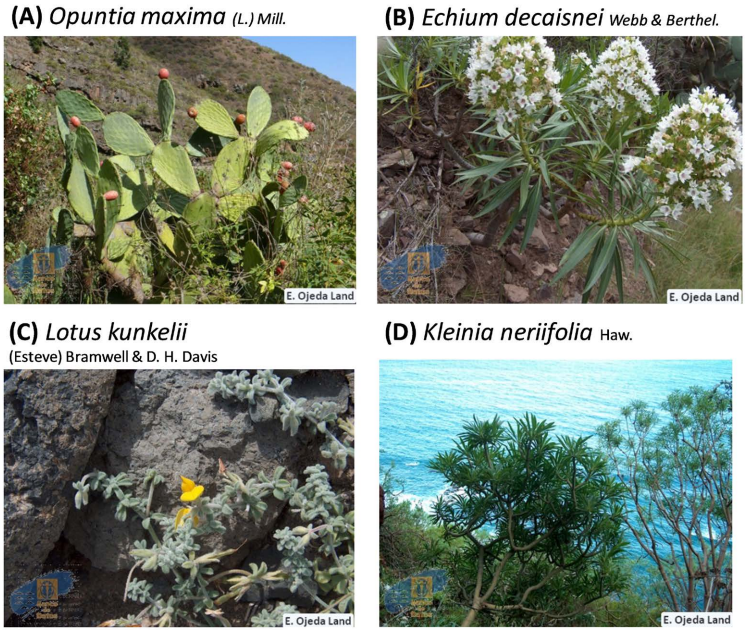

Figure 2. Species of plants and shrubs sampled in this study: (A) *Opuntia maxima*; (B) Echiumdecaisnei; (C) Lotus kunkelii; and (D) Kleinianeriifolia (Banco de datos de biodiversidad de Canarias; https://www.biodiversidadcanarias.es/).

Three plant specimens per each species were sampled using three sterile swabs for each specimen. A 3 cm^2 area of each plant specimen was scraped with each swab. The swabs were transported in a cool box to the laboratory and stored at 4 ˚C, as described previously. For analysis, prior to inoculation, each swab was placed in a tube containing 3 mL of 0.9% NaCl (w/v) solution, incubated at 25˚C for 20 minutes and finally homogenised by vortexing.

2.2. Fungal Isolation and Identification

An aliquot of 1 mL of each supernatant from the sand sample and 1 mL of each homogenate from the plant sample was plated on Petri dishes (15 cm Ø) containing the following culture media: Sabouraud Dextrose Agar, normally used for the cultivation of filamentous fungi and dermatophytes of clinical interest (SDA; 65.5 g; Sigma-Aldrich, St. Louis, MO, United States; in 1 L of ddH_2O), or Staib Agar, used for the selective isolation and differentiation of *Cryptococcus* spp. and yeasts (SA; 68.5 g; Thermo Fisher Scientific, Waltham, Massachusetts, United States; in 1 L of ddH_2O). Each medium was supplemented with antibiotics (Chloramphenicol 0.5 g L^{-1} and Actidione 1 g L^{-1}; Sigma-Aldrich, St. Louis, United States).

Three replicates were performed per medium and per sample. A total of 120 plates were incubated at 25˚C for 21 days. Colony-forming units per gram of sand dry weight (CFU g^{-1}dw) and per cm^2mL^{-1} of homogenate plant sample (CFU cm^2mL^{-1}) were recorded. Fungal strains were isolated and maintained in pure culture.

Fungi were identified by combining morpho-physiological and molecular studies. Following the preliminary determination of genera according to macroscopic

and microscopic features [13] [14] [15], fungal isolates identified as being ofhigh clinical importance (*i.e.*, groups *Aspergillus* spp., black yeasts, and *Cryptococcus* spp.) were transferred to the media recommended by the authors of these selected genus monographs [16] [17] and identifiedto the species level using molecular techniques.

2.3. Molecular Assessment of Isolated Fungi of Clinical Interest

DNA from the pure strains of each target fungus of high clinical importance was extracted using the CTAB procedure described by [18] and modified by [19]. Fresh mycelium of *Aspergillus* and black yeastsstrains were gently scraped from Malt Extract Agar (MEA; 33.6 g; Sigma-Aldrich, St. Louis, MO, United States; in 1 L of ddH_2O) plates, *Candida* strain was harvested from CHROMagar Candida (ChCan; 47.7 g; CHROMagar™, St. Louis, Paris, France; in 1 L of ddH_2O), and *Cryptococcus* spp. strains were scraped from Staib Chloramphenicol Agar (Sch; 68.5g; Thermo Fisher Scientific, Waltham, Massachusetts, United States; supplemented with 0.5g L^{-1} of Chloramphenicol in 1 L of ddH_2O). Fungal strains were individually homogenised in liquid nitrogenand separately transferred to a 2 mL microtube.

Next, the extraction buffer was prepared using CTAB 2% (w/v), PVPP 0.1% (w/v), TRIS-HCl 100 mMat pH 8.6, SDS 10%, EDTA 0.5 M at pH 8, NaCl 4 M, and β-Mercaptoethanol 2% (v/v). Next, 800 µL of extraction buffer was added to each sample. The samples were then kept in a bath at 65˚C for 1 h and gently mixed via inversion three times, in addition to the incubation step. Later, 800 µL of CIA (chloroform-isoamyl alcohol, 24:1 (v/v)) was added, and the samples were centrifuged for 20 min at 3000 rpm in a VWR Micro Star 17R centrifuge (VWR International Eurolab, BCN, Spain). Successive washes in CIA and centrifugations were carried out until the supernatant became whitish. To continue, 2/3 of isopropyl alcohol at −20˚C was added, and centrifugation at 15,000 × g for 30 min was carried out. Afterwards, the isopropyl alcohol was removed and 20 µL of ethanol (80%) was added. Finally, the samples were centrifuged at 15,000 × g for 5 min. The supernatant was discarded and the pellets were re-suspended in 20 µL of DNase-Free ddH_2O and stored at −20˚C until further use. The yield and purity of genomic DNA were calculated from the A260/A280 ratio, measured using a Nano Drop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA extraction was performed in triplicate for each fungal strain.

Molecular identification was conducted based on the partial sequences of specific molecular markers (**Table 1**) through amplification performed in a thermocycler MyCyclerTM (Biorad, Hercules, CA, USA). β-tubulin was amplified using Bt2a/Bt2b [20], while the calmodulin gene was amplified using CL1/CL2A [21]. Both molecular markers were used for the identification of *Aspergillus*s trains. NL1/NL4 were used to amplify the D1/D2 region of the large subunit ribosomal ribonucleic acid (LSU rRNA) [22] to identify *Cryptococcus* and *Candida*s trains, together with amplification of the internal transcribed spacer, including the 5.8S rDNA gene (nrITS), amplified using the ITS1/ITS4 pair primer [23]. Moreover, ITS1/ITS4 was used to identify the strains belonging to the black yeast group.

Table 1. Sequences of primers used in this study.

Target region	Primer names	Sequence (5'-3')	Reference
Internal transcribed spacer	ITS1 ITS4	TCC GTA GGT GAA CCT TGC GG TCC TCC GCT TAT TGA TAT GC	[21]
β-tubulin	Bt2a Bt2b	ACCCTCAGTGTAGTGACCCTT GGTAACCAAATCGGTGCTGCT	[20]
Calmodulin gene	CL1 CL2A	AGCAAGTCTCCGAGTTCAAGG CTTCTGCATCATCAYCTGGACG	[21]
D1/D2	NL1 NL4	GCATATCAATAAGCGGAGGAA GGTCCGTGTTTCAAGACGG	[22]

Each PCR reaction mixture contained 0.5 U Takara Ex Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), 2.5 mM of each dNTP, 10 µL Takara Ex Taq PCR buffer with MgCl2, 10 µM forward and reverse primers (Table 1), and 90 - 95 ng DNA template. The PCR protocol consisted in an initial denaturation at 95˚C for 5 min, followed by 30 cycles at 94˚C for 45 s, 58˚C for 40 s min, and 72˚C for 1 min. The final elongation was carried out at 72˚C for 10 min. The PCR amplification products were checked through agarose (1%) electrophoresis at 75 V. The amplifications were carried out in triplicate.

PCR products were purified using the QIAEX agarose gel extraction kit (Qiagen Inc., Hilden, Germany) and sequenced at Sistemas Genómicos (SYNLAB Group, Valencia, Spain). The resulting ABI chromatograms were visually inspected, trimmed, and assembled to obtain consensus sequences using the Sequencer 5.0 software (Gene Codes Corporation, Ann Arbor, MI, United States). The newly generated sequences were compared through BLASTn analyses (default settings) with those available in public nucleotide databases provided by the NCBI (Bethesda MD, United States) and the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). Similarity values ≥98% (e-value > e-100) were accepted as indicating a credible identification.

2.4. Data Analysis

Data analysis and visualisation were performed using the IBM® SPSS® Statistics v27.0 software (IBM, NY, USA). A Venn diagram was created to compare the proportion of isolated fungi of high clinical importance between beach sand and shoreline plant samples using the Venny 2.1.0 software (BioinfoGP Service, CNB-CSIC, Spain) [24].

3. Results and Discussion

3.1. Fungal Identification and Taxonomic Distribution among Sampling Zones

All studied samples of beach sand and plants were colonised by fungi. Fungal growth per incubated plate ranged from 14 to 38 CFU g^{-1} dw in sand samples

and from 21 to 54 CFU cm^{2-1} in plant samples. Overall, 115 isolates belonging to three phyla were recovered from the 4 beach sand and 4 plant species samples (Figure 3).

As expected, the dominant phylum was Ascomycota (for sand beach min. 54.54% in Playa del Águila to max. 100% in Las Canteras, Figure 3(A); for plants min. 47.37% in *Echiumdecaisnei* to max. 100% in *Lotus kunkelii*, Figure 3(B)), followed by Basidiomycota (for sand beach min. 0% Las Canterasto max. 27.27% in Playa del Águila, Figure 3(A); for plants min. 0% in *Lotus kunkelii* to max. 52.63% in *Echium decaisnei*, Figure 3(B)). Mucororomycota were only detected in sand samples, ranging from 13.64 to 18.18% (Figure 3(A)). In total, 19 genera were isolated and identified through morpho-physiological studies (Figure 4).

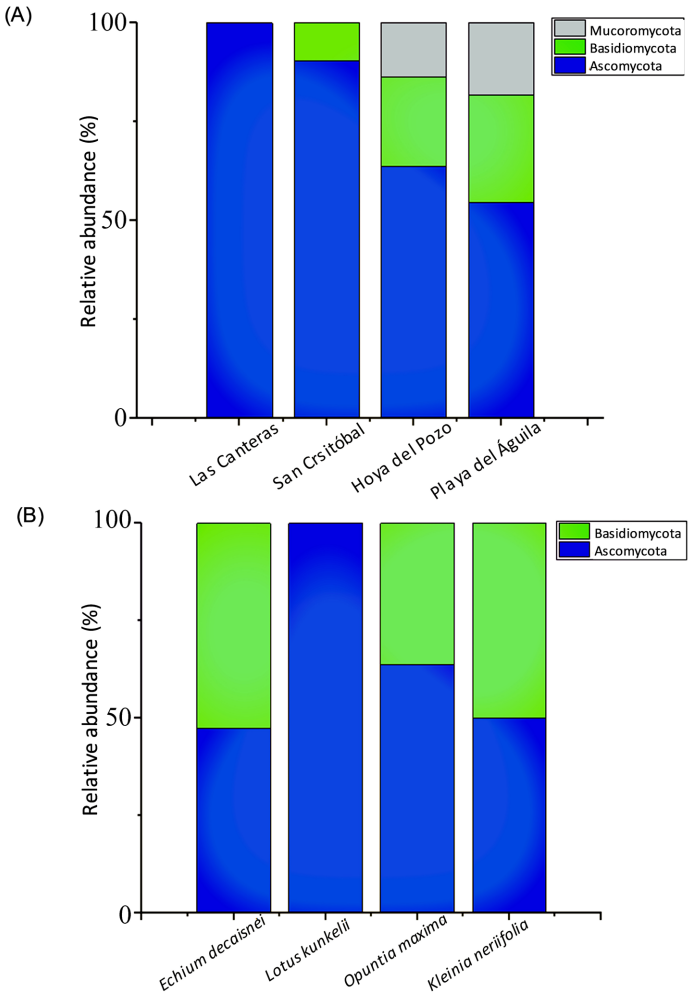

Figure 3. Relative abundance of fungi phyla retrieved from four sampling zones of Gran Canary Island (Spain): (A) Distribution in phyla of fungi isolated from sand beach harvested from Las Canteras, San Cristóbal, Hoya del Pozo, and Playa del Águila beaches; and (B) Distribution in phyla of fungi isolated from the plants sampled along the coastline (*i.e.*, *Echium decaisnei*, *Lotus kunkelii*, *Opuntia maxima*, and *Kleinia neriifolia*).

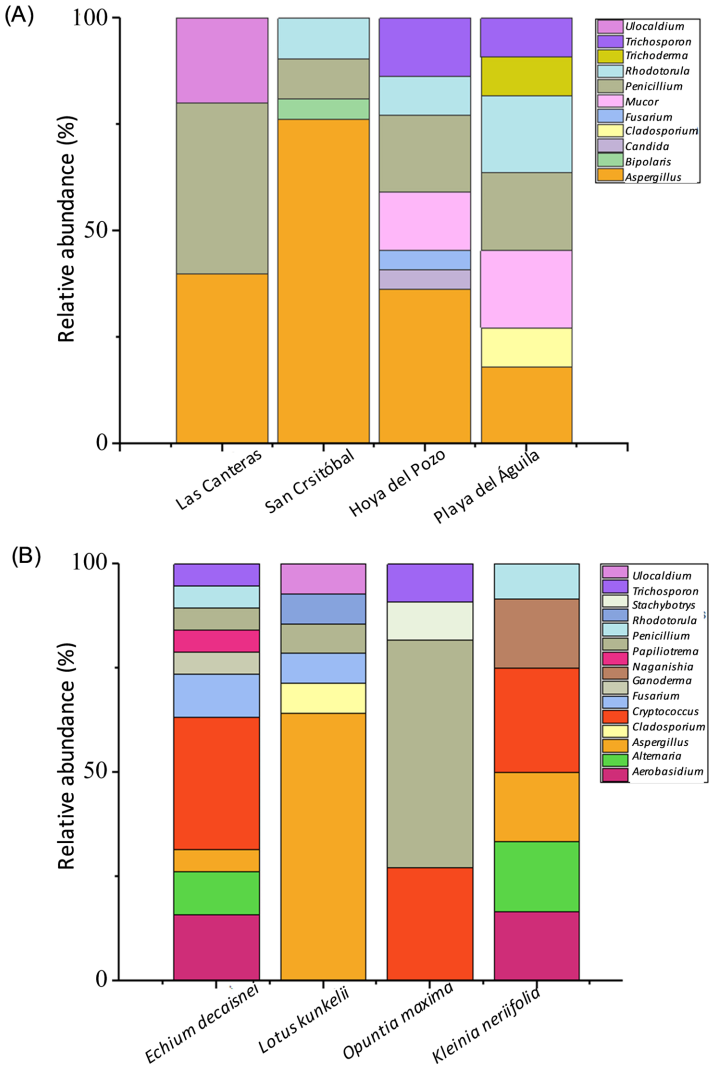

Figure 4. Relative abundance of fungi retrieved from four sampling zones of Gran Canary Island (Spain): (A) distribution in genera of fungi isolated from sand beach harvested from Las Canteras, San Cristóbal, Hoya del Pozo, and Playa del Águila beaches, and (B) distribution in genera of fungi isolated from the plants sampled along the coastline (*i.e.*, *Echium decaisnei*, *Lotus kunkelii*, *Opuntia maxima*, and *Kleinia neriifolia*).

For beach sand, the *Aspergillus* genus was dominant, followed by *Penicillium*. This result is consistent with the evidence reported for the Aspergillaceae family, which is commonly described in marine environments worldwide—probably due to its high adaptation to specific chemical-physical conditions [25]. Moreover, a *Candida* sp. strain was isolated from beach sand. The remaining isolated fungi taxa, which are the major part of the sand fungal community—except for San Cristóbal, where *Aspergillus* genus species represented >70% of the fungal community (**Figure 4(A)**)—were identified as common saprophytes or plant pathogens from the mycobiota associated with beach sand, such as *Fusarium* spp. [4].

To the contrary, for plant samples, the dominance of genera with potential high clinical importance was observed, namely, *Aspergillus*, *Aureobasidium*, and *Cryptococcus* and their related genera *Naganishia* and *Papiliotrema* (**Figure 4**). In particular, more than half of the mycobiota studied—with the exception of the fungal community associated with *Opuntia maxima*—were determined as potential human pathogens (**Figure 4(B)**). The remaining genera were identified as saprophytes (e.g., *Stachybotrys* spp. [26]) or plant pathogens (e.g., *Ganoderma* spp. [27]).

3.2. Fungi of High Clinical Interest

A deeper study through molecular techniques was carried out to identify the isolates affiliated to *Aspergillus*, *Aureobasidium*, *Candida*, *Cryptococcus*, *Naganishia,* and *Papiliotrema* genera at species level, given that they are recognised etiological agents of human diseases [7] [10] [28].

The molecular identification was performed by amplifying and sequencing the appropriate genetic marker for each taxon, as previously described [20] [21] [22] [23] (**Table 1**), which allowed for the identification of seven species: *Aspergillus fumigatus*, *Aspergillus terreus*, *Aureobasidium pullulans*, *Cryptococcus neoformans*/*gattii* species complexes, *Naganishia albida*, *Naganishia globosa,* and *Papiliotrema flavescens* (**Table 2**).

In detail, more taxa with significant pathogenicity were found in plant samples in this study, including *Cryptococcus neoformans*/*gattii* species complexes, *Naganishia albida*, *Naganishia globose*, *Papiliotrema flavescens*, *Aureobasidium pullulans*, and two species belonging to *Aspergillus* genus (**Figure 5**). This result was observed for several reasons.

The first, *Cryptococcus* species have been described as the most common pathogenic yeasts associated with trees [8]. Moreover, the ability of *Cryptococcus* spp. to grow, mate, and produce infectious propagules in association with plants has been reported, maintaining their genetic diversity and virulence factors through these abilities [29]. In addition, these virulence factors make some *Cryptococcus* species, such as *C. neoformans*/*gattii* species complex, the predominant infectious agents of cryptococcosis—a leading cause of death in adults living with HIV [28]. However, clinical series have been published of infections caused by *Naganishia* and *Papiliotrema* species [30]. It has recently been reported that *Papiliotrema flavescens* (formerly *Cryptococcus flavescens*) could produce respiratory system infections that can coexist with lung cancer [31]. Additionally, *Naganishia albida* (formerly *Cryptococcus albidus*) has been reported as a causative agent of some skin infections [32], as well as *Naganishia globosa* (formerly *Cryptococcus saitoi*), which has been described as an environmental yeast with potential opportunistic pathogenicity [33].

Second, *Aureobasidium pullulans* is a dematiaceous fungus that is found mostly in plants, plant debris, and wood, which can cause opportunistic infections [5] [8]. However, deeper research is required as its pathogenicity remains limited and infections are rare, which affect mainly immune-compromised hosts [34].

Table 2. Fungal taxa isolated from beach sand and plants harvested along the coastline of Gran Canary Island (Spain).

Taxa	From beachsand[a]	From plants[b]
Alternaria sp.	-	E; K
*Aspergillus fumigatus**	SC	L
Aspergillus section Nigri	LC; SC; HP; PA	E; L; K
Aspergillus series *Versicolores* (section Nidulantes)	SC	-
*Aspergillus terreus**	SC; HP	L
*Aureobasidium pullulans**	-	E; K
Bipolaris sp.	SC	-
Candida tropicalis	HP	-
Cladosporium sp.	PA	L
Cryptococcus neoformans/gattii species complexes*	-	E; O; K
Fusarium sp.	HP	E; L
Ganoderma sp.	-	E
Mucor sp.	HP; PA	-
*Naganishia albida**	-	K
*Naganishia globosa**	-	K
*Papiliotrema flavescens**	-	E
Penicillium sp.	LC; SC; HP; PA	E; L; O
Rhodotorula sp.	SC; HP; PA	E; K
Scopulariopsis sp.	-	L
Stachybotrys sp.	-	O
Trichoderma sp.	PA	-
Trichosporon sp.	SC; HP; PA	E; O
Ulocladium sp.	LC	L

[a]Las Canteras (LC); San Cristóbal (SC); Hoya del Pozo (HP); and Playa del Águila (PA).
[b]*Echium decaisnei* (E); *Lotus kunkelii* (L); *Opuntia maxima* (O); and *Kleinia neriifolia* (K).
-Not isolated from this matrix. *Molecularly identified.

Third, *Aspergillus fumigatus* is a ubiquitous pathogenic mould that causes various diseases, including mycotoxicosis, allergic reactions, and systemic diseases (invasive aspergillosis) [35]. *Aspergillus terreus*, for its part, is mostly isolated from plant material and soil, being the third-most common filamentous fungus in respiratory infections [6]. In line with this scientific evidence, *A. fumigatus* and *A. terreus* were also isolated from the beach sand samples studied in this paper (**Figure 5**).

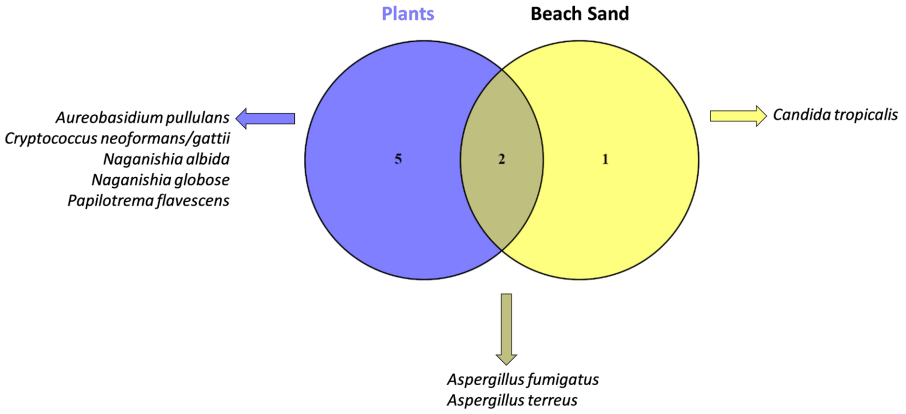

Figure 5. Venn diagram showing the total number of taxa and shared taxa between beach sand and plants from the Gran Canary Island coastline samples.

On the other hand, it is notable that sand samples harboured fewer fungal species of high clinical interest, when compared to those of plant origin (**Figure 5**). Nonetheless, it is noteworthy that the only *Candida* strain (*i.e.*, *Candida tropicalis*) was isolated from the sands of Hoya del Pozo beach, which is characterised by a high anthropogenic pressure and low sand cleanliness (**Figure 1(F)** and **Figure 4(B)**). In this regard, it has been widely reported that most species of *Candida*—both clinical and environmental—can produce candidemia, involving the presence of *Candida* species in the blood, which is the most common fungal bloodstream infection in hospitalised patients [9] [10].

4. Conclusion

In this work, we detailed and compared the cultivable fungal community associated with sand and plants from the marine coastline of Gran Canary Island (Spain). Our results highlight the important role of plants in the immediate vicinity of marine bathing areas and beach sand as a natural reservoir for human pathogenic fungi. Clinically relevant species of *Cryptococcus* and their related genera, such as *Naganishia* and *Papilotrema,* were isolated from shoreline plants; strains of Candida were isolated from beach sand; and strains of *Aspergillus fumigatus* and *Aspergillus terreus* were isolated from both types of samples (*i.e.*, plants and beach sand). Hence, the monitoring of beach sand and shoreline plants is recommended for inclusion in the assessment of the quality of marine coastal systems. Our results provide a framework to study the role of the natural marine environment in depth, including its significant role in the epidemiology of infectious diseases, as pathogens persist and evolve in environmental niches from which they can be transferred to new hosts.

Author's Contributions

M.C.-A. and P.G.-J. conceived, designed, and wrote the manuscript. M.C.-A. conducted the microbiological and molecular assays. All authors have read and approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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