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# Indoor wet cells harbour melanized agents of cutaneous infection

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The biota of black fungi in humid indoor environments was established using a protocol that consisted of non-selective and selective isolation procedures. In total, 113 samples were taken from bathrooms of residences in The Netherlands, Germany and Austria. Samples were processed either (i) directly by culturing on agar media, or (ii) by pre-incubating samples for enrichment in mineral solutions with perlite granules under constant toluene atmosphere for three months. Dilutions from the latter were then cultured and incubated as were those directly plated to agar media. Black colonies were selected and identified by sequencing the rDNA Internal Transcribed Spacer (ITS) region. Twenty-eight strains of black fungi were found in 26 positive samples without enrichment, and 42 strains were isolated from 38 positive samples after enrichment in toluene. The great majority of black fungal species were members of the order *Chaetothyriales*, which is the main order of melanized human opportunistic pathogens. *Cladosporium* species (*Capnodiales*) were the most frequent isolates when no enrichment was applied, as opposed to *Exophiala* species (*Chaetothyriales*) with enrichment. The enrichment method provides insight into a fungal biota commonly occurring in homes which has previously been overlooked. Several species have been previously known only from cutaneous infections and could suggest that bathrooms are a likely reservoir of these fungi.

**Keywords** black fungi, bathroom, indoor environment, toluene, monoaromates

## Introduction

Studies of indoor fungi are mostly devoted to rapidly growing species with dry, airborne propagules. In wet areas such as bathrooms, where *Aspergillus* species may be common [1], water currents may aid in splash dispersal of their hydrophobic conidia [2]. However, fungal biofilms on moist surfaces of bathrooms in human residences, hospital environments and public bathing facilities, may harbour entirely different fungal biota, species having slimy propagules being preponderant. In addition to rapidly growing *Fusarium* and *Acremonium* species, black fungi may emerge in culture but only after several days of incubation [3]. Genera commonly encountered in nutrient-poor, groundwater-derived drinking water are *Phialophora* (now *Cadophora*), *Phoma* and *Exophiala*. The latter genus and its relatives belong to the fungal order *Chaetothyriales*, which is of interest as it contains numerous species that are involved in human infections, ranging from mild cutaneous [4,5] to deep, neurotropic, disseminated and fatal diseases [6,7].

Chaetothyrialean black yeasts are not commonly isolated from the environment, indicating their specific but hitherto unrevealed role in the ecosystem. A large number of selective methods has been developed, among which are pre-incubation in acid and at high temperature [8], with mineral oil [9] and after enrichment with toluene [13]. The latter was based on the discovery that these fungi are capable of degrading volatile organic compounds (VOCs) and tend to survive in environments rich in BTEX and related monoaromatic toxins [10,11]. Thus far these fungi have particularly been isolated from polluted environments such

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as soil under gas stations or creosote-treated railway ties. Zhao *et al.* (unpublished results), however, sampled unpolluted habitats such as berries and noted that black yeasts could be recovered with a toluene-based enrichment protocol while isolation of these fungi had failed with direct sampling. The authors hypothesized that *Exophiala* black yeasts live on trace amounts of precursors of tannins and other polyaromates. The fungi concerned are oligotrophic [12] and hence may reside in habitats without being noticed when routine methods are used. In indoor environments, assimilation of trace amounts of VOCs would be an option. The aim of the present paper was to analyze the potentially pathogenic black yeast mycobiota of indoor wet cells, comparing direct isolation with results of a toluene enrichment protocol.

## Methods and materials

### *Samples and isolation*

A total of 113 samples were taken from bathrooms of residences in three European countries (The Netherlands, Germany and Austria) between January and June, 2008. Sampling locations included permanently moist corners of bathroom walls (17 samples), shower tubes (25), shower filters (24), shower drains (42) and washing bowls (5). Samples were taken from visibly clean surfaces except for the shower drains, which had not been cleaned on a regular basis. Sampling areas were less than 25 $cm^2$. Samples were taken with sterile cotton swabs and immediately put in sterile tubes and the latter were sealed and stored at room temperature. Samples were transferred to media in the lab within three to seven days. The swabs were inoculated into 1 ml demi-water to prepare cell suspensions, and 0.5 ml aliquots were either (i) directly plated onto malt extract agar (MEA) and incubated at 30°C, or (ii) subjected to enrichment in toluene atmosphere. Colony development in directly inoculated MEA cultures was monitored daily in the first week and later three times a week for a total of three weeks. Black yeast-like colonies were isolated and provisionally identified by morphology.

### *Enrichment and isolation in toluene atmosphere*

The solid state-like batch culture technique in a toluene atmosphere [13] was employed to select fungi able to grow with toluene as sole carbon and energy source. Serum flasks of 100 ml were filled with 25 ml of perlite granules saturated in mineral medium [14]. A portion of the cell suspension noted above was transferred to the flasks. Flasks were closed with cotton-wool plugs covered with aluminium foil and put inside desiccators, where a gaseous phase of toluene was prepared by dissolution in 5% (v/v) dibutyl-phthalate. A solution of 140 g NaCl/L was added at the bottom of the desiccator to maintain an internal 90% humidity. The desiccators were incubated at 30°C for at least 3 months. One ml suspensions with 1, 10 or 100 time dilutions from each sample were plated on MEA with penicillin and streptomycin, and incubated at 30°C. Growth of colonies was observed daily in the first week and later three times a week for a total of three weeks. Black colonies were isolated, transferred to fresh MEA plates for purification, and provisionally identified by morphology.

### *Molecular identification*

DNA extraction of the isolates was performed using UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories). Amplification and sequencing were performed according to de Hoog *et al.* [15]. For molecular identification the sequences were adjusted using the program SeqMan ∏ of Lasergene software (DNAStar, Wisconsin, USA) and aligned iteratively using Ward's averaging in the BioNumerics package v. 4.5 (Applied Maths, Kortrijk, Belgium). Nearest neighbours were found by local Blast searches using a black yeast database for research purposes maintained at CBS containing sequences of ex-type strains of all described species in the *Chaetothyriales* known in culture.

### *Statistical analysis*

Statistical analyses were carried out using the $\chi^2$ test, $P < 0.05$ was considered to be statistically significant.

## Results

### *Fungal biota in bathrooms*

While black fungi were isolated by direct culturing from 26 out of 113 samples (Table 1), 38 samples were found to be positive for these fungi after enrichment. In samples from shower tubes, shower filters and shower drains, the number of CFU per isolation plate was higher after enrichment. Wash bowl samples were positive for *Alternaria* without enrichment but negative with enrichment. Among different locations sampled, moist corners of bathrooms and surfaces of shower tubing, although visually clean, yielded large numbers of black fungi. The shower drain showed the largest proportion of chaetothyrialean black yeasts. The positive percentage before enrichment was 19.0%, and increased significantly ($0.01 < P < 0.05$) after enrichment. Most chaetothyrialean black fungi were found in shower drains and filters rather than on other locations in the bathrooms (Table 2).

**Table 1** Percentage of sampled locations before and after enrichment

| Location | Positive samples before enrichment | Positive percentage (%) | Positive samples after enrichment | Positive percentage (%) |
|---|---|---|---|---|
| Corner of bathroom wall | 8/17 | 47.1 | 8/17 | 47.1 |
| Shower tube | 5/25 | 20 | 6/25 | 24 |
| Shower filter | 4/24 | 16.7 | 6/24 | 25.0 |
| Shower drain[a] | 8/42 | 19.0 | 18/42 | 42.9 |
| Wash bowl | 1/5 | 20.0 | 0/5 | 0 |
| Total | 26/113 | 23.0 | 38/113 | 33.6 |

[a] $0.01 < P < 0.05$.

### *Species identification before and after enrichment*

The 28 positive samples before enrichment yielded seven fungal genera comprising 13 species (Table 2), three of which were only isolated without enrichment, i.e., *Alternaria alternata, Petriella sordida* and *Ochroconis cf. constricta. Cladosporium* was the dominant genus, while *Cladosporium halotolerans* was the prevalent species without enrichment. Some species were isolated at comparable frequency with and without enrichment. Two *Pyrenochaeta* species were encountered which were previously known to occur on human skin with two isolates recovered without and one with enrichment. Eleven chaetothyrialean strains were obtained without enrichment, *Exophiala* being the second dominant genus after *Cladosporium*.

The strains after enrichment belonged to four fungal orders (with the phylogenetic position of *Ochroconis* still being undetermined). The number of chaetothyrialean isolates increased threefold after enrichment, which was highly significant ($P < 0.01$). The following five species were isolated only after enrichment; *Cladophialophora boppii, Cladophialophora* spp. (an undescribed species), *Cyphellophora laciniata, Exophiala phaeomuriformis* and *Rhinocladiella similis*. Among 33 members of the order *Chaetothyriales* encountered, *Exophiala* was the dominant genus with the recovery of 20 isolates, and *Exophiala lecanii-corni* was the most frequent species.

## Discussion

Saprobic black fungi with dry conidia, such as species of *Alternaria* and *Cladosporium* are commonly reported from dry indoor environments [16,17]. These species are only rarely involved in human infection. In contrast, melanized species with slimy conidia are only rarely reported as part of the indoor fungi biota but are relatively frequent etiologic agents of human infections. Two species of the coelomycete genus *Pyrenochaeta* were isolated, which are also involved in cutaneous infections [25]. Other black fungal species, preponderantly having slimy conidia and found to be relatively common, belong to the genera *Exophiala, Aureobasidium* or *Ochroconis* [3,18,19]. These fungi share oligotrophic behavior [12]. In our study we found that isolates that not only belonged to *Exophiala*, but others of phylogenetically-related genera, i.e., members of the same order, the *Chaetothyriales*. Species of *Cladophialophora* are exceptional in the *Chaetothyriales*, being the only group within this order which has hydrophobic propagules [12].

Members of *Chaetothyriales*, with either wet or dry conidia or a combination thereof, share a number of remarkable features in their ecology. In addition to oligotrophy, members of the order are frequently associated with environmental monoaromates, which numerous species of the group are able to assimilate [11]. Further, a large share of chaetothyrialean fungi has been reported from human opportunistic infections. Until now, *Cladophialophora boppii, Cyphellophora laciniata, Exophiala phaeomuriformis, Phialophora europaea* and *Rhinocladiella similis* have exclusively been recovered from clinical samples, mostly of skin and nails [4,20–22 and unpublished data]. Their presence in residential wet environments has previously been overlooked due to the application of inadequate isolation methods. On routine media they grow much slower than highly competitive *Aspergillus* and *Penicillium* species, which are suppressed by application of toxic VOCs (volatile organic compounds) as a sole source of carbon and energy. Human skin softened during bathing might be more vulnerable to infection by fungi. Humid environments such as bathrooms and swimming pools are a reservoir for fungi that are known to be involved in human infections [23]. Our data suggest that interaction with these contaminated sites may lead to cutaneous or nail infection.

Toluene enrichment is based on the unique ability of chaetothyrialean fungi to assimilate toxic monoaromates. VOCs are usually present in indoor environments in trace amounts and due to their toxicity they are among the major pollutants in indoor air [24]. Comparing isolation with and without enrichment, the percentage of species from the order *Chaetothyriales* increased threefold when toluene was provided as bait. It may be concluded that chaetothyrialean fungi are stimulated by VOCs. Five species of the order were isolated exclusively when enrichment was

**Table 2** Species before and after enrichment in toluene

| Species | Number before enrichment | Percentage (%) | Number after enrichment | Percentage (%) | Voucher strains CBS | GenBank |
|---|---|---|---|---|---|---|
| ***Pleosporales*** | (4) | | (1) | | | |
| *Alternaria alternata** | 2 | 7.1 | 0 | 0 | | |
| *Pyrenochaeta* sp.* | 1 | 3.6 | 1 | 2.4 | | |
| *Pyrenochaeta unguis-hominis** | 1 | 3.6 | | | | |
| ***Microascales*** | (1) | | (0) | | | |
| *Petriella sordida* | 1 | 3.6 | 0 | 0 | CBS 124169 | GQ426957 |
| ***Capnodiales***[a] | (10) | | (5) | | | |
| *Cladosporium cladosporioides* | 3 | 10.7 | 3 | 7.1 | | |
| *Cladosporium halotolerans* | 7 | 25.0 | 2 | 4.8 | | |
| ***Unestablished*** | (2) | | (3) | | | |
| *Ochroconis cf. constricta** | 1 | 3.6 | 0 | 0 | CBS 124172 | GQ426969 |
| *Ochroconis humicola** | 1 | 3.6 | 3 | 7.1 | CBS 124178 | GQ426961 |
| | | | | | CBS 124179 | GQ426963 |
| | | | | | CBS 124191 | GQ426978 |
| ***Chaetothyriales***[b] | (11) | | (33) | | | |
| *Cladophialophora boppii** | 0 | 0 | 1 | 2.4 | CBS 124175 | GQ426956 |
| *Cladophialophora* sp. | 0 | 0 | 2 | 4.8 | CBS 124183 | GQ426967 |
| | | | | | CBS 124189 | GQ426976 |
| *Cyphellophora laciniata** | 0 | 0 | 1 | 2.4 | CBS 124187 | GQ426974 |
| *Exophiala* sp.* | 1 | 3.6 | 4 | 9.5 | CBS 124180 | GQ426964 |
| | | | | | CBS 124181 | GQ426965 |
| | | | | | CBS 124173 | GQ426968 |
| | | | | | CBS 124192 | GQ426979 |
| *Exophiala lecanii-corni** | 5 | 17.8 | 8 | 19.0 | CBS 124176 | GQ426959 |
| | | | | | CBS 124177 | GQ426960 |
| | | | | | CBS 124188 | GQ426975 |
| | | | | | CBS 124193 | GQ426980 |
| *Exophpiala oligosperma** | 1 | 3.6 | 2 | 4.8 | CBS 124174 | GQ426958 |
| | | | | | CBS 124184 | GQ426971 |
| | | | | | CBS 124190 | GQ426977 |
| *Exophiala phaeomuriformis** | 0 | 0 | 4 | 9.5 | CBS 124182 | GQ426966 |
| | | | | | CBS 124194 | GQ426970 |
| *Exophiala xenobiotica** | 1 | 3.6 | 2 | 4.8 | CBS 124170 | GQ426962 |
| *Phialophora europaea** | 3 | 10.7 | 4 | 9.5 | CBS 124186 | GQ426973 |
| *Rhinocladiella similis** | 0 | 0 | 5 | 11.9 | CBS 124185 | GQ426972 |
| Total[a] | 28 | 100.1 | 42 | 100 | | |

*Repeatedly observed in human infection.
[a]$0.01 < P < 0.05$.
[b]$P < 0.01$.

applied, while species that were only found without enrichment all belonged to other orders than *Chaetothyriales*. Chaetothyrialean fungi mostly infect immunocompetent humans. Of the fungi belonging to remaining orders, *Alternaria alternata* (*Pleosporales*) regularly causes skin lesions in patients [25], but the human hosts are invariably immunocompromised.

*Cladosporium cladosporioides* has the ability to utilize VOCs and remove phenanthrene from soil [26]. The species is a very common saprobe but extremely rarely found as an agent of superficial and systemic infections [25,27,28]. Kantarcioğlu *et al.* [29] once isolated the fungus from human cerebrospinal fluid and a brain biopsy. In our data *Cladosporium halotolerans* was a dominant species without enrichment and was occasionally found after enrichment. The species was recently segregated from *C. sphaerospermum*, due to it being an osmotolerant fungus commonly isolated from hypersaline water of salterns [30] and other environments with low water activity such as peanut shells. We regularly encounter the species on bathroom walls (unpublished data).

*Exophiala phaeomuriformis* has been repeatedly isolated from public bathing facilities such as saunas and bathrooms [31]. The species has a close phylogenetic relationship to *Exophiala dermatitidis* [32], a colonizer of Turkish steam baths, as well as lungs of patients with cystic fibrosis [5], and is able to cause severe brain and disseminated infections in humans [20,33].

*Exophiala lecanii-corni* has been used for biofiltration and is able to remove toluene from air streams with a very high elimination capacity [34]. Gunsch *et al.* [35] proved that the gene ElHDO (homogentisate-1,2-dioxygenase) was

involved in the fungus' ability to degradate VOCs. The species has also been reported from systematic human infections, e.g., respiratory tract, stomach and intestines, and was also involved in cutaneous and subcutaneous cases [5].

*Exophiala oligosperma* environmental strains have been found on low-nutrient or sugary substrates, such as honey or silicone, and on damp inert materials in saunas and swimming pools [36]. The species has the ability to use VOCs such as toluene [37] and phenylacetonitrile [38] as sole sources of nitrogen. The species may cause phaeohyphomycosis [39], which are as a group frequently involved in deep infections. Isolates have been recovered from lung, pleural fluid, stomach, intestine, heart, spleen, lymph node [5], blood [40] and also brain, in the latter case being fatal to the patient [6].

*Exophiala xenobiotica* is a recent segregant of *E. jeanselmei*, mainly differing at the molecular level. Environmental strains are frequently found in habitats rich in monoaromatic hydrocarbons and alkanes, and also isolated from moist environments such as dialysis fluid and bathroom floors [15]. The species is a remarkably common agent of infection with an obvious clinical potential [5]. Most clinical strains were isolated from eye, wound, cyst and from cutaneous and subcutaneous lesions, suggesting environmental inoculation.

*Phialophora europaea* was introduced by de Hoog *et al.* [4] as a species in the *Phialophora verrucosa* complex. The species is regularly isolated from human skin samples and may cause cutaneous and nail infections [G.S. de Hoog, unpublished data].

*Cladophialophora* sp. was isolated only after enrichment in toluene. The two strains attributed to this species had sequences which were not similar to any existing species of *Cladophialophora*. The species has an ability to grow with toluene as the sole source of carbon and energy [13]. Many described *Cladophialophora* species are agents of cutaneous infections, chromoblastomycosis, or fatal systematic infection such as encephalitis [21,41,42], with *C. bantiana* recognized as being obviously neurotropic [42,44].

*Rhinocladiella similis* has been isolated from a foot lesion and from a severe blood infection [7]. The species was found to be consistently different from *E. jeanselmei* in ITS sequences and morphology [36].

*Cyphellophora laciniata* was first isolated from human skin [45] and has thus far not been encountered outside the human body. Several species of the same genus were involved in cutaneous infection in humans [22].

*Ochroconis cf. humicola* is a relative of the neurotropic species *O. gallopavum,* involved in severe brain infections in mainly immunocompromised patients [46–48]. *O. humicola* is a recurrent pathogen on fish [49] and has occasionally been involved in phaeohyphomycosis of cats [50]. The CBS culture collection includes strains from human nails and skin. The taxonomy of this genus is still unresolved.

From the data presented above it is obvious that a large share of the oligotrophic black fungi encountered in wet indoor environments, particularly those isolated after toluene enrichment, have a significant potential to cause human infection. The two species of *Pyrenochaeta* that were isolated had been exclusively known from cutaneous human infections. The fungi that were recovered which belong to the order *Chaetothyriales* are highly significant because this group contains numerous agents of human (sub)cutaneous and systemic, potentially fatal infections, frequently in immunocompetent individuals. The species isolated from wet environments in the course of the present study had previously been reported from cutaneous infections. Due to unsuitable isolation methods routinely employed in laboratories involved in hygiene, and due to difficulties in species identification, black fungi have seldom been reported from indoor and hospital environments. Today, several selective isolation methods are available [8,9,13] and in this paper we explored a methodology based on VOC assimilation which is particularly effective for isolation of black yeasts and relatives, which comprise one of the main groups of agents of cutaneous and other infections [51,52]. Because of insufficient awareness among dermatologists of their clinical significance, black fungi have mostly been discarded as contaminants in dermatological practice. This has led to underdiagnosis of these organisms, and probably to underestimation of their potential health risk. With increasing numbers of immunocompromised individuals as a result of organ transplantation, Cushing's syndrome, collagen vascular disease, hematological malignancies, and AIDS, the prevalence of black fungi in hospital wet cells should be emphasized as a potentially serious health problem.

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