Environmental isolation of black yeast-like fungi involved in human infection

V.A. Vicente1, D. Attili-Angelis2, M.R. Pie3, F. Queiroz-Telles4, L.M. Cruz5, M.J. Najafzadeh6, G.S. de Hoog6*, J. Zhao7 and A. Pizzirani-Kleiner8

1Department of Basic Pathology, Federal University of Paraná, Curitiba, PR, Brazil; 2UNESP Department of Biochemistry and Microbiology, Institute of Biosciences, Rio Claro, SP, Brazil; 3Zoolology Department, Federal University of Paraná, Curitiba, PR, Brazil; 4Clinical Hospital, Federal University of Paraná, Curitiba, PR, Brazil; 5Biochemistry Department, Federal University of Paraná, Curitiba, PR, Brazil; 6CBS Fungal Biodiversity Centre, P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands; 7Department of Dermatology, Fujian Medical University Affiliated Union Hospital, Fuzhou, P.R. China; 8“Luiz de Queiroz” Superior College of Agriculture, University of São Paulo, Piracicaba SP, Brazil

*Correspondence: G.S. de Hoog, de.hoog@CBS.knaw.nl

Abstract: The present study focuses on potential agents of chromoblastomycosis and other endemic diseases in the state of Paraná, Southern Brazil. Using a highly selective protocol for chaetothyrialean black yeasts and relatives, environmental samples from the living area of symptomatic patients were analysed. Additional strains were isolated from creosote-treated wood and hydrocarbon-polluted environments, as such polluted sites have been supposed to enhance black yeast prevalence. Isolates showed morphologies compatible with the traditional etiological agents of chromoblastomycosis, e.g. Fonsecaea pedrosoi and Phialophora verrucosa, and of agents of subcutaneous or systemic infections like Cladosiphialophora bantiana and Exophiala jeaneselmei. Some agents of mild disease were indeed encountered. However, molecular analysis proved that most environmental strains differed from known etiologic agents of pronounced disease syndromes: they belonged to the same order, but mostly were undescribed species. Agents of chromoblastomycosis and systemic disease thus far are prevalent on the human host. The hydrocarbon-polluted environments yielded yet another spectrum of chaetothyrialean fungi. These observations are of great relevance because they allow us to distinguish between categories of opportunists, indicating possible differences in pathogenicity and virulence.

Key words: Black yeasts, Chaetothyriales, chromoblastomycosis, enrichment, environmental isolation, opportunists, phaeohyphomycosis, virulence.

INTRODUCTION

Knowledge of natural ecology and evolution is essential for a better understanding of pathogenicity and opportunism. Members of different fungal orders and families tend to be differentially involved in human mycoses. Among melanised fungi, for example, etiologies of members of Dothideaceae and Herpotrichiellaceae show basic differences (de Hoog 1993, 1997). Of these families, only species belonging to the Herpotrichiellaceae (black yeasts and relatives) are associated with recurrent, clearly defined disease entities such as chromoblastomycosis and neurotropic dissemination in immunocompetent individuals. In contrast, members of Dothideaceae show coincidental opportunism, whereby the infection is largely dependent on the portal of entry and the immune status of the host.

Among the diseases caused by chaetothyrialean fungi (teleomorph family Herpotrichiellaceae), chromoblastomycosis and other traumatic skin disorders are the most frequent (Attili et al. 1998, Zeng et al. 2007). Although the agents are supposed to originate from the environment, their isolation from nature is difficult. This is probably due to their oligotrophic nature, low competitive ability, and in general insufficient data on their natural habitat. Several selective techniques have been developed enabling recovery of these fungi (de Hoog et al. 2005; Dixon et al. 1980, Prenafeta-Boldú et al. 2006, Satow et al. 2008, Zhao et al. 2008, Sudhadham et al. 2008). These investigations indicated that opportunism of these fungi must be explained from the perspective of unexpected environments such as rock, creosote-treated wood, hydrocarbon-polluted soil, and hyperparasitism of fungi and lichens (Sterflinger et al. 1999, Wang & Zabel 1997, Lutzoni et al. 2001).

In the present study we tried to find recover chaetothyrialean fungi from the natural environment in the State of Paraná, Southern Brazil, where chromoblastomycosis and phaeohyphomycosis are frequent in endemic areas. In addition, human-made substrates like creosote-treated wood and hydrocarbon-polluted soil were sampled. Strains morphologically similar to etiological agents of chromoblastomycosis, such as Fonsecaea pedrosoi and Phialophora verrucosa, and to agents of subcutaneous and systemic infections, such as Exophiala jeaneselmei and Cladosiphialophora bantiana, were selected. The aim of this investigation was to clarify whether these fungi were identical to known etiologic agents of disease. Isolates were compared with clinical reference strains on the basis morphological, physiological and molecular parameters.

MATERIALS AND METHODS

Study area and strains

Samples were obtained from 28 localities belonging to three different geographical regions in the State of Paraná, Southern Brazil (Fig. 1). Locations were chosen on the basis of known records of chromoblastomycosis in the “Hospital de Clínicas” of the Paraná Federal University (HC-UFPR). The climate in the region is subtropical, with relatively regular rainfall throughout the
Fig. 1. Sampling locations in the State of Paraná, S.P., Brazil.

Fig. 2. A. Sampling location in the area of ‘FE’ series of samples in the first plateau at Colombo (Paraná) with native species (arrows) dominated by cambara tree (*Gochnathia polymorpha*) and stem palm (*Syagrus romanzoffiana*); B. Sampling location of FE5P4 of *Fonsecaea monophora*, from decaying cambara wood; C. Sample incubated at room temperature in sterile saline solution, containing antibiotics added with sterile mineral oil after vigorous shaking; D. Black yeast colonies on Mycosel medium.
yr (average 12500 mm/yr). The isolates obtained were compared to clinical isolates from the hospital of the Universidade Federal do Paraná, as well as to reference strains from the Centraalbureau voor Schimmelcultures (CBS, The Netherlands; Table 1). Samples from Eucalyptus wood in the “Navarro de Andrade” forest and creosote-treated railway ties near Rio Claro, and from hydrocarbon-polluted soil from the oil refinery of Paulinia, Paulinia state of São Paulo, Brazil, were also investigated (Table 1).

**Fungal isolation**

In each location, fragments of litter and decaying wood showing the presence of black spots, as well as soil samples, were randomly collected. Approximately 20 g from each sample were processed for fungal isolation, with 10 replicates per sample. Each sample was incubated at room temperature for 30 min in 100 mL sterile saline solution containing 200 U penicillin, 200 µg/L streptomycin, 200 µg/L chloramphenicol, and 500 µg/L cycloheximide (Fig. 2C). After the initial incubation, 20 mL of sterile mineral oil was added to the solution, followed by vigorous 5 min shaking and the flasks were left to settle for 20 min. The oil-water interphase was carefully collected, inoculated onto Mycosel agar (Difco) and incubated for 4 wks at 36 ºC (Dixon & Shadowy 1980, Iwatsu et al. 1981). The grown dark colonies were then isolated and stored on Mycosel agar (Fig. 2D).

**Morphology**

Preliminary identification was carried out based on macro- and microscopic features of the colonies after slide culturing on Sabouraud’s dextrose agar at room temperature (de Hoog et al. 2000a). In addition, vacuum-dried samples were mounted on carbon tape and sputtered with gold for 180 s for SEM. Observations were done in a Zeiss DSM 940 A microscope, operated at 5 kV.

**Nutritional physiology**

Some isolates with cultural and morphological similarity to known agents of disease were selected for physiological testing. Growth and fermentative abilities were tested in duplicate, negative controls were added. The fungi were incubated at 28 and 36 ºC on the following culture media: Mycosel, Potato Dextrose Agar (PDA), Minimal Medium (MM), Complete Medium (CM), and Malt Extract Agar (MEA). Assimilation and fermentation tests were carried out in liquid medium according to de Hoog et al. (1995). Halotolerance was tested in a liquid medium at 2.5, 5 and 10 % (w/v) NaCl and MgCl₂. Cycloheximide tolerance was determined in liquid medium at 0.01, 0.05 and 0.1 % (w/v).

**DNA extraction**

About 1 cm² mycelium of 20 to 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 300 µL CTAB (cetyltrimethylammonium bromide) buffer [CTAB 2% (w/v), NaCl 1.4 M, Tris-HCl 100 mM, pH 8.0; EDTA 20 mM, b-mercaptoethanol 0.2% (v/v)] and about 80 mg of a silica mixture (silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Cellte 545, Machery, Düren, Germany, 2.1, w/v). Cells were disrupted manually with a sterile pestle for approximately 5 min. Subsequently 200 µL CTAB buffer was added, the mixture was vortexed and incubated for 10 min at 65 ºC. After addition of 500 µL chloroform, the solution was mixed and centrifuged for 5 min at 20 500 g and the supernatant transferred to a new tube with 2 vols of ice cold 96 % ethanol. DNA was allowed to precipitate for 30 min at −20 ºC and then centrifuged again for 5 min at 20 500 g. Subsequently the pellet was washed with cold 70 % ethanol. After drying at room temperature it was resuspended in 97.5 µL TE-buffer plus 2.5 µL RNAse 20 U mL⁻¹ and incubated for 5 min at 37 ºC, before storage at −20 ºC (Gerrits van den Ende & de Hoog 1999).

**Sequencing**

rDNA Internal Transcribed Spacer (ITS) was amplified using primers V9G and LS266 (Gerrits van den Ende & de Hoog 1999) and sequenced with ITS1 and ITS4 (White et al. 1990). Amplicons were cleaned with GFX PCR DNA purification kit (GE Healthcare, U.K.). Sequencing was performed on an ABI 3730XL automatic sequencer. Sequences were edited using the Seqman package (DNASTar, Madison, U.S.A.) and aligned using BioNumerics v.4.61 (Applied Maths, Kortrijk, Belgium). Sequences were compared in a research data database of black fungi maintained at CBS, validated by ex-type strains of all known species.

**RESULTS**

Eighty-one isolates from a total of 540 showed morphologies compatible with the traditional etiological agents of chromoblastomycosis and phaeohyphomycosis. Twenty-six strains were selected and processed for taxonomic studies and listed in Table 1 with additional strains from hydrocarbon-polluted soil and wood (natural and creosote-treated).

Isolate FE9 was morphologically very similar to Cladophialophora bantiana. Physiological testing demonstrated ability to assimilate ethanol, lactose and citrate, but it was unable to grow at 40 ºC (Table 2). Sequence data proved identity to C. immunda (Table 1). Strain F10PLB was physiologically similar to FE9 of C. immunda (Table 2), which was confirmed by molecular data (Table 1). F10PLA showed physiological characteristics close to the FE9, differing only by growth in the presence of creatine and creatinine (Table 2); also this strain was identified by ITS sequence data as C. immunda. The isolate FP4IIIB was capable of growing with 0.1 % cycloheximide, showed reduced growth in the presence of ethanol and had a maximum growth temperature of 37 ºC (Table 2). It presented ellipsoidal fusiform conidia originating from denticles, consistent with Cladophialophora devriesii. However, molecular data identified the strain as C. saturnica (Table 1). FP4IIA, phenetically identified as Cladophialophora sp. and physiologically similar to FP4IIIB was identified as C. saturnica by ITS sequencing (Table 1). FE11IA and F11PLA had fusiform conidia in chains. FE11IA was unable to assimilate galactitol, developed poorly in the presence of D-glucorionate, but was able to grow in a medium with ethanol; F11PLA assimilated glucorionate having a weak development in the presence of ethanol (Table 2). With ITS sequencing two undescribed Cladophialophora species appeared to be concerned (Table 1).

Strain FE5P4 was isolated from decaying cambara wood (Fig. 2B) in an area of native species (Fig. 2A) dominated by cambara trees (Gochnathia polymorpha) and stem palm (Syagrus romanzoffiana) near Colombo city (Fig. 1). This isolate was morphologically identified as Fonsecaea pedrosi. Physiologically it differed from F. pedrosi by assimilation of L-sorbose, melibiose,
| Morphological ID | Final ITS ID | CBS   | dH  | Vicente / Attili | Origin                      | Source                                      |
|------------------|--------------|-------|-----|------------------|-----------------------------|---------------------------------------------|
| Fonsecaea pedrosoi | Fonsecaea pedrosoi | 18223 | Fp28II | Marmeleiro       | Chromoblastomycosis          |                                             |
| Fonsecaea pedrosoi | Fonsecaea pedrosoi | 18331 | Queiros | São Paulo       | Subcutaneous, compromised    |                                             |
| Fonsecaea pedrosoi | Fonsecaea pedrosoi | 102244 | 11608 | Ipora            | Chromoblastomycosis         |                                             |
| Fonsecaea pedrosoi | Fonsecaea pedrosoi | 102245 | 11610 | Ampere           | Chromoblastomycosis         |                                             |
| Fonsecaea pedrosoi | Fonsecaea monophora | 102248 | 11613 | Piraquara        | Chromoblastomycosis         |                                             |
| Fonsecaea pedrosoi | Fonsecaea monophora | 102246 | 11611 | Campo Largo      | Chromoblastomycosis         |                                             |
| Fonsecaea pedrosoi | Exophiallaa monophora | 102243 | 11607 | Ibituva          | Chromoblastomycosis         |                                             |
| Cladophialophora bantiana | Cladophialophora immunda | 102227 | 11586 | FE9 = 9EMB       | Colombo                     | Stem palm (Syagrum romanzoffianum)          |
| Cladophialophora bantiana | Cladophialophora sp. 3 | 102231 | 11592 | FE11IA          | Colombo                     | Rotten Gochnia polymorpha stem             |
| Cladophialophora devriesii | Cladophialophora satumica | 102230 | 11591 | FP4IIB          | Piraquara                   | Plant litter                               |
| Cladophialophora sp. | Cladophialophora satumica | 102228 | 11589 | FP4IIA          | Rotten wood                 |                                             |
| Cladophialophora sp. | Fonsecaea monophora | 102229 | 11590 | FP8D = 8DPIRA   | Piraquara                   | Plant litter                               |
| Cladophialophora sp. | Cladophialophora sp. 2 | 102236 | 11600 | F11PLA          | Telémaco Borba              | Plant litter                               |
| Cladophialophora sp. | Cladophialophora sp. 2 | 102237 | 11601 | F10PLA          | Telémaco Borba              | Plant litter                               |
| Exophialla lecanii-corni | Exophialla xenobiota | 102232 | 11594 | FE4IIIB         | Colombo                     | Rotten wood                                |
| Exophialla jeaneselmei | Exophialla bergeri | 102241 | 11605 | F14PL           | Cianorte                    | Soil under coffee tree                      |
| Exophialla sp. | Capronia semi-immersa | 102333 | 11595 | FE5IIIB         | Colombo                     | Rotten Araucaria trunk                     |
| Fonsecaea pedrosoi | Fonsecaea sp. 3 | 102223 | 11583 | FE3             | Castro                       | Rotten root                                |
| Fonsecaea pedrosoi | Fonsecaea sp. 1 | 102224 | 11584 | F9PRRA          | Terra Roxa                   | Grevillea robusta wood                     |
| Fonsecaea pedrosoi | Fonsecaea monophora | 102225 | 11585 | FE5P4           | Colombo                     | Rotten wood (Gochnia polymorpha)           |
| Fonsecaea pedrosoi | Fonsecaea sp. 1 | 102254 | 11619 | FE5P6           | Colombo                     | Rotten wood                                |
| Fonsecaea pedrosoi | Fonsecaea sp. 2 | 102226 | 11587 | FE5II           | Colombo                     | Rotten Araucaria trunk                     |
| Phiallophora verrucosa | Phiallophora sp. | 102234 | 11596 | FE3             | Colombo                     | Lantana camara rhizosphere                 |
| Rhinocladiella sp. | Rhinocladiella sp. 1 | 102235 | 11597 | F9PR            | Terra Roxa                   | Grevillea robusta wood                     |
| Rhinocladiella sp. | Cladophialophora immunda | 102249 | 11614 | F10PLB          | Sarandi                     | Rotten Cinnamomum trunk                    |
| Rhinocladiella sp. | Cladophialophora chaetospira | 102250 | 11615 | F3PLB           | Sertanópolis                 | Plant litter                               |
| Rhinocladiella sp. | Exophialla xenobiota | 102251 | 11616 | F3PLC           | Sertanópolis                 | Plant litter                               |
| Rhinocladiella sp. | Fonsecaea sp. 3 | 102239 | 11603 | FE11IB          | Colombo                     | Rotten Lantana camara stem                 |
| Rhinocladiella sp. | Fonsecaea sp. 3 | 102252 | 11617 | FE110IB         | Colombo                     | Plant litter                               |
| Rhinocladiella sp. | Rhinocladiella sp. 1 | 10225 | 11618 | FE101IB1        | Colombo                     | Plant litter                               |
| Rhinocladiella sp. | Rhinocladiella sp. 1 | 102240 | 11604 | F9PRC           | Terra Roxa                   | Podocarpus lamberti branch                 |
| Rhinocladiella sp. | Exophialla xenobiota | 102255 | 11621 | F20PR3          | Jacutinga                   | Soil                                        |
| Rhinocladiella sp. | Fonsecaea monophora | 102238 | 11602 | F1PLE           | Rio Tibagi                  | Soil                                        |
| Exophialla sp. | Exophialla bergeri | 122844 | 18627 | D0009           | Rio Claro                   | Railway tie treated with creosote 15 yr ago |
| Exophialla sp. | Exophialla bergeri | 122843 | 18629 | D0020           | Rio Claro                   | Railway tie treated with creosote 15 yr ago |
| Exophialla sp. | Exophialla bergeri | 122842 | 18636 | D0035           | Rio Claro                   | Railway tie treated with creosote 15 yr ago |
| Exophialla sp. | Exophialla bergeri | 122841 | 18643 | D0201           | Rio Claro                   | Railway tie treated with creosote 15 yr ago |
| Exophialla sp. | Exophialla bergeri | 122840 | 18654 | D0213           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122839 | 18635 | D0029b          | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122838 | 18646 | D0204a          | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122837 | 18651 | D0210           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122836 | 18648 | D0206           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122835 | 18652 | D0211           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122834 | 18653 | D0212           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida | 122833 | 18656 | D0215           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla dermatidida (mel-mut) | 122830 | 18650 | D0209           | Rio Claro                   | Railway tie treated with creosote 16 yr ago |
| Exophialla sp. | Exophialla xenobiota | 122832 | 18647 | D0205           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla xenobiota | 122910 | 18638 | D0044           | Rio Claro                   | Eucalyptus wood                            |
| Exophialla sp. | Exophialla xenobiota | 122831 | 18655 | D0214           | Rio Claro                   | Eucalyptus wood                            |
***Table 1. (Continued).***

| Morphological ID | Final ITS ID       | CBS       | dH        | Vicente / Attili | Origin                  | Source                                      |
|------------------|--------------------|-----------|-----------|------------------|-------------------------|---------------------------------------------|
| Exophiala sp.    | Exophiala xenobiotica<sup>a</sup> | 122829   | 19831     | D0023            | Rio Claro               | Railway tie treated with creosote 15 yr ago |
| Exophiala sp.    | Exophiala xenobiotica<sup>a</sup> | 122846   | 19832     | D0024            | Rio Claro               | Railway tie treated with creosote 15 yr ago |
| Exophiala sp.    | Exophiala xenobiotica<sup>a</sup> | 122828   | 19833     | D0007            | Rio Claro               | Railway tie treated with creosote 15 yr ago |
| Black fungus sp. | Veronaea botryosa<sup>a</sup>     | 122826   | 19839     | D0045            | Rio Claro               | Railway tie treated with creosote 20 yr ago |
| Black fungus sp. | Veronaea botryosa<sup>a</sup>     | 122824   | 19840     | D0047            | Rio Claro               | Railway tie treated with creosote 20 yr ago |
| Black fungus sp. | Veronaea botryosa<sup>a</sup>     | 122822   | 19841     | D0060            | Rio Claro               | Railway tie treated with creosote 20 yr ago |
| Black fungus sp. | Veronaea botryosa<sup>a</sup>     | 122825   | 19842     | D0063            | Rio Claro               | Railway tie treated with creosote 20 yr ago |
| Black fungus sp. | Veronaea botryosa<sup>a</sup>     | 122823   | 19828     | D0017            | Rio Claro               | Railway tie treated with creosote 20 yr ago |
| Aureobasidium sp.| Aureobasidium pullulans<sup>a</sup> | 122827   | 19857     | D0216            | Paulinia               | Polluted soil, Replan Co.                   |

Abbreviations used: CBS = Centraalbureau voor Schimmelcultures; dH = G.S. de Hoog working collection.

Known agent of: *systemic and disseminated disease; mycetoma; chromoblastomycosis; mild cutaneous disease; systemic disease and pulmonary colonization*

<sup>a</sup>Known opportunistic agent (including chromoblastomycosis).

ribitol, xylitol, myo-inositol, glucono-6-lactone, D- and L-lactate, succinate, nitrite, urease and tolerance to 5% NaCl (Table 2). This physiological profile was similar to that of clinical strains FP65 and FP82 (Table 2) originating from symptomatic patients of the same geographic region (first plateau, Fig. 1). With ITS sequencing FE5P4 was identified as *Fonsecaea monophora*. Environmental isolate FP8D morphologically was cladophialophora-like but was identified as *F. monophora* based on molecular data. It had physiological similarity with clinical strain FP82 of *F. monophora* and was isolated from the same location where the patient, a carrier of chromoblastomycosis, had acquired his infection (Pirapuara city, Fig. 1). All strains grew at 37 °C but not at 40 °C, similar to known *Fonsecaea* species (de Hoog et al. 2004). Isolate F1PLE was recovered from soil, located on the second plateau (Fig. 1). It showed similar morphology to *Rhinocladiella* but through molecular data it was identified as *F. monophora* (Table 1). Strains FE5P6, FE5II and FCL2 strains appeared to represent undescribed species of the genus *Fonsecaea* (Table 1).

In the same region isolate (FE3) was recovered which was morphologically identified as *Phialophora verrucosa* on the basis of pronounced funnel-shaped collarettes from which the conidia were released. The isolate did not assimilate glucose, ribose and inulin but was capable of L-lysine assimilation (Table 2). Using molecular data, they were identified as an undescribed *Rhinocladiella* species (Table 1). Strains FE10IIB and FE10IIIB were initially thought to be *Rhinocladiella* or *fusaeicaea-like* species. FE10IIB did not assimilate inulin and was physiologically similar to *Rhinocladiella atrovirens* (CBS 264.49 and CBS 380.59). No close molecular match was found for either of these strains (Table 1).

**DISCUSSION**

Chaelothyrialean black yeasts and relatives are interesting microorganisms from ecological as well as clinical points of view. The recurrent and consistent infections cause by many representatives of the order indicates a possible adaptation of the fungi to the human host. In the environment they occupy specific micro-habitats, probably due to their low competitive ability towards co-occurring microorganisms. Their oligotrophism (Satow et al. 2008) enables them to thrive and maintain at low density in adverse substrates where common saprobes are absent (de Hoog 1993, 1997). An eventual potential as an environmental pathogen may involve a composite life cycle of the fungi concerned. However, the invasive potential is polyphyletic and differs significantly between species (Badali et al. 2008). Recurrent, consistently identifiable diseases are caused by relatively few species, which may be morphologically very similar to environmental counterparts which in many cases seem to be undescribed (Table 1). Therefore a reliable taxonomic system is mandatory to obtain better understanding of the link between clinical disease and environmental ecology.

The state of Paraná in southern Brazil is an endemic region for chromoblastomycosis. *Fonsecaea pedrosoi* is supposed to be responsible for more than 95% of the clinical cases, mainly infecting agricultural laborers (Queiroz-Telles 1997). This species is now known to comprise two cryptic entities, causing the same disease but seemingly differing in virulence (de Hoog et al. 2004). Out of five clinical strains tested from Paraná, two appeared to be

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Table 2. Physiological test results of Brazilian isolates.

| Isolate | D-Glucose | D-Galactose | D-Sorbitol | D-Rhamnose | L-Sorbose | GlcA | D-Glucosamine | D-Arabinose | D-Arabinose | Sucrose | Maltose | α,β-Trehalose | Methyl-α-D-Glucoside | Cellobiose | Salicin | Arbutin | Ribitol | Xylitol | L-Arabinose | Galactose | Mannitol | Galactose | L-Arabinose | D-Myo-Inositol | Glucosone-δ-Lactone | Glucono-δ-Lactone | D-Ketogluconate | D-Glucuronate | D-Arabinose | L-Arabinose | L-Arabinose | Methyl-α-D-Glucoside |
|---------|-----------|-------------|------------|-------------|-----------|------|---------------|-------------|-------------|----------|---------|-------------|------------------|-----------|-------|----------|--------|---------|-------------|-----------|---------|----------|-------------|--------------|-------------------|-------------------|---------------------|---------------|--------------|-------------|--------------|--------------|-------------------|-------------------|
| 1       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 2       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 3       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 4       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 5       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 6       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 7       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 8       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 9       | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 10      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 11      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 12      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 13      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 14      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 15      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 16      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 17      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 18      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 19      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 20      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 21      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 22      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 23      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |
| 24      | +         | +           | +          | +           | +         | +    | +             | +           | +           | +        | +       | +           | +                | +         | +     | +        | +      | +       | +           | +         | +       | +        | +           | +              | +                 | +                 | +                   | +             | +            | +            | +              | +              |

**Note:** + indicates positive test result; - indicates negative test result.
F. monophora (Table 1). Our extensive environmental sampling in 56 locations in the state of Paraná showed that Fonsecaea pedrosoi was not isolated from nature, but instead we repeatedly encountered F. monophora. The natural source and route of infection of F. pedrosoi therefore still remains a mystery.

Several chaetothyrialean opportunists were isolated which are known to be associated with mild disorders, such as the cutaneous species Cladophialophora saturnica (Badali et al. 2008) and Exophiala xenobiotica (de Hoog et al. 2006). None of the systemic pathogens, such as Cladophialophora bantiana, were found. Several species listed in Table 1 concern hitherto undescribed, apparently saprobic representatives of the order Chaetothyriales that have never been reported as agents of human or animal disorders. The discrepancy of molecular identification and morphological and physiological results that were validated by analysis of ex-type strains of chaetothyrialean fungi (de Hoog et al. 1995) indicated that a vast number of saprobic species still awaits discovery and description.

The hydrocarbon-polluted environments yielded another spectrum of chaetothyrialean fungi. Exophiala dermatitidis is a fairly common opportunist, occasionally causing fatal, systemic disease. Exophiala bergeri, E. xenobiotica, E. angulospora and Veronaea botryosa are exceptional and/or low-virulent opportunists. Exophiala bergeri has thus far rarely been reported as an agent of disease, but was abundantly isolated when monoaromatic hydrocarbons were used for enrichment. The presence of aromatic compounds in...
the sample increases colony density and diversity of black yeasts. The ecological and physiological patterns of species concerned suggests an evolutionary connection between the ability to develop on alkylbenzenes and the ability to cause diseases in humans and animals (Prenafeta-Boldú et al. 2006).

The present study was an attempt to verify whether infections caused by Fonsecaea pedrosoi and other agents of human mycosis are likely to be initiated by traumatic inoculation of environmental strains, and, more in general, to find the source of infection of invasive black yeasts-like fungi. Our results showed that this link is complex: environmental strains cannot always be linked directly to clinical cases. This is illustrated above by the genus Fonsecaea, known from two clinically relevant species. Mostly *F. monophora* or unknown *Fonsecaea* species were isolated. The apparently more virulent species *F. pedrosoi* is likely to require special, hitherto unknown parameters for isolation, such as the use of an animal bait (Dixon et al. 1980, Gezuele et al. 1972). Thus far it only has been encountered on the human patient, always causing chromoblastomycosis when the host is immunocompetent. In contrast, *F. monophora* can be isolated from the environment without an animal bait, and is a less specific opportunist (Surash et al. 2006). In general, pathogenicity and virulence of chaetothyrialean black yeasts may differ between closely related species. The group can be divided in three ecological groups, as follows. (1) Saprobies not known from vertebrate disorders, such as the majority of undescribed strains reported in Table 1; (2) Low-virulent opportunists that can directly be isolated from the environment, such as *F. monophora*, and (3) Highly specific pathogens that cannot be isolated from the environment directly but require a living mammal, resp. a human host. This suggests that isolation efficiencies differing between species reflect different pathogenic tendencies in pathogenic adaptation of the species.

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