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

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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 *Cladophialophora bantiana* and *Exophiala jeanselmei*. 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 jeanselmei* and *Cladophialophora 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.

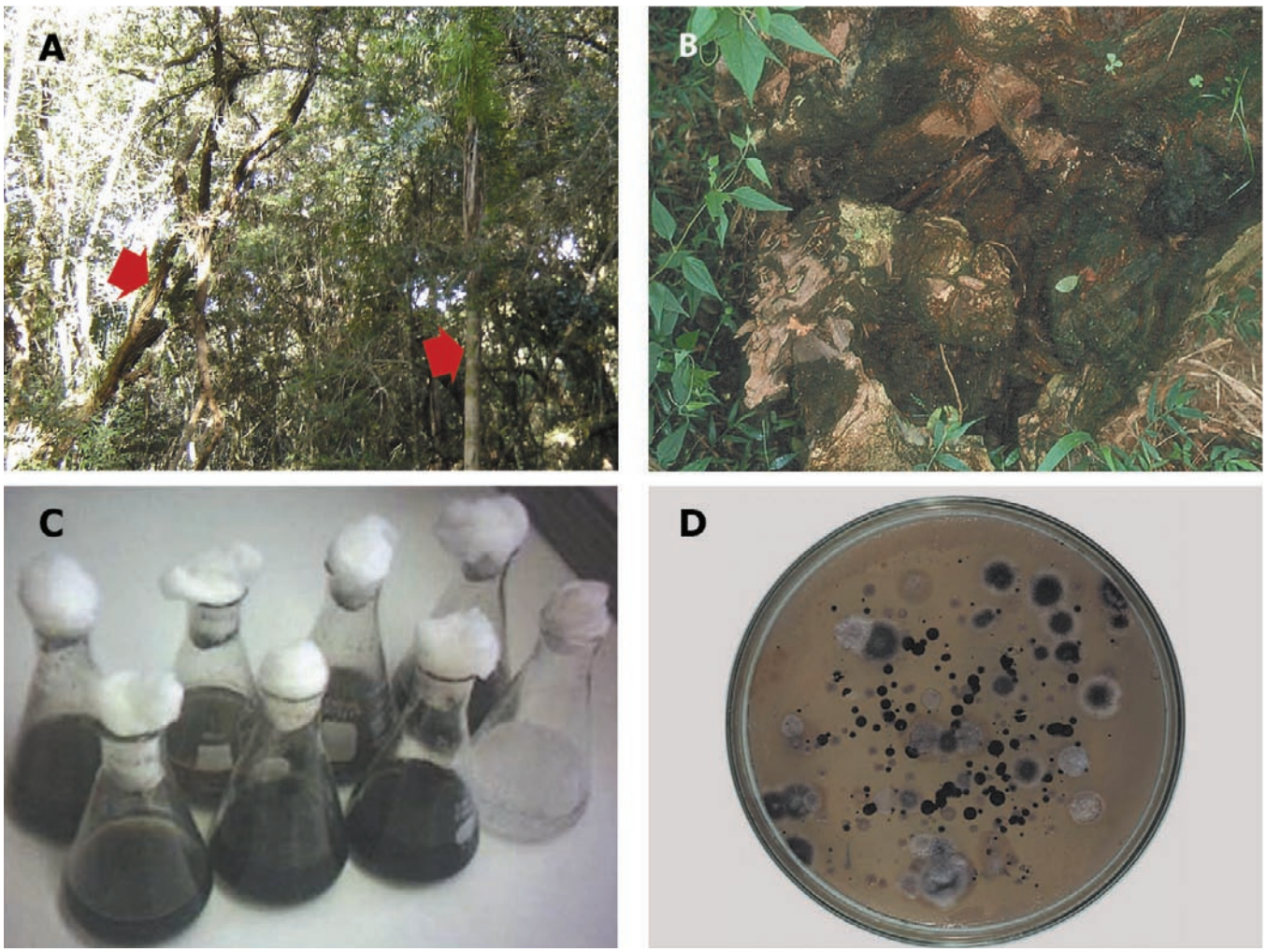

Fig. 2. A. Sampling location in the area of "FE" series of samples in the first plateau at Colombo (Paraná) with native species (arrows) dominated by cambara tree (*Gochnathia polymorpha*) and stem palm (*Syagrus romanzoffiana*); B. Sampling location of FE5P4 of *Fonsecaea monophora*, from decaying cambara 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 Paulínia, Paulínia 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 & Shadomy 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 °C 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 $MgCl_2$. Cycloheximide tolerance was determined in liquid medium at 0.01, 0.05 and 0.1 % (w/v).

DNA extraction

About 1 cm^2 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 Celite 545, Machery, Düren, Germany, 2:1, w/w). 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 °C. 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°C 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 °C, before storage at −20 °C (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 °C (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 FP4IIB was capable of growing with 0.1 % cycloheximide, showed reduced growth in the presence of ethanol and had a maximum growth temperature of 37 °C (Table 2). It presented ellipsoidal to 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 FP4IIB was identified as *C. saturnica* by ITS sequencing (Table 1). FE1IIA and F11PLA had fusiform conidia in chains. FE1IIA was unable to assimilate galacticol, developed poorly in the presence of D-glucoronate, but was able to grow in a medium with ethanol; F11PLA assimilated glucoronate 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 pedrosoi*. Physiologically it differed from *F. pedrosoi* by assimilation of L-sorbose, melibiose,

Table 1. Isolates from clinical and environmental sources in southern Brazil.

Morphological ID	Final ITS ID	CBS	dH	Vicente / Attili	Origin	Source
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*		18223	Fp28III	Marmeleiro	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*		18331	Queiros	São Paulo	Subcutaneous, compromised
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*	102244	11608	Fp37	Ipora	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*	102245	11610	Fp36I	Ampere	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102248	11613	Fp82	Piraquara	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102246	11611	Fp65	Campo Largo	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102243	11607	Fp31I	Ibituva	Chromoblastomycosis
Cladophialophora bantiana[1]	*Cladophialophora immunda*	102227	11588	FE9 = 9EMB	Colombo	Stem palm (*Syagrum roman zoffianum*)
Cladophialophora bantiana	*Cladophialophora* sp. 3	102231	11592	FE1IIA	Colombo	Rotten *Gochnatia polymorpha* stem
Cladophialophora devriesii[1]	*Cladophialophora saturnica*[4]	102230	11591	FP4IIB	Piraquara	Plant litter
Cladophialophora sp.	*Cladophialophora saturnica*	102228	11589	FP4IIA	Piraquara	Rotten wood
Cladophialophora sp.	*Fonsecaea monophora*[5]	102229	11590	FP8D = 8DPIRA	Piraquara	Plant litter
Cladophialophora sp.	*Cladophialophora* sp. 2	102236	11600	F11PLA	Telêmaco Borba	Plant litter
Cladophialophora sp.	*Cladophialophora immunda*	102237	11601	F10PLA	Telêmaco Borba	Plant litter
Exophiala lecanii-corni[2]	*Exophiala xenobiotica*[4]	102232	11594	FE4IIB	Colombo	Rotten wood
Exophiala jeanselmei[2]	*Exophiala bergeri*[4]	102241	11605	F14PL	Cianorte	Soil under coffee tree
Exophiala sp.	*Capronia semi-immersa*	102233	11595	FE6IIB	Colombo	Rotten *Araucaria* trunk
Fonsecaea pedrosoi[3]	*Fonsecaea* sp. 3	102223	11583	FCL2	Castro	Rotten root
Fonsecaea pedrosoi	*Fonsecaea* sp. 1	102224	11584	F9PRA	Terra Roxa	*Grevillea robusta* wood
Fonsecaea pedrosoi	*Fonsecaea monophora*[5]	102225	11585	FE5P4	Colombo	Rotten wood (*Gochnatia polymorpha*)
Fonsecaea pedrosoi	*Fonsecaea* sp. 1	102254	11619	FE5P6	Colombo	Rotten wood
Fonsecaea pedrosoi	*Fonsecaea* sp. 2	102226	11587	FE5II	Colombo	Rotten *Araucaria* trunk
Phialophora verrucosa[3]	*Phialophora* sp. 1	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.	*Exophiala xenobiotica*[4]	102251	11616	F3PLC	Sertanópolis	Plant litter
Rhinocladiella sp.	*Fonsecaea* sp. 3	102239	11603	FE1IIB	Colombo	Rotten *Lantana camara* stem
Rhinocladiella sp.	*Fonsecaea* sp. 3	102252	11617	FE10IIB	Colombo	Plant litter
Rhinocladiella sp.	*Rhinocladiella* sp. 1		11618	FE10IIB1	Colombo	Plant litter
Rhinocladiella sp.	*Rhinocladiella* sp. 1	102240	11604	F9PRC	Terra Roxa	*Podocarpos lambertii* branch
Rhinocladiella sp.	*Exophiala xenobiotica*[4]	102255	11621	F20PR3	Jacutinga	Soil
Rhinocladiella sp.	*Fonsecaea monophora*[5]	102238	11602	F1PLE	Rio Tibagi	Soil
Exophiala sp.	*Exophiala bergeri*[4]	122844	18627	D0009	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122843	18629	D0020	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122842	18636	D0035	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122841	18643	D0201	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122840	18654	D0213	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122839	18635	D0029b	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122838	18646	D0204a	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122837	18651	D0210	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122836	18648	D0206	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122835	18652	D0211	Rio Clarov	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122834	18653	D0212	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122833	18656	D0215	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis* (mel⁻ mut)	122830	18650	D0209	Rio Claro	Railway tie treated with creosote 16 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122832	18647	D0205	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala xenobiotica*[4]	122910	18638	D0044	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala xenobiotica*[4]	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*[4]	122829	18631	D0023	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122846	18632	D0024	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122828	18637	D0037	Rio Claro	Railway tie treated with creosote 15 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122826	18639	D0045	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122824	18640	D0047	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122822	18641	D0060	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122825	18642	D0063	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122823	18628	D0017	Rio Claro	Railway tie treated with creosote 20 yr ago
Aureobasidium sp.	*Aureobasidium pullulans*[4]	122827	18657	D0216	Paulinia	Polluted soil, Replan Co.

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

Known agent of: [1]systemic and disseminated disease; [2]mycetoma; [3]chromoblastomycosis; [4]mild cutaneous disease; [6]systemic disease and pulmonary colonization

[5]Known opportunistic agent (including chromoblastomycosis).

ribitol, xylitol, myo-inositol, glucono-δ-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* (Table 2) and was isolated from the same location where the patient, a carrier of chromoblastomycosis, had acquired his infection (Piraquara city, Fig. 1). All strains grew at 37 °C but not at 40 ºC, 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), a result that is consistent with the physiological characteristics of the *P. verrucosa* reference strain (de Hoog *et al.* 1999). With molecular ID a hitherto undescribed *Phialophora* species was found (Table 1).

Strain FE6IIB, morphologically identified as *Exophiala* species (Table 1) physiologically differed from reference strains of *Exophiala* (de Hoog *et al*. 2000a) by positive responses to lactose, L-arabinose, myo-inositol, D-gluconate and DL-lactate, by being able to assimilate D-ribose, and also presenting weak assimilation of glucoronate. By molecular identification was identified as the anamorph of Capronia semi-immersa. Isolate F14PL was preponderantly yeast-like and was provisionally identified as *Exophiala jeanselmei*, but ITS sequence data suggested *E. bergeri* (Table 1). Isolate FE4IIB showed morphological similarity to *E. lecanii-corni*, but differed from reference strains (de Hoog *et al*. 2000a) by positive assimilation of lactose, L-arabinose, myo-inositol, D-glucuronate and DL-lactate and by being able to assimilate D-ribose and D-glucoronate. Molecular data suggested identity with *E. xenobiotica*.

Isolates F20PR3 and F3PLC, morphologically having the appearance of *Rhinocladiella* species were identified as *Exophiala xenobiotica* by ITS data. These strains were unable to ferment glucose, to assimilate methanol, to grow at 40 ºC and were citrate negative. None of the strains analysed produced extracellular DNAse. Rhinocladiella-like strains F9PR and F9PRC were physiologically similar, differing only in assimilation of glycerol and L-lysine (Table 2). Using molecular data, they were identified as an undescribed *Rhinocladiella* species (Table 1). Strains FE10IIB and FE10IIB1 were initially thought to be Rhinocladiella- or fonsecaea-like species. FE10IIB1 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

Chaetothyrialean 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

Table 2. Physiological test results of Brazilian isolates.

	FE3 *Phialophora* sp. 1	FE9 *C. immunda*	F10PLA *C. immunda*	FE1IIA *Clad.* sp. 3	FP4IIB *C. saturnica*	FP4IIA *C. saturnica*	F10PLB *C. immunda*	F11PLA *Clad.* sp. 2	F14PL *E. bergeri*	FE4IIB *E. xenobiotica*	FE6IIB *Cap. semiimmersa*	F20PR3 *E. xenobiotica*	F3PLC *E. xenobiotica*	F9PR *Rhin.* sp. 1	F9PRC *Rhin.* sp. 1	FE10IIB1 *Rhin.* sp. 1	Fp82 *F. monophora*	Fp65 *F. monophora*	FE5P4 *F. monophora*	FP8D *F. monophora*	F1PLE *F. monophora*	F9PRA *Fonsecaea* sp. 1	FE10IIB *Fonsecaea* sp. 3	FE1IIB *Fonsecaea* sp. 3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	+	w	+	w	+	+	+	+	+	w	+	-	w	+	w	w	w	+	+	w	w/+	w	w	w
D-Glucosamine	w/+	w	w	+	w	w	w	w	w/+	+	w	+	+	w	w/-	-	w	+	w	w	w	w/-	+	+
D-Ribose	-	+	+	+	+	+	+	+	+	+	w	+	+	w	-	w/-	w/-	w/-	w/-	w/-	-	-	+	+
D-Xylose	+	+	+	+	+	+	+	w	+	+	+	+	+	+	w/+	+	w/+	+	+	w/+	w/+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	w/+	+	+	+
D-Arabinose	+	w	+	+	w	w	+	w	+	w/+	w	+	+	w	-	-	-	+	w	-	w/-	-	+	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	w/+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	w/+	+	+	w/+	w/+	+	+	+
α,α-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-α-D-Glucoside	w	+	+	-	+	+	+	+	-	-	+	w	-	+	+	+	w/-	+	w	w/-	w	+	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	w/+	+	w	w/+	w/+	+	+
Salicin	+	+	+	+	+	+	+	+	w/-	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+
Arbutin	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+
Melibiose	-	+	+	+	w	w	+	+	-	+	+	+	+	+	w/+	+	+	+	+	+	w/+	w/+	+	+
Lactose	-	+	+	-	+	+	+	+	w/-	-	-	-	w	+	+	+	w/-	+	w	w/-	w/-	w/+	-	-
Raffinose	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	w	-	-	-	-	-	w/-	w	w	w	-	-	-	-	-	-	-	-	-	w	w
Sol. starch	w	w	w	w/+	-	-	w	-	-	w/-	-	w/+	w/+	-	w/-	w/-	w/-	w/-	-	w/-	w/-	w/-	w/+	w/+
Glycerol	+	+	+	+	+	+	+	w	+	+	w	+	+	w	+	+	w	+	w	w	w	+	+	+
meso-Erythritol	+	+	+	+	+	+	+	+	w/-	+	+	+	+	+	+	+	w/-	+	w	w/-	w	+	+	+
Ribitol	+	w	+	+	+	+	+	+	+	+	+	+	+	w	w	w/+	w/-	+	w	w/-	w	w/-	+	+
Xylitol	-	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+	-	+	w	-	w/-	+	+	+
L-Arabinitol	w	+	+	w/+	w	w	+	+	+	+	w	w/+	w/+	w	w	w/+	w/+	+	+	w/+	+	w/+	w/+	w/+
D-Glucitol	+	+	+	+	w	w	+	+	+	+	+	+	+	+	w	w/+	w/+	+	+	w/+	w/+	w/+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	+
Galactitol	w	+	+	-	w	w	+	w	-	-	w	-	-	w	+	+	w/-	+	w	w/-	w	+	-	-
myo-Inositol	+	+	+	+	+	+	+	+	+	+	w	+	+	-	-	+	-	w/+	+	-	w	-	+	+
Glucono-d-Lactone	+	+	+	+	+	+	+	w	w/+	w/+	w	+	+	+	w	w	w	+	+	w	w	w	+	+
D-Gluconate	+	w	w	w	+	+	w	w	-	w	+	w	-	w	w	w	w	+	+	w	w	w/-	w	w
D-Glucuronate	+	w	w	w	+	+	w	+	w	w	w	w	w	w	w	w	w	+	+	w	w/-	w/-	w	w
D-Galacturonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/-	w/-	w	w/-	w	w/+	+	+
DL-Lactate	+	+	+	+	w	w	+	w	+	+	w	+	+	w	+	+	w	-	w	w	w/+	w	+	+
Succinate	+	w	+	+	+	+	+	+	w	w/+	w	+	+	+	w	w	w	w/+	w	w	w	w	+	+
Citrate	+	w	w	-	w/-	w/-	w	+	-	-	-	-	-	w	w/-	w/-	w	w	w	w	w	w/-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	w/+	w/+	+	+	w	w	+	W	+	w/+	w	+	+	w	+	w/+	w/+	+	w	w/+	w/+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. (Continued).

	FE3 P*hialophora* sp. 1	FE9 *C. immunda*	F10PLA *C. immunda*	FE1IIA *Clad.* sp. 3	FP4IIB *C. saturnica*	FP4IIA *C. saturnica*	F10PLB *C. immunda*	F11PLA *Clad.* sp. 2	F14PL *E. bergeri*	FE4IIB *E. xenobiotica*	FE6IIB *Cap. semiimmersa*	F20PR3 *E. xenobiotica*	F3PLC *E. xenobiotica*	F9PR *Rhin.* sp. 1	F9PRC *Rhin.* sp. 1	FE10IIB1 *Rhin.* sp. 1	Fp82 *F. monophora*	Fp65 *F. monophora*	FE5P4 *F. monophora*	FP8D *F. monophora*	F1PLE *F. monophora*	F9PRA *Fonsecaea* sp. 1	FE10IIB *Fonsecaea* sp. 3	FE1IIB *Fonsecaea* sp. 3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Nitrite	+	w/-	+	+	w	w	+	w	+	+	+	+	+	w	+	w	+	+	w	+	+	w/+	+	+
Ethylamine	+	+	w/+	w/+	w	w	w/+	+	w/+	+	w	+	w/+	w	w/+	w/+	w	w	w	w/+	w	w/+	w/+	w/+
L-Lysine	+	w	w/+	w	-	-	w/+	w	w/-	w/+	-	-	w	-	w/+	w/+	+	+	w	+	w/+	w/+	w	w
Cadaverine	+	+	+	+	+	+	+	w	+	+	+	-	+	+	+	+	+	+	+	w/+	+	+	+	+
Creatine	+	+	w	+	-	-	w/+	w	-	+	w	+	+	w	+	w	w/+	w/+	w	+	w/+	w/+	+	+
Creatinine	+	+	w	+	-	-	w/+	w/-	-	+	w	+	+	w	+	-	w/+	w/+	w	w/+	w	w	+	+
2.5 % MgCl2	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.0 % MgCl2	+	+	w/+	+	w/+	w/+	w/+	w/+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+
10 % MgCl2	w	+	w/+	+	w/+	w/+	w/+	w/+	+	+	+	+	+	w/+	w/+	+	w/+	+	+	w/+	+	w/+	+	+
2.5 % NaCl	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	w/+	+	+	w/+	+	+	+	+
5.0 % NaCl	w	+	w	+	w/+	w/+	W	w/+	+	+	+	+	+	+	w/+	+	-	+	+	-	+	+	+	+
10 % NaCl	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.1 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.05 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.01 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	+	-	-	-	-	-	-	-	+	+	w	w/+	+	+	-	+	+	+	-	+	+
30 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40 ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycosel	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatine	-	-	-	-	w	w	-	w	-	-	w	-	-	w	-	-	-	-	w	-	w	-	-	-
DNAse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	?	?	w/+	w/-	w/+	w/+	w/+	w/+	-	w/+	-	w/-	-	-	-	-	-	-	-	-	-	-	w/-	w/-

Abbreviations used: + = growth; w = weak growth; - = no growth; ? = ambiguous or unknown.

C. = *Cladophialophora; Cap* = *Capronia; E* = *Exophiala; Phial* = *Phialophora; Rhin* = *Rhinocladiella*. 1*Phialophora* sp.1; 2-8 *Cladophialophora* species; 9-13 *Exophiala* species; 14 -16 *Rhinocladiella.* sp; 17-18 *Clinical strain*: (m*) Fonsecaea monophora* and (p) *F. pedrosoi*; 19−21 (m) *Fonsecaea monophora*; 22-24 (sp) *Fonsecaea* sp. (molecular identifications partly according to Najafzadeh *et al.* 2008)

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) Saprobes 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 bait, 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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