Black Yeast Biota in the Mangrove, in Search of the Origin of the Lethargic Crab Disease (LCD)

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Abstract Knowledge of natural ecology is essential for a better understanding of pathogenicity and opportunism in black yeast-like fungi. Although etiological agents of diseases caused by these fungi 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, overall, insufficient data on their natural habitat. We obtained environmental samples from mangrove areas where mortalities by lethargic crab disease (LCD) are reported and areas without disease recorded. Isolation of chaetothyrialean black yeasts and relatives was performed using a highly selective protocol. Species-specific primers were used to determine if these isolates represented *Exophiala cancerae* or *Fonsecaea brasiliensis*, two proven agents of LCD, in order to test hypotheses about the origin of the disease. Isolates, identified by morphology as *Fonsecaea*- or *Exophiala*-like, were tested specific diagnostic markers for the fungi associated with LCD. Although several black fungi were isolated, the main causative agent of the LCD, *E. cancerae*, was not found. Molecular markers for *F. brasiliensis* revealed 10 positive bands for isolates from biofilms on mangrove leaves, branches, and aerial roots, of which four were confirmed by ITS sequencing. The absence of *E. cancerae* in environmental samples suggests that the species is dependent on the crab, as a genuine pathogen, different from *F. brasiliensis*, which is probably not dependent on the host species, *U. cordatus*. However, we did not attempt isolation from the marine water, which may represent the pathway of dispersion of the black yeast species between neighbor mangroves.

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Introduction

The mangrove land crab, *Ucides cordatus* (Brachyura/
Ocypodidae), plays a crucial role in a variety of
ecosystem processes in its environment, being
involved in the decomposition of organic materials
[1, 2]. In Brazil, the animal is an important component
in the economy of several underprivileged communi-
ties that depend on it for their subsistence.

Since the year 1997, an extensive epizootic causes
high mortalities of *U. cordatus* along the Brazilian
coast [1], causing a reduction of up to 85 % of the
collection rates in some regions in the northeastern
region. Affected crabs share several symptoms, such
as lethargy, poor motor control, and inability to return
to the upright position when turned upside down. The
disease was termed Lethargic Crab Disease (LCD) by
Boeger et al. [1] because of clinical signs of ataxia
observed in moribund organisms [1, 2]. Evidence from
a variety of sources (light and electron microscopy,
behavioral and experimental tests [1], molecular
phylogenetics [1], and experimental infection [3])
indicates that LCD signs are associated with the
presence of a black yeast-like fungus, *Exophiala
cancerae* recently described by de Hoog et al. [4].
However, occasional co-infection may take place by
another black yeast-like fungus, viz. a species of
Fonsecaea recently described by Vicente et al. [5] as
F. brasiliensis. Since the first record of mortalities
likely associated with LCD in Goiana, Pernambuco
State, Brazil, the disease has spread from Northern to
Southern estuaries along to South Atlantic Coast in a
wave-like pattern [6]. Currently, LCD is apparently
absent from the majority of estuaries where it was
reported in the past, having remained solely in the
mangroves of Espirito Santo State.

Knowledge of natural ecology and evolution is
essential for a better understanding of pathogenicity
and opportunism of black yeast-like fungi. Although
etiological agents of most diseases caused by black
yeast-like fungi 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 to enable recovery of these fungi
[7–12]. Several studies indicate that opportunism of
these fungi might be explained from the perspective of
their unusual niches [13–15]. Recurrent and consistent
infections caused by many representatives of the order
Chaetothyriales indicate a possible specialization of
the fungi to the animal hosts.

In this study, we obtained environmental samples
from mangrove areas in which mortalities by LCD are
reported and areas where the disease is not recorded.
Isolation of chaetothyrialean black yeasts and relatives
was performed using a highly selective protocol.
Species-specific diagnostic primers were used to
determine if these isolates represented *E. cancerae*
or *Fonsecaea brasiliensis*, in order to monitor the
spread of agents of LCD along the Brazilian coast.

Materials and Methods

Hypothesis Testing

The sampling design of this study is focused on the
hypothesis that the fungi associated with LCD are
opportunistic, that is, emerging from the environment.
The null hypothesis is accepted if the fungus species is
found in the environment, at least in areas where LCD
outbreaks occur. The alternative hypothesis suggests
that the fungus is a real pathogen. This hypothesis will
be supported if the fungus consistently associated with
LCD cannot be isolated from environmental samples
in mangroves where LCD is prevalent.

Fungal Strains

Strains analyzed (52) are listed in Table 1. Stock
cultures were maintained on 2 % malt extract agar
(MEA, Difco) and oatmeal agar (OA) slants at 24 °C.
For morphological studies, MEA slide cultures were
prepared and mounted in aniline blue.

Study Areas and Sampling Strategy

Locations chosen for this study include three mangrove
areas where mortalities of mangrove land crabs puta-
tively caused by LCD have been reported in the state of
Bahia, viz. Acupi, Saubara, and two areas in Canasvie-
iras indicated as −1 and −2 (Fig. 1; Table 1). In each

Table 1 Strains analyzed

CBS number	dH number	Taxon name	GenBank (ITS)	Other collection number	Substrate	Country and locality
CBS 128149	dH 21444	*Cladophialophora* sp.	KC445244	R25D2	Dead leaf from mangrove environment	Brazil; Bahia state, Canavieiras Control
		Fonsecaea pedrosoi	KC445245	R03B1	Branch	Brazil; Bahia state, Canavieiras Farm
		Mycosphaerella sp.	KC445246	R07B5	Leaf of living tree	Brazil; Bahia state, Canavieiras Farm
		Mycosphaerella sp.	KC445247	R08D4	Leaf of living tree	Brazil; Bahia state, Canavieiras Farm
CBS127890	dH21424	*Cladophialophora* sp.	KC445248	R24C1	Leaf of living tree	Brazil; Bahia state, Canavieiras Control
CBS127891	dH21419	*Cladophialophora* sp.	KC445249	R26A2	Branch	Brazil; Bahia state, Canavieiras Control
CBS127892	dH21442	*Cladophialophora* sp.	KC445250	R26A4	Branch	Brazil; Bahia state, Canavieiras Control
CBS127893	dH21420	*Cladophialophora* sp.	KC445251	R26B1	Branch	Brazil; Bahia state, Canavieiras Control
CBS127894	dH21425	*Cladophialophora* sp.	KC445252	R26B3	Branch	Brazil; Bahia state, Canavieiras Control
CBS127895	dH21426	*Cladophialophora* sp.	KC445253	R26D1	Branch	Brazil; Bahia state, Canavieiras Control
CBS127896	dH21421	*F. brasiliensis*	KC445254	R27A1	Branch	Brazil; Bahia state, Canavieiras Control
CBS127897	dH21443	*Cladophialophora* sp.	KC445255	R30C1	Leaf of living tree	Brazil; Bahia state, Canavieiras Control
CBS127898	dH21422	*F. brasiliensis*	KC445256	R32A1	Root	Brazil; Bahia state, Canavieiras Control
		Cladophialophora sp.	KC445257	R32E1	Root	Brazil; Bahia state, Canavieiras Control
		Exophiala oligosperma	KC445258	R32E2	Root	Brazil; Bahia state, Canavieiras Control
CBS127899	dH21427	*Exophiala* sp.	KC445259	R32E3	Root	Brazil; Bahia state, Canavieiras Control
		Cladophialophora sp.	KC445260	R32E4	Root	Brazil; Bahia state, Canavieiras Control
CBS127900	dH21445	*Cladophialophora* sp.	KC445261	R33C1	Soil	Brazil; Bahia state, Canavieiras Control
		Cladophialophora sp.	KC445262	R33D2	Soil	Brazil; Bahia state, Canavieiras Control
		Cladophialophora arxii	KC445263	R34A2	Soil	Brazil; Bahia state, Canavieiras Control
CBS127901	dH21428	*Cladophialophora* sp.	KC445264	R34A3	Soil	Brazil; Bahia state, Canavieiras Control
CBS127902	dH21429	*Cladophialophora* sp.	KC445265	R34B1	Soil	Brazil; Bahia state, Canavieiras Control
CBS127903	dH21430	*Cladophialophora* sp.	KC445266	R37B1	Crab hole	Brazil; Bahia state, Canavieiras Control
		Exophiala sp.	KC445267	R41B1	Dead leaf	Brazil; Bahia state, Acupi
CBS127904	dH21431	*F. brasiliensis*	KC445268	R42B1	Branch	Brazil; Bahia state, Acupi

Table 1 continued

CBS number	dH number	Taxon name	GenBank (ITS)	Other collection number	Substrate	Country and locality
		Cladophialophora sp.	KC445269	R42C1	Branch	Brazil; Bahia state, Acupi
		Cladophialophora sp.	KC445270	R42E1	Branch	Brazil; Bahia state, Acupi
CBS127905	dH21423	*F. brasiliensis*	KC445271	R43B1	Branch	Brazil; Bahia state, Acupi
		Cladophialophora sp.	KC445272	R43C3	Branch	Brazil; Bahia state, Acupi
CBS127906	dH21432	*Fonsecaea multimorphosa*	KC445273	R43E2	Branch	Brazil; Bahia state, Acupi
		Cladophialophora sp.	KC445274	R45A3	Leaf of living tree	Brazil; Bahia state, Acupi
		Cladophialophora devriesii	KC445275	R45C4	Leaf of living tree	Brazil; Bahia state, Acupi
		Cladophialophora devriesii	KC445276	R45E1	Leaf of living tree	Brazil; Bahia state, Acupi
		Cladophialophora sp.	KC445277	R26D2	Branch	Brazil; Bahia state, Canasvieiras Control
CBS127907	dH21433	*Cladophialophora* sp.	KC445278	R49A3	Soil	Brazil; Bahia state, Acupi
	dH21462	*Cladophialophora* sp.	KC445279	R49C1	Soil	Brazil; Bahia state, Acupi
CBS127908	dH21434	*Cladophialophora* sp.	KC445280	R52B1	Crab hole	Brazil; Bahia state, Acupi
		Cladophialophora sp.	KC445281	R63E1	Branch	Brazil; Bahia state, Saubara
CBS128037	dH21435	*Cladophialophora* sp.	KC445282	R54E2	Crab hole	Brazil; Bahia state, Acupi
		Exophiala xenobiotica	KC445283	R57A4	Leaf of living tree	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445284	R57B2	Leaf of living tree	Brazil; Bahia state, Saubara
		Phialophora sp.	KC445285	R57C2	Leaf of living tree	Brazil; Bahia state, Saubara
CBS127909	dH21436	*Exophiala xenobiotica*	KC445286	R57C3	Leaf of living tree	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445287	R57D1	Leaf of living tree	Brazil; Bahia state, Saubara
		Exophiala xenobiotica	KC445288	R57D3	Leaf of living tree	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445289	R67D1	Dead leaf	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445290	R68A1	Branch	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445291	R68C1	Branch	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445292	R68E1	Branch	Brazil; Bahia state, Saubara

Table 1 continued

CBS number	dH number	Taxon name	GenBank (ITS)	Other collection number	Substrate	Country and locality
	dH 21463	*Exophiala spinifera*	KC445293	R68E2	Branch	Brazil; Bahia state, Saubara
		Exophiala spinifera	KC445294	R68E3	Branch	Brazil; Bahia state, Saubara
		Phialophora verrucosa	KC445295	R70D1	Leaf of living tree	Brazil; Bahia state, Saubara

location, fragments of plant material and decaying wood showing macroscopic presence of black biofilms, as well as soil samples, were obtained randomly for a total of approximately 1 kg of samples per site. Approximately 20 g from each sample was processed for fungal isolation, with 50 replicates/sample.

Fungal Isolation

Each sample was incubated at room temperature for 30 min in 100 mL of a sterilized saline solution containing 200 U of penicillin, 200 μg/L of strepto-mycin, 200 μg/L of chloramphenicol, and 500 μg/L of cycloheximide. After initial incubation, 20 mL of sterilized mineral oil was added to the solution, and the container was vigorously shaken for 5 min and left to settle for 20 min. The oil–water interphase was then collected and inoculated on Mycosel agar (Difco) for 4 weeks at 36 and 30 °C [8, 16, 17]. Dark colonies were selected then isolated and stored on Mycosel agar.

Identification

Identification was carried out using macro- and microscopic features of the colonies after culture on Sabouraud Dextrose Agar (SDA, Difco) at room temperature. The isolates were analyzed by molecular methods using ITS markers [17]. Cardinal growth temperatures were determined on 2 % malt extract agar (MEA, Difco). Plates were incubated in the dark for 3 weeks at temperatures of 21–36 °C at intervals of 3 °C; growth was also recorded at 37 and at 40 °C.

DNA Extraction

Approximately 1 cm^2 of 14–21-day-old cultures was transferred to a 2 ml Eppendorf tube containing 400 μL TEx buffer (pH 9.0) and glass beads (Sigma G9143). The fungal material was homogenized with MoBio vortex for 1 min. Subsequently, 120 μL SDS 10 % and 10 μL proteinase K were added and incubated for 30 min at 55 °C, and the mixture was vortexed for 3 min. After addition of 120 μL of 5 M NaCl and 1/10 vol CTAB 10 % (cetyltrimethylammonium bromide) buffer, the material was incubated for 60 min at 55 °C. Then, the mixture was vortexed for 3 min. Subsequently, 700 μL 24:1 chloroform/isoamylalcohol was added, mixed carefully by hand and centrifuged for 5 min at 4 °C at 20,400g force value. The supernatant was transferred to a new Eppendorf tube with 225 μL 5 M NH$_4$-acetate, mixed carefully by inverting, incubated for 30 min on ice water, and centrifuged again for 5 min at 4 °C at 20,400g force value. The supernatant was then transferred to another Eppendorf tube with 0.55 vol. isopropanol and centrifuged for 5 min at 20,400 g force value. Finally, the pellet was washed with 1000 μL ice cold 70 % ethanol. After drying at room temperature, it was re-suspended in 100 μL TE buffer (Tris 0.12 % w/v, Na-EDTA 0.04 % w/v).

PCR Diagnosis of Fungi Associated to LCD

Species-specific PCR-primers for *E. cancerae* [18] and *F. brasiliensis* [5], designed on the basis of their ITS rDNA sequence, were applied to the DNA extracts to determine whether the isolate from the environment represented either species. PCR products were electrophoresed on 1.5 % agarose gels, stained with ethidium bromide and were photographed under UV light. The samples were determined to be positive for the yeast strain if they had an amplicon of *450 or *396 bp, which corresponds to the expected product size of *E. cancerae* (strain CBS 120420) and *F. brasiliensis* (strain CBS 119710).

Fig. 1 Collection sites along the coast of Bahia State. Mangroves where samples were taken are indicated with closed circles

DNA Amplification and Sequencing

Sequencing was done for strains that were positive in *F. brasiliensis* or *E. cancerae* specific PCRs. ITS amplicons were generated with primers V9G and LS266 [19] and were sequenced with primers ITS1 and ITS4. PCR was performed in a 25 μL volume of a reaction mixture containing 7 μL Go Taq master mix (Promega, Breda, The Netherlands) containing dNTPs, $MgCl_2$, reaction buffer, 1 μL of each primer (10 pmol), and 1 μL rDNA. Amplification was performed in an ABI PRISM 2720 (Applied Biosystems, Foster City, USA) thermocycler as follows: 94 °C for 2 min, followed by 30 cycles consisting of

94 °C for 30 s, 94 °C for 30 s and 65 °C for 1 min, and a delay at 72 °C for 5 min. Amplicons were cleaned with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK). Concentrations of amplicons were estimated on gel, photographed and analyzed by the Gel Doc XR system (Biorad, Veenendaal, The Netherlands), with Smart-Ladder (Eurogentec, Seraing, Belgium) as size and concentration marker. Amplicons were subjected to direct sequencing, with PCR was as follows: 96 °C for 1 min, followed by 35 cycles consisting of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and sequencing

was done using ABI prism BigDye™ terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3730XL Sequencer. Sequences were edited using SᴇQMᴀN in the Lasergene software (DNASTAR, Wisconsin, USA). Iterative alignment was performed by hand with BioNumerics version 4.61 (Applied Maths, St.-Martens-Latem, Belgium).

Results

A total of 64 environmental samples were processed, 36 of which were positive for black fungi (56.3 %). Among these, samples from branches of mangrove trees presented higher frequencies of isolation (87.5 %; Tables 2, 3). Fifty-two isolates, identified by macro- and micro-morphology as *Fonsecaea*- or *Exophiala*-like, were submitted to specific diagnostic markers for the fungi associated to LCD (Fig. 2). Despite the large numbers of black fungi isolated, no positive reaction was obtained for any of the environmental samples when analyzed with specific markers for the main causative agent of the LCD, *E. cancerae* (Fig. 2A). However, the same isolates submitted to molecular markers developed for *F. brasiliensis*, considered previously as a secondary agent of the disease [5], resulted in 10 positive bands (Fig. 2B) for the isolates from mangrove tree leaves (Canasvieiras1: CBS 127897; Acupi: R45A3I), mangrove branch (Canasvieiras 2: CBS 127891, CBS 127892, CBS 127893, CBS 127896; Acupi: CBS 127904, CBS 127905, R42C1), and aerial root (Canasvieiras 2: CBS 127898). Samples of areas with or without previous record of LCD mortality outbreaks presented isolates positively identified as *F. brasiliensis*. Among 10 isolates positive to molecular markers developed for *F. brasiliensis*, only four were confirmed by ITS sequence (Table 1) suggesting cross-reaction of the marker.

Discussion

Many reports of epizootics from the older literature, for example, [20–22], reviewed by de Hoog et al. [4], clearly demonstrate that black yeast infection is a relatively common phenomenon in cold-blood vertebrates. Particularly cold-blooded waterborne vertebrates are susceptible to a diversity of *Exophiala*

Table 2 Coordinates of collecting sites on the coast of Bahia state

Locality	Coordinates
Acupi	12°40′29.5″
	38°44′97″
Saubara	12°43′59.08″
	38°45′12.21″
Canasvieira 1	15°35′43.4″
	38°57′26″
Canasvieira 2	15°40′34″
	38°56′22.2″

species [25]. Recent studies have shown that different pathogenic species are involved [23–25], which are morphologically very similar. Some of these are host-specific to, for example, particular fish taxa [24]. Chaetothyrialean black yeasts and relatives are interesting microorganisms from ecological as well as clinical points of view. Most pathogenic species belong to *Exophiala*, *Cladophialophora*, or *Fonsecaea* (Table 1), while also severe infections are observed in *Veronaea* [4, 5, 26]. Among the most serious pathogenic *Exophiala* species, eventually leading to disseminated, fatal infections in healthy hosts, are the neurotrope *Exophiala dermatitidis* [12], the osteotrope *E. spinifera* [27], and a species tending to cause disseminated infection, *E. asiatica* [29]. As virulence factors the ability to assimilate alkylbenzenes, present in sweat and nervous tissues of mammals and in the toxic skin of amphibians, has been suggested [4, 9]. The recurrent and consistent infections caused by many representatives of the order indicates a possible adaptation of the fungi to the vertebrate host. In the environment, they occupy specific microhabitats, probably due to their low competitive ability toward co-occurring microorganisms [16]. Their oligotrophism [10] enables them to thrive and maintain at low density in adverse substrates where common saprobes are absent [25, 28].

The mangrove crab epizootic [1, 2] underlines the ability of black yeasts to infect a larger spectrum of animals, also involving invertebrates. Recently [5] the etiology of the disseminated LCD in mangrove land crabs was clarified, describing the epidemiology of the disease and establishing a relative virulence in the etiological agents. As in most black fungi, an environmental source of infection is hypothesized,
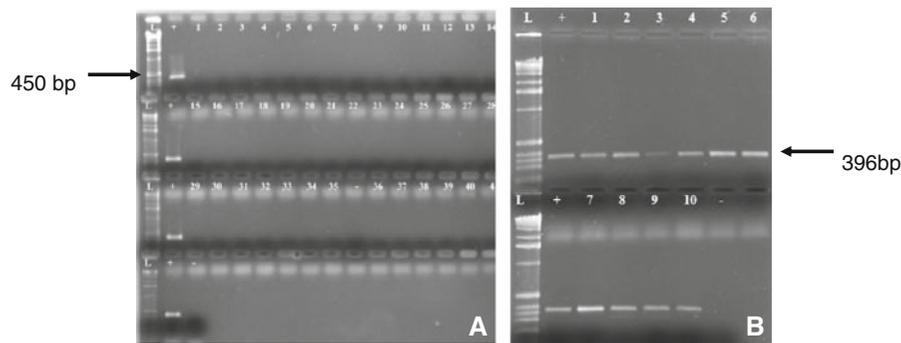
Fig. 2 Specific PCR marker for the LCD etiological agents. **a** DNA extracts with amplification of black fungi using *E. cancerae* (main LCD agent) specific fragment. L. Molecular size marker (100 bp ladder); +positive control (*E. cancerae* CBS 120420); 1–41. DNA extracts of black fungi isolated from coast of Bahia state; −negative control. **b** 1–10. DNA extracts with amplification of black fungi using *F. brasiliensis* (secondary LCD agent) specific fragment. L. Molecular size marker (100 bp ladder); +positive control (*F. brasiliensis* CBS 119710); −negative control

Table 3 Isolates from mangrove areas along northeast Atlantic coast in Brazil

Regions	Acupi	Saubara	Canasvieira 1	Canasvieira 2	TOTAL	% Isolation of black fungi by sample
Substrates	*Presence of black yeasts/number of samples analyzed*					
Tree leaf	02/02	01/02	02/02	01/02	06/08	75
Leaves ground	02/02	01/02	01/02	02/02	06/08	75
Branch	02/02	02/02	01/02	02/02	07/08	87.5
Aerial root	02/02	01/02	01/02	01/02	05/08	62.5
Soil	02/02	0/2	01/02	02/02	05/08	62.5
Crab burrow	04/05	01/04	0/10	02/05	06/24	29.17
Total by region	14/15	06/14	06/20	10/15	36/64	–
% Isolation of black fungi by region	99.33	42.85	30	66.66	56.25	–

similar to, for example, agents of chromoblastomycosis, a disease found in humans and frogs [4]. Molecular siblings of common pathogenic species may be found in association with living plants, growing on debris of tannin-rich cactus spines; therefore, plants were supposed to be the vehicle of introduction into the human body [30].

In our study, *F. brasiliensis*, considered to be a secondary agent of lethargic crab disease, was frequently isolated from the crab habitat, whereas *E. cancerae* could never be isolated or detected in the mangrove environment. De Hoog et al. [4] established that the optimum growth temperature of *E. cancerae* is 24–27 °C, with a maximum at 33 °C, while the maximum of the *F. brasiliensis* lies at 37 °C, suggesting that temperature tolerance could be an important factor determining host preference.

According to de Hoog et al. [4], the infection pattern of members of Chaetothyriales suggests the existence of intrinsic factors enhancing vertebrate invasion, but infection is probably not a prime factor in the natural habitat of these fungi. In waterborne species, a slight correlation was observed between the maximum growth temperature of the fungus and the natural habitat of the host. Fungi with maximum growth temperatures below 33 °C tend to cause diseases in animals in cold waters. This is a remarkable feature, since elsewhere in the fungal kingdom, species lacking thermotolerance generally are saprobes without infectious abilities. Some species with maximum growth temperatures around 33 °C exceptionally up to 36 °C may cause zoonotics in cold-blooded animals living in shallow tidal zones in the subtropics. A striking example is the emerging

Lethargic Crab Disease in crabs inhabiting mangrove areas along the east coast of Brazil [1, 2].

The absence of *E. cancerae* in the environmental samples evaluated supports the alternative hypothesis of this study, namely that the species would be dependent on the crab species, with direct transmission, thus likely representing a genuine pathogen [1, 4, 25]. In contrast, *F. brasiliensis* was isolated at least four times from mangrove trees with historical records of LCD outbreaks, providing strong support for the null hypothesis of this study, that is, LCD with an environmental origin. *F. brasiliensis* is likely an opportunistic species and is not dependent of the host animal, *U. cordatus*. Epidemiological records of LCD outbreaks indeed indicate that while *E. cancerae* is systematically detected in sick, moribund, and dead crabs, whereas *F. brasiliensis* has only been detected in few mortality events. These results support the earlier conclusion [3] that ascribed responsibility for the LCD to *E. cancerae* alone.

However, two points do not match with this conclusion. First, since *E. cancerae* has not been detected in the mangrove compartments evaluated in this study, it is not clear how the fungus dispersed from a central point of initial outbreak to mangroves south and north along the Brazilian coast. Our study focused on solid substrates because waterborne black yeasts quickly tend to form biofilms [31], but we have not sampled marine water, and sampling was done during a period without LCD outbreaks. It seems reasonable to postulate that the dispersion of propagules of *E. cancerae* might occur using the coastal water and currents and probably is more extensive during disease outbreaks. Second, the conclusion that *E. cancerae* would be an obligate pathogen is negated by the fact that de Hoog et al. [4] listed strains from different environments including water, fruit drinks, and human skin. Strain Cunningham 179/99 mentioned in the same study was involved in a systemic zoonosis in toads in Israel (A. Cunningham, pers. comm.).

During disease outbreaks, the organs and tissues of moribund and dead crabs are overwhelmed by fungal growth [2]. Fungal cells are likely to be released upon decomposition of crab carcasses and carried away into open and coastal water by tide currents, which are responsible for dispersion into neighboring mangrove areas. This hypothesis was suggested previously by Smith [32] based on the distribution of dead animals in the same mangrove area as sampled in the present study, Canasvieiras. Isolation of black yeasts from seawater during and between periods of active LCD outbreaks may provide the evidence for testing this putative mode of dispersion.

References

1. Boeger WA, Pie MR, Ostrensky A, Patella L. Lethargic crab disease: multidisciplinary evidence supports a mycotic etiology. Mem Inst Oswaldo Cruz. 2005;100:161–7.
2. Boeger WA, Pie MR, Vicente VA, Ostrensky A, Hungria D, Castilho GG. Histopathology of the mangrove land crab *Ucides cordatus* (Ocypodidae) affected by lethargic crab disease. Dis Aquat Organ. 2007;78:73–81.
3. Orélis-Ribeiro R, Boeger WA, Vicente VA, Chammas M, Ostrensky A. Fulfilling Koch's postulates confirms the mycotic origin of Lethargic Crab Disease. Antonie Van Leeuwenhoek. 2011;99:601–8.
4. de Hoog GS, Vicente VA, Najafzadeh MJ, Harrak JM, Badali H, Seyedmousavi S. Waterborne *Exophiala* species causing disease in cold-blooded animals. Persoonia. 2011;27:46–72.
5. Vicente VA, Orélis-Ribeiro R, Najafzadeh MJ, Sun J, Guerra RS, Miesch S, Ostrenky A, Meis JF, Klaassen CH, de Hoog GS, Boeger WA. Black yeast-like fungi associated with Lethargic Crab Disease (LCD) in the mangrove-land crab, *Ucides cordatus* (Ocypodidae). Vet Microbiol. 2012;158:109–22.
6. Ferreira CP, Pie MR, Esteva L, Mancera PFA, Boeger WA, Ostrensky A. Modelling the lethargic crab disease. J Biol Dyn. 2009;3:620–34.
7. de Hoog GS, Göttlich E, Platas G, Genilloud O, Leotta G, van Brummelen J. Evolution taxonomy and ecology of the genus *Thelebolus* in Antarctica. Stud Mycol. 2005;51:33–76.
8. Dixon DM, Shadomy HJ, Shadomy S. Dematiaceous fungal pathogens isolated from nature. Mycopathologia. 1980;70:153–61.
9. Prenafeta-Boldú FX, Summerbell RC, de Hoog GS. Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard. FEMS Microbiol Rev. 2006;30:109–30.
10. Satow MM, Attili-Angelis D, de Hoog GS, Angelis DF, Vicente VA. Selective factors involved in oil flotation isolation of black yeasts from the environment. Stud Mycol. 2008;61:157–63.
11. Zhao J, Zeng J, de Hoog GS, Attili-Angelis D, Prenafeta-Boldú FX. Isolation of black yeasts by enrichment on

atmospheres of monoaromatic hydrocarbons. Microb Ecol. 2010;60:149–56.

12. Sudhadham M, Dorrestein GM, Prakitsin S, Sivichai S, Chaiyarat R, Prakitsin S, Menken SBJ, de Hoog GS. The neurotropic black yeast *Exophiala dermatitidis* has a possible origin in the tropical rain forest. Stud Mycol. 2008;61:145–55.

13. Sterflinger K, de Hoog GS, Haase G. Phylogeny and ecology of meristematic ascomycetes. Stud Mycol. 1999;43:5–22.

14. Wang CJK, Zabel RA. Identification manual for fungi from utility poles in the Eastern United States. Lawrence: Allen Press; 1990.

15. Lutzoni F, Pagel M, Reeb V. Major fungal lineages are derived from lichen symbiotic ancestors. Nature. 2001;411:937–40.

16. Iwatsu T, Miyaji M, Okamoto S. Isolation of *Phialophora verrucosa* and *Fonsecaea pedrosoi* from nature in Japan. Mycopathologia. 1981;75:149–58.

17. Vicente VA, Attili-Angelis D, Pie MR, Queiros-Telles F, Cruz LM, Najafzadeh MJ, de Hoog GS, Pizzirani-Kleiner A. Environmental isolation of black yeast-like fungi involved in human infection. Stud Mycol. 2008;61:137–44.

18. Pie MR, Boeger W, Patella L, Vicente VA, Orélis-Ribeiro R, Ostrensky A. Specific primers for the detection of the black-yeast fungus associated with lethargic crab disease (LCD). Dis Aquat Organ. 2011;94:73–5.

19. De Hoog GS, Gerrits van den Ende AHG. Molecular diagnostics of clinical strains of filamentous basidiomycetes. Mycoses. 1998;41:183–9.

20. Fijan N. Systemic mycosis in channel catfish. Wildl Dis. 1969;5:109–10.

21. Richards RH, Holliman A, Helgason S. *Exophiala salmonis* infection in Atlantic salmon *Salmo salar* L. J Fish Dis. 1978;1:357–68.

22. Reuter RE, Hutchinson W, Ham J, Davis S. *Exophiala* sp. infection in captured King George whiting (*Sillaginodes punctata*). Bull Eur Assoc Fish Pathol. 2003;23:128–34.

23. Manharth A, Lemberger K, Mylniczenko N, Pinkerton M, Pessier AP, Kammeyer P, de Hoog GS. Disseminated phaeohyphomycosis due to *Exophiala* species in a Galapagos tortoise, *Geochelone nigra*. J Herpetol Med Surg. 2005;15:20–6.

24. Nyaoke A, Weber ES, Innis C, Stremme D, Dowd C, Hinckley L, Gorton T, Wickes B, Sutton D, de Hoog S, Frasca S Jr. Disseminated phaeohyphomycosis in weedy, *Phyllopteryx taeniolatus*, and leafy, *Phycodurus eques*, seadragons caused by species of *Exophiala*, including a novel species. J Vet Diagn Invest. 2009;21:69–79.

25. De Hoog GS. Evolution of black yeasts: possible adaptation to the human host. Antonie Van Leeuwenhoek. 1993;63:105–9.

26. Bonifaz A, Badali H, de Hoog GS, Padilla-Desgarennes C, Vázquez-González D, Navarrete G, Meis JF. Severe disseminated phaeohyphomycosis in an immunocompetent patient caused by *Veronaea botryosa*. Mycopathologia. 2013 (in press).

27. Li DM, Li RY, de Hoog GS, Wang YX, Wang DL. *Exophiala asiatica*, a new species from a fatal case in China. Med Mycol. 2009;47:101–9.

28. De Hoog GS. Significance of fungal evolution for the understanding of their pathogenicity illustrated with agents of phaeohyphomycosis. Mycoses. 1997;40 Suppl 2:5–8. doi:10.1111/j.1439-0507.1997.tb00555.x.

29. Li DM, de Hoog GS, Lindhardt Saunte DM, Gerrits van den Ende AHG, Chen XR. *Coniosporium epidermidis* sp. nov., a new species from human skin. Stud Mycol. 2008;61:131–6.

30. de Hoog GS, Nishikaku AS, Fernández Zeppenfeldt G, Padín-González C, Burger E, Badali H, Gerrits van den Ende AHG. Molecular analysis and pathogenicity of the *Cladophialophora carrionii* complex, with the description of a novel species. Stud Mycol. 2007;58:219–34.

31. Heinrichs G, Hübner I, Schmidt GK, de Hoog GS, Haase G. Compositional analysis of dark fungal biofilms from water taps using Tag Encoded FLX Amplicon Pyrosequencing. Mycopathologia. 2013 (in press).

32. Schmidt AJ. Estudo da dinâmica populacional do caranguejo-uçá, *Ucides cordatus* (Linnaeus, 1763) (Crustacea-Decapoda-Brachyura), e dos efeitos de uma mortalidade em massa desta espécie em manguezais do Sul da Bahia. Dissertation (in Portuguese). Universidade de São Paulo, Brazil. 2006.