

Fulfilling Koch's postulates confirms the mycotic origin of Lethargic Crab Disease

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Received: 31 August 2010 / Accepted: 12 November 2010 / Published online: 9 December 2010
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Abstract In the northeast region of the Brazilian coast, a disease has been causing massive mortalities of populations of the mangrove land crab, *Ucides cordatus* (L.) since 1997. The clinical signs of this disease, which include lethargy and ataxia, led to the disease being termed Lethargic Crab Disease (LCD). Evidence from a variety of sources indicates that there is an association between LCD and a new species of black yeast, *Exophiala cancerae* de Hoog, Vicente, Najafzadeh, Badali, Seyedmousavi & Boeger. This study tests this putative correlation through in vivo

experiments. Disease-free specimens of *U. cordatus* were experimentally infected with *Exophiala cancerae* (strain CBS 120420) isolate. During the 30-day experimental period, only a single death was observed within the control crabs. However, at the end of this period, crabs that were inoculated once or three-times with mycelial elements and hyphae of *E. cancerae* had a 60% and 50% mortality rates, respectively ($n = 6$ and $n = 5$). These results support that the fungal agent is pathogenic and is the causative agent of LCD. Species-specific molecular markers confirm the presence of *E. cancerae* (strain CBS 120420) in recovered colonies and tissue samples from the infected animals. The experimentally infected crabs manifested signs (lethargy, ataxia and tetany) that were consistent to LCD-affected animals in the environment. These results fulfil Koch's postulates and the hypothesis that the tested strain of *Exophiala cancerae* is a causative agent of LCD is accepted.

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Keywords Lethargic crab disease · *Ucides cordatus* ·
Black yeast · *Exophiala cancerae*
(strain CBS 120420) · Pathogenicity · Koch's
postulates · Systemic phaeohyphomycosis

Introduction

Populations of the Brazilian mangrove land crab *Ucides cordatus* (Linnaeus 1763) (Brachyura: Ocypodidae) have been submitted to massive mortalities

since 1997. These mortality outbreaks spread northward and southward, significantly reducing populations of *U. cordatus* in 8 of the 17 coastal states, particularly in the northeast (states of Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia) and southeast regions (state of Espírito Santo) (Boeger et al. 2005, 2007).

Crabs that are affected by this epidemic share several common signs, such as increasing ataxia (especially of the pereiopods and chelae), lethargy, poor balance, and, ultimately, death. Dead animals are usually found outside but by their individual burrows. In some cases, fishermen have also reported episodes of tetany. The disease was termed lethargic crab disease (LCD) because of these reported set of specific clinical signs (Boeger et al. 2005, 2007).

The economic impacts of LCD are extensive, with reductions in the fishing yields of 84% and 97.6% in mangroves of Paraíba and Bahia, respectively (Alves and Nishida 2003; Schmidt 2006). Such depressions in local stocks are of particular concern given that this species represents an important fishery resource for artisanal fisheries along the Brazilian coast. Prior to the disease outbreak, annual yields could reach more than 7 tons of crabs per km² of mangrove forest (Alves and Nishida 2003; Araújo 2006; Glaser 2003). The environmental impact of LCD is likely severe. *Ucides cordatus* is considered a keystone species of West Atlantic mangroves, since its feeding activity accelerates decomposition of mangrove litter and, thus, nutrient remineralisation and energy transfer into the sediment (Nordhaus 2003).

Although several potential etiological agents of LCD have been informally proposed, such as viruses, bacteria, environmental pollution, and chemicals used in shrimp farming, relatively few scientific studies have directly addressed the cause of LCD (Boeger et al. 2005, 2007). In those studies, evidences from a variety of sources (light and electron microscopy, behavioral tests, histopathology, and molecular phylogenetics) indicated that LCD signs were associated with the presence of a black yeast, a new species of *Exophiala* (Herpotrichiellaceae; Chaetothyriales). Such species was recently described as *Exophiala cancerae* de Hoog, Vicente, Najafzadeh, Badali, Seyedmousavi & Boeger (MycoBank 515720).

In this study, we test the hypothesis that this black yeast is the causative agent of LCD. According to Koch's postulates, four criteria must be fulfilled for

an infectious agent to be considered the causative agent of a disease (Koch 1884). The first two criteria—the presence of the putative agent in all affected crabs, as well as isolation of the pathogen and growth in pure culture—have been fulfilled by Boeger et al. (2005, 2007) and Pie et al. (in press). Thus, the final two criteria—that the agent causes disease when introduced into healthy animals and are reisolated from these hosts—were tested in this study.

Materials and methods

Healthy *U. cordatus* crabs (5.8–7.8 cm total carapace width) were collected from a LCD-free region in Ilha das Peças (Antonina Bay, State of Paraná; Fig. 1). The experimental setup consisted of a series of plastic aquariums (50 L) that contained seawater (15 L, salinity 25 psu) with continuous aeration, controlled temperature (around 25°C), and photoperiod (12 h light: 12 h dark). The crabs were individually housed and were fed ad libitum with fish meat; approximately half of the seawater was changed every 2 days. Sex ratio of crabs used in the experiments was always maintained at 1:1.

Two weeks prior to experiment initiation, fresh subcultures of *Exophiala cancerae* (number 120420 from the Centralbureau voor Schimmelcultures collection, Netherlands) isolated from sick crabs during LCD outbreaks in the state of Sergipe, Brazil



Fig. 1 Location sites of uninfected *U. cordatus* (filled triangle, Ilha das Peças, Antonina Bay, Paraná State). The thick dashed line on the Brazilian coastline represents the areas subjected to LCD epidemics

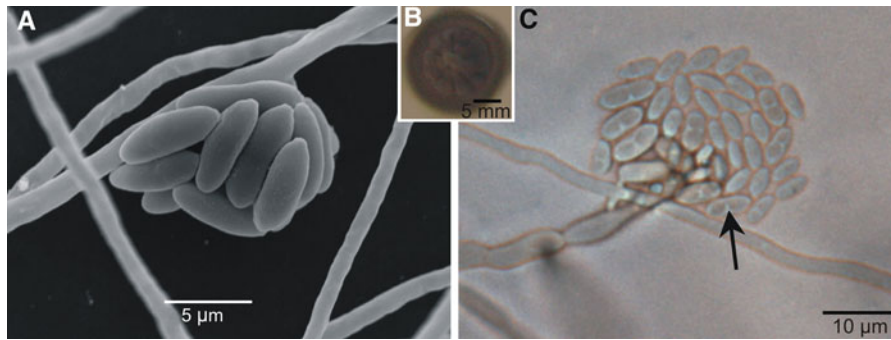


Fig. 2 Macro- and micromorphological characteristics of *Exophiala cancerae* (strain CBS 120420) **a** Scanning electron micrograph of a conidiophore. **b** Colony surface growth on

(2004–2005) were plated on Brain Heart Infusion Agar (BHI) and incubated at 25°C (Fig. 2). Conidia and hyphal elements were collected from the surface of fresh colonies and suspended in a saline solution (2.5%, *U. cordatus* hemolymph physiological salinity) (Harris and Santos 1993) with 1% Tween-20 (Promega, USA). A suspension that contained 2×10^7 elements per millilitre was prepared for inoculation (adapted from Iwatsu et al. 1981).

Pathogenicity test

Disease-free *U. cordatus* were maintained under the experimental conditions for 1 week prior to the start of the experiments to ensure that the crabs were acclimated to their surroundings and did not have any overt disorders. The experimentally infected crabs were divided into two groups: 26 infected once, at the beginning of the trial; and 26 infected three times along the trial period (days 1, 14, and 20 of the experiment). Thus trying to assess differences in mortality rate related to a hypothesis of extended exposure of naturally-infected crabs to the putative pathogen. The experimental groups were further subdivided into 3 groups (1 treatment and 2 controls). Of the 26 total crabs, 10 crabs were injected with 1 mL of the yeast inoculum solution, 8 crabs were injected with 1 mL of 2.5% saline solution, and 8 crabs had a mock injection in which no solution was introduced into the animal. Dose level of yeast suspension was chosen in order to optimize the development of the putative pathogenicity in a viable experimental time. The injections (Injex syringe, 5 mL) were administered to the arthrodistal membrane at the juncture of the pereopod basis and carapace.

Mycosel, incubated for 14 days at 25°C. **c** Light microscopy image of conidia; the *black arrow* indicates the internal septum

The decision to use injection as the infection method simply reflects the lack of knowledge on the natural invasion pathways of the agent but ensured that the fungal elements would be introduced into the crab system.

The health status of each experimental crab was monitored daily, turning each individual crab with the cephalothorax down and measuring the time (in seconds) until their return to the upright position (Boeger et al. 2005, 2007). Samples from the gills, heart, hemolymph, hepatopancreas, and thoracic ganglion were collected by the dissection of dead or euthanised crabs. Euthanasia was performed by placing the crab in cold freshwater at the end of the experiments. The tissue samples were immediately prepared for the appropriate assays as follows: fixation in Davidson's AFA (alcohol, formalin and acetic acid) (Humason 1979) for histological analysis of samples from the gills, heart, hemolymph, hepatopancreas, and thoracic ganglion; storage in salt-saturated DMSO/EDTA buffer (Seutin et al. 1991) for molecular studies with DNA extract from the heart and hepatopancreas (Pie et al. unpublished); or stored at 2–4°C for microbiology studies (e.g., fungal recovery from the heart, hepatopancreas and thoracic ganglion portions). The crab carcasses were incinerated in a bioterium and the contaminated habitat water was decontaminated with 1% sodium hypochlorite (NaClO).

Histological preparations

Histological sections from the euthanised animals were used for prospecting the fungi elements and to record host tissue and cell response to the fungus. Sections of animals that died during the experiments

were processed similarly to prospect fungal elements only. Samples fixed in AFA were embedded in paraffin after dehydration through an ethanol series. Sections (5 μm thick) were prepared with a rotary microtome (Leica RM2125RT) and stained with Harris' haematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) (Humason 1979). The processed sections were examined with standard light microscopy (Olympus BX51) and photographed with a QColor 5 digital camera. The magnification scales were added with the Image J software (Rasband 2006).

Molecular diagnosis

Tissue samples were suspended in 480 μL of digestion buffer (50 mM Tris—pH 8.0, 100 mM EDTA, 0.5% SDS) with 20 μL of proteinase K. The samples were subsequently incubated at 55°C for 3 h to digest the host tissue. Removal of crab DNA was performed using the initial part of the protocol of the EZ-DNA kit (Biosystems, Brazil). The precipitate that contained the conidia was resuspended in 500 μL of double distilled water, centrifuged, and the supernatant was discarded; this process was repeated three times to remove any trace crab DNA. The final precipitate was resuspended in 500 μL of double distilled water and the cells were disrupted by maceration in liquid nitrogen. A Dneasy kit (Qiagen, GmbH, Germany) and a standard animal tissue protocol were used to extract the yeast DNA (adapted from Boeger et al. 2005). Species-specific polymerase chain reaction (PCR) primers for *E. cancerae* (Pie et al. in press), designed based on the ITS rDNA sequence, were applied to the DNA extracts to detect the presence of the pathogenic agent of LCD using the authors recommendations.

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 bp, which corresponds to the expected product size of *E. cancerae* (strain CBS 120420).

Microbiology assays and molecular identification of colonies recovered from infected tissue

Re-isolation of fungus from dead crab tissue was performed by culturing the sample in a Mycosel

medium (Pronadisa, Spain) for 2 weeks at 25°C. The resulting colonies were analysed for their morphology and were PCR-amplified with species-specific primers to confirm the presence of the pathogen, as described above.

The samples were cultured in Sabouraud Agar (Himedia, India) at 25°C, and the DNA was subsequently extracted. Briefly, a mycelium piece (approximately 1 cm^2) was collected and transferred to a microtube with 300 μL of CTAB (cetyltrimethylammonium bromide) and a powder silica mixture (silica gel Merck/Celite 2:1). After maceration, 200 μL of CTAB was added, and the mixture was vortexed and incubated for 10 min at 65°C. Chloroform (500 μL) was added, and the mixture was centrifuged (14,000 $\times g$). The aqueous phase was extracted, two equivalent volumes of cold ethanol 96% (Merck) were added, and the mixture was incubated at -20°C for 30 min or until the DNA precipitated. The precipitated samples were centrifuged again and the supernatant was discarded. The precipitate was washed with cold 70% ethanol, centrifuged for 1 min, and then dried. The resulting DNA pellet was dissolved in 50 μL of TE buffer, incubated for 2 h at ambient temperature, and the DNA concentration was determined spectrophotometrically (Vicente 2000). The resulting DNA extract was submitted to the species-specific PCR markers (as described above).

Results

Mortality rates associated with experimental infection

Patterns of mortalities exhibited by experimental infection trials (Figs. 3, 4), single- and multiple infections, were consistent with a high virulent degree of the studied fungus. Crabs from the single-infection experiment began to die 11 days after infection; 20% of the infected crabs had died by this time ($n = 2$). Approximately 50% of the specimens were dead by day 29 and 60% of the crabs were dead at the end of the 30-day period ($n = 6$). In this experiment, no crabs that received mock injections died and only one death was observed on the group inoculated with saline (Fig. 3). No conspicuous difference of mortality was detected between sexes.

Fig. 3 Mortality curves for crabs that were subjected to a single experimental infection. The *filled circles* represent the infected group (with *E. cancerae*—strain CBS 120420); the *open triangles* represent the control group (saline solution); and the *filled squares* represent the control group (mock injection)

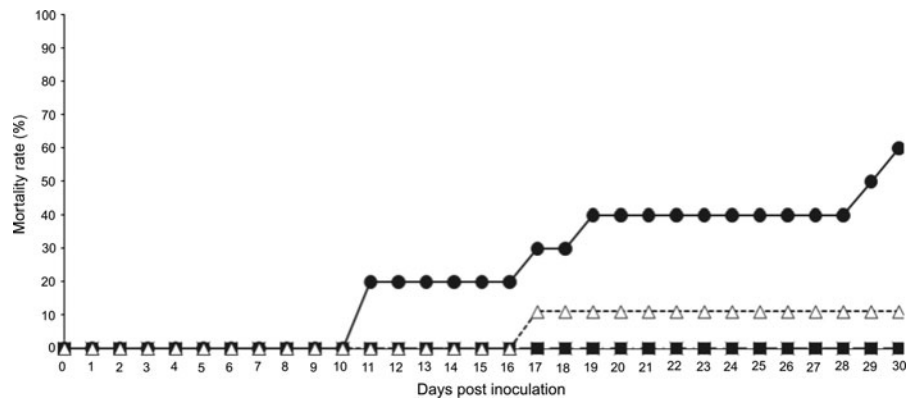
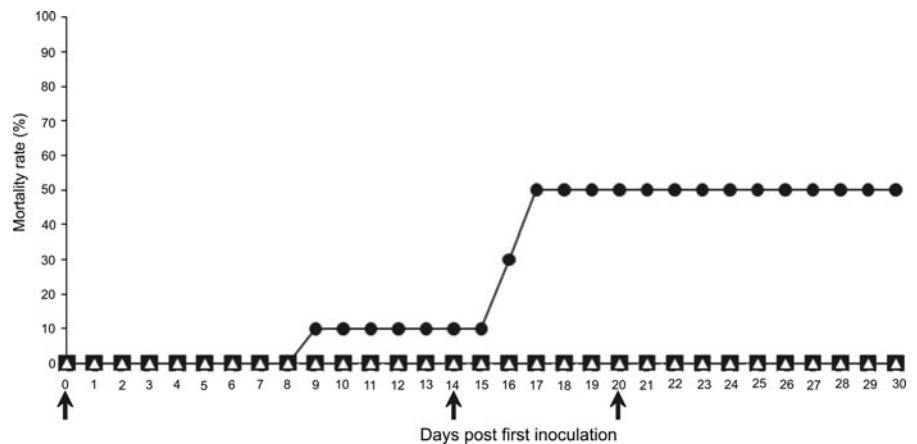


Fig. 4 Mortality curves for *U. cordatus* specimens that were subjected to multiple experimental infections. The *filled circles* represent the infected group (with *E. cancerae*—strain CBS 120420); the *open triangles* represent the control group (saline solution); and the *filled squares* represent the control group (mock injection). The *arrows* indicate inoculation on the corresponding days



In the multiple-infection trial, artificially infected *U. cordatus* began to die by day 9. A second injection was performed on day 14 and by day 17, 50% of the crabs were dead. This mortality rate did not change after the third injection, on day 20. Only half of the artificially-infected crabs survived the entire 30 days, whereas neither control groups exhibited mortality (Fig. 4). In both experiments, artificially-infected crabs showed LCD-related signs of (lethargy, ataxia, and tetany) prior to death.

Histology

Several fungal elements were observed in the tissue of deceased, artificially infected crabs. Fungal elements were also detected in all crabs euthanized at the end of the experiment but that did not express the disease. In the deceased crabs, the fungal elements observed included yeast-like cells, moniliform hyphae, and/or distorted hyphae. The experimentally infected crabs that were sacrificed at the end of the

study displayed similar histopathological patterns. Hemocytic agglutinations (granulomas) were often present in the gills and were located mainly in the hypobranchial arteries, hemal sinuses, and in the afferent vessel of the gill lamellae. Additionally, there were numerous immune cells with melanin deposits and encapsulated fungal elements. Moreover, melanised nodules were seen extensively in the heart. Two types of yeast-like morphotypes were observed in the heart: a larger size, which was positive for PAS-staining (slightly reddish) and a smaller one, which stained blue, that was only observed in hearts of crabs from the multiple-infection experiment. The smaller blue nodules were abundant in damaged hearts and were associated with necrotic areas. Several granulomas were also present close to cells that comprise the blind-ending tubules of the hepatopancreas. However, no fungal elements were observed in the numerous tubule lumens that constitute this organ. Histological sections of the thoracic ganglia showed that there was hemocytic

infiltration, which was associated with the fungal infection. The granulomas, which were highly concentrated and contained hyphae and yeast-like cells, were found outside the neurilemma, which is a membrane that may act as a barrier against infection. Additionally, there were signs of tissue degeneration near the nodules.

Tissue samples collected from infected crabs that manifested LCD-like signs have several characteristics that may relate to the disease signs. As in naturally infected crabs, infection within the heart was limited to the myocardium and no fungal elements were seen in the peri- and epicardial tissue. As reported for LCD (Boeger et al. 2007), hemocytic encapsulations (surrounding hyphae and yeast-like cells) compressed the nerve fibres in the thoracic ganglion complex.

Molecular and microbiology diagnoses—infected tissue and recovered colonies

The application of the specific molecular markers for *E. cancerae* (CBS 120420) confirmed the identity of the fungus established in the tissues of the artificially infected *U. cordatus* that died in the experiment. The fungus present in the tissue samples of all these crabs belongs to the *Exophiala* strain originally isolated from naturally infected crabs and cultivated in the laboratory.

Tissue samples were collected from crabs that died during the experiment and were plated in Mycosel medium; after 2 weeks, colonies that had characteristics similar to the fungus isolated from moribund *U. cordatus* with LCD were observed. The colonies were brown and velvety in the centre and black and smooth near the edge. The application of the PCR-diagnosis using specific marker to the DNA extracts of these colonies demonstrated that these fungi represent the original strain associated to LCD (Fig. 5) and used in the experimental infections.

Discussion

Black yeasts (Herpotrichiellaceae, Ascomycota) are known to cause a wide diversity of clinical profiles that vary from subcutaneous to cerebral infections in warm- and cold-blooded vertebrates (Carmichael 1966; De Hoog and Guarro 1995; De Hoog et al.

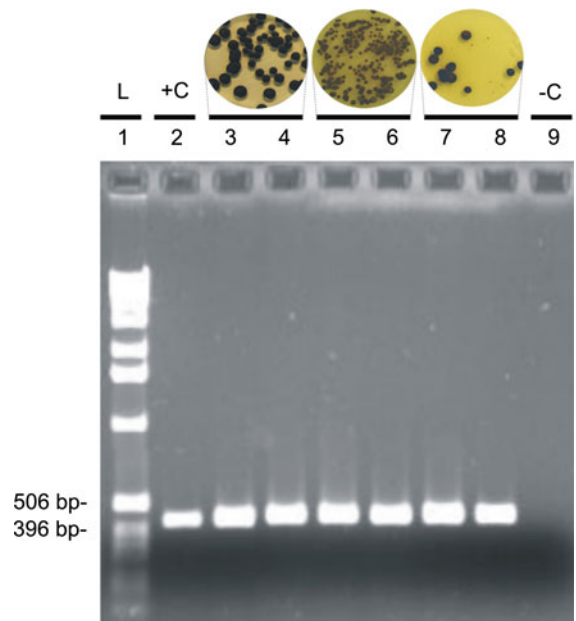


Fig. 5 Amplification of an *Exophiala* sp. (strain CBS 120420) specific fragment, which was used to screen colonies that were recovered from the tissue of artificially infected crabs. Lane 1: molecular size marker (1 kb ladder) (L); 2: *E. cancerae* (strain CBS 120420) (positive control) (+C); 3–8: DNA extract of colonies that were recovered from the heart (3 and 4), hepatopancreas (5 and 6) and thoracic ganglion (7 and 8); and 9: negative control (–C). The pictures above the gel are the respective agar plates with colony growth

1998; Otis et al. 1985). According to Koch's postulates, the data from this study show that LCD is caused by systemic phaeohyphomycosis associated with *E. cancerae* (strain CBS 120420) infection. Our results agree with the previous hypothesis of Boeger et al. (2005, 2007) concerning the etiology of this disease.

Generally, the histopathological patterns observed in the artificially infected crabs are similar to those that were seen in moribund *U. cordatus* during LCD outbreaks (Boeger et al. 2007), although there are some differences. In the naturally infected crabs, the free yeast-like cells only expressed a morphotype that was significantly larger than what was seen in the *U. cordatus* from the multiple-infection experiment. Furthermore, free hyphae were seen within the tissues (mainly cardiac tissue) of sick crabs that were collected from the mangroves and these elements were only observed in extremely debilitated crabs. According Oujezdsky et al. (1973), this scenario may be associated to the fact that fungal cells in stable

environments are in the stationary phase, i.e. accumulate lipids that trigger the conversion to hyphae. This result suggests that the artificially infected crabs did not develop a high degree of immune weakness and, probably due to the continuous stress of the trial confinement associated with handling and with the impossibility of burrowing, died prematurely.

Vertebrate models (healthy mice and fish) that were artificially infected with dematiaceous fungi (black yeast) and later sacrificed (after an average of 2 weeks) (Espinell–Ingroff et al. 1982; Iwatsu et al. 1981; Otori et al. 2005) had similar immune response as the *U. cordatus* specimens in this study. Although the vertebrate immune system is unique in that it has adaptive memory, immunoglobulins that recognize specific epitopes and specialised cells (e.g., lymphocytes), the non-specific immune responses (e.g., phagocytes and giant cells) are similar to the crustacean immune system. Non-specific immune responses recognise the non-self elements within the organism and subsequently phagocytise the foreign object or promote immune cell infiltration (Söderhäll and Cerenius 1992). Similarities of infections in vertebrates and invertebrates models are also present in the type of fungal elements found in the infected tissues (yeast-like cells, moniliform, or distorted hyphae). Another remarkable feature shared with the present (invertebrate) and vertebrate trials refer to the nervous system affinity of these elements, which can be facilitated by the level of free iron in this tissue (De Hoog 1993).

The experimental model of infection in this study also reproduced the clinical signs associated with naturally-infected *U. cordatus* specimens. In nature, infected crabs become increasingly lethargic and ataxic as the disease develops, resulting in reduced feeding dexterity and a decreased ability to escape from predators or crab fishermen. Moreover, LCD-affected *U. cordatus* show signs of tetany (Boeger et al. 2005), which suggests that the disease damages the thoracic ganglion. Accordingly, the results from this study showed that infection resulted in the formation of nodules that compressed the nerve fibres, which could result in tetany (see also Boeger et al. 2007). Infected *U. cordatus* specimens from mangroves rapidly die after capture and transport, and their rapid death may be correlated with their inability to respond to stress, as well as heart failure, starvation, and suffocation. Similarly, the experimental animals

were subjected to captivity stress and probably suffocated because of the disease-associated nodules, which obstructed the haemal sinuses and veins, as well as extensively damaged the surrounding tissue.

Because of their melanised cells, dematiaceous fungi can live in extreme conditions, such as high temperatures or exposure to ultraviolet radiation (Wheeler and Bell 1988). In addition to melanin, these fungi can synthesise carotene when light is present (Geis and Szanislo 1984). Some studies suggest that these pigments also neutralise free radicals and prevent phagocytosis within the host tissue (Dixon and Polak-Wyss 1991). Feng et al. (2001) used an immunocompetent mouse model system and *Exophiala dermatitidis* (Kano), which is phylogenetically close to the causative agent of LCD to study the correlation between melanin production and strain virulence. Inoculation with wild type strains resulted in a high mortality of mice (from 80 to 100%) compared to strains that could not produce melanin (0–10%). Because herpotrichiellaceous black yeast are greatly virulent, De Hoog (1993) hypothesised that these species can share factors needed for growth and dispersal in their natural niche, which may also facilitate their survival inside the host. Because the *E. cancerae* strain used in our study was kept under favourable culture conditions during storage, survival adaptations were likely not induced; hence the overall mortality was comparatively reduced for the experimental conditions.

Exophiala species are ubiquitous and have been isolated from polluted water, decayed wood and leaves (De Hoog and Guarro 1995), and even from *Rhizophora mangle* (red mangrove) leaves in the Puerto Rico mangroves (Nieves-Rivera 2005). According to Nordhaus (2003), 61.2% of the *U. cordatus* diet is *R. mangle* leaves, mostly senescent and decomposed. The crabs additionally feed on algae and, because green and brown algae have favourable carbon/nitrogen (C:N) ratios (measure of nutritional value of the diet), these may be important components of the crab diet and may compensate for the poor C:N ratio that is found in the red mangrove leaves. The LCD-associated fungus may be naturally present in algae and/or decaying plant components and may, thus, infect crabs through the natural feeding cycle. However, additional environmental studies are needed to confirm this hypothesis. Moreover, additional studies are necessary to better

understand the disease epidemiology, prospect the pathogenic agent in distinct environmental compartments, and test its mechanism of infection and transmission.

Acknowledgments Financial support for this research was provided by the Companhia de Desenvolvimento Industrial e de Recursos Minerais de Sergipe (CODISE), the Secretaria de Estado da Ciência, Tecnologia e Ensino Superior do Estado do Paraná (SETI), and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. The following people provided either field or laboratory support to this study: M. Pie, D. Hungria, G. Castilho, J.F. de Oliveira Neto, R. Pilchowsky, and R.V. de Souza. S.C. de Hoog of the Centraalbureau voor Schimmelcultures, Netherlands confirmed the identity of the fungus species. Antonio Ostrensky and Walter A. Boeger are research fellows of CNPq.

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