

Is Marine Dispersion of the Lethargic Crab Disease Possible? Assessing the Tolerance of *Exophiala cancerae* to a Broad Combination of Salinities, Temperatures, and Exposure Times

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Received: 13 April 2017 / Accepted: 17 June 2017 / Published online: 28 June 2017
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Abstract Since 1997, an emergent fungal disease named lethargic crab disease (LCD) has decimated stocks of the edible mangrove land crab *Ucides cordatus* (Linnaeus, 1763) (Brachyura: Ocypodidae) along the Brazilian coast, threatening the mangrove ecosystem and causing socioeconomic impacts. Evidence from a variety of sources suggests that the black yeast *Exophiala cancerae* (Herpotrichiellaceae, Chaetothyriales) has been responsible for such epizootic events. Based on the spatiotemporal patterns of the LCD outbreaks, the well-established surface ocean currents, and the range of ecological traits of *Exophiala* spp., a marine dispersal hypothesis may be proposed. Using in vitro experiments, we tested the survival and growth of *E. cancerae* CBS 120420 in a broad combination of salinities, temperatures, and

exposure times. While variation in salinity did not significantly affect the growth of colony-forming units (CFUs) ($P > 0.05$), long exposure times visibly influenced an increase in CFUs growth ($P < 0.05$). However, higher temperature (30 °C) caused a reduction of about 1.2-fold in CFUs growth ($P < 0.05$). This result suggests that sea surface temperatures either above or below the optimum growth range of *E. cancerae* could play a key role in the apparent north–south limits in the geographical distribution of LCD outbreaks. In light of our results, we conclude that a fundamental step toward the understanding of LCD epidemiological dynamics should comprise a systematic screening of *E. cancerae* in estuarine and coastal waters.

Keywords *Exophiala cancerae* · Black yeasts · Lethargic crab disease · Marine dispersal

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Introduction

Since 1997, outbreaks in populations of the mangrove land crab *Ucides cordatus* Linnaeus, 1763 (Brachyura: Ocypodidae) (popularly known in Brazil as “caranguejo-uçá”), have threaten the mangrove ecosystem [1] and caused drastic socioeconomic impacts on artisanal fishing communities [2, 3]. Evidence from a variety of sources (light and electron microscopy, histopathological studies of sick crabs, behavioral tests, experimental infections,

molecular phylogenetics, mathematical modeling, and epidemiological assessments) strongly suggest that a black yeast species of fungus, named *Exophiala cancerae* de Hoog, Vicente, Najafzadeh, Badali, Seyedmousavi & Boeger, 2011 (Herpotrichiellaceae, Chaetothyriales) (strain CBS 120420, MycoBank MB515720), is responsible for the epizootic events [1, 4–11]. Based on the clinical signs of sick crabs, this infirmity has been named lethargic crab disease (LCD) [1].

Mortalities caused by LCD have been reported from a considerable fraction of the distribution of *U. cordatus*, occurring on approximately 2700 km of the Brazilian coastline [1, 4–11]. Since the first record of mortalities in the year 1997, near the city of Goiana (state of Pernambuco, Brazil), LCD has spread northward (i.e., states of Paraíba, Rio Grande do Norte, Ceará, and Piauí) and southward (i.e., states of Sergipe, Bahia, and Espírito Santo) in a seemingly wave-like pattern in the same direction of the surrounding surface ocean currents, hence affecting crab populations of eight out of the total 17 Brazilian coastal states [1, 4–11] (Fig. 1). Given that *U. cordatus* inhabits mangroves in an extensive area in the Occidental Atlantic, from the state of Florida (USA) to the coast of the state of Santa Catarina (Brazil) [13], available evidences suggest that such dispersion of LCD outbreaks has reached an apparent north and south limit. The northernmost confirmed mortality event caused by LCD was reported from a single event in the state of Piauí in 2003, whereas the southernmost records comprise several episodes in mangroves of the state of Espírito Santo, since 2006 [7, 10].

Although the original habitat of the strain of *E. cancerae* associated with the LCD outbreaks still remains to be elucidated [11], several lines of evidence have supported a waterborne origin for the disease. The genus was proposed by Carmichael [14] based on *Exophiala salmonis* Carmichael, 1966, responsible for a systemic infection in trout. Subsequently, two other pathogens of fish were reported, *E. pisciphila* [15] and *E. psychrophila* [16]. Recent molecular phylogenetic analyses have indicated that *E. cancerae* (CBS 120420) belongs to a particular clade of waterborne species that infect a range of cold-blooded animals, including the previously mentioned fishes as well as turtles, and frogs [7]. Interestingly, strains of *E. cancerae* isolated to date are characterized by a cosmopolitan distribution and

have been regularly recovered from water sources, showing meso-/psychrophilic and halophilic tendencies [7]. Regarding specifically the strain associated with the LCD, Schmidt [17] suggested that the most likely dispersion pathway is the water, because during his studies, mangrove crabs near the lower intertidal zones had a more significant decrease in population densities.

Confronting the spatiotemporal patterns of the LCD events, the well-established surface ocean currents, and the range of ecological traits of *Exophiala* spp (Fig. 1), an epidemiological scenario may be proposed. In such context, if *E. cancerae* CBS 120420 is capable to withstand relatively long exposure to pronounced variations in environmental conditions (i.e., water surface salinity and temperature), this etiological agent may persist in the estuarine, inshore, and offshore waters and the disease could spread over relatively long distances (≈ 100 km) affecting naive populations of *U. cordatus* in adjacent estuaries. Alternatively, if fungal forms' viability is hampered by exposure to some specific salinity and temperature regime, similar to any particular condition observed in the Brazilian estuarine and coastal environments, factors limiting the dispersion of the disease outbreaks could be elucidated.

Herein, we tested the survival and growth of *E. cancerae* using in vitro experiments that emulate a varied combination of environmental conditions in space and time. The understanding of the dispersal pathways of this etiologic agent is fundamental for the improvement of predictive models in epidemiological studies, risk assessment, and management of the LCD.

Materials and Methods

Experimental Strain: Source and Preparation

The strain of *Exophiala cancerae* CBS 120420 (Fig. 2) evaluated in this study was isolated through oil flotation technique [18] from tissues of moribund crabs collected during mortality events in the states of Bahia and Sergipe (Brazil) (beginning of 2004 and 2005) [1]. Prior to the experiments, fresh subcultures of *E. cancerae* were plated on Sabouraud agar (HiMedia) and incubated at 25 °C. Conidia and hyphal elements collected from the surface of fresh colonies were re-suspended in saline solution (2.5%, the same physiological salinity of *U. cordatus* hemolymph [19]) with Tween[®]20 (1%) [7, 20]. Subsequently, a test

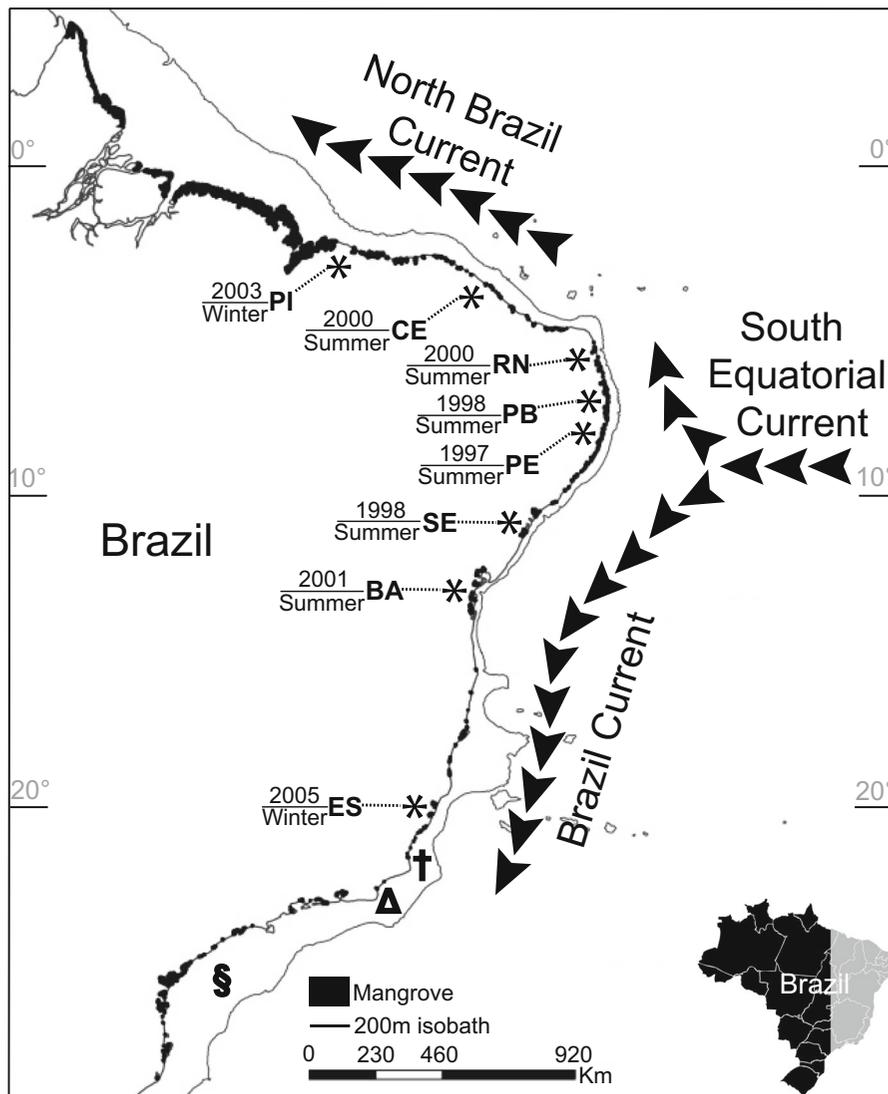


Fig. 1 Timeline of LCD outbreaks depicting a schematic distribution of the main sea surface currents, mangrove areas, and 200-m isobath. Asterisk: localities affected by LCD

solution of about 2×10^7 fungal elements per mL was obtained with the aid of a Neubauer chamber (Optik Labor) under a phase-contrast microscope (Olympus BX51) (adapted from [18]). Manipulations were performed in a horizontal laminar airflow workstation (LabCon Co., Purifier Class II).

Experimental Design

Test solution with *E. cancerae* was exposed at simultaneous combinations of seawater salinities, temperatures, and exposure times estimated to accurately

outbreaks; Dagger Cape São Tomé; triangle: Cape Frio, and section sign São Paulo Bight. Map modified from Magris and Barreto [12]

simulate the conditions faced by this black yeast in a putative scenario of marine dispersal among estuaries. A total of 24 treatments were delineated to quantify the growth of *E. cancerae* at combinations of four salinities (0, 10, 25, and 38 ppt) and two temperatures (25 °C and 30 °C), over a period of 1, 24, and 168 h (Table 1). Each experimental treatment was replicated five times. Specifically, aliquots (50 μ L) of the diluted (tenfold dilution) test solution were added to 1 mL of sterilized seawater with different levels of salinity, adjusted through evaporation and dilutions (with sterile double-distilled water). Salinity of the samples was measured in

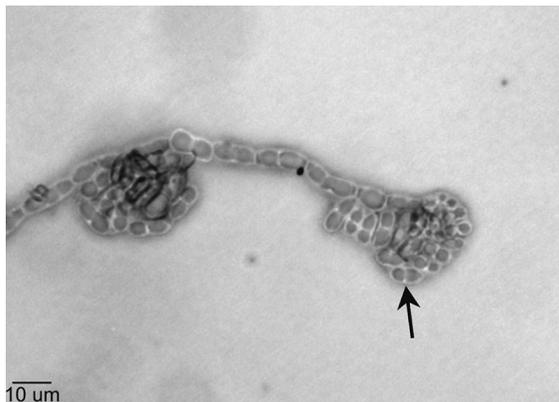


Fig. 2 *Exophiala cancerae* CBS 120420 isolated from *Ucides cordatus* showing signs of the Lethargic Crab Disease. Light microscopy micrograph of conidiophores and conidia, many evidencing internal septum (arrow), an important diagnostic feature

a scale of parts per thousand (ppt) using a hand refractometer (PCE-0100, UK). The resulting solutions comprising 0, 10, 25, and 38 ppt were distributed into 1.5-mL Eppendorf tubes, totaling 30 replicates per salinity level. Subsequently, each of these levels was divided into two subgroups, one incubated at 25 °C and another at 30 °C. Tubes of each subgroup were kept in these temperatures for 1, 24, and 168 h. Subsequently, their contents were carefully spread onto Mycosel agar (Pronadisa, Spain) plates with the aid of a Drigalski spatula. Noteworthy is that the chosen seawater salinities and temperatures approximated the average monthly values reported in the Brazilian coastal zone (<https://www.nodc.noaa.gov/>) [21, 22]. Also, selected exposure times are based on average speed of sea surface currents [23, 24] and thus assessed the possibility of survival and growth of colony-forming units (CFUs) of *E. cancerae* when transported within and between estuaries.

Growth of Colonies and Statistical Method

After 14 days of incubation, plated Petri dishes (90 × 15 mm) were photographed under equal light and camera parameters using a digital camera Sony MVC-CD500. These images were used to evaluate the area covered by the CFUs with the aid of the analysis tools provided by SigmaScan[®] Pro 5.0 software (Systat Software Inc., USA). All images were converted to gray scales; only elements with the intensity threshold

Table 1 Experimental design

Treatment #	Temperature (°C)	Exposure time (h)	Salinity (ppt)
1	25	1	0
2	25	1	10
3	25	1	25
4	25	1	38
5	25	24	0
6	25	24	10
7	25	24	25
8	25	24	38
9	25	168	0
10	25	168	10
11	25	168	25
12	25	168	38
13	30	1	0
14	30	1	10
15	30	1	25
16	30	1	38
17	30	24	0
18	30	24	10
19	30	24	25
20	30	24	38
21	30	168	0
22	30	168	10
23	30	168	25
24	30	168	38

higher than 60 pixels were considered [20]. The influence of the different salinities, temperature, and exposure times (predictive variables) on the growth (dependent variable) of *E. cancerae* (CBS 120420) was analyzed by employing the general linear model (GLM) procedure with STATISTICA 6.0 (StatSoft Inc., USA). Significance level was accepted as $P < 0.05$.

Results and Discussion

Our experimental results support the hypothesis of the marine dispersion of the etiologic agent of LCD between adjacent Brazilian estuaries. *E. cancerae* CBS 120420 grew in all experimental treatments, as indicated by the profiles of average CFUs growth per

treatment (Figs. 3, 4). GLM summarized well the overall data, in which 55% (multiple coefficient of determination) of the variation observed in CFU growth was accounted for the measured variables (Table 2). Regarding a potential osmotic stress, *E. cancerae* showed a remarkable tolerance to a wide spectrum of salinity levels, persisting and growing for a relatively extended period of exposure (1 week) under a gradient that include both very low and very high salinities (0 or 38 ppt, respectively) (Figs. 3, 4). As such, while no significant effect was correlated with this variable ($P > 0.05$), the analysis confirmed that CFU growth was significantly dependent upon exposure time and temperature (Table 2; Figs. 3, 4). Regarding the time variable, the extended periods of

exposure clearly influenced an increase in CFU growth ($P < 0.05$) (Figs. 3, 4). Regardless of the influence exerted by the other tested variables, *E. cancerae* could withstand the required conditions to be transported between estuaries by ocean currents. However, temperature increases (25–30 °C) have produced a significant depletion, about 1.2-fold, in such growth ($P < 0.05$) (Table 2; Figs. 3, 4, 5). This result corroborates the optimum growth between 24 and 27 °C reported by de Hoog et al. [6] in the species description. Accordingly, it seems plausible to suggest that sea surface temperatures either above or below this optimum growth range could play a key role in the apparent north–south limits in the geographical distribution of LCD events.

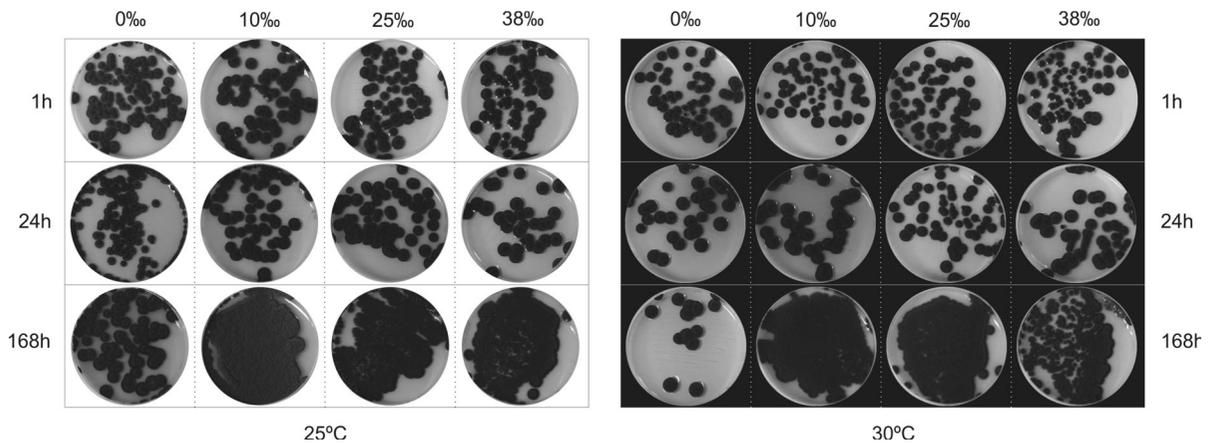


Fig. 3 *Exophiala cancerae* CFUs growth profiles before exposition to simulated experimental conditions: salinity (0, 10, 25, 38 ppt), temperature (25 and 30 °C), and exposure time (1, 24, and 168 h)

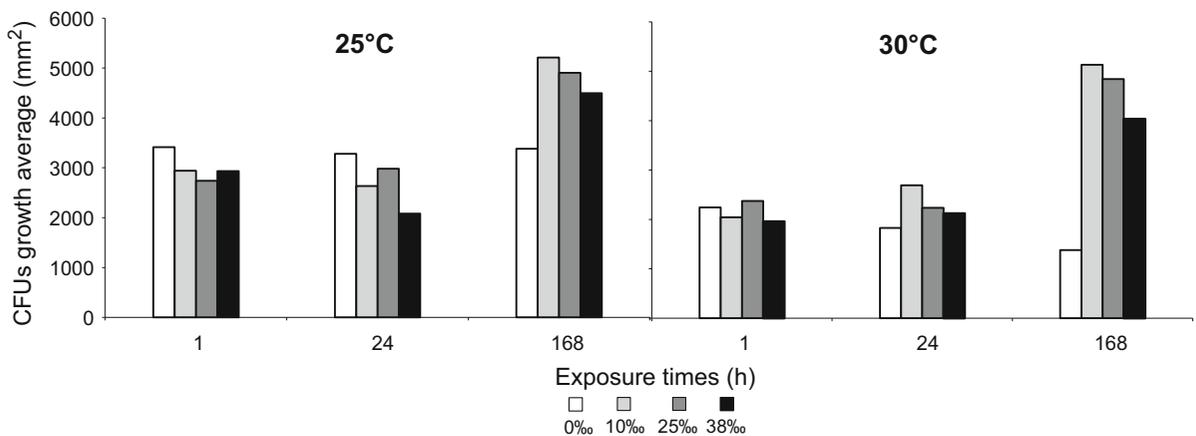


Fig. 4 Average of *Exophiala cancerae* CFUs growth under different salinities (‰), temperatures (°C), and exposure times (h)

Table 2 General linear model (GLM) of *Exophiala cancerae* CFUs growth as a function of salinity, temperature, and exposure times

Source	df	GLM of <i>E. cancerae</i> growth			
		Adj SS	Adj MS	F	P
Intercept	1	180,929,659	180,929,659	282.9459	***
Salinity	1	210,522	210,522	0.3	0.6
Exposure time	1	70,125,793	70,125,793	109.7	***
Temperature	1	9,689,058	9,689,058	15.2	***
Error	106	67n781,670	639,450		

df degrees of freedom, Adj SS adjusted sums of squares, Adj MS adjusted mean of squares, F F-distribution

* Significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$

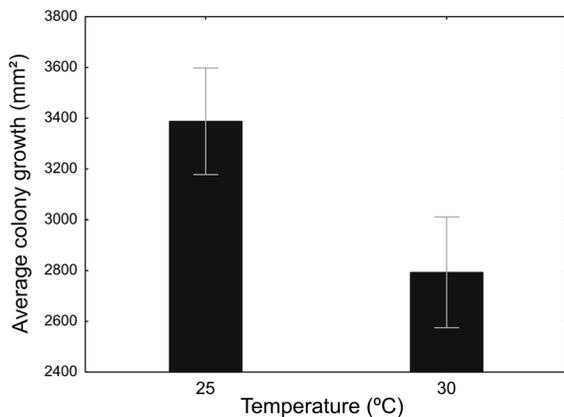


Fig. 5 *Exophiala cancerae* CFUs growth emphasizing the influence of experimental temperatures (25 and 30 °C). Bars represent standard deviations

In the north, the last record of LCD outbreaks is from the state of Piauí in 2003 (Fig. 1). Since then, no evidence exists that the disease has stricken mangrove land crab populations in any neighboring estuarine area located up north. Noteworthy is that this particular estuary comprises part of the world's largest continuous mangrove area [25] (see Fig. 1). However, under the hypothesis of marine dispersion of LCD, our data suggest that the high water temperature observed near to the Equatorial line, approximately 30 °C (<https://www.nodc.noaa.gov/>) [21, 22], could promote a reduction in the propagule pressure of *E. cancerae* conidia (non-motile asexual fungal spores), consequently affecting the contact rate between infective stages of this black yeast and susceptible mangrove crabs.

While our data did not indicate any clear evidences on the apparent southernmost limit of LCD outbreaks, direction and temperature of surface ocean currents as

well as rarefaction and discontinuity of mangrove areas (see Fig. 1) may offer clues on the factors contributing to the present distribution. Near to the last reports of LCD-associated mortalities in Espírito Santo, in the vicinity of both capes, São Tomé and Frio (20°S and 23°S, respectively) (see Fig. 1) occur abrupt changes in the Brazilian coastline direction that turns sharply westward [26]. To conserve angular momentum, the Brazil Current (BC) develops a convoluted pattern of clockwise meanders that pitches off the current away from the continental shelf [27, 28]. This phenomenon is also associated with upwelling events [27], known to affect algal distribution [29] and the position of sardine spawning areas [30]. Additionally, drops in the sea surface temperature from 23 °C to 15 °C have been recorded in Cape Frio [31]. By applying a similar logic as that for the north limit hypothesis previously mentioned, we assume that these temperature declines associated with upwelling systems could also impact on fungal forms' dispersal.

Building on knowledge derived from our unpublished results, a mathematical model on epidemiological aspects of LCD has corroborated our hypothesis of marine dispersion. In an attempt to understand LCD transmission between mangrove areas, Avila et al. [10] formulated a model incorporating the dispersion of *E. cancerae* in the ocean. Noteworthy is that this assumption was based on a personal communication of the preliminary (unpublished) results of the present study that pointed out the tolerance of this etiologic agent to the range of salinities found in the ocean. The model indicated the existence of traveling wave solutions connecting the disease-free and affected populations of the mangrove land crab *Ucides*

cordatus. Interestingly, the numerical simulations suggested that the direction of the outbreaks could be influenced by the ocean currents [10]. In a previous mathematical modeling study, Ferreira et al. [5] successfully recovered the cyclic pattern observed in nature of the mortality events within a mangrove area. Although both models predicted that the observed oscillations may be a consequence of the relationship between demographic and epidemiological parameters, thereby rejecting the influence of an external forcing parameter (e.g., stress of *U. cordatus* during mating season) [5], the seasonality present in the records of LCD outbreaks still remains to be clarified. Most epizootic events have occurred in the summer, also known as the rainy season. Given that, we believe that the formulation of future models under the scenario of water dispersion of *E. cancerae* should incorporate seasonal environmental drivers related to rainfall in order to elucidate the timing for exportation out of estuary of fungal infective stages. These variables could comprise tidal amplitude, freshwater riverine discharge rates, as well as density and distribution of populations of mangrove land crab within mangrove intertidal zones.

In conclusion, route of infection within a single host individual as well as transmission between mangrove land crabs within a population is a significant knowledge gap that needs closure. Despite the epidemiological concerns, these data could also provide basis to understand whether the LCD-associated strain of *E. cancerae* is a true pathogen only found in its host or this particular lineage represents an opportunistic strain colonizing a new host via a resource tracking process [32–36]. To date, no study had assessed the presence of *E. cancerae* in either estuarine or coastal (inshore and offshore) waters. Guerra et al. [11] screened for *E. cancerae* in plant and soil material derived from mangrove areas affected by LCD. No strain of the etiological agent of LCD was isolated from these environmental compartments. This observation aligns with the marine dispersal hypothesis and stresses the need for environmental screenings that focus efforts on recovering isolates from exclusively this particular source.

Acknowledgements We thank Robert W. Pilchowski (GIA, UFPR) for supplying the seawater used in the in vitro experiments, and Marcio R. Pie (Department of Zoology, UFPR) for advice in the experimental design and statistical analysis. This study was supported by the Companhia de

Desenvolvimento Industrial e de Recursos Minerais de Sergipe (CODISE) (Sergipe, Brazil), the Secretaria de Estado da Ciência, Tecnologia e Ensino Superior do Estado do Paraná (SETI) (Paraná, Brazil), and also by a grant to WAB and a Postdoctoral Fellowship to ROR under the project entitled “Exploring a new paradigm for the evolution of host-parasites associations,” number 404344/2013-5 of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil) (<http://www.cnpq.br>).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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