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NOTE

Specific primers for the detection of the black-yeast fungus associated with lethargic crab disease (LCD)

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ABSTRACT: Lethargic crab disease (LCD) is an emerging infirmity that has been causing extensive mortalities in populations of the mangrove land crab *Ucides cordatus* (Ocypodidae) along the Atlantic coast of Brazil. Previous studies have indicated that LCD is associated with a dematiaceous fungus, *Exophiala cancerae* de Hoog et al. In the present study, we sequenced the internal transcribed spacer (ITS) of the rDNA region of this black yeast species and developed species-specific PCR primers. Sensitivity tests indicated that the developed protocol is capable of detecting very small amounts of target DNA. Also, the application of the protocol to a variety of other dematiaceous fungi did not generate any false positives. The specific primers provided in the present study represent an important tool for rapidly surveying a large number of crab individuals, as well as environmental samples. Such knowledge will be instrumental in understanding the epidemiological dynamics of LCD.

KEY WORDS: Lethargic crab disease · Diagnosis · *Exophiala cancerae*

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Lethargic crab disease (LCD) has been responsible for repeated outbreaks that have severely depressed local populations of the mangrove land crab *Ucides cordatus* (Decapoda: Ocypodidae) along the Brazilian coast since it was first reported in 1997 (Boeger et al. 2005). The main symptom of LCD is an increasingly weak motor control, particularly of pereiopods and chelae, causing lethargy and poor balance, followed by death of the sick individual. Fisheries of the mangrove land crab have been heavily affected by LCD outbreaks. Some mangroves in the states of Paraíba and Bahia have experienced reductions in the fishing yields of 84 and 97.6%, respectively (Nóbrega & Nishida 2003, Schmidt 2006), causing severe socioeconomic problems in the affected regions.

Evidence from a variety of sources (light and electron microscopy, behavioral tests, and molecular phylogenetics) indicated that LCD symptoms were associated with the presence of a dematiaceous fungus (Boeger et al. 2005). Since then, further studies involving artificial infection experiments (Orélis-Ribeiro et al. 2011), as well as histopathological study of infected crabs (Boeger et al. 2007), sustain that LCD is associated with *Exophiala cancerae* de Hoog et al.. Although still unknown to science, the species was isolated earlier from other substrates (de Hoog et al. in press).

In the present study, we sequenced internal transcribed spacer (ITS) of the rDNA region of this black yeast species and developed specific markers that can be used in the diagnosis of sick individuals, in the molecular identification of cultured isolates, and in the prospecting of environmental samples.

Crabs with symptoms of LCD were obtained during outbreaks that occurred in the state of Sergipe in 2004 and 2005. The extraction of fungal DNA from the host tissues followed the protocol of Boeger et al. (2005),

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with the following modification: instead of submitting the yeast-like cells to cellular disruption by ultrasound, they were homogenized using a mortar and pestle in liquid nitrogen. The ITS of the rDNA was amplified using the primers ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990) in 25 µl reactions with the following final concentrations: 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 1× reaction buffer, 2.5 U of *Taq* polymerase (Biotools), 2 µM of each primer, and 50 to 75 ng of template DNA using the following program: 4 min at 95°C, followed by 30 cycles of 1 min at 94°C, 2 min at 58°C, 1 min at 72°C, and a final extension of 1 min at 72°C. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide for band visualization. Positive reactions were purified using the Minelute® (Qiagen) kit, and cycle sequencing was carried out using the following final concentrations: 0.16 µM of primer, 1× reaction buffer, 0.5 µl of BigDye v.3, and 40 ng of template DNA. Thermocycling conditions included an initial denaturation of 1 min at 96°C, followed by 35 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Products were purified using G-50 Sephadex, and both strands were sequenced on an ABI 3130 Automatic Sequencer. A representative sequence was deposited in GenBank under accession number HQ659023, and reference material has been submitted to the Centraalbureau voor Schimmelcultures, The Netherlands (CBS120420).

The obtained sequences were compared to those of several other species of black yeast and fungi to locate regions of maximum dissimilarity (particularly transversions and indels), which were used to design specific primers (Fig. 1). Several primer pairs were tested using both sensitivity and specificity tests. Sensitivity tests were carried out by using increasingly smaller amounts of template DNA (from 5 to 0.005 ng) to determine the extent of 'detectability' of the method. Specificity tests were conducted by testing the designed primers against samples of several other fungus species, including *Ramichloridium atrovirens* (CBS685.76), *R. atrovirens* (CBS677.76), *Ramichloridium* sp. (CBS102238), *Fonsecaea pedrosoi* (CBS253.49), *Cladophialophora immunda* (CBS102237), *C. saturnica* (CBS118724), *Exophiala spinifera* (HC-EML), and *E. jeanselmei* (HC-EJ4) (CBS and HC correspond to reference strains from the Centraalbureau voor Schimmelcultures, The Netherlands, and the Hospital de Clínicas of the Universidade Federal do Paraná, Brazil). The reaction with the specific primers that showed the best performance included the primers LCDF5 (5′-CCC TCT GGC CCG CGC TCG TCG ACA GC-3′) and LCDR3 (5′-GAG AAA GAT TCT CCC TGA GGG GAG GCA G-3′) in 25 µl reactions with the following final concentrations: 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 1× reaction buffer, 2.5 U of Platinum *Taq* polymerase (Invitrogen), 2 µM of each primer, and varying amounts of template DNA (see above). The following thermocycling conditions were used: initial denaturation for 5 min at 95°C, followed by 25 cycles of 40 s at 94°C, 30 s at 70.5°C, 30 s at 72°C, and a final extension of 2 min at 72°C. Electrophoresis and band visualization were carried out as indicated above.

The results from sensitivity tests are presented in Fig. 2, showing that the specific primers provided positive results reliably with as little as 0.05 ng of fungal DNA, although a faint discernible band was also present in the reaction with 0.005 ng. No unspecific band was visible when only DNA from *Ucides cordatus* was present in the reaction. Moreover, none of the tests using 2 ng μl^{-1} of other fungal species provided false-positive results (Fig. 3).

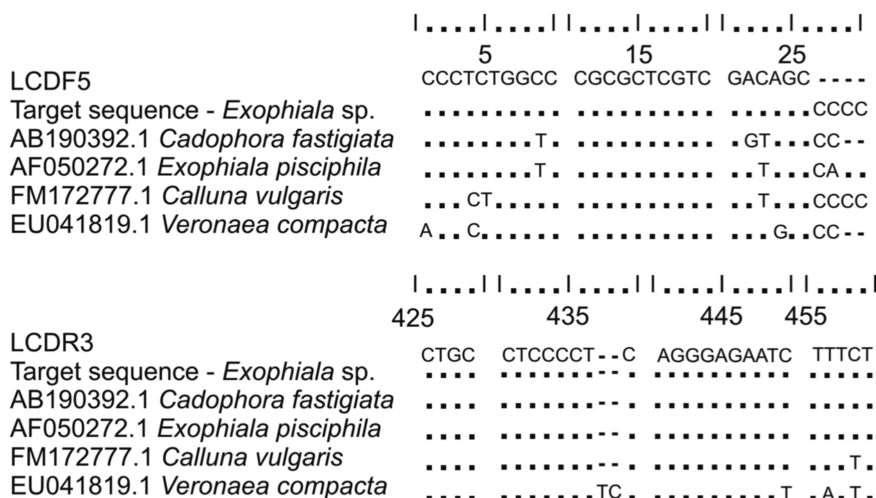

Fig. 1. Multiple alignment of the sequenced internal transcribed spacer fragment of *Exophiala cancerae* (CBS120420) and closely related species indicating the location of the new specific primers LCDF5 and LCDR3. GenBank accession numbers are indicated next to species names

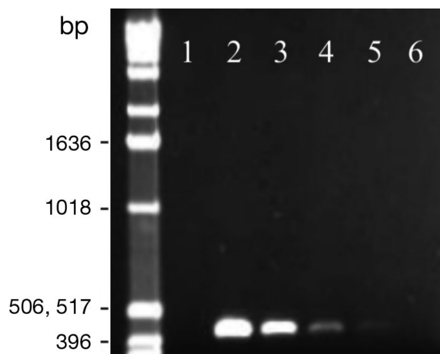

Fig. 2. Sensitivity tests of the specific primers for the detection of the *Exophiala*-like black yeast associated with lethargic crab disease. Template DNA for the PCR in each lane: Lane 1, genomic DNA of *Ucides cordatus*; Lanes 2–5, respectively, 5, 0.5, 0.05, and 0.005 ng of fungal DNA; Lane 6, negative control

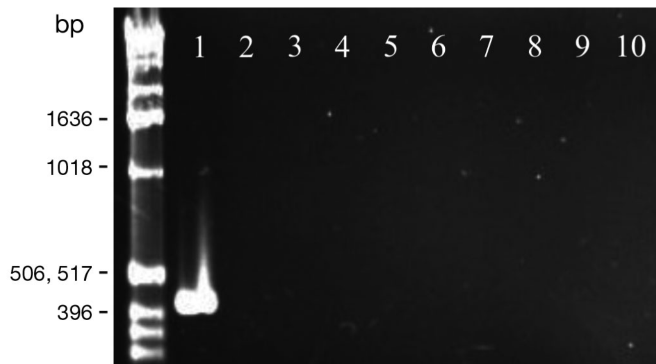

Fig. 3. Specificity tests of the specific primers for the detection of the *Exophiala*-like black yeast associated with lethargic crab disease. Template DNA for the PCR in each lane: Lane 1, *E. cancerae* (CBS120420); Lane 2, *Ramichloridium atrovirens* (CBS685.76); Lane 3, *R. atrovirens* (CBS677.76); Lane 4, *Ramichloridium* sp. (CBS102238); Lane 5, *Fonsecaea pedrosoi* (CBS253.49); Lane 6, *Cladophialophora immunda* (CBS102237); Lane 7, *C. saturnica* (CBS118724); Lane 8, *E. spinifera* (HC-EML); Lane 9, *E. jeanselmei* (HC-EJ4); Lane 10, negative control

The protocol using these specific primers was also applied in laboratory experiments (Orélis-Ribeiro et al. 2011), in the diagnosis of diseased crabs, and on *Exophiala cancerae* isolates obtained from outbreaks in other states (Bahia and Espírito Santo), confirming the adequacy of the method. Moreover, this method was used in an environmental screening of isolates from mangroves in the state of Bahia (R. S. Guerra unpubl. results). The results of this screening study corroborate the usefulness of the ITS region in the development of species-specific primers, as has been shown in a wide variety of fungal groups (e.g. LoBuglio & Taylor 1995, Amicucci et al. 1998, Redecker 2000).

The unpredictable nature of LCD outbreaks, in both space and time, is an important limitation to the study of this disease. The specific primers provided in the present study represent an important tool for rapidly surveying a large number of crab individuals, as well as environmental samples. Such knowledge is instrumental in understanding the epidemiological dynamics of LCD and will allow management efforts of this important fishery to be optimized.

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