Cryptococcus neoformans carried by *Odontomachus bauri* ants

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Cryptococcus neoformans *is the most common causative agent of cryptococcosis worldwide. Although this fungus has been isolated from a variety of organic substrates, several studies suggest that hollow trees constitute an important natural niche for* C. neoformans. *A previously surveyed hollow of a living pink shower tree (*Cassia grandis*) positive for* C. neoformans *in the city of Rio de Janeiro, Brazil, was chosen for further investigation.* Odontomachus bauri *ants (trap-jaw ants) found inside the hollow were collected for evaluation as possible carriers of* Cryptococcus *spp. Two out of 10 ants were found to carry phenoloxidase-positive colonies identified as* C. neoformans *molecular types VNI and VNII. The ants may have acted as a mechanical vector of* C. neoformans *and possibly contributed to the dispersal of the fungi from one substrate to another. To the best of our knowledge, this is the first report on the association of* C. neoformans *with ants of the genus* Odontomachus.

Key words: *Cryptococcus neoformans* - *Odontomachus bauri* - insect association - hollow tree habitat

Hollows from tree trunks have been investigated as potential sources of *Cryptococcus gattii* and *Cryptococcus neoformans*, the two causative agents of cryptococcosis, a life-threatening systemic mycosis affecting humans and a wide range of animals (Kwon-Chung & Varma 2006, Lin & Heitman 2006). Interest in this ecological niche began with the isolation of *C. gattii* from bark and wood debris collected from the hollow of a *Eucalyptus* tree in Australia (Pfeiffer & Ellis 1992) and the isolation of *C. neoformans* from wood and plant debris collected from the hollow of a *Syzygium jambolanum* tree (java plum tree) from an urban environment in the city of Rio de Janeiro, Brazil (Lazéra et al. 1993). Following this study, the hollows of several species of living trees in the same geographical region were investigated and numerous strains of *C. neoformans* were found (Lazéra et al. 1996). In addition to these two surveys, there have been other investigations on the hollow trunks of living trees in different regions of Brazil and in other countries, such as Colombia, India and Argentina, from which several representative colonies of *C. gattii* and *C. neoformans* were isolated, demonstrating that tree hollows are a suitable ecological niche for both species (Lazéra et al. 1998, 2000, Fortes et al. 2001, Granados & Castañeda 2006, Baltazar & Ribeiro 2008, Costa et al. 2009, Kumar et al. 2009, Refojo et al. 2009, Mitchell et al. 2011).

These pathogenic capsulated yeasts, which have as their teleomorphic state the genus *Filobasidiella*, belong to the class Tremellomycetes of the phylum Basidiomycota (Kirk et al. 2008). Like other members of this phylum, these yeasts also produce laccase, a ligninolytic enzyme that allows them to grow on decaying wood and other lignified substrates (Lazéra et al. 1998, Chan & Tay 2010).

Fungi can also be associated with many insect species. The exoskeletons of some insects can harbour several species of yeasts, promoting their survival or dispersal to new substrates (Rosa et al. 2003, Pagnocca et al. 2008). Species of *Odontomachus* ants, a predatory genus from the subfamily Ponerinae (family Formicidae), known as trap-jaw ants, are highly common in neotropical countries, where they interact with plants (Passos & Oliveira 2004, Gibernau et al. 2007, Spagna et al. 2009).

To test the hypothesis that ants can act as carriers of *C. neoformans*, we examined 10 of these insects collected from a hollow of a living pink shower tree (*Cassia grandis*) that has been positive for this pathogen for nearly 15 years (Lazéra et al. 1996).

MATERIALS AND METHODS

Ant collection - The ants were collected in sterilised tubes from the hollow of a pink shower tree (*C. grandis*) located on the campus of the Oswaldo Cruz Foundation (Fiocruz) in the city of Rio de Janeiro (Fig. 1). The ants were transferred to the laboratory for immediate fungal isolation.

Fungal isolation from ant exoskeletons - Each ant was allowed to walk for 24 h on niger seed agar (NSA) plate supplemented with chloramphenicol (400 mg l^{-1}) and amikacin (800 mg l^{-1}). The plates were incubated at 25ºC and examined daily for four days. During this period, phenoloxidase-positive yeast colonies were isolated and pure cultures were obtained after serial transfers on NSA. The isolates were subcultured on Sabouraud dextrose agar, stored at 4ºC and cryopreserved in 15% glycerol at -70ºC.

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Ant characterisation - The ants collected from the studied hollow were morphologically characterised and identified according to the literature (Kempf 1972, Brown 1976, Baccaro 2006, Bolton et al. 2006).

Yeast identification - Morphological and physiological characterisations were conducted, including phenoloxidase production on NSA, thermotolerance at 37ºC, cycloheximide sensitivity, urease production and assimilation tests performed with the VITEK-32 (bioMérieux, France). Canavanine-glycine-bromothymol blue medium was used to identify the species as *C. gattii* and *C. neoformans* (Kwon-Chung et al. 1982).

The molecular types were determined by restriction fragment length polymorphism (RFLP) of the gene *URA5* according to Meyer et al. (2003). The following standard strains representing each molecular type of *C. neoformans* (VN) and *C. gattii* (VG) were included in the analysis: WM 148 (serotype A, VNI/ AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, VNIII/AFLP2), WM 629 (serotype D, VNIV/ AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP6), WM 175 (serotype B, VGIII/ AFLP5) and WM 779 (serotype C, VGIV/AFLP7).

Genomic DNA was extracted as previously described by Ferrer et al. (2001) with some modifications. Half of an inoculation loop of culture was frozen at -20ºC for 1 h and incubated at 65ºC for 1 h in 0.5 mL of extraction buffer (50 mM Tris-HCl, 50 mM ethylenediamine tetraacetic acid, 3% sodium dodecyl sulphate, 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was recovered by isopropanol precipitation and then washed with 70% ethanol and diluted in sterile water.

The polymerase chain reaction (PCR) of the *URA5* gene was performed in a final volume of 50 µL. Each reaction contained 50 ng of DNA, 1 x PCR buffer [160 mM $(NH_4)^2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25ºC), 0.1% Tween-20 (Bioline)], 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH), 3 mM magnesium chloride, 1.5 U BioTaq DNA polymerase (Bioline) and 50 ng each of the primers URA5 (5'ATGTCCTCCCAAGCCCTCGACTCCG 3') and SJ01 (5'TTAAGACCTCTGAACACCGTACTC 3') (Meyer et al. 2003). The target sequence was amplified in a Perkin-Elmer thermal cycler (model 480) using the following cycling program: 94ºC for 2 min (initial denaturation), 35 cycles of 45 s at 94ºC (denaturation), 1 min at 61ºC (annealing) and 2 min at 72ºC (extension) and a final extension cycle for 10 min at 72ºC. A total of 30 µL of the PCR products was double digested with *Sau*96I (10 U/µL) and *Hha*I (20 U/µL) for 3 h and the fragments were separated by 3% agarose gel electrophoresis at 100 V. The RFLP patterns were assigned visually by comparison with patterns obtained from the standard strains described above.

RESULTS

Two out of the 10 collected ants, later morphologically identified as *Odontomachus bauri* Emery, 1892, were found to carry phenoloxidase-positive yeasts. Two NSA plates allowed the growth of one dark brown colony each, later identified as *C. neoformans* molecular types VNII and VNI (Fig. 2). These isolates were deposited in the Pathogenic Fungal Collection (CFP), Clinical Research Institute Evandro Chagas, Fiocruz, as CFP 216 and CFP 217, respectively.

Fig. 1: pink shower tree hollow (*Cassia grandis*) located in the campus of Oswaldo Cruz Foundation in the city of Rio de Janeiro, Brazil, from where the ants were collected.

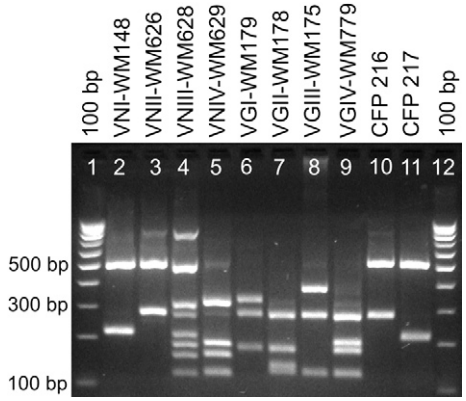

Fig. 2: banding patterns generated by *URA5*-restriction fragment length polymorphism. Lanes 2-9: standards strains of the major molecular types; 10-11: the two strains isolated from ants {Pathogenic Fungal Collection (CFP) 216 [*Cryptococcus neoformans* (VNII)] and CFP 217 (VNI)}. VG: *Cryptococcus gattii*; WM: Wicland Meyer Culture Collection.

DISCUSSION

Fourteen hollows of living trees were formerly investigated at the Fiocruz campus. Serial scrapings taken from the inner surfaces of the hollows of two living *C. grandis* trees (family Fabaceae, subfamily Caesalpinioideae), popularly known as pink shower trees, yielded isolates of *C. neoformans* (Lazéra et al. 1996). The pink shower tree that provided the most isolates of *C. neoformans* (hollow A) and was continuously positive for *C. neoformans* until recently (M Lazéra, unpublished observations) was chosen for the current investigation. These yeasts are commonly found in this habitat, probably due to their ligninolytic enzyme laccase, which allows the yeasts to grow on decaying wood (Lazéra et al. 1998, Chan & Tay 2010).

Fungi can be associated with different insects, such as ants, as previously demonstrated by Pagnocca et al. (2008), who isolated several species of yeasts, including *Cryptococcus laurentii*, from the exoskeletons of two ant species, *Atta laevigata* and *Atta capiguara*. In an earlier study, other representatives of the *Cryptococcus* genus were isolated from stingless bees (Rosa et al. 2003) and from the guts of insects belonging to the order Neuroptera (Nguyen et al. 2007). In another investigation, two different molecular types of *C. gattii* (VGI and VGII) were isolated from fresh insect frass (order Lepidoptera, family Oecophoridae) in a shallow cavity in the bark of a living *Eucalyptus tereticornis* tree (Kidd et al. 2003).

To date, there have been no reports on the association of fungi with the genus *Odontomachus*, although the association of these ants with bacteria has been studied (Caetano et al. 2009, dos Santos et al. 2009). The role of ants, including *Odontomachus* spp, as mechanical vectors of pathogenic bacteria within hospitals was recently demonstrated by dos Santos et al. (2009), uncovering a public health problem because these bacteria can be directly associated with nosocomial infections. In another study, bacteria were found in the digestive tract of *O. bauri*, the same species of ant as investigated in the present study, suggesting the participation of these bacteria in the food digestion of this ant (Caetano et al. 2009).

The finding that only two out of the 10 ants were positive for *C. neoformans* may be due to the non-homogenous presence of these pathogens in the hollow tree; these pathogens likely have a focal distribution. During sample collection, either by swabbing or scraping, it is very common to find only 10-25% that are positive. In addition, it is necessary to consider the detection limitations of the method, which can be low, in addition to the competition with other microorganisms (Mitchell et al. 2011).

The fact that the *C. grandis* tree from which the *O. bauri* ants were collected (hollow A) is very close to another *C. grandis* tree (hollow B) previously studied that is also positive for *C. neoformans* (Lazéra et al. 1996) suggests that the ants could be acting as mechanical vectors of *C. neoformans* and may be responsible for the dispersal of these yeasts from one tree to another. In any case, this yeast-ant association deserves more research.

In Teresina, the capital of the state of Piauí, Northeast Region, Brazil, the *C. neoformans* strains isolated from several trees in a single downtown square showed a high level of molecular similarity values (> 95%) in their amplified fragment length polymorphism patterns, suggesting that cryptococcal populations may be clonally dispersed among neighbouring trees and nearby animal-related habitats (Trilles et al. 2003).

Based on molecular typing, *C. neoformans* can be subdivided into the VNI, VNII, VNIII and VNIV types (Meyer et al. 1999). The *C. neoformans* isolates presently investigated were characterised as molecular types VNI and VNII. However, Trilles et al. (2008) molecularly typed 5 isolates of *C. neoformans* originating from two *C. grandis* trees investigated by Lazéra et al. (1996), one of which was the tree used in this study and found that all five isolates were molecular type VNI. Thus, from the first sampling (Lazéra et al. 1996) to the more recent sampling in the same hollow tree, a change in the community may have occurred, with *C. neoformans* VNII being added to the previously identified *C. neoformans* VNI. In their study, Trilles et al. (2008) analysed 320 *C. neoformans* isolates and 123 *C. gattii* isolates from various parts of Brazil, demonstrating that the most common molecular type in the southern region, which includes Rio de Janeiro, was VNI (64%) and that VNII had a minor incidence (5%), occurring exclusively in this region from clinical and environmental sources.

This study indicates the need for a thorough investigation with more samplings of insects, inside and around hollow trees, over an extended period of time at regular intervals to confirm that *O. bauri* and/or other insects are acting as mechanical vectors of *C. neoformans* and are responsible for the dispersal of these yeasts to other substrates. The interaction of fungi with hollow trees and insects forms a system that may be useful for understanding their ecology.

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