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Possible primary ecological niche of *Cryptococcus neoformans*

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To study hollows of living trees as natural habitats of *Cryptococcus neoformans* in an endemic area of cryptococcosis in the northeastern region of Brazil, samples of decaying wood were collected inside 32 hollows of living trees and plated on niger seed agar. Identification of *C. neoformans* was based upon morphological and physiological tests. Canavanine-glycine-bromothymol medium was used to screen the varieties and Crypto Check Iatron Kit to serotype the isolates. A total of 123 *C. neoformans* colonies were recovered from samples of six (18·5%) out of 32 hollow trees. *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* were found occurring alone (pink shower tree, fig tree and pottery tree) or sharing the same hollow (pink shower tree). Long lasting positivity (19–36 months) and significant number of cfu of *C. neoformans* per gram of decaying wood ($0{\cdot}15–21{\cdot}7\times 10^3$ cfu g^{-1}) inside hollows of pink shower tree, fig tree and pottery tree were observed, indicating colonization of these habitats by the fungus. For the first time, *C. n.* var. *neoformans* and *C. n.* var. *gattii* were found sharing the same natural biotope, thus establishing a possible link between them in their life cycle in nature and suggesting the primary natural niche for the species.

Keywords *Cryptococcus neoformans*, hollow trees, primary natural niche, wood decay

Introduction

The isolation of virulent strains of *Cryptococcus neoformans* from decomposing manure, old nests and roosting sites of pigeons was reported in 1955 [1]. Since then, studies on the environmental sources of the fungus were directed toward avian droppings and their roosting sites. As a result, the ecology of *C. neoformans* var. *neoformans* is well known, mainly in urban areas. Meanwhile, in 1972 and later on, a vegetal biotope for the fungus was suggested [2–4].

A close association between the occurrence of *C. neoformans* var. *gattii* and the flowering period of *Eucalyptus camaldulensis* was evidenced in 1990, in Australia [5]. Prompt confirmation of this observation in USA [6], Brazil [7], Mexico [8] and Italy [9] demonstrated that this association was not geographically restricted. In 1992, also in Australia, *C. n.* var. *gattii* was isolated from bark and wood debris within a hollow of *E. tereticornis* [10]. In 1993, casual isolation of *C. n.* var. *gattii* from bat guano, in Brazil [11], from fragments of a communitary wasp's nest, in Uruguay [12], and recently the isolation of serotype C from debris of almond trees in Colombia [13] were reported.

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In Brazil, in 1993, *C. n.* var. *neoformans* was isolated from decaying wood within a hollow of *Syzygium jambolana* tree [11], and in 1996, from hollows of three other species of living trees (*Cassia grandis*, *Senna multijuga* and *Ficus microcarpa*) [14]. From 1993 until the present, we have investigated the presence of *C. neoformans* in hollows of living trees in the city of Teresina, state of Piauí, northeast of Brazil, where cryptococcosis caused by *C. n.* var. *gattii* is endemic [15]. As a result, we recently reported the isolation of this variety from a hollow of *Moquilea tomentosa* [16]. In this paper we will present the last results of our investigation.

Material and methods

The investigation was carried out in the city of Teresina, capital of the state of Piauí, during the period of September 1993 to March 1997. Hollows with decomposing wood from 32 living trees were studied: 17 pottery trees (*M. tomentosa*), seven pink shower trees (*C. grandis*), three fig trees (*Ficus* sp.), one mango tree (*Mangifera indica*) and four unidentified trees. Samples were collected by scraping the fibrous discolored wood in advanced stage of decay inside the hollows. Repeated samplings were performed from positive hollows.

Each sample was suspended in physiological saline and inoculated into petri dishes containing niger seed agar medium (NSA). All dark brown colonies obtained were subcultivated and identified separately, as previously described [14]. The variety of the *C. neoformans* strains was ascertained by culture on canavanine-glycine-bromthymol blue (CGB) medium and the serotype by the 'Crypto Check Iatron' RM 304-K (Iatron Laboratories, Tokyo, Japan).

The number of cfu of *C. neoformans* per gram of decaying wood was estimated on the basis of growth of dark brown colonies on the NSA medium. To assess the significant differences between mean cfu for each variety, the Wilcoxon two-sample test was used. In the only two hollows where *C. n.* var. *neoformans* and *C. n.* var. *gattii* were simultaneously isolated, the cfu for each variety were proportionally distributed according to the number of colonies initially identified.

Air sampling was performed in August 1995, using the Biotest RCS™ centrifugal air sampler (Biotest Ag, Frankfurt, Germany) [14]. Forty-eight samples (960 l of air) were collected under the canopies of positive trees.

Results

A total of 123 dark brown colonies were recovered from NSA media seeded with the samples of six (18·5%) out of the 32 hollow trees studied. All colonies were identified as *C. neoformans*. They were composed of encapsulated yeasts, thermotolerant to 35 °C, sensitive to cycloheximide, and urease positive. Inositol, maltose and xylose assimilation tests were positive. Lactose and potassium nitrate assimilation tests were negative and the lactose fermentation test was negative. All strains identified as *C. n.* var. *neoformans* were serotype A and all strains identified as *C. n.* var. *gattii* were serotype B (Table 1).

The positive hollows are located in a downtown square, with trees over 100 years old, most of them pink shower and fig trees, some pottery and mango trees. These trees are roosting sites for birds (sparrows, turtle-doves, tyrant-fly-catchers and pigeons) and bats. The pink shower-4 and the pottery tree hollows were inhabited by bats. Occurrence of the fungus in the hollows was not related to the flowering period of the trees. Isolation

Table 1 Distribution of 123 colonies of *C. neoformans* isolated from six hollow trees in a square of Teresina, Piauí, according to variety of the fungus, time of collection and estimated density of *C. neoformans* by sample, 1993–1997

Tree identification	Number of *C. neoformans* colonies studied according to variety (estimated *C. neoformans* density in 10^3 cfu g^{-1} by sample)			
	September 1993	February 1995	August 1995	March 1997
Pottery tree	21 *gattii* (1·5)	2 *gattii* (1·0)	1 *gattii* (1·0)	2 *gattii* (1·0)
Pink shower-1	NC	2 *gattii* (1·0)	Negative	Negative
Pink shower-2	NC	7 *gattii* (1·0)	Negative*	6 *gattii* (0·3) and 1 *neoformans* (0·05)
Pink shower-3	NC	NC	37 *neoformans* (2·0)	25 *neoformans* (21·7)
Fig tree	NC	NC	3 *gattii* (0·15)	Negative
Pink shower-4	NC	NC	NC	10 *gattii* (2·6) and 6 *neoformans* (1·6)

*, burned.
NC, not collected.

of the fungus from the same hollow, when repeated, was not uniformly obtained during the investigation period (Table 1).

From 1993 to February 1995, only *C. n.* var. *gattii* was recovered from the hollows. In August 1995, *C. n.* var. *neoformans* was isolated from another hollow (pink shower-3). In 1997 both varieties were obtained from the hollows of two cassia trees (pink shower-2 and pink shower-4). One of these hollows (pink shower-2), colonized by *C. n.* var. *gattii* in February 1995, was damaged some months later by fire (Fig. 1), became negative in August 1995 and in March 1997 was found to be colonized by both varieties (Table 1).

The density of *C. neoformans* observed in the positive hollows, estimated for each period of collection, was quite variable (Table 1). *C. n.* var. *neoformans* (mean $9{\cdot}3 \pm 9{\cdot}4 \times 10^3$ cfu g^{-1}) was found in significant higher concentrations ($P < 10^{-6}$) than *C. n.* var. *gattii* (mean $1{\cdot}6 \pm 1{\cdot}3 \times 10^3$ cfu g^{-1}) in the decaying wood sampled.

Five dark brown colonies were obtained on NSA strips from the air collected near the hollows of two cassia (pink shower-1 and pink shower-4). Only one of these colonies could be subcultivated due to overgrowth of other fungi. This colony was identified as *C. n.* var. *gattii* serotype B.

Discussion

A previous investigation into the isolation of *C. neoformans* from decaying wood inside hollows of living trees in the city of Rio de Janeiro, southeastern Brazilian region, recovered *C. n.* var. *neoformans* from eight (25·8%) out of 31 hollows of three ubiquitous tree species in the urban environment [14]. The present study, however, performed in the city of Teresina, northeastern Brazilian region, recovered *C. neoformans* from six (18·7%) hollows pertaining to three genera of trees: *C. n.* var. *gattii* from three hollow trees, *C. n.* var. *neoformans* from one, and both varieties from two. These environmental findings correlate closely with the epidemiology of cryptococcosis in both investigated regions. In Teresina, *C. n.* var. *gattii* is the causative agent of cryptococcosis in 91·2% of the non-human immunodeficieny virus (HIV) patients [15], whereas in Rio de Janeiro, this variety only sporadically causes the mycosis [17]. Furthermore, cases of cryptococcosis by *C. n.* var. *gattii* diagnosed in Rio de Janeiro occur mainly in patients native of the northeastern Brazilian region [17].

Two hollows found positive for *C. n.* var. *gattii* became negative. *C. n.* var. *neoformans* was found in significantly higher density than *C. n.* var. *gattii*, as judged by cfu values. We speculate that *C. n.* var. *neoformans*, once established in these hollows, reproduces better than *C. n.* var. *gattii*. Burning of a hollow colonized by *C. n.* var. *gattii* (Fig. 1) in a fire caused transient negativity, followed afterwards by the detection of both varieties within the same hollow. Recolonization by exogenous propagules may be supposed in this case. Nevertheless, the possibility of an endogenous source inside the tree, such as persistent inoculum in cracks and fissures, must be considered. An interconnecting

Fig. 1 (a) Fig tree hollow positive for *C. neoformans* var. *gattii* in 1995; (b) pink shower tree-2 with a hollow burnt in 1995, positive for both varieties of *C. neoformans* in 1997.

system of hollowed-out areas within the solid xylem cylinder of each tree may support prolonged survival and escape of *C. neoformans* during fires. Moreover, under unfavorable conditions, different genotypes may shelter as dormant fungal propagules for long periods [18]. Such propagules are not necessarily detected in the studied samples.

Another aspect to be considered is the finding of a propagule of *C. n.* var. *gattii* in the air collected at the entrance of one hollow tree. Disturbances at cavity entrances caused by wind or small animals may disperse propagules into the air. Moreover, fallen trees and deforestation activities in endemic areas may be related to human infection. Further epidemiologic studies are necessary to establish these habitats as possible sources for human infection by the varieties of *C. neoformans*.

For the first time C. *n.* var. *neoformans* and *C. n.* var. *gattii* were found sharing the same natural habitat. Until now, each variety has been considered to occur in a distinct habitat: one geographically restricted, associated with eucalyptus trees; and the other one cosmopolitan, associated with various organic substrata. Our findings point a common niche for both varieties suggesting a possible link in their life cycle in nature.

Considering that *C. neoformans* produces laccase [19], an enzyme involved in lignin degradation in other basidiomycetes [20,21], and that *C. neoformans* probably evolved from the *Tremella* lineage [22–24], in which some species such as *T. mesenterica* are associated with wood decay [25], our present findings let us speculate on the primary ecological niche of this organism. *C. neoformans* and its teleomorph are possibly associated with wood decay, part of the succession of decomposers of lignified substrata. Inside the fissures and cracks within the decaying wood, dry, semi-dry and wet conditions may be found. The anamorphic yeast form and also the mycelial form produced by haploid fruiting of *C. neoformans* mating type α [26] or by heterothallic crossing of compatible strains may occur across a range of these habitats. Woody substrata seem to be favorable to *Filobasidiella neoformans* growth once the mycelial phase has been produced *in vitro* in medium with a low nitrogen content [26]. Further studies are necessary to evaluate the possible role of *C. neoformans* and its teleomorphic phase in wood decay.

Wood decay within living trees is distinct from decay in dead trunks, branches, logs and stumps [27]. Probably the niche of *C. neoformans* is not restricted to decay within living trees, but these biotopes represent a natural model to study *C. neoformans* diversity and ecology.

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References

1 Emmons CN. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columba livia*). *Am J Hyg* 1955; **62:** 227–232.
2 Staib F, Thielke C, Randhawa HS, Senska M, Kulins G. Colonisation of dead plants by *Cryptococcus neoformans*. *Zbl Bakt Hyg I Abt Orig A* 1972; **225:** 113–124.
3 Bauwens L, Swinne D, De Vroey C, De Meurich W. Isolation of *Cryptococcus neoformans* var. *neoformans* in the aviaries of the Antwerp Zoological Garden. *Mykosen* 1986; **29:** 291–294.
4 Swinne D. Ecology of *Cryptococcus neoformans* and epidemiology of cryptococcosis in the old world. In: Torres-Rodriguez JM, ed. *Proceedings of the X Congress of the International Society for Human and Animal Mycology*, Barcelona, 1988. Barcelona: Prous Scientific, 1988: 113–119.
5 Ellis DH, Pfeiffer TJ. Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J Clin Microbiol* 1990; **28:** 1642–1644.
6 Pfeiffer TJ, Ellis DH. Environmental isolation of *Cryptococcus neoformans* var. *gattii* from California. *J Infect Dis* 1991; **163:** 929–930.
7 Wanke B, Lazera M, Nishikawa MM, *et al.* Isolamento ambiental de *Cryptococcus neoformans* var. *gattii* relacionado a *Eucalyptus camaldulensis* no Brasil. *III Congresso do Pavilhão Pereira Filho*, *I Congresso Brasileiro de Micologia*, Porto Alegre, Brasil, 1995; Porto Alegre, Brazil: Santa Casa da Misericordia de Porto Alegra, 1995: 136–137.
8 Licea BA, Garza DG, Zúñiga MT. Aislamiento de *Cryptococcus neoformans* var. *gattii* de *Eucalyptus tereticornis*. *Rev Iberoam Micol* 1996; **13:** 27–28.
9 Montagna MT, Tortorano AM, Fiore L, *et al.* First isolation of *Cryptococcus neoformans* var. *gattii* from Italy. *3rd International Conference on Cryptococcus and Cryptococcosis*, Paris 1996; Paris: Institute Pasteur, 1996: 159.
10 Pfeiffer TJ, Ellis D. Environmental isolation of *Cryptococcus neoformans* var. *gattii* from *Eucalyptus tereticornis*. *J Med Vet Mycol* 1992; **30:** 407–408.
11 Lazera MS, Wanke B, Nishikawa MM. Isolation of both varieties of *Cryptococcus neoformans* from saprophytic sources in the city of Rio de Janeiro, Brazil. *J Med Vet Mycol* 1993; **31:** 449–454.
12 Gesuele E, Calegar L, Sanabria D, Davel G, Civila E. Isolation in Uruguay of *Cryptococcus neoformans* var. *gattii* from a nest of the wasp *Polybia occidentalis*. *Rev Iberoam Micol* 1993; **10:** 5–6.
13 Callejas A, Ordoñez N, Rodrigues MC, Castañeda E. First isolation of *Cryptococcus neoformans* var. *gattii* serotype C, from the environment in Colombia. *Med Mycol* 1998; **36:** 341–344.

14 Lazera MS, Pires FDA, Camillo-Coura L, *et al*. Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J Med Vet Mycol* 1996; **34:** 127–131.
15 Cavalcanti MAS. *Criptococose e seu agente no Meio Norte, estados do Piauí e Maranhão, Brasil*. PhD Thesis, Fundação Instituto Oswaldo Cruz-Universidade Federal do Piauí, 1997.
16 Lazéra MS, Cavalcanti MAS, Trilles L, Nishikawa MM, Wanke B. *Cryptococcus neoformans* var. *gattii* in a pottery tree hollow — evidence for a natural habitat related to decaying wood. *J Med Vet Mycol* 1998; **36:** 119–122.
17 Rozenbaum R, Gonçalves AJR. Clinical epidemiological study of 171 cases of cryptococcosis. *Clin Infect Dis* 1994; **18:** 369–380.
18 Frankland JC. Fungal succession — unravelling the unpredictable. *Mycol Res* 1998; **102:** 1–15.
19 Williamson PR. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: Identification as a laccase. *J Bacteriol* 1994; **176:** 656–664.
20 Thurson CF. The structure and function of fungal laccases. *Microbiology* 1994; **140:** 19–26.
21 Kirk TJ, Farrel RL. Enzymatic 'combustion': microbial degradation of lignin. *Ann Rev Microbiol* 1987; **41:** 465–505.
22 Kwon-Chung KJ, Chang YC. Gene arrangement and sequence of the 5S rRNA in *Filobasidiella neoformans* (*Cryptococcus neoformans*) as a phylogenetic indicator. *Int J Syst Bacteriol* 1994; **44:** 209–213.
23 Petter R, Varma A, Boekhout T, *et al*. Molecular divergence of the virulence factors of *Filobasidiella neoformans* found in other heterobasidiomycetous species. *3rd International Conference on Cryptococcus and Cryptococcosis*, Paris 1996; Paris: Institute Pasteur, 1996: 36–38.
24 Boekhout T, Fonseca A, Sampaio JP, Golubev WI. Classification of heterobasidiomycetous yeasts: characteristics and affiliation of genera to higher taxa of heterobasidiomycetes. *Can J Microbiol* 1993; **39:** 276–290.
25 Alexopoulos CJ, Mims CW, Blackwell M (eds). Phylum Basidiomycota. Other Basidiomycetes. *Introductory Mycology*. 4th edn. New York: John Wiley & Sons, Inc., 1996: 658–682.
26 Wickes BL, Mayorga ME, Edman U, Edman JC. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the α-mating type. *Proc Natl Acad Sci USA* 1996; **93:** 7327–7331.
27 Rayner ADM, Todd NK. Population and community structure and dynamics of fungi in decaying wood. *Adv Bot Res* 1979; **7:** 333–420.