

Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil

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The genetic affiliation of a large number of isolates of the *Cryptococcus neoformans* species complex from environmental sources in Brazil has been investigated using amplified fragment length polymorphism (AFLP). The strains of *C. neoformans* isolated from a single tree, as well as from neighbouring trees, showed high similarity values (>95%) of their AFLP patterns, thus suggesting considerable genetic homogeneity. The majority of isolates of *C. neoformans* belonged to AFLP genotype 1, and had serotype A and mating type α (= *C. neoformans* var. *grubii*). Three isolates belonged to AFLP genotype 2, with serotype D and mating type α (= *C. neoformans* var. *neoformans*). One isolate, obtained from a building in Rio de Janeiro inhabited by pigeons, belonged to the AD hybrid AFLP genotype 3. All isolates from trees of *C. neoformans* var. *gattii* (= *C. gattii*) belonged to AFLP genotype 6, and their banding patterns showed relatively low genetic homogeneity with a similarity value of about 76%. Isolates of this genotype occupy an environmental niche in the Americas, and they may cause disease in non-AIDS and AIDS patients as well.

Keywords *Cryptococcus gattii*, *Cryptococcus neoformans*, ecology, genotyping, trees, yeast

Introduction

Cryptococcus neoformans is a clinically important basidiomycetous yeast belonging to the order Tremellales (jelly fungi) of the Hymenomycetes [1], a group of fungi commonly occurring on woody substrates. The species is heterothallic and has a bipolar mating system with two mating type loci, MAT α and MAT β . The sexual state is only known from the laboratory, and in nature, the fungus occurs, to our present knowledge, only in the asexual state. Currently, three varieties are being distinguished, namely *C. neoformans* var. *neoformans* (= serotype D), *C. n.* var. *grubii* Franzot *et al.* (= serotype A), and *C. n.* var. *gattii* Vanbreuseghem & Takashio (= serotypes B and C) [2–7]. Based on

electrophoretic karyotypes, amplified fragment length polymorphism (AFLP) and intergenic spacer (IGS) sequences, we recently suggested that the complex is comprised of at least two species: first, *C. neoformans*, with two AFLP and IGS genotypes corresponding to *C. n.* var. *neoformans* (serotype D) and *C. n.* var. *grubii* (serotype A), as well as a genotype corresponding with serotype AD hybrids; and second, *C. bacillisporus* Kwon-Chung (= *C. n.* var. *gattii*) with three AFLP and IGS genotypes [8–11]. For the latter species, a formal nomenclatural proposal to conserve the name *Cryptococcus gattii* against obscure, earlier valid names has been submitted. For clarity, however, we refer to this species throughout this paper as *C. n.* var. *gattii*.

C. neoformans can cause life-threatening infections in humans, mainly in immunocompromised hosts. It occurs worldwide, and is known from a variety of environmental sources, namely organic substrata and related habitats, avian excreta, bat organs, guano and caves, barns, cockroaches, fruits, fermenting fruit juice, horse intestinal flora, milk, bovine mastitis, rabbit pens,

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soil, and hollows of various trees [12,13]. Recent findings strongly suggested that trees constitute a natural niche for *C. neoformans*. It has been isolated from hollows of the pink shower tree (*Cassia grandis*), fig tree (*Ficus microcarpa*), java plum (*Syzygium jambolana*) and November shower tree (*Senna multi-juga*) [13–16]. It is also known from eucalyptus wood [17]. The trees from which *C. neoformans* has been isolated in this study occurred in urban and rural areas in north and south Brazil [13–18]. However, the yeast has also been isolated from tropical jungle trees in the Amazonian forest, such as *Miroxylon peruiferum* and *Theobroma cacao* [19].

C. n. var. gattii, best known as an agent of primary systemic cryptococcosis, is mainly found in tropical and subtropical areas, and frequently associated with *Eucalyptus* species. It is believed that dissemination of the fungus occurs as an aerosolized inoculum such as basidiospores or desiccated yeast cells [20]. Recently, serotype C has been found to occur in native almond (*Terminalia catappa*) trees in Colombia [21]. In contrast to *C. n. var. neoformans*, *C. n. var. gattii* associated with eucalyptus shows a strong seasonality in its occurrence, which coincides with the periods of flowering [18,22]. In northeastern Brazil, *C. n. var. gattii* has also been isolated from hollows of a pottery tree (*Moquilea tomentosa*), fig tree (*Ficus microcarpa*) and a pink shower tree (*Cassia grandis*) [13]. Other tree genera were found to harbour *C. n. var. gattii* included a native tree (*Guettarda acreana*) from a wild environment in the Amazonian rainforest [19,23].

Almost no information is available about the genotypic relationships of cryptococcal isolates occurring in the environment. Therefore, we used AFLP to investigate the genetic affiliation of a large number of environmental isolates, mainly isolated from various tree species in Brazil.

Material and methods

The isolates studied are listed in Table 1. Isolates were obtained by plating the woody material on niger seed agar medium (NSA) [16]. Isolates of a single tree are indicated with the same series number, e.g. LMM 562, which indicates isolates from a single pink shower tree. All dark-coloured colonies seeded on a single NSA plate were identified separately. Isolates from the series LMM 547 came from a single pottery tree, and LMM 484-1, 484-2 and 484-3 came from the same fig tree. These two trees grow less than 20 m apart on an old square in the city of Teresina (Piauí, NE Brazil), and are visited by various birds and bats. The clinical

isolates LMM9, 21, 23, 29, 34, 706, 715, 736, 739, 744, 866 and 916 were included as a reference.

Identification and serotyping of the isolates were done with the 'Crypto Check Iatron' RM 304-K (Iatron Laboratories, Tokyo, Japan) as previously described [16]. The MAT α and MATa pheromones were determined by PCR according to Chaturvedi *et al.* [24] with some modifications. The specific primers for mating type α were 5'-CTT CAC TGC CAT CTT CAC CA-3' and 5'-GAC ACA AAG GGT CAT GCC A-3', and those for mating type a were 5'-CGC CTT CAC TGC TAC CTT CT-3' and 5'-AAC GCA AGA GTA AGT CGG GC-3'. Amplification was performed in 25 μ l containing 25 ng of target DNA, 50 mM KCl, 10 mM Tris HCl pH 8.4, 2 mM MgCl₂, 0.25 μ l dNTP 20 mM, 1.25 U of Taq DNA polymerase (Amersham Pharmacia Biotech, Little Chalfont, UK), 12 ng of each primer. Initial denaturation was done at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 57.5 °C for 1 min, amplification at 72 °C for 1 min, and final extension at 72 °C for 7 min. The presence of a 101-bp fragment in the agarose gel was considered positive for the mating type α , and the presence of a 117-bp fragment was considered positive for the mating type a. During the PCR assay, reference strains (ATCC [American Type Culture Collection, Manassas, VA, USA] 28958 and ATCC 28957) were included.

Isolation of the DNA and the application of AFLP were performed as described in Boekhout *et al.* [9], and additional reference isolates from all six major AFLP genotypes were also taken from this publication.

Results and discussion

The AFLP patterns of the cryptococcal isolates clustered in two main clusters showing a low similarity value of about 5.5%, corresponding to *C. n. var. neoformans* and *C. n. var. gattii* (Fig. 1). Both taxa may occur on a single tree. *C. n. var. neoformans* serotype D with mating type a was isolated together with *C. n. var. gattii* serotype B mating type α from a single pottery tree, but no evidence was found for the exchange of genetic material between these species.

The *C. n. var. neoformans* isolates came from pink shower trees in Rio de Janeiro and Teresina (state of Piauí), the tropical lowland forest trees *Miroxylon peruiferum* and *Theobroma cacao* from Amazonas, soil from an armadillo burrow in Teresina, a house contaminated by pigeons in Rio de Janeiro and some clinical cases from Rio de Janeiro, Mato Grosso do Sul and Rio Grande do Sul (Fig. 2). The majority (82%) of the *C. n. var. neoformans* isolates belonged to serotype

Table 1 Origin of isolates of *C. neoformans* var. *neoformans* (serotypes A, D, AD) and *C. neoformans* var. *gattii* (= *C. bacillisporus* = *C. gattii*), serotypes B, C, BC) studied: asterisks indicate strains of clinical origin

Strain number	Serotype	Mating type	AFLP genotype	Source	Geographical origin
LMM 9*	AD	α	1A	Bone marrow/HIV +	Rio de Janeiro, SE Brazil
LMM 21*	B	α	6	Urine/HIV +	Rio de Janeiro, SE Brazil
LMM 23*	B	α	6	Cerebrospinal fluid/HIV –	Piauí, NE Brazil
LMM 29*	B	?	6	Cerebrospinal fluid/HIV –	Piauí, NE Brazil
LMM 34*	A	α	1A	Cerebrospinal fluid/HIV +	Rio de Janeiro, SE Brazil
LMM 379	A	a	1A	House dust	Rio de Janeiro, SE Brazil
LMM 388	D	Not determined	3	Building	Rio de Janeiro, SE Brazil
LMM 414	B	a	6	Pottery tree hollow	Piauí, NE Brazil
LMM 484-1	B	a	6	Fig tree hollow	Piauí, NE Brazil
LMM 484-2	B	a	6	Fig tree hollow	Piauí, NE Brazil
LMM 484-3	B	a	6	Fig tree hollow	Piauí, NE Brazil
LMM 489	A	a	1	Pink shower tree hollow	Rio de Janeiro, SE Brazil
LMM 495	A	a	1	Pink shower tree hollow	Rio de Janeiro, SE Brazil
LMM 496	A	a	1	Pink shower tree hollow	Rio de Janeiro, SE Brazil
LMM 498	B	a	6	Pink shower entrance [air]	Piauí, NE Brazil
LMM 526	A	a	1	<i>Miroxylon peruiferum</i> hollow	Amazonas, N Brazil
LMM 527	A	a	1	<i>Theobroma cacao</i> hollow	Amazonas, N Brazil
LMM 544-1	A	α	1	Pink shower tree	Rio de Janeiro, SE Brazil
LMM 544-2	A	α	1	Pink shower tree	Rio de Janeiro, SE Brazil
LMM 547-1	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-2	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-3	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-4	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-5	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-6	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-7	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-9	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-10	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-11	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-13	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-14	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-15	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-16	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-17	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 547-18	D	a	2	Pottery tree hollow	Piauí, NE Brazil
LMM 547-19	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 561-5	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 561-6	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-1	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-2	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-3	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-4	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-5	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-6	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-7	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-9	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-10	BC → B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-11	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-12	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-13	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-15	BC → B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 562-16	B	α	6	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-9	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-10	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-11	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-14	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-17	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-19	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-21	A	α	1	Pink shower tree hollow	Piauí, NE Brazil

Table 1 (Continued)

Strain number	Serotype	Mating type	AFLP genotype	Source	Geographical origin
LMM 564-26	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 564-27	A	α	1	Pink shower tree hollow	Piauí, NE Brazil
LMM 655	B	α	6	Pottery tree hollow	Piauí, NE Brazil
LMM 706*	A	α	1	Clinical	Rio Grande do Sul, S Brazil
LMM 715*	A	α	1	Clinical	Rio Grande do Sul, S Brazil
LMM 736*	A	α	1	Cerebrospinal fluid/HIV+	Mato Grosso do Sul, SW Brazil
LMM 739*	A	α	1	Cerebrospinal fluid/HIV+	Mato Grosso do Sul, SW Brazil
LMM 744*	A	α	1	Cerebrospinal fluid/HIV+	Mato Grosso do Sul, SW Brazil
LMM 866*	B	α	6	Cerebrospinal fluid/HIV-	Roraima, N Brazil
LMM 876-1	D	A	2	Soil from armadillo burrow	Piauí, NE Brazil
LMM 876-2	D	A	2	Soil from armadillo burrow	Piauí, NE Brazil
LMM 878-3	A	α	1	Soil from armadillo burrow	Piauí, NE Brazil
LMM 878-4	A	α	1	Soil from armadillo burrow	Piauí, NE Brazil
LMM 916*	C	α	5	Sputum	Rio de Janeiro, SE Brazil

A, AFLP genotype 1 with MAT α , three isolates (9%) belonged to AFLP genotype 1A with MAT α and had either serotype A or AD, and three (9%) isolates with serotype D and MAT α belonged to AFLP genotype 2. Isolate LMM 388 with serotype D, isolated from a building in Rio de Janeiro inhabited by pigeons, belonged to the hybrid AFLP genotype 3 (results not shown). The clinical isolates LMM 9, 34, 706, 715, 736, 739 and 744 clustered within the environmental isolates (Fig. 1).

The isolates of *C. n. var. neoformans* obtained from different, but neighbouring trees, in Teresina (Piauí) formed a single cluster with a high similarity value of 96%. Three isolates from trees from Rio de Janeiro (i.e. LMM 489, 495, 496) occurred in the same cluster as the Teresina isolates, whereas two (LMM 544-1 and 544-2) were found to be somewhat different. Isolates obtained from a single tree showed very high similarity values of their AFLP patterns (Figs. 3–5). Six strains (LMM 562 series) isolated from the same pink shower tree formed a cluster with a similarity value of about 98% (Fig. 3). A cluster of eight isolates (LMM 564 series) from another pink shower tree showed two subclusters with similarity values of 97.7 and 99.7, respectively (Fig. 4). Two isolates (LMM 544-1 and 544-2) from a pink shower tree in Rio de Janeiro showed 97% similarity. Two isolates (LMM 876-1, 876-2) of AFLP genotype 2 from an armadillo burrow had a 99.2% similarity value, and showed 96% similarity with an isolate (LMM 547-18) from a pottery tree, all from Teresina. Two samples collected from the same hollow over a 3-year interval, LMM 544-1 and 544-2 (1992) and LMM 496 (1995) showed 85.8% similarity only (results not shown). The result suggests that microevolution occurs in nature, or that the tree hollows may become inhabited by different populations over time.

Our results confirm that different genera of trees and woody substrates, as well as animal-related habitats may be a source for *C. n. var. grubii*, which is commonly involved in AIDS-related disease. The variety is already known from pine needles (Australia), woody debris of *Eucalyptus* in the USA, plants from India and wood from Zaire [9]. Our genotypic data suggest that trees may be inhabited by either a single clone of *C. n. var. grubii* (e.g. in the LMM 562 series) or multiple clones (e.g. in the LMM 564 series).

A similar picture emerges for *C. n. var. gattii*, but the overall similarity value within the cluster containing the Brazilian tree isolates was only 76%. The main cluster comprised isolates from a pottery tree, a pink shower tree and a fig tree from Teresina (Piauí), as well as some clinical isolates from Rio de Janeiro, Piauí and Roraima (Fig. 1). No differences were observed in genotypes of environmental and clinical isolates. Although some polymorphism is apparent in the AFLP patterns, all the isolates seem genetically related. Mating type analysis using PCR primers showed that all *C. n. var. gattii* isolates had MAT α . The majority (97%) of tree isolates of this species belonged to AFLP genotype 6 [9], which may be similar to the genotype VGII as observed by PCR fingerprinting [25]. Ninety four per cent of the isolates were serotype B, but two isolates (LMM 562-10 and 562-15) showed a positive reaction with the agglutination factors 1, 5 and 6 of the Iatron kit, and hence are assigned to serotype BC. This analysis was repeated twice in the same year with the same results, but after a few years, the serotype switched to the B serotype. One clinical isolate from Rio de Janeiro, LMM 916, belonged to AFLP genotype 5 and had serotype C.

The isolates of *C. n. var. gattii* from Teresina are probably not all clonally related, as they differ at the

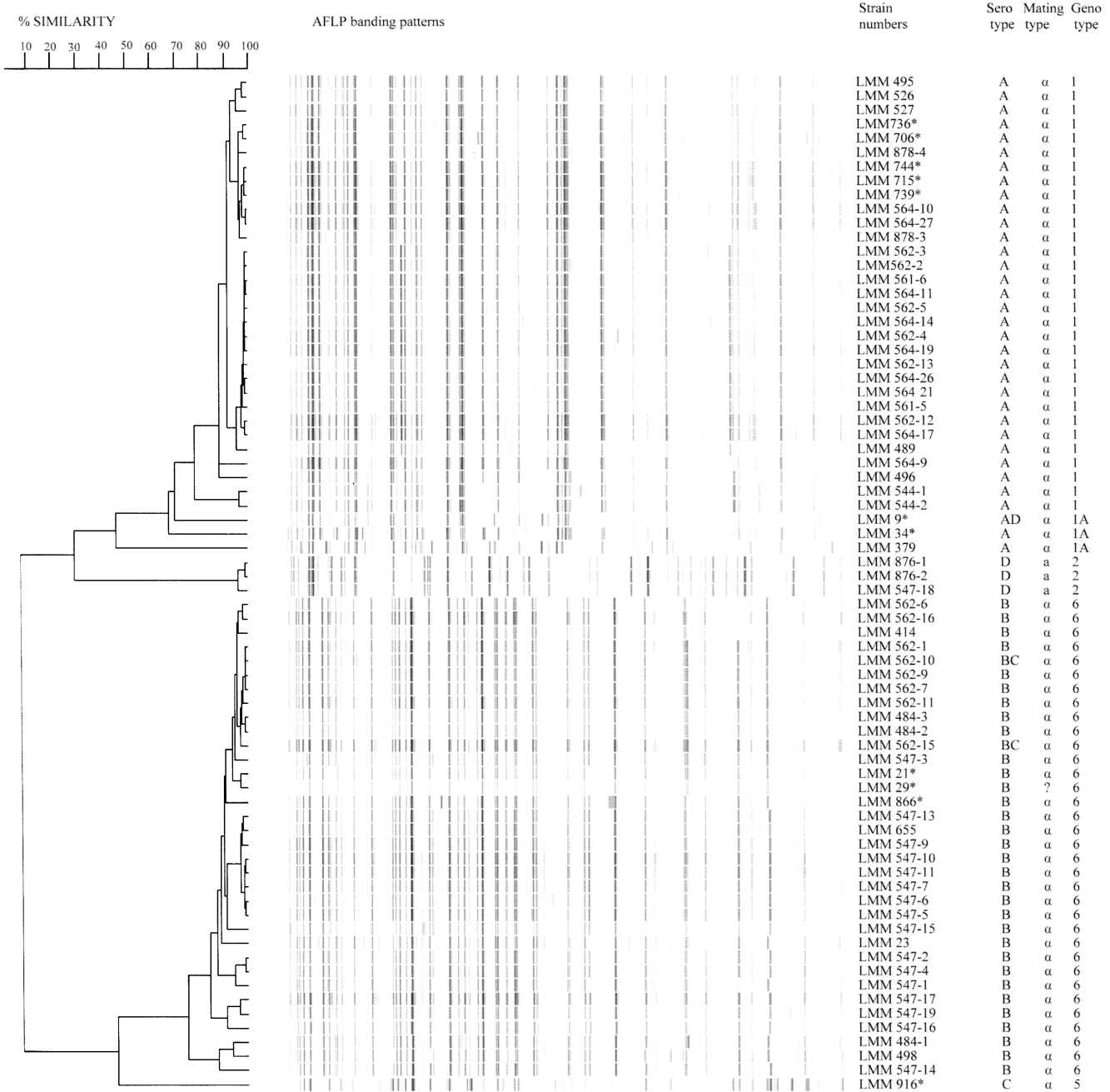


Fig. 1 UPGMA tree of AFLP banding patterns from Brazilian environmental isolates of the *C. neoformans* species complex. Strains indicated with an asterisk originate from clinical sources and are included as a reference.

82% similarity level, which is close to the 80% similarity we previously observed for a cluster of genotype 6 comprising four isolates from Uruguay, Aruba and the USA [9]. Until recently, only one isolate of this genotype was known from an arboreal habitat, namely isolate CBS 7750 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), which was isolated from bark debris of *Eucalyptus*. The present work

demonstrated that AFLP genotype 6 occupies an arboreal habitat in the Americas. However, strains of this genotype are able to cause disease in non-AIDS and AIDS patients.

In short, our results indicate that: (a) two *Cryptococcus* species may occur on a single tree; (b) isolates of AFLP genotype 6 occur widely on trees in Brazil, and probably other Latin American countries; (c) isolates of

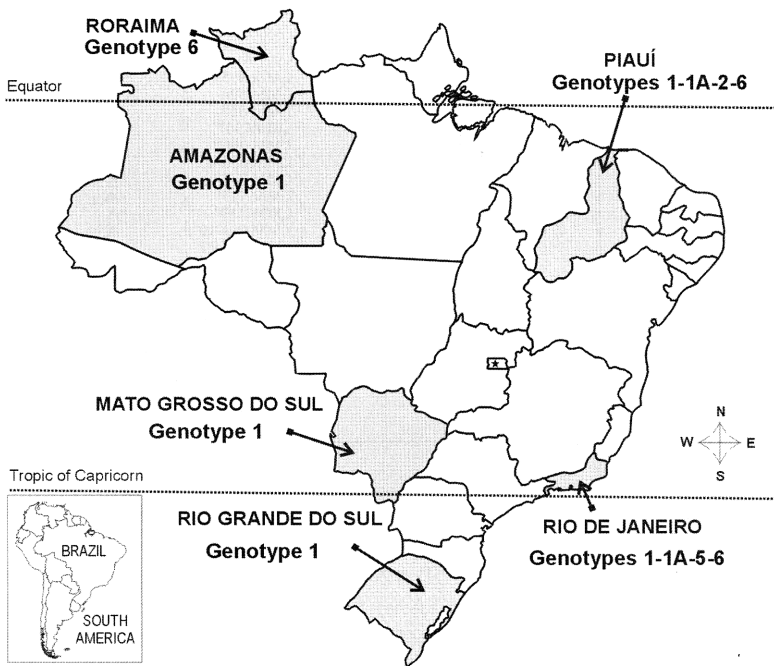


Fig. 2 Map of Brazil showing the geographic distributions of AFLP genotypes of the *C. neoformans* species complex.

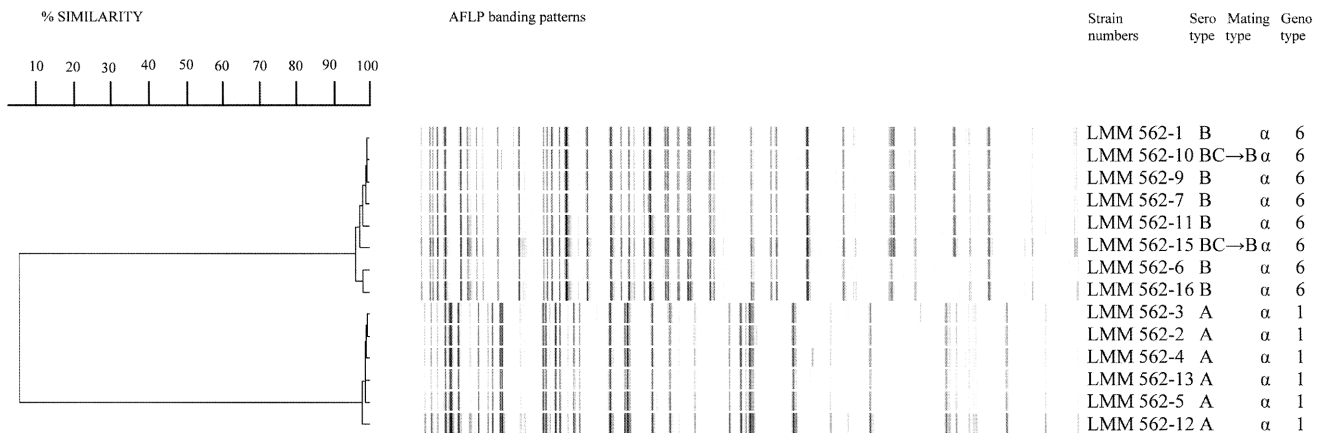


Fig. 3 UPGMA tree of AFLP banding patterns of *C. neoformans* var. *neoformans* (serotype A) and *C. neoformans* var. *gattii* (serotypes B and BC) isolated from a single pink shower tree (LMM 562).

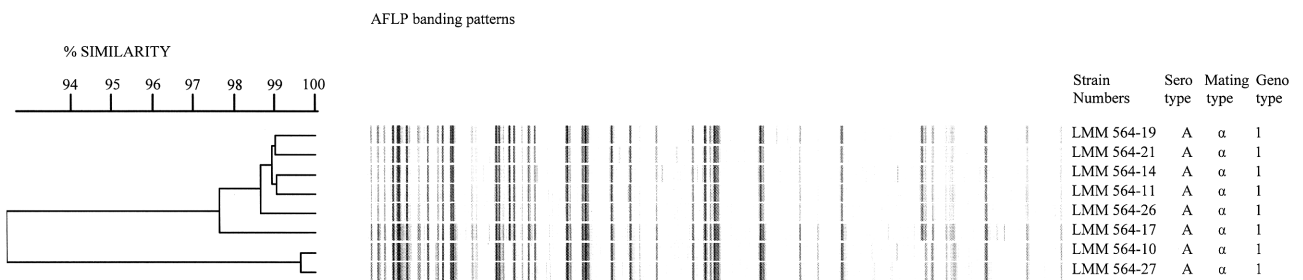


Fig. 4 UPGMA tree of AFLP banding patterns of *C. neoformans* var. *neoformans* isolated from another single pink shower tree hollow (LMM 564).

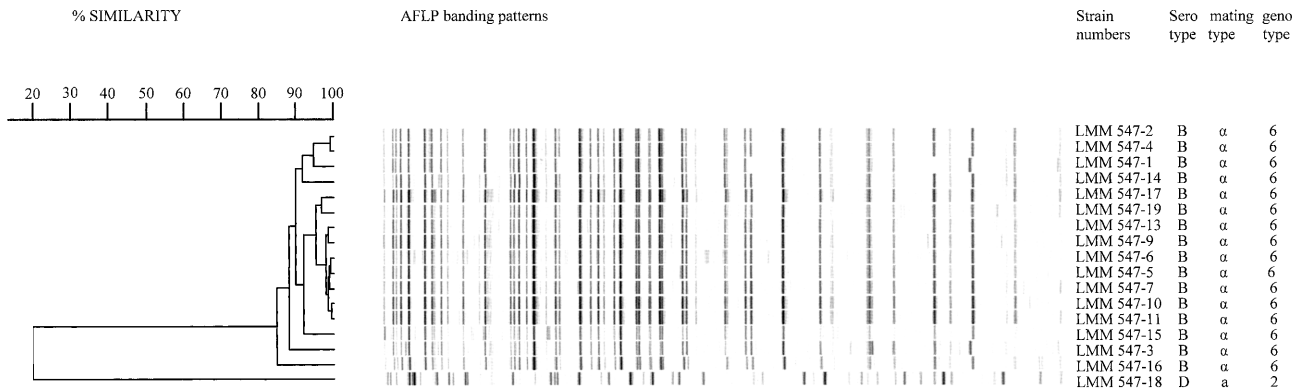


Fig. 5 UPGMA tree of AFLP banding patterns of *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *gattii* (serotype B) isolated from a single pottery tree hollow (LMM 547).

each species obtained from a single tree tend to be genetically closely related, but not necessarily genetically identical; (d) clonal expansion can occur on a single tree; and, (6) isolates from neighbouring trees may be genetically similar or even identical, thus suggesting that cryptococcal populations may be clonally dispersed among neighbouring trees and nearby animal-related habitats. The occurrence of clonal expansion is consistent with the presence of only one mating type within each AFLP genotype isolated from a given tree.

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