

## *Cryptococcus neoformans* Shows a Remarkable Genotypic Diversity in Brazil

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**The genotypic diversity of Brazilian *Cryptococcus neoformans* strains was analyzed. The majority of the samples were  $\alpha$ A (65%), followed by  $\alpha$ B (17.5%),  $\alpha$ D (9%),  $\alpha$ AaD hybrids (5%), and  $\alpha$ C (3.5%). A considerable genotypic diversity occurred within *C. neoformans* var. *grubii*, and a new amplified fragment length polymorphism genotype, 1B, was recognized.**

*Cryptococcus neoformans* is frequently implicated in meningoencephalitis in immunocompromised patients (17, 18) and is commonly associated with pigeon droppings and plant materials (1, 3, 4, 6, 7, 13, 14, 16–18, 19, 23).

Amplified fragment length polymorphism (AFLP) genotyping has been applied to evaluate the genetic structure of *C. neoformans* (2, 12). In this report, we analyzed genotypes, serotypes, and mating types of 58 Brazilian isolates of the *C. neoformans* complex, including 21 isolates from patients diagnosed prior to the onset of the AIDS pandemic, 10 isolates from AIDS patients, and 27 isolates from environmental sources (Fig. 1). These strains belong to the Culture Collection of the Biomedical Science Institute, University of São Paulo, São Paulo, Brazil. Strains ICB 59 (= CBS 132, serotype AD), ICB 162 (= NIH 444, serotype C) and ICB 163 (= NIH 442, serotype D) were used as reference strains.

Serotypes were determined by the Crypto Check kit (Iatron, Tokyo, Japan) (11) and by PCR using serotype- and mating-type-specific primers (Table 1). For *C. neoformans* (= *C. neoformans* var. *neoformans* and *grubii*), primers were based on *STE12* and *GPA1* genes (15), as well as some newly developed primers obtained by using *STE20* gene sequences. PCR was performed under the following conditions: primer pair JOHE1671/1672, 25 cycles of 96°C for 30 s, 65°C for 30 s, and 72°C for 30 s; primer pairs JOHE3240/JOHE2596 and JOHE3241/JOHE2596, 25 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and primer pairs JOHE7264/JOHE7265, JOHE7267/JOHE7268, JOHE7270/JOHE7272, and JOHE7273/JOHE7275, 30 cycles of 96°C for 30 s, 66°C for 30 s, and 72°C for 60 s. The mating types of the *Cryptococcus gattii* isolates

were determined by using primers designed by Chaturvedi et al. (5) and Halliday et al. (9).

Using serotype-specific antibodies, 38 isolates were serotype A, 10 were serotype B, 6 were serotype D, 2 were serotype C, and 1 was serotype AD (Fig. 1). Serotypes of *C. neoformans* (serotypes A, D, and AD) analyzed by PCR were in most cases concordant with results obtained by the agglutination test. Isolates ICB 160 and ICB 155, however, were found to be serotype AD by the PCR-based method, while the Iatron kit gave serotype A. These findings agree with the previously observed serotype variation among AD hybrid strains analyzed by agglutination (10, 15). Our results showed a predominance of serotypes A (66.7%) and B (17.5%) in the Brazilian populations studied, thus confirming earlier reports (4, 19, 20–22).

AFLP analysis, performed according to Boekhout et al. (2), revealed two main genotypic clusters, corresponding with the species *C. neoformans* (AFLP genotypes 1 to 3) and *C. gattii* (= *C. bacillisporus* = *C. neoformans* var. *gattii*, AFLP genotypes 4 to 6). Thirty-seven strains were AFLP genotype 1, including 1 AFLP genotype 1A isolate (ICB 154); 5 isolates were AFLP genotype 2; 3 were AFLP genotype 3; 2 were AFLP genotype 4; 3 were AFLP genotype 5; and 7 were AFLP genotype 6 (Fig. 1). Strains ICB 155 and 160, which were found to be serotype A by agglutination, belonged to AFLP genotype 3, thus supporting the AD hybrid nature (2). At the genotypic level, our results agree with recent observations published by Casali et al. (4). However, using AFLP, we were able to demonstrate considerable genotypic divergence within the Brazilian isolates of the *C. neoformans* complex, because many polymorphic AFLP markers occurred. Therefore, our AFLP data seem to conflict with previous genotypic data for Brazilian isolates of *C. neoformans* (8). In particular, isolates of *C. neoformans* var. *grubii* (serotype A, AFLP genotype 1) showed an unexpected diversity (Fig. 1 and 2). Five serotype A *MAT $\alpha$*  isolates showed only approximately 50% similarity to the remaining serotype A

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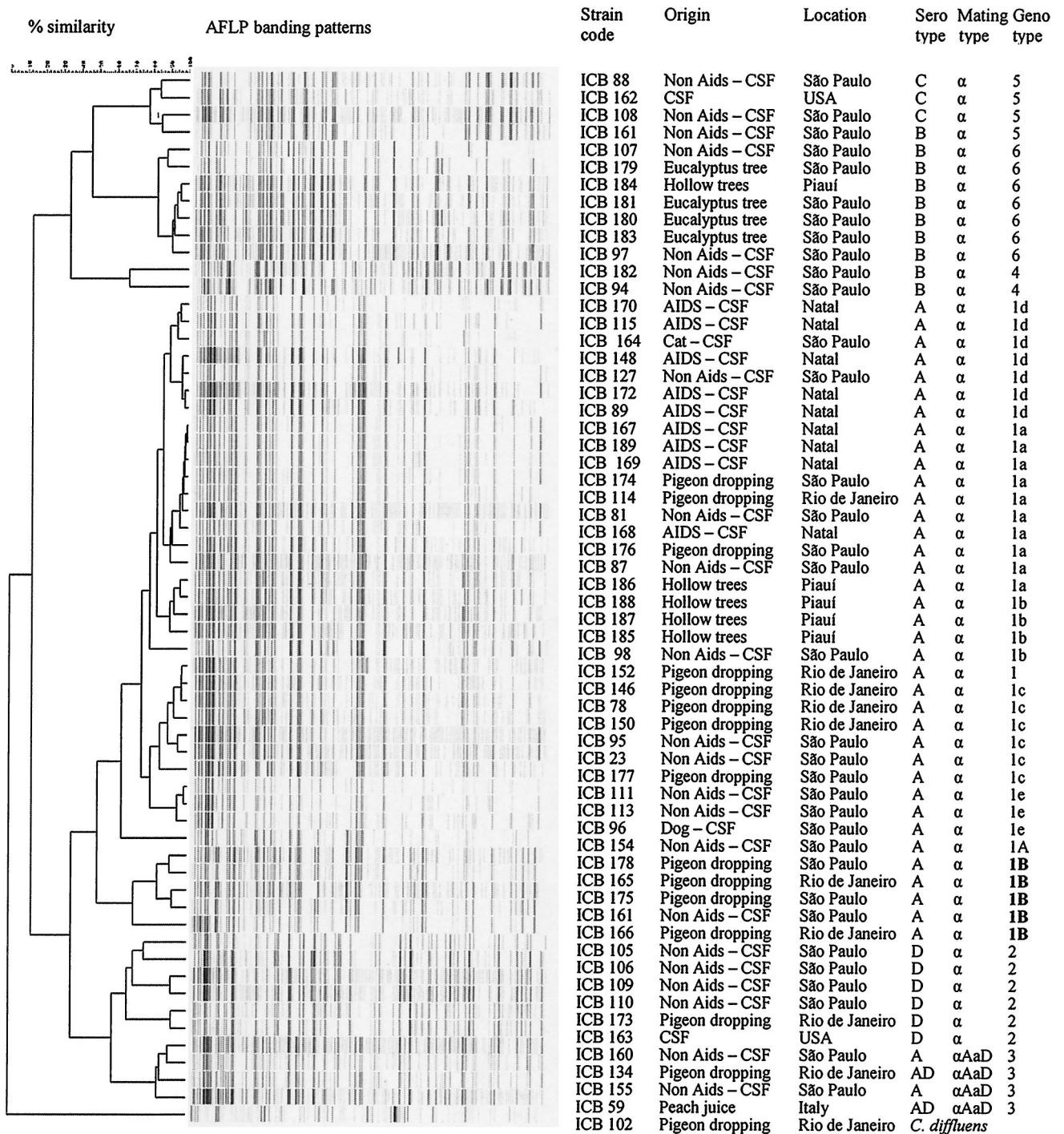


FIG. 1. Clustering of AFLP banding patterns of Brazilian isolates of *C. neoformans* and *C. gattii* (*C. bacillisporus*). (ICB 102 was demonstrated to represent *Cryptococcus diffluens*). The new AFLP genotype, 1B, is indicated in boldface.

TABLE 1. New primer combinations used for mating and serotype determination by PCR

Gene or allele	Primer	Sequence	Size (bp)
<i>MAT</i> $\alpha$ serotype A (STE20 $\alpha$ A)	JOHE 7264	5'-AGCTGATGCTGTGGATTGAATAC-3'	1,200
	JOHE 7265	5'-GTTCAATTAATCTCACTACCTGTAG-3'	
<i>MAT</i> $\alpha$ serotype D (STE20 $\alpha$ D)	JOHE 7267	5'-ATAGGCTGGTGTCTGTGAATTAAG-3'	1,200
	JOHE 7268	5'-GTTCAAGTAATCTCACTACATGCG-3'	
<i>MAT</i> a serotype A (STE20aA)	JOHE 7270	5'-ATCAGAGACAGAGGAGGAGCAAGAC-3'	870
	JOHE 7272	5'-TCCACTGGCAACCCTGCGAG-3'	
<i>MAT</i> a serotype D (STE20aD)	JOHE 7273	5'-GTTTCATCAGATACAGAGGAGTGG-3'	870
	JOHE 7275	5'-CTCCACTGTCAAACCTACGGC-3'	

*MAT* $\alpha$  strains and represent a hitherto unknown genotype, which we labeled AFLP genotype 1B (Fig. 1 and 2). Four of these isolates came from pigeon droppings collected in São Paulo and Rio de Janeiro, and one came from a non-AIDS patient in São Paulo (Southeast region). Genotypes 1, 1A, and 1B share a number of AFLP bands: some bands occur only in pairs of these genotypes, and a few bands appear to be a specific genotype (Fig. 2). Hence, unique AFLP markers may discriminate populations of *C. neoformans* var. *grubii*. These populations may be geographically limited (e.g., isolates of AFLP genotype 1B from the Southeast states of Brazil) or limited to different ecological niches (e.g., pigeon droppings and non-AIDS patients). It would be interesting to identify the molecular nature of *C. neoformans* var. *grubii* markers in order to understand their distribution and transmission and to assess a possible function of the corresponding genes, if any, in yeast biology.

Within the main AFLP cluster 1, five subclusters (labeled 1a to 1e) could be identified showing 85 to 94% similarity within the subclusters and 62 to 88% similarity among the subclusters (data not shown). Isolates from AIDS patients, all originating

from the Northeast region, belonged to AFLP genotypes 1a and 1d. Isolates from non-AIDS patients isolated from the Southeast region before the onset of the AIDS pandemic belonged to all five subtypes. Unfortunately, it is not clear whether strains belonging to AFLP genotypes 1a and 1d preferentially occur in AIDS patients, because all of our isolates originating from AIDS patients came from a geographically restricted area (Northeast region). Brazilian populations of *C. neoformans* var. *grubii* differ geographically to some extent, because AFLP type 1 subtypes 1c and 1e were observed only in the Southeast states.

Three genotypes occurred within *C. gattii* (Fig. 1). Interestingly, seven isolates from the Northeast and Southeast states with serotype B *MAT* $\alpha$  belonged to AFLP genotype 6 (Fig. 1). The occurrence of AFLP genotype 6, which is similar to genotype VGII of W. Meyer, has only recently been reported from Brazil (17, 23). Isolates of this genotype are known from non-AIDS patients and environmental samples—mainly in North and South America, but also in Northern Australia (2, 4, 17, 23). Because this genotype is known to be implicated in a recent outbreak of *C. gattii* on Vancouver Island, Canada (T.

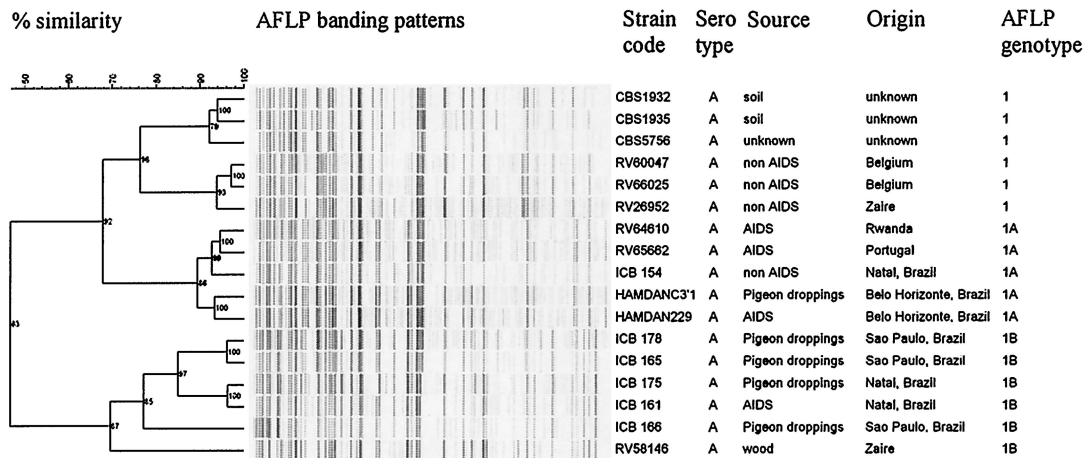


FIG. 2. AFLP patterns of AFLP genotypes 1, 1A, and 1B showing the presence of genetic markers co-occurring in all pairs of these genotypes. Isolates 125.91, RV64610, RV65662, HamdanC3'1, Hamdan229, and RV58146 were included as a reference. The numbers given at the branches represent the cophenetic values.

Boekhout et al., unpublished observations), some concern seems justified with respect to the virulence of this genotype, because two Brazilian isolates originated from non-AIDS patients.

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