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Genetic Relationship between *Cryptococcus neoformans* var. *neoformans* Strains of Serotypes A and D

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Cryptococcus neoformans **serotypes A and D are responsible for the overwhelming majority of infections in patients with AIDS. The genetic relationship between the serotypes is poorly understood, but there are significant differences in the epidemiology and clinical presentation of serotype A and D infections. We evaluated the genetic relationship between reference *C. neoformans* strains belonging to serotypes A and D by analyzing their *URA5* sequences and restriction fragment length polymorphisms (RFLPs) with the *C. neoformans* repetitive element 1 (CNRE-1) probe. The results were compared to those previously obtained for isolates from Brazil and New York City by the same typing methods, and dendrograms were generated. Serotype A and D strains produced distinct RFLP patterns consistent with their separation into two major clusters in the dendrogram generated on the basis of RFLP data. Similarly, serotype A and D strains clustered independently on the basis of the nucleotide sequences of their *URA5* genes. Pairwise comparisons revealed average numbers of nucleotide differences within serotypes A and D of 3.0 ± 1.7 and 7.2 ± 3.4, respectively ($P < 0.0001$), and between serotypes A and D of 41.9 ± 2.7. In summary, our results indicate phylogenetic differences between the two serotypes of *C. neoformans* var. *neoformans* and suggest that these serotypes could probably be considered different varieties of *C. neoformans*.**

Cryptococcus neoformans is an encapsulated yeast that can cause severe meningoencephalitis in immunocompromised patients, especially those with AIDS. On the basis of the antigenic composition of the capsular polysaccharide, *C. neoformans* has been divided into four serotypes: serotypes A, B, C, and D. The four serotypes have been further divided into two varieties, *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gattii* (serotypes B and C), on the basis of biochemical, morphological and genetic characteristics (22, 24). *C. neoformans* var. *neoformans* is primarily the etiologic agent of cryptococcosis in patients with AIDS, and serotype A comprises the overwhelming majority of clinical isolates. Infections due to strains belonging to serotype D are more prevalent in certain geographic areas, including France, Italy, and Denmark (2, 12, 24). In France, serotype D causes 21% of cases of cryptococcosis (11, 13). Serotype D infections are more likely than serotype A infections to occur in older patients, to result in skin involvement, and to be associated with corticosteroid therapy (11, 13).

The genetic relationship between isolates classified as serotypes A and D is uncertain. Guého et al. (17) found a relatively large phylogenetic distance between serotypes A and D by analysis of partial 26S rRNA sequences. Meyer et al. (23) used PCR fingerprinting analysis to demonstrate that strains of serotypes A and D could be distinguished from each other. Similarly, Varma and Kwon-Chung (31) reported the isolation of a DNA probe (UT-4p) that was able to discriminate between these serotypes. Brandt et al. (4) demonstrated that serotypes A and D could be distinguished by their multilocus enzyme electrophoresis profile. Serotypes A and D also show consistent differences in their electrophoretic karyotypes (26, 27). Hence, there is evidence that serotypes A and D belong to genetically distinct groups, but they remain within a single varietal classification because occasional strains of A and D isolates have been successfully mated (20). The uncertainty regarding the genetic relationship between serotype A and D strains is compounded by extensive genetic heterogeneity for strains grouped within a serotype.

The field of cryptococcal research is relatively small, and independent groups tend to work with different strains. This raises the concern that findings with a particular strain may not be generalizable. In this study we used two molecular typing techniques, DNA fingerprinting with the *C. neoformans* repetitive element 1 (CNRE-1) probe and analysis of the nucleotide sequence of the *URA5* gene, to investigate the genetic relationship between reference *C. neoformans* strains belonging to serotypes A and D. For the purpose of this study we defined a reference strain as one that has been used in more than one study and/or more than one laboratory. Our results indicate phylogenetic differences between the two serotypes of *C. neoformans* var. *neoformans* and the genetic relationship between commonly used laboratory strains.

MATERIALS AND METHODS

C. neoformans **strains.** A detailed list of the reference strains used in this study is presented in Table 1. Clinical and environmental isolates from Brazil (designated C and E followed by a number, respectively) were described previously (16). Clinical strains from New York (J isolates) were also reported earlier (6). All strains were kept in 50% glycerol in a freezer at −80°C and were grown overnight on Sabouraud's broth at 30°C for DNA isolation.

Serotyping. The serotype classifications for the reference strains are listed in Table 1. Recent isolates from Brazil (isolates C5, C7, C24, C25, C31, C33, RJ1, RJ2, E3, E4, E5, E6, E9, and E12) and New York (isolates J15, J17, J19, J24, J25, and J26) were serotyped by the slide agglutination test with sera containing cryptococcal antigen factors 1, 5, 6, 7, and 8 (Iatron Laboratories, Inc., Tokyo, Japan). On the basis of the patterns of agglutination, the results were interpreted as follows: serotypes A, B, C, D, and AD reacted with antigen factors 1, 7; 1, 5; 1, 6; 1, 8; and 1, 7, 8, respectively.

The reactivity of monoclonal antibody (MAb) 13F1 with the recent isolates from Brazil and New York was also examined by indirect immunofluorescence

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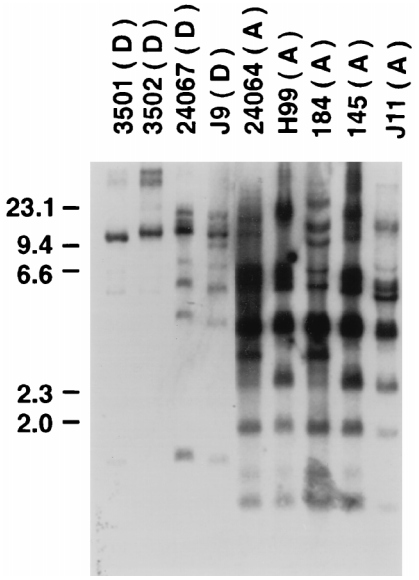

FIG. 1. CNRE-1 RFLP patterns obtained from reference strains of *C. neoformans* of serotypes A and D. The serotype of each strain is indicated in parentheses. The numbers on the left are in kilobases.

(IF). Previously, we reported that IF with MAb 13F1 discriminates between most serotype A and D strains by producing annular and punctate binding patterns, respectively (9). IF studies were performed as described previously (9). Briefly, stationary-phase cells were washed and incubated with MAb 13F1 at 10 μg/ml for 2 h at room temperature. MAb binding was detected with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin M (Southern Biotechnology, Birmingham, Ala.). Samples were viewed with a Zeiss (Thornwood, N.Y.) Axiophot microscope equipped with a fluorescein isothiocyanate filter. All the reference strains listed in Table 1 were previously examined by IF (9).

CNRE-1 RFLP analysis. All strains were typed by Southern blot analysis with CNRE-1 as described previously (16). Briefly, genomic DNA was extracted from protoplasts (16) and was digested with *Sac*I (Boehringer Mannheim, Indianapolis, Ind.), and the resulting fragments were probed with CNRE-1 labeled with [α-^{32}P]dCTP. The bands were visualized by autoradiography.

The CNRE-1 restriction fragment length polymorphism (RFLP) patterns obtained for each strain were compared by using the Molecular Analyst/PC Fingerprinting software (Bio-Rad, Hercules, Calif.). Band positions were normalized by equating the *Hin*dIII-digested bacteriophage λ DNA molecular weight marker (Boehringer Mannheim), and Dice coefficients of similarity (number of shared bands × 2/total number of fragments in the two strains) were calculated for each pair of strains compared, generating a matrix of similarity coefficients. Dendrograms based on these matrices were then generated by the unweighted pair-group method of average linkage (28). When two patterns were compared, a match was recorded if the normalized molecular size of the fragment in the first pattern was within a window of ±1.5% of the molecular size of a fragment in the second pattern. The CNRE-1 RFLP patterns of Brazilian and New York City isolates reported earlier (10, 16) were also scanned and compared to those of the reference strains.

***URA5* nucleotide sequencing.** *URA5* DNA was amplified from genomic DNA by PCR as described previously (16) and was cloned into the pCR 2.1 vector of the TA cloning system (Invitrogen, San Diego, Calif.). *Escherichia coli* transformants were selected on plates containing 50 μg of kanamycin per ml, and plasmid DNA was purified with Midi-Prep columns (Qiagen, Chatsworth, Calif.). The insert was sequenced in the DNA Sequencing Facility of the Albert Einstein College of Medicine with automated sequencing instrument models ABI373A and ABI377 (Perkin-Elmer, Foster City, Calif.). Samples were analyzed by fluorescent cycle sequencing with dye-labeled primers.

The *URA5* sequences obtained from the reference strains were compared with sequences previously obtained for seven New York City clinical isolates (GenBank accession no. L38582 to L38858, respectively), 10 Brazilian clinical and environmental isolates (GenBank accession no. U67723 to U67732, respectively), isolate B-3501 (GenBank accession no. M34606), and *C. neoformans* var. *gattii* (GenBank accession no. M93026). All sequences were first aligned by using Clustal V (18), and phylogenies were then estimated by use of the DNAPENNY (branch and bound parsimony), CONSENSE, and SEQBOOT programs of the PHYLIP package, version 3.5c (15).

Nucleotide sequence accession numbers. The *URA5* DNA sequences of the reference strains have been deposited in GenBank, and the accession numbers are listed in Table 1.

RESULTS

Serotyping. Of the 20 recent isolates from Brazil and New York, all but isolates J25 and J26 presented agglutination patterns consistent with their assignment to serotype A. Isolates J25 and J26 showed positive reactions with antigenic factors 1, 7, and 8 characteristic of serotype AD. IF with MAb 13F1 produced an annular pattern of binding typical of serotype A strains for all recent isolates, thus confirming the results obtained with the sera containing cryptococcal antigen factors.

CNRE-1 RFLP analysis. To determine whether the DNA fingerprints of the reference strains correlated with their serotypic status, Southern hybridization with the CNRE-1 probe was performed with serotype A and D isolates of *C. neoformans* var. *neoformans*. As seen in Fig. 1, hybridization of CNRE-1 to *Sac*I-digested genomic DNA from serotype A isolates generated complex patterns of bands ranging from 11 to 16 restriction fragments with various intensities. A strongly hybridizing band at approximately 3.5 kb was present in all serotype A isolates. No two serotype A isolates had identical CNRE-1 RFLPs, although the dendrogram generated on the basis of the RFLP data grouped isolates 24064 and 184 together because it considered a band position tolerance of 1.5% (Fig. 2). This dendrogram also showed that isolates H99 and 145 were highly related, with 76% similarity. CNRE-1 hybridized to fewer bands and with a lower intensity for serotype D

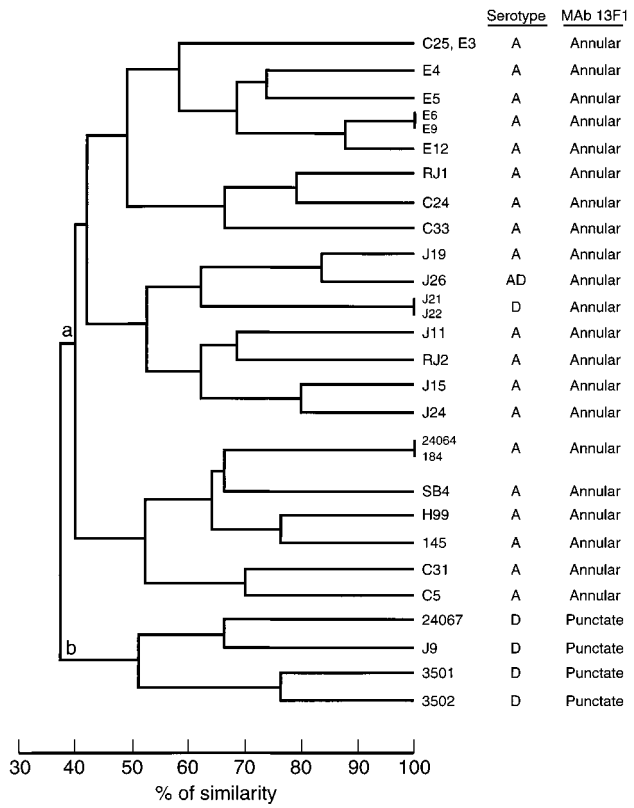

FIG. 2. Dendrogram generated from the Dice coefficients computed from the CNRE-1 patterns for reference strains of *C. neoformans* and recent isolates from Brazil (C and E isolates) and New York City (J isolates). The serotypes and binding patterns obtained by IF with MAb 13F1 are presented on the right.

TABLE 1. Details for some *C. neoformans* var. *neoformans* strains used in this study[a]

Strain	Source	Serotype	Original source or reference	GenBank accession no. (*URA5* gene)
3501	ATCC (ATCC 34873)	D	K. J. Kwon-Chung, NIH	M34606
3502	ATCC (ATCC 34874)	D	K. J. Kwon-Chung, NIH	AF032430
24067	ATCC	D	Clinical isolate (32)	AF032431
24064	ATCC	A	Clinical isolate (32)	AF032432
145	S. Levitz (BU)	A	Clinical isolate	AF032433
184	J. Murphy (UOklahoma)	A	Clinical isolate (25)	AF032434
H99	J. Perfect, DUMC	A	Clinical isolate from a lymphoma patient	AF032436
SB4	E. D. Spitzer, SUNY	A	Clinical isolate from an AIDS patient (30)	AF032435
J9	A. Casadevall (AECOM)	D	Clinical isolate from an AIDS patient (30)	—[b]
J11	A. Casadevall (AECOM)	A	Clinical isolate from an AIDS patient (30)	—[c]
J21	A. Casadevall (AECOM)	D	Clinical isolate from an AIDS patient (10)	L38585
J22	A. Casadevall (AECOM)	D	Clinical isolate from an AIDS patient (10)	—[d]

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); NIH, National Institutes of Health (Bethesda, Md.); BU, Boston University (Boston, Mass.); UOklahoma, University of Oklahoma (Oklahoma City); DUMC, Duke University Medical Center (Durham, N.C.); SUNY, State University of New York (Stony Brook); AECOM, Albert Einstein College of Medicine (Bronx, N.Y.).
[b] —, the *URA5* sequence is the same as that of strain 24067.
[c] —, the *URA5* sequence is the same as that of strain H99.
[d] —, the *URA5* sequence same as that of strain J21.

isolates (Fig. 1). The number of restriction fragments produced ranged from 5 to 11. Isolates 3501 and 3502 were very similar, clustering together with 76% similarity. Overall, serotype A and D isolates produced distinct RFLP patterns consistent with their separation into two major clusters (clusters a and b), as demonstrated in the dendrogram presented in Fig. 2. All recent isolates from Brazil and New York were placed among the reference serotype A strains.

***URA5* sequence analysis.** Partial sequences of the *URA5* gene for serotype A and D strains are presented in Fig. 3 along with the partial sequence of strain 3501 (14). Isolates 24067 and J9 had identical sequences, as did isolates J11 and H99. Among the isolates belonging to serotype D, the number of nucleotide substitutions averaged 7.2 ± 3.4 (n = 15 pairwise comparisons). The *URA5* genes from strains J21 and J22 showed the greatest number of base differences (between 9 and

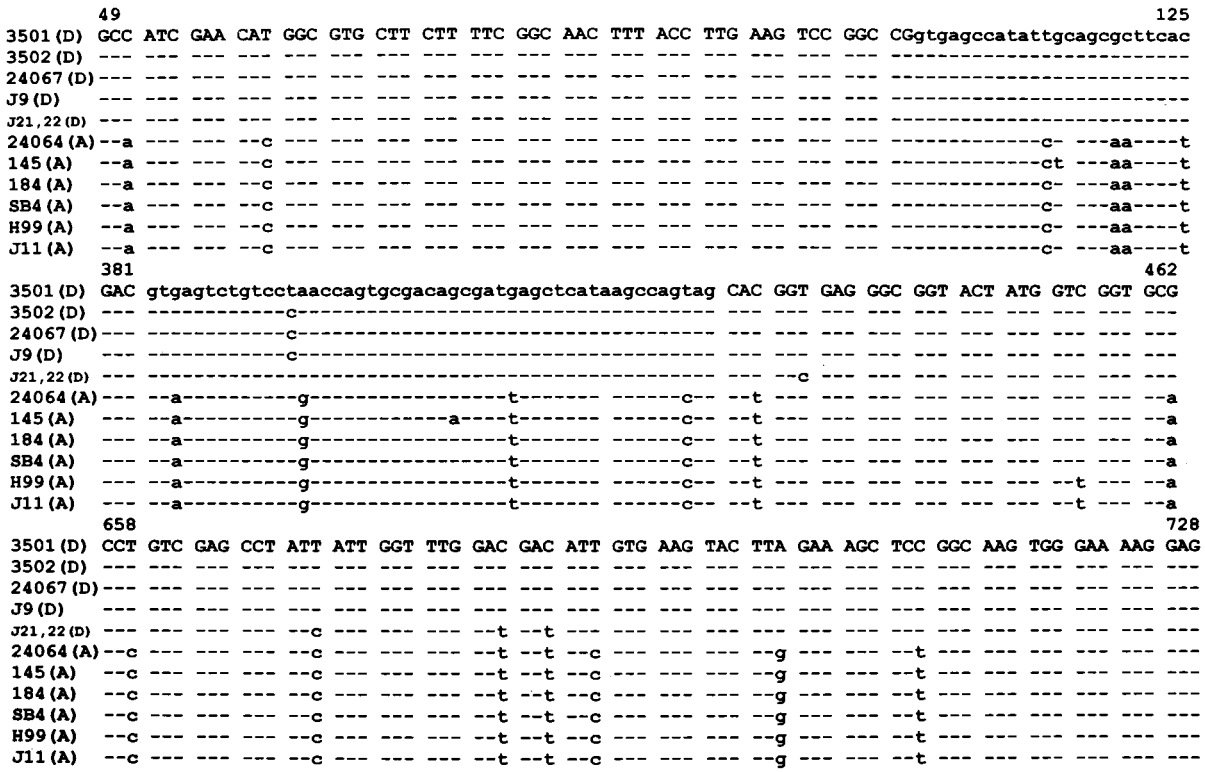

FIG. 3. Partial nucleotide sequence of the *URA5* genes from reference strains of *C. neoformans* serotypes A and D. Serotypes are indicated in parentheses. A hyphen indicates that the base is identical to that in strain 3501 (14). A space implies that the base was not present in the allele. Lowercase letters indicate synonymous substitutions. The complete sequences are available in GenBank under the accession numbers listed in Table 1.

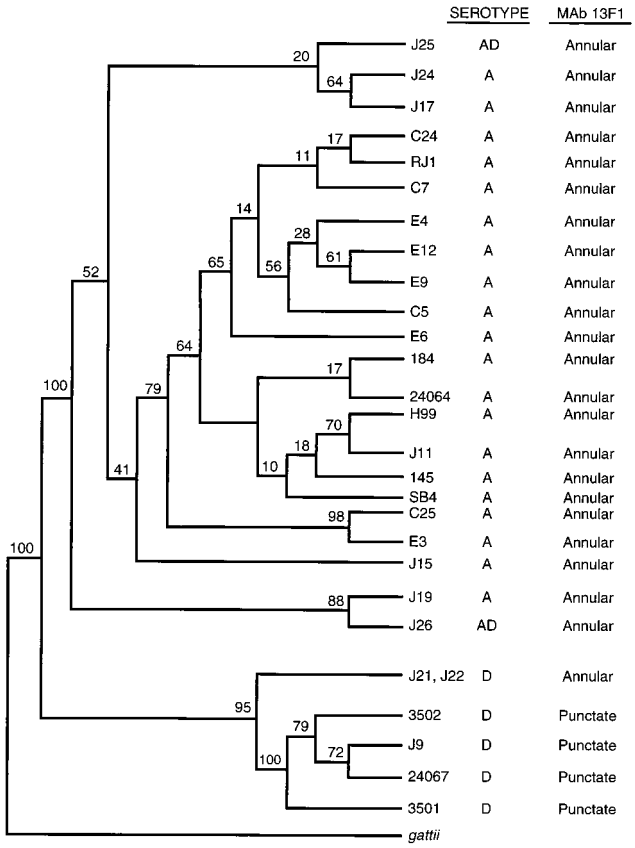

FIG. 4. Relationship of reference strains of *C. neoformans* serotypes A and D and recent isolates from Brazil and New York City obtained from phylogenetic analysis of *URA5* sequence data. The tree was obtained by use of DNAPENNY and CONSENSE of the PHYLIP program. The numbers at each branch point were generated by use of SEQBOOT and indicate the percentage of bootstrap replications. *C. neoformans* var. *gattii* was designated the outgroup. The serotypes and binding patterns obtained by IF with MAb 13F1 are presented on the right. The sequences of isolates C31, E5, and RJ2 were identical to the sequence of isolate C7 (16). Isolate C33 had the same sequence as isolate C5 (16).

10 substitutions) compared to the numbers for the other serotype D isolates. The nucleotide sequences of the serotype A isolates differed from each other by an average of 3.0 ± 1.7 base differences (n = 15 pairwise comparisons). The average number of *URA5* base differences obtained by pairwise comparisons between serotype A and D isolates was 41.9 ± 2.7 (n = 30). Parsimony analysis (with bootstrapping) of these sequences identified a consensus tree that confirmed the dendrogram obtained from the CNRE-1 RFLP data, indicating that isolates of serotypes A and D clustered independently (Fig. 4). Remarkably, the separate clusters obtained for these serotypes occurred in 100% of the bootstrap replicates. Again, recently isolated strains from Brazil and New York were placed in the cluster that contained reference serotype A isolates.

DISCUSSION

To better understand the genetic relationship between serotypes A and D of *C. neoformans* var. *neoformans*, we have determined the CNRE-1 RFLP profiles and the nucleotide sequences of the *URA5* genes from a set of reference strains. The CNRE-1 RFLP profiles of serotype A strains were more complex than those of serotype D strains, with average band numbers of 14 ± 2.1 and 8.2 ± 2.4 bands, respectively. Spitzer and Spitzer (29) also observed that CNRE-1 hybridized less intensely and to fewer bands for a serotype D isolate. In our study the CNRE-1 RFLP patterns for serotype D strains were also less intense than those for serotype A strains. This difference in intensity most likely represents a quantitative difference in CNRE-1 copy number in the genome and suggests that serotype D isolates contain fewer repetitive elements within one restriction fragment.

Genetic differences between serotypes A and D were also demonstrated by the nucleotide sequence of the *URA5* gene. Within a serotype, the base replacements occurred mostly at the same nucleotide position. The only exceptions were isolates J21 and J22, which showed a few substitutions (5 of 22) that were more typical of serotype A isolates than D isolates (Fig. 3). The RFLP patterns of strains J21 and J22 also contained more bands than those of the other serotype D isolates tested (data not shown). Interestingly, these strains are classified as serotype D, but it was later shown for isolate J22 that it has a novel GXM triad structure not found in serotype A or D isolates (8). Studies with a MAb that discriminates between serotype A and D isolates on the basis of the fluorescence pattern revealed that strain J22 is like serotype A isolates (9). Hence, strain J22 appears to be an unusual strain with certain unique characteristics.

Overall, our results indicate that serotypes A and D segregated into two groups which appear to be phylogenetically distant from each other, as demonstrated by the independent clusters observed in the trees generated from RFLP and sequencing data. Our findings are in agreement with those of Guého et al. (17), who also found a relative phylogenetic difference between serotypes A and D on the basis of the sequence of the 26S large subunit of rRNA. We were also able to detect phylogenetic distance between serotypes A and D by investigating a gene that has been shown to be highly polymorphic among cryptococcal isolates (5, 6). In addition, a comparison between the reference isolates and those from Brazil and New York suggested that the observed phylogenetic distance of serotype A and serotype D isolates is independent of the geographic background of the isolate.

Analysis of several commonly used *C. neoformans* laboratory strains revealed significant phylogenetic differences. Among the reference serotype A strains, strains H99 and 145 and strains 24064 and 184 were most closely related by both *URA5* sequence and CNRE-1 RFLPs. Nevertheless, each of the isolates was distinguishable by DNA typing, suggesting the possibility of different biological traits. Considering the existence of significant genetic differences among isolates classified within a serotype and among reference strains, one should be cautious in generalizing conclusions on the basis of data obtained for one strain. Our results suggest that it may be prudent to include both serotype A and D strains when conducting biological studies with *C. neoformans*.

The taxonomy of *C. neoformans* and its teleomorph, *Filobasidiella neoformans*, has been the subject of several studies. Initially, two different species were recognized: *C. neoformans* (serotypes A and D) and its sexual state, *F. neoformans* (20), and *C. bacillispora* (serotypes B and C) and its teleomorph, *F. bacillispora* (21). Further taxonomic studies with *Cryptococcus* species and their teleomorphs reclassified these two species into *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* on the basis of successful interspecific crosses and DNA relatedness (1, 22). Although the current classification of *C. neoformans* remains at the varietal status, more recent studies have brought into question this proposed scheme. Boekhout et al. (3) demonstrated that *C. neoformans* var. *neoformans* and

C. neoformans var. *gattii* differ in their genetic makeups and may represent separate species. Genetic differences between serotypes A and D, as described in this report and by others (see the introduction), provide additional evidence that the taxonomy of *C. neoformans* may need to be reinvestigated. Consistent with this, phenotypic differences between serotypes A and D have also been reported. These differences include the chemical structure of the capsular polysaccharide (7, 19) and qualitative and quantitative antigenic dissimilarities among serotype A and D strains (9). In one study, it was suggested that serotypes D and A may not have the same virulence, tropism, and/or ecological niche (13).

Recently, we have proposed that the population structure of *C. neoformans* is clonal on the basis of analysis of *URA5* sequences, electrophoretic karyotypes, and CNRE-1 RFLPs (16). The results of this study are consistent with that proposal and extend it by suggesting the divergence of serotype A and D strains along different lineages. *C. neoformans* mating types **a** and α have been described for serotype D strains, but no serotype A **a** strain has ever been recovered from clinical or environmental sources. *C. neoformans* strains which are efficient mating pairs have all been serotype D. The divergence of serotype A and D lineages combined with a high mating efficiency restricted to serotype D suggests that future population structure studies should consider strains of serotype A and D separately.

In summary, our findings revealed genetic unrelatedness between serotypes A and D of *C. neoformans* and indicate that these serotypes could probably be considered different varieties of *C. neoformans*. Our results suggest that *C. neoformans* var. *gattii* and *C. neoformans* var. *neoformans* could be regarded as separate species, as originally proposed, and serotypes A and D and serotypes B and C would then represent different varieties of each species. Additional genetic studies will be necessary to further define the exact genetic relationship between the serotypes of *C. neoformans*, including those belonging to serotypes B and C.

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