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Cryptococcus neoformans var. *grubii*: Separate Varietal Status for *Cryptococcus neoformans* Serotype A Isolates

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***Cryptococcus neoformans* var. *neoformans* presently includes isolates which have been determined by the immunologic reactivity of their capsular polysaccharides to be serotype A and those which have been determined to be serotype D. However, recent analyses of the *URA5* sequences and DNA fingerprinting patterns suggest significant genetic differences between the two serotypes. Therefore, we propose to recognize these genotypic distinctions, as well as previously reported phenotypic differences, by restricting *C. neoformans* var. *neoformans* to isolates which are serotype D and describing a new variety, *C. neoformans* var. *grubii*, for serotype A isolates.**

Mycology has traditionally relied upon phenotypic features, e.g., morphology, biochemistry, and the presence and nature of reproductive structures, to evaluate the relationship of fungi and to determine the appropriate identification of isolates (7, 22). However, such characteristics may show wide variation within species and may change as isolates are maintained in culture (17). Therefore, molecular techniques such as DNA fingerprinting, DNA sequence analysis, electrophoretic karotyping, and others have gained increasing importance in clarifying the taxonomy of several fungal genera and species (22, 23). In the present study we have used molecular tools to re-evaluate the taxonomic status of *Cryptococcus neoformans* var. *neoformans*.

C. neoformans (Sanfelice) Vuillemin is a heterothallic yeast frequently associated with systemic infections in immunocompromised patients, especially those with AIDS (6, 30). Isolates of the fungus have been divided into four distinct serotypes, A, B, C, and D, on the basis of the immunologic properties of their capsular polysaccharides (15, 35). Subsequent studies proposed that the serotypes be grouped into two separate varieties; serotypes A and D were designated as *C. neoformans* var. *neoformans*, while serotypes B and C were designated as *Cryptococcus neoformans* var. *gattii* Vanbreuseghem et Takashio (28, 33). A number of biochemical, epidemiological, and molecular characteristics have been used to distinguish these varieties, including their assimilation of L-malic acid (3) and D-proline (14), creatinine metabolism (26), and electrophoretic karyotypes (4, 34). While the taxonomic status of the two varieties has been generally accepted by mycologists, recent molecular typing of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* has suggested that they may be diverging into separate species (4).

While several investigators have focused on differences between the two varieties of *C. neoformans*, molecular analyses conducted by two of the present authors have demonstrated significant genotypic differences between the serotypes which comprise *C. neoformans* var. *neoformans* (16). The first evidence of such genetic division was obtained from fingerprint analyses with a *C. neoformans* repetitive element 1 (CNRE-1) probe, which hybridizes with moderately repetitive DNA sequences dispersed throughout the yeast's genome (32). The hybridization of CNRE-1 to *Sac*I-digested DNA from A and D isolates resulted in such distinct restriction fragment length polymorphism (RFLP) patterns that they separated into two independent clusters in a dendrogram generated by distance analysis. The CNRE-1 probe hybridized effectively with DNA recovered from serotype A isolates and yielded 11 to 16 bands of various intensities. In contrast, the same probe hybridized poorly with DNA from D serotypes and yielded 5 to 11 bands, which were generally less intense than those obtained with serotype A isolates.

The second indication of molecular differences between the two serotypes was obtained through analyses of the nucleotide sequence of the *URA5* gene. The *URA5* gene of each of six isolates of serotypes A and D was amplified by PCR, and the nucleotide sequence of the cloned gene was determined for both strands (submitted to the GenBank database [16]). The nucleotide sequences of the *URA5* gene of serotype A isolates differed from each other by an average of 3.0 ± 1.7 bases (15 pairwise comparisons). Alternatively, the number of nucleotide differences among D serotype isolates averaged 7.2 ± 3.4 ($n = 15$). The number of *URA5* base differences obtained by pairwise comparisons between serotype A and D isolates was 41.9 ± 2.7 ($n = 30$). When bootstrap analyses were performed on phylogenetic trees based on these sequence data, the test isolates of serotype A and D clustered into two distinct and separate groups in 100% of the trees generated (16).

These findings are consistent with those reported by Guého et al. (18), who examined the phylogenetic relationships of *C. neoformans* by comparing the partial sequences of the most variable domain (D2) of the large subunit rRNA. These workers reported that B and C serotypes had identical sequences but the D2 sequences of A and D serotypes were significantly different. In fact, Guého and coworkers found that the sequence of this segment of the large subunit rRNA of D serotype isolates was closer to the corresponding sequences in B and C serotypes than to those of A serotype test strains.

While A and D serotypes are genetically dissimilar from each other, they are members of the same species. For exam-

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TABLE 1. Differences among the three varieties of *C. neoformans*[a]

Characteristic	*C. neoformans* var. *grubii*	*C. neoformans* var. *neoformans*	*C. neoformans* var. *gattii*	Reference(s)
Serotype(s)	A	D	B, C	2, 6, 15, 35
Main geographic distribution	Worldwide	Northern Europe	Tropical and subtropical	2, 6, 12
Mating type(s) isolate	α	**a** and α	**a** and α	6, 24, 25, 29
Perfect state	*F. neoformans* var. *neoformans*	*F. neoformans* var. *neoformans*	*F. neoformans* var *bacillispora*	24, 25, 28
Factor serum reactivity	1, 2, 3, 7	1, 2, 3, 8	1, 2, 4, 5 (serotype B); 1, 4, 6 (serotype C)	19
Reactivity with MAb 13F1	Annular	Punctate	NA	10
CGB medium	No growth	No growth	Growth	27
GCP medium	No growth	No growth	Growth	31
CDBT medium (thymine assimilation)	No growth	Growth (orange color change)	Growth (blue-green color change)	21
Hybridization to CNRE-1	11–16 bands	5–11 bands	Very weak	16, 32
Average chromosome number	121	12.8	13	34
MEE pattern	ET1–ET4	ET8–ET12	ET13–ET19	5
Dermatotropism	Frequent	Less frequent	Not determined	13

[a] Abbreviations: NA, not available; CGB, canavanine-glycine-bromothymol blue; GCP, glycine-cycloheximide-phenol red; CDBT, creatinine-dextrose-bromothymol blue-thymine; MEE, multilocus enzyme electrophoresis; ET, electrophoretic type.

ple, Aulakh et al. (1) conducted reassociation studies of the DNAs from these two serotypes and reported 88 to 94% relatedness. Kwon-Chung (24) demonstrated that serotype A and D isolates are able to mate with each other and produce fertile progeny, i.e., the teleomorphic, basidiomycetous yeast *Filobasidiella neoformans* Kwon-Chung var. *neoformans*.

Despite the significant genetic differences, isolates of A and D serotype can be distinguished by only a few phenotypic characteristics (Table 1). For example, the two serotypes differ in their ability to assimilate thymine (21). When isolates of serotype D are grown on a medium containing creatinine, dextrose, bromothymol blue, and thymine, they convert the pyrimidine to β-isobutyric acid. In contrast, serotype A isolates cannot utilize thymine, and colonies remain cream colored when grown on the same medium (21). Additionally, there are the antigenic properties of the polysaccharide capsule resulting from differences in the extent of xylose present in the glucuronoxylomannan capsular backbone (8). However, serotyping of isolates is an involved procedure not readily performed in the average clinical microbiology laboratory. Then, too, Cleare and Casadevall recently observed an annular pattern of immunofluorescence binding to the immunoglobulin M monoclonal antibody (MAb) 13F1 with serotype A isolates but a punctate pattern with serotype D isolates (10). While the differentiation of *C. neoformans* isolates on the basis of their serotypes is widely accepted, Cherniak et al. recently demonstrated that the glucuronoxylomannan structure and serotype of isolates recovered from clinical specimens can change during the course of infection (9). Therefore, the serotype of an isolate may not be as stable a phenotypic character as had previously been perceived.

Although relatively few serotype-specific serological reagents are available, several methods for serotype discrimination have been described (reviewed in reference 6). Ikeda et al. (20) developed commercially available serological reagents which discriminate the serotypes based on the presence of one or more of eight antigenic factors (Table 1). MAbs that react with specific serotypes have also been generated. Dromer et al. (11) developed MAb E1, which reacts primarily with serotype A isolates, while Ikeda et al. (19) isolated a MAb specific for serotype D strains. As mentioned above, the differential binding of MAb 13F1 to the capsular material of serotype A and D strains indicates that it can be used as another reagent for serotype discrimination (10).

There are also epidemiological and clinical differences between the two serotypes. While the overwhelming majority of isolates recovered from AIDS patients throughout the world are serotype A, infections due to serotype D in such individuals are more prevalent in isolated geographic areas, especially France, Italy, and Denmark (2, 12). Moreover, infections due to serotype D are more strongly correlated than those caused by serotype A to older patients, the skin, and the use of corticosteroids (13). Finally, despite the fact that the teleomorph *F. neoformans* is considered to be a heterothallic, basidiomycetous yeast composed of two mating types, **a** and α, serotype A isolates recovered from clinical cases and the environment have all proven to be α mating types. Observation of both mating types and successful mating pairs has been restricted to serotype D isolates.

Therefore, we propose on the basis of this phenotypic and genotypic evidence to separate serotype A and D isolates into distinct varieties of the same species. Since the original description of *C. neoformans* var. *neoformans* was based on a D serotype (24), we suggest retaining this variety for isolates of this serotype. We propose a new variety, *Cryptococcus neoformans* var. *grubii*, to include only A serotypes. The descriptions of these two varieties are as follows.

Cryptococcus neoformans (Sanfelice) Vuillemin var. *neoformans* emend. Franzot, Salkin, et Casadevall. The same characteristics as those previously described for *Cryptococcus neoformans* var. *neoformans*, except that this emendation restricts the variety to D serotypes of the anamorphic species as determined by the reactivity of their capsular polysaccharide with rabbit sera. The variety may be further distinguished by the RFLP patterns of 5 to 11 bands of limited intensity obtained with the CNRE-1 probe of *Sac*I-digested genomic DNA. Additionally, the variety is characterized by 7.2 ± 3.4 nucleotide differences in the *URA5* gene as determined through PCR amplification and sequencing of the cloned gene. Teleomorph: *Filobasidiella neoformans* var. *neoformans*. Holotype: Isolated from peach juice and deposited in Centraalbureau voor Schimmelcultures as CBS 132.

Cryptococcus neoformans var. *grubii* Franzot, Salkin, et Casadevall, var. nov. Anamorphus ut in *Cryptococcus neoformans* var. *neoformans* simili sed ab hac varietate differt: capsulae "polysaccharide" serotypum A vocatum continentes; RFLP ordinatione 11–16 fasciarum ab exemplo CNRE-1 vocato, secundum concoctionem DNA enzymato *Sac*I vocato; gens *URA5*

vocata 3.0 ± 1.7 "nucleotide" diversitate secundum PCR et DNA seriem praebens. Teleomorphys est *Filobasidiella neoforans* var. *neoformans*. Holotypus: In fluido ossis posterioris ex homo sapiens morbo Hodgkin's vocato lectus est, apud Durham, in Durham comitatu North Carolinsium finium, in imperio United States CSF cultura 898, 15 February 1978, e DUMC 135.97, cultura H99, NYSD 1649. In Museo Noveboracensis finibus herbario.

The same characteristics as those previously described for *Cryptococcus neoformans* var. *neoformans*, except that *Cryptococcus neoformans* var. *grubii* is restricted to "A" serotypes of the anamorphic species as determined by the reactivity of their capsular polysaccharide with rabbit sera. The variety may be further distinguished by the RFLP patterns of 11 to 16 bands of variable intensity obtained with the CNRE-1 probe of *Sac*I-digested genomic DNA. Additionally, the variety is characterized by 3.0 ± 1.7 nucleotide differences in the *URA5* gene as determined through PCR amplification and sequencing of the cloned gene. Teleomorph: *Filobasidiella neoformans* var. *neoformans*. Holotype: Isolate H99 recovered from Hodgkin's disease patient and deposited under accession no. NYSD 1649, New York State Herbarium, Albany, N.Y. Etymology: The specific epithet *grubii* was selected to honor David Gruby, 19th-century scholar, physician, and scientist, who was the first to recognize and prove that dermatophytic infections are caused by fungi.

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REFERENCES

1. **Aulakh, H. S., S. E. Straus, and K. J. Kwon-Chung.** 1981. Genetic relatedness of *Filobasidiella neoformans* (*Cryptococcus neoformans*) and *Filobasidiella bacillispora* (*Cryptococcus bacillispora*) as determined by DNA base composition and sequence homology studies. Int. J. Syst. Bacteriol. **31:**97–103.
2. **Bennett, J. E., K. J. Kwon-Chung, and D. Howard.** 1984. Epidemiologic differences among serotypes of *Cryptococcus neoformans*. Am. J. Epidemiol. **105:**582–586.
3. **Bennett, J. E., K. J. Kwon-Chung, and T. S. Theodore.** 1978. Biochemical differences between serotypes of *Cryptococcus neoformans*. Sabouraudia **16:** 167–174.
4. **Boekhout, T., A. van Belkum, A. C. Leenders, H. A. Verbrugh, P. Mukamurangwa, D. Swinne, and W. A. Scheffers.** 1997. Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. Int. J. Syst. Bacteriol. **47:**432–442.
5. **Brandt, M. E., S. L. Bragg, and R. W. Pinner.** 1993. Multilocus enzyme typing of *Cryptococcus neoformans*. J. Clin. Microbiol. **31:**2819–2823.
6. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans*. ASM Press, Washington, D.C.
7. **Check, W. A.** 1994. Molecular techniques shed light on fungal genetics. ASM News **60:**593–596.
8. **Cherniak, R., and J. B. Sundstrom.** 1994. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. Infect. Immun. **62:**1507–1512.
9. **Cherniak, R., L. C. Morris, T. Belay, E. D. Spitzer, and A. Casadevall.** 1995. Variation in the structure of glucuronoxylomannan in isolates from patients with recurrent cryptococcal meningitis. Infect. Immun. **63:**1899–1905.
10. **Cleare, W., and A. Casadevall.** 1998. The different binding patterns of two immunoglobulin M monoclonal antibodies to *Cryptococcus neoformans* serotype A and D strains correlate with serotype classification and differences in functional assays. Clin. Diagn. Lab. Immunol. **5:**125–129.
11. **Dromer, F., E. Guého, O. Ronin, and B. Dupont.** 1993. Serotyping of *Cryptococcus neoformans* by using a monoclonal antibody specific for capsular polysaccharide. J. Clin. Microbiol. **31:**359–363.
12. **Dromer, F., S. Mathoulin, B. Dupont, A. Laporte, and the French Cryptococcosis Study Group.** 1996. Epidemiology of cryptococcosis in France: a 9-year survey (1985–1993). Clin. Infect. Dis. **23:**82–90.
13. **Dromer, F., S. Mathoulin, B. Dupont, L. Letenneur, O. Ronin, and the French Cryptococcosis Study Group.** 1996. Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. Clin. Infect. Dis. **23:**91–96.
14. **Dufait, R., R. Velhol, and C. De Vroey.** 1987. Rapid identification of the two varieties of *Cryptococcus neoformans* by D-proline assimilation. Mykosen **30:**483.
15. **Evans, E. E.** 1950. The antigenic composition of *Cryptococcus neoformans*. I. A serologic classification by means of the capsular and agglutination reactions. J. Immunol. **64:**423–430.
16. **Franzot, S. P., B. C. Fries, W. Cleare, and A. Casadevall.** 1998. Genetic relationship between *Cryptococcus neoformans* strains of serotypes A and D. J. Clin. Microbiol. **36:**2200–2204.
17. **Franzot, S. P., J. Mukherjee, R. Cherniak, L. C. Chen, J. S. Hamdan, and A. Casadevall.** 1998. Microevolution of *Cryptococcus neoformans* in various laboratory environments resulting in differences in virulence and other phenotypes. Infect. Immun. **66:**89–97.
18. **Guého, E., L. Improvisi, R. Christen, and G. S. de Hoog.** 1993. Phylogenetic relationships of *Cryptococcus neoformans* and some related basidiomycetous yeasts determined from partial large subunit rRNA sequences. Antonie van Leeuwenhoek **63:**175–189.
19. **Ikeda, R., S. Nishimura, A. Nishikawa, and T. Shinida.** 1996. Production of agglutinating monoclonal antibody against antigen 8 specific for *Cryptococcus neoformans* serotype D. Clin. Diagn. Lab. Immunol. **3:**89–92.
20. **Ikeda, R., T. Shinoda, Y. Fukuzawa, and L. Kaufman.** 1982. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. J. Clin. Microbiol. **16:**22–29.
21. **Irokanulo, E. A. O., C. O. Akueshi, and A. A. Makinde.** 1994. Differentiation of *Cryptococcus neoformans* serotypes A and D using creatinine dextrose bromothymol blue thymine medium. Br. J. Biomed. Sci. **51:**100–103.
22. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35:**1216–1223.
23. **Kurtzman, C. P., and H. J. Phaff.** 1987. Molecular taxonomy of yeasts, p. 63–94. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, 2nd ed., vol. 1. Academic Press, New York, N.Y.
24. **Kwon-Chung, K. J.** 1975. A new genus *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia **67:**1197–1200.
25. **Kwon-Chung, K. J.** 1976. A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. Mycologia **68:**942–946.
26. **Kwon-Chung, K. J.** 1991. The discovery of creatinine assimilation in *Cryptococcus neoformans*, and subsequent work on the characterization of the two varieties of *C. neoformans*. Zentralbl. Bakteriol. **275:**390–393.
27. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J. Clin. Microbiol. **15:**535–537.
28. **Kwon-Chung, K. J., J. E. Bennett, and J. C. Rhodes.** 1982. Taxonomic studies on *Filobasidiella* species and their anamorphs. Antonie van Leeuwenhoek **48:**25–38.
29. **Madrenys, N., C. De Vroey, C. Raes-Wuytack, and J. M. Torres-Rodriguez.** 1993. Identification of the perfect state of *Cryptococcus neoformans* from 195 clinical isolates including 84 from AIDS patients. Mycopathologia **123:**65–68.
30. **Mitchell, T. G., and J. R. Perfect.** 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin. Microbiol. Rev. **8:**515–548.
31. **Salkin, I. F., and N. J. Hurd.** 1982. New medium for differentiation of *Cryptococcus neoformans* serotype pairs. J. Clin. Microbiol. **15:**169–171.
32. **Spitzer, E. D., and S. G. Spitzer.** 1992. Use of a dispersed repetitive DNA element to distinguish clinical isolates of *Cryptococcus neoformans*. J. Clin. Microbiol. **30:**1094–1097.
33. **Vanbreuseghem, R., and M. Takashio.** 1970. An atypical strain of *Cryptococcus neoformans* (San Felice) Vuillemin 1894. Part II. *Cryptococcus neoformans* var. *gattii* var. nov. Ann. Soc. Belge Med. Trop. **50:**695–702.
34. **Wickes, B. L., T. D. E. Moore, and K. J. Kwon-Chung.** 1994. Comparison of the electrophoretic karyotypes and chromosomal location of ten genes in the two varieties of *Cryptococcus neoformans*. Microbiology **140:**543–550.
35. **Wilson, D. E., J. E. Bennett, and J. W. Bailey.** 1968. Serologic grouping of *Cryptococcus neoformans*. Proc. Soc. Exp. Biol. Med. **127:**820–823.