JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2003, p. 5572–5576 Vol. 41, No. 12
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.12.5572–5576.2003

Molecular Analysis of *Cryptococcus neoformans* Mitochondrial Cytochrome *b* Gene Sequences

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Received 13 May 2003/Returned for modification 4 July 2003/Accepted 20 September 2003

Mitochondrial cytochrome *b* genes (*cyt b*) of 40 strains of *Cryptococcus neoformans* were partially sequenced to determine the genetic relations. With the exception of the type strain of *C. neoformans* var. *neoformans*, all strains contained introns in their sequences. Analysis of 386 bp of coding sequence from each strain under investigation revealed a total of 27 (6.99%) variable nucleotide sites and categorized isolates of *C. neoformans* into nine *cyt b* types. *C. neoformans* var. *gattii* included *cyt b* types I to V, and *C. neoformans* var. *neoformans* comprised types VI to IX. *cyt b* types were correlated with serotypes. All strains with *cyt b* types I, IV, and V were serotype B. All other strains except IFM 5878 (serotype B) with *cyt b* types II and III were serotype C. Serotype D strains had *cyt b* types VI and IX, and serotype A strains were *cyt b* type VIII. Of four serotype AD strains, one was *cyt b* type VII and the remaining three were type VIII. The phylogenetic tree based on deduced amino acid sequences divided the strains only into *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. These results indicate that *cyt b* sequences are effective for DNA typing as well as phylogenetic analysis of *C. neoformans*.

Cryptococcus neoformans is an encapsulated basidiomycetous yeast and is the causative agent of cryptococcosis. The incidence of cryptococcosis, which was formerly a relatively rare disease, has increased markedly in recent years because of the increases in the numbers of AIDS patients and organ transplantation patients. Meningitis and, to a lesser extent, pneumonia are the most frequent life-threatening manifestations of cryptococcosis (6, 15, 18, 21).

Isolates of *C. neoformans* from patients with cryptococcosis have been divided into five serotypes, A, B, C, D, and AD, on the basis of the immunologic properties of the capsular polysaccharides (9, 15, 16, 20). These five serotypes have been grouped into two separate varieties: *C. neoformans* var. *neoformans* (serotypes A, D, and AD) and *C. neoformans* var. *gattii* (serotypes B and C) (11–13, 23).

The issue of *C. neoformans* nomenclature is still unsettled. Franzot et al. (7) proposed that *C. neoformans* var. *neoformans* strains be subdivided into two varieties, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), on the basis of sequence fingerprint data for the *C. neoformans* repetitive element 1 and nucleotide sequence analyses of the *URA5* gene. Sequence analysis of the *CAP59* gene (19) revealed a phylogenetic separation between serotypes A and D. The two serotypes can also be differentiated by analysis of mating type (*MAT*) genes, *MAT*α and *MAT***a** (3). However, analysis of the D1/D2 region of the large-subunit ribosomal DNA (rDNA), which is widely used for phylogenetic analysis, revealed that the sequences of serotype A (CBS 132; DDBJ/EMBL/GenBank accession no. AF075484) and serotype D (CBS 882; DDBJ/EMBL/GenBank accession no. AF189845) strains were identical. There is only one nucleotide difference between the D1/D2 sequences of *Fidobasidiella neoformans* var. *neoformans* and *F. neoformans* var. *bacillispora* (5). Analysis of the sequence of the intergenic spacer associated with rDNA suggested that *C. neoformans* var. *grubii* (serotype A) should not be considered a separate variety and instead that *Cryptococcus* isolates should be considered two separate species, *C. neoformans* (serotypes A, D, and AD) and *Cryptococcus bacillisporus* (serotypes B and C, synonymous with *C. neoformans* var. *gattii*) (4).

Mitochondrial (mt) genes are an attractive marker for inferring phylogeny of closely related species because of the rapid evolution of the mt genome, the lack of recombination, and the strict maternal inheritance (17). Restriction fragment length polymorphism (RFLP) analysis of mtDNA has been shown to be useful for estimating the relations between fungi (8, 10, 24). Xu et al. (29) showed that the origin of mitochondria in *C. neoformans* is uniparental. RFLP analysis of the mt large rRNA gene and NADH dehydrogenase subunit 2 gene allowed efficient screening of the mtDNA of *C. neoformans* (28).

We have reported that the mt cytochrome *b* gene (*cyt b*) is useful for identification, classification, and phylogenetic analysis of fungi (25–27, 31). We have also shown that mt *cyt b* sequences are effective for identifying and studying the phylogenetic relations of closely related yeasts such as *Candida albicans* and *Candida dubliniensis* (30). *cyt b* is useful for typing isolates of *Candida albicans* and differentiating such isolates from *Candida stellatoidea* (1). We have also shown that the *cyt b* phylogeny of basidiomycetous yeasts correlates with cell wall biochemistry and septal ultrastructure (2). However, similar techniques have not been used to characterize *C. neoformans*.

In the present study, mt *cyt b* genes of 40 strains of *C. neoformans* (15 strains of *C. neoformans* var. *gattii* and 25

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TABLE 1. *C. neoformans* strains used in this study

Variety	IFM no.	Serotype	DNA type	AA[a] type	Exon position(s) (nt)	Source	Accession no.
C. neoformans var. *gattii*	5815	B	I	I	1–46, 461–800	Patient	AB105913
C. neoformans var. *gattii*	48634	B	I	I	1–46, 461–800	CBS 6289	AB105914
C. neoformans var. *gattii*	48636	B	I	I	1–46, 454–793	CBS 7229	AB105915
C. neoformans var. *gattii*	48819	B	I	I	1–46, 461–800	CBS 6998	AB105916
C. neoformans var. *gattii*	48820	B	I	I	1–46, 461–800	CBS 8273	AB105917
C. neoformans var. *gattii*	48821	B	I	I	1–46, 454–793	CBS 7749	AB105918
C. neoformans var. *gattii*	5856	C	II	I	1–46, 317–632, 1148–1171	NIH 18	AB105919
C. neoformans var. *gattii*	5873	C	II	I	1–46, 317–632, 1148–1171	Patient	AB105920
C. neoformans var. *gattii*	5875	C	II	I	1–46, 317–632, 1148–1171	Patient	AB105921
C. neoformans var. *gattii*	5878	B	II	I	1–46, 317–632, 1121–1144	Patient	AB105922
C. neoformans var. *gattii*	48635	C	III	I	1–46, 317–632, 1162–1185	CBS 6955^T	AB105923
C. neoformans var. *gattii*	5855	B	IV	I	1–46, 317–632, 1162–1185	NIH 112	AB105924
C. neoformans var. *gattii*	47258	B	V	I	1–46, 1175–1514	Patient	AB105925
C. neoformans var. *gattii*	48818	B	V	I	1–46, 1175–1514	CBS 6956	AB105926
C. neoformans var. *gattii*	48822	B	V	I	1–46, 1175–1514	CBS 7750	AB105927
C. neoformans var. *neoformans*	5844	D	VI	II	1–46, 1190–1529	Patient	AB105928
C. neoformans var. *neoformans*	5845	D	VI	II	1–46, 1190–1529	Patient	AB105929
C. neoformans var. *neoformans*	5857	D	VI	II	1–46, 1190–1529	NIH 52	AB105930
C. neoformans var. *neoformans*	5881	D	VI	II	1–46, 1190–1529	Patient	AB105931
C. neoformans var. *neoformans*	46082	D	VI	II	1–46, 1190–1529	Patient	AB105932
C. neoformans var. *neoformans*	46090	D	VI	II	1–46, 1190–1529	Patient	AB105933
C. neoformans var. *neoformans*	48640	D	VI	II	1–46, 1190–1529	CBS 6901	AB105934
C. neoformans var. *neoformans*	48641	D[b]	VI	II	1–46, 1190–1529	CBS 6995	AB105935
C. neoformans var. *neoformans*	48642	D	VI	II	1–46, 1190–1529	CBS 7697	AB105936
C. neoformans var. *neoformans*	48643	D	VI	II	1–46, 1190–1529	CBS 7698	AB105937
C. neoformans var. *neoformans*	5889	AD	VII	II	1–46, 1190–1529	Patient	AB105938
C. neoformans var. *neoformans*	5505	A	VIII	II	1–46, 1189–1528	Patient	AB105939
C. neoformans var. *neoformans*	5506	A	VIII	II	1–46, 1189–1528	Patient	AB105940
C. neoformans var. *neoformans*	5854	A	VIII	II	1–46, 1189–1528	CDC 551	AB105941
C. neoformans var. *neoformans*	45708	A	VIII	II	1–46, 1189–1528	Patient	AB105942
C. neoformans var. *neoformans*	45737	AD	VIII	II	1–46, 1189–1528	Pigeon droppings	AB105943
C. neoformans var. *neoformans*	45756	AD	VIII	II	1–46, 1189–1528	Pigeon droppings	AB105944
C. neoformans var. *neoformans*	46132	AD	VIII	II	1–46, 1189–1528	Pigeon droppings	AB105945
C. neoformans var. *neoformans*	46554	A	VIII	II	1–46, 1189–1528	Patient	AB105946
C. neoformans var. *neoformans*	46572	A	VIII	II	1–46, 1189–1528	Patient	AB105947
C. neoformans var. *neoformans*	46729	A	VIII	II	1–46, 1189–1528	Pigeon droppings	AB105948
C. neoformans var. *neoformans*	46734	A	VIII	II	1–46, 1189–1528	Pigeon droppings	AB105949
C. neoformans var. *neoformans*	48638	A	VIII	II	1–46, 1189–1528	CBS 996	AB105950
C. neoformans var. *neoformans*	48639	A	VIII	II	1–46, 1189–1528	CBS 5756	AB105951
C. neoformans var. *neoformans*	48637	D	IX	II	1–386	CBS 132^T	AB040655[c]

[a] AA, amino acid.
[b] IFM 48641 is regarded as a serotype A strain in the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands), but repeated checking in our laboratory showed serotype D.
[c] Biswas et al. (2).

strains of *C. neoformans* var. *neoformans*) were analyzed to determine the genetic relations. Isolates of *C. neoformans* were divided into nine *cyt b* types; however, the deduced amino acid sequences suggested the existence of only two varieties: *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*.

MATERIALS AND METHODS

***C. neoformans* strains and serotyping.** The *C. neoformans* strains, both environmental and clinical isolates, and the reference cultures used in this study are listed in Table 1. Cultures were grown on YPD (1% [wt/vol] yeast extract, 2% [wt/vol] polypeptone, 2% [wt/vol] glucose) slants. The serotypes of the clinical and environmental strains were determined by slide agglutination tests (Crypto Check; Iatron Laboratories, Inc., Tokyo, Japan).

Isolation of DNA. One loopful of cells from each YPD slant was suspended in 1 ml of sterile distilled water and used for extraction of total cellular DNA with the Gen Toru Kun kit (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) as described previously (30).

PCR primers and amplification of the *cyt b* gene. PCR primers E1M4 (5′-TGRGGWGCWACWGTTATTACTA-3′) and E2M4 (5′-GGWATAGMWSKTAAWAYAGCATA-3′) (R, A or G; W, A or T; M, A or C; S, C or G; K, G or T; Y, C or T) were designed as described previously (25). One microliter of extracted DNA was used as the template for amplification of the mt *cyt b* with a TaKaRa Ex *Taq* PCR amplification kit (Takara Shuzo). Reactions were performed in a final reaction volume of 50 μl containing 10 pmol of each primer, 4 μl of 2.5 mM (each) deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.0 U of TaKaRa Ex *Taq* polymerase, and 5 μl of 10× reaction buffer (Takara Shuzo). Amplification conditions were 94°C for 2 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and extension for 1 min at 72°C, with a final extension at 72°C for 10 min.

Sequencing. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Both strands of PCR products were sequenced directly with an ABI Prism 377 or 310 DNA sequencer with a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Japan Co., Ltd., Tokyo, Japan). Amino acid sequences were deduced from the DNA sequences with the yeast mt genetic code.

Molecular phylogenetic analysis. With the exclusion of the portions of the sequences that included the primers, DNA and amino acid sequences were aligned with GENETYX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). This software also generated phylogenetic trees by the unweighted pair group method with arithmetic mean (UPGMA).

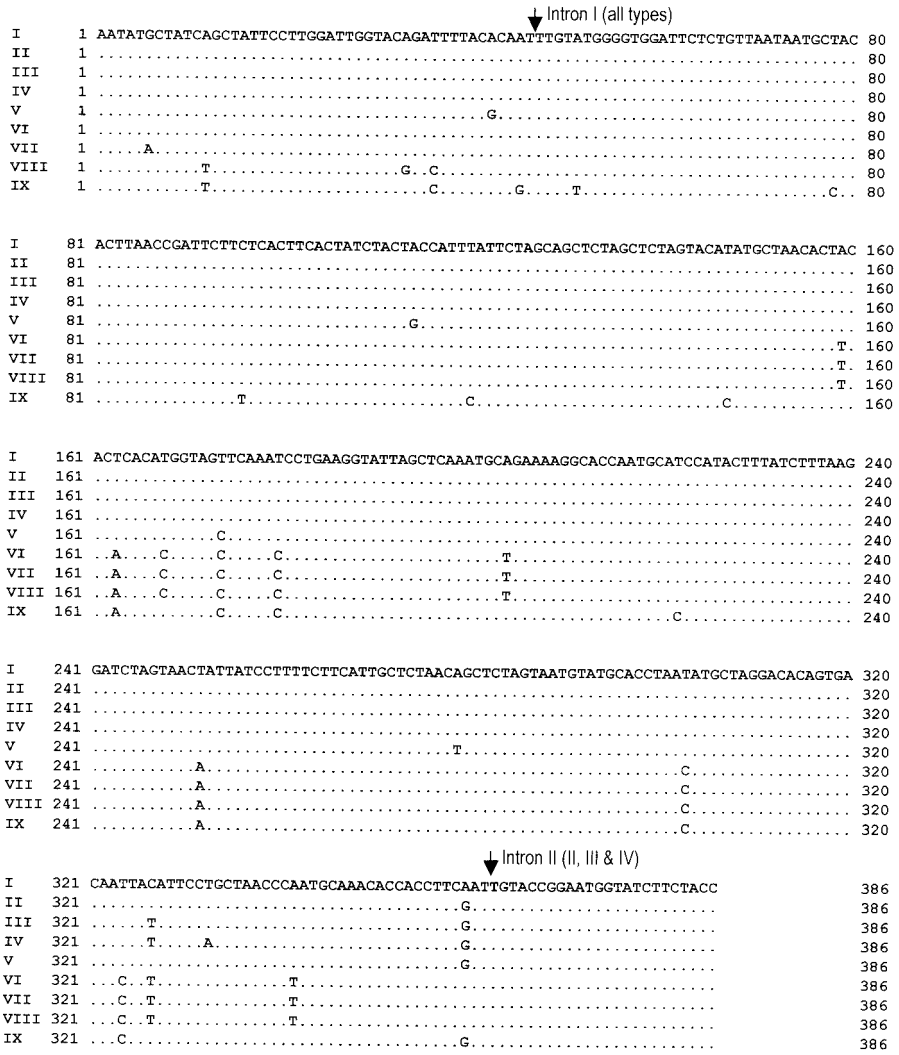

FIG. 1. Comparison of coding sequences of the mt *cyt b* genes of various *C. neoformans* isolates. Dots, nucleotides that are identical to those of *C. neoformans cyt b* type I; arrows, positions of introns. *Cyt b* types are in parentheses.

Nucleotide sequence accession numbers. Sequences of the *cyt b* genes of the *C. neoformans* strains sequenced in this study have been deposited in DDBJ/EMBL/GenBank under the accession numbers listed in Table 1.

RESULTS

The 386-bp fragment corresponding to nucleotides (nt) 445 to 830 of the *Candida glabrata cyt b* coding sequence (GenBank accession no. X53862) was analyzed in this study. The type strain of *C. neoformans* var. *neoformans*, IFM 48637, had no introns in this region of *cyt b*. However, all other strains investigated contained one intron that started after nt 46 of the sequence (Fig. 1); the size of this intron varied from 270 to 1,143 bp. Some isolates of *C. neoformans* var. *gattii* contained a second intron that began at nt 633 of the *cyt b* sequence (Table 1) or nt 363 of the coding sequence (Fig. 1). The size of the second intron varied from 515 to 529 bp. The sizes and locations of these introns were determined as described previously (2) through comparison of strains with and without introns to maximize amino acid identities. Isolates of *C. neoformans* var. *neoformans* contained the longest introns, which were 1,142 and 1,143 bp. Among isolates of *C. neoformans* var. *gattii*, IFM 47258, IFM 48818, and IFM 48822 contained introns similar in size (1,128 bp) to that of *C. neoformans* var. *neoformans* and with greater than 96% sequence identity. Although the intron sizes were variable, they were fixed for specific *cyt b* types (Table 1), with the exception of strains IFM 48636 and 48821 (*cyt b* type I) and IFM 5878 (*cyt b* type II).

Analysis of 386 bp of coding sequence of *cyt b* revealed 27 (6.99%) variable nucleotide sites (Fig. 1). To ensure that these variations were not due to polymerase errors, we used the TaKaRa Ex *Taq* polymerase, which has an approximately four-fold-lower error rate than standard *Taq* DNA polymerase. Moreover, we sequenced both strands of each PCR product and repeated each PCR and sequencing reaction. On the basis of these differences in *cyt b*, the *C. neoformans* strains we analyzed were divided into nine types: *C. neoformans* var. *gattii* comprising *cyt b* types I to V and *C. neoformans* var. *neoformans* comprising *cyt b* types VI to IX (Table 1 and Fig. 1). All

FIG. 2. Comparison of the deduced amino acid sequences encoded by the *cyt b* genes of various *C. neoformans* isolates. Dots, amino acids that are identical to those of *C. neoformans cyt b* type I; arrows, inserted positions of introns. *cyt b* types are in parentheses.

strains with *cyt b* types I, IV, and V were serotype B; all strains with *cyt b* types II and III except IFM 5878 (serotype B) were serotype C. Serotype D strains had *cyt b* types VI and IX, and serotype A strains were *cyt b* type VIII. Of four serotype AD strains, one was *cyt b* type VII and the remaining three were *cyt b* type VIII. Although the *cyt b* sequences contained 27 variable nucleotides (Fig. 1), the deduced amino acid sequences revealed that only one of these substitutions was nonsynonymous. *C. neoformans* var. *gattii* isolates, with *cyt b* types I to V, had identical amino acid sequences (type AA-I). Similarly, isolates of *C. neoformans* var. *neoformans*, with *cyt b* types VI to IX, had identical amino acid sequences (type AA-II). Therefore, isolates of *C. neoformans* var. *neoformans* differed from those of *C. neoformans* var. *gattii* only at amino acid position 55 (Thr instead of Ser) (Fig. 2).

The phylogenetic trees (UPGMA) generated from the mt *cyt b* DNA and amino acid sequences are shown in Fig. 3. The strains of *C. neoformans* were distributed according to nine DNA types. The type strain of *C. neoformans* var. *neoformans* (IFM 48637) was the outgroup in the phylogenetic tree. IFM 48637 contained the most variable nucleotide sites in *cyt b* (Fig. 1) and showed only a distant relation with other strains, even those of *C. neoformans* var. *neoformans*. Strains with *cyt b* type V (*C. neoformans* var. *gattii* IFM 47258, IFM 48818, and IFM 48822) contained introns similar in both length and content to that of *C. neoformans* var. *neoformans*, and they were phylogenetically closer to *C. neoformans* var. *neoformans*. Analysis of mt *cyt b* sequences suggested the existence of nine DNA types of *C. neoformans*; however, the phylogenetic tree based on the deduced amino acid sequences divided the strains into only two varieties: *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*.

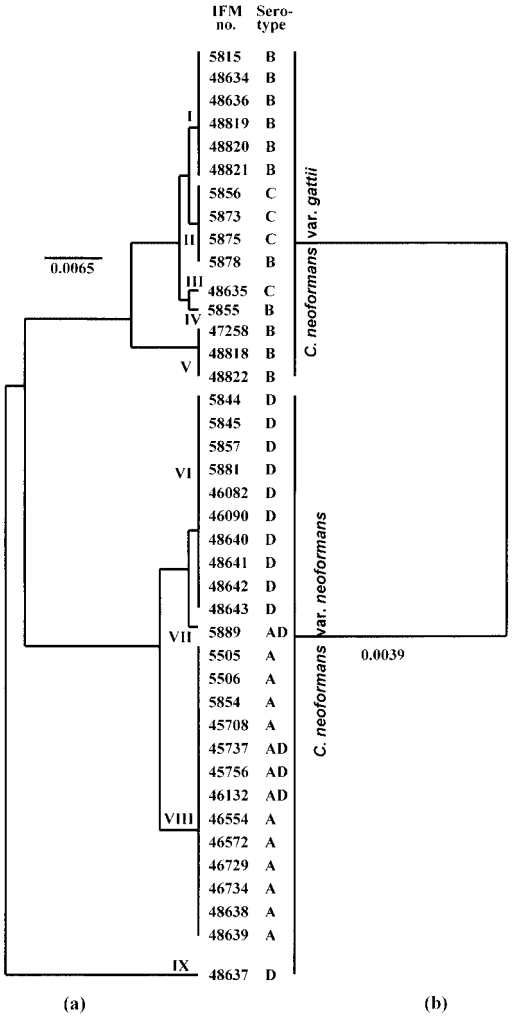

FIG. 3. UPGMA-based trees showing the relations of various *C. neoformans* isolates generated from nucleotide sequences of the *cyt b* gene (exon) (a) and deduced amino acid sequences (b). Bar, number of nucleotide and amino acid substitutions per nucleotide site and amino acid site.

DISCUSSION

To our knowledge this is the first study where mt *cyt b* sequences were used to analyze the genetic relations of strains of *C. neoformans*. Isolates of *C. neoformans* var. *gattii*, the anamorphic state of *F. neoformans* var. *bacillispora*, had five types (I to V) for mt *cyt b*; however, the deduced amino acid sequences were identical. Similarly, the amino acid sequences encoded by mt *cyt b* genes of isolates of *C. neoformans* var. *neoformans*, which is the anamorphic state of *F. neoformans* var. *neoformans*, were identical although there were four *cyt b* types (VI to IX). This result is consistent with the varieties of *C. neoformans* var. *neoformans* and var. *gattii* distinguished by D-proline assimilation or reaction on L-canavanine-glycine-bromthymol blue agar (11, 14).

The present study also revealed that one serotype D strain (*cyt b* type IX) had no intron in the region sequenced; however, all other strains of *C. neoformans* contained one intron, and some isolates of *C. neoformans* var. *gattii* contained a second intron (Table 1 and Fig. 2). A recent study of the mt *COX1* gene of *C. neoformans* indicated that the presence or absence of introns in *COX1* is not serotype specific (22), which is similar to the outcome of our study. The same group also reported that serotype D strains contain more introns in *COX1* than do serotype A strains. However, in our study of the *cyt b* gene, all serotype A and D strains except IFM 48637 contained a single intron and the introns from all strains were similar in size and

nucleotide sequence. The first intron in *cyt b* of *C. neoformans* started at nt 47 (Fig. 2), which is the same position as intron 2 of *Neurospora crassa cyt b* gene. *Rhodotorula acheniorum* and *Rhodotorula ferulica*, two other basidiomycetous yeasts, have introns in the same location (2). Some isolates of *C. neoformans* var. *gattii* (*cyt b* types II, III, and IV) contained a second intron that began at nt 633 (Table 1), which is similar to the location of intron 5 of the *Saccharomyces cerevisiae cyt b* gene. These findings suggest two possible evolutionary events. The first possibility is that these introns appeared in these locations prior to the separation of these species and that some species lost introns over time. The second is that these introns appeared in these locations after separation of these species.

Analysis of the mt large rRNA gene and NADH dehydrogenase subunit 2 of *C. neoformans* revealed that serotype AD strains had either the serotype A or serotype D mtDNA genotype (29). In the present study, we obtained almost similar results for the distribution of *cyt b* gene types in serotype AD strains. Of four serotype AD strains, three had serotype A-specific *cyt b* (type VIII) and one had a unique specific cyt *b* (type VII) that was nearly identical to serotype D-specific *cyt b* (type VI), with only 1 nt difference.

In conclusion, we have shown that isolates of *C. neoformans* represent nine *cyt b* types; however, the deduced amino acid sequences indicate that there are only two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*.

ACKNOWLEDGMENT

This study was performed as part of the program Frontier Studies and International Networking of Genetic Resources in Pathogenic Fungi and Actinomycetes (FN-GRPF) through the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2001).

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